AxioVision User's Guide

Release 4.9.1 SE64

March 2013
000000-1968-162

Carl Zeiss draws the User's attention to the fact that the information and references contained in these documents may be subject to technical modifications, in particular due to the continuous further development of Carl Zeiss's products. The documents enclosed do not contain any warranty by Carl Zeiss with regard to the technical processes described in the documentation or to certain reproduced product characteristics. Furthermore, Carl Zeiss shall not be held liable for any possible printing errors or other inaccuracies in this documentation, unless proof can be furnished that any such errors or inaccuracies are already known by Carl Zeiss or that these are not known to Carl Zeiss due to gross negligence and that furthermore Carl Zeiss has for these reasons refrained from eliminating these errors or inaccuracies appropriately. Carl Zeiss hereby explicitly draws the User's attention to the fact that this manual only contains a general description of the technical processes and information, the implementation of which in any individual case may not be appropriate in the form described here. In cases of doubt, we recommend the User to consult Carl Zeiss.

This manual is protected by copyright. Carl Zeiss has reserved all rights to this documentation. It is prohibited to make copies, partial copies, or to translate this manual into any other language, except for personal use.

Carl Zeiss explicitly draws attention to the fact that the information contained in this manual will be updated regularly in compliance with the technical modifications and supplements carried out in the products and furthermore that this manual only reflects the technical status of Carl Zeiss's products at the time of printing.

Disclaimer

The author is not responsible for any contents linked or referred to from his pages - unless he has full knowledge of illegal contents and would be able to prevent the visitors of his site fromviewing those pages. If any damage occurs by the use of information presented there, only the author of the respective pages might be liable, not the one who has linked to these pages. Furthermore the author is not liable for any postings or messages published by users of discussion boards, guestbooks or mailinglists provided on his page.

Carl Zeiss Microscopy GmbH
Carl-Zeiss-Promenade 10
07745 Jena, Germany

microscopy@zeiss.com
www.zeiss.com/microscopy



Carl Zeiss Microscopy GmbH
Königsallee 9-21
37081 Göttingen, Germany

© Carl Zeiss Microscopy GmbH, 1999

Microsoft, Windows, and Excel are registered trademarks of Microsoft Corporation in the United States and/or other countries.

Adobe und Acrobat Reader are trademarks or registered trademarks of Adobe Systems Incorporated in the United States and other countries.

QuickTime is a trademark of Apple Computer, Inc.

QuickTime Software can be downloaded from Internet under www.apple.com.

Notice of the Producer

This Software product ("AxioVision") was designed, realized, verificated, validated and released in a certificated process environment. The quality management system is certified following the rule of DIN EN ISO 9001.

The fields of application of the Software are common tasks and applications in microscopy respectively imaging (so called "Off-The-Shelf Software"). Though the user acknowledges that in any kind of use the end user of the Software is responsible for the validation of the Software for the end user's dedicated intend of use considering all requirements of law and standards (e. g. FDA/21 CFR part 11, IvDD, etc.). If necessary the end user has to establish, to document, to implement and to maintain a special process to fulfill all the requirements to be conform with the validate rules of law and standards. It is pointed out that displayed measure values (eg length measurement) may not be used directly as analytical values for diagnostic results.

CARL ZEISS DOES NOT WARRANT THAT THIS SOFTWARE IS USABLE FOR SPECIAL PURPOSES OTHER THAN IN THE FIELDS OF APPLICATION DEFINED ABOVE.

Safety



Refer to the safety notes and instructions in the manuals of all necessary devices (e.g. microscope peripherals, cameras, computers, computer additionals, etc.) before installing and using the software (see also "Safety Notes AxioVision, AxioVision LE, SAP 000000-2054-368").

Table of Contents

1 Introduction 1-1
1.1 The Concept 1-1
Image Acquisition 1-1
Microscope Control 1-1
Incubation 1-1
Image Processing and Annotations 1-2
Image Analysis 1-2
Documentation 1-2
Configuration 1-3
1.2 The Modules 1-3
Image Acquisition Modules 1-3
Autofocus 1-3
Extended Focus 1-3
Mark&Find 1-4
Fast Acquisition 1-4
Fast MosaiX 1-4
Multichannel Fluorescence 1-4
MosaiX 1-4
Panorama 1-4
Time Lapse 1-5
Z-Stack 1-5
Digital High Speed Recorder 1-5
Dual Camera 1-5
HDR Imaging (High Dynamic Range) 1-5
Image Processing Modules 1-6
ApoTome 2 1-6
Imaging Plus 1-7
2D Deconvolution 1-7
3D Deconvolution 1-7
Inside4D 1-7
Widefield Multichannel Unmixing 1-8
Image Analysis Modules 1-8
Interactive Measurement 1-8
AutoMeasure (Automatic Measurement) 1-8
AutoMeasure Plus 1-8
Calotte Grinding Measurement 1-9

TIC Measurement *1-9*
Particle Analyzer *1-9*
NMI *1-10*
Grains *1-10*
Multiphase *1-11*
Comparative Diagrams *1-12*
Graphite *1-12*
QuantiFISH *1-13*
Ratio *1-13*
Tracking *1-13*
TMA (Tissue Micro Arrays) *1-13*
Colocalization *1-14*
Topography *1-14*
Online Measurement *1-14*
3D Measurement *1-14*
Physiology *1-15*
SFM *1-16*
Comparative Diagrams *1-16*
ASSAYbuilder *1-17*
Shuttle&Find *1-17*
Correlative Particle Analysis (CAPA) *1-18*
Archive Modules 1-18
Asset-Archive *1-18*
Configuration Modules 1-18
Commander *1-18*
VBA *1-19*
1.3 AxioVision and Microsoft® Windows 1-19
1.4 User's Guide and Online Help 1-19
1.5 Backup Copies 1-20

2 Overview and Operating 2-1
2.1 Overview 2-1
2.2 Workarea 2-2
2.3 Workflow 2-3
2.4 Own Toolbars and Dialogs 2-5
2.5 Shortcut Menus 2-6
2.6 Properties Window 2-7

3 Image Acquisition 3-1

3.1 General 3-1
3.2 First Steps 3-2
3.3 Conditions 3-2
The Standard workflow 3-3
Step by step to the first image 3-4
3.4 Practical Notes on Operation in AxioVision 3-10
Speed of the live image 3-10
Notes on Optimum Color Reproduction in AxioVision 3-11
3.5 HDR - High Dynamic Range 3-13
General 3-13
HDR imaging 3-14

4 Microscope Control 4-1
4.1 General 4-1
4.2 Configurating microscopes with Microscope Configuration 4-2
4.3 Configurating microscopes with MTB2004 Configuration 4-3
4.4 Activating microscope configurations 4-4
4.5 The Settings Editor 4-5
How Do I Generate a Hardware Setting? 4-6
4.6 Definite Focus 4-9
Overview 4-9
Example 1: Automatic focus-stabilization at one position of a culture dish over time (Multidimensional Acquisition) 4-9
Example 2: Automatic focus-stabilization at one position of a culture dish over time (Fast Acquisition) ... 4-12
Example 3: Automatic focus stabilization during acquisition of a MosaiX image 4-12
Example 4: Automatic z-position adjustment at multiple positions in experiments using multiwell plates or similar sample carriers. 4-14
Example 5: Automatic z-position adjustment and stabilization over time at multiple positions in experiments using multiwell plates or similar sample carriers. 4-15
Example 6: User-defined Definite Focus operation 4-18
4.7 TIRF 4-21
Performing automatic calibration 4-21

5 Image Processing 5-1
5.1 General 5-1
The different ways of processing images 5-1
Image formats 5-2
5.2 Annotations 5-3
5.3 Working with the Image Processing Functions 5-4
General 5-4
Smoothing an image (basic) 5-4
Shading Correction (basic) 5-9
Correction of XY pixel shift in multichannel images (basic) 5-11
Correction of z pixel shift in multichannel z-stack images .. 5-15
5.4 Enhancing the Image Display on the Monitor 5-19
5.5 Drawing in and Formatting Annotations 5-19
5.6 Specifying Standard Settings for Annotations 5-21

6 Image Analysis 6-1
6.1 General 6-1
Possible ways to perform measurements 6-1
Conditions for measurements 6-1
Allocation of scalings 6-1
If the selected image does not have a scaling 6-2
Displaying measurement data 6-2
Saving data 6-2
Saving measurement data 6-2
Saving as separate data table 6-3
Configuring measurement tools 6-3
6.2 Measurement workflow: easy and save measurement 6-4
Measure workflow: overview 6-5
Executing the Interactive Measurement Function 6-6
Display and export measurement data 6-7
6.3 Sorting and performing calculations with measurement data . 6-8
6.4 Creating data reports 6-9
Modifying and creating report templates with the file extension ZDR 6-9
Report templates toolbars 6-10
Modifying a report template 6-10
Creating a report template 6-16
Creating a report with several opened images 6-30

Creating reports with the extension ZDR, printing them and saving them as a PDF file 6-34
Creating a report for an image 6-34
Adopting a new image into an open report template ... 6-36
Printing a report 6-37
Saving a report as a PDF file 6-37
Creating a report for a multichannel fluorescence image 6-38
Creating a report for a time lapse image with two fluorescence channels 6-40
Creating a report with an image, data table and histogram 6-43
Creating a report with saved data tables 6-47
Creating a report with files from an archive 6-51
6.5 Creating charts 6-57
Properties of charts 6-57
Creating data tables with a script 6-57
Creating a bar chart 6-59
Creating an X-Y point chart 6-63
Creating a X-Y line chart 6-66
Saving and resetting chart settings 6-70
Loading and adapting chart settings for any table 6-71
Creating an image from a chart 6-71
Exporting a chart as a meta file 6-71

7 Documentation 7-1
7.1 General 7-1
7.2 AxioVision ZVI - the Image Format for Digital Microscopy 7-1
Advantages of the ZVI format 7-1
Saving 2D images 7-2
Saving multidimensional images 7-3
7.3 Managing Images with the Image File Browser 7-4
The Image File Browser window 7-5
The functions of the Image File Browser toolbar 7-6
Context menu of the Image File Browser 7-7
Context menu of the slide show 7-8
Change the form for data entries 7-9
7.4 Generate, print and export reports with the file extension ZVR 7-10

Generating new reports *7-11*
Printing a report *7-12*
Exporting a report *7-13*
Templates for forms and reports 7-14
Settings for editing a template 7-15
Moving and deleting fields *7-18*
Inserting new fields and changing field values *7-19*
Changing field attributes *7-21*
7.5 Editor for Multidimensional Images 7-22
7.6 Creating an Asset Archive 7-25
Creating new projects 7-26
Creating contacts 7-26
Adding an asset to an archive 7-27
Working with value lists 7-29
Views 7-29
Categories 7-29
Searching 7-30
Keyword search *7-30*
Creating a user-defined search *7-30*
Managing archives 7-32
Setting a password *7-32*
Field management *7-32*
Specifying the storage location *7-33*
Changing project properties *7-34*

8 Configuration 8-1
8.1 General 8-1
8.2 Overview: Setting up AxioVision 8-1
Installation 8-1
Setting up AxioVision 8-2
Backing up the configuration and restoring it for individual users 8-2
Making user files available for all users 8-3
8.3 User and Data Administration 8-3
Microsoft Windows user administration 8-3
User files *8-4*
The shared account "All Users" *8-6*
Creating new users *8-6*
Data administration under AxioVision 8-8

Data files *8-8*
AxioVision images *8-9*
Temporary files *8-9*
AxioVision configuration files *8-10*
Moving the "My Documents" folder *8-10*
Moving the folder for temporary files *8-12*
8.4 Scalings 8-12
General 8-12
Generate scalings 8-13
8.5 Adapting the User Interface 8-17
Creating toolbars 8-17
Define key combinations for functions 8-20
Create own dialogs 8-22
Define workflows 8-25
8.6 General Settings 8-27
8.7 Exporting and Importing User Configurations 8-28
General 8-28
Export 8-29
Import 8-29
Importing the configuration files *8-29*
Importing user files *8-30*
8.8 Creating various AxioVision configurations for a user 8-30
Background 8-30
Creating individual configurations 8-31

9 Image Acquisition Modules 9-1
9.1 ApoTome 2 9-1
General 9-1
Fringe projection imaging principle 9-2
Why is the resulting image an optical section? *9-5*
Hardware configuration 9-5
ApoTome 2 Configuration for Axio Imager, Axio Observer or Axiovert 200 ..... *9-6*
ApoTome 2 Calibrations 9-8
Calibration of phase position *9-9*
Step 3: Grid Focusing *9-12*
Calibration of the grid focus *9-18*
Step 2: Optical Focusing *9-20*
Step 3: Grid Focusing *9-21*

Calibration mode....9-23
Image acquisition....9-24
Selecting the camera....9-25
Single image acquisition....9-25
Multidimensional acquisition with the ApoTome 2....9-30
Accelerated image acquisition with the ApoTome 2 (burst mode)....9-31
Objective – grid allocation....9-34
Axio Imager 1 / Axio Imager 2....9-35
Axiovert 200 and Axio Observer....9-36
Axioplan 2 imaging e....9-38
Other ApoTome 2 Settings....9-38
Conversion of ApoTome 2 images from raw data mode....9-39
ApoTome 2: HDR Mode....9-42
9.2 Autofocus....9-43
General....9-43
Calibrating Autofocus....9-44
Preparation....9-44
Calibration....9-44
Using Autofocus....9-45
9.3 Multidimensional Acquisition....9-48
How do I create an experiment?....9-49
Multichannel Fluorescence module....9-52
How do I create a multichannel experiment?....9-52
How do I manage channel settings (working with the channel pool)?....9-58
Z-Stack module....9-62
How do I create a z-stack?....9-63
Time Lapse module....9-66
How do I create a time lapse series?....9-66
Advanced image acquisition: Mark&Find....9-68
How do I create a Mark&Find experiment?....9-69
9.4 Extended Focus....9-75
General....9-75
Acquiring images with Extended Focus....9-75
Using z-stack images for Extended Focus....9-77
9.5 Mark&Find....9-79
Overview....9-79

How do I generate a Mark&Find list? 9-80
Example 1: slide 9-80
Example 2: multiwell plate (96) 9-92
9.6 Panorama 9-98
General 9-98
System requirements 9-99
Special features of Panorama images 9-100
Acquisition of Panorama images 9-100
Creating Panorama images from individual files 9-105
Converting a tile image into a regular AxioVision image 9-108
9.7 MosaiX 9-110
General 9-110
System requirements 9-113
Special features of MosaiX images 9-113
Multidimensional MosaiX images 9-114
Setting up the camera and scanning stage 9-115
Image orientation 9-115
Directions of movement of the stage 9-116
Alignment of camera and stage 9-119
Acquisition of MosaiX images 9-121
Stitching parameters 9-127
Converting a tile image into a regular AxioVision image 9-130
9.8 TMA 9-132
Background 9-132
Application 9-132
Functions and integration into AxioVision 9-133
Make Map 9-134
Detect Cores 9-134
Prerequisites 9-134
9.9 Fast Acquisition 9-135
Acquisition principles 9-135
Experiment property page 9-136
C (Channels) property page 9-140
Extended parameters 9-141
Enable 9-142
Name 9-142
Channel Color 9-142
Exposure Time [ms] 9-142

Dye 9-143
Display settings 9-144
Exposure 9-147
Fast Acquisition Setting Editor 9-148
Channel Actions 9-151
Z property page (Z-stack) 9-151
T (Time) property page 9-153
Fast Acquisition dialog 9-155
Fast Acquisition Document Cutter 9-156
Information for Fast Acquisition 9-160
9.10 Physiology 9-161
Introduction 9-161
Acquisition principles 9-162
Acquisition 9-164
Physiology Setup 9-167
Regions property page 9-167
C (Channel) property page 9-174
Markers property page 9-174
Physiology Acquisition 9-179
Physiology Acquisition Document Cutter 9-187
Cutter Physiology 9-187
Physiology Measurements 9-194
Information for Physiology 9-210
Information 9-211
9.11 FRET Analysis in the Physiology Module 9-212
Method 9-212
Software package 9-212
FRET determination using correction methods 9-213
FRET determination using acceptor bleaching method 9-216
FRET determination using formula generator 9-217
Examples of FRET formulae for the formula generator 9-218
Samples 9-219
Basic settings for image acquisition 9-219
Image acquisition for AxioVision FRET analyses 9-220
FRET measurement in the AxioVision Physiology module 9-222
FRET measurement according to Youvan 9-224
Functions of the Physiology window for FRET measurements 9-225

Function keys for FRET measurement 9-228
Other pre-programmed FRET methods 9-236
Donor Ratio 9-236
Acceptor Ratio 9-238
Gordon 9-239
Xia 9-241
Siegel (FRET calculation via acceptor bleaching) 9-243
Analysis of long time lapse images 9-245
User-defined FRET measurements (formula generator) 9-246
Example of a 2-channel measurement 9-246
Example of an f0 measurement 9-248
FRET online measurements 9-250
Literature 9-258
9.12 Dual Camera 9-259
Dual Camera setup hints 9-259
Dual Camera property pages 9-262

10 Image Processing Modules 10-1
10.1 Imaging Plus 10-1
General 10-1
Image enhancement 10-1
Gray morphology 10-2
Image arithmetics 10-2
Fourier transformation 10-3
Color space transformation 10-3
Working with image processing functions 10-4
Reducing or enlarging images or image regions 10-4
Copying various regions from an image and generating a new image 10-11
Working with individual color channels 10-18
Converting a color image into a black/white image 10-23
10.2 Inside4D (3D View) / 3D Measure 10-32
The viewing modes Shadow, Transparency, Surface, Mixed and Maximum 10-38
Measure 3D Mode 10-39
Settings for the viewing modes 10-40
Data property page 10-40
Shadow mode 10-41
Transparency mode 10-42

Surface mode *10-43*
Maximum mode *10-44*
Mixed mode *10-45*
Illumination property page 10-47
Shadow mode *10-49*
Transparency mode *10-49*
Surface mode *10-50*
Maximum mode *10-50*
Mixed mode *10-50*
Series property page 10-51
View property page 10-54
Shadow mode *10-54*
Transparency mode *10-57*
Surface mode *10-59*
Maximum mode *10-62*
Mixed Mode *10-65*
Info property page 10-67
Clipping property page 10-69
Stereo property page 10-72
Special property page 10-72
10.3 Deconvolution 10-75
How do I perform deconvolution using the Regularized Inverse Filter? 10-76
10.4 Widefield Multichannel Unmixing 10-84
Background 10-84
The Principle of unmixing 10-85
The Widefield Multichannel Unmixing function 10-89
Working with the basic functions 10-90
Unmixing with reference samples *10-90*
Unmixing of a multichannel image using ACE *10-98*
10.5 Colocalization 10-104
Background 10-104
Control elements 10-104
Regionn Select 10-105
Channel Select 10-105
Threshold 10-106
Mask 10-107
Graphical Display 10-107

11 Image Analysis Modules 11-1
11.1 Interactive Measurement 11-1
General 11-1
Working with interactive Measurement Programs 11-1
Generating measurement programs 11-2
Loading and executing measurement programs 11-3
11.2 AutoMeasure (Automatic Measurement) 11-5
General 11-5
The concept of the AutoMeasure module 11-8
The wizard for generating measurement programs 11-9
Program management 11-10
Image enhancement 11-11
Segmentation 11-12
Object Separation 11-13
Processing the measurement mask (binary image editor) 11-14
Set Measurement Propeeties 11-15
Evaluation 11-17
Executing measurement programs 11-18
11.3 AutoMeasure Plus (Advanced Automatic Measurement) 11-19
General 11-19
The concept of the AutoMeasure Plus module 11-19
Working with the segmentation functions 11-20
Dynamic segmentation 11-21
Automatic segmentation of a fluorescence channel n a multichannel z-stack image 11-25
Edge detection 11-28
Performing binary image processing 11-35
Processing a binary image (smoothing, removing artifacts, filling gaps) 11-35
Separation and/or reconstruction of structures using watersheds 11-39
Masking structures/Using Boolean functions 11-45
Working with the Functions for automatic Measurement 11-53
Generation of Measurement Properties Files and Measurement 11-53
11.4 Particle Analyzer Projects 11-60
General 11-60

The system variants *11-60*
Accessories for particle analysis *11-62*
The concept 11-65
Operation and configuration *11-65*
Projects and test specifications *11-69*
Preparing the system 11-70
Introduction *11-70*
General AxioVision Settings *11-72*
Special settings for particle analysis *11-74*
Creating test specifications 11-77
General *11-77*
Importing and exporting test specifications *11-78*
The Change Test Specification dialog *11-79*
Testing test specifications *11-80*
Hardware settings *11-80*
Image Source *11-81*
Input form *11-82*
Evaluation scripts *11-83*
Modifying report templates 11-87
Optional settings *11-88*
Performing measurements 11-90
11.5 NMI (Non-Metallic Inclusions) 11-92
General 11-92
The system variants *11-92*
The concept 11-94
Operation and configuration *11-94*
Projects and test specifications *11-96*
Preparing the system 11-97
Introduction *11-97*
General AxioVision Settings *11-98*
Special settings for NMI *11-99*
Creating test specifications 11-102
General *11-102*
Importing and exporting test specifications *11-104*
The Change Test Specification dialog *11-105*
Testing test specifications *11-106*
Hardware settings *11-106*
Image source *11-108*

Input forms *11-108*
Evaluation script *11-109*
Modifying report templates *11-112*
Optional settings *11-112*
Performing measurements 11-114
How do I create an NMI archive? *11-120*
How do I create a test specification? *11-122*
How do I set up image acquisition for several samples? *11-125*
11.6 Grains 11-131
The concept 11-133
Operation and configuration 11-134
Preparing the system *11-134*
The Material Analysis archive *11-136*
Projects and test specifications *11-139*
Importing and exporting test specifications *11-140*
Modify Test Specifications *11-141*
Hardware settings *11-142*
Image source *11-142*
Input forms *11-143*
Modifying report templates *11-144*
Options – Auto *11-144*
Options – Chord *11-144*
The measurement procedure 11-144
Performing measurements *11-145*
The results document *11-146*
Generating reports *11-147*
Creating and performing a grains analysis 11-148
How do I create an archive? *11-148*
How do I create a test specification? *11-150*
How do I perform a measurement? *11-153*
11.7 Multiphase 11-155
The concept 11-156
Operation and configuration 11-157
Preparing the system *11-157*
The Material Analysis archive *11-159*
Projects and test specifications *11-162*
Importing and exporting test specifications *11-163*

The Change Test Specification dialog *11-164*
Hardware settings *11-165*
Image source *11-165*
Input forms *11-166*
Modifying report templates *11-167*
The measurement procedure 11-167
Performing measurements *11-167*
The results document *11-169*
Generating reports *11-170*
Creating and performing a multiphase analysis 11-171
How do I create an archive? *11-171*
How do I create a test specification? *11-173*
How do I perform a measurement? *11-176*
11.8 Comparative Diagrams 11-178
The concept 11-179
Operation and configuration 11-180
Preparing the system *11-180*
The Material Analysis archive *11-182*
Projects and test specifications *11-184*
Importing and exporting test specifications *11-185*
The Change Test Specification dialog *11-186*
Hardware settings *11-187*
Image source *11-188*
Input forms *11-188*
Modifying report templates *11-189*
Performing a comparative-diagram comparison *11-189*
Generating reports *11-190*
Creating and performing a comparative-diagram comparison 11-190
How do I create an archive? *11-190*
How do I create a test specification? *11-193*
How do I perform a comparative-diagram comparison? *11-195*
11.9 Graphite 11-197
The Concept 11-198
Operation and configuration 11-199
Preparing the system *11-199*
The Material Analysis archive *11-201*

Projects and test specifications *11-204*
Importing and exporting test specifications *11-205*
Modify Test Specifications *11-205*
Hardware settings *11-206*
Image source *11-207*
Input forms *11-207*
Modifying report templates *11-208*
The measurement procedure 11-208
Performing measurements *11-208*
The results document *11-210*
Generating reports *11-211*
Creating and performing a graphite analysis 11-211
How do I create an archive? *11-211*
How do I create a test specification? *11-214*
How do I perform a measurement? *11-216*
11.10 Layer Thickness Measurement 219
The concept 219
Operation and configuration *219*
Projects and test specifications *220*
Preparing the system 222
Introduction *222*
General AxioVision Settings *223*
The Material Analysis archive *224*
Creating test specifications 228
General *228*
Importing and exporting test specifications *229*
The Change Test Specification dialog *230*
Testing test specifications *231*
Hardware settings *231*
Image source *233*
Input forms *233*
Preprocessing script *235*
Modifying report templates *238*
Optional settings *238*
Performing measurements 240
11.11 Calotte Grinding Measurement 241
General description 241
Performing a calotte grinding measurement 241

Describing the sample *242*
Opening an image *243*
Drawing circles *244*
Evaluation *246*
Appendix A – Example of a Report 247
11.12 Microhardness Measurement 248
General 248
Workflow 250
Performing a microhardness measurement 251
Glossary 255
Appendix A – Example of a report 256
11.13 TIC Measurement 257
Automatic TIC measurement 258
Interactive TIC measurement 262
Causes of errors during interactive measurement 263
TIC scaling 265
11.14 SFM (Scanning Fluorescence Microscope) 270
SFM Evaluation - Original Datatable 273
SFM Histogram 276
SFM Scatter Plot 282
SFM Gallery 289
SFM Gated Datatable 295
11.15 Shuttle&Find 299
Overview 299
Setting up Shuttle&FindSetting up 299
Procedure for using Shuttle&Find 307
Overlaying images 323
Creating new holders 325
Fine calibration 328
The SEM image 329
Application examples for Shuttle&Find 330
Example 1: from light microscope (LM) to electron microscope (SEM) 330
Example 2: from electron microscope (SEM) to light microscope (LM) 334
Notes on operation 338
Tips to ensure accuracy 339
On the light microscope *339*

On the electron microscope *339*
General points *340*
General Information on Accuracy 340
Troubleshooting 342
Calibration *342*
Recovery/Find *345*
Overlay *347*
SEM *347*
Shuttle&Find Checklist 348
11.16 Correlative Particle Analysis (CAPA) 352
Overview 352
Setting up Correlative Particle Analysis (CAPA) 354
Activating the toolbar *354*
Setting the stage and camera on a light microscope *357*
Setting CAPA options *360*
Setting calibration options *362*
General calibration settings 363
Creating new holders/importing/exporting 366
Editing holders 368
Adjusting specimen holders *369*
Setting SEM options *369*
SEM Live 371
SEM Acquisition 372
Workflow with Correlative Particle Analysis 372
Correlative workflow on a light microscope *372*
(1) Open an archive - see section 7.6. 373
(2) Start the particle analysis using the Correlative Particle Analysis test specification. 373
(3) Complete the input mask as far as necessary 389
(4) 3-point calibration 390
Fine calibration 397
(5) Structure the rest of the workflow for particle analysis using Particle Analyzer Projects 399
Correlative workflow on an electron microscope *399*
(1) Import the particle results data 400
(2) Calibration on an electron microscope 401
(3) Particle selection 407
(3.1) Presettings for Correlative Particle Analysis 408

(3.2) Optionen property page: Selection of SEM/EDS parameters 408
(3.3) Options property page: Selection of results documentation 409
(3.4) Particle data property page 410
Documentation of results 412
Report and results view 412
Report 417
Comparison of data between light and electron microscope 421
Save to Archiv 421
Overlaying 422
EDS mapping of a particle 422
Notes on operation 422
On the light microscope 423
On the electron microscope 423
General information on accuracy 424
Troubleshooting 425
Calibration 425
Relocation 429
Correlative Particle Analysis checklist 431

12 Configuration Modules 12-1
12.1 Commander 12-1
Introduction 12-1
Special points relating to the creation of scripts 12-2
Batch processing - application of a script to several images 12-2
Limitations of scripts 12-3
Use of scripts by several users of a system 12-3
Installation of scripts on other systems 12-5
Recording a script 12-5
Inserting commands into an existing script 12-10
Changing the way individual commands are executed, selecting images to be displayed 12-14
Executing a script automatically 12-17
Preparing a script for batch processing 12-18
Repeating scripts 12-21
12.2 VBA 12-24
General 12-24

First steps 12-24
The VBA online help 12-25
Welcome to Standard Visual Basic documentation 12-25
Help with the Visual Basic user interface 12-25
Visual Basic concepts 12-25
Help with Visual Basic procedures 12-26
Visual Basic language directory 12-26
Visual Basic add-in model 12-26
Reference for Microsoft Forms 12-26
Welcome to AxioVision Visual Basic documentation ... 12-26
Managing and running macros 12-26
Running macros via workflows, toolbars and shortcut keys 12-27
12.3 Kiosk Mode 12-29
General 12-29
Different Kiosk Mode user profiles 12-29
Setting up Kiosk Mode 12-31
Switching off Kiosk Mode 12-34

1 Introduction

1.1 The Concept

AxioVision is a modular image-processing and analysis system for modern microscopy. The basic functionality for image acquisition and microscope control, image processing and annotations, image analysis, documentation and configuration can also be expanded by integrating additional modules into the system for specific tasks (see section 1.2 "The Modules").

The basic version comprises the following functionalities:

Image Acquisition

A range of different camera types can be used with AxioVision, from simple TV cameras through to high-resolution and high-sensitivity cameras. The cameras of the Carl Zeiss AxioCam family guarantee optimum integration. As the cameras are integrated seamlessly into the AxioVision software, you are also able to generate complex images and image series with a simple mouse-click.

Microscope Control

With AxioVision it is possible to control any motorized Carl Zeiss microscope fully automatically and interactively (you can of course also use manual standard microscopes). Software control offers the following advantages: the microscope's setting parameters can be saved as required, and are therefore available for reuse during future tests, magnifications are determined automatically, and complex procedures are significantly faster and can be easily reproduced at any time.

Incubation

AxioVision controls and monitors the Zeiss incubation components. Incubation parameters like temperature and CO_2- or O_2 concentrations can be stored as hardware settings and be reused for new experiments. All incubation parameters are accessible as measurement parameters for further evaluation of acquired multidimensional time series.

Image Processing and Annotations

The acquired image is immediately displayed on the monitor. It can then be optimized using a wide range of techniques:

- Contrast, brightness and color adjustment
- Noise suppression, smoothing and contour enhancement
- Sharpness enhancement/emphasizing of details
- Correction of illumination influences and white balance

AxioVision can also be used to add any annotations that you may require to the images. All elements, from scale bars and colored markings through to text and graphics, have been integrated into the program.

Image Analysis

You are able to perform simple interactive measurements in the basic program. The measured values (e.g. lengths, areas and angles) are made available in a data table, and can be processed further using spreadsheet programs, such as Microsoft Excel. The interactive measurement can be executed via a special dialog, or freely via a menu or a toolbar.

Documentation

Besides the image itself, the AxioVision image format ZVI also saves additional data, such as the image number, date of acquisition, microscope settings, exposure values, size and scale details, contrast procedures used etc. Annotations and measured values are also saved with the image. Rather than being permanently "burnt" into the image, annotations are present in the form of a separate plane. This means that no image information is concealed, and changes can be made at any time. Consequently, an image can be reproduced years later under identical conditions.

The Image File Browser supports the management of the content of large databases. With this browser you can navigate quickly within your file folders, and obtain a clear display of all the key data acquired with the image.

For the generation of reports or presentations, AxioVision offers you predefined report layouts. These can be modified or completely regenerated at any time on an individual basis.

Configuration

AxioVision offers you a great deal of freedom in terms of the arrangement of your individual work environment. To speed up your work, it is possible to execute any commands using freely definable key combinations. You can configure your own toolbars and group functions together on your own menus. Relevant control elements for camera and microscope control in the form of individual dialogs are grouping together with just a few clicks of the mouse. For recurring routine processes, workflows make simple operation possible. As a result, operating errors can by and large be eliminated.

1.2 The Modules

The following modules are currently available as extensions to the basic system:

Image Acquisition Modules

Autofocus

This module allows the optimum focus position to be calculated with motorized microscopes. It can be used in reflected light, transmitted light, bright field, dark field and fluorescence, and works with all cameras and frame grabbers controlled directly by AxioVision.

Extended Focus

Needle-sharp images are essential for carrying out precise analyses. Depending on the object and the setting, a microscope's depth of field is often insufficient to generate a single image which is sharp over the entire plane. The AxioVision Extended Focus module solves the problem. A number of images are acquired from different focus planes, and the sharp details of each individual image are combined to produce an image that is sharp throughout.

Mark&Find

This module is used to record and automatically relocate various positions on slides, in culture plates and in multiwell plates using a motorized x/y stage.

Fast Acquisition

This module expands the functionality of the modules multichannel fluorescence, z-stack and time lapse allowing image acquisition to be done in high speed.

Fast MosaiX

Fast MosaiX expands AxioVision with the option of acquiring a large number of high-resolution individual images in a very short time. The use of a flash for exposure ensures that your samples are protected, and, thanks to the optimum interaction between hardware and software, several hundred images per minute can be acquired.

Multichannel Fluorescence

This module allows you to acquire multichannel fluorescence images with up to 32 channels. It can be combined with the Z-Stack, Time Lapse, MosaiX and Mark&Find modules.

MosaiX

MosaiX is developed to analyze large surfaces - MosaiX permits the area of a specimen to be scanned in order to generate one single large image. This electronically created image can thus serve as a map to navigate the specimen and provides a basis for further analyses.

Panorama

Panorama is ideal for specimens which do not fit into the image frame. High-resolution panorama or overview images can be formed with pixel accuracy from individual shots. Even overlapping images can be combined so precisely that all the important details of your specimen are recorded in a single image.

Time Lapse

This module allows you to acquire time lapse images. It can be combined with the Z-Stack, Multichannel Fluorescence, MosaiX and Mark&Find modules.

Z-Stack

This module allows you to acquire z-stack images. It can be combined with the Multichannel Fluorescence, Time Lapse, MosaiX and Mark&Find modules.

Digital High Speed Recorder

With this module time lapse sequences can be acquired in high speed and without compression artifacts. The raw data are streamed to a dedicated hard disk. Either the complete sequence or selected parts of the sequence can be converted into the ZVI image format using a Cutter function.

Dual Camera

This module enables Fast Acquisition to use two identical cameras simultaneously. This effectively doubles the speed of acquisition. Also, if configured with two different emission filters, acquisition of two colors can happen at the same time avoiding motion artifacts which may occur when acquiring images sequentially.

HDR Imaging (High Dynamic Range)

The HDR module is available in AxioVision to allow you to test out its function. This means that you can now try out this acquisition technique at your leisure and examine the positive effect it has for your specific application. Following image acquisition, a watermark is burnt into your HDR image.

Once you have licensed this module, it is available to you without any restrictions.

HDR Imaging enables you to produce images with a greater dynamic range than the camera you are using would technically allow.

In certain acquisition situations the dynamics of an image scene, i.e. the range of intensity differences present, can exceed the dynamic range offered by even a good digital camera. An example here would be the acquisition of polished steel sections in the field of materials science. Acquiring images of structures in the reflective surface and in black inclusions at the same time is no easy task. Another example could be taken from neurobiology, where you may wish to simultaneously capture in an image not only the brightly fluorescing GFP-labeled cell nucleus of a neuron, but also its axons and dendrites, which emit much darker signals.

With the HDR technique, image sequences are generated during acquisition using a constantly increasing exposure time. An initial image that is not overexposed is taken as a starting point. The number of images and the extent of the increase in the dynamics can be adjusted.

Image content that is concealed by noise in the first image is certain to be shown with good contrast in an image acquired using a longer exposure time. On the other hand, image content that is displayed clearly in the image with the shortest exposure time will be overexposed in an image for which a longer exposure time is used.

By means of the weighted superimposition of the component images it is therefore possible to generate a resulting image that offers excellent contrast both in dark image regions and in bright zones.

As pixels are saved with a maximum of 16 bits, 65,535 gray levels is the greatest possible dynamic range that can be stored in an image.

With the help of the adjustable display characteristics curve in AxioVision, even data with such a high dynamic range can be presented in a meaningful way on a monitor.

Image Processing Modules

ApoTome 2

An ApoTome 2 system allows you, on the basis of the principle of fringe projection, to generate optical sections through fluorescence samples. The parts of the image that are out of focus are then removed, and an increase in

- image sharpness
- the signal to noise ratio (contrast)
- and resolution in the axial direction

can be achieved.

Imaging Plus

The Imaging Plus module offers you all the main digital image processing techniques. This module comprises functions for image enhancement, gray morphology, image arithmetic and color space transformation.

2D Deconvolution

Using this module you can improve the quality of 2-dimensional fluorescence images in both the lateral and axial direction in accordance with a predefined or self-generated Point Spread Function (PSF).

3D Deconvolution

This module enables you to improve the quality of z-stack fluorescence images using deconvolution algorithms. Using the Point Spread Function (PSF) this 3D Deconvolution calculates out of focus light from all z planes back to its origin.

Four methods are available:

- Nearest Neighbor for fast contrast enhancement results
- Regularized Inverse Filter for access to genuine 3D filtering
- Fast iterative algorithm for high speed restoration
- Iterative Maximum Likelihood Algorithm – the high-end method for maximum light efficiency and increased resolution

Inside4D

The Inside4D module allows you to display z-stack images in three dimensions in a very simple way. Three-dimensional structures can also be viewed as animations over time, and exported as digital video films into AVI format or in MOV format (compatible to QuickTime). Five projection methods are available: shadow, transparency, surface, maximum projection, and mixed. You also

have access to a wide range of image processing options (display of cutting planes, anaglyph view, iterative 3D measurement). The zoom factor, for instance, can be freely selected, which also means that you can zoom into structures to view them from the inside.

Widefield Multichannel Unmixing

Widefield Multichannel Unmixing is a module for the removal of crosstalk between fluorescent dyes in multichannel images. Crosstalk is a phenomenon which occurs, whenever dyes are excited by excitation light of more than one filter combination. Up to eight fluorescence channels using a traditional epifluorescence microscope can be separated using unmixing matrices determined either by automatic component extraction (ACE) or by measuring crosstalk in appropriate reference samples.

Image Analysis Modules

Interactive Measurement

This module allows the interactive measurement of a variety of object parameters, such as size, circumference, number etc. Circle measurements can also be performed simply by clicking on several contour points. The measured data are saved together with the image, and are therefore available for additional evaluations.

AutoMeasure (Automatic Measurement)

With this module, measurement programs can be generated very quickly and simply with the help of a wizard. All of the functions required for an image analysis procedure are called up, one after the other. The parameters for the functions can then be set for the measurement task in question, and saved at the end as a measurement program. Once these measurement programs have been defined, you can measure any number of images by performing series analyses.

AutoMeasure Plus

This module expands the functions of the AutoMeasure module to include a wide range of additional functions for single segmentation and binary image

processing. Here the functions are not executed in a wizard, but are available individually as menu functions. They can be freely combined, allowing you to create your individual measurement routines.

Calotte Grinding Measurement

During the process of coating materials, quality is controlled by measuring the thickness of layers in accordance with the DIN V ENV 1071 standard. The Calotte Grinding Measurement application module allows you to determine the thickness of calotte section layers. To prepare the calotte section samples, a grinding ball with a defined diameter generates a calotte-shaped hollow in the coating of the sample. This results in circular or elliptical indentations, the diameters of which are determined by drawing in measurement circles. The layer thickness is calculated automatically from the diameters of the measurement circles and the diameter of the grinding ball.

TIC Measurement

The TIC module (**T**otal **I**nterference **C**ontrast – only in combination with the TIC slider from Carl Zeiss) allows precise, contact-free and extremely simple determination of the optical height and thickness of object structures over a range from just a few nanometers through to several micrometers. The advantage of the TIC method over conventional thin-layer thickness measuring instruments (profilometers, atomic force scanning microscopes) lies in the combination of short measurement and analysis times with a high degree of accuracy. The use of circularly polarized light makes the orientation of the structures on the sample irrelevant, eliminating the need for stage rotation. It is even possible to analyze samples with large surface areas.

Particle Analyzer

The Particle Analyzer module allows you to create and carry out particle analysis projects. The aim is to classify particles based on a selectable measurement parameter (in most cases: largest diameter). This classification takes place on the basis of predefined norms or classes determined by the user. Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed out in the form of a report. Work using this module can be divided into two task areas:

- Generating project templates to determine the sequence of an analysis: type of image acquisition (MosaiX, single images etc.), template for input form and report, sequence of image processing and measurement etc. (for administrators only).
- Carrying out the measurement (for all users).

NMI

The NMI module allows you to determine the content of non-metallic inclusions in steel. Several samples with an area of 200 mm² can be analyzed in a single measurement. Analysis can be performed in accordance with up to 5 standards at the same time.

- ASTM E45-05 methods A and D
- DIN 50 602:1998 methods K and M
- JIS G0555:2003; methods A and B
- ISO 4569:1998; methods A and B
- EN 10247:2007; methods K, P and M

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).
- Carrying out the measurement (for all users).

Grains

The module Grains determines Grain sizes according to current international standards. There is a choice of three different modes: interactive, by means of a direct comparison with overlaid comparative images (Compare); semi-automatic intercept method, using various chord patterns (chord) and a fully automatic analysis, based on a reconstruction of even complex grain boundaries (Auto).

- Auto: automatic reconstruction of grain boundaries – DIN EN ISO 643, ASTM E 112, ASTM 1382
- Chord: Intercept method – DIN EN ISO 643, ASTM E 112, ASTM 1382
- Compare: Comparison with comparative diagrams – SEP151061, ASTM – Plate IA, ASTM– Plate IB, ASTM– Plate II, ASTM– Plate III, ASTM– Plate IV, BS4990

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).
- Carrying out the measurement (for all users).

Multiphase

This module is used for the analysis of multiphase samples. It makes it possible to detect any number of phases in gray-level or color images. The following measurement parameters are available for measurement and classification:

- area, diameter, maximum feret
- perimeter, form factor, ellipse (major and minor)
- volume of sphere, volume of ellipsoid, gray value, color value

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).
- Carrying out the measurement (for all users).

Comparative Diagrams

The Comparative Diagrams module allows the comparison of a micrograph with comparative diagrams interactively.

The result is a table containing the comparative diagram numbers relating to each image and a table containing statistical analyses. You can also have a micrograph displayed with an overlaid comparative diagram.

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).
- Carrying out the measurement (for all users).

Graphite

The Graphite module automatically analyzes the size of graphite particles in cast iron samples in accordance with EN ISO 945 and SAE J 1887. The following analyses are available:

- Spherolite: Size-Analysis of spheroidal graphite according to EN ISO 945
- Lamellar: Size-Analysis of lamellar graphite according to EN ISO 945
- SinterCast: nodularity analysis according to SAE J 1887

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).
- Carrying out the measurement (for all users).

QuantiFISH

The FISH technique (FISH = Fluorescence-In-Situ Hybridization) is used in the fields of cytogenetics and tumor research to localize specific DNA or RNA sequences in chromosomes. The QuantiFISH module offers you the quantitative determination of FISH signals in DAPI-stained, interphase cell nuclei of multichannel images or multichannel z-stack images. The results of the FISH analysis are displayed both in the image and in data lists.

Ratio

To observe processes inside cells, use is often made of fluorescent dyes. This technique makes it possible to measure the concentration of messengers, e.g. Ca^{2++}, over a certain period of time. The Ratio module offers you a suitable analysis method by means of the ratiometric processing of two fluorescence channels in multichannel time lapse images. The calculated ratio image can be displayed as a pseudo color image, making it possible to draw conclusions about the concentration, for example. In addition, the module allows you to measure the intensity of the fluorescence of selected cells over time. The results are displayed graphically and in data lists after the measurement has been performed.

Tracking

It is often necessary to observe the movement of cells or other objects during biological processes. In many cases the processes in question are transport or growth processes that require quantitative analysis. With the Tracking module you can track moving objects in time lapse sequences using either an automatic or an interactive method. The calculated tracking parameters (e.g. distance, speed, direction etc.) are displayed in data lists.

TMA (Tissue Micro Arrays)

Tissue micro arrays consist of numerous small, circular tissue samples with a defined diameter, which are fixed on a single slide. The TMA module makes it possible to acquire a composite image of the tissue micro arrays. And you can save the positions in a Mark&Find List.

Colocalization

The acquisition of fluorescence images in several channels makes it possible to visualize the relationship between biological structures. The Colocalization module provides you with a tool that enables you to determine colocalization quantitatively. It is always the colocalization of two channels that is analyzed. For images with more than two channels the user must make a selection. Colocalization results from the pixel-by-pixel comparison of intensities per channel.

Topography

With this module you can generate a height map (topographic image) from a z-stack and carry out roughness measurements in accordance with EN ISO 4287. To allow you to display the various aspects of the topographic image, a rotation and a tilt mode are available in addition to four different 3D views (texture, projection, grid, surface).

Online Measurement

This module allows you to perform measurements in an online image using interactive measurement tools. You can therefore generate measurement values very quickly without having to acquire the image. In addition, predefined or generated grids can be inserted into the online image and compared with the structures in the image (e.g. to inspect components).

3D Measurement

This module offers you a range of possibilities for the measurement of three-dimensional objects. The image stacks are presented as a 3D volume model, which allows the user to define the surfaces of interesting objects interactively. A segmented data set is then generated from these settings. Using the binary image processing function you can further process the objects obtained, e.g. by means of interactive object separation. The measurement of the 3D objects then takes place automatically for the entire image stack or interactively by clicking on individual objects in the 3D view. The function calculates morphometric parameters (e.g. barycentric coordinates in x, y and z, volume or surface content) and densitometric parameters (such as mean density of the object or standard deviation of mean

density of gray values) for all objects. In addition, field-specific parameters can be calculated for the entire 3D image, e.g. number of 3D objects in the image, total volume of all 3D objects or sum of the surfaces of all 3D objects.

Physiology

The Physiology module enables you to acquire time lapse images at a speed that is only limited by the technical limitations of the devices you are using (camera, light source) and the selected camera settings. To achieve such speeds, the Physiology module automatically uses a "streaming" technique, which involves the raw camera data being written directly to their own fast streaming hard disk (or a suitable empty partition).

The results of the acquisition process are therefore a streaming document (*.ziAR) and the data measured during acquisition (online).

At the same time, enough memory is reserved so that the measurement data can also be managed. Physiology calculates the maximum possible number of individual images that can be acquired from the number of measurement regions drawn in and the memory available on the streaming hard disk. As this value is dependent on numerous factors (binning, ROI, number of measurement regions drawn in, demands made on available free memory by other applications etc.), the entire hard-disk space that is theoretically available cannot always be used.

In a second step, which is separate from the acquisition process, the streaming document is converted into a regular ZVI image document. With the aid of a sort of video editor (Cutter) interesting regions in the image as well as interesting time sections can be selected and converted potentially reducing the data volume dramatically. Converted ZVI files are required for all further processing steps in AxioVision.

Thirdly, a range of evaluation methods allows direct measurements of gray intensities or ratiometric measurements of various kinds (exitation- and emission ratio imaging) as well as a range of FRET (Förster resonance energy transfer) measurements. In addition a custom formula editor allows the generation of user defined evaluation formulas.

SFM

This module allows you to analyze multichannel fluorescence images of cytospin specimens. It also offers a predefined workflow for detecting and measuring the cells that can be adapted flexibly to the sample in question. The measurement results can be displayed in the form of an image gallery, data table, histogram or scatterplot and provide additional information to accompany the results of flow cytometry.

Comparative Diagrams

Using the Comparative Diagrams module it is possible to compare micrographs with comparative diagrams interactively. Various views can be used:

- Micrograph and one comparative diagram
- Micrograph and two comparative diagrams
- Micrograph and four comparative diagrams
- Micrograph and eight comparative diagrams

The result you obtain takes the form of a table, along with the micrographs, if required. Comparative diagrams are possible for up to five freely definable dimensions.

Every measurement is managed within an archive as an independent project. The results are analyzed graphically and statistically immediately after the measurement and can be printed in the form of a report. Work using the module can be divided into two task areas:

- Generating project templates to determine the workflow for an analysis: type of image acquisition (single images from a camera or loaded from a file etc.), template for input form and report, workflow for image processing and measurement etc. (only for administrators).
- Carrying out the measurement (for all users).

ASSAYbuilder

The ASSAYbuilder module is a so-called "high content analysis" module and efficiently generates high-quality and biologically relevant data from your image material.

ASSAYbuilder describes your fluorescence images in the form of numerous key parameters and provides objective, quantitative data that can be used as a basis for decisions relating to the planning of further experiments or to support published results. The module is operated using guided workflows and an intuitive graphical user interface. No programming knowledge is required.

Five analyst modules support you with solutions that have been specially tailored to different biological tasks: Physiology Analyst, Morphology Analyst, Membrane Analyst, CellCycle Analyst, Motility Analyst. Examples of supported applications include cell-based toxicity tests, transcription factor activation, cell-cycle analyses, cell-movement analyses and morphology determination.

Shuttle&Find

Shuttle&Find links the workflow between a light microscope and an electron microscope. With this module it is possible to correlate information acquired on a light microscope with that obtained on an electron microscope. Shuttle&Find is a hardware and software solution that allows you to analyze a sample on a light microscope, for example, and to save the position of the points you are observing. When you perform a subsequent analysis, e.g. on a scanning electron microscope, the previously saved points are automatically retrieved with a high degree of accuracy. This saves you from having to perform a time-consuming search for the region in question and enables you to correlate the different results. On the electron microscope detailed images of exactly the same point can be acquired with magnifications in the nanometer range and in this way the functional data can be supplemented and combined/overlaid with structural information. Further explanations can be found in section 11.15.

Correlative Particle Analysis (CAPA)

Correlative Particle Analysis is used to quickly relocate individual particles on an electron microscope which have previously been analyzed using a light microscope. The aim is to perform an automatic EDS (Energy-Dispersive X-ray Spectroscopy) analysis of selected particles. For this Correlative Particle Analysis refers back to the results data of the light microscopy analysis by means of Particle Analyzer Projects. These data are used as the basis for further materials analysis on the electron microscope. Further explanations can be found in section 11.16.

Archive Modules

Asset-Archive

With the Asset-Archive module you can save images, measurement data lists and reports etc. as "assets" in a hierarchically structured database. You have the option of creating various project types in which you can then save your assets. A contact management function allows you to allocate projects to different clients etc. You are also able to classify and structure your assets within your own customized categories. The option of a full-text search enables you to find individual data sets quickly. In addition, asset- or project-related filters allow you to carry out targeted searches for information in individual data fields.

Long-term archiving is also possible, and gives you the option of transferring your projects and the corresponding assets onto CD, DVD or magnetic tape.

Configuration Modules

Commander

The Commander module allows you to record steps for image acquisition, processing, and measurement in a single command – a script. Finally a script can be executed on any number of images automatically.

VBA

Should any users find that even the wide-ranging functions offered by AxioVision do not meet all of their requirements, they can increase the available options still further and adapt the program to their needs. This process is based on **V**isual **B**asic for **A**pplications. VBA offers a complete, integrated development environment with which programmers are familiar. As VBA has been directly integrated into AxioVision, it offers the advantage of fast interaction and the possibility of developing solutions without additional programs.

1.3 AxioVision and Microsoft® Windows

AxioVision fully supports the usual Microsoft Windows standards:

Files are stored in accordance with Microsoft Windows rules in the folders provided for the purpose ("My Documents", "Documents and Settings"), so it is easy to locate the files you need.

In multi-user environments, there is no need to make separate settings for AxioVision in order to keep the configurations and data of individual users separate and to protect them from unauthorized access. User administration is also based entirely on the settings in Microsoft Windows.

1.4 User's Guide and Online Help

The AxioVision manual and online help should not be regarded as separate entities. When used in combination, they offer you immediate access to comprehensive information. The User's Guide contains a broad overview of all program modules, with detailed step-by-step instructions covering all the basic functions and describing the activities that arise most frequently.

At the beginning of each chapter you will find information describing the background to the relevant section of the program or the relevant module in more detail. This may be conceptional information or a description of theoretical details. The introduction also contains a step-by-step description of operation. Due to the extremely wide range of functions offered by

AxioVision, it is not possible to describe every conceivable operational situation in detail. However, once you have worked through the steps described in the manual, you should be able to apply these procedures to your specific task.

The Online Help provides full details of the parameters and setting options of a function. It is therefore the ideal complement to the manual. If you have a question about a specific parameter, press the **F1** key to obtain further information on it. The online help does not, however, contain descriptions of procedures of the type found in the manual.

We strongly recommend that you familiarize yourself with the program using the examples described in the manual before you start working on concrete projects with AxioVision. Once you are familiar with the general operating procedures, you will find all the information you require in the online help.

1.5 Backup Copies

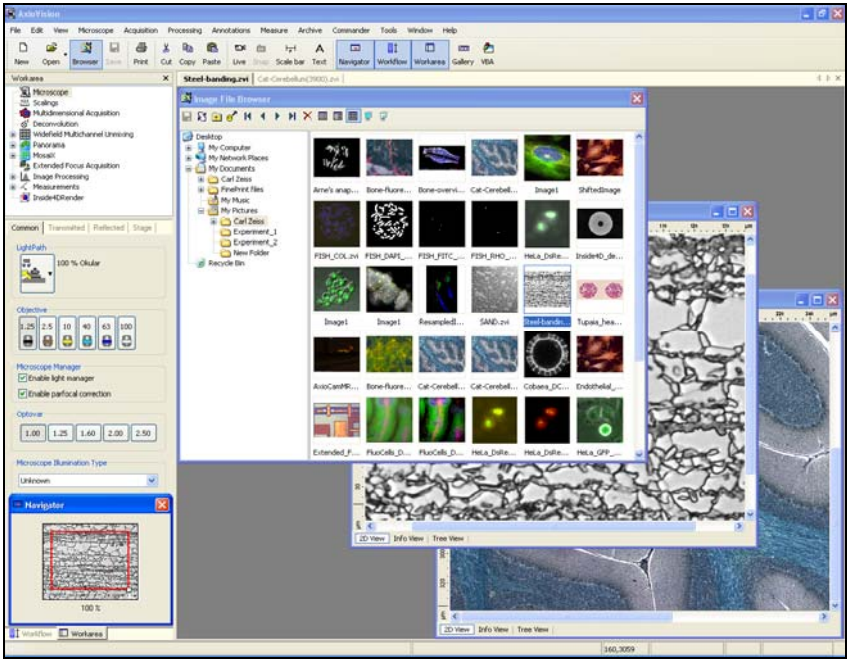
We strongly recommend that all users save the data they create, such as images, measurement data, archives, reports, forms and documents, at regular intervals on an external medium. Otherwise it cannot be excluded that access to this data may be lost as a result of operational errors or hardware defects. Carl Zeiss and Carl Zeiss Vision accept no liability for consequential damage resulting from insufficient data protection.

2 Overview and Operating

2.1 Overview

AxioVision's main window is divided into two main areas: the **Workarea** and **Workflow** on the left-hand side, and the document area on the right-hand side. The elements of the **Workarea** and the **Workflow** are used to select and operate image processing functions and functions for controlling the camera and microscope.

In the document area, all document types (images, archives, reports, forms) are displayed and edited. The main window also contains the Microsoft Windows specific control elements, such as the main menu, toolbar and status bar.



AxioVision's main window

2.2 Workarea

The elements within the workarea (see figure below) can be used to

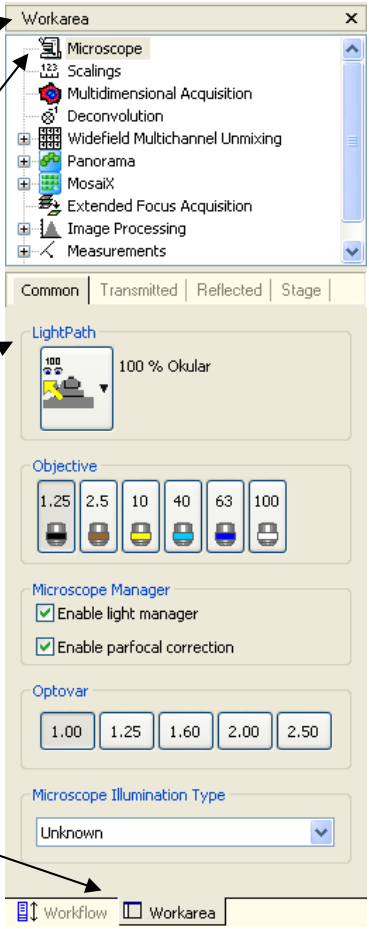
- select processing functions on the work tab.
- set parameters for processing functions on the property pages.

By dragging the title bar you can separate the **Workarea** and the Workflow, or move one on top of the other.

Activated **Microscope** property page

Setting of functional parameters.

Tabs for switching between **Workflow** and **Workarea**.



Notes:

- All of the workarea's functions can also be found in AxioVision's main menu.

- The workarea and workflows are displayed one on top of the other by default. To switch between them, use the tabs at the bottom of the workarea.

- If you have hidden the workflows, when displayed again they are shown next to the workarea. You can, however, move them back on top of the workarea by dragging the title bar. This gives you more room to display documents.

- You can show and hide the workarea using two different methods: either via the menu commands **View** ⇒ **Windows** ⇒ **Workarea** or via the button on the **Standard** toolbar 

.

- If the **Standard** toolbar is hidden, select the menu commands **View** ⇒ **Windows** ⇒ **Standard**.

2.3 Workflow

One very simple way to operate AxioVision is to use workflows for routine tasks that need to be performed again and again. All you have to do is click on the functions in the workflow one after the other. You will then achieve the desired results quickly and smoothly.

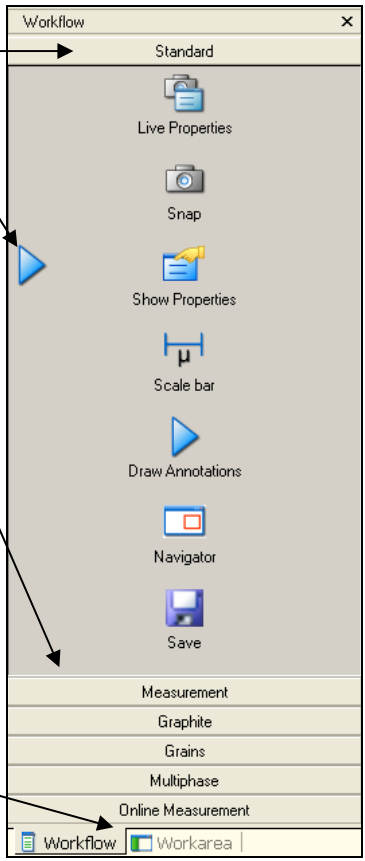
If you also hide all the elements that are not required, such as the workarea and toolbars, you can concentrate fully on the work you are actually performing.

Activated **Standard** workflow.

The last function executed is indicated with an arrow.

To activate other workflows, click on them. They then scroll upwards.

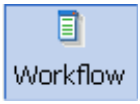
Tabs for switching between the **Workarea** and **Workflow**.



Notes:

- All the functions available in AxioVision can be used in a workflow (see also chapter 8 "Configuration").
- The workarea and workflows are displayed one on top of the other by default. To switch between them, use the tabs at the bottom of the workarea.
- If you have hidden the workflows, when displayed again they are shown next to the workarea. You can, however, move them back on top of the

workarea by dragging the title bar. This gives you more room to display documents.

- You can show and hide the workarea using two different methods: either via the menu commands **View** ⇒ **Windows** ⇒ **Workflow** or via the button on the Standard toolbar: 

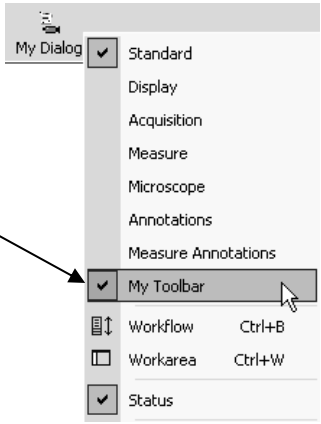
.

- If the Standard toolbar is hidden, select the menu commands **View** ⇒ **Windows** ⇒ **Standard**

2.4 Own Toolbars and Dialogs

You have the option of generating your own toolbars and dialogs to create a system that is tailored entirely to your own needs. If the range of setting options in the dialogs for camera and microscope control is too detailed for the work you are performing, you can therefore (with just a few clicks of the mouse) generate your own dialog containing only those control elements that you require for your work. This dialog can then be placed on a self-generated toolbar, and all the other default elements can be hidden. You then have a unique system that is optimized to meet your own individual requirements.

You can call up the **My Dialog** function here via **My Toolbar**.

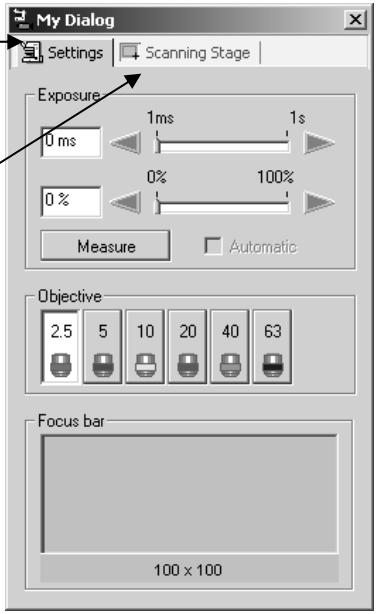


Notes:

- You can generate as many personal toolbars as you want.
- Your own dialogs can also be used on default toolbars.

The **My Dialog** dialog contains options for **Setting** the exposure time and changing the objectives, and for the focus bar.

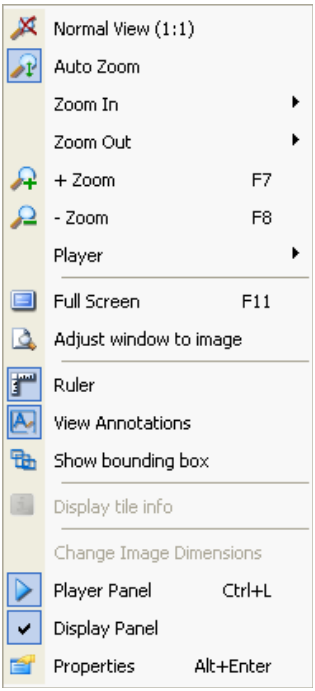
Functions for controlling the scanning stage can be found on a second tab sheet: **Scanning Stage**.



2.5 Shortcut Menus

Shortcut menus contain functions that are frequently required in connection with documents (e.g. images) and certain elements of the AxioVision interface. They are the easiest way to select these functions.

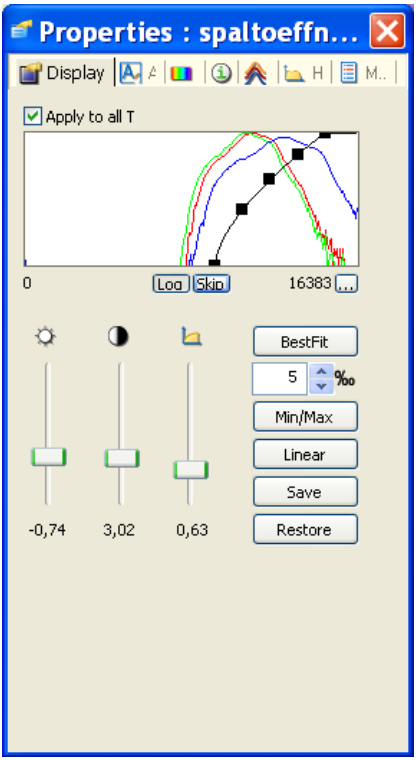
Shortcut menus are available for every element of the user interface. They can be activated at any time by right-clicking on an element (see *shortcut menu for an image* below):



2.6 Properties Window

The **Properties** window is required whenever you want to look over or to change the appearance, the behavior or certain additional information relating to a document (e.g. an image) or a particular element of the AxioVision interface. In this window, behind the tab sheets (**Display**, **Attributes, Colors**, ...), you will find a list of the relevant information (e.g. at an image) and/or will be given the option of entering or modifying certain elements.

This may be information on the date of saving and the location where an image file is to be saved, for example, but may also relate to the formatting (**Attributes**) of text, lines and other objects you have drawn into the image (**Annotations**). See *Property window of an image* below:



As the property window plays a central role in the operation of AxioVision, it can be opened in a number of different ways:

- Via the **Properties** function in the shortcut menu (see previous section).
- Via the **Properties** function in the **View** menu.
- By pressing the key combination **Alt**+**Enter**.

3 Image Acquisition

3.1 General

In order to process images using a computer, you must first acquire them in a form which the PC can understand. This requires an electronic camera, which acquires the image information from the microscope and converts it into electrical signals. These signals are digitized and then processed and displayed on the computer.

Compared to analog camera technology, modern digital camera technology offers advantages in terms of the signal quality and camera resolution that can be achieved. With this technology, the data are digitized directly in the camera.

To transfer the data to the PC, you need to use frame grabbers, interface boards or standardized digital interfaces. These are operated using special software programs (known as drivers). With these device drivers integrated into AxioVision it is now possible to acquire images via them.

When installing framegrabbers, interface boards and interfaces, use the corresponding drivers supplied by Carl Zeiss and the associated manuals/the manuals from the respective manufacturers.

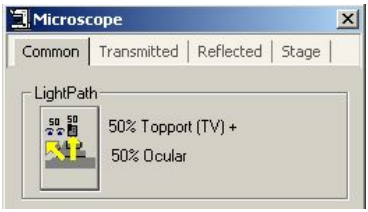
The description below assumes that the frame grabber or interface board and the corresponding driver have been successfully installed.

Detailed information on the cameras/framegrabbers supported by AxioVision can be found in the current AxioVision online help, in the AxioCam HR, MR and MRc5 installation and reference manuals, and in the PDF files of the manuals, which can be found on the product DVD.

3.2 First Steps

When taking your first steps with the system, if possible use a simple sample so that it will be relatively easy for you to determine whether the resulting image is good or bad.

Place the sample under the microscope, and set the microscope in such a way that you can see a good image through the eyepiece. Switch the microscope's beam path to the TV adapter, e.g. **50% Topport** and **50% Ocular**.



Notes:

- If you are not yet familiar with your camera's functions, make sure that you have the respective manuals to hand so that you can look up any relevant information.

- This text describes the main procedure for image acquisition. For explanations of the functions and setting options of all the cameras supported by AxioVision, please refer to the manual for the camera concerned. The description for the Carl Zeiss AxioCam product family serves as an example for all supported cameras.

3.3 Conditions

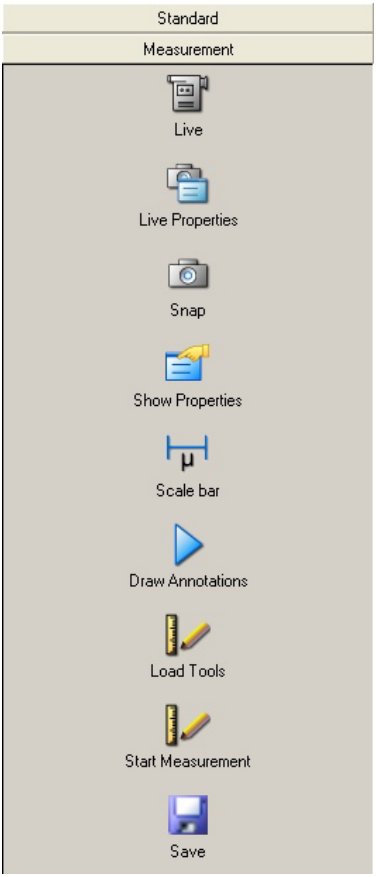
The text below describes how to achieve your first image in AxioVision with just a few clicks of the mouse (here with the AxioCam HR). The **Standard** workflow is the easiest way to control image acquisition. If the workflows are not displayed, in the **View** menu select the **Windows** function, and there select the **Workflow** command.

Note:

- The workarea may in fact be open but covered by the workarea. If that is the case, simply click on the **Workflow** tab at the bottom edge of the workarea 

.

The Standard workflow



Live starts and closes the live window.

Live Properties opens and closes the window for adjusting the display characteristic curve and for controlling the camera (exposure time, white balance etc.).

Snap acquires an image using the active camera.

Show Properties adapts the image display using a characteristic curve and shows the parameters of the image.

Scale bar inserts a scale bar.

Draw Annotations inserts a caption and scale bar into the acquired image.

Navigator opens the navigator window for zooming.

Save saves the image.

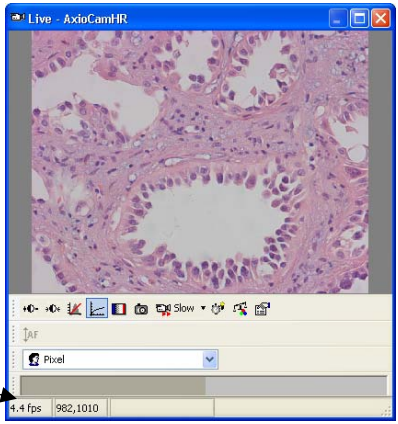
As the icons are identical on all menus, the following description applies both to operation via the toolbar and to the workflow.

Step by step to the first image

- Select the camera you want to use (if only one camera is installed, this step is not necessary).
- Set the light path to the camera.
  Then click on the **live image** icon.
  The live window is opened to display the camera image.



- This function can also be called up via the **Acquisition** ⇒ **Live** menu.
- Now focus the camera image and select the frame you want to acquire.
- In the footer you can select a suitable scaling for the objective you are using from the **List of available scalings.**



- Clicking on this icon overexposed (too bright) areas will be displayed in red in the live image.



- Clicking on the gamma icon in the bottom line of the live image accomplishes the optimum color reproduction.



- In this drop-down list box you can select the speed of the live image (**Slow, Middle, Fast**).

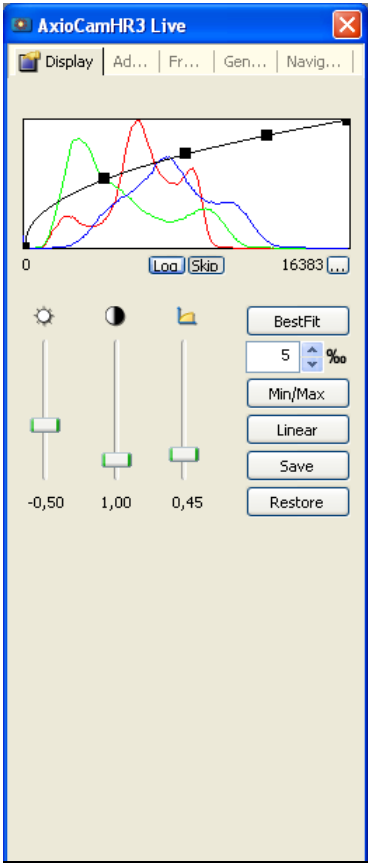


➢ Click on the icon **Live Properties** to open the live properties.

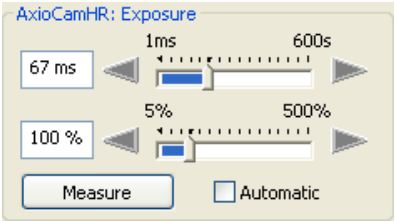


Here you can see the settings for the display of the image like Brightness, Contrast and Gamma. Settings made here are passed on the acquired image.

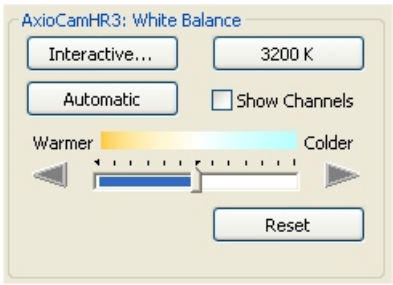
Note: Only the display is adapted, the camera data remain unchanged.



Make changes for the camera settings on the following controls:

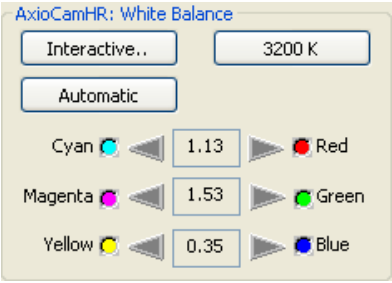


- The central element is the setting for the exposure time on a digital camera. The **Measure** button can be used to determine an optimum exposure time. Check the quality of the exposure in the live image.
- The arrow keys and sliders can be used for fine adjustment of the exposure time.
- Exposure time can be edited via the keyboard.
- **Auto Snap** performs an exposure measurement before the acquisition of each image. **Auto Live** adjusts the exposure time continuously in the live image.

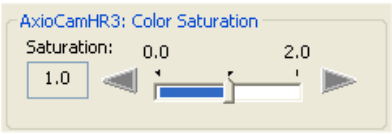


- If you are using a color camera, you need to perform a white balance for the image. If you select the automatic balance (**Automatic** button), the camera tries to determine an optimum value itself.
- Using the **Warmer/Colder** slider you can shift the target value of the white balance to warmer (→ redder) or colder (→ bluer) tones.
- The **Show Channels** check box allows you to switch to an alternative setting option if you wish to influence the target value of the white balance manually for each channel.

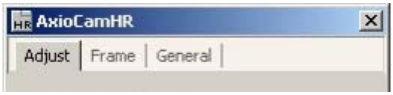
- If you are using a color camera, you need to perform a white balance for the image. If you select the automatic balance (**Automatic** button), the camera tries to determine an optimum value itself.



- Move the slider to increase or decrease the color intensity of the acquired images.



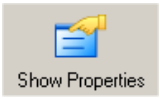
- Enter further settings for the selected camera on the **Adjust**, **Frame** and **General** property pages.



- Click on the camera icon to acquire an individual image, or in the **Acquisition** menu click on **Snap**.

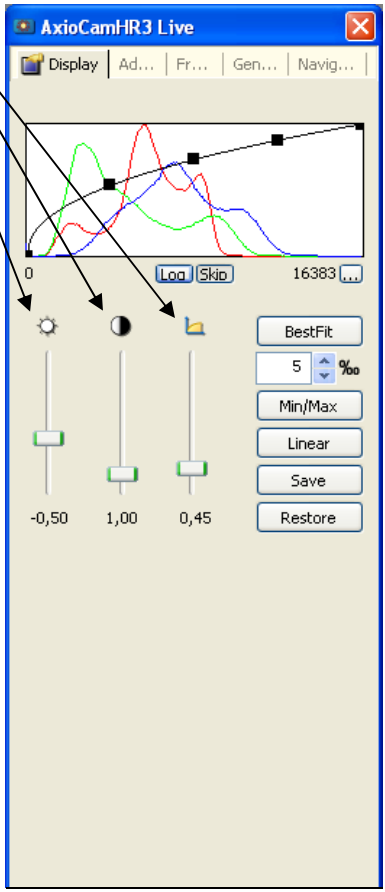


- Click on the **Show Properties** icon and activate the **Display** tab. Any changes you make here are displayed in real time in the image window.



- Move the sliders

Gamma
Contrast
Brightness



up or down to adjust the image. Good color reproduction on a monitor is achieved with a gamma value of around 0.45.

- To find the optimum setting for contrast and brightness, click on **Min/Max**.

- To undo all your changes, click on **Linear**.

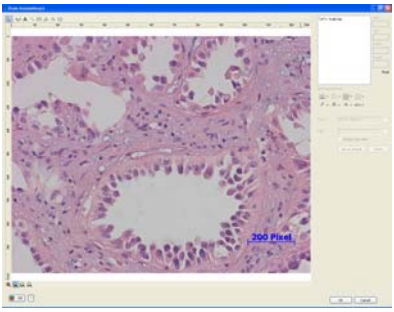
- You can use this icon to insert a scale bar into your image. The appropriate scaling must have been generated and selected in advance.



- This icon is used to open the window for generating annotations.



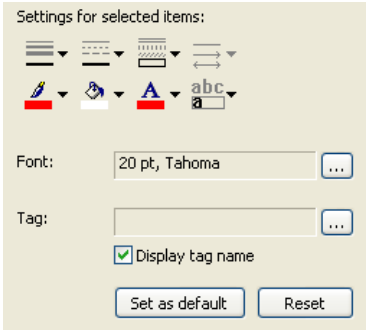
- Lettering and marking can be inserted in the **Draw Annotation(s)** window.



- In the dialog window **Draw Annotation(s)** the icons **scalebar**, **text**, **line**, **rectangle**, **outline**, **curve** und **ellipse** are available in the menu line.



- In the dialog window **Draw Annotation(s)** a field for **Settings for selected items** as well as for **Font** are available. If **Display tag name** is activated, you can use the ... icon in the drop-down list box **Tag**. The dialog window **Select tag** opens where parameters for lettering can be selected.

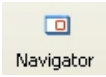


- In the dialog window **Draw Annotation(s)** zoom symbols and two buttons to toggle the color channels are available at the bottom of the image window.





- Using the navigator you can magnify or reduce the image and position the displayed image area in the overview window.



➢ Click on this icon to save the image you have generated to the computer's hard drive.



Notes:

- Once you have found a good camera setting, you can save it with the **Settings Editor** in the **Tool** menu and load it again automatically in the future. This allows you, for example, to acquire particular samples under the same conditions every time.

- To allow correct scalings to be selected in the footer of the live image during acquisition, you need to have generated these before you start your work. If you are using a motorized or encoded microscope, the scalings can be allocated automatically. For further information see Chapt. 9 "Configuration".

3.4 Practical Notes on Operation in AxioVision

Below you will find a few practical notes on the speed of the live image, color adjustment, working with exposure times, fluorescence images and configurable toolbars.

Speed of the live image

There are three speed modes available for the AxioCam HR live image. If you use the pointer to click on the 

icon at the bottom edge of the live image, a pop-up menu with the possible setting options appears. The current setting is displayed at the bottom edge of the live image.

Each mode has its own particular compromises.

Live mode	Description
Fast	Greatly reduced resolution, fast frame rate
Medium	Medium resolution, moderate frame rate
Slow	High resolution, slow frame rate

The image rate can be further increased by deactivating certain AxioVision features:

- Deactivating the focus bar.
- Deactivating the live image scaling (1:1 representation of the image).
- Activating a frame for the live image (**Size**) on the camera property page **Frame**.
- Set image orientation to original on the **General** property page (no rotation).

Notes:

- Note that the above mentioned notes are only valid for short exposure times, as for very long exposure times the speed of the acquisition is mainly dominated by the exposure time.

- For very long exposure times, the live image will appear grainy, as automatic amplification is active. However, this will not influence the quality of the final image.

Notes on Optimum Color Reproduction in AxioVision

Below you will find a detailed description of how best to set up AxioVision to achieve optimum color reproduction with the AxioCam HR.

Note:

- For an optimum color reproduction the monitor first has to be adjusted. To do so please use a well-suited image.

Adjusting the display characteristic curve in AxioVision:

In order to achieve optimum reproduction of color images of the AxioCam HRc data on a monitor, the display characteristic curve must be used to set gamma compensation. The advantage here is that only the display is influenced and the actual data remain unchanged. Further details on this can be found below. The settings can be done as follows:

➢ With the mouse click on the icon 

in the footer of the live image. This adjusts the display characteristic curve automatically for an optimum color reproduction of the monitor (gamma = 0.45).

Or in an already acquired image:

➢ In the **Properties** dialog window, set the gamma characteristic curve in the histogram image on the **Display** property page.

Notes:

- This also influences the reproduction of the color saturation.
- For fluorescence images, background correction can also be carried out simply by shifting the offset of the characteristic curve.
- Clicking on this icon 

returns to a linear display of the data.

3.5 HDR - High Dynamic Range

General

The HDR module is available in AxioVision to allow you to test out its function. This means that you can now try out this acquisition technique at your leisure and examine the positive effect it has for your specific application. Following image acquisition, a watermark is burnt into your HDR image.

Once you have licensed this module, it is available to you without any restrictions.

HDR imaging offers you the possibility of increasing the camera-specific dynamic range. If you want to capture all the information from objects that are extremely rich in contrast, it is usually necessary to acquire several images, which need to be adapted to the relevant exposure situation. Either the very bright or the dark structures can be correctly exposed. With the HDR module it is possible to document all the information in a single image.

To achieve this effect, depending on the parameters set, several images are automatically acquired with different exposure times and subsequently merged into a single image with an increased contrast range.

To achieve the best possible result with your image, it is crucial to set the exposure time precisely. No position on the specimen may appear overexposed. This is the only way to guarantee that the information acquired in the brightest structures is differentiated and contributes to the HDR image. In this case a simple measurement to determine the correct exposure time prior to acquisition is not suitable, as this involves performing average metering that is subject to tolerances. Instead, the overexposure warning should be activated and the display characteristics in the non-logarithmized display should be adjusted in such a way that there genuinely is no overexposure of any pixel.

The advantages of the technique depend to a great extent on the specimen and on the microscopy technique being used. A sample in transmitted-light bright field generally has only a relatively flat dynamic range. This means that the effect you notice from the use of the HDR method will not be an increase in contrast, but merely a reduction in image noise. Images of specimens

acquired using other contrast techniques, such as dark field, reflected light or fluorescence, exhibit a stronger increase in contrast, depending on the structure in question, as they often have a higher dynamic range than that of the cameras used.

In many cases, it is not possible at first sight to see a clear improvement in the acquired HDR image compared to a normal image. This is due to the limited display depth of monitors, which usually have a contrast range of 8 bits. As no further tone mapping is performed in AxioVision to map the increased dynamic range to a monitor, an adjustment can only be made with the help of the gamma setting (non-linear bending of the display curve).

The additional information gained from expanding the dynamic range is most noticeable when performing measurements. Thanks to the more differentiated display, more brightness gradations are available to the user, which means that as well as a distinction in bright structures there is also nuancing in dark areas of the image. This allows segmentations to be performed more precisely and measurement results to be refined.

Note:

- For detailed information on the individual functions and parameters, please simply refer to the online help (F1 key).

HDR imaging

In the following sections you will see that acquiring an HDR image using an AxioCam is extremely simple. By bearing a number of fundamental aspects in mind, you will quickly achieve good results.

If you are using a camera from another manufacturer, it may be necessary to change parameters in **HDR Setup**, even for simple HDR imaging. This will be mentioned at the relevant points and the procedure will be explained in more detail under the final point “Basic settings in HDR Setup”.

HDR Mode

The quickest way to obtain an HDR image using an AxioCam is to use **HDR Mode**.

This mode is independent of the other image acquisition modules and can be activated individually for all acquisition functions.

Using the HDR button 

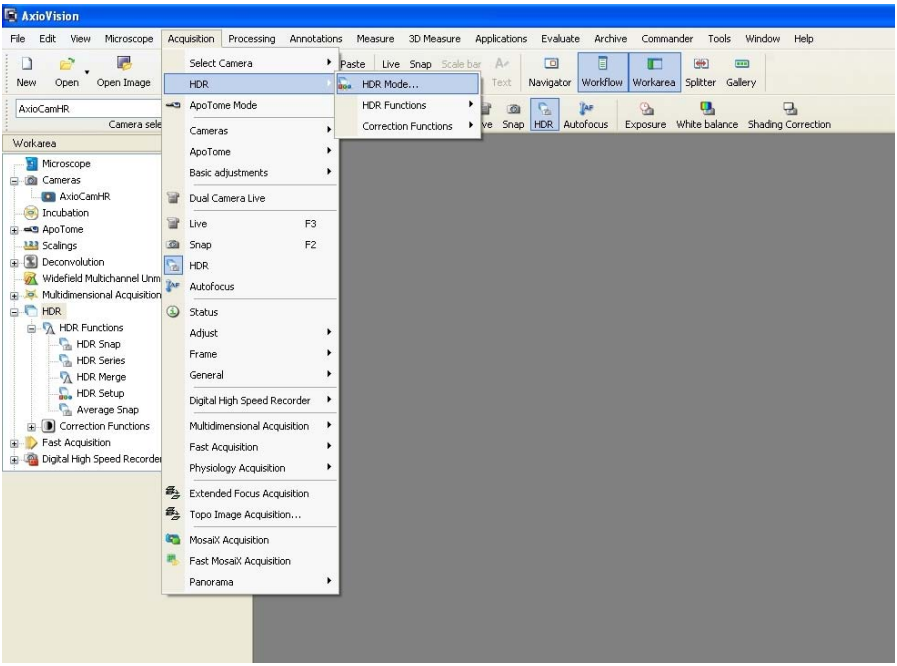
on the **Acquisition** menu you can activate and deactivate the HDR Mode very simply.

This button can make your work with the HDR module much easier as it also informs you of the current status of the function. When the HDR Mode is active, the HDR button is highlighted in blue. If, on the other hand, the module has been deactivated, the button is no longer highlighted.

Tip:

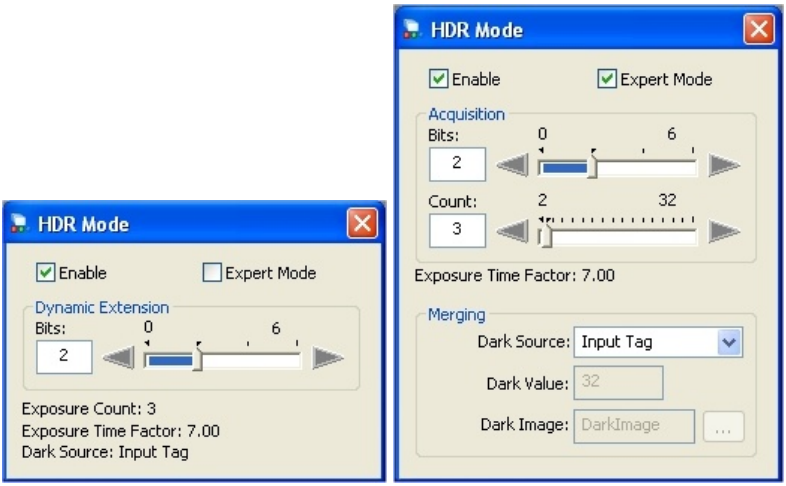
- The button for the HDR Mode can be found on the **Acquisition** toolbar. To allow you to maintain a constant overview of the HDR function and, consequently, avoid any undesired extension of the acquisition time, you can place the button on your own predefined toolbar using the **My AxioVision** function (**Tools** menu ⇒ **My AxioVision**).

You will find this function under the following menu item: **Acquisition** ⇒ **HDR** ⇒ **HDR Mode**.



If you activate HDR Mode, all subsequent images will be generated with an increased dynamic range.

HDR Mode can be operated in two different settings. On the one hand, there is Simple Mode, in which only the dynamic extension can be set. In this case the other information required is completed automatically. Beneath the slider you will find the information on the number of images used, the source for merging the dark value and the extension factor. This is therefore a good way to get to know HDR Mode without having to set a large number of parameters. Expert Mode, on the other hand, contains all the setting options required to individually adapt the HDR image.



Simple Mode **Expert Mode**

The acquisition parameters should be selected carefully, especially in Expert Mode, as these define the duration of the exposure and therefore the burden placed on the sample. To give you an idea of the exposure time to be expected, a factor for the extension of the overall acquisition time is shown underneath the acquisition parameters.

As a rule of thumb for the correct setting of HDR Mode, the following should apply: a dynamic extension of 2 bits should result in the exposure count increasing by 2. This guarantees good information density in the HDR image.

The predefined settings with an extension by 2 bits using 3 images and the source “Input Tag” for merging the dark value are a good starting point for working with an AxioCam.

Note:

- If you are using a camera from another manufacturer, it cannot be guaranteed that an “input tag" for merging the dark value will be transferred to AxioVision and consequently that the result will be free of errors. If the source "Input Tag" is not available for the calculation, the dark value set is used instead (see also the section "Basic settings in HDR Setup").

Simplest way to generate an HDR image

Following this brief explanation of the different versions of HDR Mode, we will now return to a description of the quick acquisition of an HDR image.

Activate HDR Mode in the simple display and set the slider to a dynamic extension of 2 bits. If you are using HDR Mode for the first time, the following data will be completed automatically and displayed in the information rows underneath the slider: an exposure count of 3 images, an exposure time extension by a factor of 7 and the dark source "Input Tag". If you have already used HDR Mode and have changed the input parameters, these values will be displayed and applied.

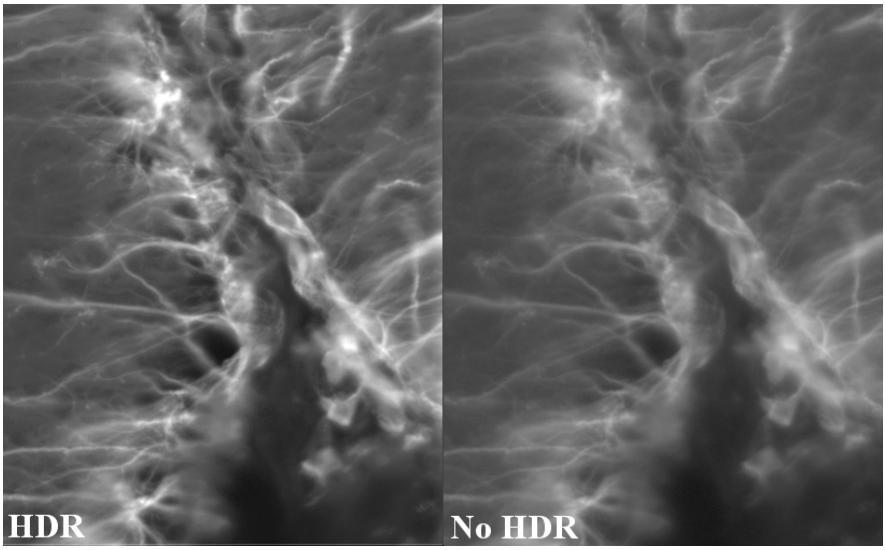
Please therefore always pay attention to the settings to ensure that your HDR image is acquired as you want it to be.

Now you need an object that is rich in contrast and must determine the correct exposure time. To do this, activate the overexposure warning. This makes it easy to determine the right acquisition time. Adjust the exposure time in such a way that no overexposed pixels are displayed. The more precisely you do this, the more gradations you will be able to differentiate later in the bright structures of the HDR image.

Once the correct exposure time has been determined, you now only need to click on 

or the F2 key.

Everything else takes place automatically and the output image will already show the HDR image with a dynamic range that has been increased by 2 bits.



An individually adapted HDR image step by step

Another way to obtain an HDR image is to make use of the individual functions.

These can be opened either via the **Acquisition** menu or in the work area under **HDR** ⇒ **HDR Functions**.

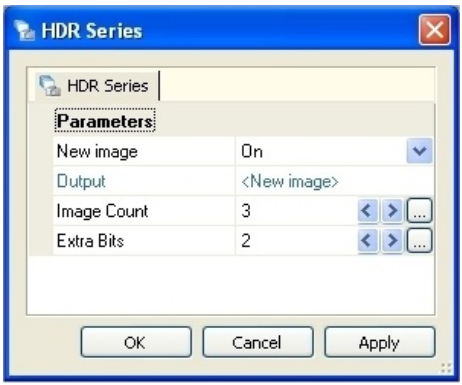
The individual functions allow you to enter individual settings for an HDR image and adapt it to the properties of the various cameras. Using the individual functions is also an excellent way to familiarize yourself with the operation of the program and the various merging and setting options.

A good HDR image can be improved even further by means of a calculation using an adapted dark value. This means that an individual black-reference image is subtracted for each exposure time. Only a few additional operations are necessary to do this. Black references are normally created before the actual acquisition of the image. In this case, due to the individually adapted exposure times, it is easier and less time-consuming to acquire the black reference after actual image acquisition. One condition for achieving a flawless result with your image is to ensure that the beam path is absolutely

free of stray light. You should therefore always make sure that the camera sensor is not receiving any light at all.

For the HDR image you must first adjust the specimen and define the appropriate exposure time. As with the use of HDR Mode, make sure that there are no overexposed areas in the image (activate overexposure warning). This is the only way to guarantee that you will gain the maximum information.

- Open the **HDR Series** function and set the **Image Count** and **Extra Bits** parameters for the increase in the dynamic range.



With these two details you have defined all the important data for acquisition itself.

- Once everything has been set to your satisfaction, click on the Apply button.

HDR Series generates an image series consisting of images with different exposure times. The various component images are displayed in a multichannel image. This means that you can view all the exposure times separately from each other, analyze them and save them for other uses.

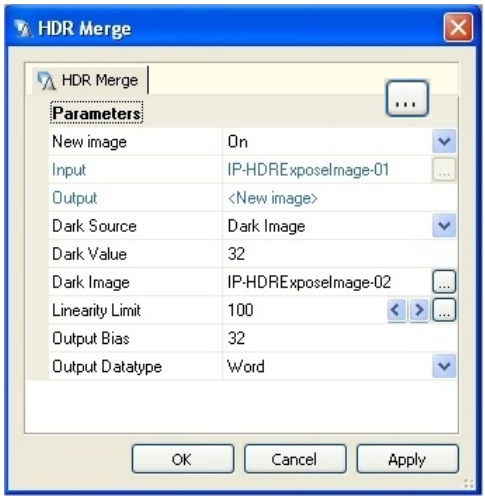
To generate the black reference you only need to close the light path to the camera and acquire a black-reference image using the same settings as before for the HDR image.

Note:

- The display of the series initially shows you an overlay of all images acquired. Click on the On icon to view the individual images.

To obtain a finished HDR image, all that you now need to do is merge the individual components. To do this, select the **HDR Merge** function.

- To merge the black-reference series previously acquired, set the parameter **Dark Image** as the dark source.
- Select the image by clicking on ... .
- Lastly, activate the previously acquired HDR image. To do this, all you have to do is click once on the image's Acquisition tab.



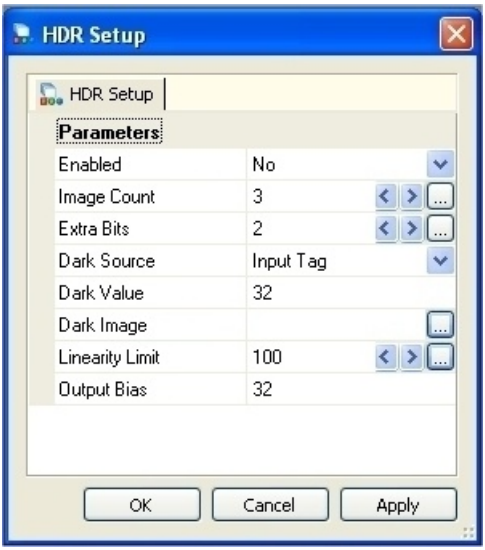
No other changes to the parameters are required if you are using an AxioCam camera. If you are operating an AxioCam ICc 1 or ICc 3 in 8-bit mode, the output bias should be reduced to the value 3 (more detailed explanations can be found in the following section or in the online help).

Apply starts the merging process. The HDR image is now finished.

Basic settings in HDR Setup

The settings in **HDR Setup** determine all the parameters that cannot be changed individually in the various HDR functions. This function is therefore of central importance.

HDR Setup can either be opened via the **Acquisition** menu or in the work area under **HDR** ⇒ **HDR Functions** ⇒ **HDR Setup**.



The default values in **HDR Setup** have been set in such a way that they can be adopted without any further changes for an AxioCam.

It may be necessary to change the dark value for the merging of the HDR image if you are using a camera from another manufacturer. To determine the actual dark value, it is helpful to take the value of the darkest areas of a simple image as a reference.

The second setting that you should change, if necessary, if you are using an AxioCam ICc 1 or AxioCam ICc 3 in 8-bit mode or a camera from another manufacturer is the **Output Bias** parameter. This determines the brightness value of the darkest pixel in the output image and should be set to the value 3 in the case of the AxioCam ICc.

4 Microscope Control

4.1 General

AxioVision has been specially designed to support motor-driven microscopes from Carl Zeiss.

The introduction of the new microscope types from the Axio Imager, Axio Observer and SteREO series means that the established Axioplan, Axiovert, Axioskop and Stemi systems are being replaced. These systems will no longer be developed or produced, but will naturally still be supported by AxioVision.

As the Axio Imager, Axio Observer and SteREO microscopes open up completely new opportunities for computer-aided microscopy, new requirements are also necessary with regard to the configuration of the systems. Therefore, a distinction is made in AxioVision between the established systems Axioplan, Axiovert, Axioskop and Stemi and the new Axio Imager, Axio Observer and SteREO systems. Various configuration programs are available for both generations of instruments. However, there is only a difference as far as operation in AxioVision is concerned where functional differences actually exist. Therefore, if you change your system, you will be immediately familiar with the new microscope without having to readapt.

The microscope is either controlled via a property page containing all configured components or via control elements for individual components.

The property page for controlling the microscopes can be activated up by clicking the **Microscope** entry in the work area or by selecting from the **Microscope** menu the function **Microscope**.

Alternatively you can select single controls from the five functions **General**, **Transmitted Light**, **Reflected Light**, **Stage/Focus** and **Extra**.

A more detailed description of the controls can be found in the Online help.

4.2 Configurating microscopes with Microscope Configuration

At the time of AxioVision's release the following Carl Zeiss microscopes are supported:

- Axioplan 2
- Axioplan 2 imaging
- Axioplan 2 imaging e
- Axioplan 2 imaging e man
- Axiovert 200 man
- Axioskop 2 mot plus
- Axioskop 2 mot

Your microscope type and its individual facilities are selected in the separate program **Microscope Configuration**. This configuration program can be found on the Microsoft Windows Desktop.



Here you can define your microscope's type and components.

4.3 Configurating microscopes with MTB2004 Configuration

The following microscopes are currently supported:

- Axiovert 100 M
- Axiovert 200 M
- Axiovert 200 M MAT
- Axiovert 200 MAT
- Axio Imager (A1, D1, M1, Z1)
- Axio Observer (A1, D1, Z1)
- Axio Observer MAT (A1m, D1m, Z1m)
- SteREO Discovery (V8, V12, V20)
- SteREO Discovery (V8, V12, V20) o.EMS
- SteREO Lumar.V12
- SteREO Lumar.V12 o.EMS

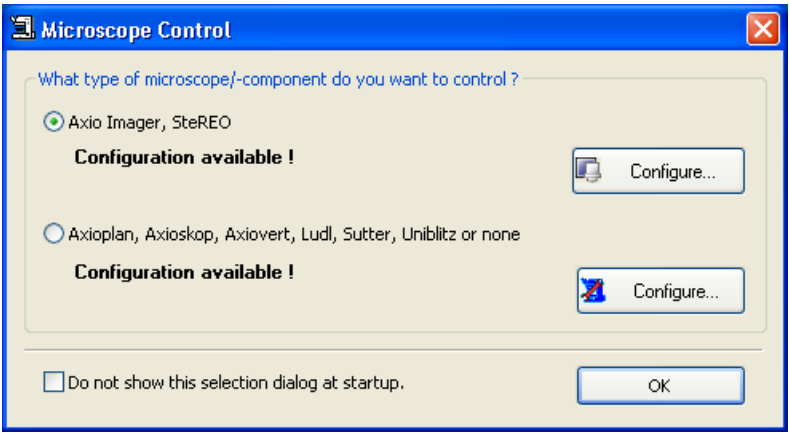
The microscope type and the individual equipment you are using on your microscope are selected in the separate **MTB2004 Configuration** program. his configuration program is located on the Microsoft Windows Desktop.



4.4 Activating microscope configurations

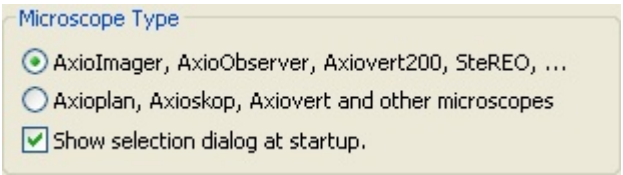
In order to activate a new microscope configuration, you must first exit AxioVision. You can then start the relevant configuration program (**Microscope Configuration** or **MTB2004 Configuration**) to activate the appropriate configuration or to make the desired changes to the current configuration.

A new feature of AxioVision release 4.3 is the opportunity to change the configuration directly at the start. The following dialog is displayed when AxioVision is started:



Here you can choose the microscope type and then start the corresponding configuration program via **Configuration**.

If this dialog is not displayed, you should check the settings in the **Tools** ⇒ **Options** ⇒ **General** function:



Here, you can specify your type of microscope and activate the **Microscope Selection** dialog by activating the **Show selection dialog at startup** check box.

In addition, for the new Axio Imager, Axio Observer and SteREO microscopes only, it is possible to change the configuration of the microscope when AxioVision is running:



The **MTB2004 Configuration** program can be started via the **Microscope ⇒ Configure microscope** function. You can then make changes to the current microscope configuration. These changes are adopted immediately.

The possibility of changing a configuration is limited, however:

- Changes to the current configuration are limited to the objectives and the reflector. You will need to restart AxioVision to make any further changes.
- Only the current configuration can be changed. You will also need to restart AxioVision in order to activate a different configuration or to generate a completely new configuration.

4.5 The Settings Editor

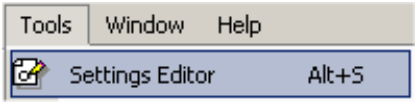
Under the **Tools** menu you can open the **Settings Editor** dialog (keyboard command **Alt+S**). In this dialog you can generate, save and retrieve settings for your current hardware, i.e. for your microscope, camera or other external components such as filter wheels or shutters. You can also apply these settings to the hardware.

These settings are intended to make it easier for you to operate your microscope. They are also used for microscope control when you are generating complex experiments, e.g. with the Multichannel Fluorescence or Z-Stack modules. The settings are saved as a document (*.zvhs).

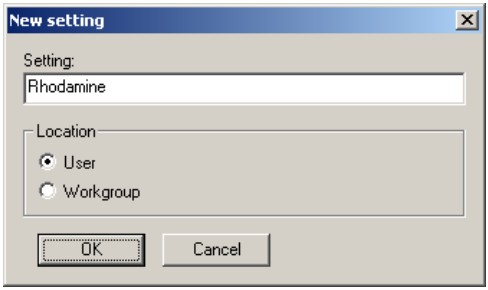
How Do I Generate a Hardware Setting?

The text below explains step by step how to generate and modify a hardware setting document and how to apply it to the microscope. In this example, the reflector nosepiece is to be switched to the Rhodamine position, and the integrated fluorescence shutter is to be opened so that light falls on the sample. The setting is to be saved as a document with the name "Rhodamine.zvhs".

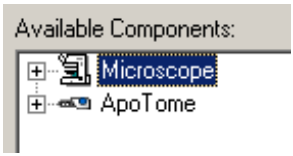
- In the **Tools** menu, open the **Settings Editor**. It can also be opened using the key combination **Alt+S**.



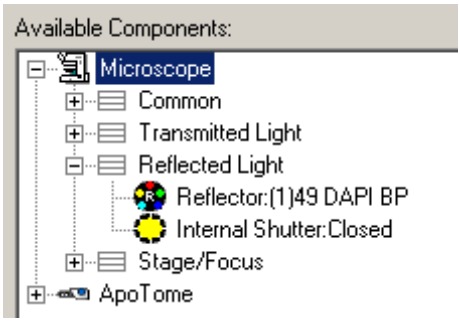
- In the window that is now open, click on **New**, enter a suitable name, such as "Rhodamine", in the dialog window, and click on **OK**. You can decide, whether you only (**User**) or other persons (**Workgroup**) can use the settings file



- Now add hardware settings to the empty document. To do so, select from the list **Available Components**.

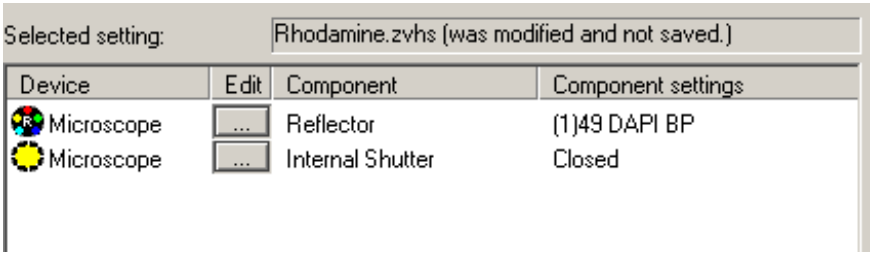


- Click on ⊞ to open the microscope branch, and then open the branch for the **Reflected Light** component group. The tree should now look like this:



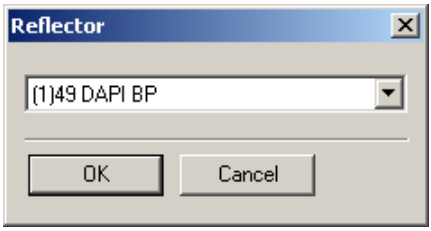
- Select the **Reflector** component, and add it to the Rhodamine setting by clicking on < Add. Repeat this step for the **Internal Shutter**. You can also add this component by using Drag&Drop.

You will now see these two components listed in the setting window:

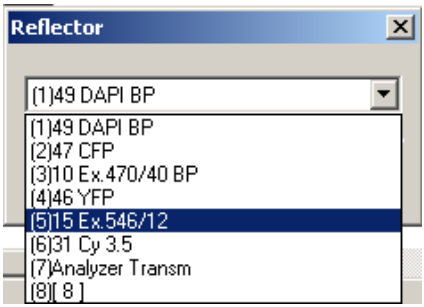


Both components contain a certain setting. In our example, the component **Reflector** is set to the filter set "[1]49 DAPI BP", and the **Internal Shutter** is **Closed**. These parameters correspond to the current setting status on your microscope. What you see may therefore differ from the examples shown here.

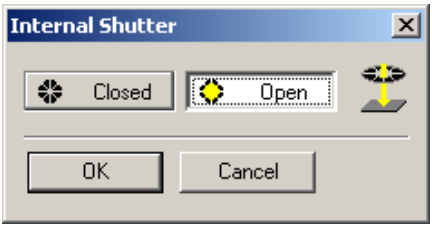
- Select the component **Reflector** and click on the ... button. This selection dialog is opened:

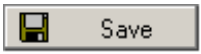


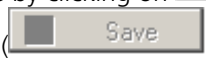
- From the drop-down list box, select the reflector position for Rhodamine (in this case it is 15 Ex.546/12 at position 3), and confirm with **OK**.



- Now select the component **Internal Shutter**, click on the ... button, and then click in the dialog on **Open**. Confirm your selection with **OK**.



- Save your settings by clicking on 

. This button then becomes inactive (

).

- Test your new hardware setting by clicking on 

. The reflector block should now swing to position 3, and the shutter for the fluorescence lamp should open.

The hardware setting "Rhodamine.zvhs" has now been saved and can be assigned to a function key (for more information, please refer to chapter 8 "Configuration" - " Define key combinations for functions") or used in an experiment as part of the acquisition of a multichannel image, for example.

4.6 Definite Focus

Overview

The Definite Focus functionality in AxioVision enables control of Definite Focus, an LED-based focus stabilizer. Definite Focus can e.g. be used to compensate changes in the distance between microscope objective and specimen caused by temperature fluctuations.

Notes:

- Definite Focus control is also possible without using AxioVision, although depending on the application, this may only be possible with limited functionality.

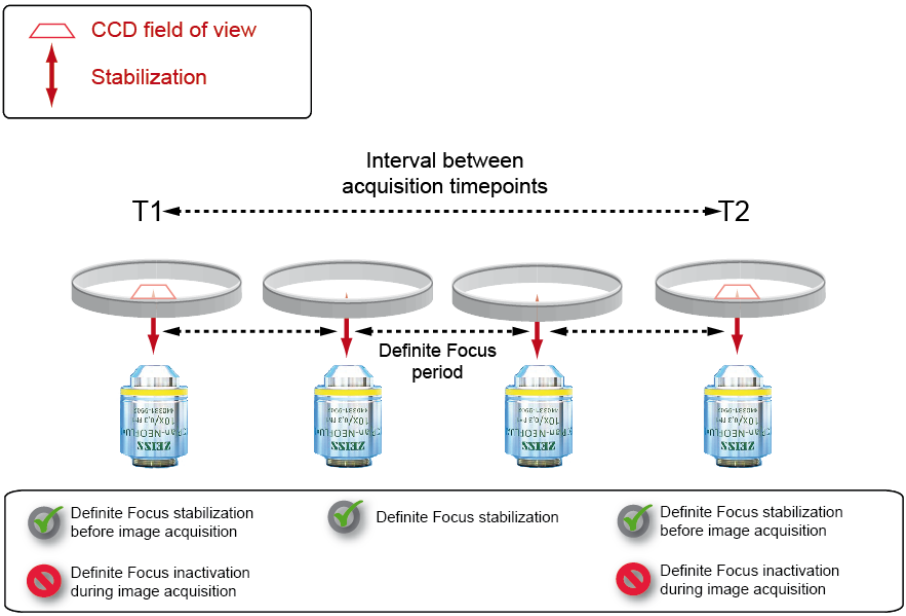
- For further information on the working principle, operation and compatibility with hardware components (e.g. objectives), please refer to the Definite Focus operating manual.

In this section several examplary use cases are given for operation of definite Focus by AxioVision. However, the quality of the actual results that can be obtained, not only depends on the software settings but also on many other parameters like e.g. type of sample, microscope objective, speed of focus-drift, etc.

Example 1: Automatic focus-stabilization at one position of a culture dish over time (Multidimensional Acquisition)

In this example Definite Focus automatically stabilizes the focus-position (Z) at a single xy-Position in a culture dish, without needing further settings or interactions made by the user.

The following schematic representation (not to scale) shows how Definite Focus operates during such an experiment.

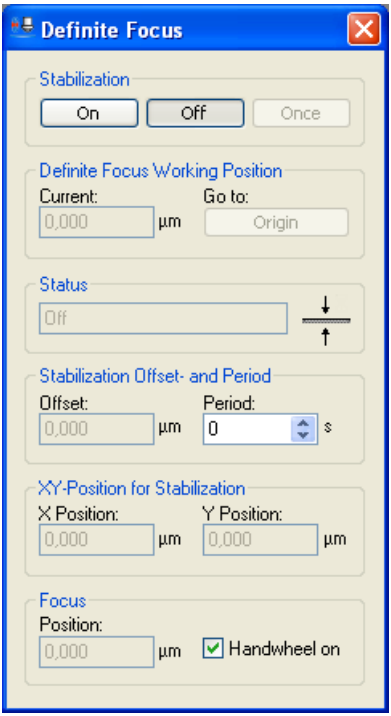


➢ Make sure that Definite Focus control by AxioVision is running in default mode. Default handling of Definite Focus is the preferred working mode.

The box ☐ Disable Default Handling of Definite Focus in the menu **Tools** ⇒ **Options…** ⇒ **Acquisition** must not be checked for this mode. Activation and deactivation of Definite Focus during the experiment will now be carried out automatically by AxioVision.

➢ Place your specimen on the microscope stage and focus it.

➢ Open the Definite Focus window in the menu **microscope** ⇒ **XYZ** ⇒ **Definite Focus**:



- Define the activity interval of Definite Focus by entering a Period value. The faster the expected focus drift is, the smaller the value has to be.

- Activate Definite Focus by clicking the 

button.

Activity of Definite Focus is displayed in the **Status** area of the Definite Focus window by a periodic indication of „Waiting…" and "Setting focus…", as well as by an animated icon  .

- Now start the image acquisition experiment as you are used to it. This can be e.g. a single- or multichannel timelapse experiment.

Note:

- Definite Focus keeps the distance from the bottom of your sample (e.g. the culture dish) constant. Please note that movement of individual cells in the culture dish can nevertheless lead to a situation where these cells are not in focus anymore. In this case use of the software- (image-) based autofocus functionality of AxioVision is recommended. The AxioVision software-autofocus can also be used additionally to Definite Focus.

Example 2: Automatic focus-stabilization at one position of a culture dish over time (Fast Acquisition)

Definite Focus operation in Fast Acquisition experiments is different from that in Multidimensional Acquisition experiments. Due to the nature of Fast Acquisition experiment, Definite Focus has to be restricted to not interfere with the highly efficient image acquisition process.

It is not recommended to use Definite Focus in Fast Acquisition Experiments including z-stacks. For Acquisition of fast timeseries only, we suggest to perform experiments aiming at the optimization of a suitable Definite Focus period for the desired image acquisition interval.

Note:

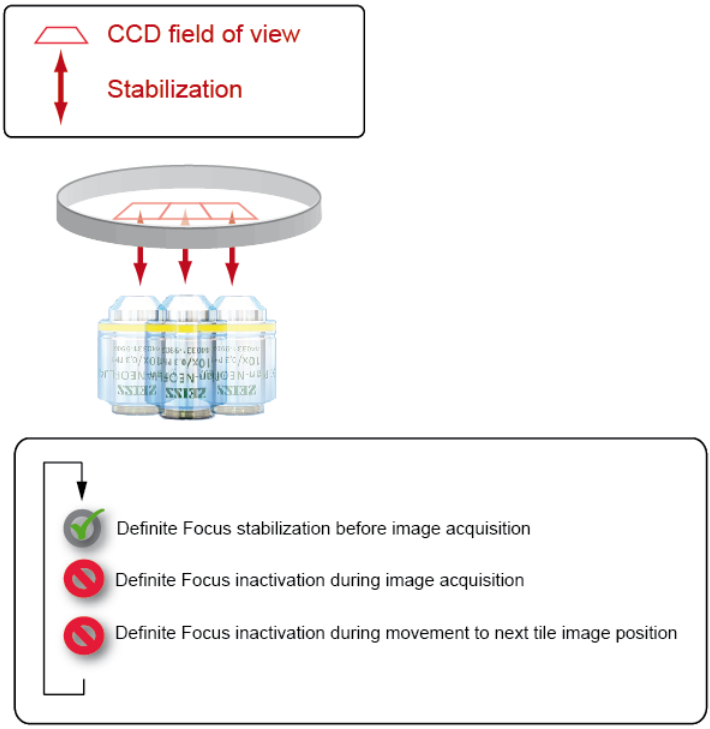
- If you should observe a continuous focus jitter, please increase the Definite Focus period to restrict the effect to a subset of (defined) images.

- In case you observe periodic changes in image brightness (especially when multibandpass- or longpass-fluorescence filters are used), please install the IR-blocking filter delivered with you Definite Focus device. Please also see the Definite Focus hardware manual for this procedure.

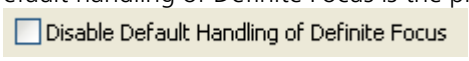
Example 3: Automatic focus stabilization during acquisition of a MosaiX image

In this example Definite Focus automatically stabilizes the focus-position (Z) at tile images of a MosaiX.

The following schematic representation (not to scale) shows how Definite Focus operates during such an experiment.



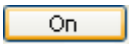
- Make sure that Definite Focus control by AxioVision is running in default mode. Default handling of Definite Focus is the preferred working mode.

  The box 

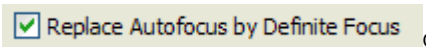
in the menu **Tools** ⇒ **Options...** ⇒ **Acquisition** must not be checked for this mode. Activation and deactivation of Definite Focus during the experiment will now be carried out automatically by AxioVision.

- Place your specimen on the microscope stage and focus it.

- Open the Definite Focus window in the menu **microscope** ⇒ **XYZ** ⇒ **Definite Focus**:

- Activate Definite Focus by clicking the 

button.

- Check the box 

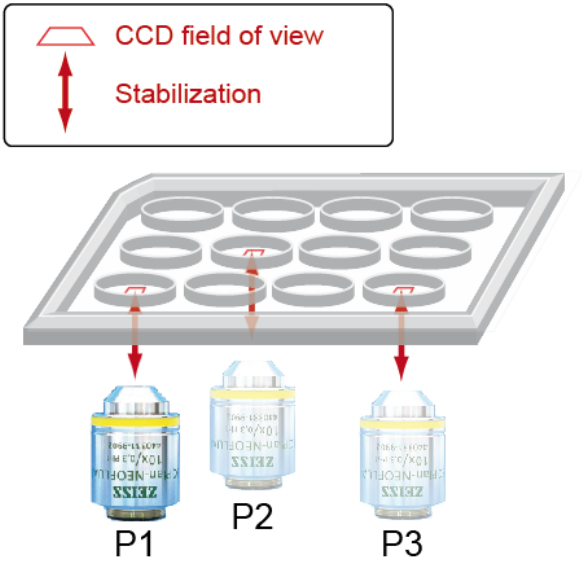
on the **MosaiX** property page. If the box is checked, Definite Focus will be activated during the MosaiX acquisition the same the software autofocus would get activated. This means you are free to choose between **Off, At Center, At 1. tile, Every nth tile**. Please see the **MosaiX** help and manual for a description of these options.

- Start the image acquisition.

Example 4: Automatic z-position adjustment at multiple positions in experiments using multiwell plates or similar sample carriers.

In this example Definite Focus automatically stabilizes the focus-position (Z) at multiple xy-positions of a multiwell plate.

The following schematic representation (not to scale) shows how Definite Focus operates during such an experiment.

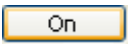


- Make sure that Definite Focus control by AxioVision is running in default mode. Default handling of Definite Focus is the preferred working mode. The box 

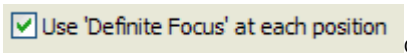
in the menu **Tools** ⇒ **Options…** ⇒ **Acquisition** must not be checked for this mode. Activation and deactivation of Definite Focus during the experiment will now be carried out automatically by AxioVision.

- Place your specimen on the microscope stage and focus it.

- Open the Definite Focus window in the menu **microscope** ⇒ **XYZ** ⇒ **Definite Focus**:

- Activate Definite Focus by clicking the 

button.

- Check the box 

on the **XY** property page. If the box is checked, Definite Focus will be activated before image acquisition at every single position of the active positionlist.

- Start the image acquisition.

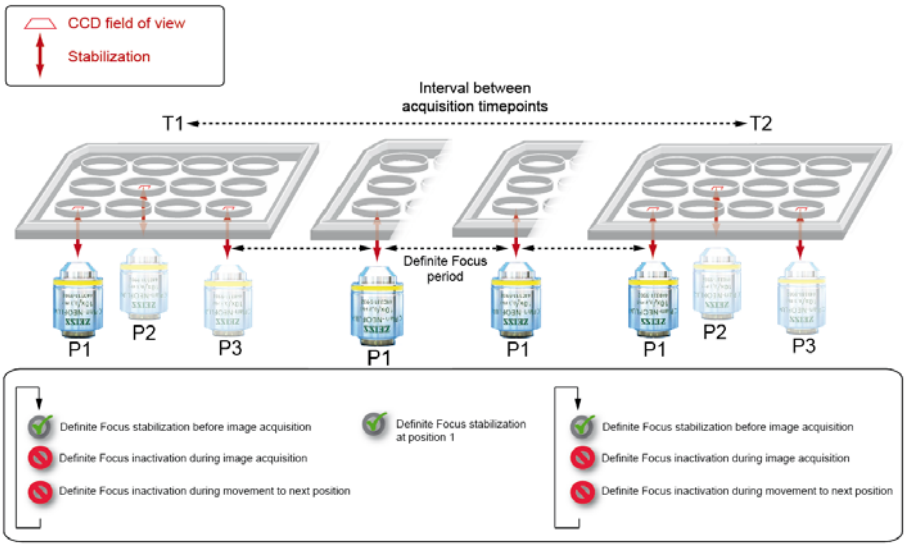
Note:

- When multiwell plates or holders are tilted too much or the bottom of the plate is very uneven, the difference in the z-position of xy-positions that are very far apart, can be greater than the lock-in range for Definite Focus with the used objective.

Example 5: Automatic z-position adjustment and stabilization over time at multiple positions in experiments using multiwell plates or similar sample carriers.

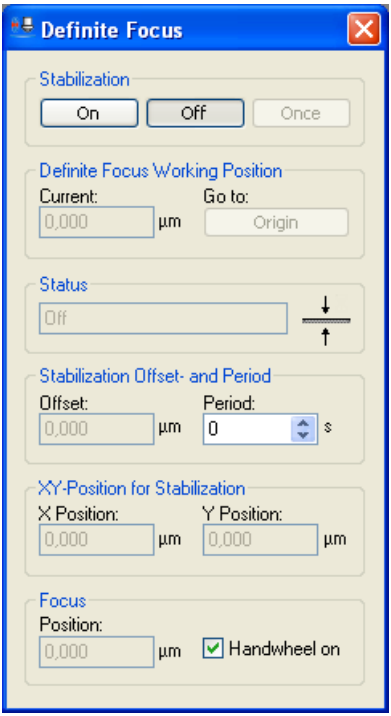
In this example Definite Focus automatically adjusts the focus-position (Z) at multiple xy-positions of a multiwell plate and at the same time compensates a z-drift over time.

The following schematic representation (not to scale) shows how Definite Focus operates during such an experiment.



➢ Proceed as described in example 4.

➢ Open the Definite Focus window in the menu **microscope** ⇒ **XYZ** ⇒ **Definite Focus**:



- Define the activity interval of Definite Focus by entering a value into the Period field. The faster the expected focus drift is, the smaller the value has to be. You should also take into account the time interval that you use for the image acquisition. When a time interval of 120 seconds is used for image acquisition, it could e.g. make sense to use a Definite Focus period of perhaps 5 or 10 seconds.

- Start the image acquisition.

Note:

- When multiwell plates or holders are tilted too much or the bottom of the plate is very uneven, the difference in the z-position of xy-positions that are very far apart, can be greater than the lock-in range for Definite Focus with the used objective.

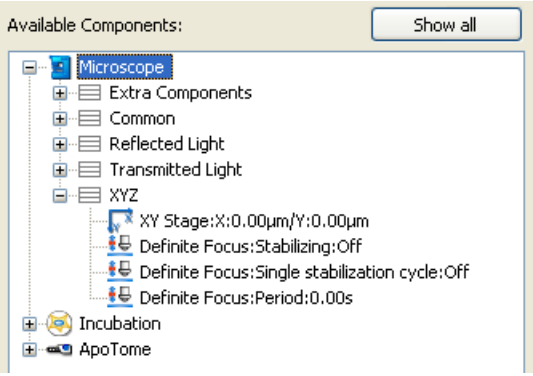
Example 6: User-defined Definite Focus operation

In this example, Definite Focus is controlled by user-defined hardware-settings. This mode is only recommended if the results obtained by the automatic Definite Focus control are not satisfying. It is also recommended that only users, experienced in generating hardware-settings are using this mode.

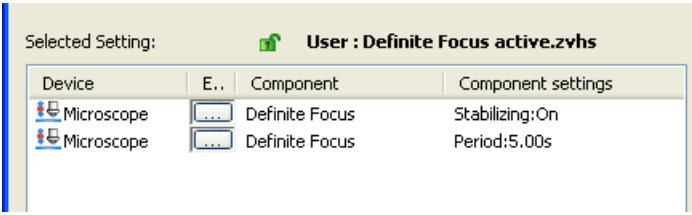
- Deactivate the default handling of Definite Focus by AxioVision by checking the box 

in the menu **Tools** ⇒ **Options…** ⇒ **Acquisition**. Activation and deactivation of Definite Focus during an Experiment is not done automatically anymore by AxioVision, but has to be defined by the user in an experiment with hardware-settings.

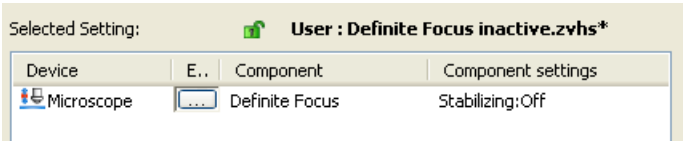
- You will find the Definite Focus Control-Components for the creation of hardware-settings in the menu **Tools** ⇒ **Settings-Editor** in the category **Microscope** ⇒ **XYZ**.



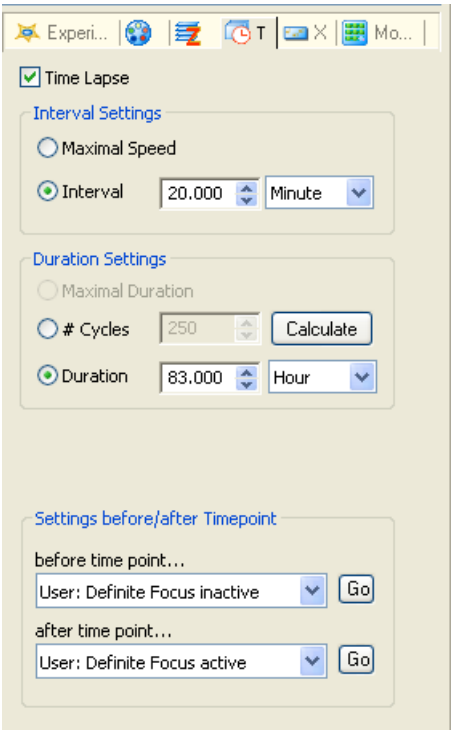
- Create a hardware-setting „Definite Focus active“ that activates Definite Focus for focus stabilization with an interval (period) of 5 seconds.



- Create a hardware-setting „Definite Focus inactive“ that deactivates Definite Focus.



- Use these hardware-settings to enable focus stabilization and correction by Definite Focus in between the timepoints of a timelapse experiment, while disabling Definite Focus immediately before image acquisition. For this use the submenu Settings before/after Timepoint in the Tab **T** of the **Multidimensional Acquisition**.



➢ You may now start you experiment. Definite Focus will correct the focus position between all timepoints every 5 seconds. During image acquisition it will be inactive to minimize the possibility of any putative negative influence of Definite Focus on image quality.

4.7 TIRF

Performing automatic calibration

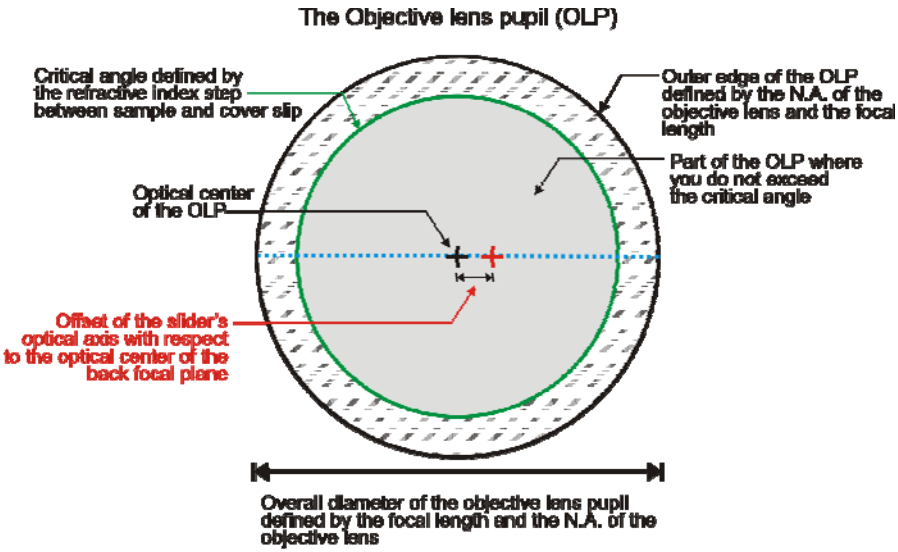
Function

In quantitative TIRF microscopy it is important to know the exact angle at which the sample is being illuminated so that additional parameters of the evanescent field and the sample (penetration depth, refractive index, critical angle of total reflection etc.) can be determined from this. The angle is generally set using a motorized deflection unit and is influenced by the opto-mechanical tolerances of the microscope components being used (e.g. filter set, objective).

It is therefore necessary to calibrate the deflection system. For this purpose, use is made of the effect that causes the depth to which the illumination light penetrates into the sample to decrease exponentially when the critical angle of total reflection is reached and exceeded. This penetration depth then amounts to just a few tens or hundreds of nanometers. The consequence of this is that the background fluorescence from parts of the sample that are further away from the cover slip drops rapidly.

It is possible to measure the intensity via the surface brightness of the objective pupil. To do this, the deflection unit is moved automatically in such a way that the laser focus passes directly across the entire objective pupil once. (The position of the laser focus in the objective pupil determines the angle at which the sample is illuminated.)

Laser focus in objective pupil

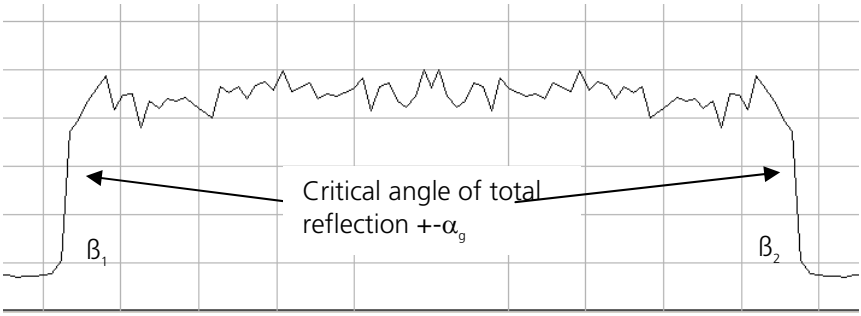


The angle setting ß of the deflection unit and the integrated intensity of the pupil image at this angle are always measured.

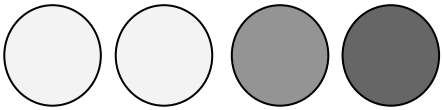
The resulting integrated intensity curve (see below) depending on the angle setting of the deflection unit is subsequently analyzed.

Two jumps in intensity can be identified in the graph. These occur at the moment the sample illumination reaches the critical angle of total reflection, when the intensity of the background fluorescence increases/decreases. The sample-illumination angles are equal in size and differ only in the sign that precedes them.

The graph shows the integrated intensity depending on the angle ß of the deflection unit:



Light intensities in the objective pupil or a plane conjugated to it:



The angle setting $ß_0$ of the deflection unit at a sample-illumination angle α of 0 degrees is therefore calculated as follows:

$$ß_0(\alpha=0°) = ß_1 - ß_2$$

$ß_1$ and $ß_2$ are the angle settings of the deflection unit at which the critical angle of total reflection is realized in the sample.

The signs preceding the angles must be observed.

The deflection unit has therefore been calibrated and there is a direct relationship between the angle setting of the deflection unit and the angle of sample illumination. In other words, you know what deflection angle results in a sample-illumination angle α of 0°.

It is now possible to set defined sample-illumination angles, as sample-illumination angle α and deflection angle ß are in a fixed relationship to each other as a result of the optical and mechanical structure.

$$\alpha = f(ß - ß_0), \qquad ß = f(\alpha) + ß_0$$

From the critical angle α_1 or α_2 you can now calculate the refractive index of the sample.

$n_1 = n_2 \times \sin (f(ß_g - ß_0))$
n_2 = refractive index of cover slip
n_1 = refractive index of sample

The angle must be calibrated for each combination of objective and fluorescence filter set, as each filter set causes slightly different deviations in the beam path due to manufacturing tolerances.

To automatically correct the TIRF slider's offset from the zero position, follow the procedure below:

- Insert your sample into the microscope's sample holder. Only aqueous samples are suitable for calibration purposes. **Fixed specimens are not suitable!**
- Focus on the lowest layer of your sample. **No air bubbles or impurities must be present in the oil.**
- Set the laser intensity to the lowest possible level.
- Move the Bertrand slider installed in front of the camera into the beam path.
- Restrict the camera frame to roughly the diameter of the imaged objective pupil.
- Switch the display characteristics of the live image to **Linear**.

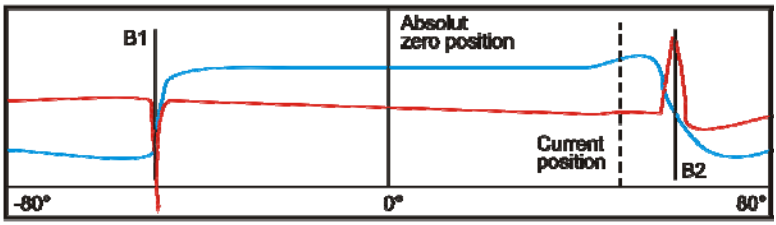
- Select the laser intensity and camera exposure time in such a way that the image is neither overexposed nor underexposed at the middle setting for the illumination angle.

- Select the combination of filter, objective and laser line that you wish to calibrate.

- Start the calibration run by clicking on the **Automatic Calibrate** button.

- Calibration now begins.

When calibration is complete, a display appears of the measured average gray values as a function of the angle set.



Red: 1st deviation of the mean image intensity
Blue: Mean image intensity

If automatic calibration fails, follow the procedure below:

- Reactivate the laser and set the angle to the left-hand detected edge, denoted as **B1**. Take note of the image displayed. Now repeat this for **B2** by changing the uncorrected angle until it is at the point of the steepest slope. Repeat the step until the image intensity has equal values. Exit the dialog by clicking on the **Apply** button.

Open the live display and set the beam to the zero position using the corresponding button for setting the illumination angle. If the fluorescence returning from the sample is at the center of the objective pupil.

5 Image Processing

5.1 General

The task of image processing essentially consists of two fields of activity: correction and optimization of the acquired image data.

In many cases digital image processing functions are required to make up for the technical limitations of the available devices. However, we should point out that often (in fact probably in most cases) image processing functions are needed to compensate for carelessness during image acquisition.

It is therefore essential to make sure that you fully utilize the capabilities of your technology both prior to and during image acquisition. This is the only time when the actual original information is available. It is also your best opportunity to ensure good image quality.

One area that is often neglected is the preparation of the sample. The principle here is that a poor sample inevitably results in a poor image. For this reason you need to be self-critical and check whether the quality of your sample is suitable to provide optimum acquisition results.

Whatever you use image processing functions for, make sure that wherever possible original data are not overwritten. You should always be able to return to the original image to ascertain whether executing a function has actually brought about an improvement or has in fact resulted in relevant image information no longer being available.

The different ways of processing images

In AxioVision there are two different ways to process images. They each have different features and have been devised for different areas of application. On the one hand, all functions can be executed via the **Processing** menu or the **Workarea**. Alternatively, via the **Properties** window, you can optimize the quality of the image display (brightness, contrast and gamma value) for your monitor using a characteristic curve.

In both cases AxioVision ensures that initially your original data are not modified:

- If you are using the functions via the **Processing** menu, by default the result is displayed in a new image document. You can then decide whether you want to save the result in a new file or overwrite the existing file.

- If you are working via the **Properties** window, the numerical values of the brightness and color information of your images are not modified. Only the display of the saved values on the monitor is adapted. As long as you save your images in AxioVision "ZVI" format, in addition to the original data it is only the settings in the **Properties** window that are saved in the file.

- When you open the file, it is then the optimized display that is initially made available. You can revert to the original display at any time via the **Properties** window. If you export the image into a different format, the settings of the **Export** function can be used to specify whether you want to save the original data or whether you want the settings in the **Properties** window to be adopted before saving. In the latter case, however, the gray or color values are changed, and you are no longer able to restore the original data.

Note:

- We recommend that initially you always save your images in ZVI format, and only export them to an external format if you need to do so.

Image formats

In the section that follows, image format should not be understood to mean the file format (ZVI, BMP, TIF etc.), but rather the way in which brightness and color are displayed in an image.

Conventional TV cameras are able to distinguish 256 gray levels. The entire spectrum from black to white is displayed using this number of brightness levels. To be able to distinguish 256 gray levels on a PC, 8 bits are required. These images are therefore known as **8 bit images**.

To display color images, three of these gray level images are required – one for red, one for green and one for blue. These images are essentially gray level images. This means that a color image is made up internally of 3x8-bit images. We therefore also talk of **24 bit images**. With these kinds of image it is possible to distinguish up to 16.8 million color values.

Modern digital cameras, however, are not only able to distinguish 256 gray levels, but can actually distinguish as many as 65536. This means that 8 bits are no longer sufficient to save the data. 16 bits are required in such cases. However, **16 bit images** are essentially only gray level images too. If three of these images are combined to form a color image, we talk of **48 bit images**.

Note:

- Some file formats are not able to save 16 and/or 48-bit images. We therefore recommend that you always save your images in ZVI format initially, and only export them to an external format if you need to do so.

5.2 Annotations

Annotations are additional drawing elements (scale bars, captions, arrows etc.) that can be drawn into an image. The word "into", however, is not strictly accurate, as the annotations are in fact drawn on top of the image. In other words, the annotations are drawn into their own plane on top of the actual image information, ensuring that initially the original information is not overwritten.

Provided that you save your images in AxioVision "ZVI" format, this plane information can be retained. The annotations are therefore saved separately from the actual image information. When you open the file, any annotations that are present can be changed, deleted or hidden or new annotations can be added.

The AxioVision ZVI format is the only format that supports this plane technique. Consequently, if you save the image in a different format, in the settings of the **Export** function you must specify whether or not you wish to "burn" the annotations into the image. You have the following options:

- If you burn the information into the image, the pixels that lie beneath the annotations are overwritten and you will then be unable to restore them.

- If you decide not to burn the annotations into the image, the annotations are lost.

- Alternatively, you can save the image twice (once in its original form and once with the annotations burnt in).

Note:
- As there are considerable limitations involved in saving images in external formats, we recommend that initially you always save your images in ZVI format and only export them to an external format if you need to do so.

5.3 Working with the Image Processing Functions

General

The text below outlines the principal method for operating the image processing functions. The steps described are identical for all functions. A description of the operation of the individual functions and their parameters can be found in the online help under the **Processing** menu entry.

The individual steps in the following examples can also be practiced using the original images that were employed. The sample images can be found on the Product DVD.

Smoothing an image (basic)

Images frequently contain noisy regions or inhomogeneous brightnesses, which impair the detection of the objects to be measured (segmentation). To achieve satisfactory segmentation, these kinds of images are processed using smoothing filters.

All filter functions work using filter matrixes, the size of which can be set using the **KernelSize** parameter. During the filtering process, the central pixel in the matrix is combined with the gray values within the matrix, and is therefore given a new value.

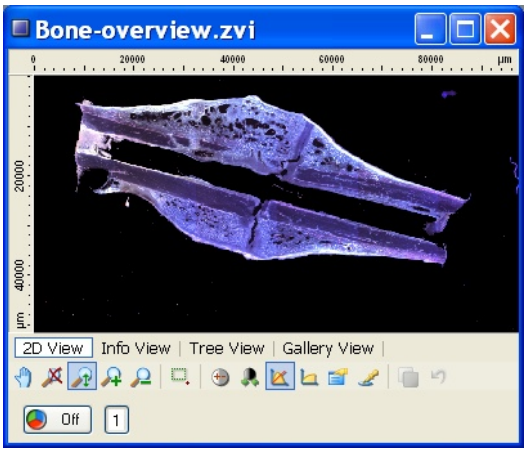
Which smoothing filter is most suitable depends on the structures that are present in the image. You should therefore try out various filters initially.

The **Sigma** function is particularly appropriate if you wish to remove uneven brightnesses from within structures without influencing the edges of these structures. Fine structures are also preserved.

To suppress noise, the **Gauss** function can be used. This reduces the dynamic range of the image and smoothes the entire image, including the object edges.

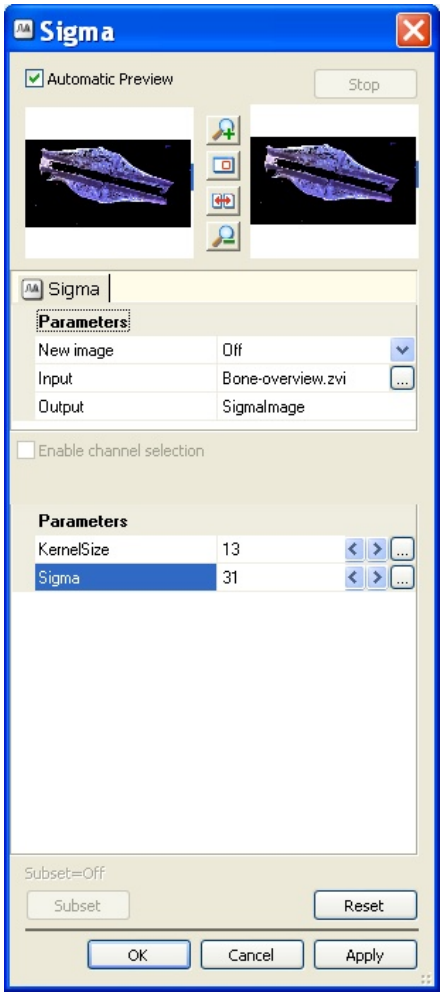
The following example explains step by step how to smooth the structures in an image using two different filter functions.

➢ From the Product-DVD load the image "Bone-overview.zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**, or acquire an image with the camera. The image is displayed in AxioVision's image window.



Select from the **Processing** menu the **Smooth** functional group and then the **Sigma** function.

- You will now see the dialog window of the **Sigma** function.
- If the **Automatic Preview** check box is activated, you will see the reduced input and output images.
- Set **CreateNew** to **Off** so that a different filter function can later be executed with the same image.
- Set the value **13** for **KernelSize** and the value **31** for **Sigma** by clicking on the arrow keys or by entering the values directly.
- Click on the **Splitter Display** icon  to display the original and the result image side by side.
- Click on **OK** to close the dialog.



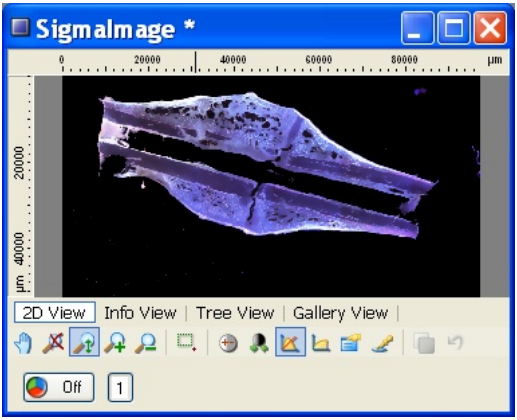
Select from the **Processing** menu the **Smooth** functional group and then the **Gauss** function.

- You will now see the dialog window of the **Gauss** function.
- If the **Automatic Preview** check box is activated, you will see the reduced input and output images.
- If you click on the button in the **Input** field, the image gallery appears. Select the original image "Bone-overview.zvi" by clicking on it.
- Set the value 13 for **KernelSize** by clicking on the arrow keys or by entering the value directly.
- Click on the **Splitter Display** icon to display the original and the result image side by side.
- Click on **OK** to close the dialog.

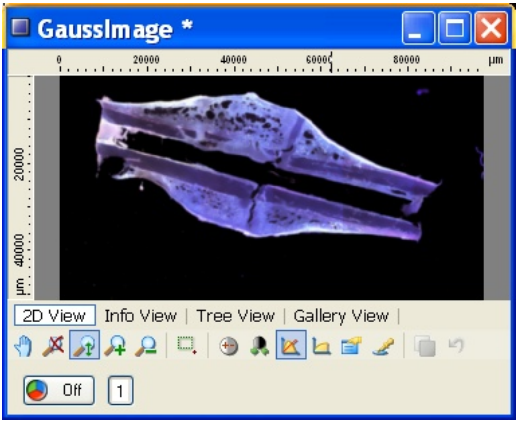


Compare the two resulting images.

➢ With the **Sigma** filter, strong smoothing has been achieved without the edges becoming blurred:



➢ With the **Gauss** filter, weak smoothing has been performed using the same **KernelSize**. The edges of the object have also been smoothed:



Note:

- Adjust the display characteristic curve in the resulting image if necessary.

Shading Correction (basic)

Uneven illumination, vignetting in the optical system or inhomogeneous sensitivity of the camera sensor can sometimes lead to a so-called shading effect in images. This means that the brightness of the image is uneven, which greatly impairs the detection of the objects to be measured.

To eliminate this disruptive effect, shading correction must be performed using a reference image. As a rule, an empty field with no structures is used as a reference image. Often, however, the image has already been acquired, and the sample is no longer available to allow the acquisition of an empty field. In this case a pseudo-shading reference image can be used. This is generated by applying particularly strong smoothing (e.g. using a strong Lowpass filter) to the original image. The smoothing can, however, take place automatically within the shading correction function, which renders the generation of a shading reference image superfluous.

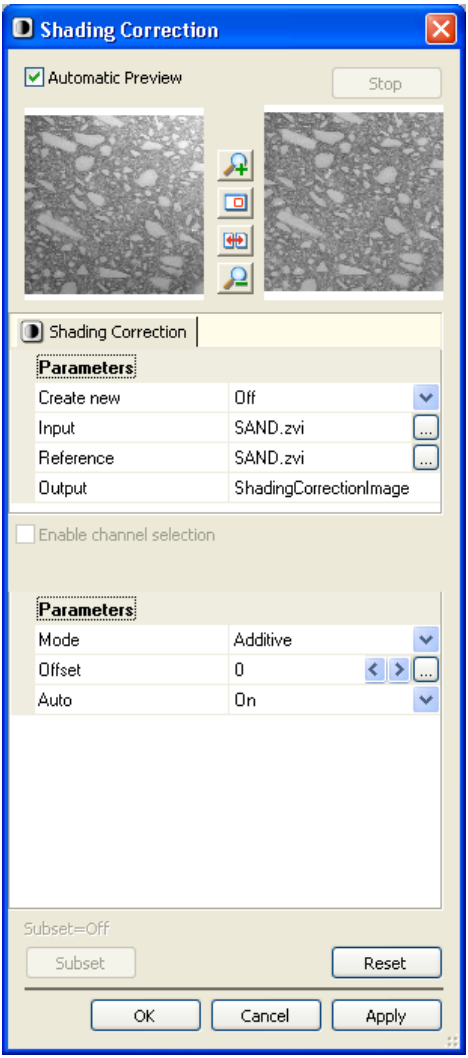
The following example explains step by step how to perform shading correction.

- From the Product-DVD load the image "SAND.ZVI" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**, or acquire an image with the camera. The image is displayed in AxioVision's image window.

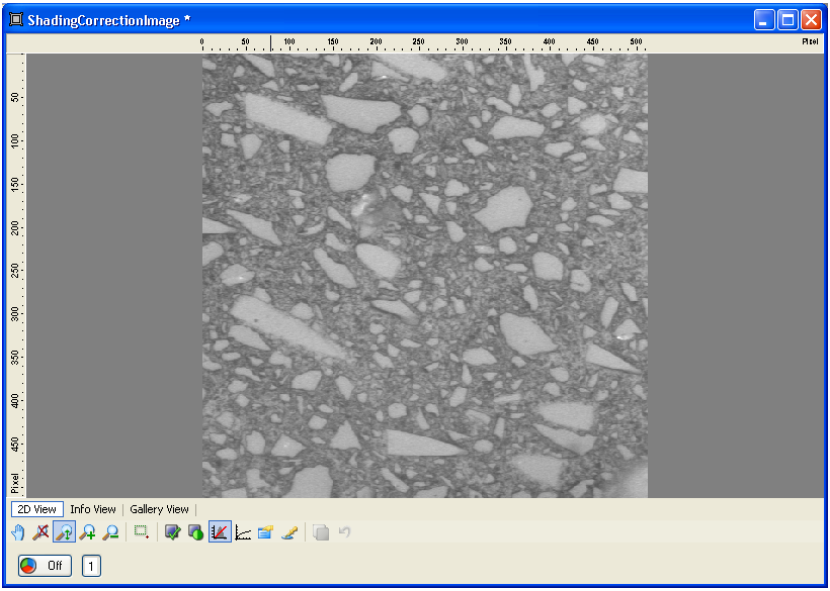


Select from the **Processing** menu the **Adjust** functional group and then the **Shading Correction** function.

- You will now see the dialog window of the **Shading Correction** function.
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.
- Set **CreateNew** to **Off** so you can select the images freely.
- Select the loaded image as **Input** and **Reference**.
- Set the **Auto** parameter to **On**. This will result in a pseudo-shading reference image being generated automatically in the background and combined with the original image.
- Click on the **Splitter Display** icon  to display the original and the result image side by side.
- Click on **OK** to close the dialog.



The resulting image then contains homogeneous illumination.



Note:

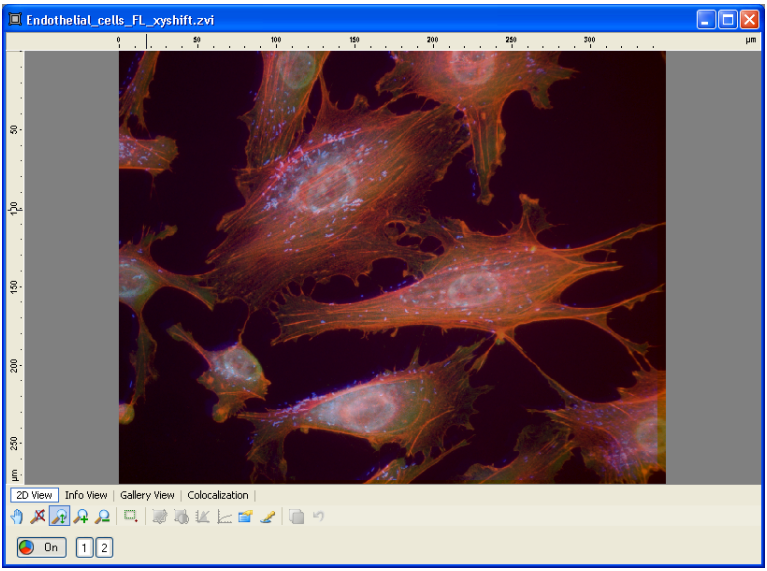
- Adjust the display characteristic curve in the resulting image if necessary.

Correction of XY pixel shift in multichannel images (basic)

During the acquisition of multichannel images using a manual microscope, you switch from one fluorescence filter to another by moving the fluorescence slider manually. In the acquired images, this can result in a shift of a few pixels between the individual channels in the horizontal and vertical directions. The individual fluorescence channels are shown in a pseudo color display. The XY pixel shift that has occurred must be subsequently corrected, to ensure that the individual fluorescence channels lie precisely on top of one another in the horizontal and vertical directions in the pseudo color image.

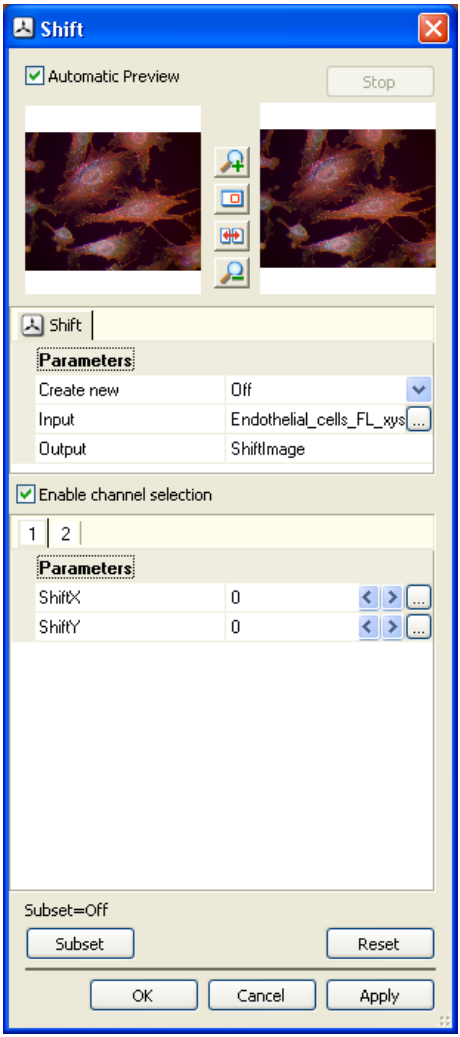
The following example explains step by step how to correct this XY pixel shift.

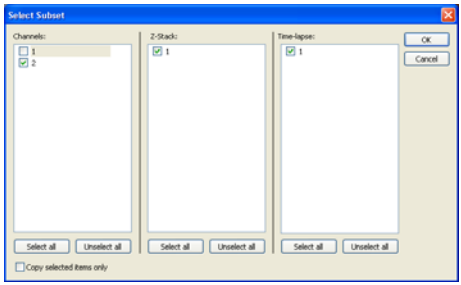
➢ From the Product-DVD load the image "Endothelial_cells_FL_shifxy.zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**, or acquire an image with the camera. The image is displayed in AxioVision's image window.



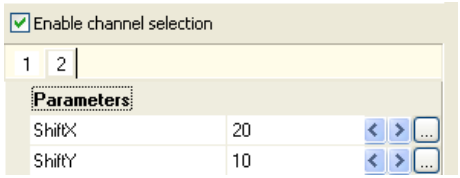
Now select from the **Processing** menu the **Geometric** functional group, and then the **Shift** function.

- You will now see the dialog window of the **Shift** function.
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.
- Activate **Enable channel selection**, so that you can enter the XY shift for an individual channel.
- Click on **Subset**.





- Activate only the channel that you want to be corrected (channel 1 in the example).
- Deactivate **Copy selected items only**, so that all channels are present in the resulting image (including the uncorrected ones), and click **OK**.



- In the dialog window, activate the channel to be corrected (channel 2 in the example).
- Set the shift for **ShiftX** and **ShiftY** by clicking on the arrow keys, or by entering the values directly.

Notes:

- The value for **ShiftX** and/or **ShiftY** always relates to the number of pixels that you want to be shifted.

- Click on the **Splitter Display** icon  to display the original and the result image side by side.

- Click **OK** to close the dialog window.

Note:

- Adjust the display characteristic curve in the resulting image if necessary.

Correction of z pixel shift in multichannel z-stack images

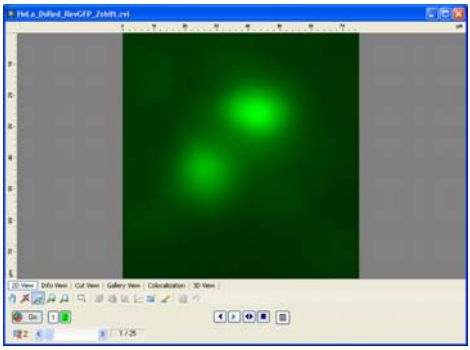
During the definition of multichannel z-stack images, the focus plane is normally set for one channel only. This means that the acquisition of the various fluorescence channels always starts with the same focus plane. When you switch from one fluorescence filter to another, the excitation and emission wavelengths, which also influence the focus plane, change. If the objective is not corrected for this chromatic aberration, the focus planes between the individual fluorescence channels in the acquired image will not correspond.

The individual fluorescence channels are shown in a pseudo color display. When you switch between the individual channels, a small shift is visible in the z direction. This can be subsequently corrected, so that in the pseudo color image the individual fluorescence channels lie exactly next to each other in the z direction.

The following example explains step by step how to balance out this z pixel shift.

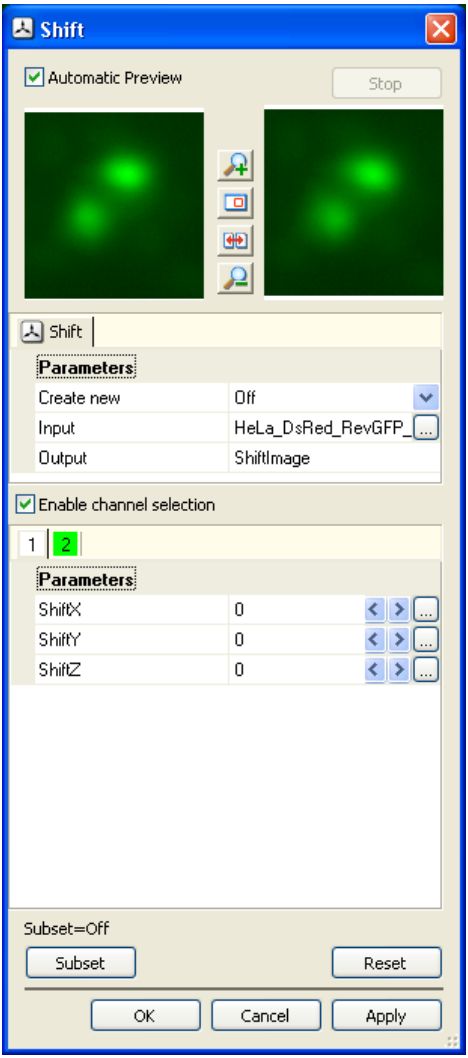
- From the Product-DVD load the image "Hela_DsRed _RevGFP_Zshift.zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**, or acquire an image with the camera.

- The image is displayed in AxioVision's image window.



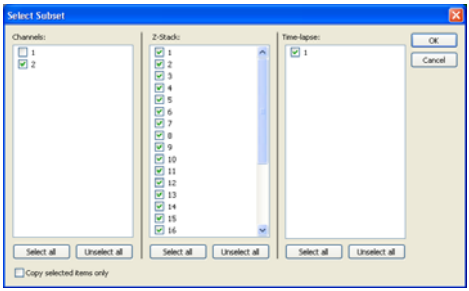
Select from the **Processing** menu the **Geometric** functional group, and then the **Shift** function.

- You will now see the dialog window of the **Shift** function.
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.
- Activate **Enable channel selection**, so that you can enter the Z shift for an individual channel.
- Click on **Subset** to enter the specific settings in the **Select Subset** window.



- Activate only the channel that you want to be corrected (channel 2 in the example).

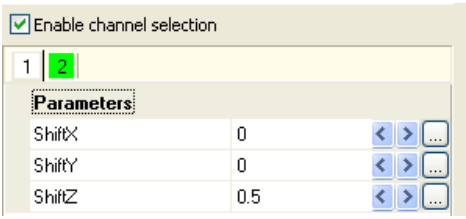
- Deactivate the **Copy selected items only** check box, so that all channels are present in the resulting image (including the uncorrected ones).



- Click on **OK**.

- In the **Shift** dialog window, activate the channel to be corrected (channel 2 in the example).

- Set the shift for **ShiftZ** by clicking on the arrow keys, or by entering the values directly.



Notes:

- The value for **ShiftZ** always relates to the number of sections by which you want the shift to be made.

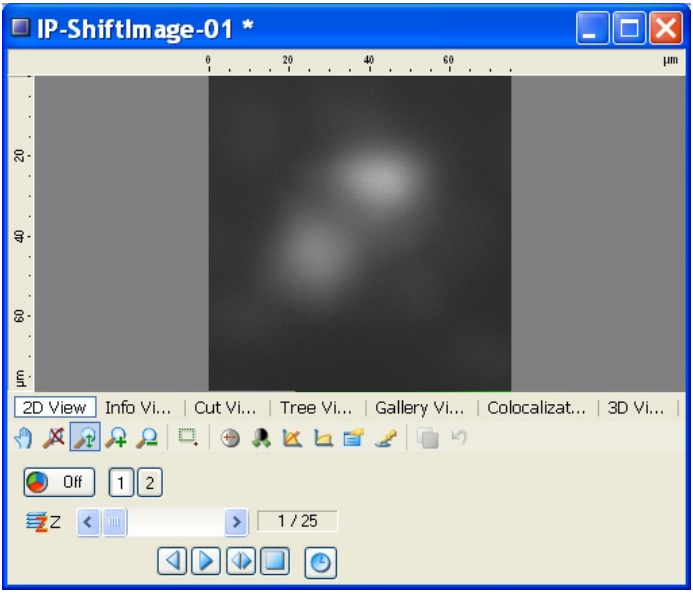
- Example: If **ShiftZ** is set to the value 5, the stack in the resulting image starts at section 6. The first 5 sections remain empty, whilst the last 5 sections of the original image are omitted.
  If a value of -5 is set, the first 5 sections of the original image are omitted. The first section of the resulting image is section 6 of the original image. The last 5 sections of the resulting image remain empty.

- It is also possible to perform a shift by a fraction of a section (e.g. 0.3). The value to be entered can be calculated from the distance between the individual sections.

➢ Click on the **Splitter Display** icon  to display the original and the result image side by side.

➢ Choose a z-level in the middle (here 13) using the player.

This is the resulting image obtained.



Note:

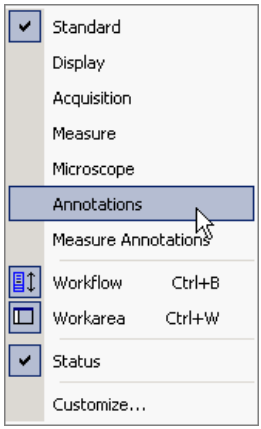
- Adjust the display characteristic curve in the resulting image if necessary.

5.4 Enhancing the Image Display on the Monitor

The fastest and easiest way to display an image that is optimally adapted to the monitor is to use the **Standard** workflow. A description of this workflow, including all the steps involved in image acquisition, can be found in chapter 3 "Image Acquisition".

5.5 Drawing in and Formatting Annotations

- Make sure that the **Annotations** toolbar is visible: right-click on the main menu or any toolbar and activate **Annotations** (a tick must be visible in front of it).



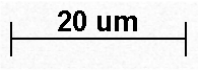
- Now activate the image into which you want to draw annotations. To do this, simply click on the image or select it via the tabs at the top of the document area.



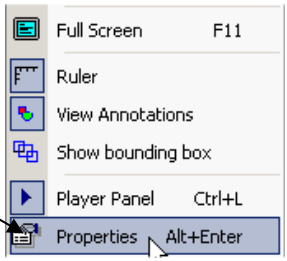
- From the **Annotations** toolbar select the tool you want to use, e.g. the **Scale Bar**.



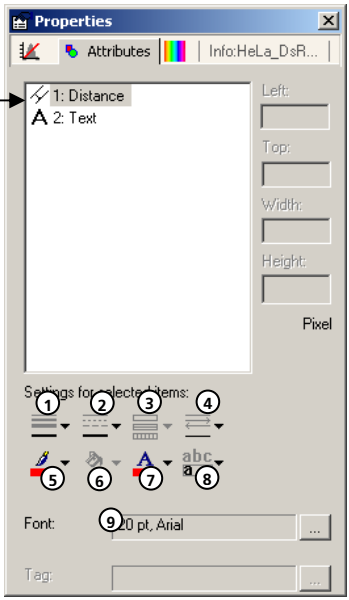
- Now draw in the scale bar: click once on the starting point, drag out the desired length and then click on the end point.



- To format the annotations (change the line color, font etc.), right-click in the image and select **Properties**.



- In the **Properties** window activate the **Attributes** tab.
- Select an element from the list.
- Change the **Settings for selected items**:
  1: Line thickness
  2: Line style
  3: Fill mode
  4: Line type
  5: Line color
  6: Fill color
  7: Text color
  8: Text alignment
  9: Font



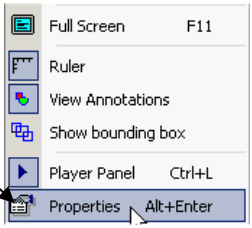
The settings only apply to the selected element. To specify standard settings, please read the next section.

5.6 Specifying Standard Settings for Annotations

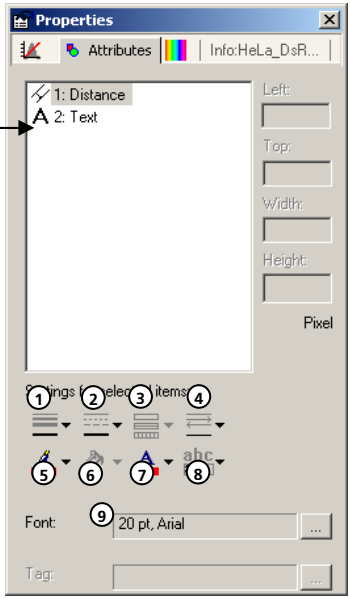
- Activate the image for which you want to specify standard settings. To do this, simply click on the image or select it via the tabs at the top of the document area.



- To enter the settings (line color, font etc.), right-click in the image and select **Properties**.



- In the **Properties** window activate the **Attributes** tab.
- Make sure that no elements are selected in the list of annotations and displayed above the **Default settings** formatting options.



- Now change the:
  1: Line thickness
  2: Line style
  3: Fill mode
  4: Line type
  5: Line color
  6: Fill color
  7: Text color
  8: Text alignment
  9: Font

Note:

- The settings are now used for all elements that are subsequently drawn in. If you save your images in ZVI format, the settings are saved with the image.

6 Image Analysis

6.1 General

With AxioVision Version 4 you are now able to perform interactive measurements in the basic program.

Possible ways to perform measurements

Measurements can, as a rule, be performed in two different ways:

- The individual measurement tools, e.g. line, circle etc., can be accessed via the **Measure** menu and/or toolbar. Individual values can be determined quickly using this method.

- Via the **Measure ⇒ Interactive Measurement ⇒ Start Measurement** function. This function contains the image to be measured with all the measurement tools in a single dialog. It is, therefore, particularly suitable for use in workflows and Commander scripts, and for this reason is also integrated into the **Measure** workflow. This method gives you the option of being led through the entire procedure, from image acquisition through to the saving of the image, via measurement and evaluation.

Conditions for measurements

Allocation of scalings

To obtain measurement values in real units, you must have allocated the correct scaling to the image you want to measure. This usually takes place during image acquisition (see chapter 3 "Image Acquisition").

If the selected image does not have a scaling

If a scaling has not been allocated to the image you have selected, you can allocate one now:

- Make sure that the image is selected (simply click on it).
- From the **Measure** menu, select the **Scalings** function.
- Select the corresponding scaling from the **Scalings Control** list.
- Click on the **Apply scaling to image** button.

Displaying measurement data

The measurement data can be displayed in different ways:

In principle, all measurements are displayed in the graphics plane as annotations. Which measured values are displayed for the relevant tool is freely configurable (please also see the "Configuring measurement tools" section). The display itself (color, line thickness etc.) can be formatted via the **View ⇒ Properties ⇒ Attributes** dialog.

If you use the **View ⇒ Properties ⇒ Measurement** dialog, the measured values are displayed in table form. In this dialog you also have a direct link between the table of measured values and the image that is currently selected. This means that when you activate a tool in the image, the corresponding values are automatically displayed in the table, and vice versa.

Saving data

Saving measurement data

The simplest and most convenient way to save the data is to save your images in AxioVision ZVI format. This is because this format saves the measurement data together with the image. Consequently, all data (pixel data, measurement data, annotations etc.) are present in the image file. There is no need to perform an additional saving procedure.

If you save your image files in an external format (BMP, JPEG etc.), the measurement data, together with all other additional information (annotations, acquisition data etc.), are saved in a separate file. As long as you open the images using AxioVision, the data in this separate file are automatically read in again and displayed. Read also section 7.2 "Saving 2D Images" in chapter 7 "Documentation".

Saving as separate data table

To save the measured values in a separate file, you should use the **Measure ⇒ Create Data Table** function. This function writes the measurements relating to the selected image in a data table. This data table is a completely normal document in AxioVision and can then be saved as a file. The data table no longer has a direct link to the image. To combine the values of several images in a single file, the **Measure ⇒ Append Data Table** function can be executed.

Configuring measurement tools

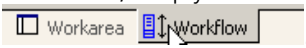
The interactive measurement tools can configured to your own requirements. Using the **Measure ⇒ Interactive Measurement ⇒ Configure Measurement Tools** function, you can generate configurations for the application scenarios of your choice. In a configuration you can determine which measurement parameters should actually be measured for a measurement tool and then displayed later in a data table, and which measurement parameters should be displayed in the graphics plane. A measurement can then be loaded via the **Measure ⇒ Interactive Measurement ⇒ Load Configuration** function. The defined settings are automatically active for a special measurement application. You can specify the default behavior of the measurement tools in the same way.

6.2 Measurement workflow: easy and save measurement

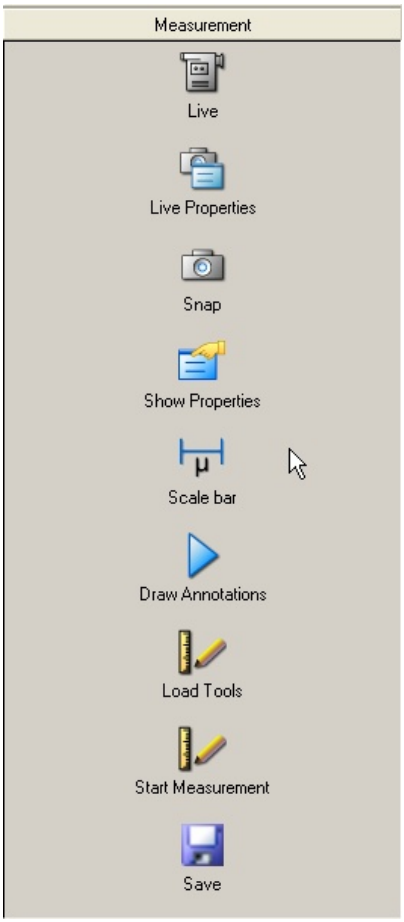
The easiest way to perform interactive measurements is to use the **Measure** workflow. This can be found on the **Workflow**. If the workflow is not displayed, in the **View** menu select the **Windows** list, and there select the **Workflow** command.

Note:

- The **Workflow** may in fact be open but covered by the **Workarea**. If that is the case, simply click on the **Workflow** tab of the work area:



Measure workflow: overview



Live opens and closes the Live window.

Live Properties displays a window containing the camera parameters and the display characteristic curve.

Snap acquires an image using the active camera.

Show Properties opens the image **Properties** dialog window with the property pages **Display**, **Attributes**, **Color**, etc.

Scale bar inserts a scale bar.

Draw Annotations inserts annotations into the acquired image.

Load Tools opens a dialog to select and activate a specific configuration.

Start Measurement starts the measurement.

Save saves the image.

Executing the Interactive Measurement Function

Go through the toolbar function by function:

- Acquire an image, or load an image from the hard drive.
- Adapt the display on the monitor.
- Insert a scale bar, if necessary.
- Draw in annotations, if required.
- Load a measurement tools configuration.

The next button starts the interactive measurement.

➢ Start the **Start Measurement** function.



➢ Perform any measurements by selecting a measurement tool from the toolbar.

1: Distance
2: Circle
3: Curve
4: Curve (Spline)
5: Angle 3
6: Angle 4
7: Events

The measurements are performed directly in the image.



➢ Now save the image with the measurement results via the **Measure** workflow.

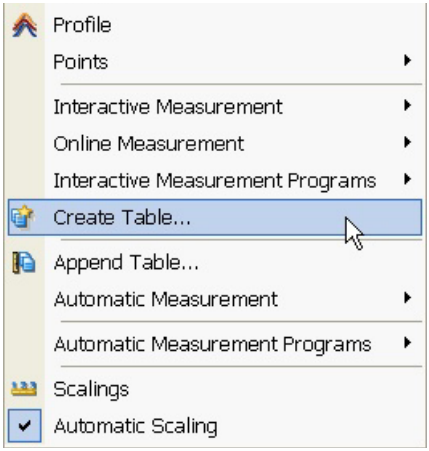


Display and export measurement data

The measurement data from an image can be

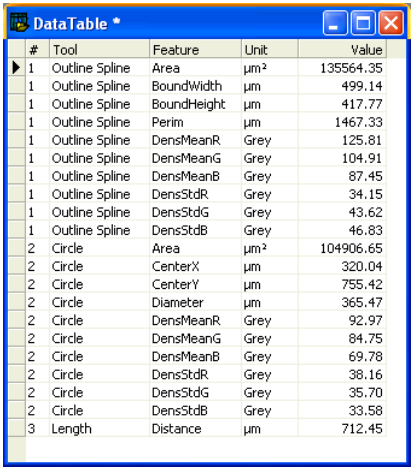
- displayed as a list;
- exported into an Excel-compatible text format; or
- combined with the measurement data from other images to perform an overall evaluation.

➢ Select the function **Measure –Create Table**.



➢ If you do not save your images in ZVI format, export the measurement data by clicking on **Save**.

➢ To exit the dialog, click on **Close**.



#	Tool	Feature	Unit	Value
1	Outline Spline	Area	µm²	135564.35
1	Outline Spline	BoundWidth	µm	499.14
1	Outline Spline	BoundHeight	µm	417.77
1	Outline Spline	Perim	µm	1467.33
1	Outline Spline	DensMeanR	Grey	125.81
1	Outline Spline	DensMeanG	Grey	104.91
1	Outline Spline	DensMeanB	Grey	87.45
1	Outline Spline	DensStdR	Grey	34.15
1	Outline Spline	DensStdG	Grey	43.62
1	Outline Spline	DensStdB	Grey	46.83
2	Circle	Area	µm²	104906.65
2	Circle	CenterX	µm	320.04
2	Circle	CenterY	µm	755.42
2	Circle	Diameter	µm	365.47
2	Circle	DensMeanR	Grey	92.97
2	Circle	DensMeanG	Grey	84.75
2	Circle	DensMeanB	Grey	69.78
2	Circle	DensStdR	Grey	38.16
2	Circle	DensStdG	Grey	35.70
2	Circle	DensStdB	Grey	33.58
3	Length	Distance	µm	712.45

6.3 Sorting and performing calculations with measurement data

The interactive and automatic measurement functions produce measurement data tables which, as a rule, contain raw data. In the AxioVision menu **Evaluate**, a range of analysis functions are available to allow you to process these data.

To measure several images in a measurement loop, you need to accumulate all the measurement data in a data table (**Append table** function). This measurement data list can be used to calculate statistical values, such as the mean and standard deviation (**Calculate statistical values** function), or to classify the measurement data (**Calculate classified table** function).

It is also possible, however, to combine individual columns from the same or from different data tables in the form of a new data table (**Concatenate table** function). In many cases you will need to filter out only specific measurement values from a data table, e.g. only the measurement values of the FITC channel (**Filter table** function), or to perform calculations using the measurement values of different columns (**Calculate table columns** function).

To obtain a clear presentation of the measurement data from time lapse images, the pivot table (**Calculate Pivot table** function) is a useful tool.

Note:

- Sample scripts of all evaluation functions can be found on the Product DVD in the folder *Demo Images and Scripts\Commander Script Examples*.

6.4 Creating data reports

Modifying and creating report templates with the file extension ZDR

A report is used for printing out images, sample information, data tables and histograms. A template, in which the layout and formatting for the individual report elements (image, data table, etc.) are defined, always forms the basis for a report.

In AxioVision, a selection of sample report templates is available. All reports have the file extension *.zdr and can be modified for a specific application. Please also read the section "Modifying a report template" and "Creating a report template" which you will find further down this section.

Newly created files or modified files are saved in the user folder *My Documents\Carl Zeiss\Templates\Data Report Templates* or in the work group folder *Shared Documents\Carl Zeiss\Templates\Data Report Templates.*

Reports will be connected to the respective documents (images, data tables):

- with an archive
- with a folder
- with a gallery

To create a report correct links are mandatory. If you move the documents, which are linked to the current reports to another location, the links in the report have to be updated accordingly.

Note:

- A description about reports with the file extension ZVR can be found in chapter 7.3 " Generate, print and export reports with the file extension ZVR".

Report templates toolbars



The meaning of each icon will be displayed when you move the cursor on one of the icons.

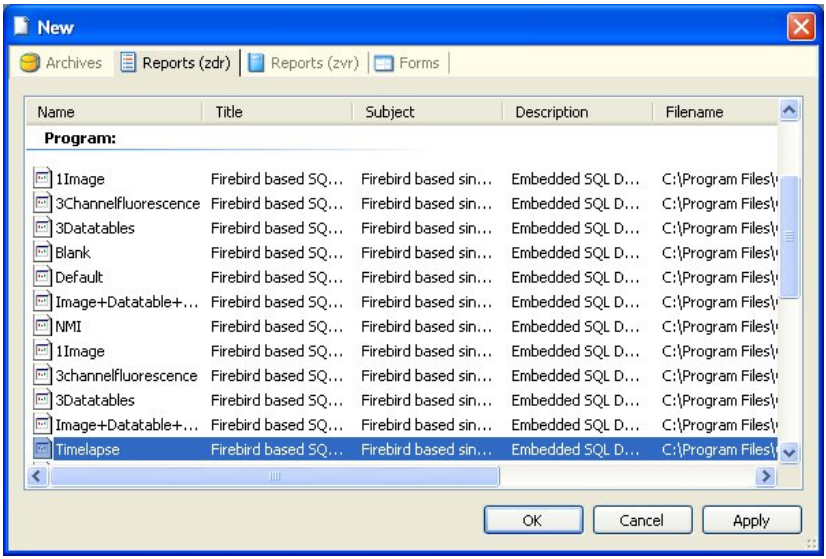
Modifying a report template

The following example will show you how to modify an existing template for another application (displaying user data and an image) and save it as a new template.

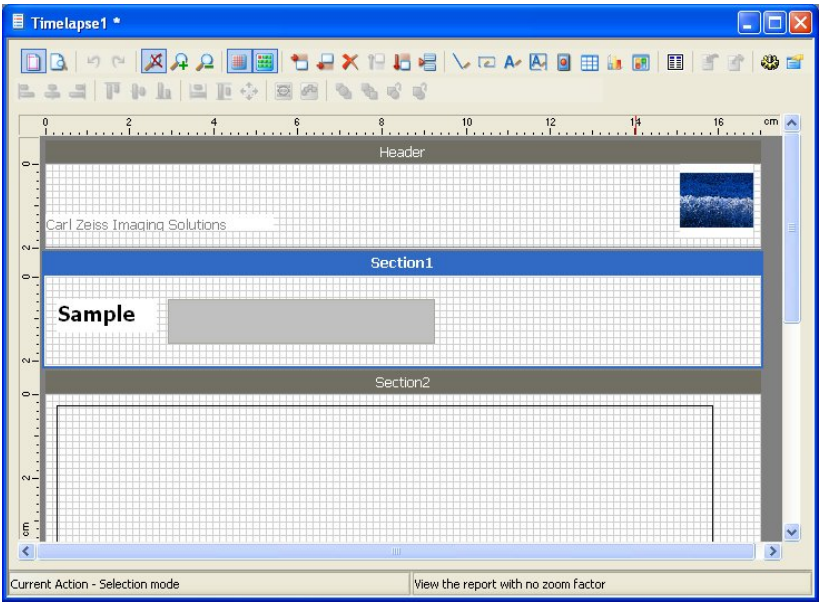
- First enter your user data on the **Tools** menu ⇒ **Options** ⇒ **ID**.

- Use the **Snap** function to acquire an image with the camera, or load the image "Cat-Cerebellum(1300).zvi" from the Product DVD from the folder *Demo Images and Scripts**AxioVision Images*\Imaging Plus* using the **Open image** function on the **File** menu.

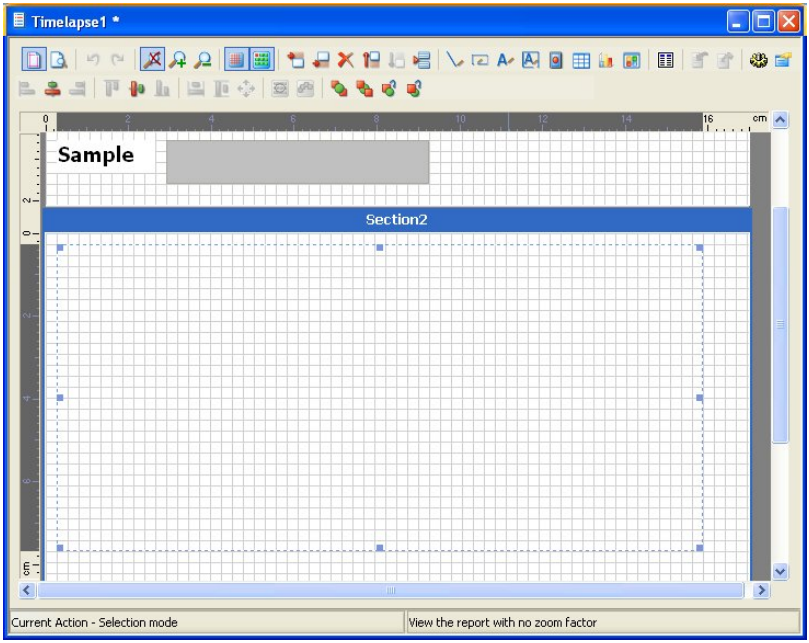
➢ From the **File** menu, select the **New** function, and then activate the **Reports (zdr)** tab sheet. Select the **Timelapse** template. Then click on **OK**.



The report is now displayed in the Edit  mode.



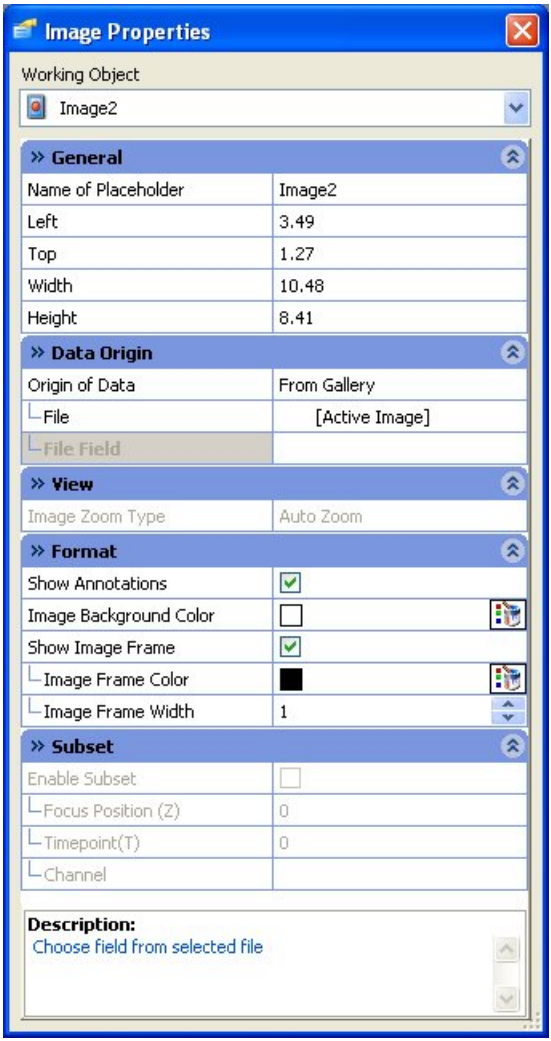
➢ With the left-hand mouse button depressed, drag out a rectangle over the object in **Section2**.



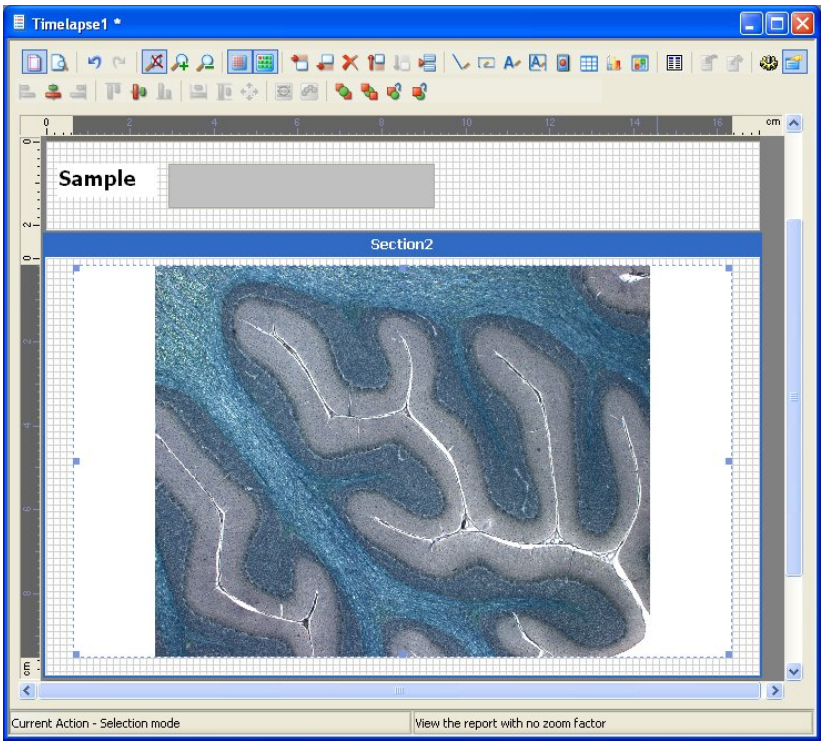
➢ Press the **Del** key to delete the selected fields. Click on  and create a large image field.

➢ Now show the property window by clicking on the  icon.

➢ In the **Data Origin** field, select "From Gallery" under **Origin of Date** and under **File** "[Active Image]". The original image now appears in the image field.



➢ Adjust the size of the image field to the size of the image.



➢ Use the  icon in the toolbar to switch to Preview mode to allow you to check the report. Then use the  icon to return to Edit mode.

The new report template can be saved and a name entered, e.g. "Image+userdata", using the **Save Report As Template** function on the **File** menu.

Note:

- The information relating to the user, company and address only appears if this has been entered on the **Tools** menu ⇒ **Options** ⇒ **ID**.

Creating a report template

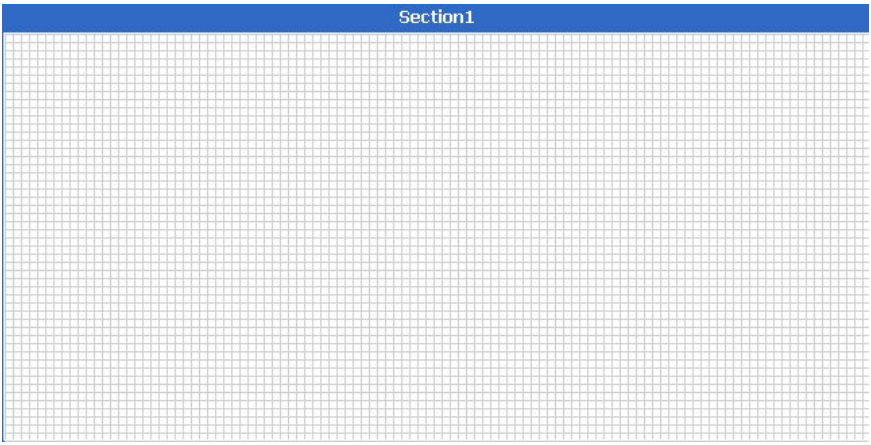
The following example shows you how to create a template for a z-stack from an empty layout. The images of individual z-planes are displayed with focus positions as annotations.

- Use the **Snap** function to acquire an image with the camera, or load the image "Anaphase-chromosomes.zvi" from the Product DVD from the folder *Demo Images and Scripts**AxioVision Images**Imaging Plus* using the **Open image** function on the **File** menu.

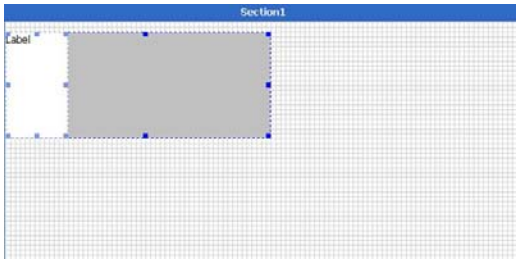
- From the **File** menu, select the **New** function, and then activate the **Reports (zdr)** tab sheet.

- Select the **Blank** template and then click **OK**. To see a preview of a template, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.

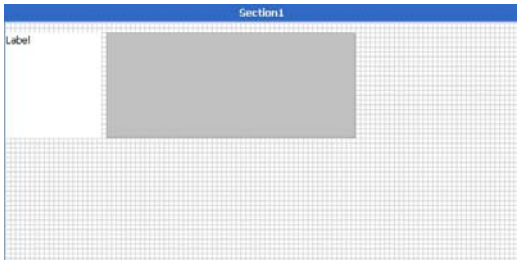
An empty layout is now displayed in Edit mode.



- Click on  , drag out a text field in **Section1** and position it in the top part of the report. A description field automatically appears next to the text field.



- Now click on any position in **Section1** outside the text field / label field.
- Click on the text field to position it in the desired location, independently of the label field, and, if necessary, to adjust the size.

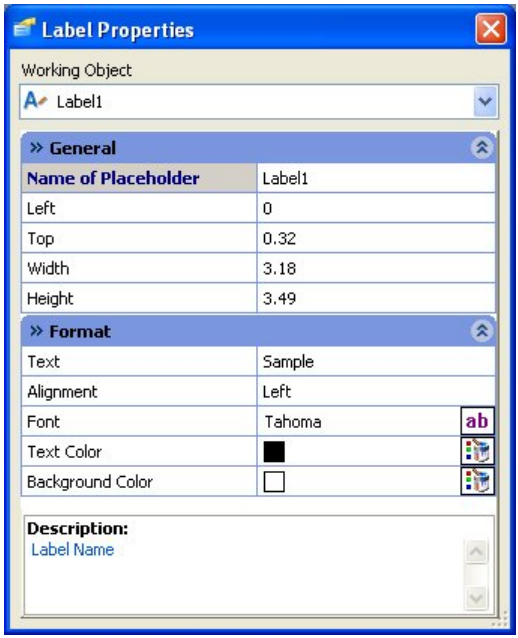


- Double-click on the text in the label field and replace it with the text "Sample". To change the display of the word "Sample" do the following:

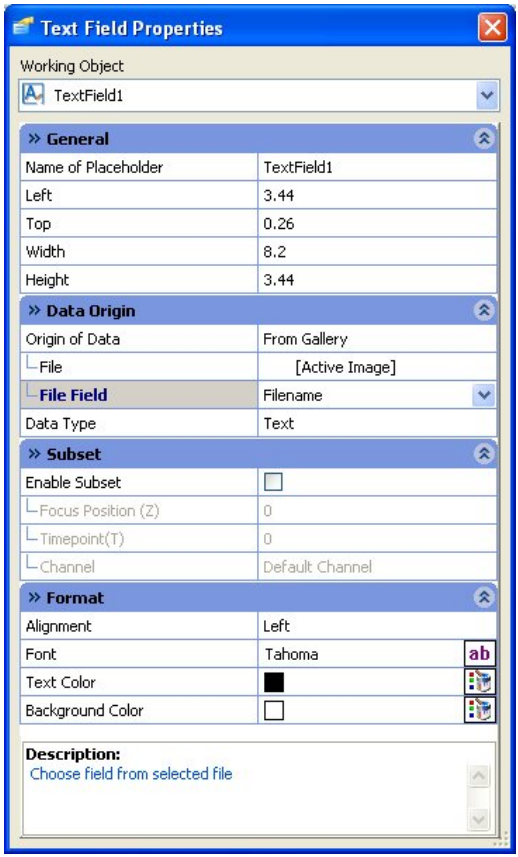


- Click on  to open the property window for the label field.

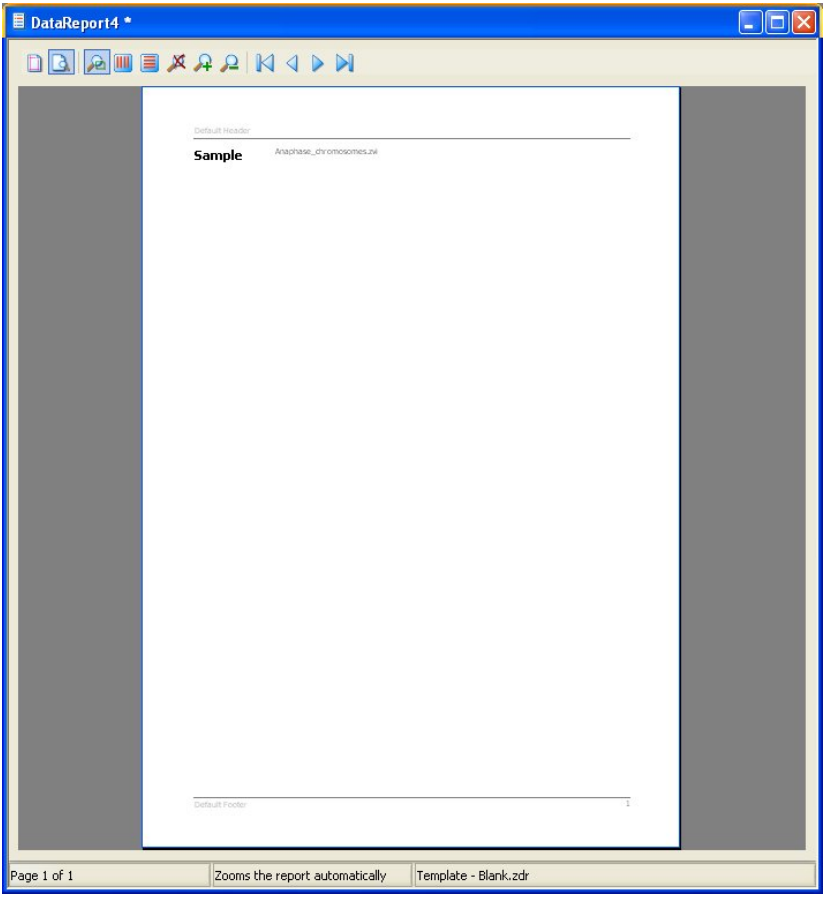
- In the **Format** field, select **14** and **Bold** under **Font**.



- In the **Working Object** field, select the **TextField1** function from the drop-down list box.
- In the **Data Origin** field, select "From Gallery" under **Origin of Date**, "[Active Image]" under **File** and the file "FileName" under **File Field**.
- In the **Format** field, select **12** and **Bold** under **Font**.



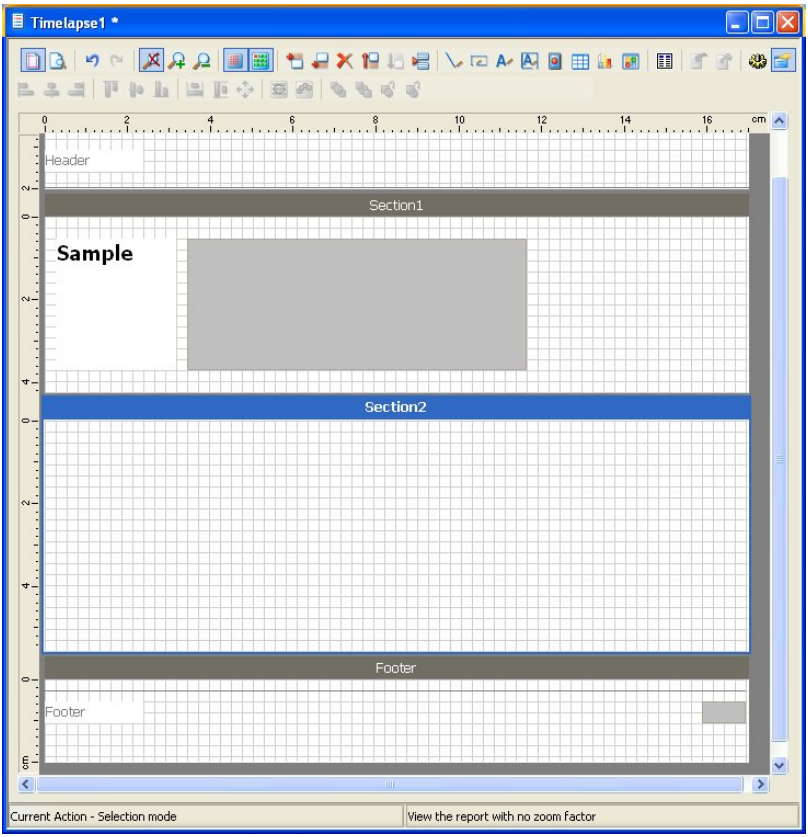
➢ Use the  icon to switch to Preview mode to allow you to check the image name in the text field.



➢ Then use the  icon to return to Edit mode.

➢ Downsize **Section1** of the report template by positioning the mouse on the bottom edge and dragging upwards with the left-hand mouse button depressed.

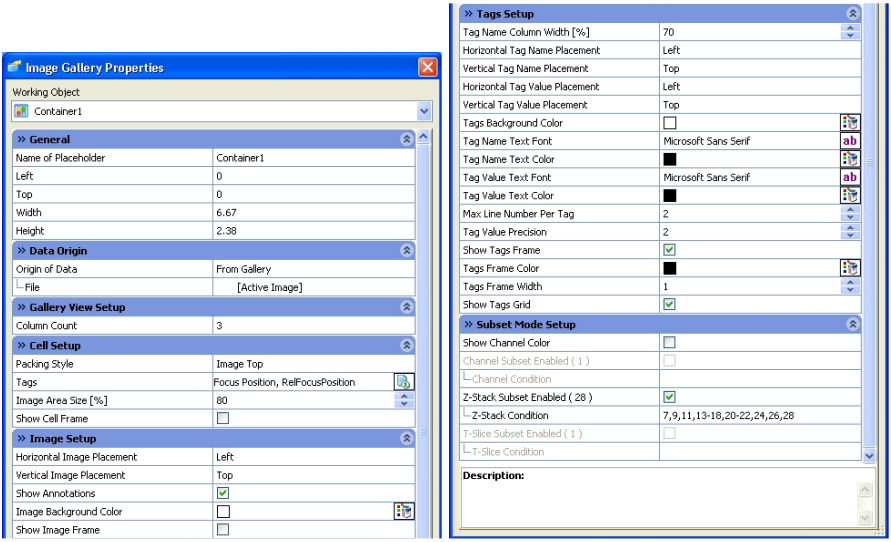
- Click on  and insert a new section below **Section1**. Now increase the size of **Section2** of the report by positioning the mouse on the bottom edge and dragging downwards with the left-hand mouse button depressed.



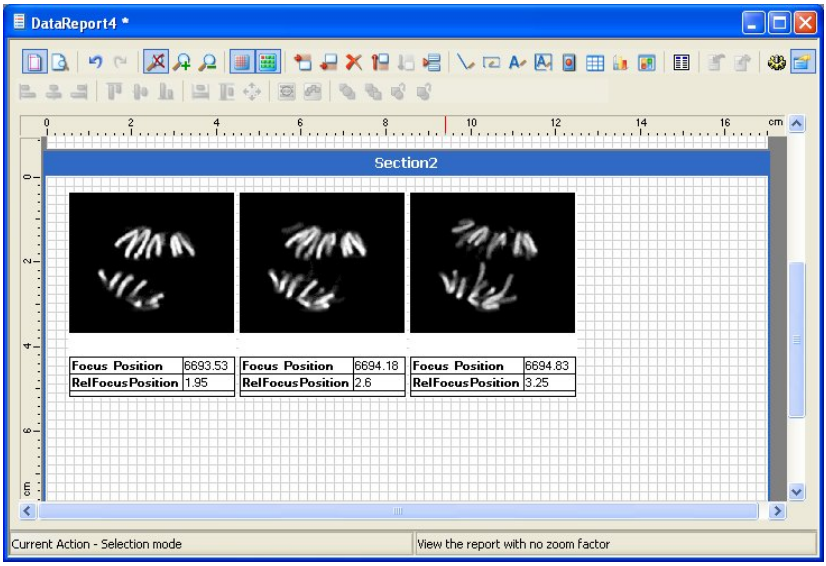
- Click on  and with the left-hand mouse button depressed, drag out an image container.

- In the **Data Origin** field, select "From Gallery" under **Origin of Data** and "[Active Image]" under **File**.

In the **Properties** window enter the following settings:

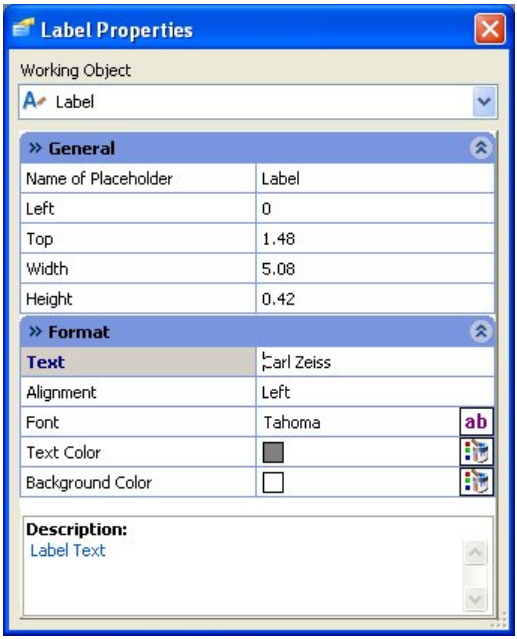


Field **General**	**Left** 0.05, **Top** 0.05, **Width** 6.51, **Height** 2.5
Field **Gallery View Setup**	**Column Count** 3
Field **Cell Setup**	**Image Area Size (%)** 80
	Show Cell Frame off
	Tags FokusPosition, RelFokusPosition
Field **Image Setup**	**Show Image Frame** off
Feld **Tags Setup**	**Tag Name Column Width (%)** 70
	Show Tags Frame on
	Show Tags Grid on
Field **Subset Mode Setup**	**Z-Stack Supset Enabled (28)** on
	Z-Stack Condition 7,9,11,13-18,20-22,24,26,28



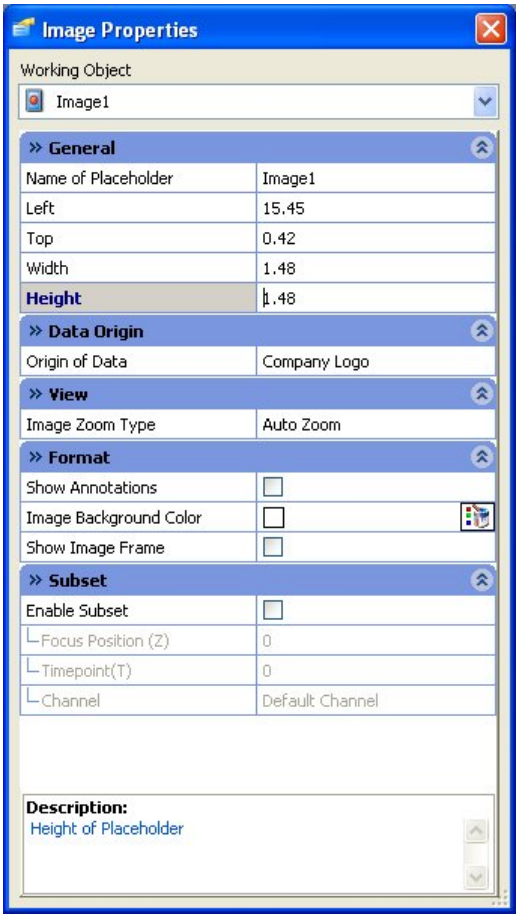
- Now click in the headline on the label field (Header) and enter in the **Properties** window any text (e.g. name of the company) in the **Format** field under **Text**.

- Adapt the size of the region field by dragging out with the left-hand mouse button depressed.



➢ Click on  and insert a new image field to position a company logo.

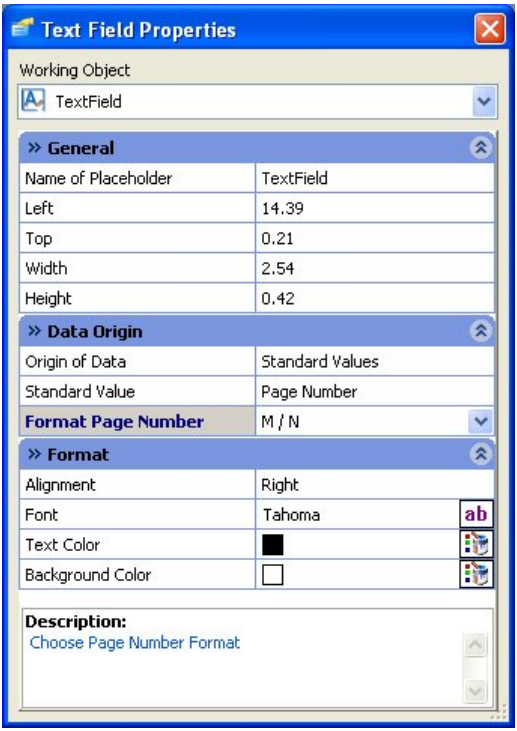
➢ In the **Properties** window enter the following settings:



Field **General**	**Left** 15.4, **Top** 0.5, **Width** 1.5, **Height** 1.5
Field **Data Origin**	**Origin of Data** Company Logo
Field **Format**	**Show Annotations** off
	Show Image Frame off

The logo selected in the **Tools** menu ⇒ **Options** ⇒ **Logo** will be displayed automatically.

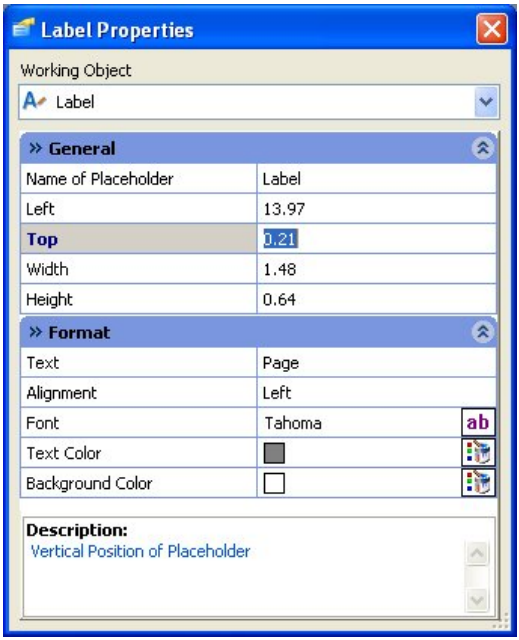
➢ In the footer click on the text field at the right edge and enter the following settings:



Field **General** **Left** 15, **Top** 0.2, **Width** 1.9, **Height** 0.6

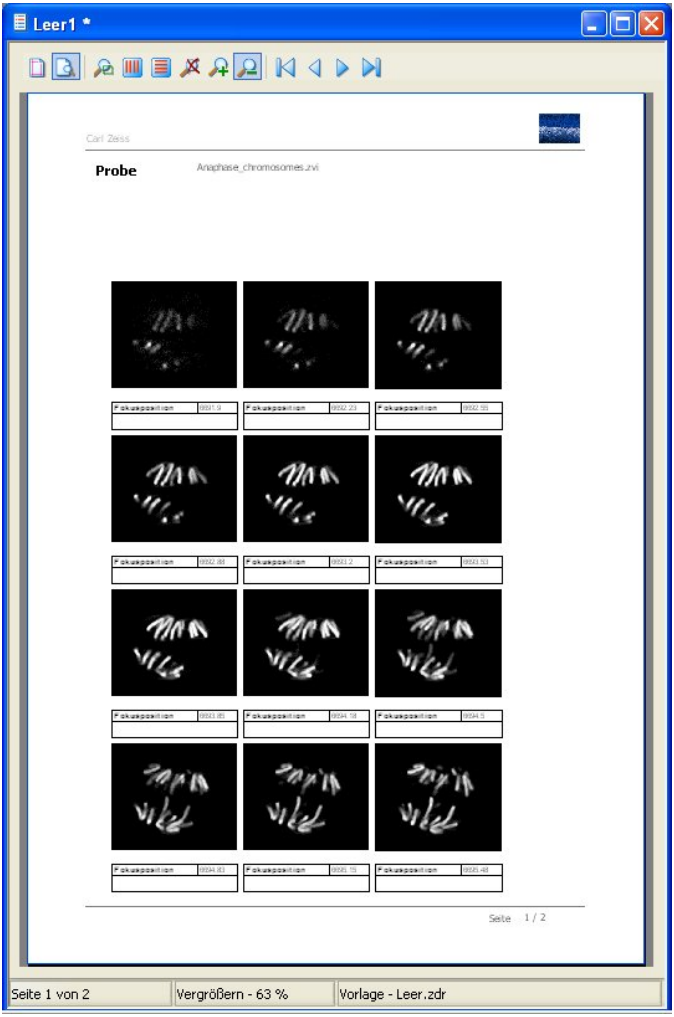
Field **Data Origin** **Format Page Number** M / N

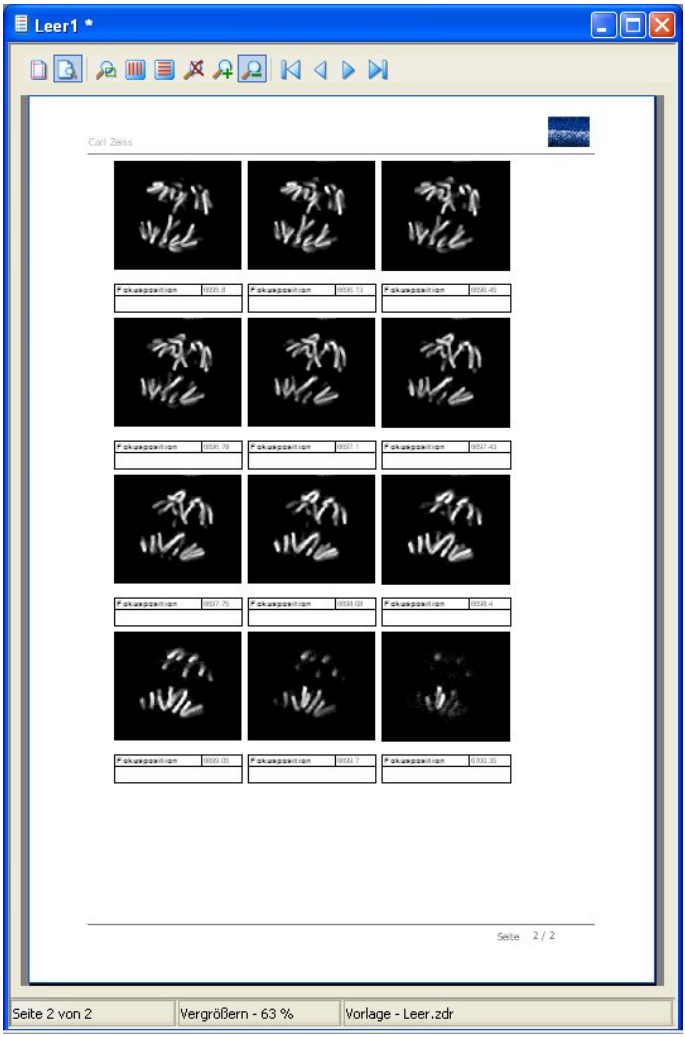
➢ Now click on the label field (**Footer**), positon it close to the text field at the right edge and enter the following settings in the **Properties** window.



Field **General**	**Left** 13, **Top** 0.2, **Width** 1.5, **Height** 0.6
Field **Format**	**Text** Page
	Alignment Left
	Text Color Black

➢ Use the  icon to switch to preview mode to allow you to check the report.





The new report template can be saved and a name entered, e.g. "z-stack", using the **Save Report As Template** function on the **File** menu.

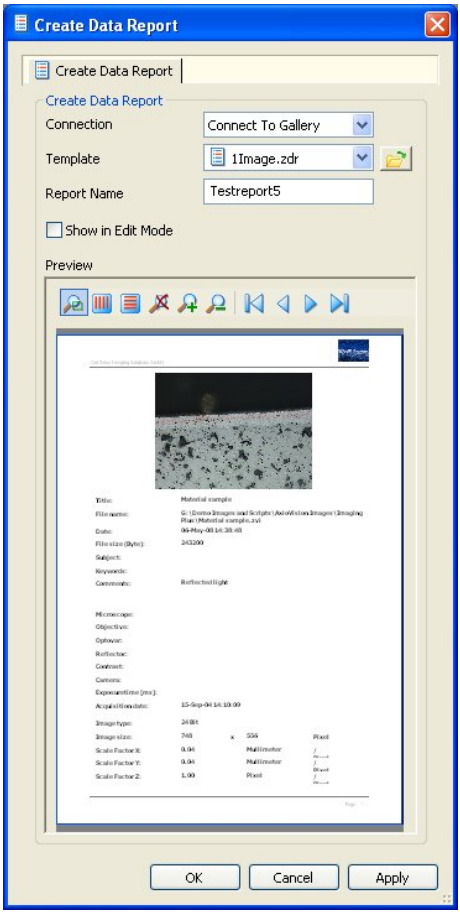
Creating a report with several opened images

The following example will show you how to display automatically in a report several images among each other together with information about the images.

- Use the **Snap** function to acquire any number of images with the camera, or load the images "graphite and ferrite with perlite obj20x 01.zvi", "lamellar graphite obj20x 01.zvi" und "spherolite graphite obj20x 01.zvi" from the Product DVD from the folder *Demo Images and Scripts\AxioVision Images\Graphite* using the **Open image** function on the **File** menu.

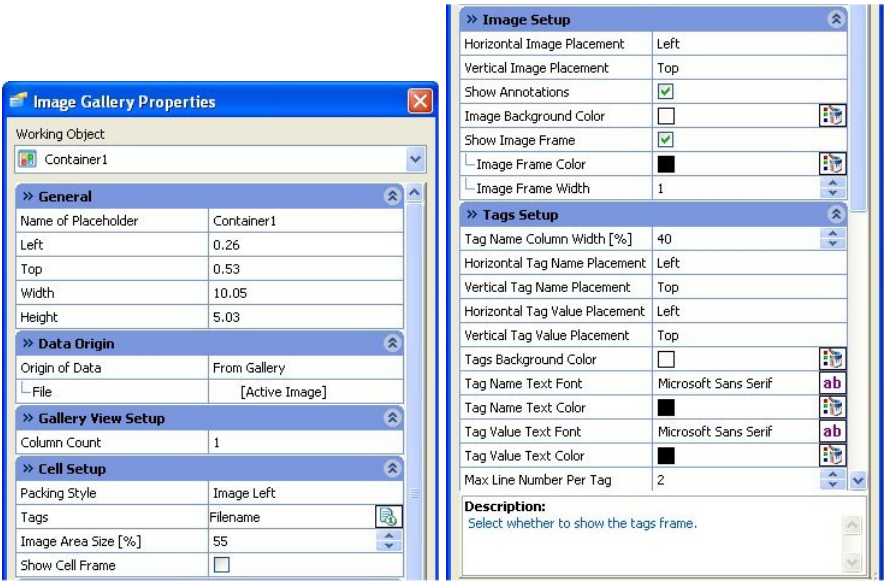
- Select the function **Create Data Report** from the **Evaluation** menu.

- In the dialog window select the function **Connect to Gallery** from the drop-down list **Connection** in the **Create Data Report** field.
- From the drop-down list **Template** select the "1Image.zdr" template.
- In the edit field **Report Name** enter a name for the report to be create, e.g. "Testreport5".
- Click the **OK** button.



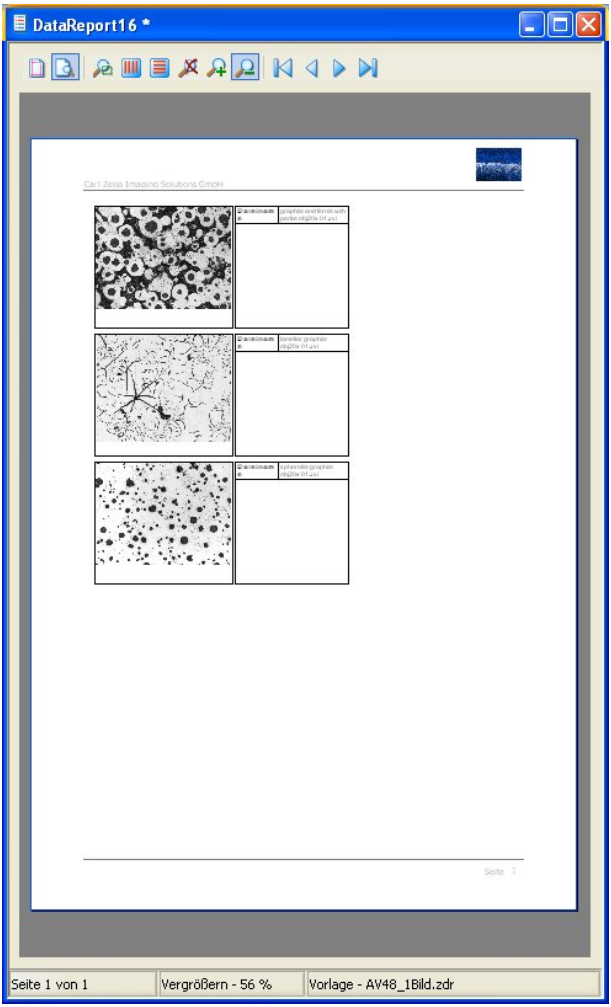
- Select one after another Section3, Section4, Section5 and delete the respective section by clicking on  .
- Click on the image in Section1 and delete this image using the **Del** key.
- Click on  and insert an image container.

In the **Properties** window enter the following settings:



Field **General**	**Left** 0.3, **Top** 0.5, **Width** 10, **Hight** 5
Field **Data Origin**	**Origin of Data** From Gallery
Field **Gallery View Setup**	**Column Count** 1 The number of culumns indicates how many images can be displayed side by side.
Field **Cell Setup**	**Packing Style** Image left **Tags** Filename **Image Area Size (%)** 55 **Show Cell Frame** off
Field **Bildeinstellungen**	**Bildrahmen anzeigen** aus
Field **Einstellungen Felder**	**Feldbezeichnung Spaltenbreite** (%) 40 **Gitternetzlinien für Felder anzeigen** aus

To check the report template click on  to select the Preview mode.



You can save the new report template and entering a name (e.g. "3images") using the **Save Report As Template** in the **File** menu

Creating reports with the extension ZDR, printing them and saving them as a PDF file

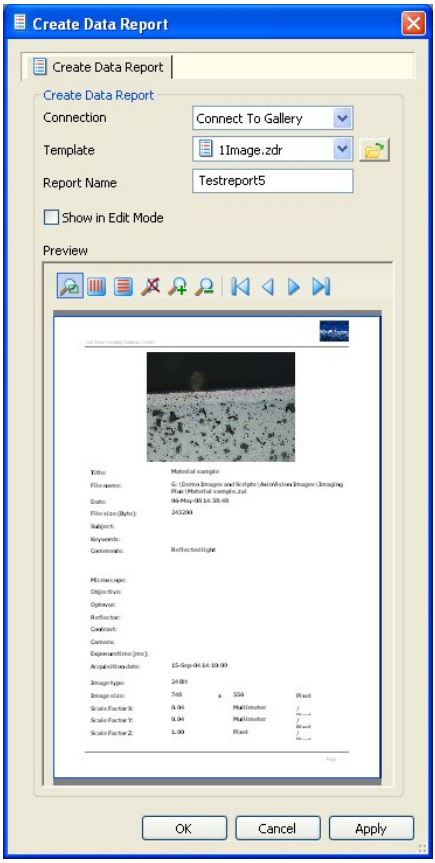
Creating a report for an image

This example shows you how to incorporate an open image into a report and display it with the associated microscope and camera information.

- Use the **Snap** function to acquire an image with the camera, or load the image "Material_sample.zvi" from the Product DVD from the folder *Demo Images and Scripts**AxioVision Images**Imaging Plus* using the **Open image** function on the **File** menu.

- To create a report, select the **Create Data Report** function on the **Evaluation** menu.

- In the dialog window, select the **Connect to Gallery** function via the **Connection** drop-down list box in the **Create Data Report** field.
- In the **Template** drop-down list box select the template **1Image.zdr**.
- In the **Report** text field enter a name for the report to be created, e.g. "Testreport5".
- Click on **OK**.
- The new report is displayed and you can save it using the **Save Report As Template** functions on the **File** menu.



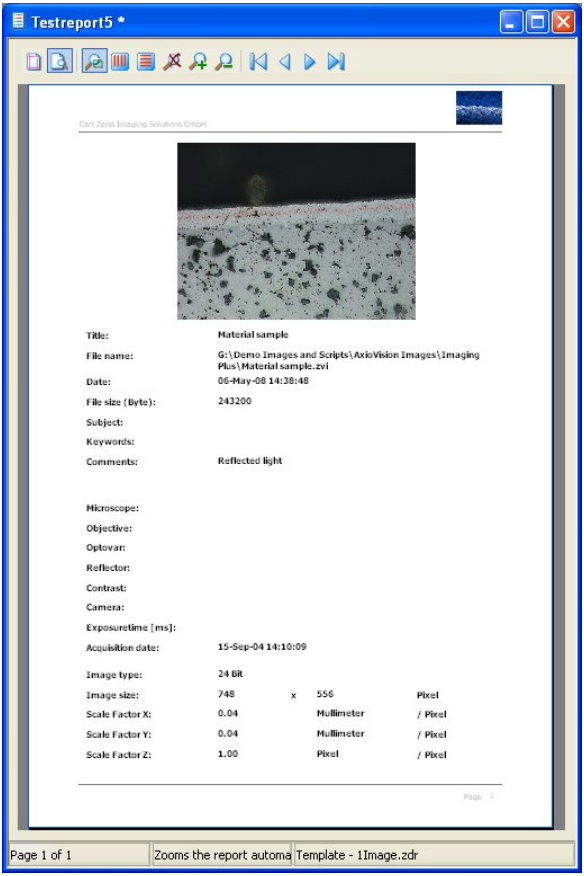
Notes:

- The microscope and camera information only appears if the camera and microscope were connected and configured in MTB2004 prior to image acquisition.
- When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Adopting a new image into an open report template

The following example shows you how to replace an image present in a report with another image.

You can work through this example after you have followed the example in the section "Creating a report for an image".



➢ Use the **Snap** function to acquire an image with the camera, or load the image "Color_grains.zvi" from the Product DVD from the folder *Demo*

Images and Scripts\AxioVision Images\Imaging Plus using the **Open image** function on the **File** menu.

- The current image will be adopted.

- The modified report can be saved using the **Save** or **Save As** functions on the **File** menu.

Printing a report

The following example shows you how to print out a saved report or one that you have just created.

- Activate the report you have created by clicking on it or open a saved report using **File** menu ⇒ **Open Report**.

- From the **File** menu, select the **Print** function to open the standard dialog for printing documents.

- Enter the desired settings and click on **Print** to print the report.

Saving a report as a PDF file

The following example shows you how to create a PDF file from a saved report or one that you have just created. This can be sent and printed out again at any time on any system.

- Activate the report you have created by clicking on it or open a saved report using **File** menu ⇒ **Open Report**.

- From the **File** menu select the **Save to PDF** function.

- Under **File name** enter the desired name and click on **Save** to save the report as a PDF file. This report can then be opened using Adobe Acrobat Reader.

Creating a report for a multichannel fluorescence image

The following example shows you how to display a pseudo color image and the images of the individual fluorescence channels with the corresponding channel names for a 3-channel fluorescence image.

- Use the **Snap** function to acquire an image with the camera, or load the image "CHO-3-channel_fluorescence.zvi" from the Product DVD from the folder *Demo Images and Scripts\AxioVision Images\ Multichannel Fluorescence Z-Stack* using the **Open image** function on the **File** menu.

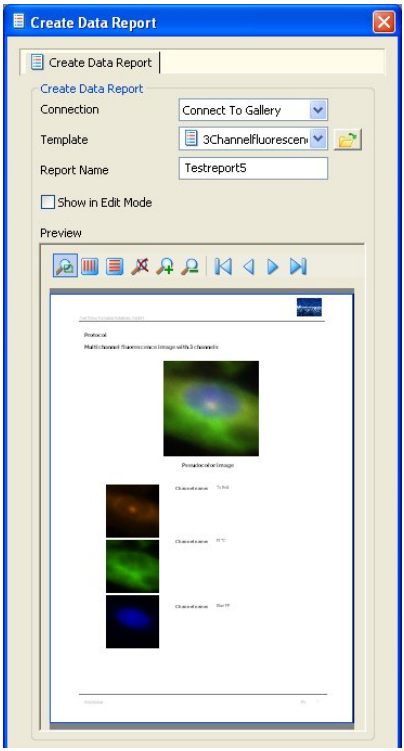
- To create a report, select the **Create Data Report** function on the **Evaluation** menu.

- In the **Create Data Report** field, select *Connect to Gallery* from the **Connection** drop-down list box.

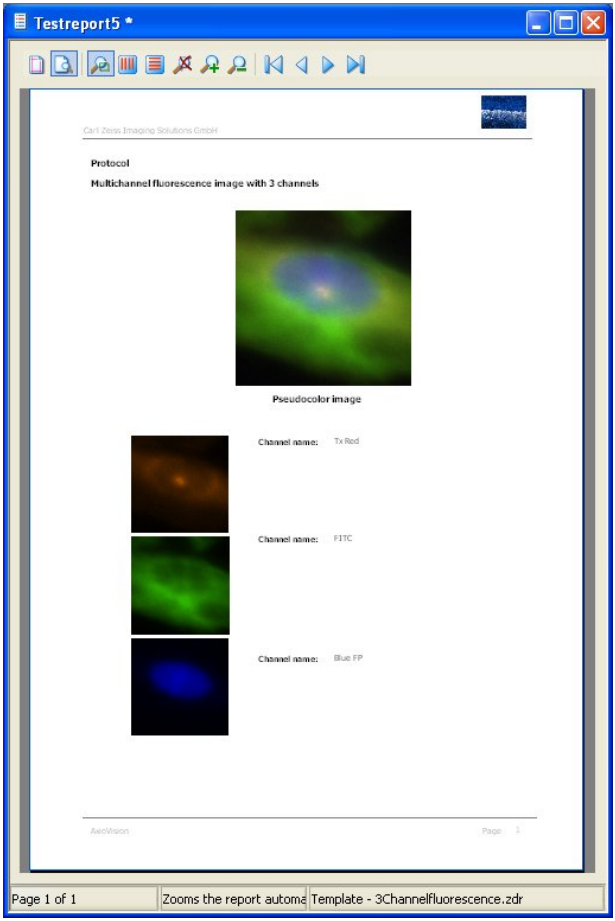
- In the **Template** drop-down list box select the template **3channelfluorescence.zdr**.

- In the **Report Name** field enter a name for the report to be created, e.g. "Testreport4".

- Click on **OK**.



➢ The new report is displayed and you can save it using the **Save** or **Save As** functions on the **File** menu.



Note:

- When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Creating a report for a time lapse image with two fluorescence channels

Note:

- The information relating to the user, company and address only appears if this has been entered on the **Tools** menu ⇒ **Options** ⇒ **ID**.

The following example shows you how to display the images of individual time points for a 2-channel time lapse image.

- Use the **Snap** function to acquire an image with the camera, or load the image "4-Channel-timelapse.zvi" from the Product DVD from the folder *Demo Images and Scripts**AxioVision Images*\ Multichannel Fluorescence Timelapse* using the **Open image** function on the **File** menu.

- To create a report, select the **Create Data Report** function on the **Evaluate** menu.

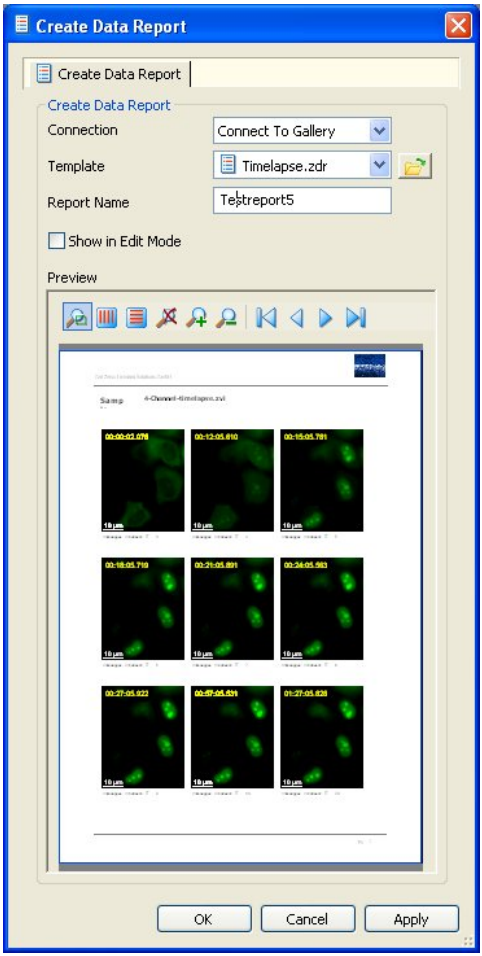
- In the **Create Data Report** field, select *Connect to Gallery* from the **Connection** drop-down list box.

- In the **Template** drop-down list box select the template **Timelapse.zdr**.

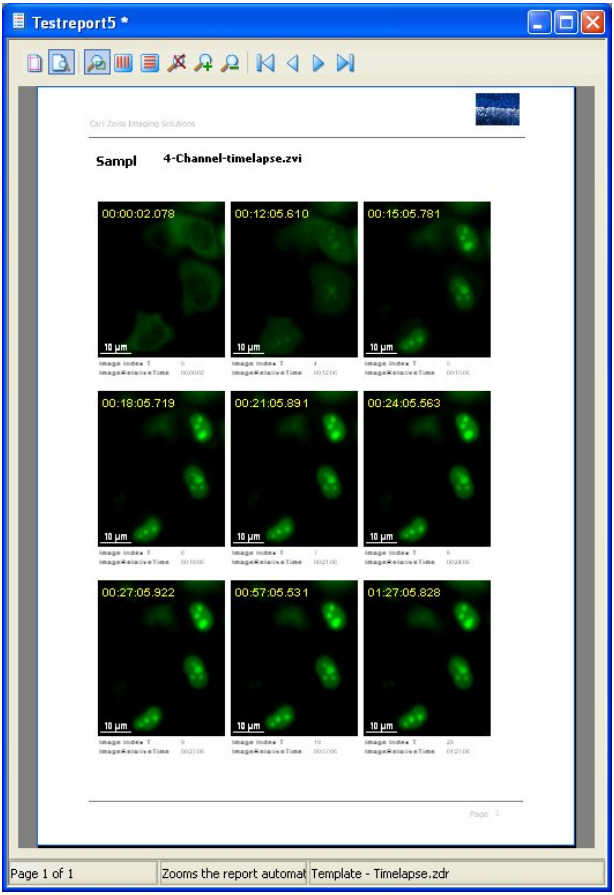
- In the **Report Name** field enter a name for the report to be created, e.g. "Testreport5".

- Click on **OK**.

For the two channels (DIC, FITC) the time points 1,5-10, 20 and 30 will be displayed in the report.



➢ The new report is displayed and you can save it using the **Save Report As Template** function on the **File** menu.



Note:

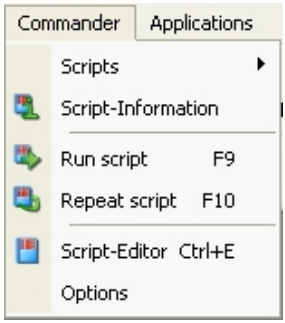
- When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Creating a report with an image, data table and histogram

Sample scripts can be installed from the *Report Script Examples* folder of the Product DVD by launching the **setup.exe** function.

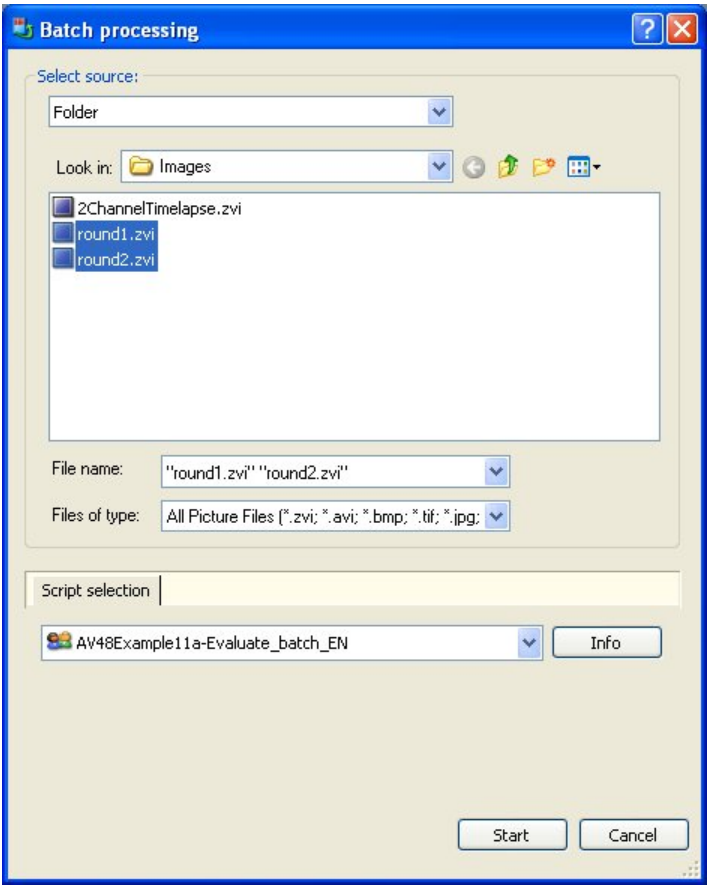
The following example shows you how to present the data of a measurement in a report with output images, a statistics table and a histogram.

- Carry out the measurement for a number of images. To do so, select the **Repeat script** function on the **Commander** menu.

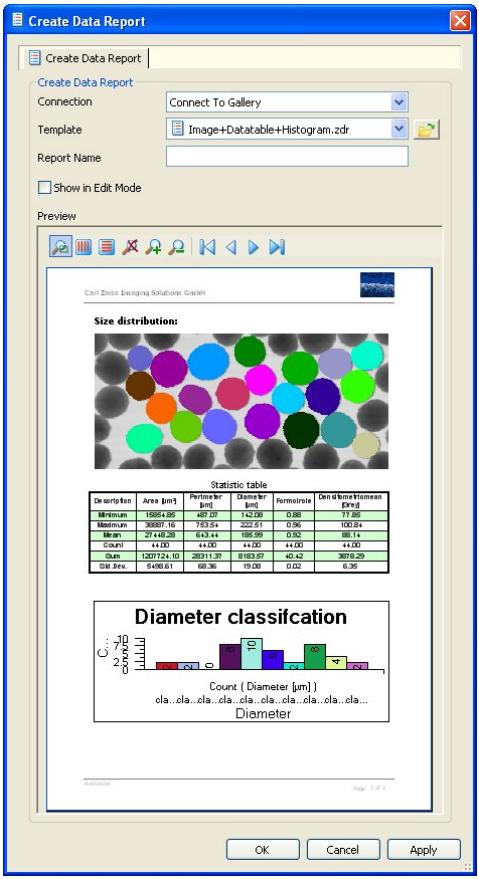


- In the **Select Source** field select from the drop-down list box **Folder**, from the drop-down list box **Look in** the directory *Shared Documents\Carl Zeiss\Images* and mark the two images "round1.zvi" and "round2.zvi".

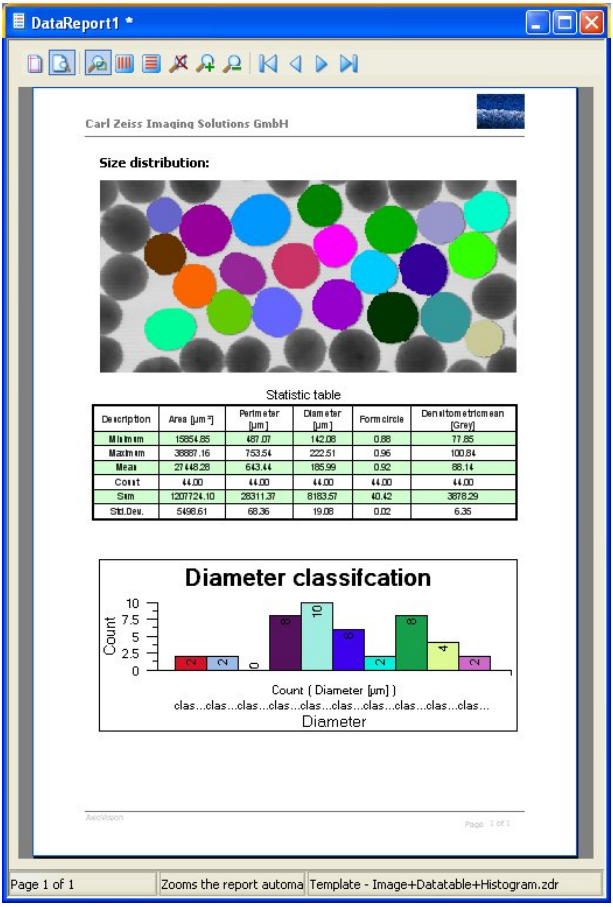
➢ Select in the **Script selection** field the sample script "AV48Example11a-Evaluate_batch_EN.ziscript" and then click on **Start**.



- To create a report, select the **Create Data Report** function on the **Evaluate** menu.
- In the **Create Data Report** field, select *Connect to Gallery* from the **Connection** drop-down list box.
- In the **Template** drop-down list box select the template **Image+Datatable+Histogram.zdr**.
- In the **Report Name** text field enter a name for the report to be created, e.g. "TestreportA".
- Click on **OK**.



The new report is displayed and you can save it using the **Save Report As Template** function on the **File** menu.



Note:

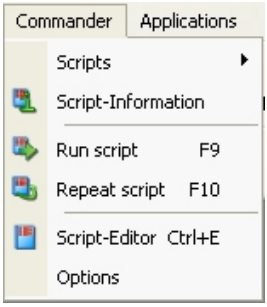
- When the report is saved, the files linked to the gallery (images, data tables) are automatically stored in a folder with the same name as the report.

Creating a report with saved data tables

Sample scripts can be installed from the *Report Script Examples* folder of the Product DVD. by launching the **setup.exe** function.

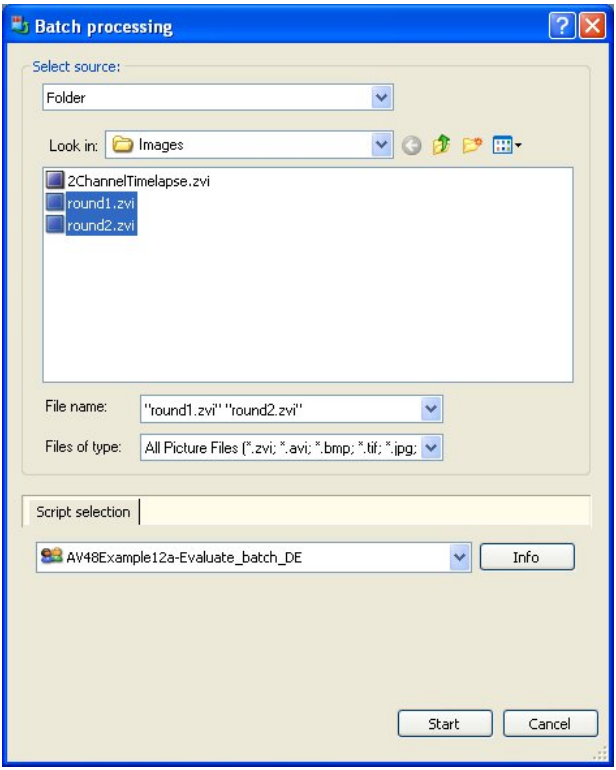
The following example shows you how to perform a measurement with a **Commander** script, display the measurement values that are saved in files in a two-page report and automatically save this report both in ZDR format and as a PDF file.

- Carry out the measurement for a number of images. To do this, select the **Repeat script** function on the **Commander** menu.

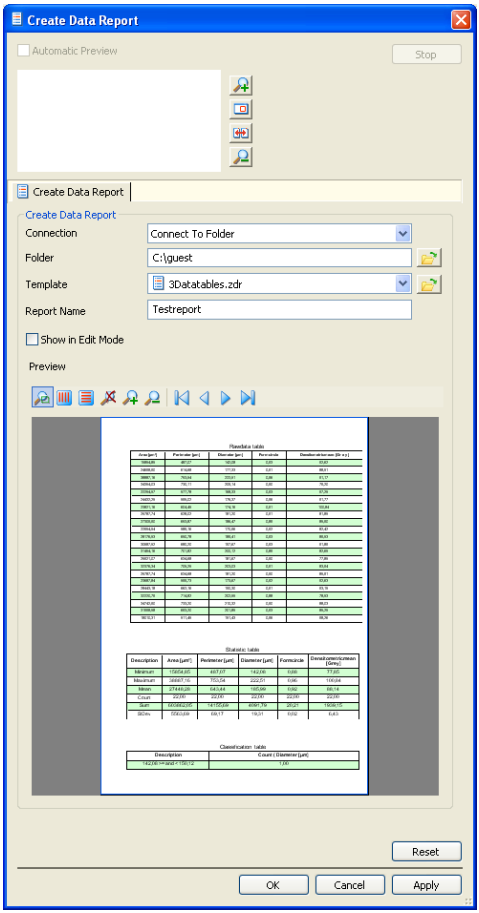


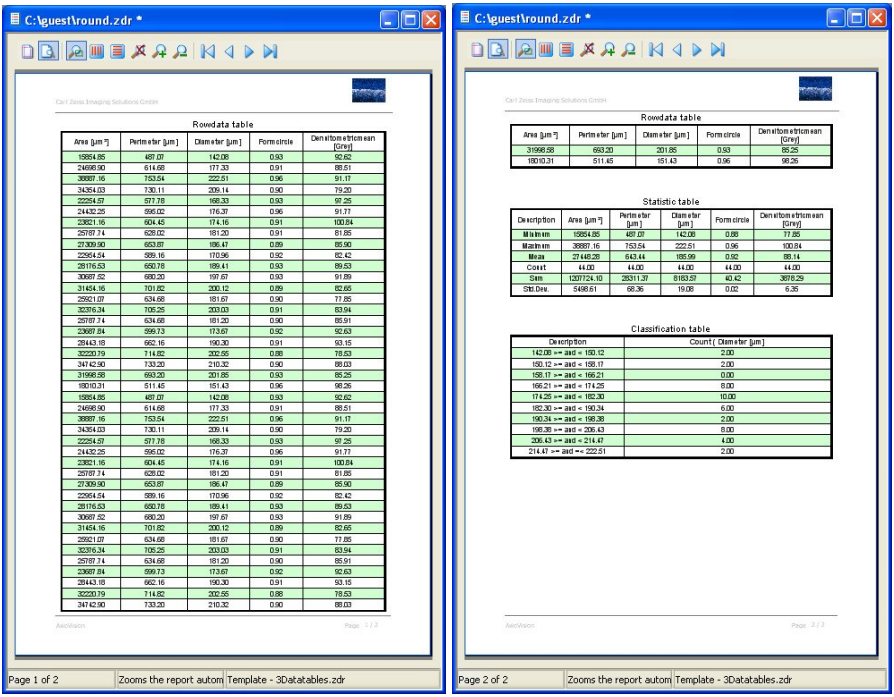
- In the **Select Source** field select from the drop-down list box **Folder**, from the drop-down list box **Look in** the directory *Shared Documents\Carl Zeiss\Images* and mark the two images "round1.zvi" and "round2.zvi".

➢ Select in the **Script selection** field the sample script "AV48Example12a-Evaluate_batch_EN.ziscript" and then click on **Start**.



- The script automatically saves the generated raw-data, class and statistics tables in the directory *C:\guest* and opens the **Create Data Report** dialog.
- In the **Template** drop-down list box select the template **3datatables.zdr**.
- A report with three data tables will be created automatically.
- Click **OK**.





The report can anytime be loaded and printed out from the folder *C:\guest*.

Note:

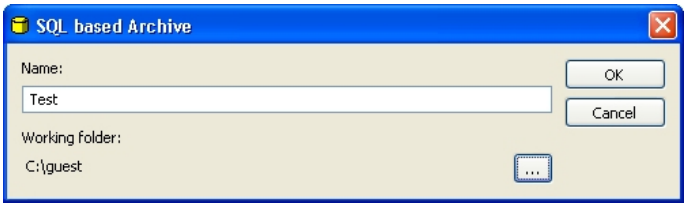
- When you are using a report template that contains file links to a folder, the linked files need to be retained if the report is to be printed out again at a later point in time.

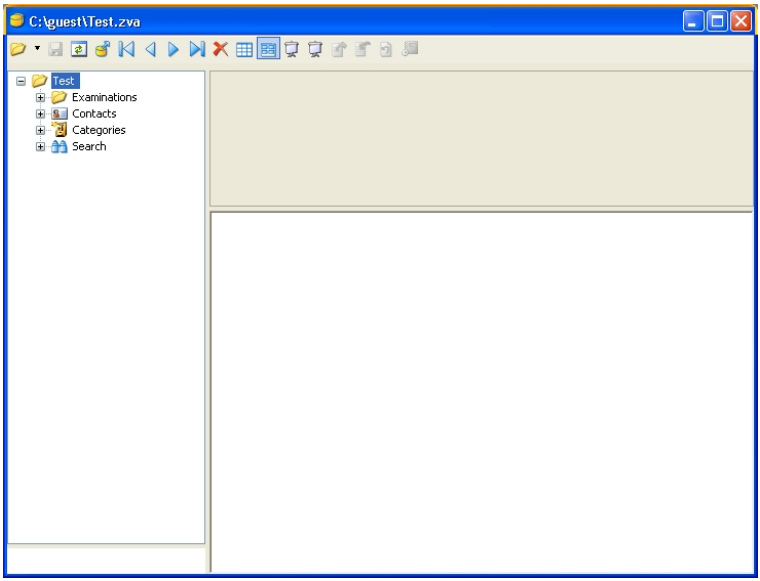
Creating a report with files from an archive

Sample scripts can be installed from the *Report Script Examples* folder of the Product DVD. by launching the **setup.exe** function.

The following example shows you how to display images and measurement values saved in an archive together in a report.

➢ First, use the **New** function on the **File** menu to create a new Asset Archive with the name "Test" in the folder *C:\guest*.

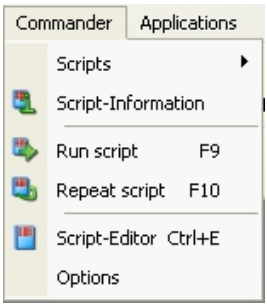




➢ Create a new project with the name "P0001" using the **Add New Project** function on the **Archive** menu, using the template **Default**.

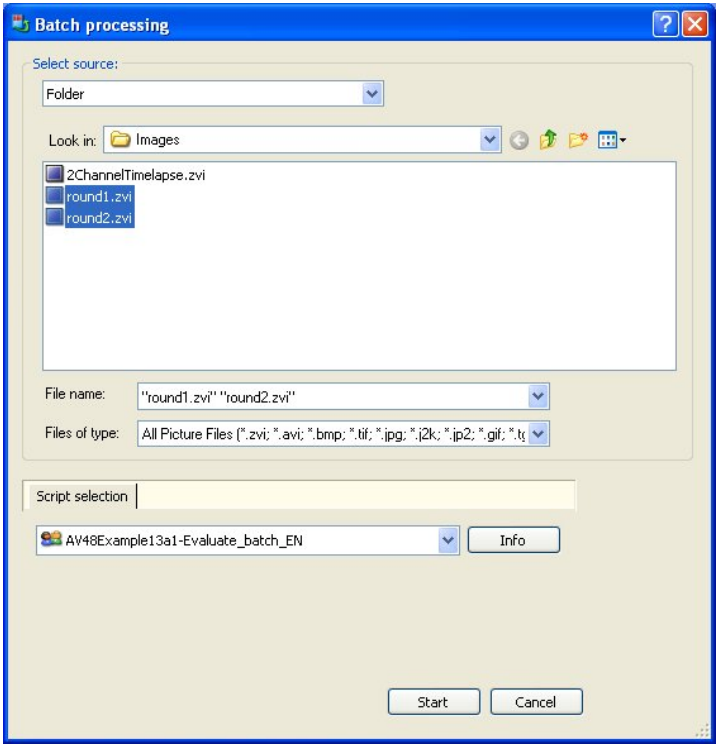


➢ Carry out the measurement for a number of images. To do this, select the **Repeat script** function on the **Commander** menu.



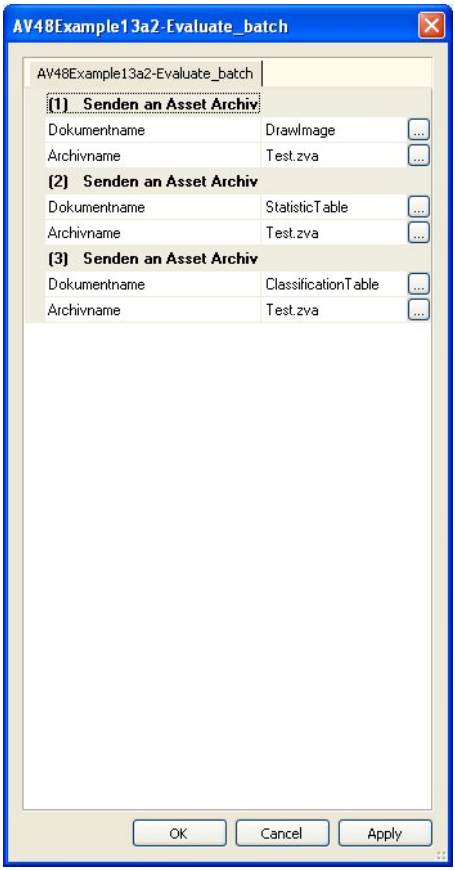
➢ In the **Select Source** field select **Folder** from the drop-down list box, under **Look in** the directory *Shared Documents\Carl Zeiss\Images"* and mark the two images "round1.zvi" and "round2.zvi".

- In the **Script selection** field select the sample script "AV48Example13a-Evaluate_batch_EN.ziscript" and then click on **Start**.



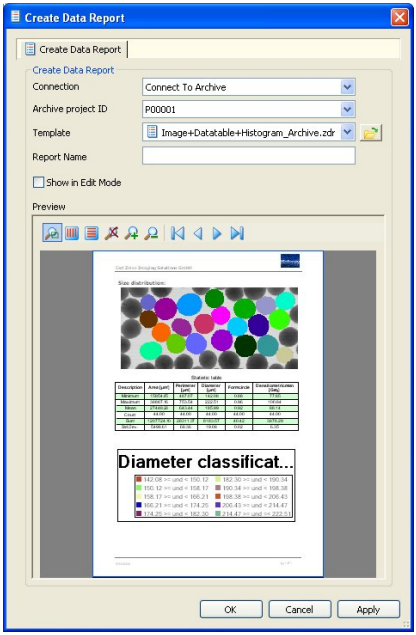
- In the menu **Commander** ⇒ **Scripts** ⇒ **Workgroup** open the file "AV48Example13a2-Evaluate_batch.ziscript"

- Click **Start**.

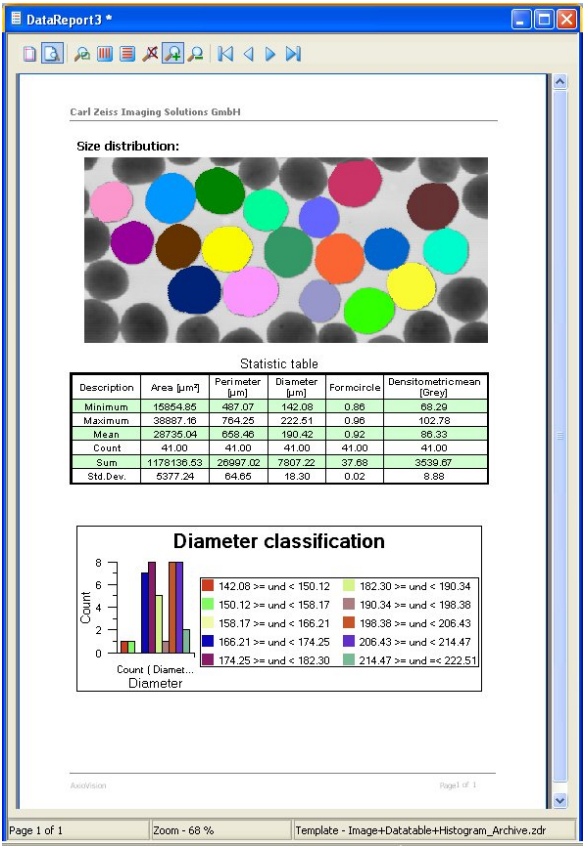


All files, created with the script are saved automatically in the project "00001" of the archive.

- To create a report, select the **Create Data Report** function on the **Evaluate** menu.
- In the **Create Data Report** field, select *Connect to Archive* from the **Connection** drop-down list box.
- In the dialog **Create Report** select in the drop-down list box **Template** "P0001".
- In the **Template** drop-down list box select the template Image+Datatable+Histogram+ Archive.zdr.
- In the **Report Name** text field enter a name for the report to be created, e.g. "TestreportB".
- Click on **OK**.



The new report is displayed and you can save it using the **Save** or **Save As** functions on the **File** menu.



Note:

- When you are using a report template (e.g. Image+Datatable+Histogram+Archive.zdr) that contains file linked to a project within an archive, the linked files need to be retained in this archive/project, if the report is to be printed out again at a later point in time.

6.5 Creating charts

Properties of charts

A chart is used for the graphic display of measurement data that are available in a data table.

In AxioVision it is possible, in the opened data table, to switch directly from the table view to the chart view. The type of chart is defined in the chart view (e.g. bar chart, line chart). From this, it is possible to generate an image or a meta file. The chart settings can be saved as well as loaded.

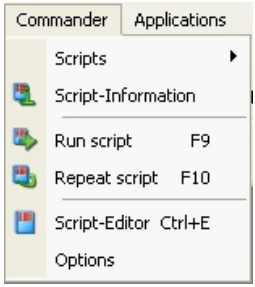
Newly created or modified chart settings are saved in the *Eigene Dateien\Carl Zeiss\Data\Evaluate* user folder or in the *Gemeinsame Dokumente\Carl Zeiss\Data\Evaluate* " work group folder with the file extension *.zdcs.

Creating data tables with a script

Sample scripts can be installed from the folders *Report Script Examples* and *Evaluate Script Examples* of the Product DVD by launching the **setup.exe** function.

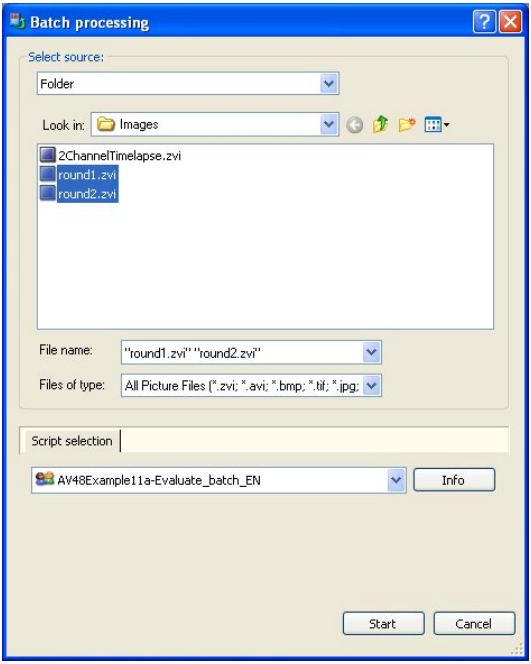
The following example shows you how to generate various data tables (raw data table, statistic table, classification table). You need these tables to carry out the bar charts and line charts, which are descriped on the following pages.

- Carry out the measurement for a number of images. To do so, select the **Repeat script** function on the **Commander** menu.



- In the **Select Source** field select from the drop-down list box the function **Folder**. Select from the drop-down list box **Look in:** the directory *Shared Documents\Carl Zeiss\Images.* Now mark the two images "round1.zvi" and "round2.zvi".

- Select in the **Script selection** field the sample script "AV48Example11a-Evaluate_batch_EN.ziscript" and then click on **Start**.



The result are three data tables:

- Raw data table (OutputTableRegion)
- Statistic table (StatisticTable)
- Classification table (ClassificationTable)

Creating a bar chart

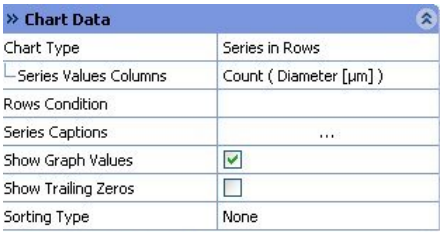
In order to create a bar chart, the data must be available in a classified table. In the example below, the number of measurement values in each class is displayed as an individual bar. First, execute the sample script described in the " Creating data tables with a script" section.

Click on the table **ClassificationTable** and switch-on the **Chart View** at the lower edge of the dialog window.

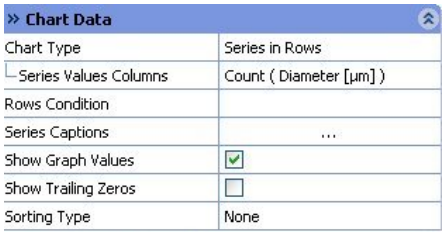
ClassificationTable *

Description	Count (Diameter [µm])
142.08 >= and < 150.12	1.00
150.12 >= and < 158.17	1.00
158.17 >= and < 166.21	0.00
166.21 >= and < 174.25	7.00
174.25 >= and < 182.30	8.00
182.30 >= and < 190.34	5.00
190.34 >= and < 198.38	1.00
198.38 >= and < 206.43	8.00
206.43 >= and < 214.47	8.00
214.47 >= and =< 222.51	2.00

- In the **Chart Data** field, select under **Chart Type** "Series in Rows".
- In the **Series Values Columns** field, select the column with the desired measurement values, in this example "Count (Diameter)". Only the bars of this column will be displayed.

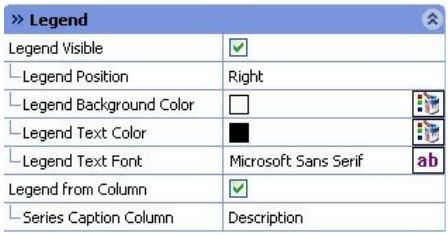


- Activate the check box **Show Graph Values** to display the measurement values in the corresponding bars.

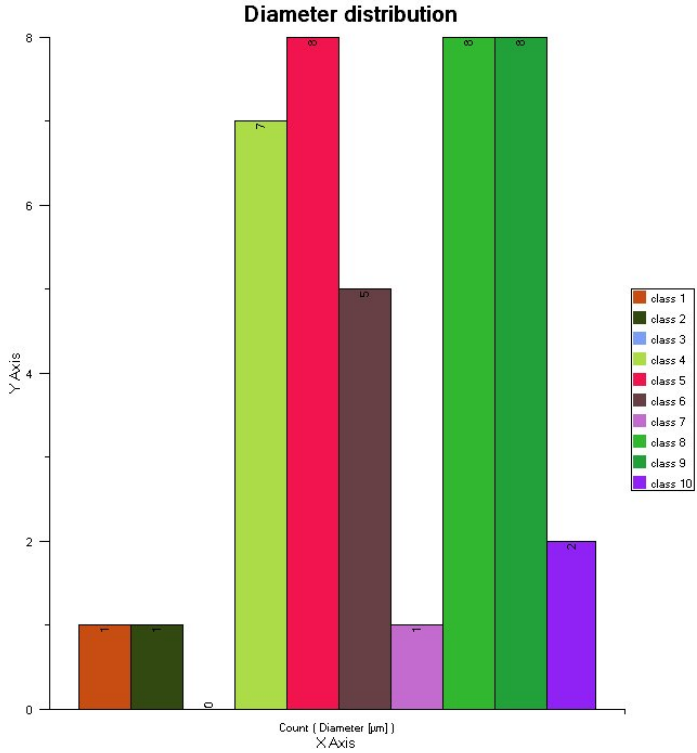


- Under **Series Captions** enter the name of the classes (e.g. Class1, Class2, Class3, …) by double-cklicking on the chart title.

- In the **Legend** field activate the check box **Legend Visible**. The name of each individual class will be displayed in the respective color at the right side of the chart.



- Alternatively you can activate the check box **Legend from Column**. Select under **Series Caption Column** the column "Description. The class boundaries will be adopted.



- Instead of the legends you can display the names of the series in rows below the bars. To do so switch off **Legend Visible** in the **Legend** field.

  In the **X Axis** field switch on **Show Series On X Axis**.

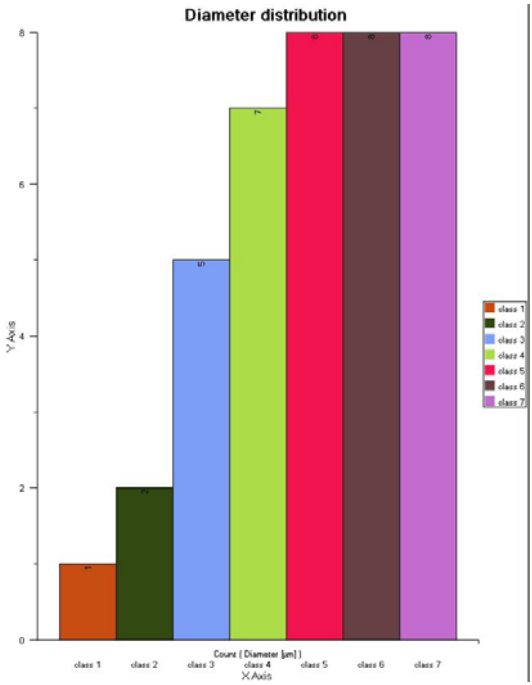
» X Axis	
X Axis Caption Visible	☑
└X Axis Caption	X Axis
└X Axis Text Color	■
└X Axis Text Font	Microsoft Sans Serif
Axis Text Orientation	Horizontal
Show X Values	☑
Show Series On X Axis	☑

- In the **Caption** field unter **Chart Type** enter a title, e.g. "Diameter distribution".

» Caption	
Caption Visible	☑
Chart Caption	Diameter distribution
Caption Position	Top
Caption Alignment	Center
Caption Color	■
Caption Font	Microsoft Sans Serif

- In the **Chart Data** field, under **Rows Condition**, enter e.g. "4-10", if you will display only a part in a sorted
- Under **Sorting Type** select "Ascending" and under **Series Values** Columns select "Count (Diameter)".

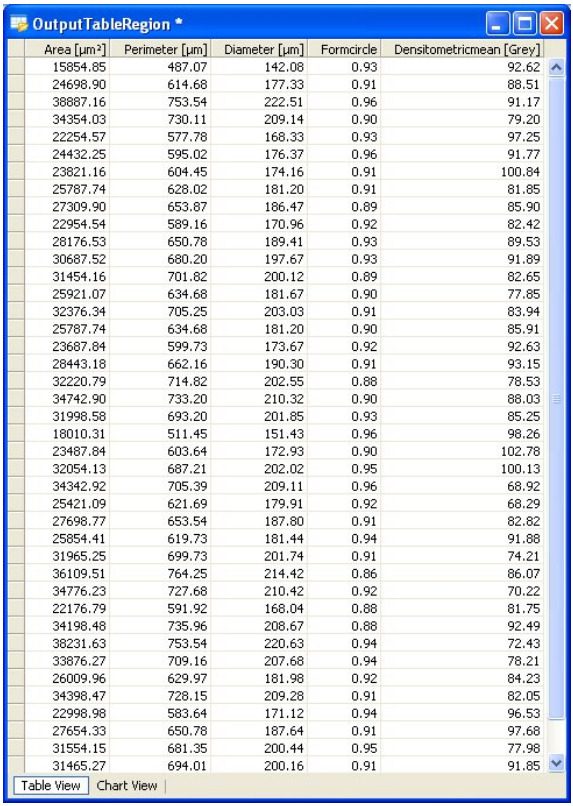
» Chart Data	
Chart Type	Series in Rows
Series Values Columns	Count (Diameter [µm])
Rows Condition	4-10
Series Captions	...
Show Graph Values	☑
Show Trailing Zeros	☐
Sorting Type	Ascending
Column to sort	Count (Diameter [µm])



The bar chart will be displayed. You can save the chart settings by clicking on the **Save As** button.

Creating an X-Y point chart

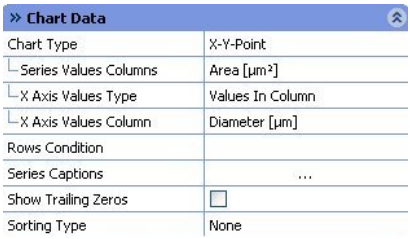
In order to create an X-Y point chart (so-called scattergram), the data must be available in a raw data table. In the example below, area and diameter are plotted against one another. First, execute the sample script described in the "Creating data tables with a script" section.



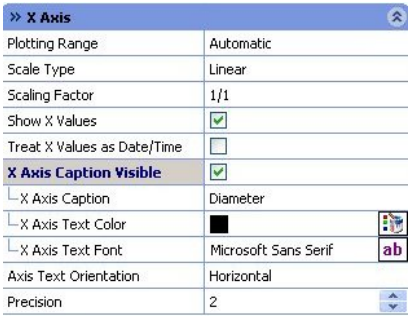
Area [µm²]	Perimeter [µm]	Diameter [µm]	Formcircle	Densitometricmean [Grey]
15854.85	487.07	142.08	0.93	92.62
24698.90	614.68	177.33	0.91	88.51
38887.16	753.54	222.51	0.96	91.17
34354.03	730.11	209.14	0.90	79.20
22254.57	577.78	168.33	0.93	97.25
24432.25	595.02	176.37	0.96	91.77
23821.16	604.45	174.16	0.91	100.84
25787.74	628.02	181.20	0.91	81.85
27309.90	653.87	186.47	0.89	85.90
22954.54	589.16	170.96	0.92	82.42
28176.53	650.78	189.41	0.93	89.53
30687.52	680.20	197.67	0.93	91.89
31454.16	701.82	200.12	0.89	82.65
25921.07	634.68	181.67	0.90	77.85
32376.34	705.25	203.03	0.91	83.94
25787.74	634.68	181.20	0.90	85.91
23687.84	599.73	173.67	0.92	92.63
28443.18	662.16	190.30	0.91	93.15
32220.79	714.82	202.55	0.88	78.53
34742.90	733.20	210.32	0.90	88.03
31998.58	693.20	201.85	0.93	85.25
18010.31	511.45	151.43	0.96	98.26
23487.84	603.64	172.93	0.90	102.78
32054.13	687.21	202.02	0.95	100.13
34342.92	705.39	209.11	0.96	68.92
25421.09	621.69	179.91	0.92	68.29
27698.77	653.54	187.80	0.91	82.82
25854.41	619.73	181.44	0.94	91.88
31965.25	699.73	201.74	0.91	74.21
36109.51	764.25	214.42	0.86	86.07
34776.23	727.68	210.42	0.92	70.22
22176.79	591.92	168.04	0.88	81.75
34198.48	735.96	208.67	0.88	92.49
38231.63	753.54	220.63	0.94	72.43
33876.27	709.16	207.68	0.94	78.21
26009.96	629.97	181.98	0.92	84.23
34398.47	728.15	209.28	0.91	82.05
22998.98	583.64	171.12	0.94	96.53
27654.33	650.78	187.64	0.91	97.68
31554.15	681.35	200.44	0.95	77.98
31465.27	694.01	200.16	0.91	91.85

Click on the table **OutputTableRegion** and switch on the **Chart View** at the lower edge of the dialog window.

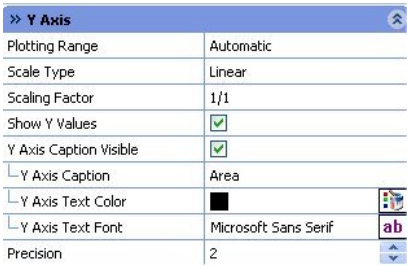
- In the **Chart Data** field select under **Chart Type** "X-Y-Point".
- Under **Series Values Columns** select this column, which displays the measurement values on the y axis, in this example "Area".
- Under **X Axis Values Type** select "Values in Column".
- Under **X Axis Values Column** select this column, which displays the measurement values on the x axis, in this example "Diameter".



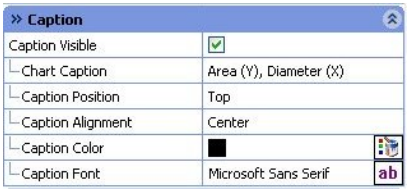
- In the **X Axis** field activate the check boxes **X Axis Caption Visible** and **X Axis Caption**.
- Under **X Axis Caption** enter "Diameter".

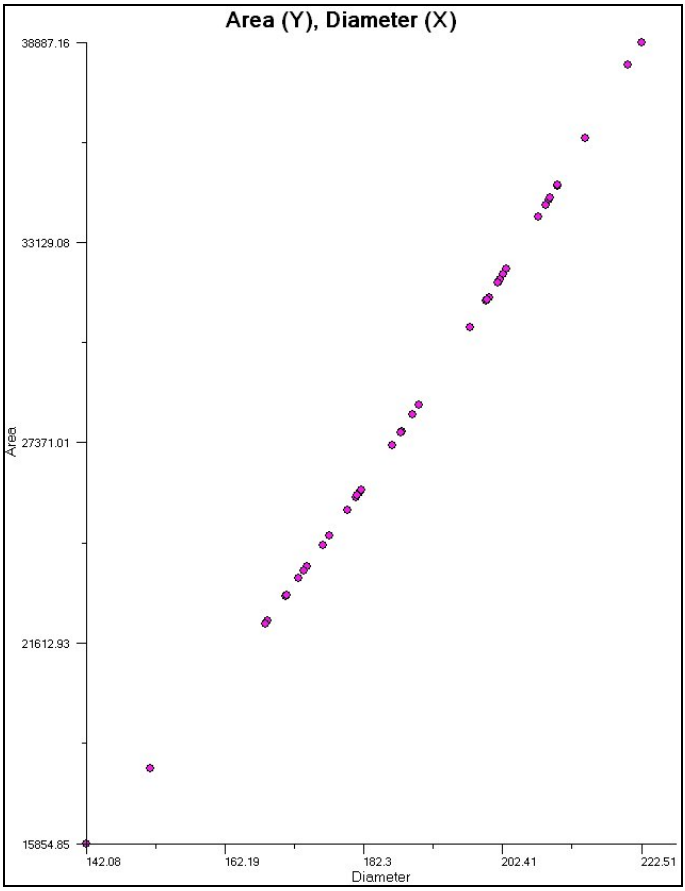


- In the **Y Axis** field activate the check boxes **Y Axis Caption Visible** and **Y Axis Caption**.
- Under **Y Axis Caption** enter "Area".



- In the **Caption** field enter a title under **Chart Caption**, e.g. "Area (Y), Diameter (X)".



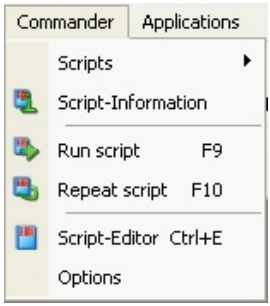


The X-Y point chart will be displayed. You can save the chart settings by clicking on the **Save As** button.

Creating a X-Y line chart

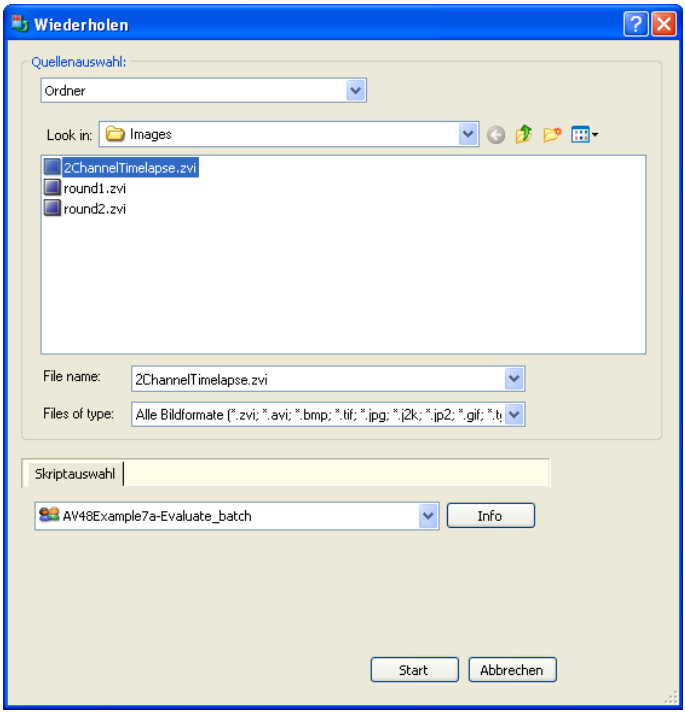
To create a X-Y line chart the data should be available in a raw data table. In the following example area the mean gray value is plotted against the time points to demonstrate the changing of the fluorescence intensity of the cells over the time.

- Carry out the measurement for a time series. To do so, select the **Repeat script** function on the **Commander** menu.

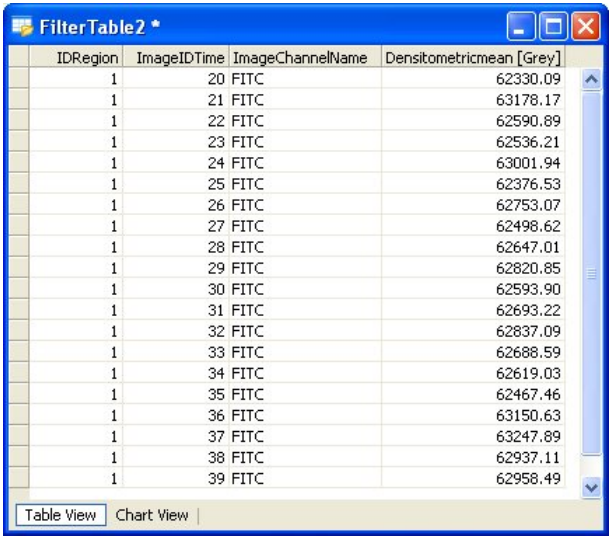


- In the **Select Source** field select from the drop-down list box **Folder**, from the drop-down list box **Look in** the directory *Shared Documents\Carl Zeiss\Images* and mark the image "2ChannelTimelapse.zvi".

➢ Select in the **Script selection** field the sample script "AV48Example7a-Evaluate_batch_EN.ziscript" and then click on **Start**.



The result is a filtered table (FilterTable2).



FilterTable2 *

IDRegion	ImageIDTime	ImageChannelName	Densitometricmean [Grey]
1	20	FITC	62330.09
1	21	FITC	63178.17
1	22	FITC	62590.89
1	23	FITC	62536.21
1	24	FITC	63001.94
1	25	FITC	62376.53
1	26	FITC	62753.07
1	27	FITC	62498.62
1	28	FITC	62647.01
1	29	FITC	62820.85
1	30	FITC	62593.90
1	31	FITC	62693.22
1	32	FITC	62837.09
1	33	FITC	62688.59
1	34	FITC	62619.03
1	35	FITC	62467.46
1	36	FITC	63150.63
1	37	FITC	63247.89
1	38	FITC	62937.11
1	39	FITC	62958.49

Table View | Chart View

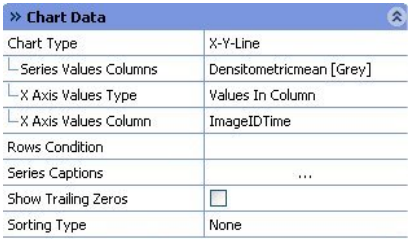
- Click on the table **FilterTable2** and switch on the **Chart View** at the lower edge of the dialog window.

- In the **Chart Data** field, select "X-Y-Line" under **Chart Type**.

- Under **Series Values Columns** select the column, which displays the measurement values on the y axis, in this example " Densitometricmean".

- Under **X Axis Values Type** select "Values in Column".

- Under **X Axis Values Column** select the column, which displays the measurement values on the x axis, in this example " ImageIDTime".



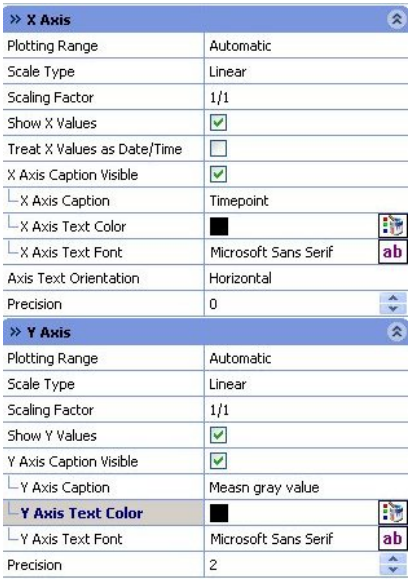
» Chart Data

Chart Type	X-Y-Line
Series Values Columns	Densitometricmean [Grey]
X Axis Values Type	Values In Column
X Axis Values Column	ImageIDTime
Rows Condition	
Series Captions	...
Show Trailing Zeros	☐
Sorting Type	None

- In the **X Axis** and **Y Axis** fields switch on the check boxes **Show X (Y) Values** and **X (Y) Axis Caption Visible**.

- In the **X Axis** field enter "Timepoint" under **X Axis Caption** and in the **Y Axis** field enter "Mean gray value" under **Y Axis Caption**.

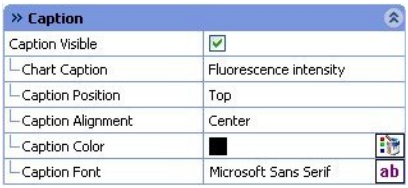
- In the **X Axis** field enter the value "0" under **Precision**, to display the timepoints as integer values.

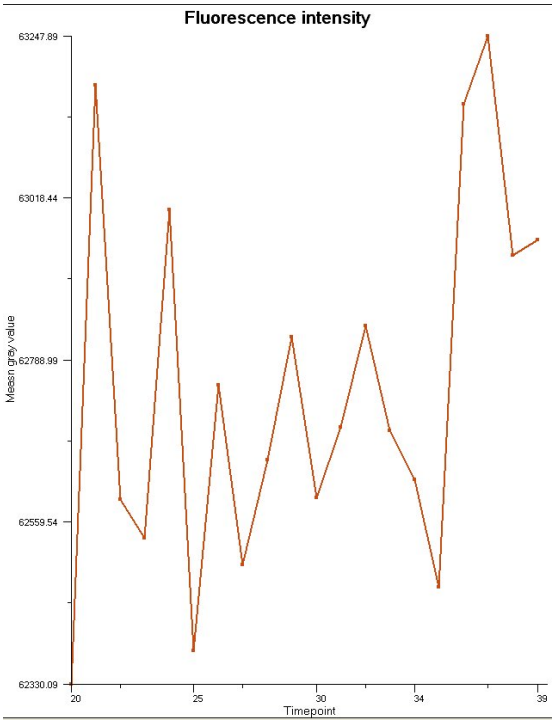


- In the **Legend** field switch off the function **Legend Visible**.



- In the **Caption** field enter a title under **Chart Caption**, e.g. "Fluorescence intensity". The mean gray values of each individual cell at each timepoint will be displayed.





The X-Y line chart will be displayed. You can save the chart settings by clicking on the **Save As** button.

Saving and resetting chart settings

- To save chart settings click on the **Save As** button in the **Chart View** of the data table. Enter a name for the chart, e.g. "line chart".
- To reset chart settings click on the **Reset** button.
- To import chart settings, use the  icon in the report's design mode.

Loading and adapting chart settings for any table

- First, generate a data table containing several measurement values, e.g. by measuring an image interactively. Click on the **Load** button in the chart view.

Alternatively you can also call up the **Create Data Chart** function from the **Evaluate** menu.

- Select "Use External Settings" under the **Data settings type** parameter.
- Then select your file containing the settings under the **External chart settings file** parameter.
- Now make the necessary adaptations.

Creating an image from a chart

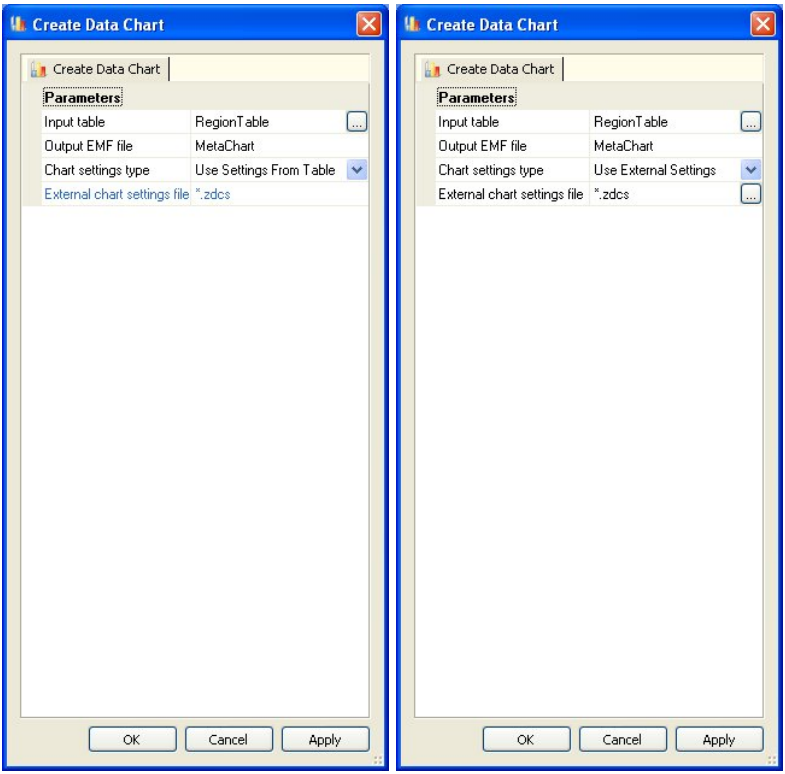
- In the **Chart View** of the data table click on the **Create Image** button and enter a name. The chart will be saved in bmp format.

Exporting a chart as a meta file

- In the chart view of the data table, click on the **Create EMF** button and enter a name. You can choose between two formats: EMF (enhanced meta file) and WMF (Windows meta file).

Alternatively you can also call up the **Create Data Chart** function from the **Evaluate** menu.

- Select "Use External Settings" under the **Chart settings type** parameter to select your file under **External chart settings file** or select "Use Settings From Table" if you wish to adopt the settings from the table.


Create Data Chart
Create Data Chart
Parameters
Input table
RegionTable
Output EMF file
MetaChart
Chart settings type
Use Settings From Table
External chart settings file
*.zdcs
OK
Cancel
Apply
Create Data Chart
Create Data Chart
Parameters
Input table
RegionTable
Output EMF file
MetaChart
Chart settings type
Use External Settings
External chart settings file
*.zdcs
OK
Cancel
Apply

7 Documentation

7.1 General

Documentation is probably the most important part of your work. This term is often used to cover a number of different procedures:

In one case it may simply be a question of saving image files under a definable name; in others it may be a question of storing additional information with the images, so that the conditions of acquisition can be traced and recreated identically, or so that individual images can be found again quickly using this additional information.

Even in the basic version of AxioVision, the **Image Browser** offers a wide range of options that enable you to manage your images efficiently. Additionally you can save your images in a folder-based archive.

Via the AxioVision module **Asset Archive**, you can archive even extremely large quantities of data.

Note:

- Important information about "Saving" can be found in chapter 8.2 "User and File Administration".

7.2 AxioVision ZVI - the Image Format for Digital Microscopy

Advantages of the ZVI format

The AxioVision ZVI image file format is central to documentation with AxioVision. Compared to external formats, such as BMP, JPG, TIF etc., the ZVI format offers you numerous advantages:

- **Saving without loss of quality.** AxioVision also saves the current display settings for brightness, contrast and gamma value, without modifying the original data. You can, therefore, reactivate the original display of the image at any time. For external formats, you either have to go through the laborious process of finding the relevant display again or

the pixel information has to be saved in modified form. However, in this case the original data are lost.

- **Incorporation of additional data** (e.g. microscope and camera configurations, comments, information on the sample etc.).

- **Separate saving of image and annotations.** This means that annotations can also be changed or deleted at a later time. When external formats are used, annotations have to be "burnt" into the image. However, they can then no longer be deleted or changed. In this case, of course, the pixel data beneath the annotations are overwritten.

- **Incorporation of measurement data for interactive measurements** into the image file.

- **Management of all data in a single file.** This makes it much simpler to handle the files, particularly when working with multidimensional images. If you use external formats, every single image has to be saved separately. This can quickly result in several hundred individual image files.

None of the above features are available with other formats. In short, the ZVI format has been specially developed for use in the field of digital microscopy. This is not the case with any of the other formats.

Saving 2D images

AxioVision gives you the option, for images saved in external formats, saving all additional information (additional data, annotations, interactive measurements etc.) separately from the actual image data. An additional file is created, containing these data, for each image. The format of the file is XML. This XML file is saved in the same folder as the image. The name of the XML file is composed of the name of the image + the extension + "XML". Therefore, if the saved image is called "Testimage.jpg", a file is generated with the name "Testimage.jpg. meat.xml", which then contains all the additional information.

When you open an image with AxioVision that has been saved in an external format, the system will always check automatically whether a file containing the corresponding additional data is present. If one is, these data are read out and displayed accordingly. Of course, this only applies if you are working in AxioVision. When you open an image such as this in a different image processing program, the image is displayed without the additional information.

To save an image with a format larger than 8 bits such that it can be displayed in another application program just like it is displayed in AxioVision, we recommend that you activate the **Apply display mappings** check box when saving the image (**File ⇒ Save As**). Please note, however, that this will change the pixel values.

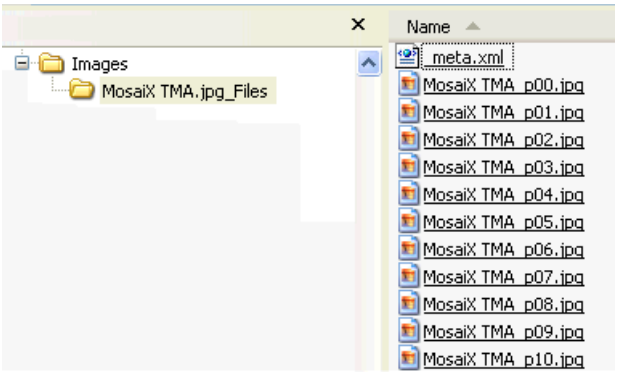
Saving multidimensional images

In principle, multidimensional images can be saved using the standard function for saving images (**File ⇒ Save As**). Images can also be saved in external formats using this function. For multidimensional images, however, all single images then have to be saved in separate files. In order to retain an overview, only the first image is saved under the specified name and folder for each multidimensional image. A separate subfolder is created for the single images that belong with it:



A MosaiX image saved as a jpg file. The single images are saved in the *MosaiX TMA.jpg_Files* folder.

An XML file with the additional information is also saved in the folder with the single images. The XML file containing the additional information always bears the name "_Meta.xml":



Subfolder containing single images and XML file

When you open an image with AxioVision that has been saved in an external format, the system will always check automatically whether such a folder is present. If one is, the single images are read out and combined to form a multidimensional image. In the course of this process, the information from the "_meta.xml" file is analyzed.

Note:

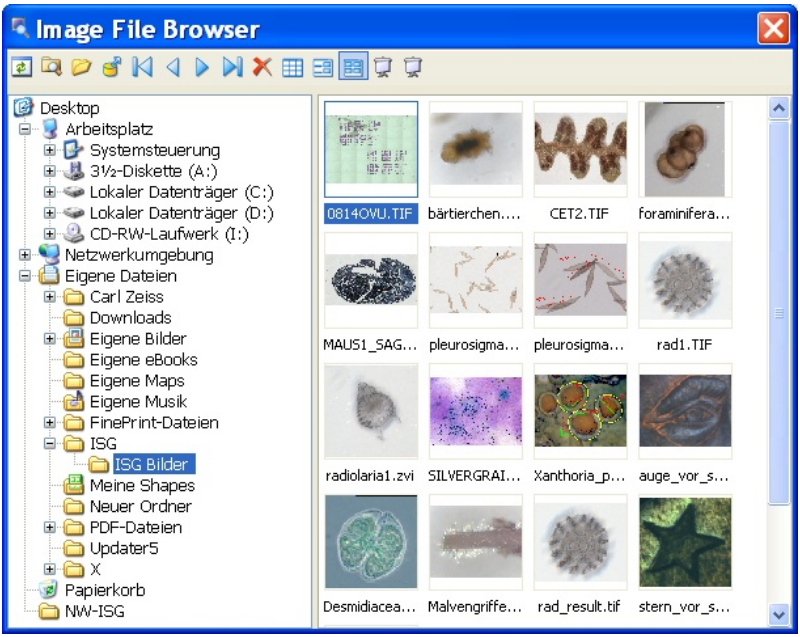
- The **File ⇒ Export** function offers special functions for saving multidimensional images. These include, for example, functions for generating video formats or special functions for saving multichannel fluorescence images.

7.3 Managing Images with the Image File Browser

The Image File Browser allows you to display the images contained in the folders on all of your PC's drives as a gallery or list, or with a form for entering additional data; you are able to search for and load images and view a slide show. The image browser is a convenient, easy-to-use tool to help you manage your images.

To open the image browser, select **File ⇒ Image File Browser**.

The Image File Browser window



The left-hand side shows your system's folder structure, in the familiar Microsoft Windows Explorer format.

The **Image File Browser** can be operated fully using the functions on the toolbar.

If you select a particular folder, all the image files it contains are displayed automatically in the selected mode (here **Gallery** mode).

The functions of the Image File Browser toolbar





Refreshes all views.



Opens the dialog window **Scan drive**.



Creates a new folder or edits an existing one.



Loads the selected image(s) from the archive.



Moves to the first record.



Moves to the previous record.



Moves to the next record.



Moves to the last record.



Deletes the currently selected record.



Selects the Data Grid mode.



Selects the Form mode.



Selects the Gallery mode.

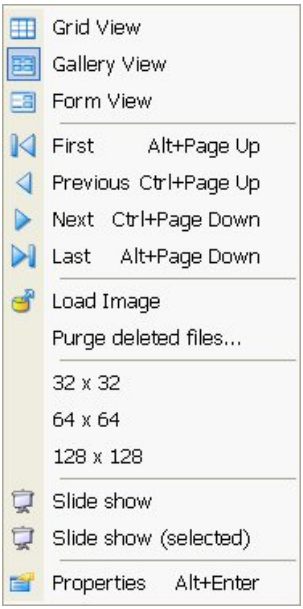


Runs the slide show using all records. To stop the slide show, press the **Esc** key or right-click in the image and deselect **Slide show**.



Runs the slide show using selected records. To stop the slide show, press the **Esc** key or right-click in the image and deselect **Slide show**.

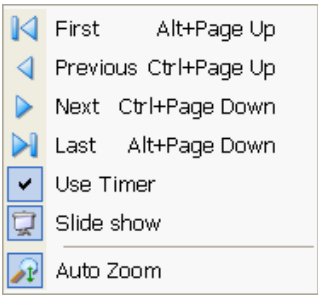
Context menu of the Image File Browser



 Grid View	Displays the image files contained in the selected folder in grid view.
 Gallery View	Displays the image files contained in the selected folder in gallery view.
 Form View	Displays the image files contained in the selected folder in form view.
 First, Previous, Next, Last	Selects the first, previous, next or last image in the selected view.
 Load Image	Loads the selected image.
Purche deleted files	Purches the deleted image files from the cache.

32x32, 64x64, 128x128	Selects the size of the image files displayed.
Slide show	Shows all image files as a slide show.
Slide show (selected)	Shows all selected image files as a slide show.
Properties	Opens the image's property window.

Context menu of the slide show



First, Previous, Next, Last	Selects the first, previous, next or last image of the selected view.
Use Timer	Activates the timer: the slide show automatically proceeds to the next image or Deactivates the timer: in this case use the arrow keys on the keyboard to go to the next (⇒) or previous (⇐) image.
Slide show	Stops the slide show.
Auto Zoom	Continuously adjusts the zoom factor of the image display to the size of the window.

Change the form for data entries

A form is used to enter data and to display information on the monitor.

To display image information and enter additional information switch to the Form view by clicking on the  icon. In the Form view, the form "Archive.zvf" is used:



The form "Archive.zvf".

This form must be in the folder *Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Form.*

To use a different form for the form view in the Image Browser, generate a new form on the basis of the installed templates, and save this form in the folder mentioned above.

- From the **File** menu select the **New** function and there the **Form** tab.
- Select one of the templates and click on **OK**. To see a preview of templates, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.
- Now select the **Save As** function from the **File** menu, and go to the data directory *"C:\Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Form"*. Open the form folder, or create it if it does not already exist, and save the template under the name "Archive.zvf".
- Now open the image browser and refresh the image gallery  . If the form view is not active, click on the  button on the image browser's toolbar.

7.4 Generate, print and export reports with the file extension ZVR

Reports are used to display and print out saved images and the additional information relating to them (title, author, comments, keywords etc.). Each report is based on what is known as a report template. Templates specify the layout and formatting for the report and its individual elements (image, data etc.).

You can modify report templates yourself, or generate completely new ones. Please also read the section "Templates for forms and reports", which you will find further down this section. The template files are saved in the folder *Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Report*.

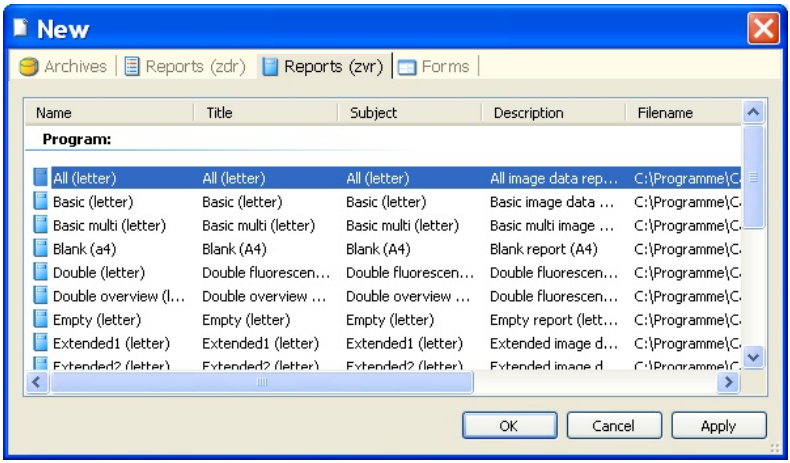
To allow reports to be incorporated into other documents (e.g. Word files), the option of exporting them is available. AxioVision offers export into the so-called Microsoft Windows Metafile Format (WMF). This format allows you to incorporate reports into virtually any Microsoft Windows program. The individual fields of a report can be modified in most cases. However, the precise range of functions depends on the program in question.

Generating new reports

➢ Activate the image for which you want to generate a report.



➢ Select from the **File** menu the **New** function, and activate the **Reports (zvr)** tab.



➢ Select one of the templates and click on **OK**. To see a preview of templates, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.



- The new report is now displayed. You can save it just like any other file via **File** ⇒ **Save** or ⇒ **Save As**.



Printing a report

- Activate the report that you want to print.
- Select from the **File** menu the **Print** function. The standard dialog for printing documents is then displayed.
- Enter the desired settings and click on **OK** to print the report.

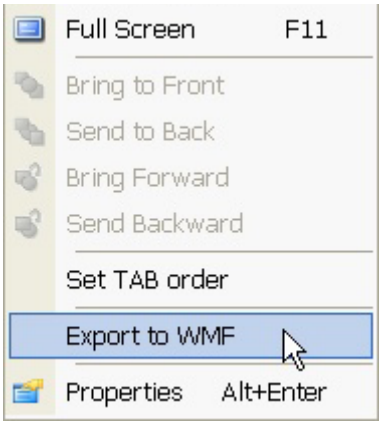


Exporting a report

- Activate the report that you want to export.



- Right-click in the report and select the **Export to WMF** function.



- A standard Microsoft Windows dialog for saving the file is then opened. Select the target folder under **Save As** and enter a file name.

- Then click on **OK** to save the report.

Templates for forms and reports

Reports are mainly used to print out saved images and the additional information relating to them (title, author, comments, keywords etc.).

Forms are used for displaying information in a structured way and for entering information on screen.

Reports and forms are based on what are known as templates. Templates specify the layout and formatting of the individual elements (image, data etc.).

You can modify templates yourself, or generate completely new ones. The template files are saved in the folder *Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Forms* or *Report.*

AxioVision is supplied with a range of different templates. This means that you do not necessarily have to generate templates yourself. Often, however, a modification will be necessary to bring the templates into line with company or project-specific requirements.

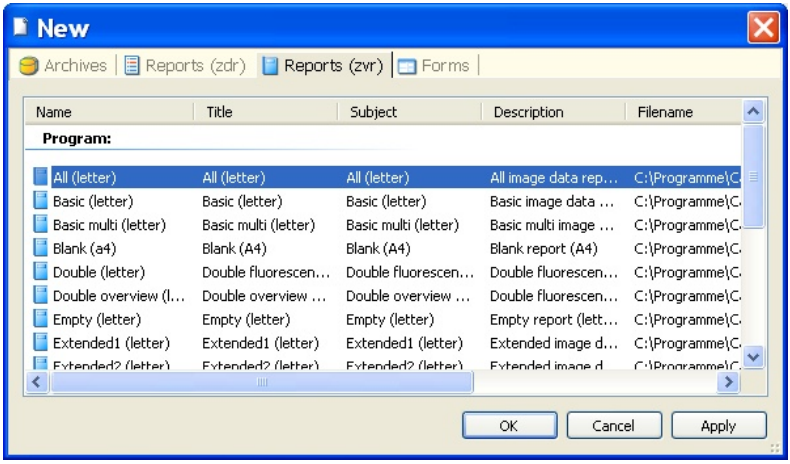
As the generation of templates can be very time-consuming, depending on their complexity, we recommend that you use an existing template as a starting point wherever possible. This avoids having to generate them from scratch (an even more time-consuming process).

Note:

- The following example runs through the steps required to edit a template, using the example of a report. These steps also apply, however, to form templates.

Settings for editing a template

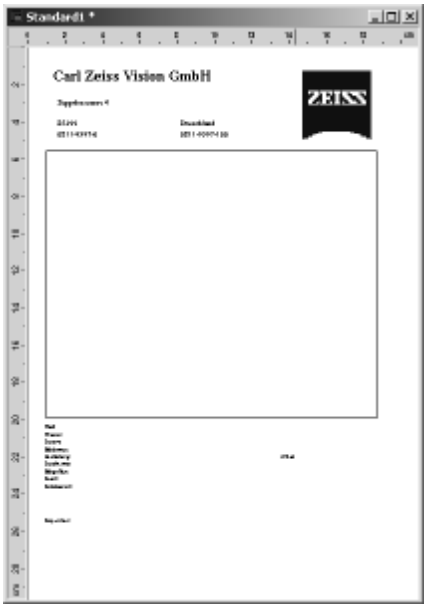
- Select from the **File** menu the **New** function, and activate the **Reports** or **Forms** tab.



- Select one of the templates (e.g. **Reports** - **Standard**) and click on **OK**. To see a preview of templates, click on **Apply**. The corresponding template is then opened without the **New** dialog being closed.



➢ The report is now displayed with its current layout.

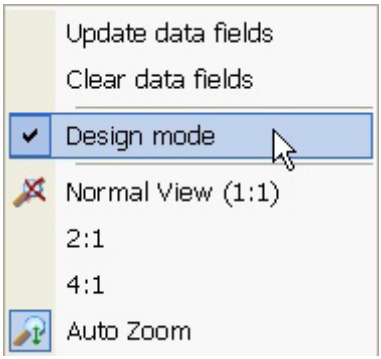


Notes for report templates:

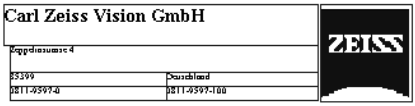
- The details in the header (logo, company name etc.) do not have to be defined as individual fields, but can be entered directly via a dialog. To do this, select **Tools** ⇒ **Options** ⇒ **ID**. Enter the corresponding data here. These data are then displayed automatically in the report's header.

- If you have not yet entered this information, this area is empty.

- To modify the template, you need to switch to **Design mode**.
- To do this, right-click in the template and activate the **Design mode** command from the shortcut menu.
- The draft mode is active if the tick can be seen to the left of the **Design mode** command.

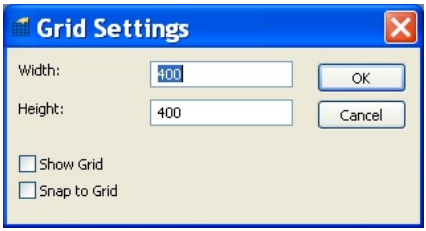


- To see the position and size of all elements, activate the **Show bounding box** option from the shortcut menu. All fields are then displayed with a blue frame.

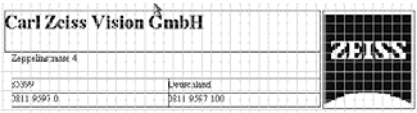


To make positioning easier, you can activate a grid, with which all elements can then be automatically aligned.

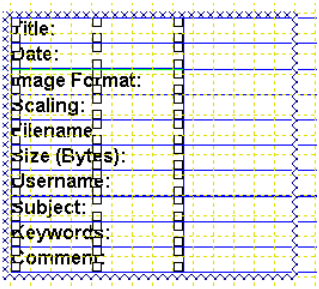
- Select from the shortcut menu the **Grid settings** function.
- Activate the **Show Grid** and **Snap to Grid** options, and click on **OK**.



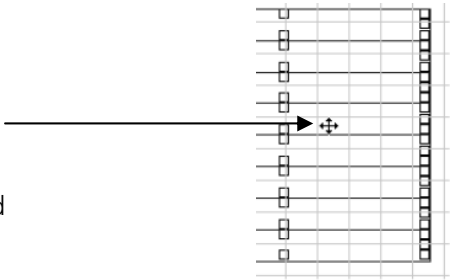
- A yellow grid is now displayed on top of the template.



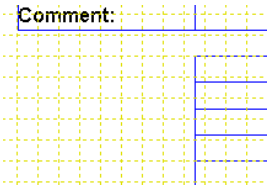
Moving and deleting fields



- To move one or more fields, you need to select the field(s) in question. To do this, click on an individual field, or drag out a frame around the fields you want to move with the left-hand mouse button depressed.
- If you hold down the **Shift** key while clicking, you can also select a number of individual fields.
- To move the fields, left-click in the selected area, and drag the fields with the left-hand mouse button depressed. The pointer changes to a cross during this process.



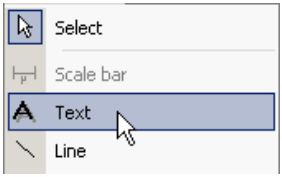
- The fields are automatically aligned with the grid.
- To delete the selected fields, simply press the **Del** key on your keyboard.



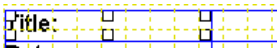
- The fields are then deleted, but can be restored via the menu function **Edit** ⇒ **Undo** provided you have not saved the template.

Inserting new fields and changing field values

- If you want to insert a new field, you can select the **Text** tool from the **Annotations** menu and then position it in the desired location.



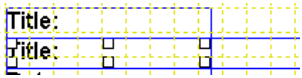
- The simplest way to do this, however, is to copy an existing field. In this case all of the attributes (size, color, font etc.) are adopted.



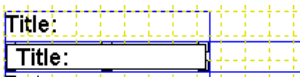
- To do this, click on an individual field, or drag out a frame around several fields with the left-hand mouse button depressed.

- If you hold down the **Shift** key while clicking, you can also select a number of individual fields.

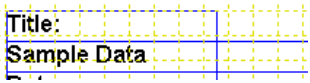
- To duplicate the field and move it at the same time, hold down the control key (**Shift** key), and left-click in one of the two selected fields. Drag it to the desired location with the left-hand mouse button depressed.



- To change the name of a field, switch to editing mode by double-clicking in the field.



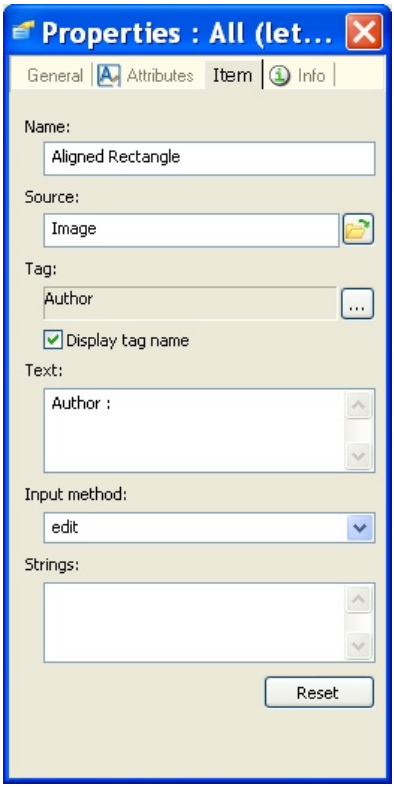
- You can now enter a new name:



- So far you have only changed the field name! Now you need to specify that relevant information should also be entered to the right of the identifier.

- To do this, select the empty field to the right of the field name, and open the **Properties** window by right-clicking in the field. Activate the **Item** tab.

- A particularly important element here is the **Tag** parameter, which displays the information available in AxioVision images. Click on the  button to make a selection.



- Now complete the other parameters according to your requirements.

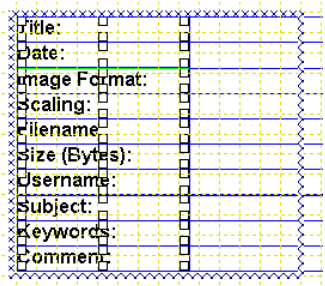
- Carry out these steps for all of the fields that you wish to use in your template.

- Then save the template in the folder *Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Report.*

The templates are now available in the list of available templates on the **File** ⇒ **New** dialog.

Changing field attributes

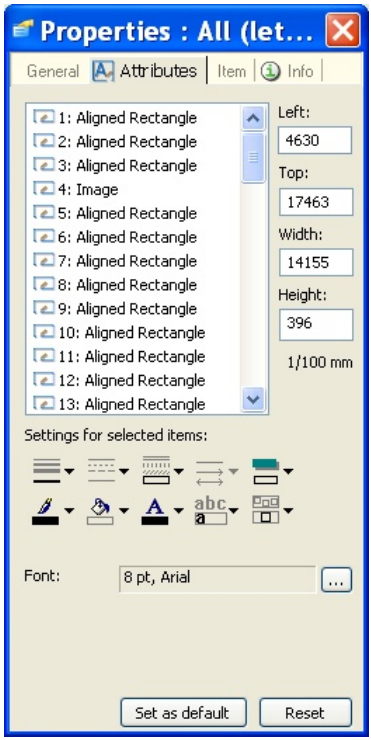
Changing field attributes means modifying the appearance (color, line thickness, text type, text size etc.) of fields.



- To change the attributes for individual or several fields, you need to select the field(s) in question. To do this, click on an individual field, or drag out a frame around the fields you want to change with the left-hand mouse button depressed.

- If you hold down the **Shift** key while clicking, you can also select a number of individual fields.

- Now right-click in the report and select from the shortcut menu the **Properties** command.
- In the **Properties** dialog, activate the **Attributes** tab.
- Now change the attributes using the tools under **Settings for selected items**.
- If necessary, select a different **Font**.
- Save the template in the folder *Programs\Carl Zeiss Vision\AxioVision4\0009\Templates\Report".*



7.5 Editor for Multidimensional Images

Using the image editor, it is possible to generate a large number of images in a targeted way from large, complex ZVI images. The result is always a new, unsaved image with reduced complexity. It is possible to influence the following dimensions:

- x/y pixel resolution
- channels
- instants of exposure
- z-stack positions

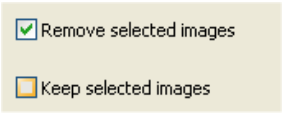
When you select the **Change Image Dimensions** function on the **Edit** menu, a wizard is opened. This wizard always uses the active image in the foreground. When you are working in the image editor, it is not possible to execute any other functions in AxioVision. Only the pages that are relevant for the image are displayed in the wizard.

- Open a multidimensional image. In this example, a 3-channel z-stack fluorescence image will be modified. Channel 1 and z-planes #1-6 are to be removed from the image, and the x/y resolution is to be reduced by a factor of 2.

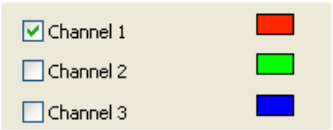
- Select from the **Edit** menu the **Change Image Dimensions** function. A dialog is opened in which the image located in the foreground is displayed. All the control elements that are required to operate the image (**Player**) can be found at the bottom image edge.



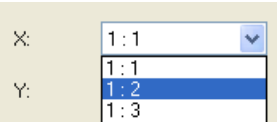
- As the first step, indicate that you want to remove the selected images, and click on **Next**.



- Now select channel 1 (which is to be removed), and click on **Next**.



- From the drop-down list box, select the desired reduction in resolution (1:2).



- This setting means that every second pixel in both the x and y dimensions is removed. This reduces the image resolution to a quarter. Click on **Next** to proceed to the next step.

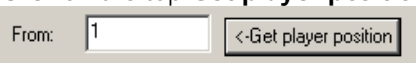
- Now define the z plane range that you want to be removed from the image.

- To do this, move the **z** slider to position 1



.

- Click on the top **Get player position** button



. Now move the slider to z-position # 6, and click on the bottom **Get player position** button



.

- To check the result, click on the 

button. A preview of the reduced image is created. If you are happy with the selection, click on 

. The wizard is closed, and a new image with the reduced complexity is generated. Save this image under a new name.

7.6 Creating an Asset Archive

What is an asset archive?

Using the Asset Archive module, you can store images, measurement data lists, reports, etc. as "assets" in a hierarchically structured database. Different projects can be created in which your assets can be stored. A contact management feature enables you to allocate the projects to different clients etc., and you can classify and structure your assets in your own customized categories. A full-text search function makes it possible to find individual data sets again quickly. Using asset or project-related filters, you can carry out targeted searches for information in individual data fields.

The long-term archiving feature allows you to transfer your projects and the associated assets onto CDs, DVDs or tape drives.

File – New – Archive

In order to archive assets, you must first create an asset archive. We recommend that you create **just one** archive and manage your assets using the organizational structures that the archive offers.

To create an archive, open the **File** menu and select the **New** command.

In the next dialog, select the **Archive** tab sheet. There, select the **Asset Archive** entry and click on **OK**.

Asset archives are SQL databases. In the next dialog **SQL-Archive**, give the new archive a name. You can also specify the work folder in which the archived images will be stored. To do this, click on the **"..."** button and select the desired folder. Naturally, you can also create a new folder.

Click on **OK** to generate the asset archive.

The new asset archive is now ready for you to use.

Creating new projects

Structuring your asset archives

The asset archive gives you the opportunity to structure your assets in different ways. On the one hand, you can divide your work into different projects. All the images, data lists and reports that you can allocate to a project can also be stored in a project in the asset archive. An asset archive allows you to create any number of projects.

If, however, you do not want to make use of this structuring option, you can archive all your assets in a single project, although this means that at least one project must exist.

Creating projects

In order to create a (new) project, execute the **Add New Project** function in the **Archive** menu. In the **Add New Object** dialog window, you can select a name for the project. It is also possible to enter a description and comments in the appropriate fields.

In addition, you can allocate a contact to the project, and the project will then also be listed under this contact. To do this, click on the "**...**" button in the **Contact** group. You can now select a contact from the **Select Record** list. You can amend this entry or carry out this selection step later at any time if, for example, you did not create any contacts when you created the project.

Creating contacts

Allocating projects to clients

Projects are often allocated to individual clients, customers or patients. It is frequently necessary to process several projects from one client. To make it possible to allocate these projects to particular clients, the asset archive gives you the opportunity to manage these clients in a list of **Contacts**.

Creating contacts

In order to create one or more contacts in an archive, execute the **Add New Contact** function in the **Archive** menu.

In the **Add New Object** dialog window, you can now enter the name of a company, as well as the surname, first name and any other personal details relating to the new contact.

If you click on **OK**, the contact is incorporated into the list of contacts and can then be allocated to individual projects.

If you now click on **Contacts** in the project tree, you will see a list of all the contacts that have been created.

Adding an asset to an archive

What are assets?

Assets are digital resources. In AxioVision, assets are simply archived files or data. An image, a list of measurement values or a report layout – these are all examples of files that are commonly managed using AxioVision.

What are data sets?

Data sets represent assets. Every data set refers to an asset. Data sets contain information about assets, such as the file size and type, storage location, creation date and much more.

Activating the project

Assets, as data sets, are always allocated to a project. This means that you are currently working on a project and add the assets to this project. Click on a project from the project list in the project tree to activate it. You can now make this project the default active project. To do this, right-click on the project and select the **Set Start Folder** function.

Adding assets

➢ Image

In order to archive an image, select it and execute the **Add to Archive** function in the **Archive** menu.

You will see a form containing the information that AxioVision already has on this image. You can now give the asset a title and a subject; keywords and comments can also be added.

If the image was not acquired using an automatic Zeiss microscope, you can also complete the fields relating to image information.

The asset archive will store this image in the allocated work folder and it will automatically be given a file name.

If you archive an image that has already been saved as a file, a second file will be created.

➢ Data list

If you have obtained measurement data from an image, this information is available in AxioVision in the form of data lists. These data lists can also be archived as assets. To do this, select the data list and execute the **Add to Archive** function in the **Archive** menu.

You can also archive data lists that have already been saved.

➢ Archiving from Microsoft Windows Explorer

As a rule, you can select any files in Microsoft Windows Explorer and use drag and drop to move them into an active project in the asset archive. Please note, however, that in this case the asset archive saves each asset archived in this way again as a file in the allocated work folder.

Working with value lists

If you click in the relevant entry fields, it is possible to select the content of the field from a value list that you have created. To do this, click in the entry field, and the "**...**" button will appear at the edge of the field. Click on this button if, for example, you wish to insert keywords from a pre-defined list.

You will find the available values in the list above. Select a value and insert it into the current data field by means of a double-click or by clicking on the **Append** button. Clicking on the **Replace** button replaces all the values that were previously contained in the field with the selected value.

You can expand the value list by clicking on **Add New**, entering the new value in the **Enter value to add** window and confirming the entry with **OK**.

A selected value can be removed from the value list using the **Delete** button.

Views

Using the **Gallery View** and **Grid View** commands, you can specify how the contents are displayed for each selection – project lists, contacts, assets within a project, categories and user-defined search queries.

Categories

Using categories, you can structure your assets in different ways. This is useful when saving similar assets in different projects and if you want to work with cross-project organizational criteria. You can define and manage the categories yourself; an asset can be allocated to one or more categories, or none at all.

Creating a category

Execute the **Add New Category** command in the **Archive** menu. Enter the name of the new category in the **Add New Object** dialog and confirm by clicking on **OK**. The category is then available and listed under **Categories** in the archive tree.

Allocating an asset to a category

To allocate an asset to a category, you first need to activate a project. Select an asset in the active project and, using the mouse, drag it to the desired category. Now, when you activate the category in the archive tree, all the assets that belong to this category will be displayed. You can also drag an asset to several categories one after the other.

Within a project it is possible to highlight several assets. To do this, hold down the **Ctrl** or **Shift** key as you highlight the assets using the mouse.

Searching

You can search for different types of data sets in an asset archive:

- You can start a keyword search across all the data fields of an asset. All the assets in which the search text has been found are then displayed.
- Using a wizard, you can generate search queries. This allows you to search through the contents of one or more data fields of assets or projects and to display the hits.

Keyword search

To search for a text in all assets, select the **Search** function in the **Archive** menu. You can then enter the keywords in the **Enter search keywords** dialog field. Click on **OK** to see the search results.

The **Search** command will now have opened in the archive tree and the **Keywords** entry will be activated. Here, you will see the result of the search query.

You can enter a new search term or switch back to all the available projects at any time.

Creating a user-defined search

To create a user-defined search, open the **Search** entry in the archive tree and right-click on the **User defined** entry. Select the **Add New Filter** command in the shortcut menu. This starts the filter wizard.

- In the first step, you can define which objects you wish to search for in the asset archive. You can search for assets or projects which should meet certain search criteria. Searching for projects results in a list of all projects which meet the search query criteria. When you search for assets, the data sets which meet the criteria of the search query are listed, irrespective of the projects. Click on **Next**.

- In this step, you must select a data-set field in which a search will be carried out for certain values. Different attributes will be displayed depending on the types of objects that you are searching for. Select the first attribute and click on **Next**.

- This step allows you to define how the values in the data field should be compared. You can choose between:
  1. An exact, case-sensitive match.
  2. A non case-sensitive match.
  3. A check to establish whether certain text is contained in a field
  4. A comparison of numerical values: "less than", "less than or equal to", "greater than", "greater than or equal to".
  5. You can then define whether the exact opposite of the formulated condition should be met by activating the "Condition MUST NOT match" check box.
  6. Click on **Next**.

- In this step, enter the value with which the comparison should be made. Click on **Next**.

- You can now choose whether you wish to add additional criteria to the search query. If you wish to add further conditions, you can select whether at least one condition ("Yes, OR additional possible conditions…") or every individual condition should be met ("Yes, AND additional required conditions…"). If you add additional conditions, proceed as in step 2. When you have completed the definition of the search query, select **No, finish filter…** and click **Next**.

- You can now define whether the search query should be stored. In the drop-down list box, you can choose between **Don't save** and **Save for all users**. If you select **Save for all users** in the drop-down list box, you can enter a name for the search query in the **Name** field.

- The search will then appear under this name in the list of user-defined searches. If you click on **Finish**, the search query is stored and the definition process is concluded.

In the archive tree, you can now open the **Search** entry and activate the newly created search under **User defined**.

Managing archives

Asset archives can be adapted specifically to the requirements of the user. In order to make such adjustments, however, you, as the user, will need special "administrator rights".

If you wish to execute one of the following functions for an asset archive, the first time you call up the function you will be asked to enter the password for the **administrator** user. The default setting for this password is **zeiss**.

Setting a password

We recommend that you first set a new password for the administrator.

To do this, start the **Change Password** function from the **Archive ⇒ Manage Archive** menu. Before you can change the password, you must first log on as the "administrator". You will then be asked to enter a new password and to confirm this a second time. After successfully executing this function, you will also be logged on as the administrator. This mode will remain active for as long as you have the current archive open.

Field management

For each data field in an Asset-Archive you can specify certain parameters. There are three types of objects: **ADDRESSES**, **IMAGES**, **PROJECTS**. Select a type first an then select a field from the list of fields. You can modify the following parameters of a field: **Name**, **Data type**, **Maximum Length**, **Input Validation Rules**. The **Input Validation Rules** allow you to specify rules which apply when a user enters data into that field.

Long-term archiving

You can also transfer your archived assets onto external storage media. The long-term archiving function is used for this purpose. If you use this function, assets are transfered onto CD, DVD or tape drives and are deleted from the hard drive of your computer. The entries relating to the individual assets and preview images will remain on your computer so that you continue to have direct access to the information about the assets.

Select the projects and/or assets in the archive that you want to transfer.

Now start the **Long Term Archivation** function in the **Archive** ⇒ **Manage Archive** menu. When you call up this function for the first time, you will be asked to identify yourself as the administrator of the archive.

In the first step of the wizard, you will see how many projects and assets you have selected to be archived and how much memory will be required for this. Click on **Next**.

In this step, select a storage medium by double-clicking on the <**New Medium**> entry in the **Media available for writing** area. If you have more than one drive for writeable media, you can select a **Drive Type** in the **New Medium** dialog window. Select an unambiguous name for the transfer medium in the **Medium Name** field. The transfer medium will later be referenced using this name should you wish to open the transferred assets in AxioVision. Click on **Next**.

In this step, you can determine whether the data carrier to which you have written the data should be compared with the original data at the end of the process. Click on "**Finish**" to start the actual transfer. Depending on the volume of data, this process may take some time.

Specifying the storage location

Using this function, you can specify the basic folder in which your archive(s) will be saved. The default setting for this is the folder for **My Pictures**.

During the creation of a new asset archive, you had the option of specifying a work folder for the new archive. Using this function, you can change this work folder.

Please note: if you have already archived assets, you must move **ALL** the files from the previous storage location to the new storage location manually.

Changing project properties

If you are logged on as the **administrator** you can change other properties of the asset archive. Activate the **Root** entry in the archive tree that displays the name of your archive and execute the **Properties** function in the **View** menu.

Activate the **General** tab sheet in the **Properties** window. You can now change the following properties:

Copy data from last record

Here you can specify how the input form of a new asset will be treated. You can copy the values from the previously added asset always or never or you can select to be asked for each new asset, whether or not the values shall be copied.

Storage format for images in archive"

Select the format in which images should be stored. You can choose from the following formats: ZVI, JPEG and TIFF.

Name for Projects Folder

If necessary, you can also replace the name "Project" with another expression.

Name for Addresses Folder

If necessary, you can also replace the name **Addresses** with another expression.

8 Configuration

8.1 General

One of the most striking new features of AxioVision Version 4 is the fact that it allows you to adapt the interface and the way you operate the program almost entirely to suit your own requirements. A great deal of attention has therefore been devoted to ensuring that the configurations of individual users can be managed separately. This is guaranteed by saving individual data, such as toolbar configurations, customized dialogs and workflows, general system settings and, of course, the data generated on an individual basis, separately from the data of other users. The Microsoft Windows user administration is used entirely. In AxioVision additional settings are not required.

The settings of an individual configuration can be exported easily and can be accessed to other users or to all users (see also chapter 8.7 "Exporting and Importing User Configurations").

8.2 Overview: Setting up AxioVision

This chapter gives you a brief overview of the steps to setup up AxioVision. In the appropriate subchapter you will find further information.

Installation

- Installation must be carried out with **administrator rights**.
- AxioVision is then available to all users of the system (including new users who are set up after installation). Installation is **not** carried out individually for each user.
- Remain in administrator mode during the following steps described below. Read also Chapter 1 and 2 of the Installation Manual *AxioVision Product Suite*.

Setting up AxioVision

AxioVision can now be set up as desired:

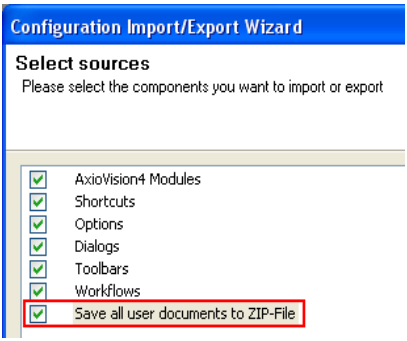
- Microscope configuration and/or MTB2004 Configuration
- Scalings
- Hardware settings files and experiments
- Toolbars, keyboard shortcuts, workflows and user dialogs
- Measurement programs

Backing up the configuration and restoring it for individual users

➢ After configuration the settings and user files can be backed up via the **Tools** menu ⇒ **Import/Export Configuration**.

➢ In **step 1** select **Export**.

➢ Important: Please ensure that **Save all user documents in ZIP-file** is also activated in **step 2 Select sources** so that the user files (scalings, hardware settings etc.) are also exported:



The result is two files with the name given to them during the export and the extensions **AVCONF** and **ZIP**. You can select the folder for these two files.

The relevant settings for individual users can be imported using the same function.

➢ To do so select **Import** in **step 2**.

Notes:

- The relevant user must naturally be logged on under Microsoft Windows during the import – not the administrator.

- The **channel pool**, used for simple definition of multichannel fluorescence images, cannot be exported. If necessary, the corresponding file must be copied via Microsoft Windows Explorer.

Making user files available for all users

➢ To make the user files available for all users, the files in the ZIP file must be extracted into the *Shared Documents* folder of the **All Users** user account.

➢ The directory structure required by AxioVision is generated automatically.

8.3 User and Data Administration

Microsoft Windows user administration

Under Microsoft Windows XP there are three fundamental levels of security that are allocated to users. These are granted to end users through their membership of the groups **Users**, **Power Users** or **Administrators**.

- The **Users** group is the most secure group, as the standard rights of this group do not allow the members to change operating system settings or the data of other users. Members of the **Users** group are able to carry out most general tasks, such as running applications, using local and network printers, as well as shutting down and blocking the work station. Users are not entitled to release directories or set up new local printers.

    - → Under certain circumstances, members of the **Users** group are not able to execute AxioVision, e.g. if cameras from other manufacturers are being used or a Matrox Meteor frame grabber is installed.

- **Power Users** are able to execute the following tasks: installing programs that do not change operating system files, or installing system services. Adjusting system-wide resources, including printers, date, time, energy options and other control panel resources. Creating and managing local user accounts and groups. Stopping and starting system services which are not started by default. **Power Users** have no access to the data of other users on an NTFS volume unless they are granted this right by the users concerned.

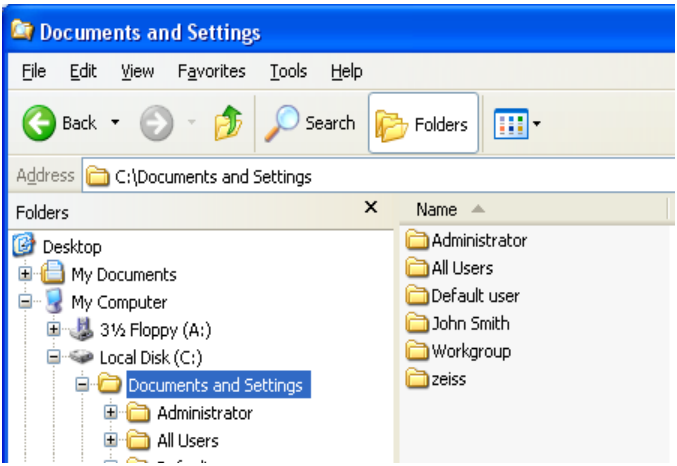
  - → In order to use AxioVision, users should always belong to the **Power Users** group.

- The **Administrators** group carries out maintenance tasks on the computers. The standard rights for this group give members complete control over the entire system. For this reason, only reliable employees should belong to this group.

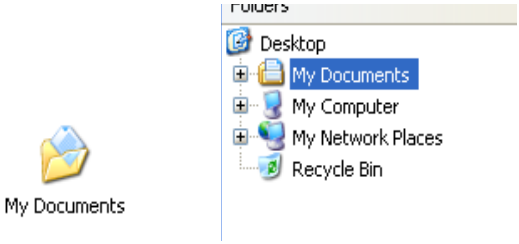
User files

Each user under Microsoft Windows XP has a personal folder for the files that he has created. These personal folders are created by Microsoft Windows for each user of the computer. If a computer is used by more than one user, the individual personal folders are indicated by the name of the corresponding user. In addition, Microsoft Windows provides the *Shared Documents* folder for files that you want to release for other users.

When a new user is created under Microsoft Windows XP, a user account is set up for each user.



Theoretically, user data should only be saved in the *My Documents* folder. This folder has a link to the Microsoft Windows Desktop as well as in Microsoft Windows Explorer. The personal folders can be accessed very easily via these links:



However, the folder is actually located within the folder structure of the user account.

For reasons of data security, however, we recommend that you separate program and data files. This means that the data created by a user should be stored on a different drive from the programs.

Advantages of this procedure:

1. After installation and configuration an image of the system partition can be created, which makes it possible to restore the system very quickly in the event of a disaster. "Images" represent a 1:1 copy of the hard drive, irrespective of whether or not there are files in the memory. An attempt is therefore made to limit the size of the system partition.

2. When you are making regular data backups, it is not always necessary to back up the complete system partition as well – only the user data needs to be backed up.

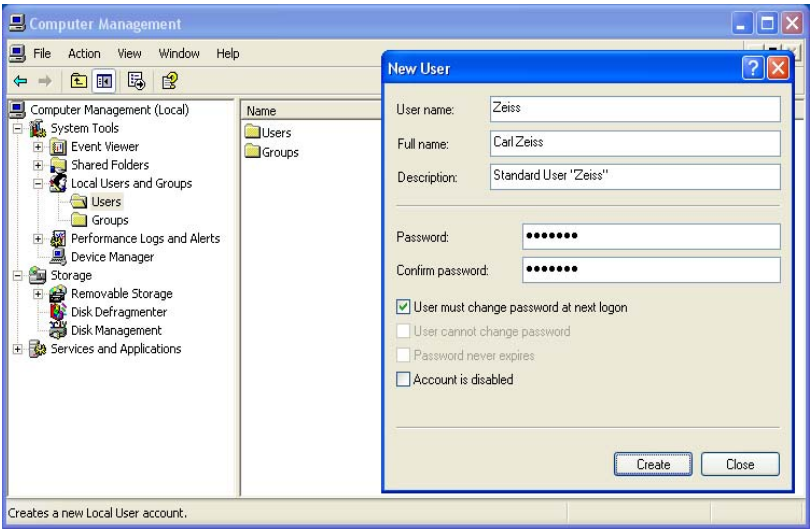
3. The data folder can be moved to a separate drive with sufficient memory, even for multidimensional images (see also point 1). If the folder is on a network drive, automatic backup by the relevant IT department is often ensured.

The shared account "All Users"

Microsoft Windows always creates a default user account with the name **All Users**. Files saved here can be used by all users of the system. This "shared account" can be used to save files (e.g. scalings and measurement programs) that are used centrally by all users, without the need to copy these files individually to each user's personal account in the event that the files are modified.

Creating new users

Set up new users (or create new user accounts) via the computer administration function (Microsoft Windows Desktop ⇒ **My Computer** shortcut menu ⇒ **Manage** ⇒ **Local Users and Groups**):



Notes:

- Administrator rights are required to create a new user or to allocate a profile.

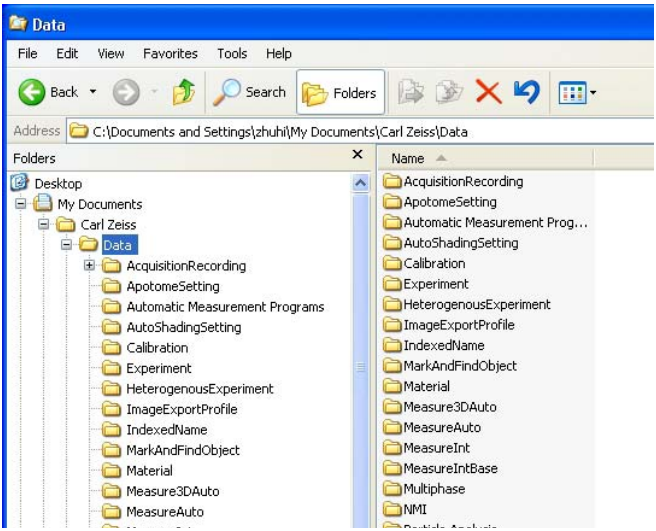
- When you add a new user, the **Users** profile is allocated by Microsoft Windows by default. A different profile can be allocated after the user has been set up.

- AxioVision is released for use with the **Power Users** profile only. This is mainly for reasons of hardware support. The Matrox Meteor drivers, for example, do not function with the **Users** profile.

Data administration under AxioVision

Data files

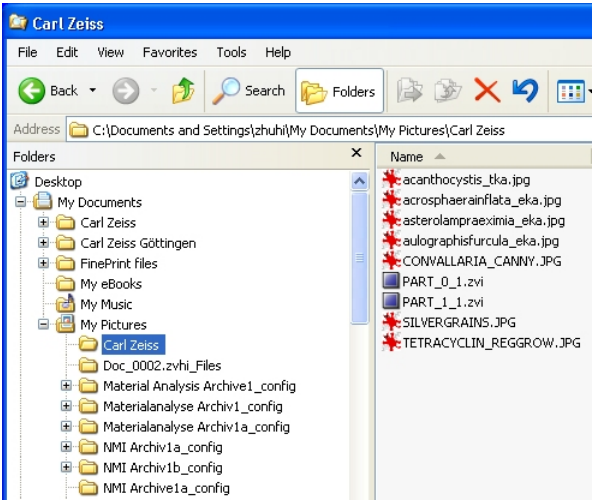
AxioVision stores all files created by the user in the *My Documents\Carl Zeiss\Data* folder. There you will find additional subfolders for the individual documents, such as automatic measurement programs, calibrations, experiments etc.:



Folder structure of the AxioVision data folder

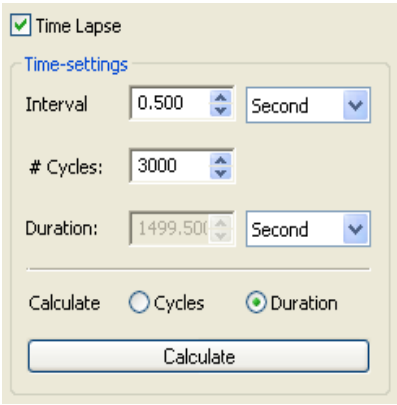
AxioVision images

Within the *My Documents* folder, you will also find the Microsoft Windows default folder *My Pictures*, which is suggested by AxioVision as a location for saving your images.

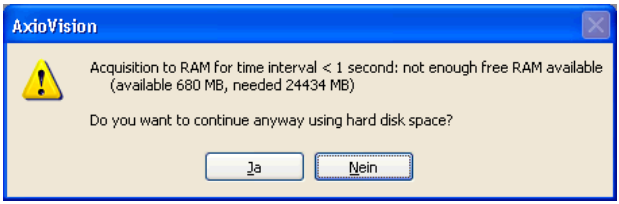


Temporary files

During the acquisition of multidimensional images using the Time Lapse Imaging module, if the time intervals are less than 1s, images are only stored in the user memory. However, if the experiment is too complex, it is possible that the user memory may not be able to hold all the images, which are then stored temporarily in the folder for temporary files.



AxioVision draws your attention to this by means of an appropriate message before acquisition:



This folder is also located within the user account. As huge amounts of data can accumulate as part of this process, this folder should also be moved.

Notes:

- Since the highest possible speed is important for this procedure, you should either create a reference to a different drive on the same hard drive, or use a different, faster, hard drive.

- On no account should you transfer the folder for the temporary files to a network drive. Data transmission is clearly too slow here.

AxioVision configuration files

Files that are important for AxioVision to function (INI files for devices etc.) are also saved within the user account in the folder *Application Data\Carl Zeiss\AxioVS40*.

This subfolder is not displayed if you are a normal user. It is only available to administrators.

Moving the "My Documents" folder

➢ Select the **Properties** function in the shortcut menu of the *My Documents* folder (right click on the desktop or in Explorer).

➢ Click on the **Move...** button in the **My Documents Properties** dialog and select the target folder. If you click on **OK**, the folder is adopted into the **Properties** dialog.

- Exit the **My Documents Properties** dialog by clicking on **OK**. All subfolders and files from *My Documents* will now be moved into the new folder.

- Repeat these steps for each user.

Notes:

- This step must be performed for each user before AxioVision is executed for the first time. AxioVision remembers this path in "plaintext" after the first program start. If the *My Documents* folder is not moved until after the first program start, AxioVision creates the folder and all subfolders in the original place again!

- The following tip should only be followed by specialists (and even specialists should be reminded to **save data *beforehand***.)
  The following action can be taken if AxioVision is accidentally started BEFORE the folder has been moved, resulting in the problem described above:

  - Close AxioVision.

  - Delete the **UserOptions.xml** file in the *Application Data\Carl Zeiss\AxioVS40\Profiles\Default* folder of the appropriate user account.

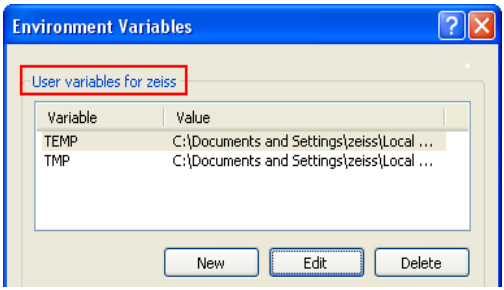
  - The file is automatically recreated and the current path settings for *My Documents* are read in again the next time AxioVision is started.

- Never simply move the *My Documents* folder via Explorer. Always follow the procedure described above, as only then will all the links and references to this folder work.

- In AxioVision the paths for user and work group files and templates are displayed in the **Tools** menu ⇒ **Options** function ⇒ **Folder** tab sheet. It is also possible to change the paths here. However, you should avoid doing this for the reason given above.

Moving the folder for temporary files

- Select **System** in the control panel.
- Click on **Environment Variables** on the **Advanced** property page.
- Repeat these steps for each user.
- Change the settings of the user variables for **TMP** and **TEMP** via **Edit** in the **Environment Variables** dialog.



8.4 Scalings

General

Scalings are required to allow you to display scale bars and measurement values in real units instead of pixel values. This requires configuration of the system for every combination of objective, optovar and camera resolution. If you are using a camera of the AxioCam family, please be aware of the following!

Notes:

- Only the basic resolution needs to be calibrated for the cameras of the AxioCam family. All other resolutions (higher resolution levels and binning modes) are then derived from this basic calibration automatically.
- For theoretical scaling there is no need using a stage micrometer, but special settings in the microscope configuration are necessary, which are described in the Online Help.

The theoretical scaling offers you the option of having scalings calculated automatically for a microscope that contains motorized or encoded elements.

This means that there is no need for the time-consuming generation of scalings using a stage micrometer.

Calibration is usually performed using a stage micrometer. The scale depicted on the stage micrometer allows you to specify a known length simply and precisely.

Each scaling is saved in its own file with the extension "ZVSC".

Notes:

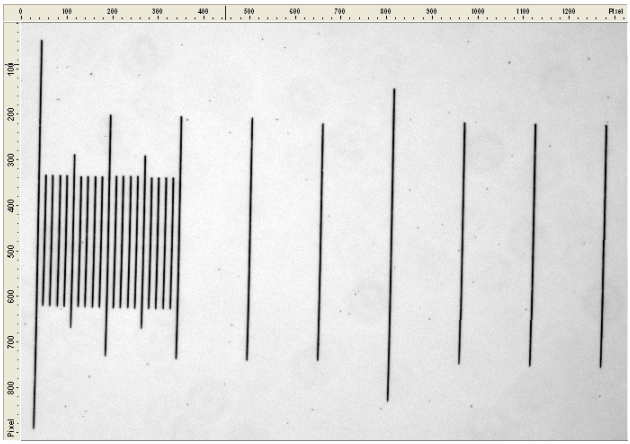
- You can also use scalings from AxioVision Version 3, simply by copying them into this folder.

- As scalings are often also of interest to other users, in some cases it may be useful to save them in the shared account *All Users* (please read section 8.7 "Exporting and Importing User Configurations").

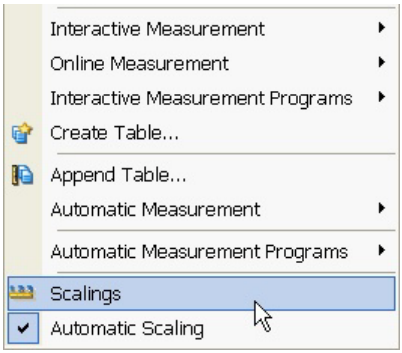
Generate scalings

- Acquire an image of the stage micrometer.

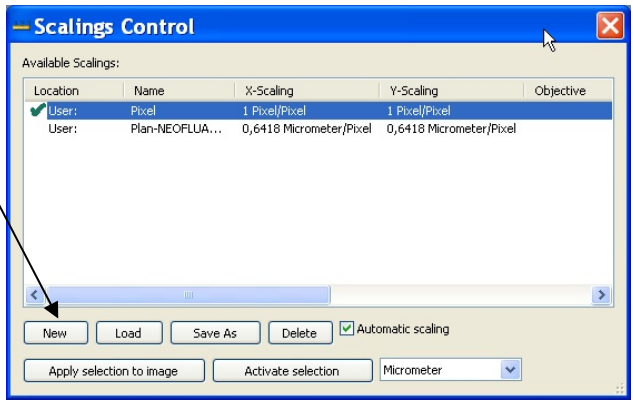
- Make sure that you position as much as possible of the scale in the image.



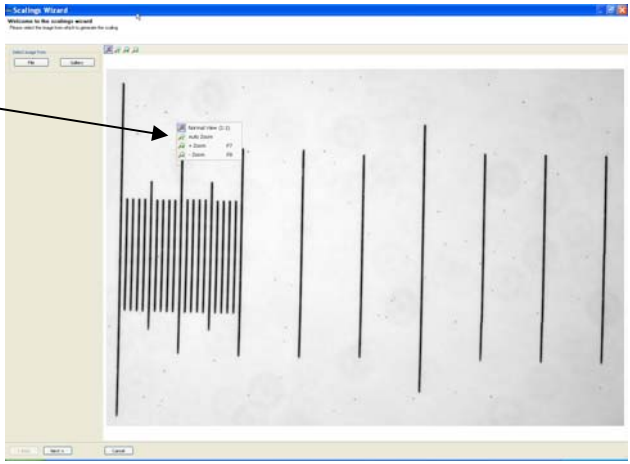
- From the **Measure** menu, select the **Scalings** function.



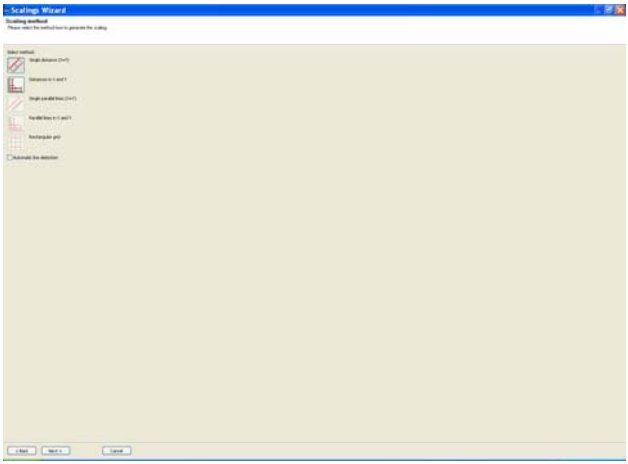
- In the **Scalings Control** window click on **New**.
- The scaling wizard is started.



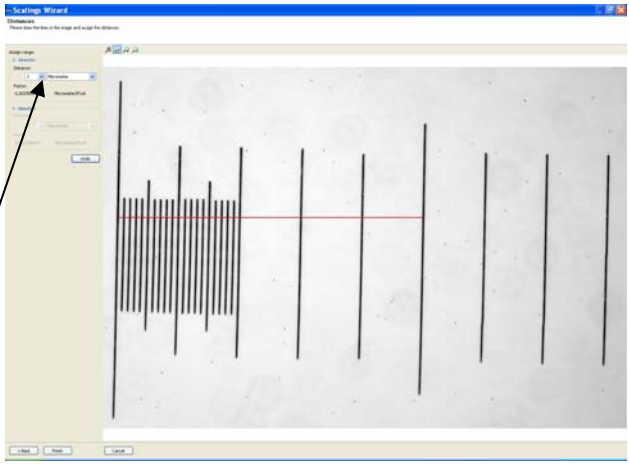
- Right-click in the image and select **Auto Zoom** to view the entire image.
- Enlarge the window, if necessary.
- Click on **Next**.



- Select the mode **Single Distance (X=Y)**.
- Click on **Next**.



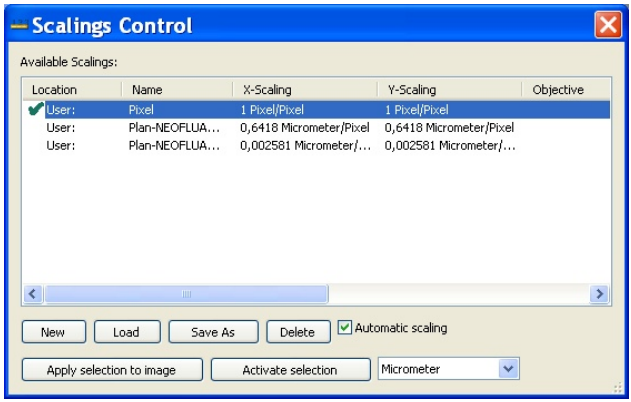
- In the image click on the start and end points of the scale.
- Enter this distance into the **Distance** field, and select the **Unit**.
- Then click on **Finish**.



- Enter a name for the scaling and click on **OK**.



- The new scaling now appears in the **Scalings Control** window, and is activated automatically.
- The scaling is used as the default for subsequent image acquisition procedures.



Notes:

- As scalings are often also of interest to other users, in some cases it may be useful to save them in the shared account *All Users* (please also read the previous section and section 8.7 "Exporting and Importing User Configurations").

- The **Save As** button is a simple way to save individual scalings for the work group.

8.5 Adapting the User Interface

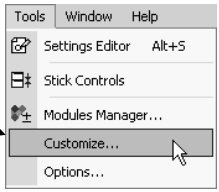
The **Customize** function in the **Tools** menu allows you to customize the user interface. This function gives you a great deal of freedom in configuring AxioVision. You can therefore design "your own" AxioVision, showing only those functions that you actually need.

Any changes made in the **Customize** dialog are immediately displayed on the AxioVision interface. The dialog does not therefore contain "traditional" buttons, such as **OK** or **Cancel**. Once you have made the desired changes, simply close it using the button in the top right-hand corner.

Creating toolbars

How do you create a toolbar for quick access to the image processing functions?

➢ From the **Tools** menu select the **Customize** function.

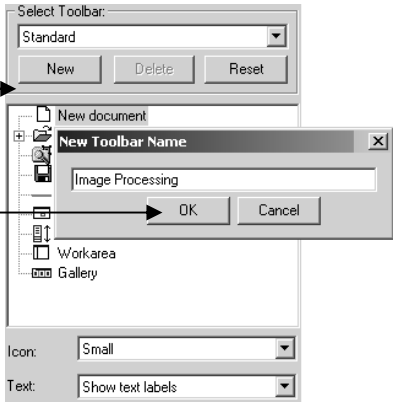


➢ Make sure that the **Toolbar** property page is active in the **Customize** dialog.



➢ To create a new toolbar:

- Click on **New**.
- Specify a name for the new toolbar.
- Click on **OK**.
- Define the size of the icons and indicate whether labels should be assigned to the buttons as well.

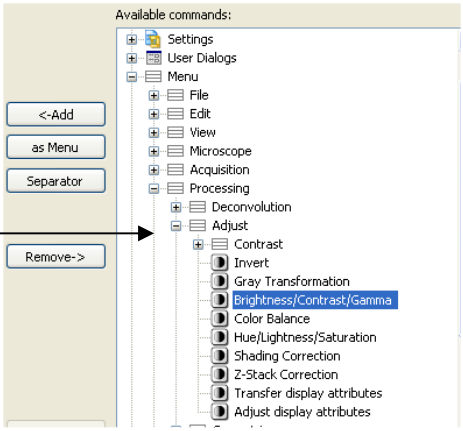


➢ In the **Available commands** field, select the commands that you would like to use on the toolbar.

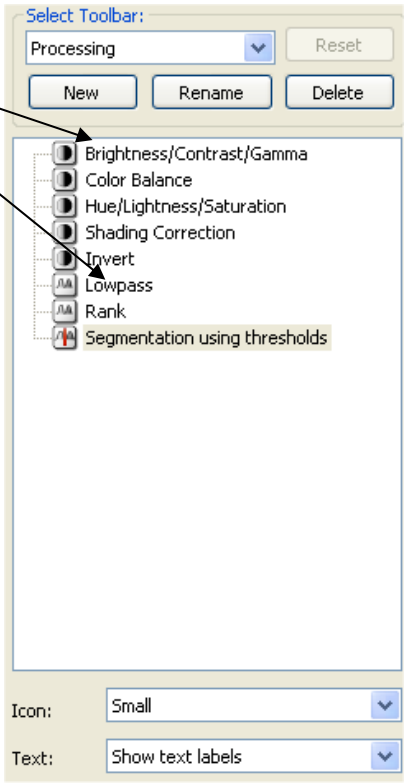
➢ Select the command **Processing ⇒ Adjust ⇒ Brightness/Contrast/Gamma**

➢ Then click on **Add** to apply it to the toolbar.

➢ Apply further functions from the **Processing** menu in the same way.



The toolbar now contains the commands displayed.



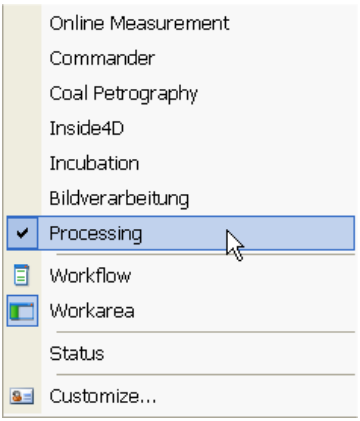
➢ To create the toolbar definitively, click on **OK**.

The **Processing** toolbar is now displayed at the top of the AxioVision window.



Notes:

- You will also find the toolbar in the toolbar list if you right-click in the main menu or on another toolbar.
- This procedure can also be used to modify existing toolbars.



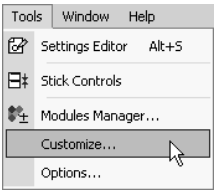
➢ To do this, simply select one of the existing toolbars in the **Select Toolbar** drop-down list and then proceed as described above.



Define key combinations for functions

You can execute any command that is available in AxioVision by pressing shortcut keys. This is particularly useful for quickly calling up commands that you require time and again, such as image acquisition or the activation of a camera or microscope setting that you often need (requires a motorized microscope).

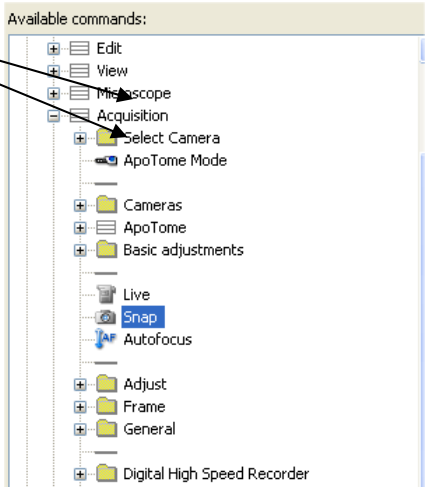
➢ From the **Tools** menu select the **Customize** function.



➢ Make sure that the **Keyboard** property page is active in the **Customize** dialog.

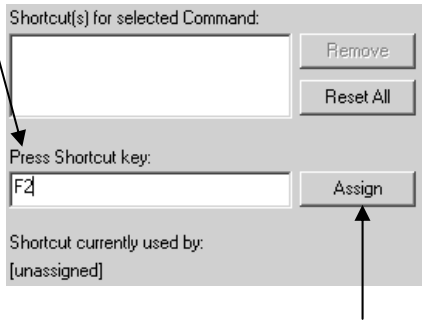


- In the **Available commands** field, select the commands that you would like to use on the toolbar.
- Select, for example, the command **Acquisition** ⇒ **Snap**.



- Click in the **Press Shortcut key** field using the mouse.
- Then press the shortcut key(s) on your keyboard that you would like to use to execute the command.

(If possible, the shortcut key should not already be assigned to any other command.)

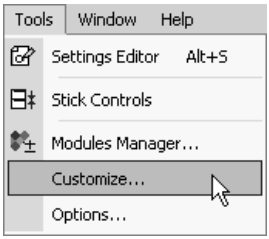


- Now click on **Assign**.
- Close the dialog by clicking on the button in the upper-right corner of the window.

Create own dialogs

The creation of your own dialogs allows you to take complex control dialogs for, say, microscope and camera operation and pick out precisely the elements that you need to operate your applications. This considerably simplifies parts of the operation. Create a dialog that enables you to control the objective nosepiece and lamp voltage on a motorized microscope as well as to set the camera's exposure time.

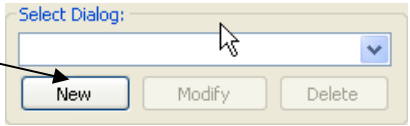
- From the **Tools** menu select the **Customize** function.



- Make sure that the **Dialogs** property page is active in the **Customize** dialog.

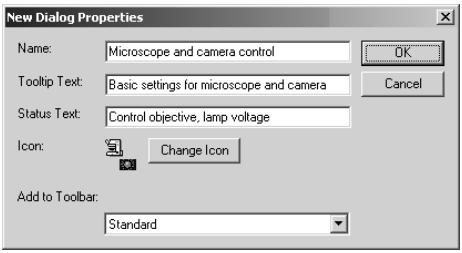


- To create a new dialog, click on **New**.



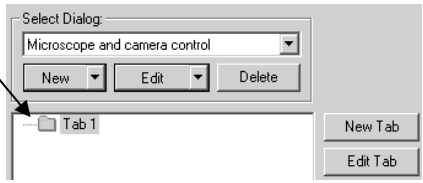
- Enter the following data:

  Name (dialog + button)
  Tooltip Text
  Status Text (status line)
  Button **Symbol**
  Toolbar, on which the button should be created to open the dialog.

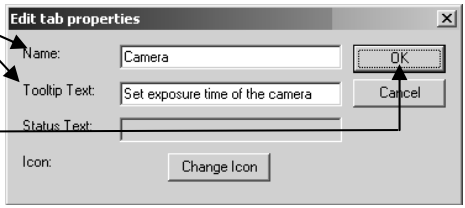


- Click on **OK** to create the dialog.

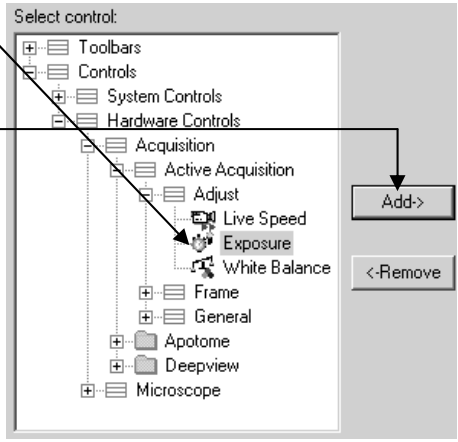
- Now select the entry **Tab 1**.
- To change the name, click on **Edit Tab**.



- Specify a **Name** and a **Tooltip Text**.
- Then click on **OK** to create the tab.



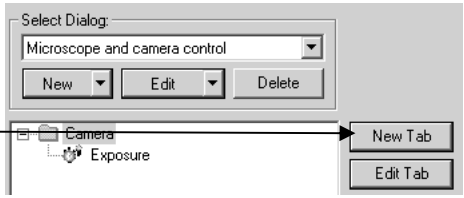
- Now select **Exposure** in the **Select control** field.
- To insert both into the dialog, click on **Add**.



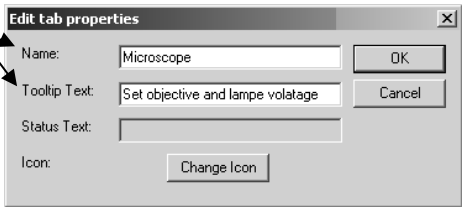
Notes:

- The control elements depend on your system configuration and may differ from those displayed here.

- To create a second property page in your dialog for controlling the microscope components, click on **New Tab**.

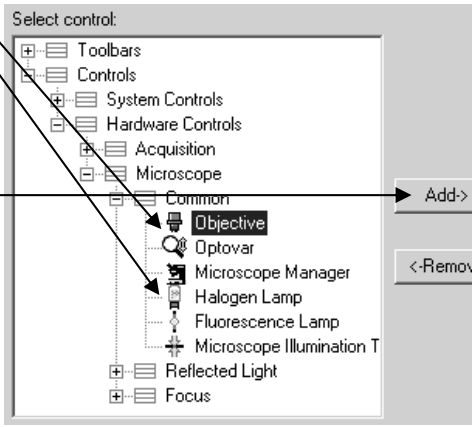


- Specify a **Name** and a **Tooltip Text**.



- Then click on **OK** to create the property page.

- Now select **Objective** and **Halogen lamp** in the control elements field.



- To insert both into the dialog, click on **Add**.

Note:

- The control elements depend on your system configuration and may differ from those displayed here.

- Close the dialog by clicking on the button in the upper-right corner of the window.



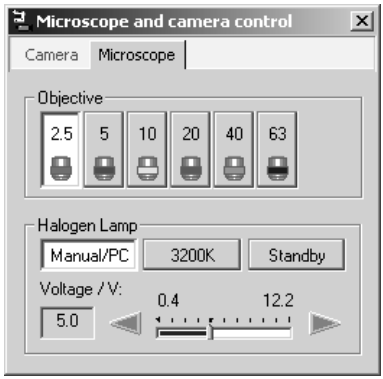
The button is now displayed on the toolbar you have chosen. The result now looks like this:

The dialog contains two property pages: **Camera** and **Microscope**.

- The **Camera** property page contains the element for controlling the **Exposure** time.



- The **Microscope** property page contains the elements for controlling the objectives and for controlling the lamp voltage.



Note:

- Also check the Tooltip text and the texts in the status line.

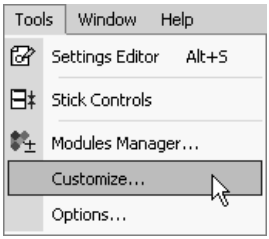
Define workflows

Workflows are the most efficient way to operate AxioVision. They are the "central element" for executing the functions required to perform a specific task. All you have to do is click on the buttons they contain one after the other to achieve the desired results quickly and without problems. If you also hide all the control elements that you do not need, you can concentrate fully on the work you are actually performing.

Note:

- You can also place dialogs you have created yourself and all the toolbars on a workflow.

- From the **Tools** menu select the **Customize** function.

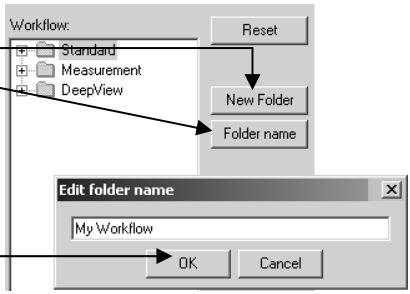


- Make sure that the **Workflows** property page is active in the **Customize** dialog.



To create a workflow:

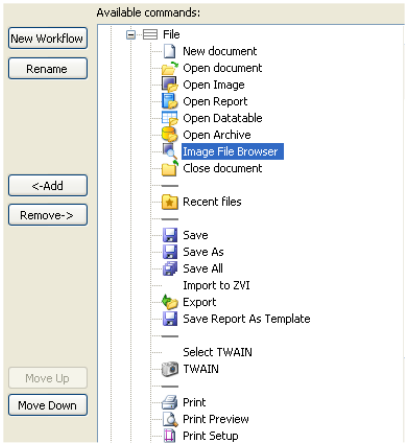
- Click on **New Folder** and then on **Folder name**.
- Specify a name for the new workflow.
- Click on **OK**.



In the **Available commands** field, select the commands that you would like to use on the toolbar.

- Select, for example, the menu command **File** ⇒ **Image File Browser**.
- Then click on **Add** to apply it to the workflow.
- Also apply the menu commands

  View ⇒ **Show Properties**
  Annotations ⇒ **Scale bar**
  File ⇒ **Save**



➢ Close the dialog by clicking on the button in the upper-right corner of the window.



The workflow is now located under the **My Workflow** bar.



8.6 General Settings

In this dialog you can enter settings for the configuration of AxioVision. The settings are grouped by topic on property pages.

The settings comprise:

- AxioVision's starting performance.
- Selection of language.
- Specification of user-specific paths for AxioVision documents.
- Image display.
- Automatic saving of images after acquisition.
- Entry of user information.
- And much more.

From the **Tools** menu select the **Options** function. The **Options** window with the **General**, **Layout**, **Folder**, **Storage**, **Acquisition**, **Naming**, **Display**, **ID** and **Commander** property pages is opened.

Details on the settings used there, and how they influence the system, can be found in the online help.

8.7 Exporting and Importing User Configurations

General

The term user configuration is understood to mean all the settings you have made to adapt AxioVision to your own personal requirements after installation.

The **Import/Export Configuration** function allows you to back up your personal work environment in a convenient way, so that you can restore it quickly and easily in the event of problems with the system. On multi-user systems, you therefore have the option of making settings that are of interest to all users (e.g. scalings, toolbars etc.) available very quickly to all users by offering access to this configuration via the shared account **All Users**.

Please also read section 8.3 "User and Data Administration", as well as the section "Importing user files" later in this section.

The following elements of AxioVision can be exported:

- All settings made in the **Customize** dialog on the **Tools** menu. This means all the toolbars, shortcut keys, workflows and dialogs you have generated. (Please also read section 8.5 "Adapting the User Interface".)

- All settings in the **Options** dialog on the **Tools** menu. (Please also read section 8.6 "General Settings".)

- You can also save a record of which modules have been activated. See the **Modules Manager** function on the **Tools** menu.

To allow you to make your personal files available to other users, if necessary, all files from the folder *My Documents\Carl Zeiss* are placed together in a compressed archive (ZIP file). The folder structure from the Carl Zeiss data folder is retained.

Export

➢ Select from the **Tools** menu the **Import/Export Configuration** function.

A wizard is started. This guides you through the individual steps of the export process.

➢ Select the elements to be exported, and the folder and file name for the target file.

This will result in two files (if you have also exported the user files) in the selected folder. One file has the extension *.avconf. This file contains all the elements of your configuration. The second file has the extension *.zip. All the user files are saved in this file.

Note:
- If you want to make your exported configuration available to other users, make sure that you save the files in a generally accessible folder.

Import

Importing the configuration files

➢ To import the configuration of another user, select from the **Tools** menu the **Import/Export Configuration** function.

A wizard is started. This guides you through the individual steps of the import process. At the end of the process, all of the other user's elements are available.

NOTES:
- A configuration can only be imported by the relevant user.

Importing user files

User data cannot be copied via the import function. This has to be performed separately by unpacking the ZIP file. The reason for this is to avoid files you have created yourself being inadvertently overwritten.

- Start Microsoft Windows Explorer, and go to the previously selected folder containing the ZIP file with the other user's files.

- Double-click on the ZIP file to unpack it.

- Now select the folder *My Documents* as the target folder. The user files are copied automatically into the correct folder structure. If the *Carl Zeiss* subfolder does not yet exist in your *My Documents* folder, it is created

Notes:

- You need appropriate software to unpack ZIP files. A program is available automatically under Windows XP Professional. Ideally contact your system administrator. Usually these kinds of tools will be present anyway.

- If you want to make the files available to all users, select the shared account *All Users (Shared Documents)* as the target folder for unpacking the files. All users of the system will then be able to share use of the files (e.g. scalings).

8.8 Creating various AxioVision configurations for a user

Background

Anyone who finds the management of users too complicated can continue to use the procedure supported under AxioVision Release 3 (see below). Other subfolders with new profiles are created for the different configurations in the *Application Data\Carl Zeiss\AxioVS40\Profiles* folder of the relevant user account, in addition to the default profile **Default**. The name of the profile is allocated while it is being created. The description from the earlier manual follows:

Creating individual configurations

➢ Right-click on the AxioVision icon on the Microsoft Windows desktop.

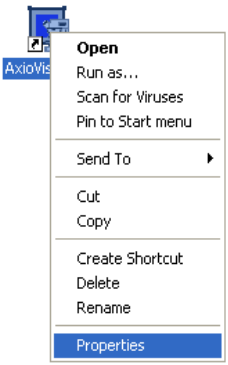
➢ Select the **Create Shortcut** function from the shortcut menu.



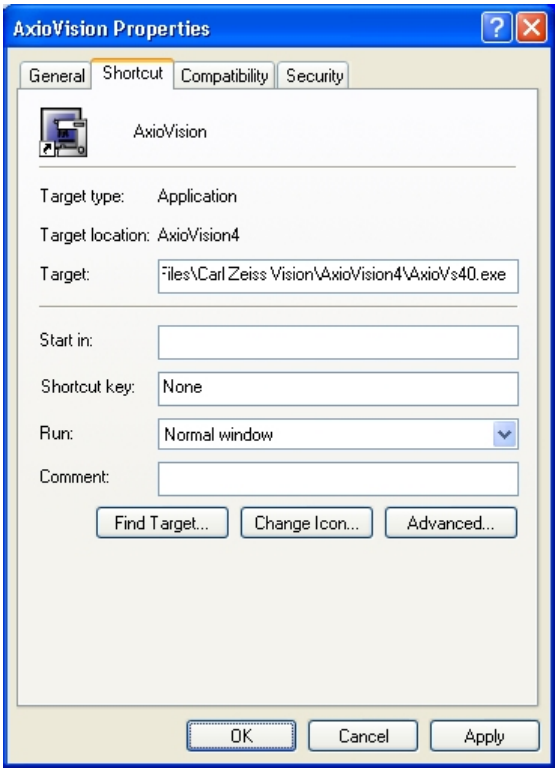
➢ A new AxioVision icon is created on the desktop.



- Now right-click on the new AxioVision icon, and select the **Properties** function from the shortcut menu.



➢ The **Properties** window is opened.



➢ Enter the following at the end of the **Target** field: -u=Name (N.B. Insert a space before -u!). Here 'Name' is the description for the configuration. Use, for example, -u=Test to create a configuration with the name Test.



➢ Adopt the setting by clicking on **OK**.

- To change the description of the new configuration on the Microsoft Windows desktop, right-click again on the new AxioVision icon, and from the shortcut menu select the **Rename** command. You can now enter a name of your choice in the icon's description field.



- Enter, for example, Test Configuration and then click on any area of the desktop to adopt the entry.



- You can now start AxioVision from the new icon and configure it accordingly.

9 Image Acquisition Modules

9.1 ApoTome 2

General

The **ApoTome 2** software module controls the ApoTome 2 hardware (control box and slider) and coordinated image acquisition using a digital camera, such as the AxioCam **MRm**. An ApoTome 2 system allows you to generate optical sections through fluorescence samples. The parts of the image that are out of focus are then removed, and an increase in

- image sharpness
- the signal to background ratio (contrast)
- and resolution in the axial direction

is achieved.

The following units are required for an ApoTome 2 imaging workstation:

- Microscope: Axio Imager.Z1, Axio Imager.D1, Axio Observer.Z1, Axio Observer.D1 or Axiovert 200
- Anti-vibration system
- Digital camera with more than 10-bit dynamic range
- ApoTome 2 control box and slider (release for Axio Imager, Axioplan Observer / Axiovert 200)
- PC with monitor
- AxioVision basic package and the optional ApoTome 2 software module

For details on setting up and using the hardware components, such as the microscope and ApoTome 2 control box/slider, please refer to the manuals enclosed with the corresponding devices.

Note:

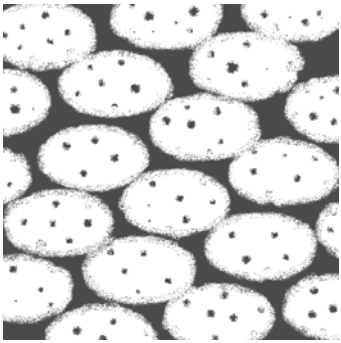
- Before using the ApoTome 2, familiarize yourself with the basic functions of the AxioVision software. You should be familiar with the operation of the camera and microscope components in particular.

Fringe projection imaging principle

The optics of a microscope are optimized for analyzing very thin samples. For a cover-glass-corrected objective, all optical calculations are performed for very thin objects that lie directly beneath the cover glass. All cover-glass-corrected objectives from Carl Zeiss are optimized for this particular usage, and exhibit an optimum Point Spread Function (PSF) for the wavelengths for which the corresponding objective has been specified.

In biological applications, however, the vast majority of samples used do not satisfy these optimum requirements. Sometimes thicker biological tissue slices are used, e.g. to analyze cells in the tissue using specific fluorescent markers.

In such cases, during microscopic analysis, and particularly during documentation, the set focus plane is hidden by parts of the image that originate from above and below the actual focus plane. As a result the image appears "faded", the contrast is reduced, and the background becomes bright. In extreme cases important structures and image details may be completely hidden.



The above representation of a microscopic image of cell nuclei in tissue shows this effect.

A number of methods can be used to prevent or reverse this effect, such as confocal laser scanning microscopy or 3D Deconvolution.

With the ApoTome 2 the principle of "fringe projection" has been employed. This technology involves inserting a grid structure with grid lines of a defined width into the plane of the field diaphragm of the reflected light beam path. As the plane of the field diaphragm is matched to the focal plane, this grid structure can be displayed on the microscope. When you look into the eyepiece you can therefore see the grid, superimposed onto the actual sample.



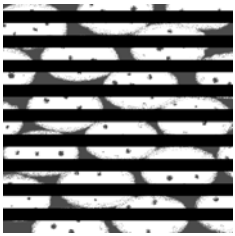
Above is a schematic representation of the reproduction of the grid. In reality the grid lines are much thinner.

A scanning mechanism in the ApoTome 2 slider is used to move the grid structure in three defined steps within the sample plane. The movement of the grid takes place very quickly (in less than 20 ms). A digital image is acquired at each grid position.

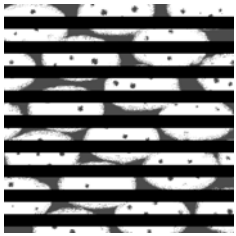
The movement of the grid is represented schematically below:



Grid Position 1



Grid Position 2



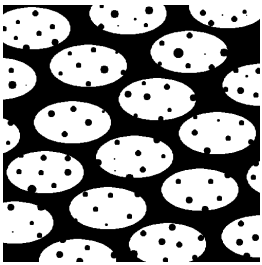
Grid Position 3

The three raw images are combined online on the PC to form a resulting image. The time it takes to process the image depends on the image size:

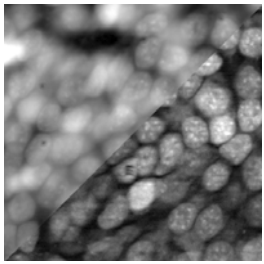
- 512 x 512: approx. 30 ms
- 1300 x 1000: approx. 100 s

The processed resulting image is an optical section through the sample with the following characteristics:

- The grid structure has been removed from the raw images.
- The parts of the image that are out of focus are no longer visible.
- The sharpness and contrast of the image have been increased.
- The image's resolution in the axial direction has been increased.



A corresponding resulting image is represented schematically above.

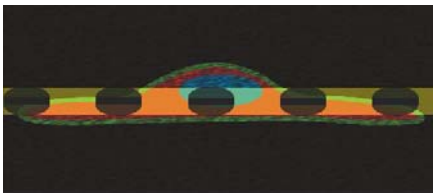


The above image is an application image of cell nuclei (tadpole brain section) in black and white.

- Top left: conventional fluorescence
- Bottom right: optical section

Why is the resulting image an optical section?

One possible way to explain this is to use the image of the grid in the sample:



The image of the grid provides the necessary information on the distance of the various sample structures from the set focal plane (see figure above). Some sample structures are in focus, while others lie above or below the focal plane, and enter the set focal plane.

The technique of grid projection makes use of the fact that the image of the grid above and below the actual focal plane is blurred, and enters the blurred areas of the sample. When the grid line is moved, significant brightness differences (= contrast) appear in the focal plane. Outside the focal plane only minor differences are produced, as the sample and the image of the grid are practically "blurred" together. The brightness differences are detected by the algorithm used to combine the three raw images, and are used to remove the parts of the image that are out of focus.

Hardware configuration

For information on setting up the devices, please refer to the manuals of the devices concerned.

- First switch on the microscope, then the fluorescence switched-mode power supply, followed by the ApoTome 2 control box and the camera.

- Then switch on the PC.

ApoTome 2 Configuration for Axio Imager, Axio Observer or Axiovert 200

➢ Open the **MTB2004 Configuration** program on the Microsoft Windows desktop by double-clicking on the icon 

.

Note:

- Configurations for various microscopes are created and managed in **MTB2004 Configuration**. Please refer to the manual or online help for **MTB2004 Configuration** for detailed information on creating configurations.

➢ For the description of the ApoTome 2 configuration below, it will be assumed that the microscope itself has already been configured.

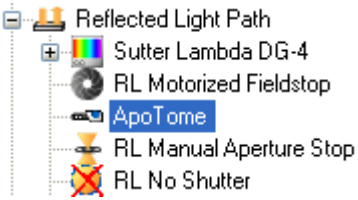
➢ Make sure that the ApoTome 2 control box is connected to the PC and is switched on.

➢ Open the configuration tree under **Reflected Light Path**.

➢ Click on the **No ApoTome 2** entry. The entry will be shown with a blue background.



➢ Select the **ApoTome 2** by clicking on **ApoTome 2** in the selection area on the right-hand side of the user interface and assign it.



➢ In the information area, you can now select the connection type (RS232 or CAN bus) and the COM port being used (port 1 to 5).



➢ Check the connection by clicking on the **Check Connection** button. If the **ApoTome 2** is found at the specified port, a green symbol is displayed next to the button.

Note:

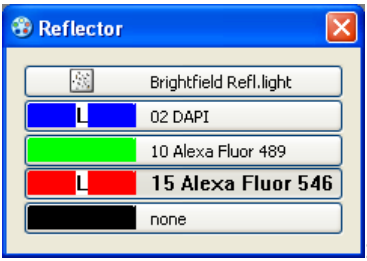
- If you activate the **Simulate Hardware** check box, for example, the function of the ApoTome 2 can be adjusted virtually in the **AxioVision** microscope control and imaging software (simulation mode).

ApoTome 2 Calibrations

➢ Start the AxioVision software by double-clicking on the 

icon on the Microsoft Windows desktop.

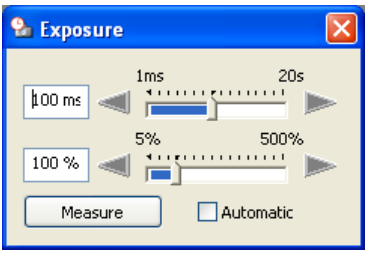
For subsequent steps it is useful to have easy access to the following control elements. Please bear in mind that, depending on the features of the device, some control elements may not be present, or may appear different:



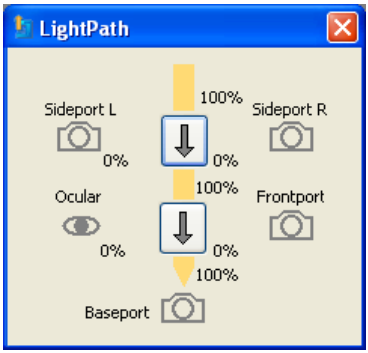
: Reflector nosepiece for switching between fluorescence wavelengths.



Internal shutter for opening and closing the shutter.



Camera exposure time.



Light path for switching the light path, e.g. with an Axiovert 200.

You can also create a user-specific dialog containing all the control elements required, including the control elements for the ApoTome 2 functions. Information on creating dialogs can be found in chapter 8 "Configuration".

Calibration of phase position

To set the optimum angle of deflection for the ApoTome 2's scanner unit, you need to carry out a fine adjustment of the scanner calibration in accordance with the system structure. The mirror sample and the special reflected light reflector cube, both of which are supplied with the ApoTome 2, are used for this purpose. Insert the Push&Click filter cube into an empty reflector nosepiece position, and enter this filter set as **Refl. BF**, for example, in the **Microscope Configuration** or the **MTB2004**. The calibration must be performed for each of the two grids provided.

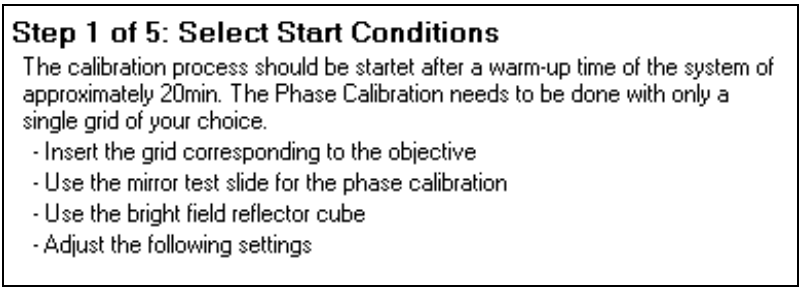
It is advisable to calibrate the grid for the low magnification range (grid marked with "L" for "Low magnification") using a 20x objective.

In the phase calibration dialog the positioning of the camera is also optimized. To achieve optimum performance, the camera horizontal should be aligned parallel to the ApoTome 2 grid lines with as much precision as possible.

The calibration process is supported by a wizard. Start the function by selecting from the **Acquisition** menu the **ApoTome 2** function, and then **Phase Calibration**.

The software wizard guides you through the calibration process in 5 steps. The most important instructions are displayed in the wizard's text field.

Step 1: Select Start Conditions



- Move the ApoTome 2 slider to the iris position (click stop position 1).
- Use the reflector position with the ApoTome 2 bright-field reflector.
- Place the mirror sample supplied under the microscope, open the reflected light shutter, and focus (directly on the microscope) on the reticle at the center of the sample.
- Now switch to operation of the software on the PC.
- In the software, enter settings for grid, reflector, and objective, if you have not already done so. Pay particular attention to ensuring that the grid is correctly selected.
- Three different transmission grids are available in ApoTome 2 that covering the whole magnification area from 10x to 100x. The software offers automatically the optimal grid. As an option you can manually selct another grid (e.g. to adopt the section thickness to the experimental conditions).
- Activate the **Automatic** check box for the automatical selection of the grid.

Note:

- You can use the ApoTome 2 in combination with a Colibri, an excitation filter wheel, a Sutter DG-4 or DG-5 or a T.I.L.L. Photonics Polychrome V. In this case, carry out the relevant settings in addition to the microscope settings (grid, reflector, objective).

➢ Click on **Next** to proceed to the next step. The live image is opened automatically.

Step 2: Optical Focusing

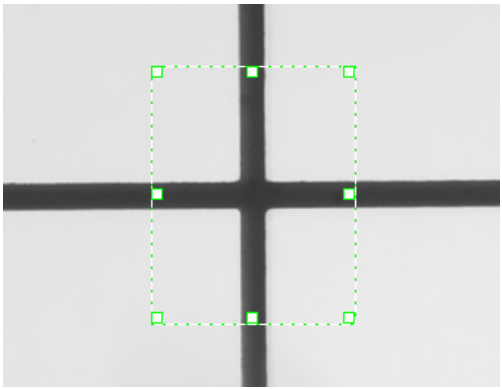
Step 2 of 5: Optical Focusing

Use the first click-stop of the ApoTome slider (iris position) to focus on the cross hair of the mirror slide

- Adjust the exposure time of the camera

➢ Set an optimum exposure time by clicking on the Measure button.

➢ Display the reticle as sharply as possible in the live image. The green focusing rectangle in the live image can help you find the optimum focus position. Position the focusing rectangle over the center of the reticle.



Notes:

- On the **Microscope** property page you can enter general settings for the microscope.

- Click on **Next** to proceed to the next step.

Step 3: Grid Focusing

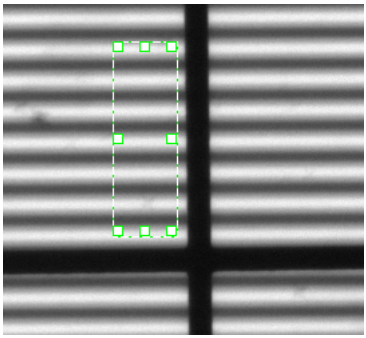
Step 3 of 5: Grid Focusing

Carefully push the ApoTome slider in the second click-stop position. Make sure, that grid lines are aligned horizontally (Camera adjustment)

- Position the green focus rectangle over approximately 10 grid lines
- Click on 'Full Scan'
- (The grid will be moved through the complete travel range)
- The grid position with the highest focus value will automatically be selected

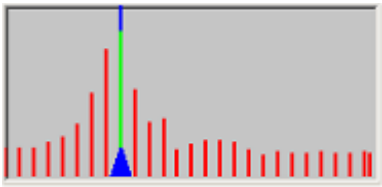
Move the ApoTome 2 slider carefully to the second click stop position (ApoTome 2 mode).

➢ Move the focusing rectangle in the Live window to a position where no part of the reticle is present.

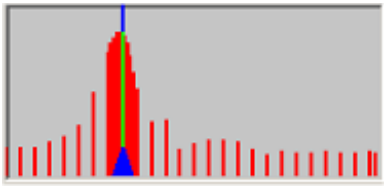


➢ Click on the **Full Scan** button. The grid will now move through its adjustment range automatically, in increments of ten steps. A sharpness value for the reproduction of the grid will be determined, and displayed

as a histogram, following each increment. When the end of the adjustment range has been reached, the grid position with the highest sharpness value will be set automatically.



➢ You can choose to carry out a fine adjustment around the grid position with the highest sharpness value by clicking on the **Local Scan** button. A local area above and below the sharpness maximum that has been identified will be covered automatically in individual steps, and the sharpness value will be determined for each individual step. The data from the local scan will be included in the histogram, and the grid position with the highest sharpness value will again be located automatically.



Note:

- You can determine the grid position freely using the buttons for coarse focusing,  and  , and fine focusing,  and  . You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

Step 4: Camera Alignment

Step 4 of 5: Camera Alignment

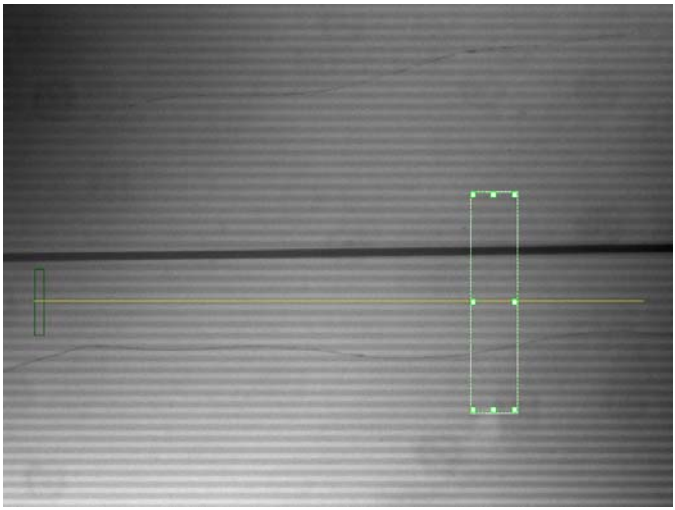
Move the cross hair out of the live image. Move the starting point of the grid detection by moving the green focus rectangle up and down.

- Rotate the camera head until you get as close to 0.00 degree as possible

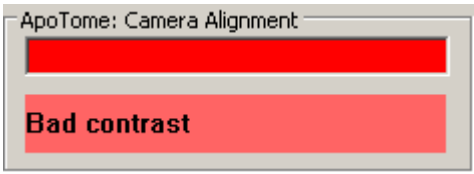
The purpose of this step is to achieve precise alignment of the camera. To achieve optimum results, the camera horizontal should be aligned parallel to the grid lines with as much precision as possible. The software automatically detects a grid line and calculates the camera horizontal's deviation from this grid line in degrees.

As the vertical line of the reticle interferes with the detection of the grid lines, please first move the reticle out of the camera's field of view.

A green rectangle marks the software's search area, a yellow line indicates a detected grid line and a small dark-green rectangle shows you the starting point for line detection.

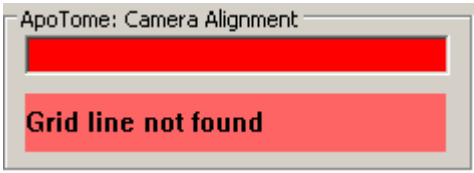


If the contrast in the image is insufficient to detect a grid line automatically, a corresponding error message appears:



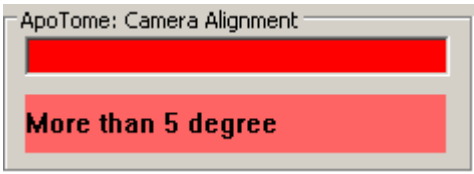
In this case you should take steps to increase the contrast (e.g. increase the exposure time, refocus the grid (step 3)).

If it is still not possible to detect a grid line, a corresponding error message appears:

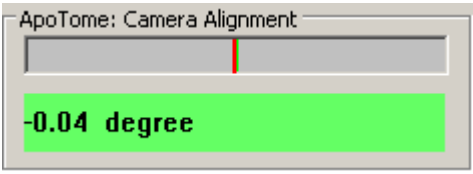


In this case the deviation of the grid lines from the camera horizontal is probably too great. Rotate the camera until the long side of the camera's field of view is roughly parallel to the grid lines. To do this, loosen the corresponding lock on the camera adapter and realign the camera.

If a grid line can be detected but the deviation is still greater than 5 °, you will see a message to this effect:



In this case, rotate the camera further to minimize the deviation. For fine adjustment you can use the red bar in the dialog. Try to achieve a deviation < 0.1 °.

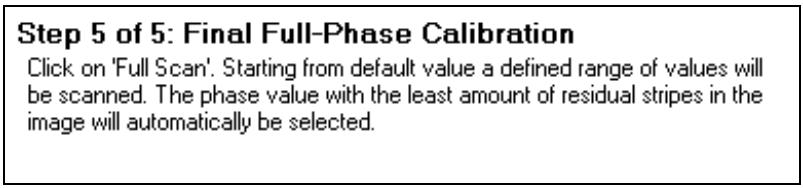


Once the camera has been aligned with sufficient precision, please lock the camera adapter in this position.

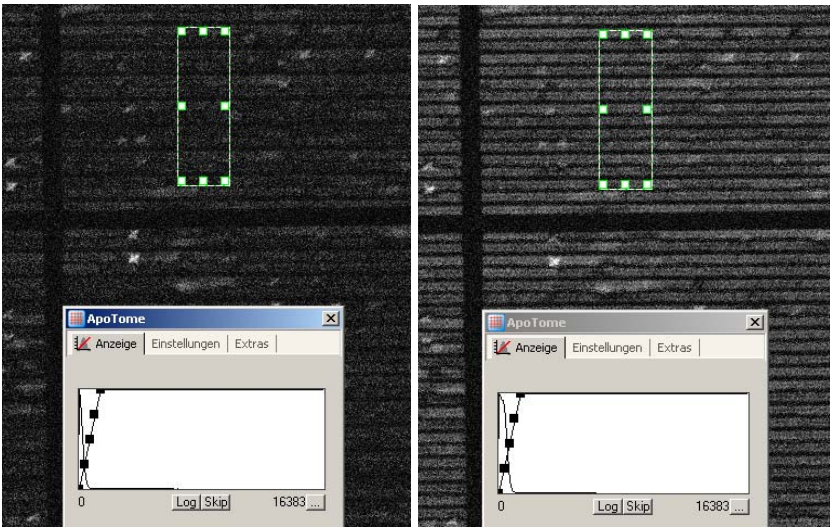
Notes:

- If you change the ApoTome 2 grid, the new grid should be inserted with the same alignment, if possible. The grid mount in the ApoTome 2 slider has a small amount of play to make it as simple as possible to change the grid. You should always align the grid, if possible, with the same edges in the grid mount. If you wish to check the camera alignment following a change of grid, you can run through the phase calibration dialog up to step 4. Should you not wish to perform a new phase calibration, cancel the dialog after step 4, once the camera has been aligned.

Step 5: Final Full-Phase Calibration

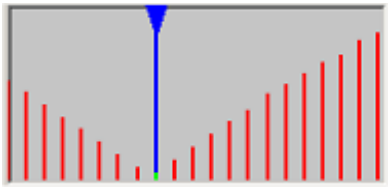


This step is used for the actual setting of the scanner calibration. In each case, two images are acquired. The grid is displaced by precisely the width of a line between the first and second image. The two images are subtracted from each other, and the resulting image is displayed. If the system is perfectly calibrated, the two images cancel each other out – a black image with a noise component is shown.

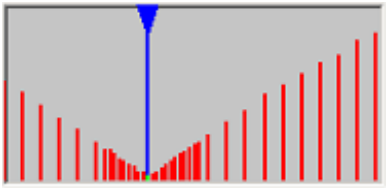


Phase calibration is performed automatically in accordance with the focus calibration of the grid, carried out in the preceding step.

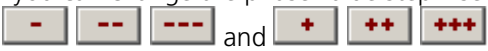
- Click on the **Full Scan** button. A predefined area around the specified phase calibration value will be covered in coarse steps. At each position, the calibration value will be determined, and displayed in the histogram. When the end of the area has been reached, the minimum value (minimum number of residual lines present in the image) will automatically be selected in the histogram.



- You can choose to perform an additional fine adjustment using the **Local Scan** button. An area close to the minimum on either side will be covered in individual steps, and the data included in the histogram. This may enable you to identify an even lower minimum, where there are even fewer residual lines in the image.



Notes:

- Optional you can change the phase value stepwise by clicking on the buttons  . You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.
- Click on the **Close** button to end the phase calibration.

➢ You will now be asked whether you want to adopt the phase calibration for the other grids.

- **Yes**: in computed mode the calibration values are transferred to the other grids.
- **No**: Each grid can be calibrated manually. To do so, repeat this procedure to calibrate the phase shifting for the second and third grid.

- Although the settings do not change once calibration has been performed, from time to time you should check that the system is perfectly calibrated. This applies in particular after dismantling and reassembling the system.

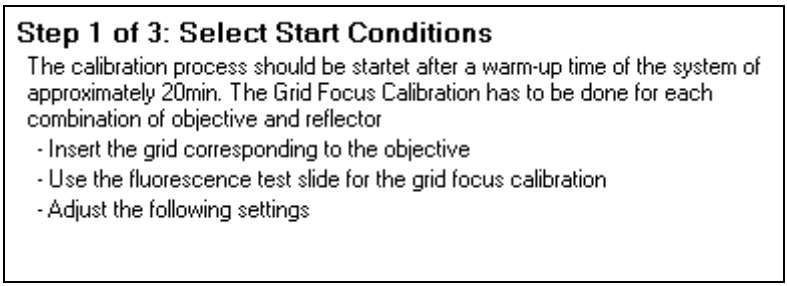
Calibration of the grid focus

For the ApoTome 2 to function optimally, the grid must be displayed precisely in the focus set on the objective. The grid's focus depends on the objective and the excitation and emission wavelengths of the fluorescence. This means that the grid focus needs to be calibrated for every fluorescent dye (e.g. DAPI, FITC, Rhodamine etc.). It is best to perform calibration using your own fluorescence sample, although this must exhibit flat fluorescence to allow the focusing of the grid. Alternatively, you can also use the fluorescence sample provided.

A wizard is available for the calibration of the grid focus. This guides you through the calibration process in 3 steps. Start the function by selecting from the **Acquisition** menu the **ApoTome 2** function, and then **Grid Focus Calibration**.

The most important instructions are displayed in the wizard's text field.

Step 1: Select Start Conditions

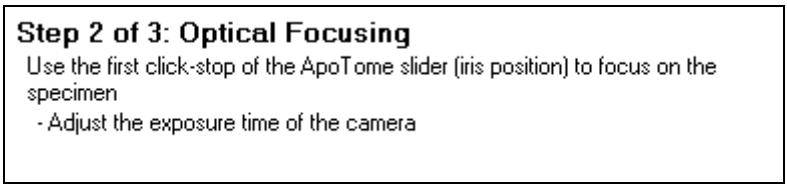


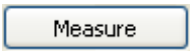
- Move the ApoTome 2 slider to the iris position (click stop position 1).
- Use a reflector position with a fluorescence filter (e.g. 10 Ex. 470/40).
- Place the fluorescence sample under the microscope, and open the reflected light shutter.
- Focus on the sample on the microscope.
- Switch to operation of the software on the PC.
- In the wizard, select the settings for **Grid**, **Reflector** and **Objective**, if you have not already done so.

Notes:

- Make sure that the correct transmission grid is selected for the objective being used. The table at the end of this chapter provides an overview explaining which transmission grid should be assigned to which objective.
- It is possible to use the ApoTome 2 in combination with a Colibri, an excitation filter wheel, a Sutter DG-4 or DG-5 or a T.I.L.L. Photonics Polychrome V. In this case, carry out the relevant settings in addition to the microscope settings (grid, reflector, objective).
- Click on **Next** to proceed to the next step. The live image is opened automatically.

Step 2: Optical Focusing



➢ Set an optimum exposure time by clicking on the 

button.

➢ Focus the sample as sharply as possible in the live image.

Note:

- On the **Microscope** property page you can enter general settings for the microscope.

➢ Click on **Next** to proceed to the next step.

Step 3: Grid Focusing

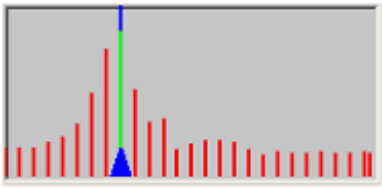
Step 3 of 3: Grid Focusing

Carefully push the ApoTome slider in the second click-stop position
- Adjust the exposure time of the camera if necessary
- Position the green focus rectangle over approximately 10 grid lines
- Click on 'Full Scan'
- (The grid will be moved through the complete travel range)
- The grid position with the highest focus value will automatically be selected

- Move the ApoTome 2 slider carefully to the second click stop position (ApoTome 2 mode).

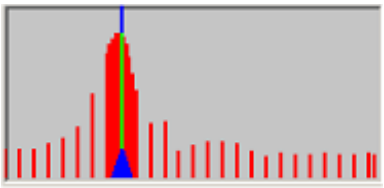
- Move the focusing rectangle in the live window to a position where the fluorescence is as flat and bright as possible.

- Click on the **Full Scan** button. The grid will now move through its adjustment range automatically, in increments of ten steps. A sharpness value for the reproduction of the grid will be determined, and displayed as a histogram, following each increment. When the end of the adjustment range has been reached, the grid position with the highest sharpness value will be set automatically.



- You can choose to carry out a fine adjustment around the grid position with the highest sharpness value by clicking on the **Local Scan** button. A local area above and below the sharpness maximum that has been identified will be covered automatically in individual steps, and the sharpness value will be determined for each individual step.

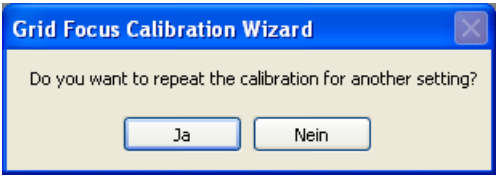
The data from the local scan will be included in the histogram, and the grid position with the highest sharpness value will again be located automatically.



Note:

- You can determine the grid position freely using the buttons for coarse focusing,  and  , and fine focusing,  and  . You may need to use this function if, for example, a sharpness maximum cannot be determined automatically.

➢ When you click on **Close** to end the calibration, the following dialog window is displayed:



➢ Click **Yes**, if you want to carry out a further calibration, or click **No** if you want to end the calibration.

Once calibration is complete, a corresponding message is displayed in the status window of the ApoTome 2 dialog window (**Acquisition** menu ⇒ **ApoTome 2** function ⇒ **ApoTome 2 Dialog** ⇒ **Settings**).

Calibration mode

There are two possibilities for managing ApoTome 2 calibration data:

- **User mode** (conventional): An individual file containing the calibration data is generated in each Windows user account, i.e. each user generates his own calibrations and then works with these. Other users do not have access to these data. In this case, the file containing the calibration data can be found in the *My Documents* folder of the user account in question under *My Documents\Carl Zeiss\Data\Apotome2Setting\Default.ini* ".

- **Administrator mode**: A user generates calibration data which are then made available to all other users. In this case, only the user who has generated calibrations in the ApoTome 2 administrator mode is able to change these or add to them. The file containing the calibration data can be found in the folder *Shared Documents\Carl Zeiss\Data\Apotome2Setting\Default.ini*.

The user mode is activated automatically following a new installation of AxioVision. If you want to change the mode, open the calibration mode dialog (**Acquisition** menu ⇒ **ApoTome 2** function ⇒ **Calibration mode**):



The administrator mode is password-protected. The default password is "zeiss" and can be replaced by a password of your choice following the first log in.



Once administrator mode has been activated, all users are only able to access the calibrations that can be found in the shared files. However, any individual calibrations that have previously been generated remain in the relevant user accounts (although they cannot be used). If you switch back from the administrator mode to the user mode, the file containing the calibration data that has been saved in the shared files will be copied to the Windows user account of the ApoTome 2 administrator. Users of other Windows accounts can then use their old calibrations again or may have to generate new calibrations.

Image acquisition

As an ApoTome 2 imaging system works with a digital CCD camera, the ApoTome 2 mode can be regarded as an expansion of the camera's functionality.

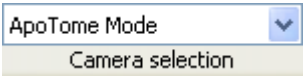
Selecting the camera

In principle, a cooled monochrome CCD camera (AxioCam MRm, AxioCam HRm etc.) is used in combination with the ApoTome 2 for fluorescence images. When you start AxioVision, a monochrome camera is automatically selected (if possible) for operation with the ApoTome 2.

If several monochrome cameras are connected to the system, you need to define the one that you want to be used in ApoTome 2 mode. To select the camera, follow the procedure below:

- Click on **ApoTome 2** in the work area or open the ApoTome 2 dialog (**Acquisition** menu ⇒ **ApoTome 2** function ⇒ **ApoTome 2 Dialog**). Select the **Extras** property page

- In the **ApoTome 2: Camera** drop-down field, select the camera that you want to use in ApoTome 2 mode.



Single image acquisition

For single image acquisition, follow the procedure below:

- For efficient operation, open the control elements for the camera exposure time (**Acquisition** menu ⇒ **Adjust** function ⇒ **Exposure**), the shutter and the reflector (**Microscope** menu ⇒ **Reflected Light** function ⇒ **Reflector** or **Internal Shutter**).

- Make sure that the ApoTome 2 slider is in the second click stop position (ApoTome 2 mode).

- Click on **ApoTome 2** in the work area or open the ApoTome 2 dialog (**Acquisition** menu ⇒ **ApoTome 2** function ⇒ **ApoTome 2 Dialog** ⇒ **Settings** property page).

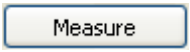
- Three different transmission grids are available in ApoTome 2 that covering the whole magnification area from 10x up to 100x. It is not necessary to choose the grid from a list, depending of the selected objective. The software offers automatically the optimal grid. As an option you can manually selct another grid (e.g. to adopt the section thickness to the experimental conditions).

- Activate the **Automatic** check box for the automatical selection of the grid.

- Select the **Camera** property page.

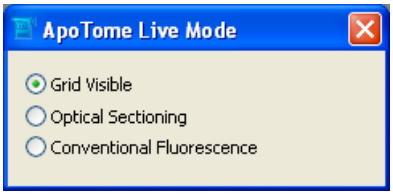
The live image is opened using the 

button on the toolbar. The shutter is opened automatically with the live image.

- Click on the 

button to set an optimum exposure time for the specimen. To achieve optimum results with the ApoTome 2, the images should be illuminated in the best possible way. Full use should be made of the camera's dynamic range. However, no pixels should be overexposed. The histogram displayed will help you achieve optimum illumination.

Various settings can be made for the ApoTome 2 mode. Three settings are available for the live image in ApoTome 2 mode (**Settings** property page in the ApoTome 2 dialog).



Grid Visible: The behavior of the live image is the same as in conventional camera mode. No processing takes place. As the grid is located in the beam path, it is also visible in the live image. The grid is moved up and down

constantly, to prevent grid lines being "burnt" into the sample as a result of the bleaching effects of the fluorescent dye.

Optical Sectioning: In this mode three images are acquired, combined online into an optical section, and displayed. As this mode requires the acquisition of three raw images, the speed of the live image is reduced accordingly.

Note:

- In this mode, if you change the focus on the microscope or the position of the sample, artifacts, in the form of lines, may appear in the live image for a short time. The same effect can occur if vibrations are passed on to the microscope. The lines disappear from the live image as soon as the sample is stabilized again.

Conventional Fluorescence: In this mode two raw images are acquired, and the grid position is displaced in each case by precisely the width of the lines. From these two raw images, an image that corresponds to the conventional fluorescence image (wide field) can be calculated. This mode enables you to make comparisons between the conventional fluorescence image and the optical section.

Image acquisition is triggered using the 

button on the toolbar.

Four settings are available for image acquisition in ApoTome 2 mode (**Settings** property page in the **ApoTome 2** dialog).

- **No Processing**: The behavior of image acquisition is the same as in conventional camera mode. No processing takes place. As the grid is located in the beam path, but is moved constantly up and down, the image acquired corresponds to a conventional fluorescence image. This setting is only used if you are carrying out automated image acquisition in several channels, and processing needs to be switched off, e.g. to acquire a transmitted light or a phase contrast image.

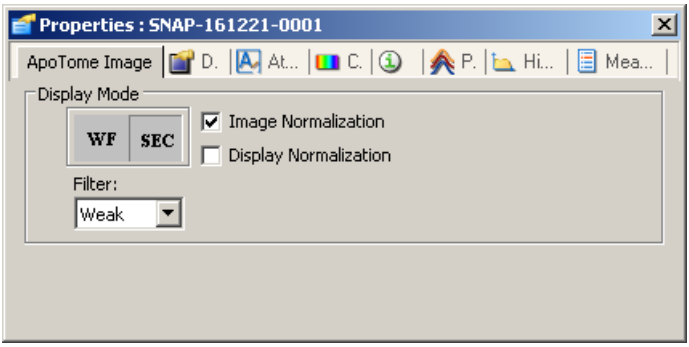
- **Optical Sectioning**: In this mode three images are acquired, combined online into an optical section, and displayed.

- **Conventional Fluorescence**: In this mode two raw images are acquired, and the grid position is displaced in each case by precisely the width of the lines. From these two raw images, an image that corresponds to the conventional fluorescence image (wide field) can be calculated. This mode enables you to make comparisons between the conventional fluorescence image and the optical section.

- **Raw Data Mode**: In this mode the raw image acquisition data are saved (i.e. the individual images with the various grid positions are saved internally). The advantage of this mode is that it allows you to switch between the algorithms (**Optical Sectioning** versus **Conventianal Fluorescence**) after image acquisition:

  - To do this, open the shortcut menu for the acquired image by clicking on the **Properties** icon at the bottom edge of the image. screen Alternatively, you can also open the shortcut menu by right-clicking in the acquired image.

ApoTome 2 Image property page:



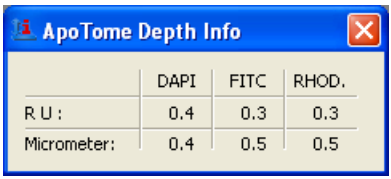
- Click on the **WF** (Widefield = **Conventional Fluorescence**) or **SEC** (Section = **Optical Sectioning**) buttons to switch between the corresponding algorithms. The filter used to remove residual lines in the image (**Filter** drop-down list box) and the display scaling (**Auto Display Scaling**) can also be adjusted. The application of the algorithm takes place purely in the display plane of the image data and does not change the original raw data.

Note:

- In order to process ApoTome 2 data in AxioVision using the 3D Deconvolution module, you must acquire the image in the **Raw Data Mode**.

Acquiring images in the **Raw Data Mode** substantially increases the memory required for the image data. In the **Averaging** default setting, for example, it is increased by a factor of 3 in comparison to an image acquired with ApoTome 2 in the **Conventional Fluorescence** or **Optical Sectioning** modes. If the **Averaging** value is increased by 2 or 3, the resulting memory requirement increases by a factor **of** 6 or 9. We therefore recommend that you use the **SIMConvert** function to convert the raw data to an optical section or a conventional fluorescence image, thereby substantially reducing the data volume again (e.g. after using 3D Deconvolution or for 3D reconstruction of an image stack using the Inside 4D module.)

The thickness of the **Optical Section** can be displayed in the **Depth Info** field on the **Extras** property page.



Notes:

- Depending on the sample, fluorescent dye, exposure time etc., fine residual lines may be visible in the resulting image in ApoTome 2 mode. These lines can be removed using the filter. You can select **Off**, **Weak**, **Medium** and **Strong**.

- The residual lines are displayed in the Fourier space as points along a vertical line. The filter "removes" a defined group of points from the Fourier spectrum.

Start with the setting **Weak**. If residual lines can still be identified in the image, increase the setting step by step. As the filter is used during the calculation of the optical section, a new image must be acquired whenever changes are made to the filter setting.

A further increase in image quality can be achieved if you use a value of 2 or higher in the **Averaging** field.



If you use the value **2**, for example, two ApoTome 2 images are acquired and averaged. This method significantly reduces the noise component. An additional effect is the reduction or removal of residual lines in the image.

Multidimensional acquisition with the ApoTome 2

The ApoTome 2 functions are fully integrated into AxioVision's automatic image acquisition modes. You can perform acquisition of multichannel fluorescence images, z-stack images, time lapse images, MosaiX and any combination of these modes using the ApoTome 2. The images are acquired in accordance with the settings made under Acquisition menu ⇒ **ApoTome 2** function ⇒ **Acquisition Mode**. A precise description of the Multidimensional Acquisition functionality can be found in section "Multidimensional Acquisition".

A prerequisite for the above is calibration of the system as described in chapter 8 under the headings "Configuration" and "Scalings". Essentially, for multidimensional acquisition the same functions as those described in the previous sections apply.

If, during multichannel image acquisition, components that are responsible for the fluorescence wavelength being used are operated automatically, for example, a focus calibration must be available for each combination. The crucial components are:

- Reflector nosepiece
- Colibri
- External excitation filter wheel (e.g. Ludl, Sutter)
- Sutter DG-4 or DG-5
- T.I.L.L. Photonics Polychrome V

If a grid focus calibration is not available for a combination of the above external components, a single image is acquired automatically without any shifting of the grid. This artifact is usually easy to identify as an image with clearly pronounced fringes. For better control of image acquisition, open the **ApoTome 2: Status** window (**Acquisition** menu ⇒ **ApoTome 2** ⇒ **Status** function). This window will inform you if, during multidimensional image acquisition, a microscope status is set for which no ApoTome 2 focus calibration is available.

Accelerated image acquisition with the ApoTome 2 (burst mode)

With the ApoTome 2, the speed of image acquisition (image rate) is limited by the following:

1. At least three raw images have to be acquired for a resulting image.

2. During image acquisition, as much as possible of the gray-value range of the camera in question should be used. This generally results in exposure times that prevent high acquisition speeds from being achieved.

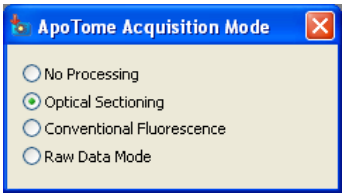
However, provided that acquisition takes place in one fluorescence channel only, it is possible to increase the image acquisition rate to a value in the region of 4 to 5 images/s. To achieve these values, follow the procedure below:

➢ Open the **ApoTome 2: Status** window (**Acquisition** menu ⇒ **ApoTome 2** ⇒ **Status** function).

➢ In the **ApoTome 2 Dialog** on the **Camera** property page, set a frame that is as small as possible (e.g. 512 x 512 pixels or smaller).

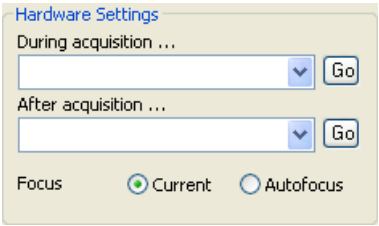
Tip:
If your requirements with regard to optical resolution allow, you can increase the binning on the camera to 2x2 (**Camera** work area⇒ **Frame** property page). This function makes it possible to reduce the camera's exposure time by around a factor of 4, at the expense of the resolution in the image.

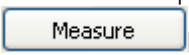
- For the **ApoTome 2: Acquisition Mode**, select the **Raw Date Mode** setting (**ApoTome 2** property page ⇒ **Settings** property page).

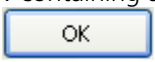


- In the work area, select the **Multidimensional Acquisition** entry and then the **C (Channels)** property page.

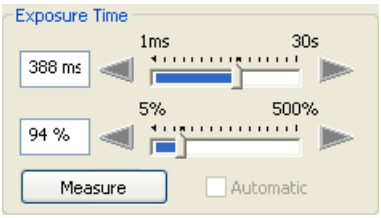
- Make sure that no **Hardware Settings** has been selected for the **During Acquisition** and **After Acquisition** drop-down list boxes.

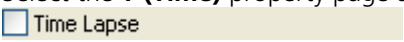


- Determine an optimum exposure time by clicking on the 

button. A pop-up window containing a live image opens. Confirm the exposure time by clicking on 

. If necessary, the measured exposure time can be reduced manually by around 10 to 20%.

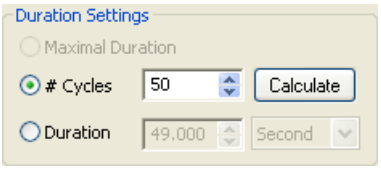


- Select the **T (Time)** property page and activate the  check box.

- Set the **Interval Settings** to **Maximal Speed** or alternatively the **Interval** to 0.



- In the **Duration Settings** field, define either the number of cycles **# Cycles** (e.g. 50) or the **Duration**.(e.g. 15s).



- Click on the  button to start acquisition of the image sequence. In the **ApoTome 2 Status** dialog the message **Calibrated Burst Mode: ON** is displayed.

  In this mode the image data are acquired as quickly as possible, with the grid being constantly shifted. The optical sections are only calculated after acquisition (and following a staggered pattern, i.e. the last 3, 6, 9 raw images etc.), according to the setting of the ApoTome 2's **Averaging** function, with different grid positions.

This technique increases the image rate by around a factor of 3, but not the temporal resolution, as rapidly moving particles on the sample can nevertheless lead to movement artifacts in the resulting image due to the exposure times selected during the acquisition of the raw data.

The image view (**Optional Sectioning** / **Conventional Fluorescence**) can be switched at any time, on the basis of the raw data acquired. The corresponding settings are made on the **ApoTome 2** property page in the shortcut menu of the acquired image.

Notes

- A further slight increase in acquisition speed can be achieved by deactivating the **Show preview at Start** check box in the **Multidimensional Acquisition** work area ⇒ **Experiment** property page.



Objective – grid allocation

The following tables provide an overview of the allocation of grids to objectives.

Axio Imager 1 / Axio Imager 2

Objective	V	NA	Immersion	Grid / Optional section thickness @ 490 nm [RE/µm]			DAPI with FS 34	DAPI with FS 49
				High grid	Middle grid	Low grid		
EC Plan-Neofluar	10x	0,3	Air	2,9 / 31,9	1,7 / 18,2	**0,9 / 9,9**	✓	✓
EC Plan-Neofluar	20x	0,5	Air	2,4 / 9,2	1,4 / 5,3	**0,7 / 2,9**	✓	✓
EC Plan-Neofluar	40x	0,75	Air	1,6 / 2,8	**0,9 / 1,6**	0,5 / 0,9	✓	✓
EC Plan-Neofluar	40x	1,3	Oil	2,5 / 2,2	1,4 / 1,2	**0,8 / 0,7**	✓	✓
EC Plan-Neofluar	63x	0,95	Air	**1,0 / 1,1**	0,6 / 0,7	0,4 / 0,4	✓	✖
EC Plan-Neofluar	63x	1,25	Oil	1,6 / 1,5	**0,9 / 0,9**	0,5 / 0,5	✓	✓
EC Plan-Neofluar	100x	1,3	Oil	**1,0 / 0,9**	0,6 / 0,5	0,4 / 0,3	✓	✓
LCI Plan-Neofluar	25x	0,8	Oil, water or glycerine	2,9 / 6,6	1,7 / 3,7	**0,9 / 2,0**	✓	✓
LCI Plan-Neofluar	63x	1,3	Water or glycerine	1,5 / 1,3	**0,9 / 0,7**	0,5 / 0,4	✓	✓
Plan-Apochromat	10x	0,45	Air	4,2 / 20,4	2,4 / 11,5	**1,3 / 6,2**	✓	✓
Plan-Apochromat	20x	0,8	Air	3,2 / 4,9	1,8 / 2,8	**1,0 / 1,5**	✓	✓
Plan-Apochromat	40x	0,95	Air	1,6 / 1,7	**0,9 / 1,0**	0,5 / 0,5	✓	✓
Plan-Apochromat	40x	1,3	Oil	2,5 / 2,2	1,4 / 1,2	**0,8 / 0,7**	✓	✓
Plan-Apochromat	40x	1,4	Oil	2,4 / 1,8	**1,4 / 1,0**	0,7 / 0,6	✓	✓
Plan-Apochromat	63x	1,4	Oil	1,6 / 1,2	**0,9 / 0,7**	0,5 / 0,4	✓	✓
Plan-Apochromat	100x	1,4	Oil	**1,0 / 0,8**	0,6 / 0,5	0,4 / 0,3	✓	✓

LD LCI Plan-Apochromat	25x	0,8	Oil, water or glycerine	2,9 / 6,6	1,7 / 3,7	**0,9 / 2,0**	✓	✓
C-Apochromat	10x	0,45	Water	4,2 / 20,4	2,4 / 11,5	**1,3 / 6,2**	✓	✓
C-Apochromat	40x	1,2	Water	2,2 / 2,0	**1,2 / 1,1**	0,7 / 0,6	✓	✓
C-Apochromat	63x	1,2	Water	1,4 / 1,3	**0,8 / 0,7**	0,5 / 0,4	✓	✓
LD C-Apochromat	40x	1,1	Water	2,2 / 2,3	**1,2 / 1,3**	0,7 / 0,7	✓	✓
α Plan-Apochromat	63x	1,46	Oil	1,5 / 1,0	**0,9 / 0,6**	0,5 / 0,3	✓	✓
α Plan-Fluar	100x	1,45	Oil	**1,0 / 0,7**	0,6 / 0,4	0,3 / 0,2	✖	✖
α Plan-Apochromat	100x	1,46	Oil	**1,0 / 0,7**	0,6 / 0,4	0,3 / 0,2	✓	✖

Axiovert 200 and Axio Observer

Objective	**V**	**NA**	**Immersion**	**Grid / Optional section thickness @ 490 nm [RE/µm]**			**DAPI with FS 34**	**DAPI with FS 49**
				High grid	**Middle grid**	**Low grid**		
EC Plan-Neofluar	10x	0,3	Air	2,9 / 31,5	1,7 / 18,5	**0,9 / 9,8**	✓	✓
EC Plan-Neofluar	20x	0,5	Air	2,3 / 9,0	**1,4 / 5,4**	0,7 / 2,9	✓	✓
EC Plan-Neofluar	40x	0,75	Air	1,6 / 2,7	**0,9 / 1,6**	0,5 / 0,9	✓	✖
EC Plan-Neofluar	40x	1,3	Oil	2,4 / 2,1	1,4 / 1,3	**0,8 / 0,7**	✓	✓
EC Plan-Neofluar	63x	0,95	Air	**1,0 / 1,1**	0,6 / 0,7	0,4 / 0,4	✓	✓
EC Plan-Neofluar	63x	1,25	Oil	1,6 / 1,5	**0,9 / 0,9**	0,5 / 0,5	✓	✖
EC Plan-Neofluar	100x	1,3	Oil	**1,0 / 0,9**	0,6 / 0,6	0,4 / 0,3	✓	✖

LCI Plan-Neofluar	25x	0,8	Oil, water or glycerine	2,9 / 6,5	1,7 / 3,8	**0,9 / 2,0**	✓	✓
LCI Plan-Neofluar	63x	1,3	Water or glycerine	1,5 / 1,3	**0,9 / 0,8**	0,5 / 0,4	✖	✖
Plan-Apochromat	10x	0,45	Air	4,2 / 20,2	2,4 / 11,7	**1,3 / 6,1**	✓	✓
Plan-Apochromat	20x	0,8	Air	3,1 / 4,8	1,8 / 2,8	**1,0 / 1,5**	✓	✓
Plan-Apochromat	40x	0,95	Air	1,6 / 1,7	**0,9 / 1,0**	0,5 / 0,5	✓	✓
Plan-Apochromat	40x	1,3	Oil	2,4 / 2,2	1,4 / 1,3	**0,8 / 0,7**	✓	✓
Plan-Apochromat	40x	1,4	Oil	2,4 / 1,8	**1,4 / 1,1**	0,7 / 0,6	✓	✓
Plan-Apochromat	63x	1,4	Oil	1,5 / 1,2	**0,9 / 0,7**	0,5 / 0,4	✓	✓
Plan-Apochromat	100x	1,4	Oil	**1,0 / 0,8**	0,6 / 0,5	0,4 / 0,3	✓	✖
LD LCI Plan-Apochromat	25x	0,8	Oil, water or glycerine	2,9 / 6,5	1,7 / 3,8	**0,9 / 2,0**	✓	✓
C-Apochromat	10x	0,45	Water	4,2 / 20,2	2,4 / 11,7	**1,3 / 6,1**	✓	✓
C-Apochromat	40x	1,2	Water	2,1 / 1,9	**1,3 / 1,1**	0,7 / 0,6	✓	✓
C-Apochromat	63x	1,2	Water	1,4 / 1,3	**0,8 / 0,7**	0,5 / 0,4	✓	✓
LD C-Apochromat	40x	1,1	Water	2,1 / 2,3	**1,3 / 1,4**	0,7 / 0,7	✓	✓
α Plan-Apochromat	63x	1,46	Oil	1,5 / 1,0	**0,9 / 0,6**	0,5 / 0,3	✓	✓
α Plan-Fluar	100x	1,45	Oil	**1,0 / 0,7**	0,6 / 0,4	0,3 / 0,2	✖	✖
α Plan-Apochromat	100x	1,46	Oil	**1,0 / 0,7**	0,6 / 0,4	0,3 / 0,2	✓	✖

Axioplan 2 imaging e

ApoTome 2 kann nicht mit Axioplan 2 Imaging e verwendet werden.

Other ApoTome 2 Settings

In addition, the following control elements can be found on the **Extras** property page:

ApoTome 2: Auto Shutter

If **Automatic Shutter Control** is activated, the shutter is controlled automatically if

1. the live image is opened or closed
2. an image is acquired

This function is useful in order to protect the sample against the bleaching of fluorescence and is activated as a default setting when the software is installed.

ApoTome 2 Display Normalization

The algorithm used to calculate the optical section reduces the image's gray value range. If the **Display Normalization** function is activated, the display characteristic curve is automatically adjusted to guarantee a bright, high-contrast display of the gray values. This function is activated as a default setting when the software is installed.

ApoTome 2: Status

This field displays status information relating to the ApoTome 2 and image acquisition with the ApoTome 2. It makes sense to have this window open or to integrate it into your own customized dialogs so that any problems with the system can be analyzed quickly.

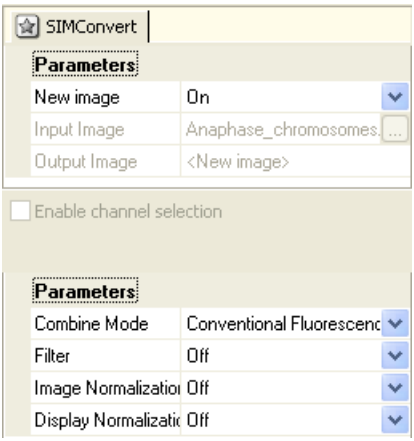
Conversion of ApoTome 2 images from raw data mode

If you select the **Raw Data Mode** for image acquisition with the ApoTome 2, the size of the data is increased by a factor of 3, 6, 9 etc. in accordance with the settings under **Averaging**. If you are acquiring z-stack images, images in several fluorescence channels or time lapse images, this can lead to enormous volumes of data that can be difficult to work with during subsequent analyses. The **SIMConvert** function converts the raw data to an optical section or a conventional fluorescence image with a substantially reduced data volume.

Use of this function is strongly recommended after you have acquired a z-stack in **Raw Data Mode** and processed it using the AxioVision 3D Deconvolution module.

To perform conversion, follow the procedure below:

- Click on the ApoTome 2 image that you want to convert.
- Click on the **ApoTome 2** entry in the work area.
- Open the function tree by clicking on screen in front of the **ApoTome 2** entry – click on **SIMConvert**. The parameters for the function are listed underneath the work area.



- Adopt the settings in the top **Parameters** field. The default settings ensure that the selected image is processed and a new image is created automatically (the original file is retained). The **Enable channel selection** check box should be deactivated, as generally all the channels in an image should be converted in the same way.

Four parameters are available in the lower area of the property page for the actual conversion **Combine Mode**, **Filter**, **Image Normalization** und **Display Normalization**:

- **Combine Mode**: This parameter determines the algorithm used to convert the image. You can select from **Conventional Fluorescence** or **Optical Sectioning**.

- **Filter**: Depending on the sample, fluorescent dye, exposure time etc., fine residual lines may be visible in the image that results from the conversion. These lines can be removed using the filter. The options **Off**, **Weak**, **Medium** and **Strong** are available.

  The filter function makes use of the fact that the residual lines are displayed in the Fourier space as points along a vertical line. The filter "removes" a defined group of points from the Fourier spectrum, thereby removing the residual lines.

- **Display Normalization**: The algorithm used to calculate the **Optional Sectioning** reduces the gray value range of the image data, depending on the specimen and the acquisition conditions. The **On** functional parameter automatically sets the image's display characteristic curve so that a bright, high-contrast image is displayed. The **Min/Max** function of the display characteristic curve is used, which sets the display characteristic curve on the basis of the darkest and brightest gray value in the image histogram. If a z-stack is converted, the image plane that is currently set in the active image is used to determine the **Min/Max** value for the entire image stack.

- **Image Normalization**: As described under "Display Normalization", the algorithm used to calculate the **Optional Sectioning** reduces the gray value range of the image data, depending on the specimen and the acquisition conditions. As the so-called floating point format is used for raw data acquisition with the ApoTome 2 and the subsequent combination of the gray value data, during the conversion from floating point back to gray value, a normalization function can be applied directly to the data to display a bright, high-contrast image. The **On** parameter activates this form of normalization.

Note:

- If **Image Normalization** is switched on, **Display Normalization** is no longer necessary, as the result of both functions has the same effect for the user. For the conversion of a z-stack, the **Image Normalization** function offers advantages, as the gray value distribution of all image planes can be taken into account.

ApoTome 2: HDR Mode

By activating the **HDR Mode**, images with an expanded dynamic range can also be acquired in ApoTome 2 mode. For further details, please refer to the description of **HDR Snap** and the AxioVision manual.

If the ApoTome 2 is operated in **HDR Mode**, the corresponding number of images will be acquired for each grid position with various exposure times and, from this, an HDR image will be calculated for each grid position. The ApoTome 2 calculation will then be performed on these three HDR images. This will ultimately result in an optical section with an expanded dynamic range.

Because in the **HDR Mode** several images are acquired with very long exposure times, residual fringes may occur in the ApoTome 2 optical section as a result of bleaching effects. In this case, it is necessary to use the ApoTome 2 filters in order to suppress the residual fringes.

The additional time required for calculating the HDR image can lead to delays in the displaying of the preview when multidimensional images are acquired. In this case, the preview should be deactivated.

9.2 Autofocus

General

Autofocus enables you to focus a sample automatically. To do this, you need a microscope with a motorized z-drive. Autofocus can be operated with all cameras that are available for use with AxioVision, with the exception of those that are only controlled via a TWAIN interface. You can use this functionality for the illumination methods reflected light, transmitted light, bright field and dark field, as well as fluorescence.

Note:

- The Autofocus functionality is not suitable for the contrasting methods Phase Contrast and DIC.

To make it possible to focus samples automatically with the different microscopy procedures and at all possible magnifications, it is necessary to carry out calibration in advance on a sample which has been focused manually. The calibration requires information on the objective and optovar magnification, the numerical aperture of the objective being used, the illumination method and, if you are using a digital camera, the corresponding resolution.

If all the components of your microscope are motorized, AxioVision determines these parameters automatically. Otherwise the missing calibration data have to be entered manually.

Note:

- We recommend that you recalibrate the system whenever microscope components have been changed.

Calibrating Autofocus

Preparation

Switch the light path to the camera. Open the live image window by clicking on the icon for the live image in the **Standard** toolbar 

. Alternatively, select the **Live** function in the **Acquisition** menu.

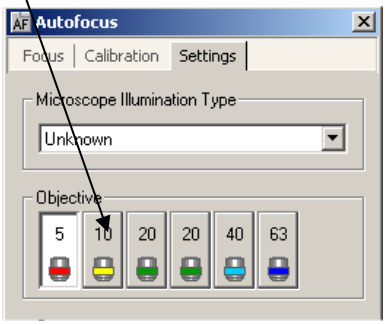
➢ Now focus the camera image and select the frame to be acquired.

Calibration

➢ Select the **Autofocus** function in the **Acquisition** menu.

➢ Activate the **Settings** property page.

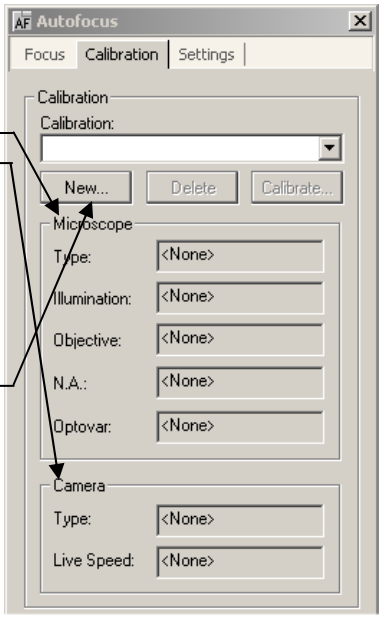
➢ Make sure that the settings for the type of **Illumination**, **Objective**, **Optovar** and live image speed match those on your system.



Note:

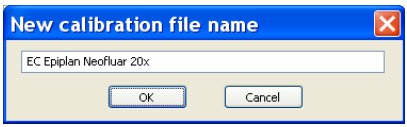
- The setting options depend on your system configuration and may differ from those shown here.

- Now activate the **Calibration** property page.
- Check the settings for **Microscope** and **Camera**.



Note:

- If individual components are not encoded or motorized, you must enter the relevant information here using the keyboard.
- Then click on **New** to create a new calibration with these settings.
- Enter a name for the calibration and click on **OK**.
  The calibration process is now started automatically.



Using Autofocus

The quickest and easiest way to use the Autofocus is to run it directly via the toolbar at the bottom of the live image window.

If the results are unsatisfactory, you can make fine adjustments via the **Autofocus** property page. A detailed description of the parameters available there can be found in the online help.

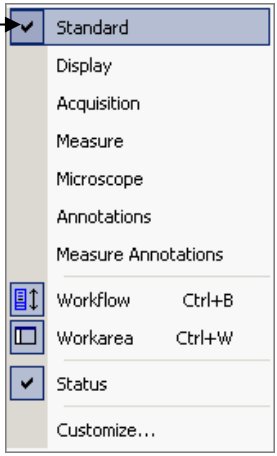
Use of the Autofocus together with the other modules for image acquisition is described in the relevant sub-sections.

Notes:

- Before the Autofocus can be used, calibration must be carried out for the microscope and camera setting you are using. See the preceding chapter.

- If problems arise during execution or if execution is not possible for certain reasons, please check the **Autofocus** function in the **Acquisition** menu. Activate the **Focus** tab and look under **Status**. There you will receive an indication as to why the execution process has failed.

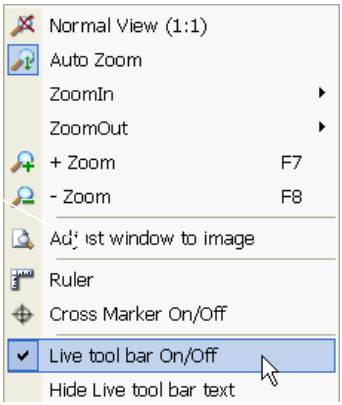
➢ Make sure that the **Standard** toolbar is displayed: right-click on the menu or any toolbar and activate **Standard** (there must be a tick in front of it).



➢ Switch the light path to the camera. Then click on the icon for the live image in the **Standard** toolbar.



- If the tool bar of the live image window is not displayed, right-click in the live image and activate the **Live tool bar On/Off** entry (there must be a tick in front of it).



- Now select the frame to be acquired on the sample, and then click on the **Autofocus** button in the live image toolbar.



- Now click on **Snap** to acquire an individual image.



9.3 Multidimensional Acquisition

Images with more than two dimensions cannot be acquired using your camera's normal acquisition function. These kind of images may consist, for example, of several channels or a time lapse series. For these images AxioVision requires additional information, such as the number of channels, z-planes etc. This collection of information is referred to within AxioVision as an "experiment".

In the work area you will find **Multidimensional Acquisition**. If you click here the **Experiment** property page opens. The control elements for the modules Multichannel Fluorescence, Z-Stack, Mark&Find and Time Lapse appear here, depending on which modules you have purchased.

The **Experiment** property page contains an overview of the modules that are installed and a selection of important control elements. From this page you can perform a wide range of functions to control, modify, save and execute a previously prepared experiment.

On this page you can re-use settings of already acquired images via the **Reuse** function.

Each module also has its own tab, on which the essential settings can be made.

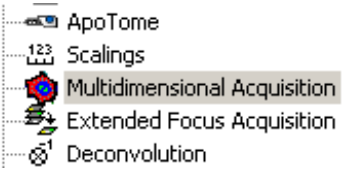
Notes:

- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased.

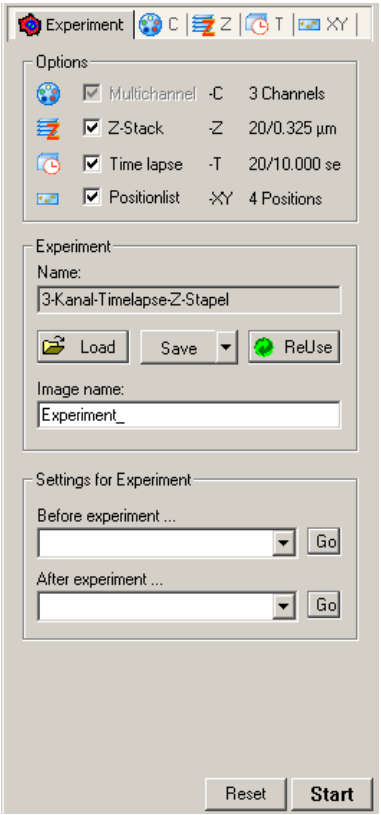
- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create an experiment?

- In the work area click on Multidimensional Acquisition.



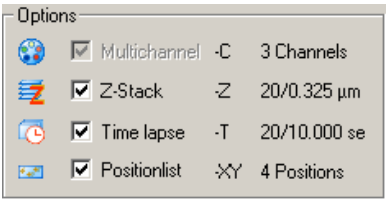
- In the work area you will now see the **Experiment** property page.



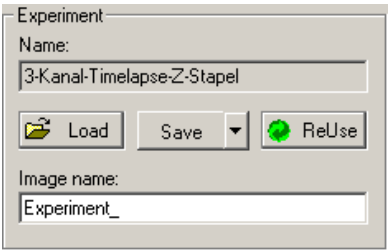
Note:

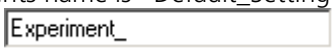
- Please bear in mind that the options listed here are only displayed if the corresponding modules are available.

➢ In the **Options** field the dimensions for your experiment can be selected.

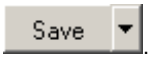


➢ In the **Experiment** window the name of the current experiment is displayed.



The default experiments name is "Default_Setting". The default name for an acquired image is 

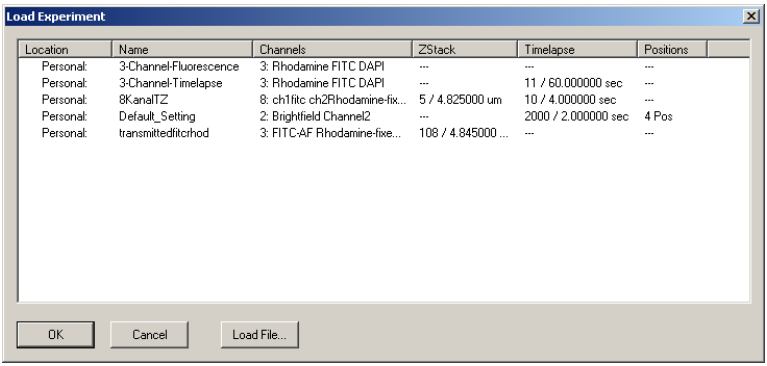
. A serial number will be attached to this name after acquisition. You can specify the basic name for the acquired image here before acquisition.

➢ Make the desired settings for your experiment and click on 

. A dialog is then opened. Save your new experiment.

➢ Click on 

, select an experiment from the list of experiments and click on **OK**.



Once this experiment has been loaded, the settings it contains are adopted.

- If you want to adopt these settings from a ZVI image that has already been saved, click on 

, select the desired image, and confirm with **OK**. Only the acquisition settings used for this image are adopted. The image itself is not loaded. This function makes it easier for you to acquire images under reproducible conditions.

- To execute the experiment, click on 

.

Multichannel Fluorescence module

With the Multichannel Fluorescence module you can define up to 8 channels to be used for acquisition. These channels are saved together in a ZVI document. This allows you, for example, to switch quickly back and forth between the various views to compare different channels.

Notes:

- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased.

- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create a multichannel experiment?

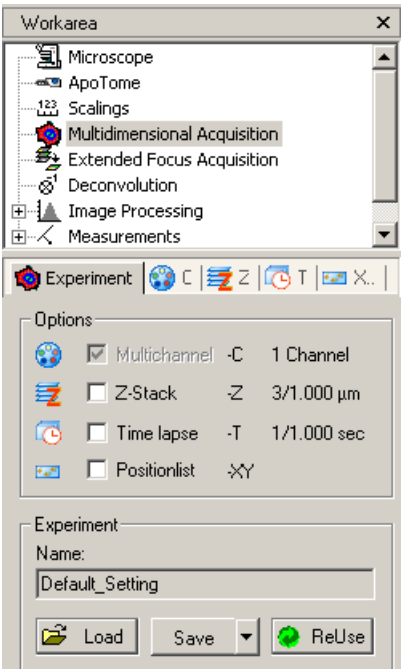
In the following example a 3 channel fluorescence image should be generated. The channels should be configured for the fluorochromes Rhodamine, FITC and DAPI. Please use a test sample to test the settings.

Notes:

- The following instructions require use of a motorized microscope.

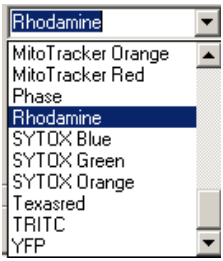
- You should also be able to generate, edit and apply settings in the **Settings Editor** and should already have generated channel-specific settings (see chapter 8 "Configuration" - "Settings Editor"). The hardware components available to you may differ from those shown in this example.

➢ In the **Workarea** activate the property pages under **Multidimensional Acquisition**:



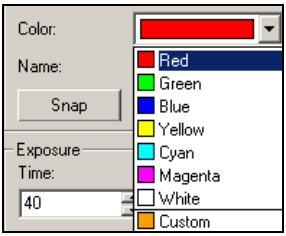
- Go to the **C** property page  . To generate various channels, you need to enter the channel-specific parameters here.

- From the **Dye** field select the Rhodamine fluorochrome.



Once the dye has been selected, a suggested channel color with wavelengths for excitation and emission maximums are assigned to the channel.

- If you do not like the suggested color, you can change it (e.g. to 100% red) by making a selection from the list:

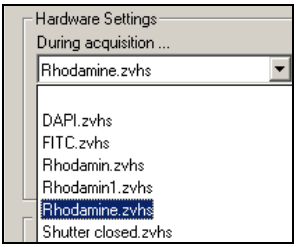


- A channel name is automatically generated from the dye 

but can be changed if needed, e.g. to reflect the type of exposure used 

.

- Now allocate a hardware setting to the channel. To do this, select "Rhodamine.zvhs" from the list in the field **During Acquisition**:



- You can test this setting by clicking on the 

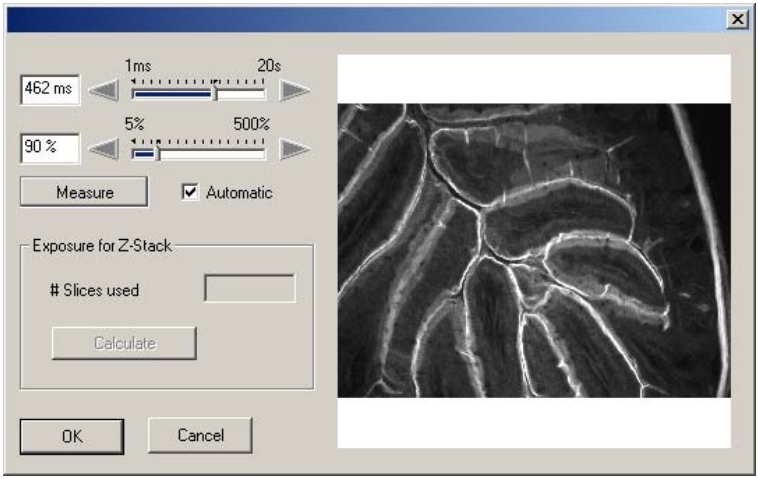
button. This opens the fluorescence shutter, and switches the reflector to the correct filter position.

- Repeat the procedure for the next field, and select the setting "shutter closed.zvhs". This ensures that the shutter is closed immediately after acquisition 

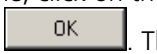
.

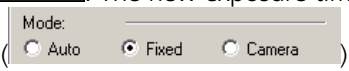
- To determine the exposure time for the Rhodamine channel, click in the **Exposure** field and then click on the 

button. The hardware settings you have selected are now applied to the microscope and a dialog is opened:



- To set the correct exposure time, click on the 

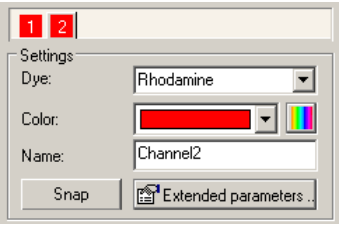
button and close the dialog by clicking on 

. The new exposure time is now entered and used for the channel (

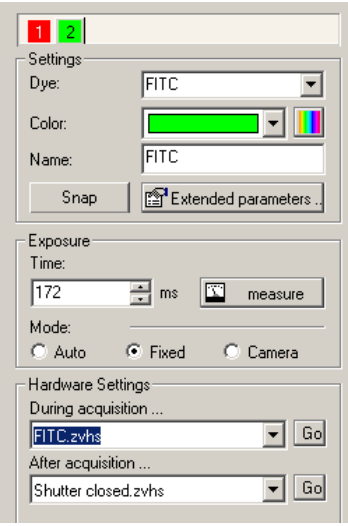
).

- Add a new channel to your experiment. To do this, click in the **Extended Channel Parameters** dialog on 

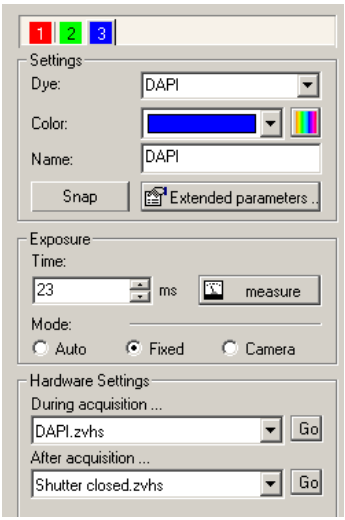
. A copy of the channel you have already configured is then added to the experiment.



➢ Now change the settings that are relevant for the new channel (channel name, dye, hardware setting during acquisition, exposure time measurement):



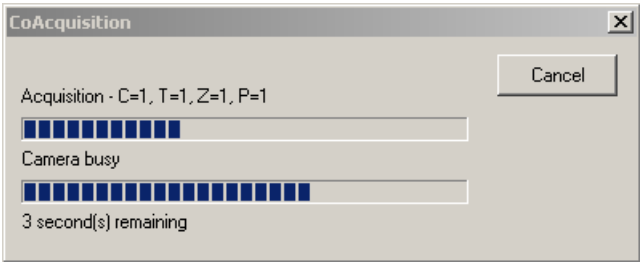
➢ Duplicate the second channel, and change it accordingly for the DAPI dye:



- You can add together up to 8 channels in this way.

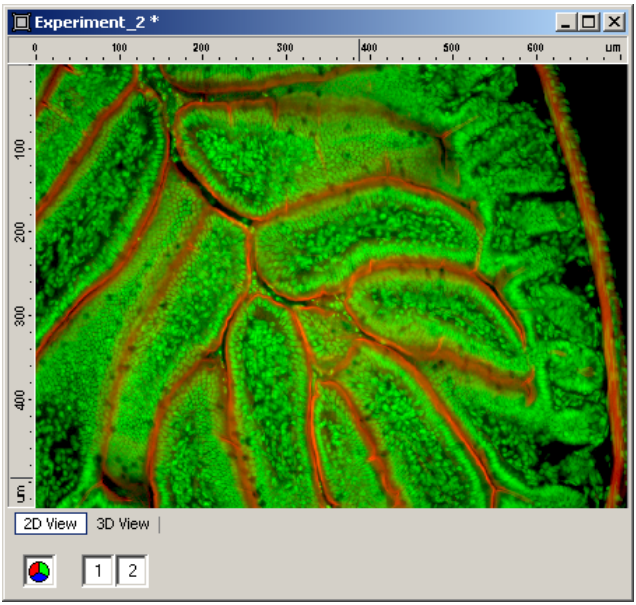
- You should now save the settings you have made as a new experiment (see above), so that you can use them again in the future.

- To acquire your 3-channel image, click on Start. During acquisition a progress bar informs you about the procedure:



- If you want to cancel the acquisition, click on **Cancel**.

The resulting image appears in the image area, where it can be operated using the player:



This image has not been saved, as you can see from the * symbol in the image name. Save the image. The program remembers the last storage location that you searched for.

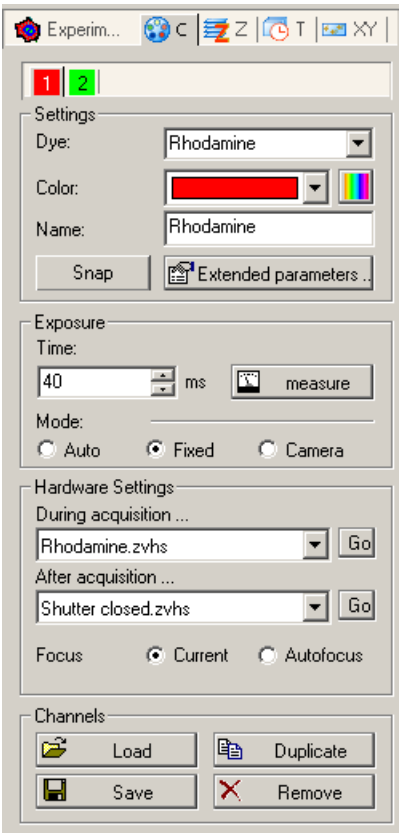
How do I manage channel settings (working with the channel pool)?

Above you have seen how to configure channels for multichannel images and save them as an experiment. An experiment therefore contains settings that are specifically intended for that particular experiment. As time goes by you will find channel settings that can be applied in many different cases. You may also wish to combine channels from different experiments. The "channel pool" has been created for these kinds of requirement. These instructions describe how to work with the channel pool.

Note:

- The following instructions require use of a motorized microscope. The hardware components available to you may differ from those shown in this example. You must also be able to generate, save and load experiments and have already created a multichannel experiment.

➢ Load an available multichannel experiment. In this example a 3-channel experiment (Rhodamine/FITC/DAPI) is loaded. If you go to the **Channel** property page, you should see e.g. the following channel parameters:

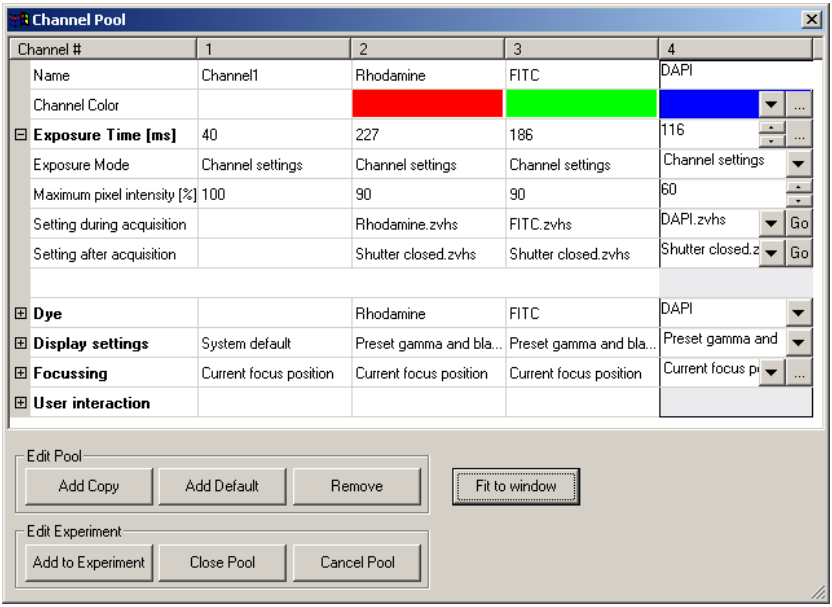


- Select the Rhodamine channel: In the **Channels** field click on Save.

- Repeat this for the other channels.

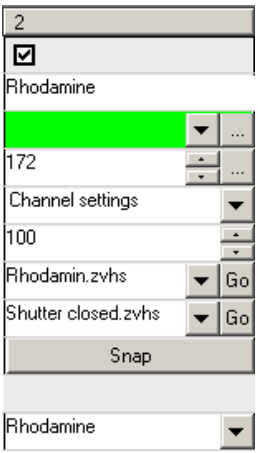
- Now remove the channels from the experiment by clicking repeatedly on Remove. The **C** (channel) property page should now only contain one channel.

- Now click on the Load button. The channel pool opens as a new dialog:

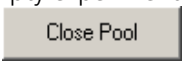


The size of this dialog can be changed to give you sufficient space to work with the channel parameters.

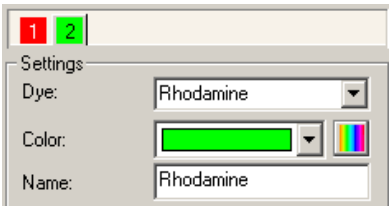
- Now select the Rhodamine channel …



and add it to the empty experiment by clicking on the 

button. Clicking on 

the channel pool is closed and the channel now appears on the **C** property page:



- To add another channel to the experiment, repeat the last steps.

- In the channel pool you can also duplicate, modify or remove channels. To do this, select the desired channel and click on the corresponding button.

Z-Stack module

Using the Z-Stack module you can acquire series of images over a defined z-focus range. The resulting image is known as a z-stack. These kinds of image are required for the calculation of three-dimensional displays.

Z-stack images are essential for the 3D Deconvolution module. The ApoTome 2 module benefits from z-stack images, as they can be used to generate impressive 3D images quickly. Using the Extended Focus module a sharp image can be calculated from a z-stack.

The AxioVision module Z-Stack allows the easy determination of the correct z-distance between individual images of a z-stack if the maximum of axial resolution of the objectives should be achieved. This optimum step size (also called the Nyquist criterion) is a function of the numerical aperture of the used objective and the recorded wavelength (in the case of fluorescence usually the emission maximum). It is calculated as follows:

The axial resolution **d** is approximated from the following formula:

$$d_{axial} \approx 2 \frac{\eta \lambda}{N.A.^2}$$

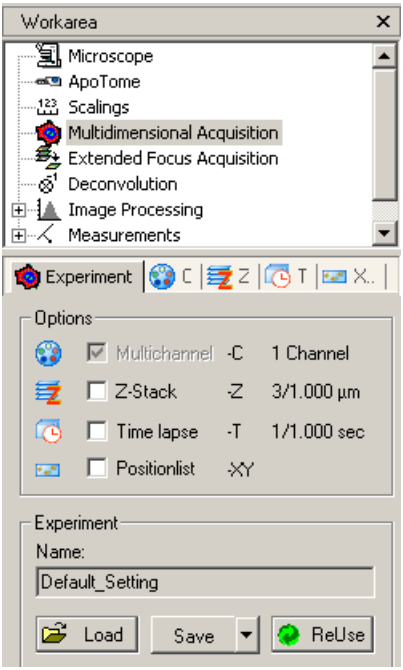
At a given wavelength λ = 540 nm and a numerical aperture **N.A**. = 1.3 at a refractive index η of 1.52 $\mathbf{d_{axial}}$= 0.970 µm. According to Nyquist a twofold oversampling (approximately 485 nm between neighboring z-planes) all available information will be collected. This sampling rule is a prerequisite for reconstructing z-stacks with the module AxioVision 3D-Deconvolution.

Notes:

- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased. In order to follow this introduction, you need to know what an AxioVision "experiment" is. Please read the documentation on experiments in section "How do I create an experiment?".
- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

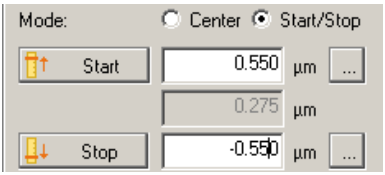
How do I create a z-stack?

- In the work area activate the property page under **Multidimensional Acquisition**.



- On the **Experiment** property page select the  (**Z-Stack**) function.

- From the two possible definition modes select the **Top/Bottom** mode:

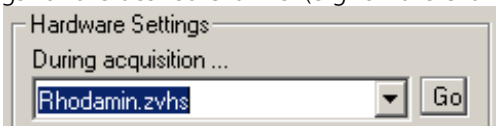


- Click on the  button in the  control element. A dialog is opened that contains a live image and the control element for focusing. Please note that light will only fall on the sample if it has been directed onto the sample manually, or if a hardware setting have been configured for the selected channel. On the microscope, focus on the area where you want the z-stack to begin. To adopt this z-position, close the dialog by clicking on **OK**. The new z-position is then automatically adopted into the start field.

- Repeat this procedure to determine the **Stop** position using the  button from the  control area.

- Now define the desired interval between the individual z-planes. You can enter any value here. To guarantee maximum resolution in the z-direction, click on the  button.

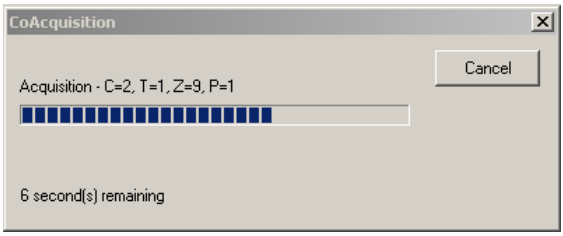
- The correct value is calculated, taking into consideration the numerical aperture of the objective and the emission wavelength of the fluorochrome (this takes place automatically if a fluorochrome has been selected from the list). If no emission wavelength is stated, a wavelength of 500 nm is taken as a basis.

- To check your selection, you can navigate through the z-stack using the z-stack navigation function. To do this, open a live image, and activate the hardware settings for the desired channel (e.g. on the channel page via the  button  .

- You can now position the focus drive at the upper  or lower  limit or the center  of the defined z-stack.

- If you are not satisfied with the definition of the z-stack, you can modify it step by step (with the set increment) using the  or  buttons.
  Adopt the new definition by clicking on the  /  buttons in the control area for z-stack settings.

- Click on  to begin acquisition. During acquisition a progress bar informs you about the procedure:



  If you want to cancel acquisition, click on **Cancel**.

- The resulting image appears in the image area, where it can be operated using the player. This image has not been saved, as you can see from the * symbol in the image name. Save the image. The program remembers the last storage location you have chosen.

Time Lapse module

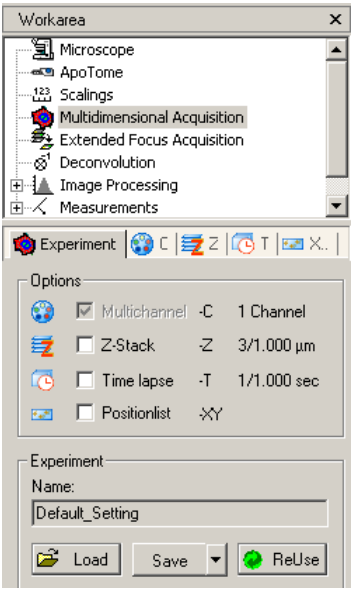
The Time Lapse module can be used to acquire series of images over a defined period of time. The resulting image is a time lapse series. These kinds of image are required for time lapse acquisition of changing processes.

Note:

- Please note that you may not have access to all of the modules and functions described here. This depends on the options you have purchased. In order to follow this introduction, you need to know what an AxioVision "experiment" is. Please read the documentation on section "How do I create an experiment?". For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

How do I create a time lapse series?

➢ In the work area activate the property pages under **Multidimensional Acquisition**.



- On the **Experiment** property page select the **Time lapse** function 

.

- Enter the desired time interval, e.g. 2 minutes. The unit can be selected from the drop-down list box.

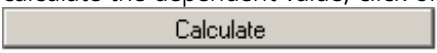
- Now enter the desired number of images (**# Cycles**). The total duration of the experiment is calculated from this.

- Alternatively, you can enter the interval and the total duration, and this information will be used to calculate the number of cycles. Select the function that you want to be calculated



.

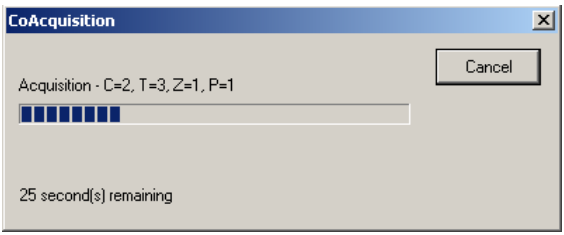
- It is also possible to enter numerical values directly into the input fields. To calculate the dependent value, click on the



button.

- Click on 

to begin acquisition. During acquisition a progress bar informs you about the procedure:



If you want to cancel acquisition, click on **Cancel**.

- The resulting image appears in the image area, where it can be operated using the player. This image has not been saved, as you can see from the * symbol in the image name. Save the image. The program remembers the last storage location that you searched for.

Advanced image acquisition: Mark&Find

The Mark&Find module allows you to acquire images of various positions on your sample, also in combination with the Multidimensional Acquisition modules. All the dimensions offered by AxioVision can also be used for a position list. The output images are saved automatically in a folder that you have defined, and from this a folder-based archive is generated. This makes it easy to manage the individual images.

Notes:

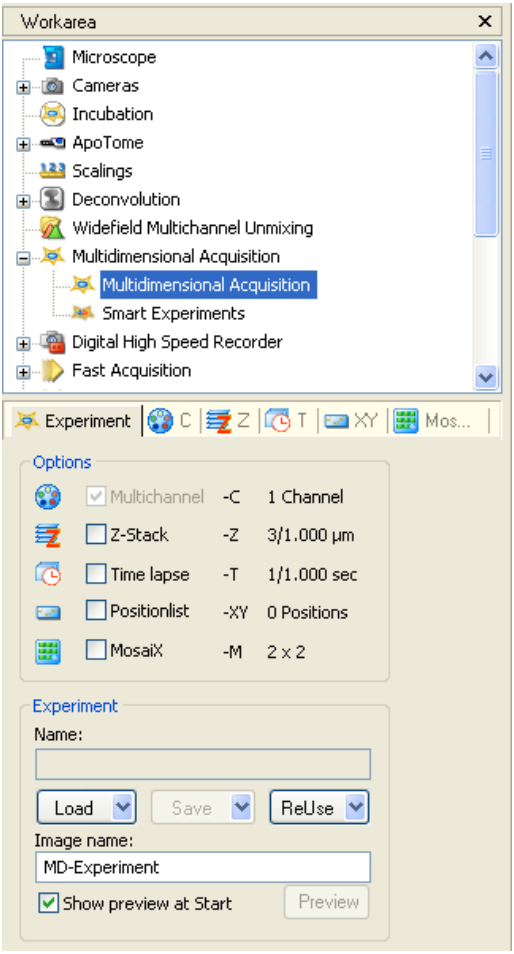
- Please note that you may not have access to all of the modules and functions. This depends on the options you have purchased.

- In order to follow this introduction, it is useful to know what an AxioVision "experiment" is. You will find the relevant information in the "How do I create an experiment?" section.

- For detailed information on the individual functions and commands, please refer to the online help (**F1** key).

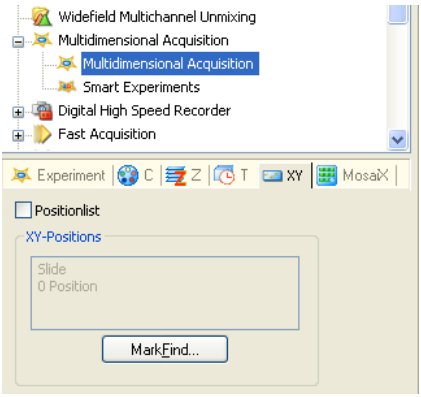
How do I create a Mark&Find experiment?

➢ In the work area, activate the **Multidimensional Acquisition** function:

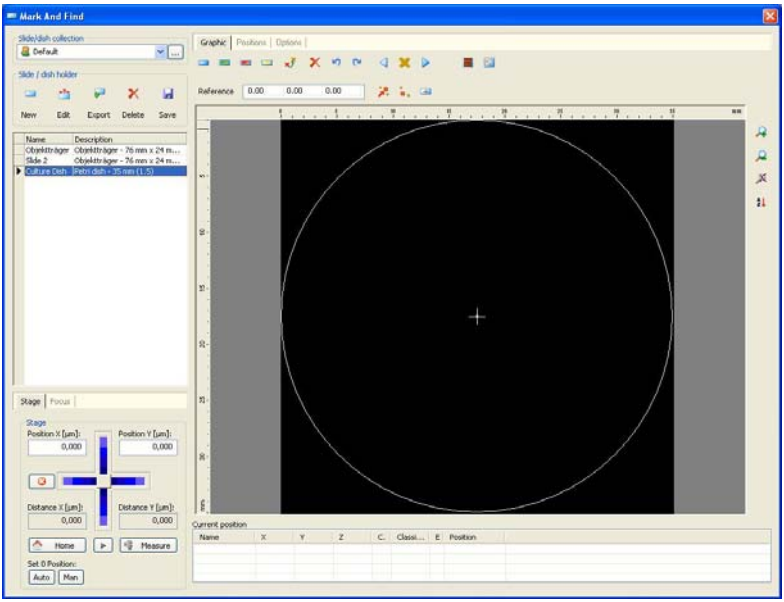


➢ Select the **Experiment** property page and load an experiment.

➢ Select the XY (**Positionlist**) tab sheet.



➢ Open the Mark&Find dialog by clicking on the MarkFind... button.

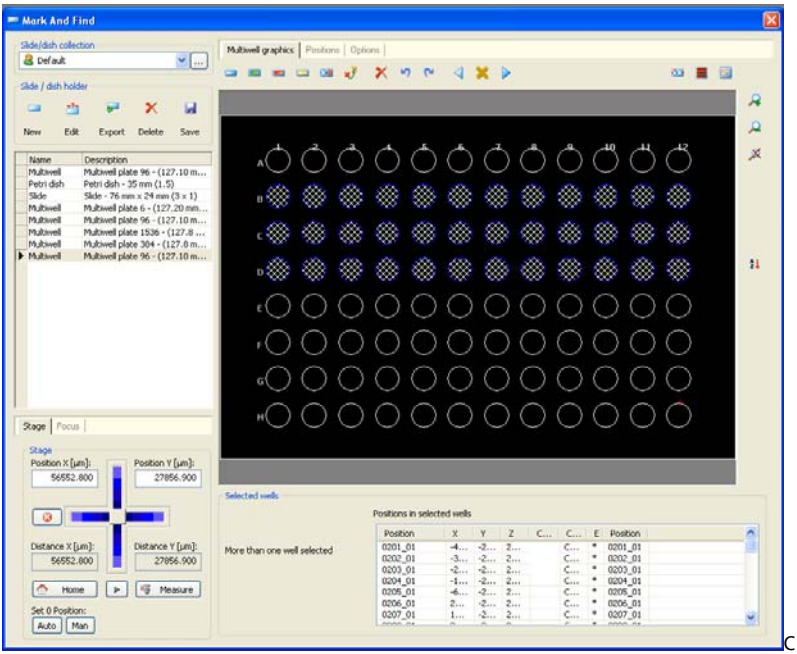


➢ Load a saved Mark&Find database from the list box

- or define a new list by clicking on **New**/modify an existing list by selecting **Edit** (please read section 9.5 Mark&Find "How do I generate a Mark&Find list?", or call up the online help (**F1** key)).



The Mark&Find control element now shows a graphic display of the selected list.

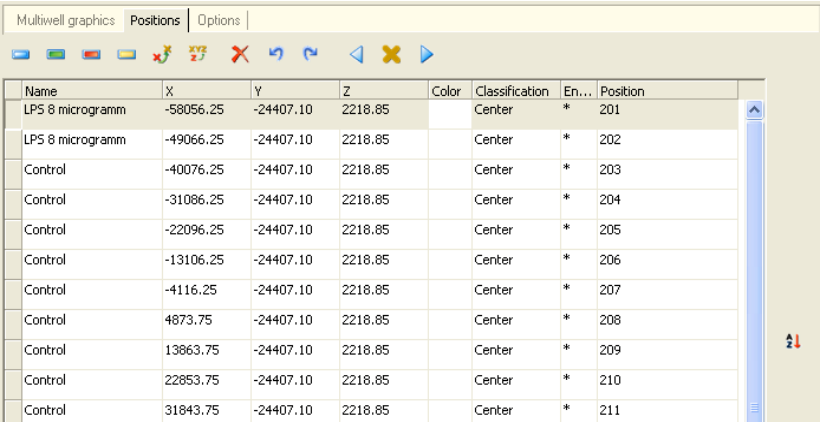


C

Note:

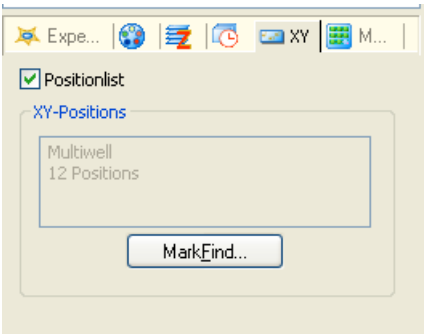
- The graphic will only have the expected appearance if both the reference point on the sample and the sample holder have been centered. Please read section 9.5 Mark&Find "How do I generate a Mark&Find list?", or consult the online help (**F1** key).

If you click on the **Positions** tab sheet, you will see a tabular display of the position list.



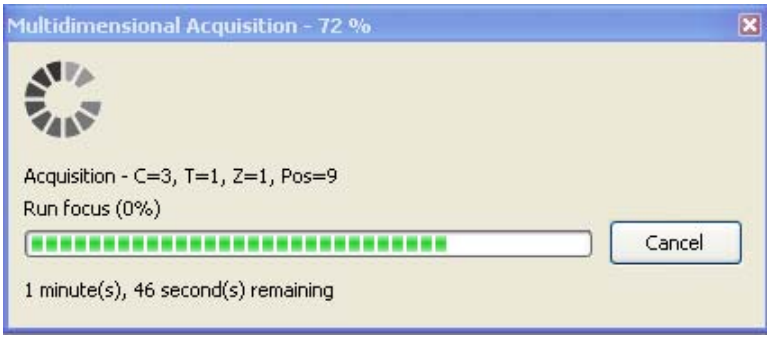
Name	X	Y	Z	Color	Classification	En...	Position
LPS 8 microgramm	-58056.25	-24407.10	2218.85		Center	*	201
LPS 8 microgramm	-49066.25	-24407.10	2218.85		Center	*	202
Control	-40076.25	-24407.10	2218.85		Center	*	203
Control	-31086.25	-24407.10	2218.85		Center	*	204
Control	-22096.25	-24407.10	2218.85		Center	*	205
Control	-13106.25	-24407.10	2218.85		Center	*	206
Control	-4116.25	-24407.10	2218.85		Center	*	207
Control	4873.75	-24407.10	2218.85		Center	*	208
Control	13863.75	-24407.10	2218.85		Center	*	209
Control	22853.75	-24407.10	2218.85		Center	*	210
Control	31843.75	-24407.10	2218.85		Center	*	211

➢ Close the dialog by clicking on  . The Mark&Find control element now displays the number of positions and the name of the position list:



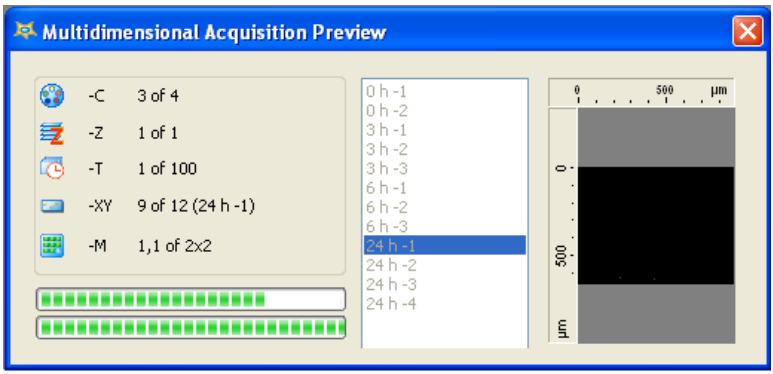
➢ Click on Start to begin acquisition.

During acquisition a progress bar informs you about the procedure:

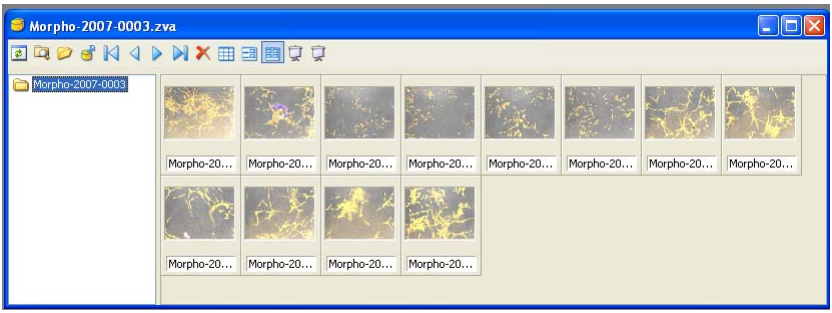


➢ If you want to cancel acquisition, click on Cancel.

A preview containing acquisition parameters is also displayed:



At the end of the above procedure, a folder-based archive showing all output images is created.



These can be loaded and handled in the same way as normal individual images.

Note:

- No images are displayed during the acquisition of a position list. To view the images, you need to exit the experiment.

9.4 Extended Focus

General

The **Extended Focus** function is available as an optional module for AxioVision. This function enables you to display the sharp areas of different focus positions of your sample in a single image.

Essentially, the **Extended Focus** module is operated interactively. You do not need a motorized microscope. Several images are acquired at different focus positions. The sharp areas are extracted from each of the images and automatically combined. You immediately obtain a sharp 2D image. The individual images of the different focus positions are not saved, but are combined straight away. The focus is driven manually across the entire z-range until all the details have been displayed sharply. There are no specifications, recommendations or formulae that say how many images have to be acquired or how large the distance between two focus positions should be. This always depends on the sample in question. Generally speaking, it can be said that it is better to acquire too many images than too few.

Alternatively, an image with extended depth of field can also be calculated using a z-stack. To do this, you need a z-stack image, the acquisition of which requires a motorized microscope and the **Z-Stack** module (see also section 9.8 "Z-Stack Images").

Acquiring images with Extended Focus

The **Extended Focus** function can be run via the work area on the left of the AxioVision window. If the work area is not displayed, activate it using the **Standard** toolbar 

.

Alternatively, select the **Windows** function in the **View** menu and then select the **Workarea** entry.

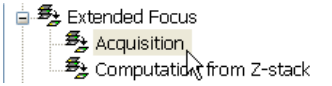
Note:

- The work area may be open but covered by the workflows. If that is the case, simply click on the **Workarea** tab at the bottom of the work area.

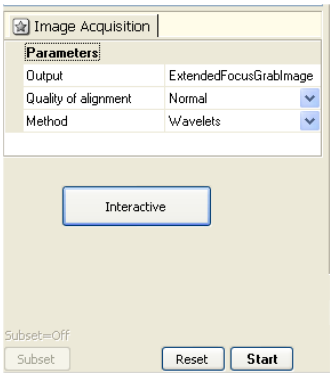
- Switch the light path to the camera. Open the live image window by clicking on the live image icon in the **Standard** toolbar. Alternatively, select the **Live** function in the **Acquisition** menu.



- In the work area select the **Acquisition** entry in the **Extended Focus** section.



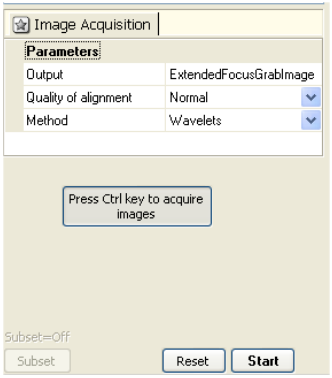
- If you capture images with a Stereo microscope, select an appropriate value for **Quality of alignment**. This will correct the lateral shift which occurs when you are focusing through the specimen.
- Select **Wavelets** as the **Method** for calculation.
- Click the **Interactive** button to capture the single images by pressing the **Ctrl** key.



Note:

- As an alternative you can click the **Start** button to capture the images.

- Press the **Ctrl** key to capture the first image
- Now go to the next focus position.
- Press the **Ctrl** key to acquire the next image.



Notes:

- The image of the second focus position is now automatically combined with the first image.

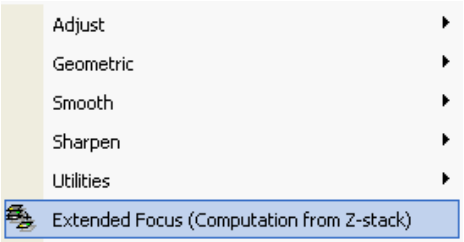
- You can now acquire further images until the result is satisfactory.

Using z-stack images for Extended Focus

➢ Activate the image to which you would like to apply the **Extended Focus** function. To do this, click on the image or select it via the tabs at the top of the document area.

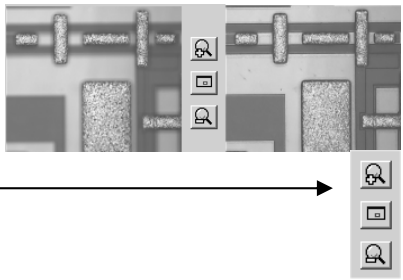


➢ Now select the **Extended Focus** (Computation from Z-stack) function in the **Processing** menu.

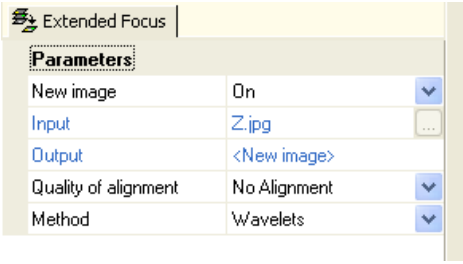


➢ The two thumbnails show the original image (left) and the resulting image (right).

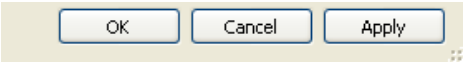
To select a specific frame, you can magnify, choose any frame from, or reduce the thumbnails.



- If the z-stack was acquired with a Stereo microscope, select an appropriate value for **Quality of alignment**. This will compensate the lateral shift which usually occurs during acquisition of the z-stack.



- Select **Wavelets** as the **Method** for calculation.
- Click on **Apply** to check the result.



- Now click on **Cancel** to exit the function.

9.5 Mark&Find

Overview

The Mark&Find module allows you to record the positions of an encoded or motorized x/y stage and relocate them in the future.

Open the Mark&Find window using the **Microscope** ⇒ **Mark And Find** menu command.

The recorded positions are saved in a database, which is able to store the position lists of several carriers. You can select the size of the carrier concerned. The following types are available: **Petri dish**, **Slide/Dish holder, Multiple slides** and **Multiwell plates**.

The module supports slides with sizes of 76 mm x 24 mm, 76 mm x 50 mm, 48 mm x 25 mm and 48 mm x 27 mm, or any user-defined size. Petri dishes can have a diameter of 35 mm, 60 mm or a user-defined diameter. Multiple-slide plates are supported in a format with 8 slides per plate or may have a user-defined format. Multiwell plates are supported in formats with 6, 12, 24, 48, 60, 96, 384 and 1536 wells per plate.

To make it possible to display the recorded positions graphically, when you first use the module you will need to define the center of the carrier on the x/y stage. If you also want to work with your carriers and the recorded positions on other Carl Zeiss microscopes using AxioVision, you will need to work with absolute stage positions for each carrier (please also refer to "Microscope" in the online help (**F1** key)).

Note:

- Many control elements in Mark&Find control both the microscope stage and the focus drive. If used incorrectly, damage can therefore be caused to objectives and/or carriers lying in the stage's traveling range.

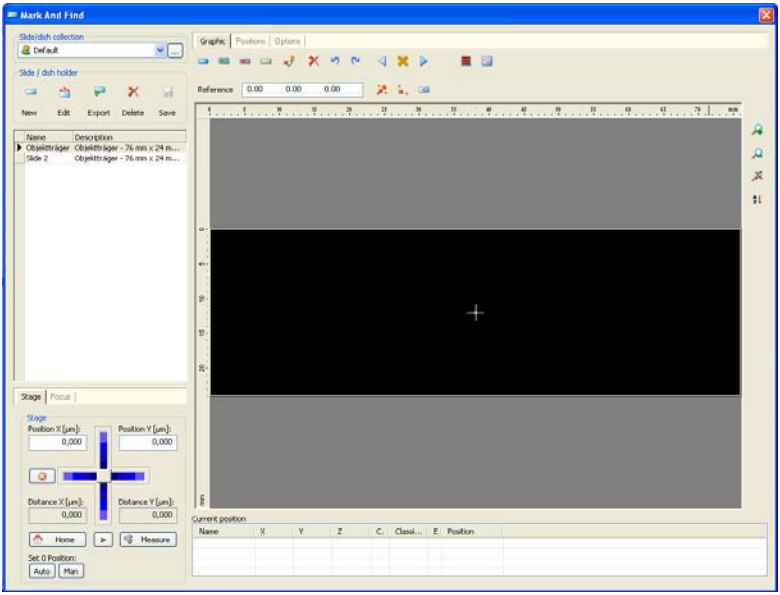
How do I generate a Mark&Find list?

The following two examples explain how to define a **Mark&Find** position list for a slide (example 1) and a multiwell plate (example 2). The same procedure should be followed for other types of carrier, e.g. Petri dishes.

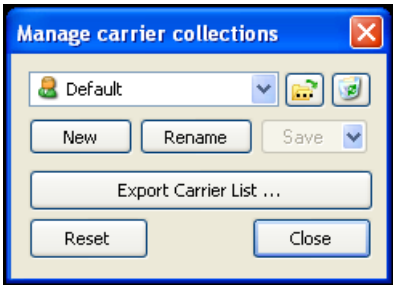
Example 1: slide

In the following example, a new database is created and 3 positions on a standard slide are created and saved. The slide is centered to ensure that a correct graphic display of the positions is achieved. A reference point is also selected on the slide. In this example positions are only recorded in relation to the center of the slide.

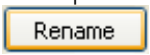
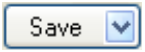
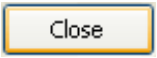
➢ Open the **Mark&Find** dialog using the **Microscope** ⇒ **Mark And Find** menu command:



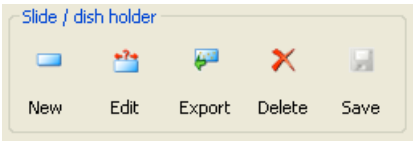
- Select a database from the drop-down list box of slide/dish collections or create a new database. To do this, click on  in the left-hand field. The **Manage carrier collections** dialog is opened:



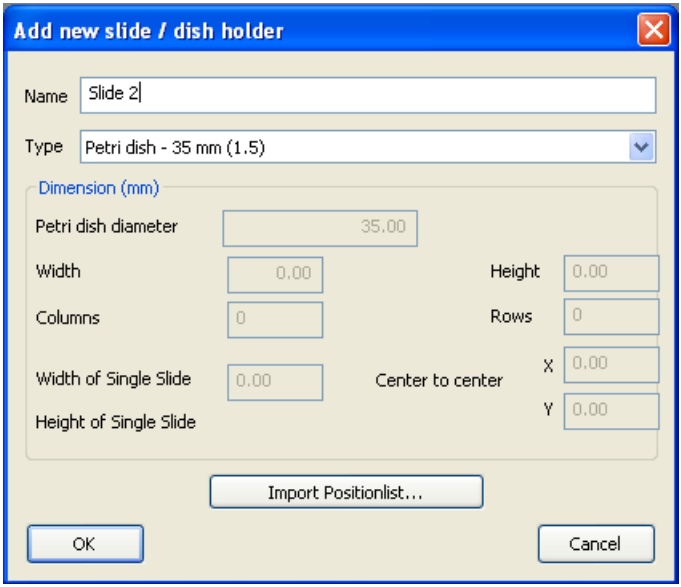
- If you click on  , a dialog is opened in which you can select a slide/dish collection file.  deletes the selected slide/dish collection.

-  opens another dialog where you can give the database a name.  allows you to change the name of the database.  saves your current database, whilst  deletes existing position lists and creates an empty database. In addition,  enables you to save the file in Excel format (.xls). Click on  or  to exit the dialog.

- Now add a new slide to the database. To do this, click on the **New** icon in the **Slide / dish holder** field:



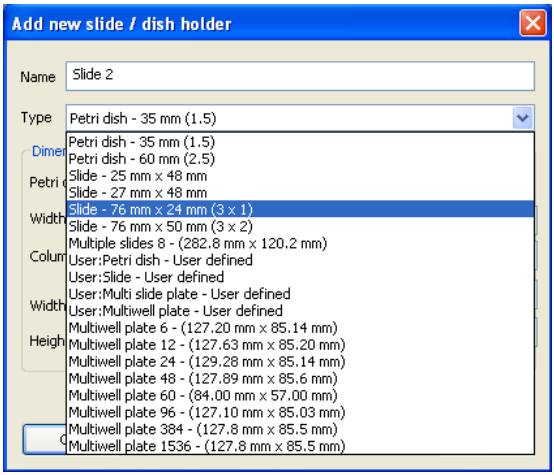
This opens the following dialog:



➢ Give the new carrier a name, e.g. "Slide 2".



➢ Select the standard slide from the **Type** drop-down list box:



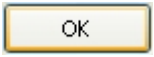
➢ It is also possible to import positions from a position list in a text document by clicking on 

. A position list to be imported should have the following structure, for example:

```
Slide,,,,
Name,Width,Height,,
Slide,76000.00,24000.00,,

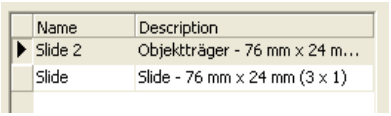
Positions,,,,
Comments,PositionX,PositionY,PositionZ,Color
"Position(1)",-11058.36,-4043.66,1.00,Blue
"Position(2)",-2382.21,5485.68,-1.55,Red
"Position(3)",10049.20,-4067.70,-2.25,Green
"Position(4)",3674.27,-1514.12,3.00,Yellow
```

To ensure that Mark&Find is able to understand this document, it must be saved in the form of a CSV document (Comma Separated Values). This export option is available in Microsoft Excel, for example.

- Confirm your selection by clicking on 

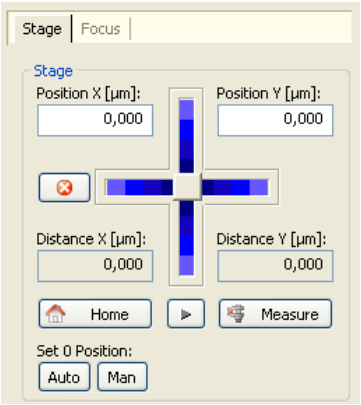
.

  You will now see the new carrier as an entry in the list of carriers in the **Mark&Find** dialog:



- Now insert the "home slide" (Carl Zeiss part number 453583) into the holder. The center of this slide is marked with a high degree of precision, and the slide has polished edges to ensure precise insertion into the holder.

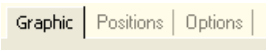
- Make sure that the control elements for stage control are displayed in the foreground:



- Manually set the stage position to 0 by clicking on the 

button.

- Make sure that you are in the graphic view of **Mark&Find**:



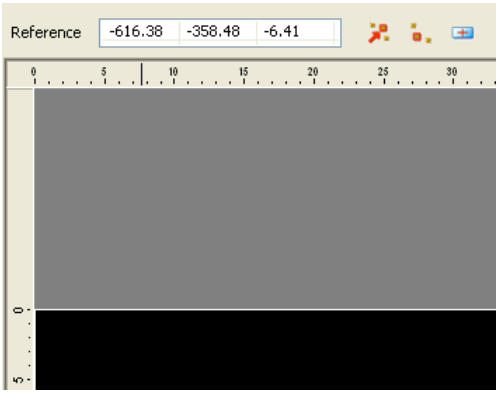
- Define the center by clicking on the button. The graphic display of the carrier should now be centered as follows:



- The white cross indicates the current stage position (in relation to the optical axis of the microscope).

- Remove the home slide and insert your carrier into the holder. Define a reference point on your carrier. This should be a conspicuous structure on your carrier that you will be able to find again easily in the future. If you do not set a reference point, all coordinates refer to the zero position of the motorized x/y stage.

- Click on the button. In the graphic view, a small gray cross indicates the current reference position. The coordinates are also displayed (in µm for the x, y and z axes):



This reference position can be automatically relocated at any time in the future by clicking on  .

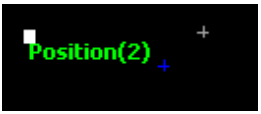
Once these preparatory steps have been completed, you can locate and save any desired positions on your carrier.

- To do this, move the stage to the desired position, bring the sample into focus (this is best done in the live image) and click on one of the colored icons  .

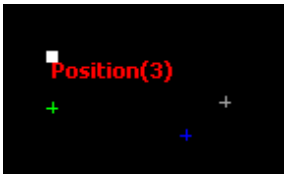
  Repeat this procedure until you have defined all the positions of interest on your carrier. The selected positions are shown in the graphic as colored crosses. The white cross indicating the current stage position covers the current position, the name of which is also visible.



Position 1

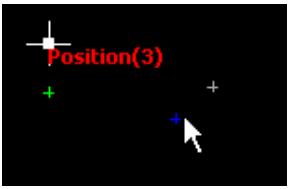


Position 2

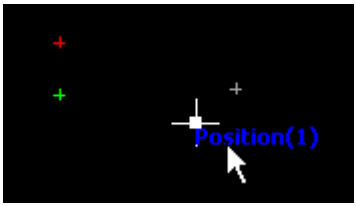


Position 3

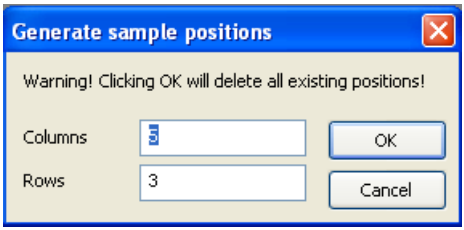
- These positions can be relocated. To do this, double-click on one of the positions:



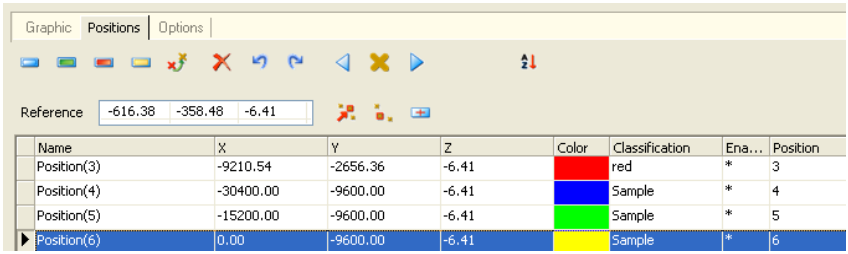
The large white cross indicating the current stage position shows the movement of the stage as it travels to the new position:



➢ Alternatively, if you click on  , you can also have a number of positions generated automatically as a sample selection. Here you only need to define the number of positions in the x and y dimensions. Please note that any previously saved positions are lost.



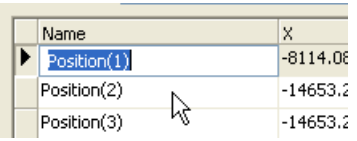
➢ Now go to the **Positions** property page:



Name	X	Y	Z	Color	Classification	Ena...	Position
Position(3)	-9210.54	-2656.36	-6.41		red	*	3
Position(4)	-30400.00	-9600.00	-6.41		Sample	*	4
Position(5)	-15200.00	-9600.00	-6.41		Sample	*	5
Position(6)	0.00	-9600.00	-6.41		Sample	*	6

All defined positions are listed here in tabular form on the basis of the parameters **Name**, **X**, **Y**, **Z**, **Color**, **Classification**, **Enabled** and **Position**.

- **Name**: name of the position. If you click in the corresponding field, you have the option of changing this name.



- **X**: the x-coordinate in µm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

- **Y**: the y-coordinate in µm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

- **Z**: the z-coordinate in µm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

- **X**: the x-coordinate in mm. The value displayed can be changed manually. To do this, click in the number field and enter the desired value.

- **Color**: indicates the color used when the position was defined. The color can be changed with the help of the color selection dialog, which opens if you click in the field.

- **Classification**: a number of different positions can be combined in the form of a group by assigning the same names to them. Change the name of the classification by clicking in the field. Division into groups allows you to sort position lists on the basis of specially defined conditions and travel to the positions they contain.

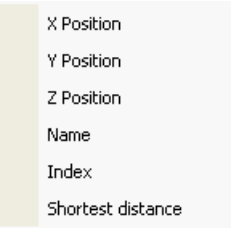
- **Enabled**: indicates the activation status of a position. Only positions that are marked with a "*****" symbol are used in the experiment. Click twice in the corresponding field to deselect the position via a check box, and to reselect it again as required.

- **Position**: indicates the sequence in which the positions were originally created.

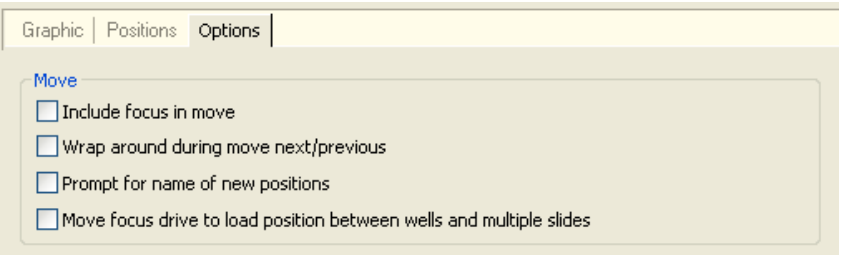
It is possible to select several positions by making a multiple selection (**Shift** + mouse click) or by selecting them individually (**Strg** + mouse click). These positions can then be modified together.

By double-clicking on the name of a parameter, you can sort the position list on the basis of that parameter.

- Once you have selected a position, you can delete it by clicking on  .
- Clicking on  undoes an action, whilst  redoes an action that has previously been undone.
- The selected position is located automatically by clicking on  .
- Clicking on  moves the stage to the previous position in the list, whilst clicking on  moves it to the next position.
- You also have the option of zooming into the graphic display by clicking on  or zooming out of it by clicking on  . Hold down the **Shift** key and move the mouse wheel to make use of a stepless zoom function. Clicking on  restores the full view of the entire slide.
- Select  to resort your position list. The criteria **X Position**, **Y Position**, **Z Position**, **Name**, **Index** and **Shortest distance** are available for selection from a drop-down menu.



- Further selection options can be found on the **Options** property page:



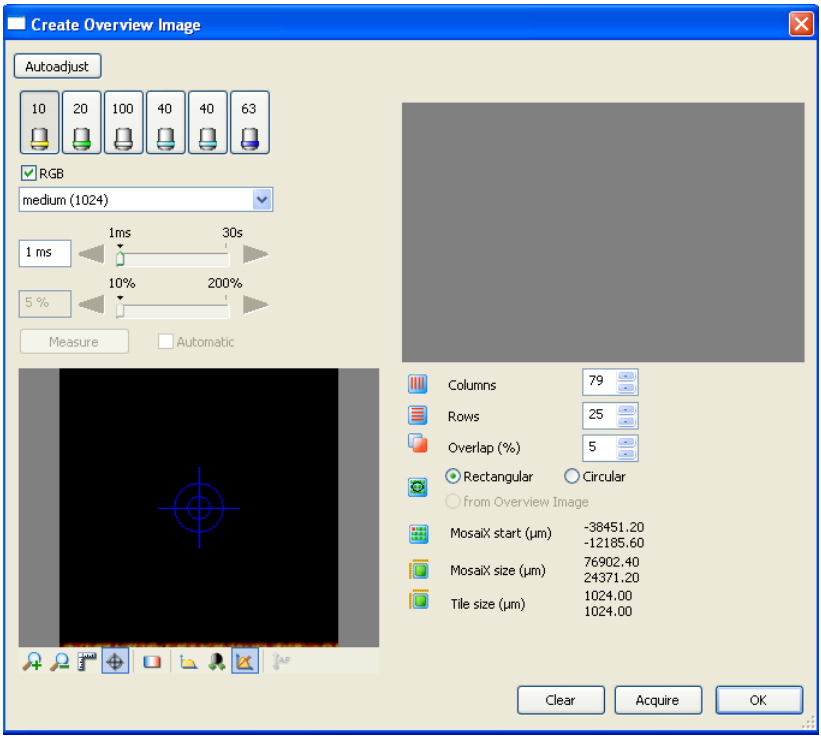
- **Include focus in move**: if this check box is activated, the z-position (focus position) saved when the position was defined is also restored when you travel to a position.

- **Wrap around during move next/previous**: if this check box is activated, when "traveling forwards" you move from the last position to the first position in the list, and when "traveling backwards" from the first position to the last position.

- **Prompt for name of new positions**: if this check box is activated, a dialog for entering the position name is displayed automatically after each new position is created.

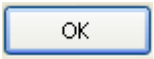
- **Move focus drive to load position between wells and multiple slides**: if this check box is activated, the objectives are moved to the load position while you are moving to another well or slide, in order to prevent damage.

➢ To make it easier to locate positions for the Mark&Find list, the option is available of having the carrier scanned fully automatically at a low magnification to obtain a preview. To do this, click on  . Provided that the MosaiX module is licensed and selected in the module list, the following dialog appears:



➢ You can now click on the 

button to generate an overview image containing the set number of columns and rows with a defined overlap. You have the option of setting the objective for acquisition, the exposure time and the size of the tile image in µm (300, 512, 1024 or 2500).

➢ Click on 

to transfer this overview to the graphic display in your **Mark&Find** dialog.

The overview dialog is otherwise operated in the same way as the **MosaiX** dialog (please also read the manual and the online help entries on **MosaiX**, **F1** key).

➢ Now save the changes. To do this, click on the 

button.

You have now successfully generated a position list and can use this at any time to relocate the saved positions. This list can also be used within the context of **Multidimensional Acquisition**.

Example 2: multiwell plate (96)

Position lists for multiwell plates are created in the same way as for slides, although there are a number of additional control elements and functions. These additional, divergent functions are presented below, without explaining all the functions that are identical to those in example 1 again. For details of the general functions, please also refer to the section "Example 1: slide".

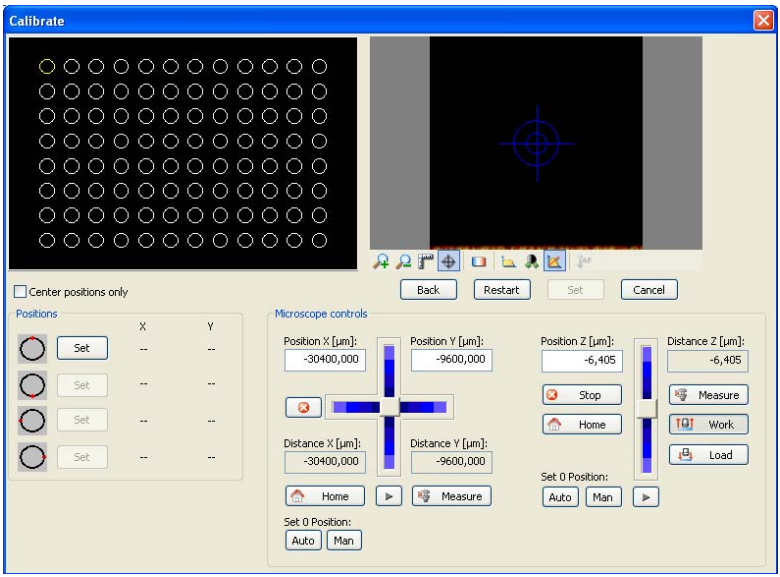
To ensure that you can work sensibly with multiwell plates, the coordinates of the plate need to be adapted to the stage coordinates by means of calibration. The more wells your plate has, the more important it is to perform precise calibration.

Note:

- If the stage has already been calibrated and a standard plate has been selected, the program tries to locate the correct position after you call up the wizard. Please first ensure, therefore, that there are no obstacles within the stage's travel range.

- Select a low objective magnification (e.g. 10x) and transmitted light illumination. Place a multiwell plate of the type that you wish to use in the holder on the microscope stage.

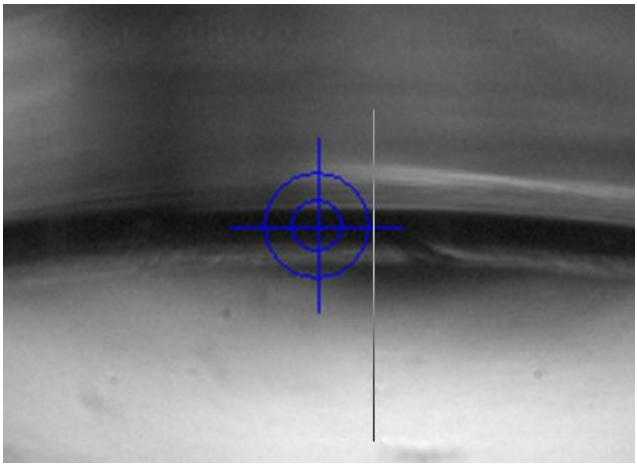
- Click on the  icon. This opens the calibration wizard. If you have already performed calibrations, you are asked whether you wish to retain the calibration data. Select **No** if you wish to perform a new calibration and reject the old calibration data. Select **Yes** to continue working with the old calibration data.



Note:

- The orientation of the camera and the stage axes must be correctly configured to allow error-free calibration. The movement of the camera's field of view must correlate with the movements of the slider (or joystick) for stage control in all four directions. The camera orientation, the camera port being used and the settings for the image orientation and axis orientation of the stage all influence this behavior.

- Use the stage control to move interactively to the positions on the multiwell plate that correspond to the well marked in yellow. The aim is to travel to the calibration points symbolized in the control area at the bottom left and to move them to the center of the live image (reticle). The first position to be located could look something like this in the live image:



- By clicking on the Setzen button, which is currently active, you can now define this calibration point. It then becomes possible to calibrate the next position. In total, the following positions need to be located and set for a calibration:

  - Top left well: 12, 6, 9 and 3 o' clock position
  - Top right well: 12 and 9 o' clock position
  - Bottom right well: 12 o'clock position

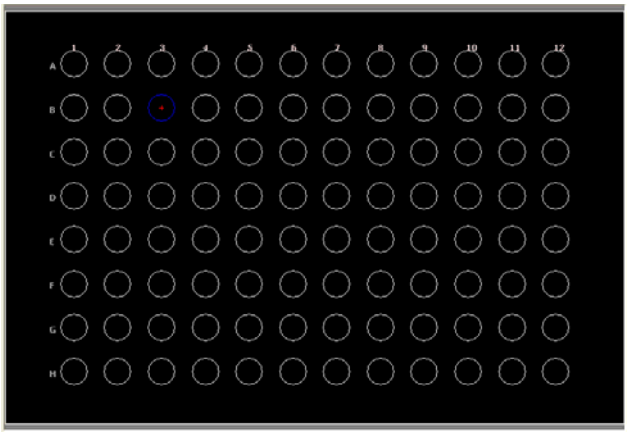
  Wells that have already been calibrated are shown green in the graphic. Wells that have yet to be calibrated are marked in yellow.

- Click on Back to go back to the previous step of the calibration.
  Click on Restart to return the calibration wizard to its original state.
  Click on Cancel to exit the wizard without saving any changes you have made.

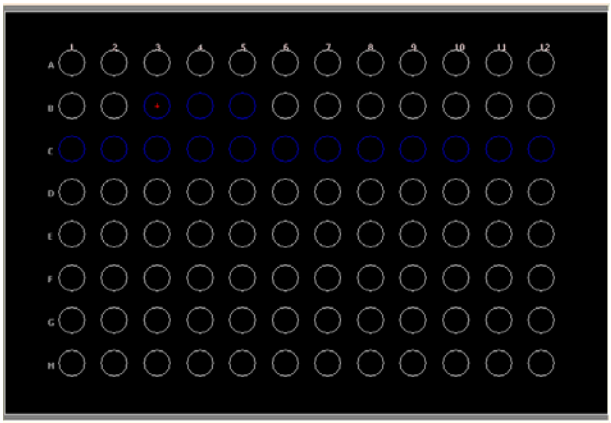
- The Set button under the live image only becomes active once all the necessary calibration steps have been performed. Click on it to save the calibration and exit the wizard.

- As an alternative to calibrating on the basis of the well edges, it is possible to calibrate using the well center only. To do this, activate the **Center positions only** check box. You then only need to define the center positions of the top left, top right and bottom right wells. You should use a low objective magnification for this that allows you to view the entire well in the live image.

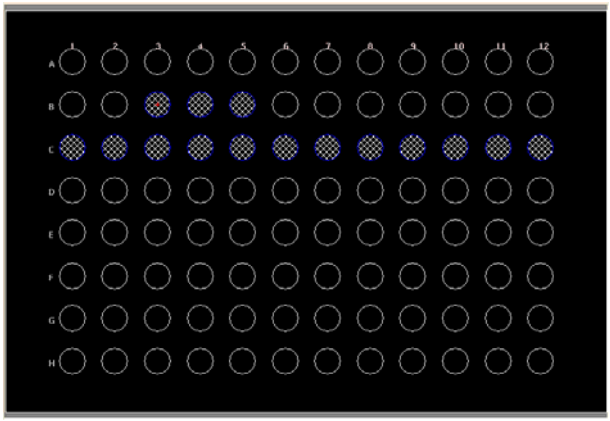
- Go to the **Multiwell graphics** property page. Double-clicking on a well automatically moves the stage to the center of the selected well. The current stage position is marked with a red cross. The selected well (B3) is outlined in blue:



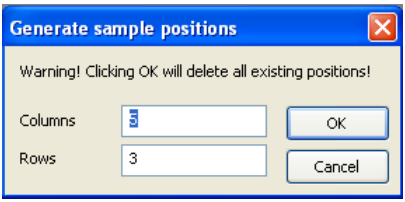
- By outlining several wells with the left-hand mouse button depressed or performing a multiple selection using the **Strg** key, combinations of different wells can also be selected (in the example B3, B4, B5, row C):



- To automatically generate positions in the selected wells and save them in a Mark&Find position list, click on  . A position at the center of the well is now generated for all selected wells, using the current focus position. Wells in which positions have been set are marked with hatching:



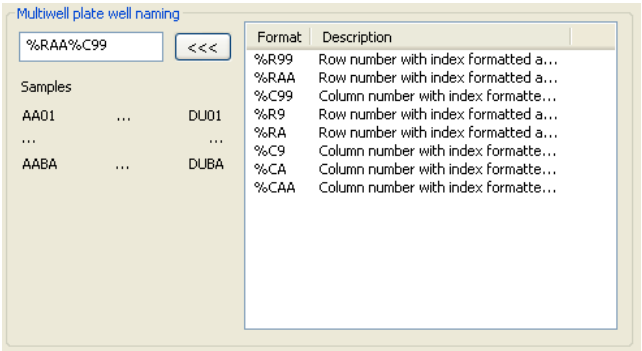
- Alternatively, by clicking on  , it is also possible to have a number of positions generated automatically for each well. Here you only need to define the number of positions in the x and y dimensions. Please note that any previously saved positions are lost.



- You can, of course, also locate positions manually and add individual positions by clicking on the  icons.

- A special function that is available for multiwell plates allows you to overwrite the focus position of the selected position by clicking on  . This function makes it possible to adapt a position list that has already been created to a multiwell plate with a different focus position (e.g. due to a different base thickness).

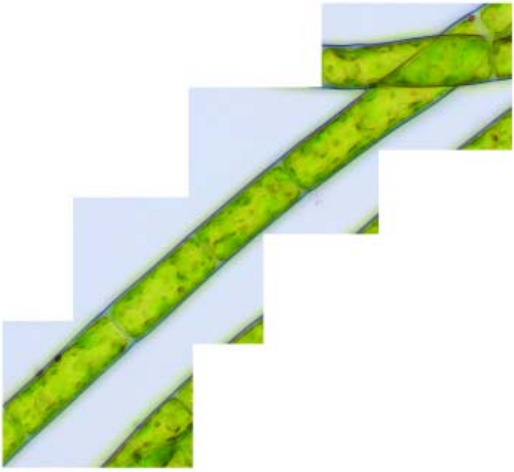
- In the case of multiwell plates, you can also define a rule on the **Options** property page that will be used to automatically name the positions in the list:



9.6 Panorama

General

The Panorama module creates large images from individual images, enabling you, for example, to display objects that would otherwise not fit into an image field. The images are combined with pixel precision, either interactively by superimposing the images, or (with support from the software) using **Stitching** (automatic image combination). It is also possible to combine displaced images so that they fit together perfectly.



Panorama image consisting of four different images.

Panorama images can be created in two different ways:

- Combining images acquired directly via a camera.
- Using already saved images.

System requirements

There are no special technical requirements for generating Panorama images. The operation of the module has been specially designed for the use of manual microscopes and single image acquisition.

Notes:

- The automatic acquisition of large composite images is possible using the MosaiX module and requires a scanning stage.

If you wish to combine images acquired directly from the camera, however, you will require a camera that has been integrated into AxioVision. It is not possible to use TWAIN devices, but you can acquire images using a camera integrated via the TWAIN interface, and then create Panorama images from the individual files.

It is not necessary to use a scanning stage, but this does make it easier to position the individual images. AxioVision will then adjust the resulting image size automatically, and suggest a position for the newly acquired image. The known stage coordinates are converted directly into the image position. A correct image can then usually be generated using **Stitching** (automatic image combination).

You should bear in mind that the resulting images may sometimes require a very large amount of storage space. In particular, if you are using high-resolution digital cameras, such as those in the AxioCam family, the Panorama images can easily take up as much as several hundred megabytes. The computer you are using should therefore have an appropriate user memory and hard disk capacity. You should also consider the possibility of external storage (network, CD/DVD writer etc.), as otherwise local hard-disk capacities may be quickly used up.

Special features of Panorama images

Generally, an image that has been acquired using the Panorama module is the same as any other – it is just larger, and made up of several component images. For Panorama images, a special view which allows you to work more efficiently with the individual images is therefore available via the image window: **Tile View**.

In this view you have access to the individual images (the "tiles") that make up a Panorama image. Here you can "pick up" the tiles individually, and change their position or alignment. You can also display information on the individual tiles, and insert circumscribing rectangles.



Pane to activate the tile view in the display window.

Acquisition of Panorama images

The **Panorama Acquisition** function can be executed via the work area on the left-hand side of the AxioVision window. If the work area is not displayed, activate it via the **Standard** toolbar 

.

Alternatively, select the **Windows** function on the **View** menu and then the **Workarea** entry.

Notes:

- The work area may in fact be open but covered by the workflows. If that is the case, simply click on the **Workarea** tab at the bottom edge of the work area 

.

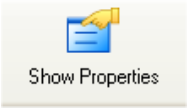
- Set the light path to the camera. Then open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.



- Select the scaling for the chosen objective from the **List of available scalings** underneath the live image, if automatic scaling is not available.



- To adjust the live image, click on the **Properties** button. Alternatively, select the **Properties** function from the **View** menu.



Notes:

- To guarantee a stitching result without annoying shading on the image border, you should execute or activate the shading correction. Go to the **General** tab in the **Properties** dialog. Make sure that the camera is viewing a completely empty image. To ensure that this is the case, move your sample on the microscope completely out of the field of view.

- Click the **Shading Correction** button to acquire the shading correction image.

- To ensure that dirt particles from the slide are not visible in the correction image, use a clean slide and a clean objective and completely defocus the microscope.

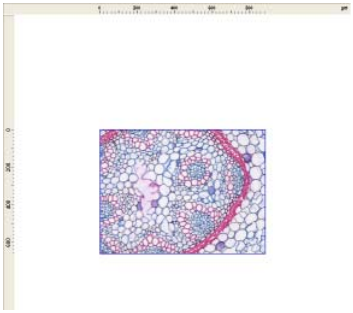
- Please note that if the objective is changed the white reference has to be generated again.

- A detailed description of the steps involved in preparing for image acquisition can be found in the section "Step by Step to the First Image" in section 3.3 "Quick Guide to the First Image".

- Close the Live display and select the **Acquisition** entry under **Panorama** in the work area.

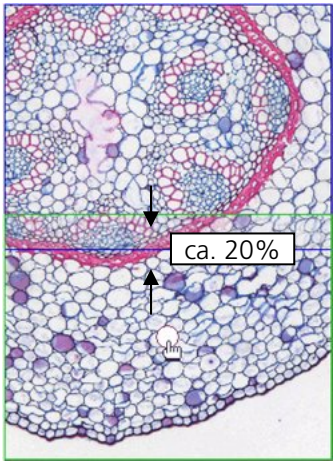


- The camera's live image is now displayed at the center of the **Panorama Acquisition** dialog. To ensure that you have sufficient space to position several images, we recommend that you reduce the display accordingly. To do this, use the buttons on the toolbar of the Panorama image.



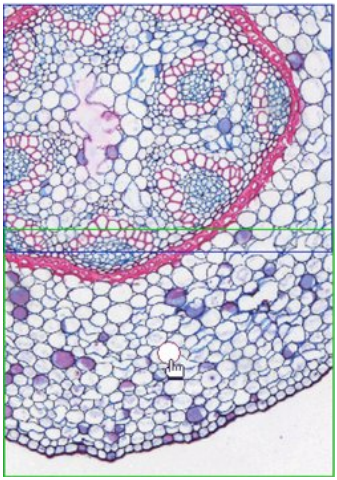
- Move the scanning stage to the starting point of the region you want to acquire, and focus. Then click on **Snap Image** or use the **F2** key of the keyboard.

- The live image (marked by a white circle in the center) and the acquired image overlap partially. To acquire the next image, drag the live image (with the left mouse button depressed) to the position where you want the next image to be acquired. The images should clearly overlap.



- Do not attempt to put adjacent images together with pixel precision. The function for automatic alignment (stitching) requires a overlap of good visible structures. The guide value is around 20%. And: There must be

- Now move the specimen to the new position. The areas where the live image overlaps with the images already acquired are displayed in semi-transparent form. This makes it easier to position the next image.



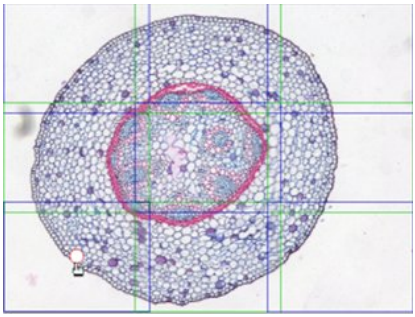
- The images do not have to be aligned precisely, as they can be placed in the correct position automatically later on using the stitching function. However, if the image regions that belong together are positioned too far away from each other, automatic stitching may not work.

- Repeat the previous steps until you have acquired the entire region.

- If the result is unsatisfactory, you can shift the individual tiles of the Panorama image manually.

- To do this, make sure that the editing mode is active. (The **Turn on/off tile mode** button at the bottom edge of the Panorama image has to be depressed.) Then click inside a tile. The tile is highlighted by a white circle at its center. Hold down the left mouse button, and move the tile to the desired position. As before, areas of overlap with other images are displayed in semi-transparent form.





- To delete individual tiles, click on a tile. Then press the **Delete selected tile** button at the bottom edge of the Panorama image.



- Click on **OK** to adopt the completed Panorama image into AxioVision.
- At the bottom of the image window click on **Tile View**.
- To align the tiles in relation to each other, click on the button for automatic tile alignment on the toolbar directly under the image window.



Alternatively, select the **Stitching** entry under **Panorama** in the work area.

Notes:

- It is also possible to add extra images to an existing Panorama image. To do this, click on the  button in the top right-hand corner of the **Panorama Acquisition** dialog window. A gallery of all images currently loaded in AxioVision is displayed. Select the desired image, and carry out the steps described to add further single images to the existing Panorama image.

- If you click on the 

button in the upper right corner of the **Panorama Acquisition** dialog, you can start generating a new Panorama image directly, without having to close the dialog first.

Creating Panorama images from individual files

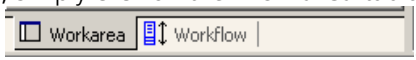
If the individual tiles of a Panorama image are already available as individual files, they can be put together manually. The most frequent problem and source of errors here is the overlap of the individual tiles. If the overlap is too large or too small, this can lead to problems when using the function that automatically combines the tiles (stitching).

In principle, we therefore recommend that you create the images using the Panorama acquisition function, and ensure that there is an appropriate area of overlap. Please note, that the images must be of the same type (color/gray values, size) to put them together.

The **Import from Files** function can be executed via the work area on the left-hand side of the AxioVision window. If the work area is not displayed, activate it via the **Standard** toolbar**.**

Alternatively, select the **Windows** function on the **View** menu and then the **Workarea** entry.

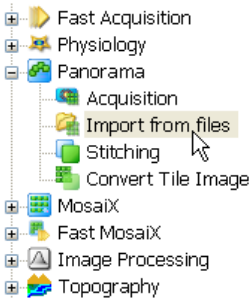
Notes:

- The work area may in fact be open but covered by the workflows. If that is the case, simply click on the **Workarea** tab at the bottom edge of the work area 

.

- All the steps involved in the generation of a Panorama image can be found in sequence in the **Panorama** workflow.

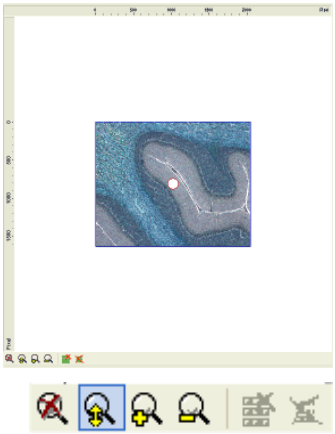
➢ Select the **Import from files** entry under **Panorama** in the workarea.



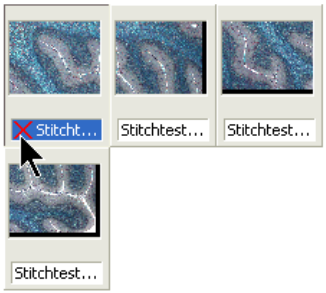
- Select the folder containing the individual tiles of your Panorama image. To do this, click on the button above the gallery. The Microsoft Windows standard dialog **Select folder** is opened. Select the desired folder and confirm by clicking on OK. All the available images are now displayed as thumbnails in the gallery.



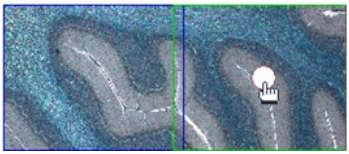
- To put the Panorama image together, drag the first image into the Panorama image area.
- The image fills the whole of the Panorama image area. To ensure that you have sufficient space to position additional images, we recommend that you reduce the display accordingly. To do this, use the buttons on the toolbar of the Panorama image.



- The selected image is now marked with a red cross in the gallery, to show that it has already been used.



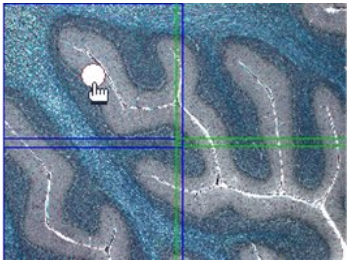
- Select the next image from the gallery and drag it to the desired position. The images should clearly overlap. The function for automatic alignment (stitching) requires a certain overlap. The guide value is around 20%.



- Drag all the other images into the Panorama image one after the other in the same way, and position them.

- If the result is unsatisfactory, you can of course subsequently shift the individual tiles of the Panorama image manually.

- Click inside a tile. The tile is highlighted by a white circle at its center. Hold down the left mouse button, and move the tile to the desired position. As before, areas of overlap with other images are displayed in semi-transparent form.



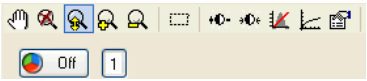
- To delete individual tiles, click on a tile. Then press the **Delete the selected tile** button at the bottom edge of the Panorama image.



- Click on **OK** to adopt the completed Panorama image into AxioVision.

- At the bottom of the image window click on **Tile View**.

- To align the tiles in relation to each other, click on the button for automatic tile alignment on the toolbar directly under the image window.



- Alternatively, select the **Stitching** entry under **Panorama** in the work area.

Notes:

- It is also possible to add extra images to an existing Panorama image. To do this, click on the  button in the top right-hand corner of the **Import from files** dialog window. A gallery of all images currently loaded in AxioVision is displayed. Select the desired image, and carry out the steps described to add it to the image.

- If you click on the **New** button, you can start generating a new Panorama image directly, without having to close the dialog first.

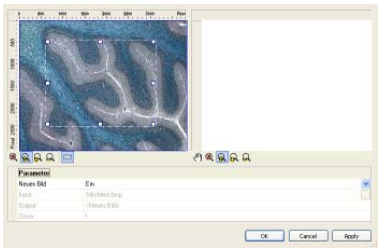
Converting a tile image into a regular AxioVision image

As tile images are a special format, they cannot be further processed or measured using the AxioVision functions. You have to convert them into a regular AxioVision image first.

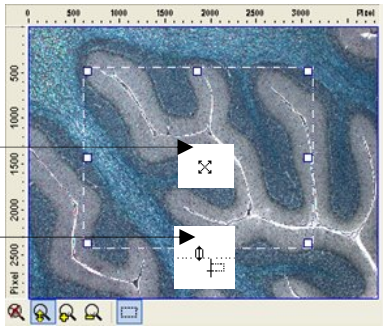
➢ Acquire a tile image, or load one from the hard drive.

➢ Select the **Convert Tile Images** function from the **Processing** menu.

➢ The current tile image is displayed, with a frame, on the left-hand side.



- You are now able to position the frame freely and change its size.
- If the mouse cursor is inside the frame, keep the left mouse button pressed and you can move the whole frame.
- If you move the mouse cursor onto one of the frame handles, keep the left mouse button pressed and resize the frame.



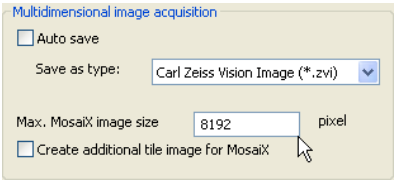
- Once you have selected the desired region, click on **OK**. A regular AxioVision image is then generated. This image can now be processed and measured in the usual way.

Note:

- The size of the resulting image is reduced to 4096 pixels by default. If the frame size set exceeds this value, the resulting image is automatically reduced accordingly. The region you have selected is always used, however. If you want to avoid reduction, you can freely configure the size of the AxioVision image.

- Select the **Options** function from the **Tool** menu. Activate the **Acquisition** tab sheet.

- Under **Multidimensional image acquisition** you can adjust the **Max. MosaiX image size** parameter accordingly. The size of the value that you can select for this setting is essentially dependent on your computer's user memory.



If the setting you select is too large, you may not be able to process the resulting images optimal. The processing speed will in any case be reduced dramatically, constricting optimal working.

9.7 MosaiX

General

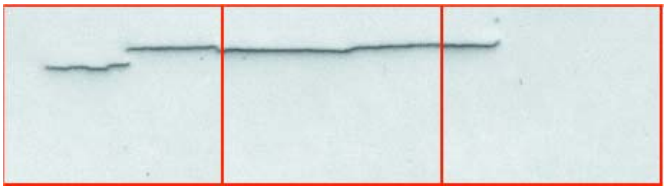
The MosaiX module has been developed to resolve a classic microscopy problem: the insufficient size of the field of view. At high magnifications in particular, the region that can be seen through the eyepiece is never large enough. If you are working with cameras, the problem is frequently exacerbated by the fact that the camera's rectangular sensor cuts off regions of the round field of view when it is viewed through the eyepiece.

The ideal situation, therefore, would be to display the entire region of interest in a single image, even at high magnifications.

A number of different application scenarios are conceivable:

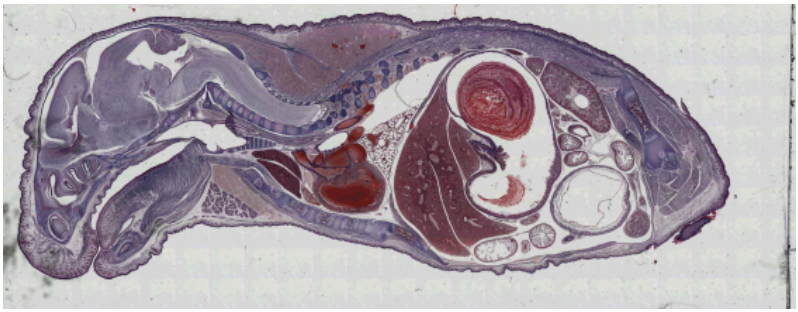
- **Acquisition of image overlapping objects**

  In the simplest case, the object of interest cannot be displayed in full in a single image at the selected magnification. This means that several individual images have to be combined to form a larger overall image, and if the number of images in question is small, or if the system you are using does not have a scanning stage, you may be well advised to make use of the Panorama module too.



- **Archiving of a complete slide ("virtual slide")**

  The archiving of specimen over al long period requires a high amount of space and its always associated with loss of quality (i.e. bleaching). The digital archiving of complete specimen is because storage media are becoming cheaper all the time is a realistic way to save them permanently with consistent quality.

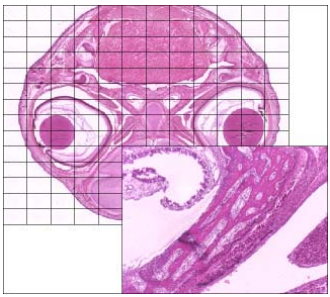


- **Correction of objects cut by the image border**

  If you want to measure large areas, usually several single images have to be acquired. In this case always the questions comes up, how to handle objects that are not visible in full in the image. These objects should not be measured, as this would distort the measurement result. Using MosaiX images, you can avoid this problem completely.

With MosaiX, a specimen is scanned with a scanning stage extensively to generate a large "virtual" representation. This electronically generated image of the specimen serves as a map for navigating around the sample, and as a basis for further analyses. The advantage of this method is that you only have to scan the specimen once, and it is then available for all subsequent processing or analysis steps.





There are two ways to combine the individual images with the highest possible pixel precision:

- Ensure that the stage calibration and camera alignment are correct. The images are then "simply" put together. No check is performed to establish whether this has been done correctly. This is a fast method, but a certain amount of care is required when you are configuring the system.

- The second option automatically guarantees that all images are correctly positioned in relation to each other. This is what is known as **Stitching**. Here the image structures of adjoining images are used as a basis to check that the boundaries actually fit together correctly. If this is not the case, the images are automatically placed in the correct position in relation to each other.

As the focus plane does not always remain the same if samples are large, or if the scanning stage has to travel large distances, automatic focusing is often required during acquisition. Two options are available here: the **Autofocus** function and **Focus Correction**.

- **Autofocus**: here every nth selected tile image is focused in accordance with the calibration performed. The focus plane found is retained for the acquisition of the following tile images, until the next nth tile is reached.

- This is **Focus Correction**: With this method a "focus landscape" is defined. Several focused positions are used to calculate the three-dimensional surface curves of the object and the individual tiles are focused accordingly. At least three positions need to be set. The larger the desired MosaiX, the more positive the effect of setting more focus positions will be on the sharpness of all the tile images.

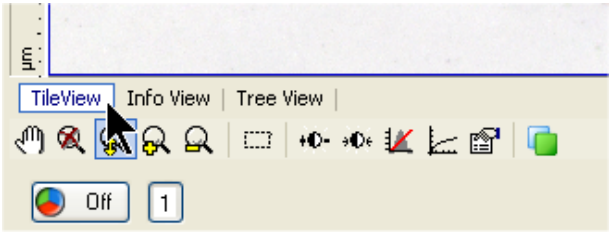
System requirements

A scanning stage is essential for generating MosaiX images. To make use of the options for automatic focusing, you will require a microscope with a motorized z-drive. The **Autofocus** function requires the AxioVision **Autofocus** module.

You should also bear in mind that the resulting images may sometimes require a very large amount of storage space. Images with several hundred MB, or even several GB, are not uncommon. This means that the computer you are using must have an appropriate user memory and hard disk capacity. You should also consider the possibility of external storage (network, CD/DVD writer etc.).

Special features of MosaiX images

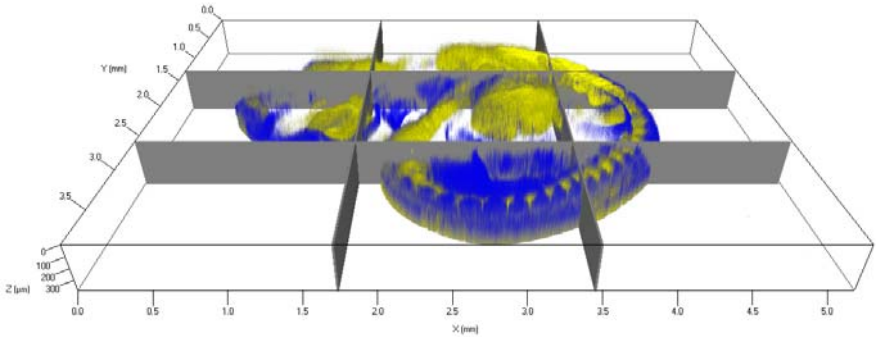
Generally, an image that has been acquired using the MosaiX module is the same as any other – it is just larger, and made up of several component images. For MosaiX images, a special view which allows you to work more efficiently with the individual images is therefore available via the image window: **TileView**. In this view you have access to the individual images (the "tiles") that make up a MosaiX image. Here you can "pick up" the tiles individually, and change their position or alignment. You can also display information on the individual tiles, and insert circumscribing rectangles.



Button to activate the tile view in the display window.

Multidimensional MosaiX images

The MosaiX module can also be used together with the modules for multidimensional image acquisition. It can therefore be combined freely with the Multichannel Fluorescence, Z-Stack, Time Lapse and Mark&Find modules. If you are using MosaiX together with the Mark&Find module, you can define a region around any position in a Mark&Find position list for which you want to acquire a MosaiX image.



Muscle specific GFP tag (yellow), nuclear stain (blue), stage 10.5 embryo, 2 channel fluorescence Z-stack ApoTome 2 acquisition of 3x3 Camerafields, stitched together with the new MosaiX module of AxioVision 4.2. Dr. Frederic Relaix and Emanuelle Perret, PFID, Pasteur Institute, Paris, France.

The Extended Focus module is only useful in combination with the MosaiX module if you are using Z-stack images. A microscope with a motorized Z-drive is required.

If you want the individual planes of a region of a specimen to be acquired manually for the extended focus, the Panorama module is also required, to put the individual images together.

Setting up the camera and scanning stage

If you want to acquire good quality MosaiX images, you need to ensure that certain requirements relating to the interaction of the camera and microscope/scanning stage are met. This is firstly to guarantee the correct movement of the stage, and secondly to prevent the individual tiles from being shifted or rotated in relation to each other during acquisition.

Image orientation

The image orientation in the eyepiece and the image on the monitor must be identically. This means that the image on the monitor must not be rotated in relation to the eyepiece display. If the alignment in the live image is wrong, it can be corrected either by rotating the camera or by adjusting the image alignment:

- Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.

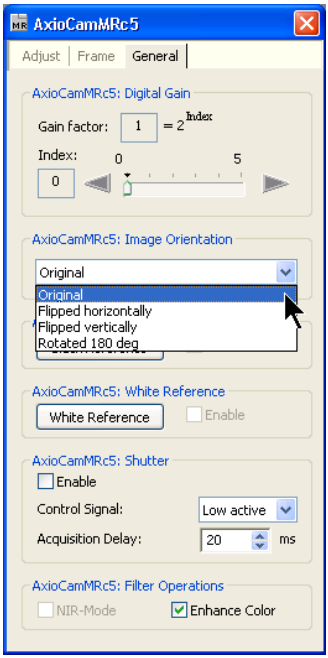


- To change the image alignment, click on the **Live Properties** button. Alternatively, select the **Live Properties** function from the **View** menu.



- In the **Live Properties** dialog, select the **General** tab.

- Select the appropriate **Image Orientation** from the drop-down list box, so that the live image and eyepiece image will correspond.



Notes:

- A detailed description of the steps involved in preparing for image acquisition can be found in the section "Step by Step to the First Image" in section 3.3 "Quick Guide to the First Image".

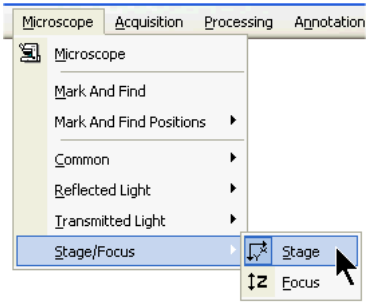
Directions of movement of the stage

To guarantee that the stage moves correctly, the direction of movement must be set in such a way that a movement of the camera is simulated when the control function in AxioVision is used to move the stage. This means that if the stage control function is moved to the right, the objects in the live image move to the left. If the stage control function is moved up, the objects move down. If the directions of movement are wrong, they can be adjusted via the **Microscope Configuration** program.

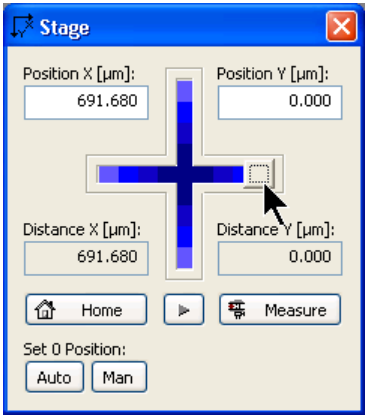
- Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.



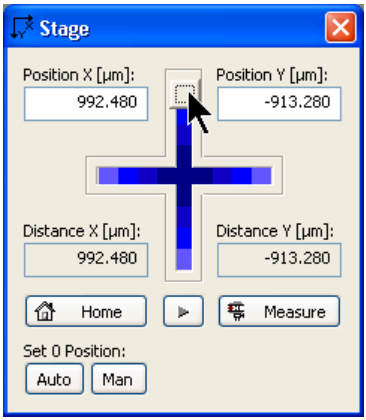
- Select the **Stage/Focus** entry from the **Microscope** menu, and open the stage control function via the **Stage** entry.



- Now check the directions of movement in the x- and y-directions.
- First move the stage to the **right**.
- The objects in the image should move to the **left**.
- If the objects also move to the right, you need to change the direction of movement via the **Microscope Configuration** program.



- Before you do this, however, test the movement in the y-direction.

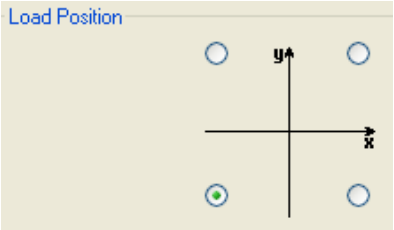


- Move the stage **up**.
- The objects in the image should move **down**.
- If, instead of moving as described above, the objects move in the exact opposite direction, the directions of movement of the stage can be reversed.
- Close AxioVision and start the **MTB 2004 Configuration** program. If the program is not available as an icon on the Microsoft Windows desktop, select **MTB 2004 Configuration** via **Start → Programs → Carl Zeiss Vision**.

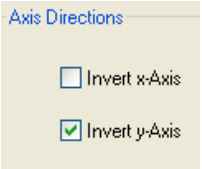


- In **MTB 2004 Configuration** select the 

drop-down menu. You can change the direction of travel by clicking on the quadrants.



- If you are using the MAC 5000 as stage controller, the movement can be reversed by the check boxes of the parameter **Invert x-Axis** and **Invert y-Axis**.



➢ Click on OK to come out of the **MTB 2004 Configuration** program and save the settings.

➢ Start AxioVision again, and follow the steps described above to check the direction of movement.

Note:

- If you are working with a joystick, you may find that the direction of movement cannot be changed when the joystick is being used. This means that the hardware and software joysticks have different directions of movement.

Alignment of camera and stage

The camera and stage must be aligned with each other in such a way that objects move parallel to the x- and y-axes when the stage is moved. Otherwise, the individual tiles of the MosaiX image would have to be rotated in relation to each other in order to combine them exactly.

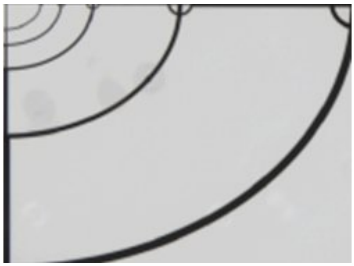


The home slide (part number 453583) offers you optimum definition conditions. The center of this slide is marked with a high degree of precision, and the slide is covered and has polished edges. This ensures that it can be placed precisely into the slide holder. Alternatively, you can use a point that stands out clearly on a slide of your choice.

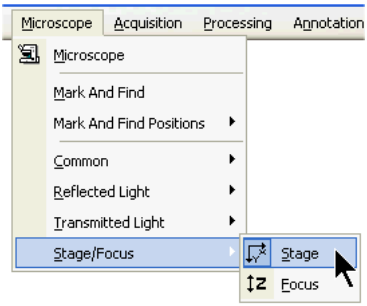
➢ Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.



- Find a point that stands out clearly on your slide, or position the center of the home slide (as shown in the example) in the top left-hand corner of the live image.



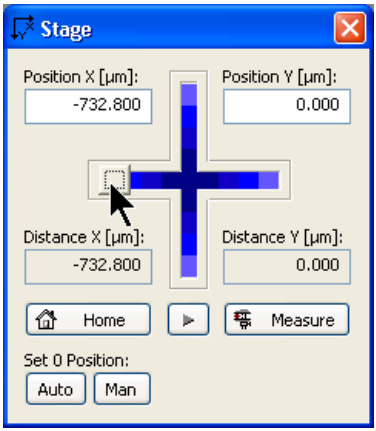
- Select the **Stage/Focus** entry from the **Microscope** menu, and open the stage control function via the **Stage** entry.



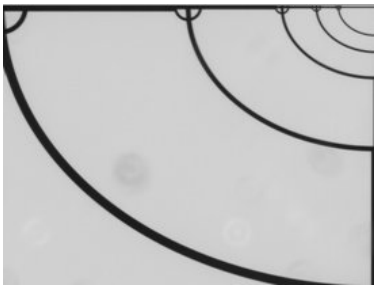
- Now move the stage to the **left**. The selected point moves to the right.

Note:

- If the point moves to the left, you need to change the direction of movement. Please refer to the previous section.



➢ The selected point should move parallel to the top edge of the image over the entire image width, up to the top right-hand corner. If it does, the edges of the stage and the camera are aligned parallel to each other.



If it does not, you need to rotate the camera slightly, and then move the point across the image again. Keep doing this until parallel movement is achieved.

Note:

- If you are no longer able to combine your MosaiX images correctly, this check should be repeated. It is quite possible that the camera has been inadvertently rotated.

Acquisition of MosaiX images

➢ Set the light path to the camera. Open the live window by clicking on the icon for the live image on the **Standard** toolbar. Alternatively, select the **Live** function from the **Acquisition** menu.



➢ Select the scaling for the chosen objective from the **List of available scalings** underneath the live image.



➢ To adjust the live image, click on the **Live Properties** button. Alternatively, select the **Live Properties** function from the **View** menu.



Notes:

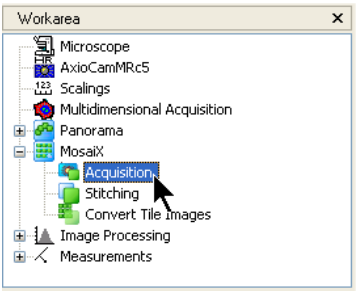
- To guarantee a stitching result without annoying shading on the image border, you should activate the shading correction. Go to the **General** tab in the **Live image properties** dialog. Make sure that the camera is viewing a completely empty image. To ensure that this is the case, move your sample on the microscope completely out of the field of view.

  To ensure that dirt particles from the slide are not visible in the correction image, use a clean slide and a clean objective and completely defocus the microscope.

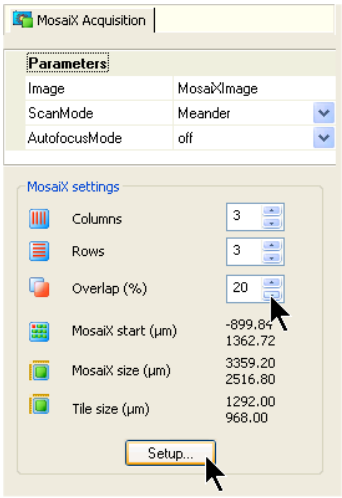
  Please note that if the objective is changed the shading correction has to be generated again.

- A detailed description of the steps involved in preparing for image acquisition can be found in the section "Step by Step to the First Image" in section 3.3 "Quick Guide to the First Image".

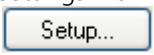
➢ Select the **Acquisition** entry under **MosaiX** in the work area.



- The **MosaiX Acquisition** property page displays the current MosaiX settings.

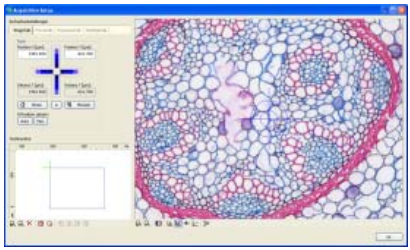


- In order for automatic alignment (stitching) to be performed successfully, the images need to be acquired with an overlap. A value of 10-20% is recommended.

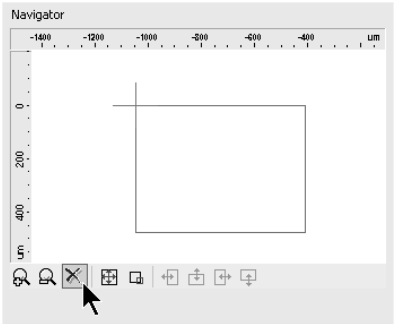
- The **MosaiX-Setup** dialog is available if you wish to adjust these settings. To open the dialog, click on 

.

- The MosaiX **Acquisition Setup** dialog is the central dialog for defining the position and size of the MosaiX image to be acquired of your specimen.



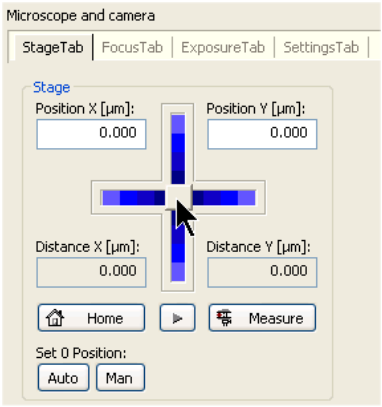
- To define a new MosaiX image, reset any existing settings. To do this, click on the  button below the **Navigator**.



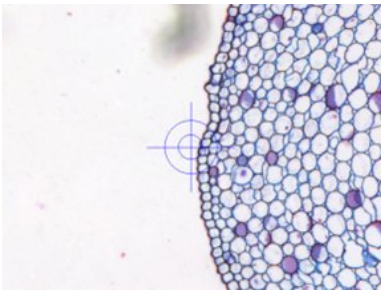
- The size is reset to one tile. The starting point is the current stage position. You can choose between the rectangle or circle mode or the overview image.

As a reference point you can choose between the tile in the top left-hand corner or the center of the entire MosaiX image.

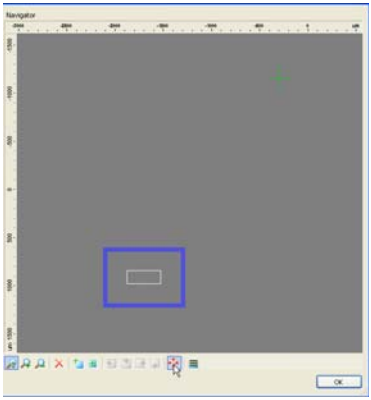
- Now, starting from the current point, move to the furthest points of the MosaiX area using the control elements for the **Stage** in the **Microscope and camera** group.



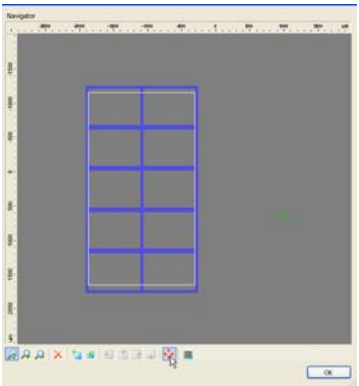
- First, move to the left edge of the area you wish to acquire, for example, or double-click on the corresponding position in the **Navigation** window.



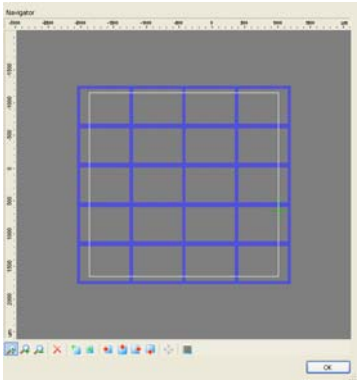
- Click on the button to enlarge the MosaiX image to the current position.



➢ The MosaiX area is now enlarged to the current position.



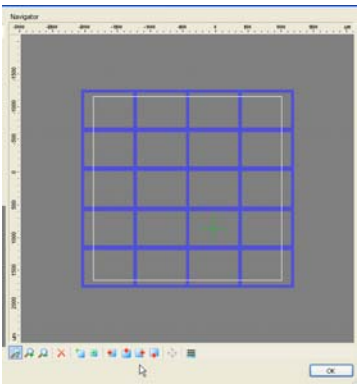
➢ Repeat these steps for the right, top and bottom edges of the area you wish to acquire.



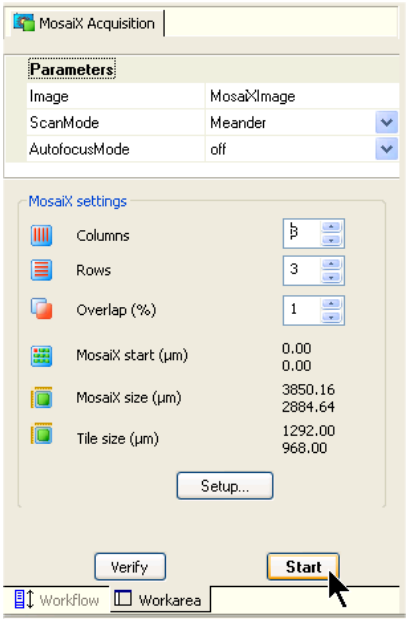
Note:

- To check that the MosaiX area has been correctly defined, you can move to individual tiles directly by double-clicking on a tile in the navigator.

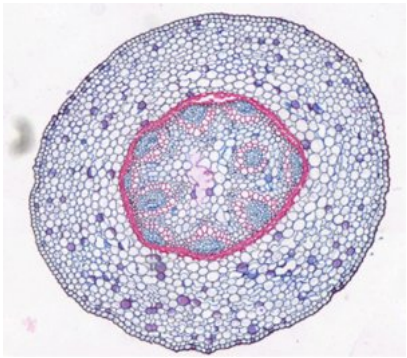
➢ If you wish to reduce the area for the MosaiX image, move to the new edge positions (top, bottom, right, left) and click on the appropriate button for clipping the MosaiX image.



- Click on **OK** to adopt the settings.
- The settings are now adopted into the **MosaiX Acquisition** property page.
- Start acquisition by clicking on **Start**.

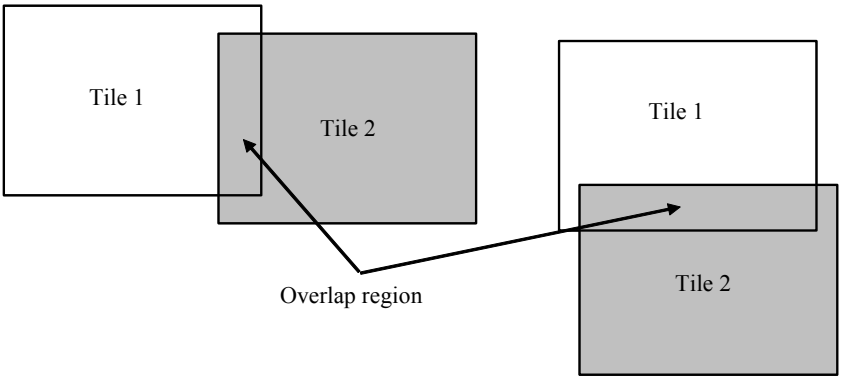


- AxioVision now covers the defined area and displays the result step by step.

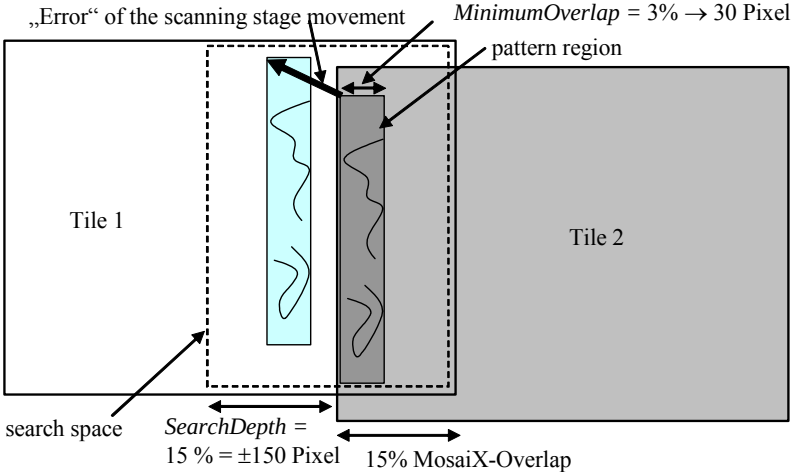


Stitching parameters

Scanning stages deviate from their exact positioning as they move. To ensure that it is nevertheless possible to assemble an overall image correctly when you are acquiring combined images (MosaiX), the single images (tiles) are acquired with overlapping regions and are subsequently fitted together using the so-called stitching method. It is essential that sufficient structures are displayed within these overlapping regions. The stitching algorithm attempts to recognize the precisely matching regions of two tile images that are located side by side and tries to position them accordingly.



The idea of the stitching algorithm is based on pattern recognition. A structure is detected in the overlapping region of a tile image. An attempt is then made to find this structure again in the overlapping region of the neighboring tile.



The tile images in the diagram overlap by 15%. The **Overlap** parameter is set in the **MosaiX Acquisition** function.

Note:

- Tiles that do not overlap cannot be fitted together!

Three parameters are important for the stitching algorithm:
MinimumOverlap, **Search Depth** and **SobelSize**.

- The **MinimumOverlap** parameter indicates the size of the pattern region. The size is given as a percentage of the tile size. If, for example, a tile image contains 1000x800 pixels and the **MinimumOverlap** has been set to 3%, this results in a pattern region of 30 pixels.

  In the diagram, this region is shaded dark gray. An attempt is now made to find the structures contained here in the overlap region of the neighboring tile (light gray rectangle).

  The stitching algorithm can only be applied successfully if visible structures are present in the search space. If only a few structures are present, you can increase the **MinimumOverlap** parameter. This means that the

pattern region that you wish to look for is expanded and the possibility of finding a clear structure is increased.

The **MinimumOverlap** region selected should not be larger than the MosaiX-Overlap, however. This means that, when acquiring objects that contain few structures, you should select a 20-30% overlap for the **MosaiX Acquisition** function so that you can subsequently set the **MinimumOverlap** to a high value, such as 10%.

The lower the value set for the **MinimumOverlap** parameter, the quicker the stitching process takes place.

- The **SearchDepth** parameter indicates the percentage of the tile size in which the best match for the defined structure should be searched for in the 1st tile image. If you use an accurate scanning stage, the **SearchDepth** can be kept low (e.g. 5%), as the pattern region can be found close to the original position.

  For stages that are less accurate, you will need to work with a larger **SearchDepth** in order to have a chance of finding the structure. However, you should bear in mind that if too large a **SearchDepth** is selected, an incorrect stitching position may be found – particularly if the specimen displays similar or recurring structure patterns.

  Remember that a lower value for the **SearchDepth** parameter speeds up the stitching process.

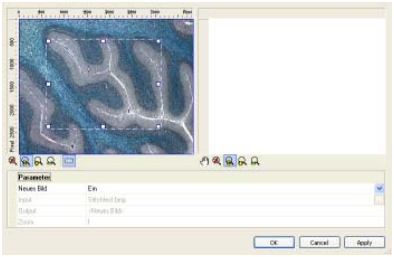
- The **SobelSize** parameter indicates the way in which the stitching algorithm deals with the structures in the defined pattern region for an optimum search result. In most cases, you can retain the preselected value **Low**.

  However, if your tile images contain blurred structures which cause stitching problems, you can set this value to **Off**. In this case, it is essential that you perform shading correction during the acquisition of the tile images. Should the problems continue with the **Low** or **Off** values, you can use the **High** parameter value. This selection then takes larger image structures into account for pattern recognition.

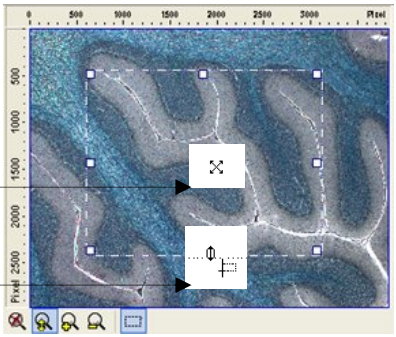
Converting a tile image into a regular AxioVision image

As tile images are a special format, they cannot be further processed or measured using the AxioVision functions. You have to convert them into a regular AxioVision image first.

- Acquire a tile image, or load one from the hard drive.
- Select the **Convert Tile Images** function from the **Processing** menu.
- The current tile image is displayed, with a frame, on the left-hand side.



- You are now able to position the frame freely and change its size.
- If the mouse cursor is inside the frame, keep the left mouse button pressed and you can move the whole frame.
- If you move the mouse cursor onto one of the frame handles, keep the left mouse button pressed and resize the frame.

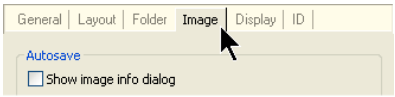


- Once you have selected the desired region, click on **OK**. A regular AxioVision image is then generated. This image can now be processed and measured in the usual way.

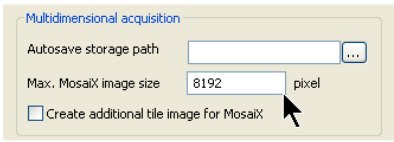
Note:

- The size of the resulting image is reduced to 8192 pixels by default. If the frame size set exceeds this value, the resulting image is automatically reduced accordingly. The region you have selected is always used, however. If you want to avoid reduction, you can freely configure the size of the AxioVision image.

➢ Select the **Options** function from the **Tool** menu. Activate the **Image** tab sheet.



➢ In the **Multidimensional acquisition** field you can adjust the **Max. MosaiX image size** parameter accordingly. The size of the value that you can select for this setting is essentially dependent on your computer's user memory.

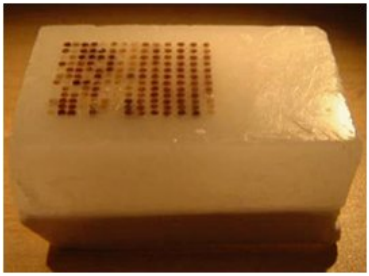


If the setting you select is too large, you may not be able to process the resulting images optimal. The processing speed will in any case be reduced dramatically, constricting optimal working.

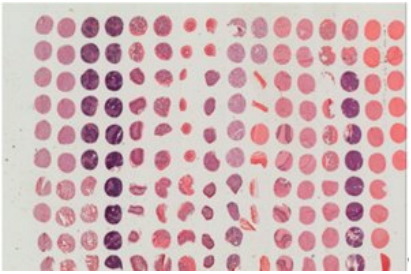
9.8 TMA

Background

Tissue microarrays consist of hundreds of small tissue samples of cores fixed on a single:



Biopsies embedded in paraffin



Composite image of a tissue microarray

This technique makes it possible to analyze a wide variety of tissue samples in parallel, under the same conditions and without variability in the staining. In connection with automated image acquisition and analysis, tissue microarrays are fed into an efficient high-throughput procedure.

The functions of the TMA module are integrated into AxioVision, expanding the software with the option of acquiring tissue microarrays. On the other hand, all the modules available in AxioVision can also be combined with TMA, e.g. with the quantitative analysis methods.

Application

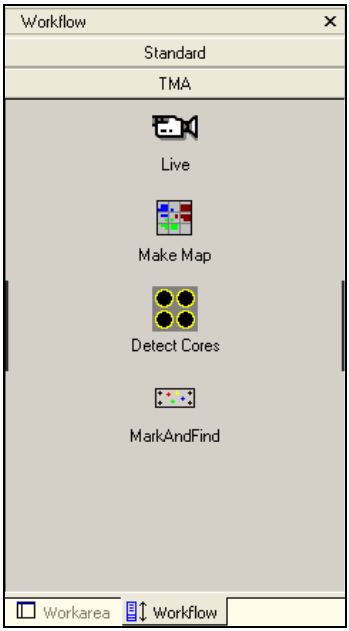
The TMA module enables the acquisition of an overview "map" of the tissue microarray. Tissue cores are automatically detected and identified with a grid position and a coordinate list is created. Using the detected core positions, TMA acquires high resolution images of immuno-histochemically stained or fluorescence labeled core samples (e.g. FISH) and can acquire images with multiple separately labeled fluorochromes.

The AxioVision measurement functions provide all the tools for counting cells and quantifying staining intensity, percent positive marked elements and other parameters.

Functions and integration into AxioVision

TMA adds two functions to AxioVision – one for generating a composite image and one for deriving a position list from the composite image. The functions are available via the **Applications** ⇒ **TMA** menu. Consequently, they can be used in your own toolbars and workflows etc. via the **Extras** ⇒ **Customize** function.

The TMA workflow can also be used for routine work.



Detailed descriptions relating to the individual functions can be found in the online help. Simply start a function and then press the F1 key.

Make Map

Captures a low magnification overview map of the array using a mosaic technique. This is a quick and easy to use function with re-usable templates for different tissue microarray layouts. **Make Map** can acquire an overview in brightfield or fluorescence illumination.

Detect Cores

Automatically detects the cores from the overview map image and assigns grid coordinates. A highly sensitive algorithm is used that can cope with distorted array patterns or missing cores. The grid coordinates of each core are used to create a Mark&Find list. Coordinates can be referenced to marker cores on the sample, allowing cores to be relocated after the slide has been replaced on the microscope. Additionally, the reference point is required for using the TMA position list correctly with other modules of AxioVision.

Prerequisites

In order to work with TMA, the Mark&Find and MosaiX modules must also be installed on your system in addition to TMA. Depending on what you wish to use the TMA module for, other modules may also be useful, e.g. Multichannel Fluorescence, Z-stack, Autofocus, AutoMeasure. Your microscope must be equipped with a scanning stage, to facilitate automatic location of the sample.

9.9 Fast Acquisition

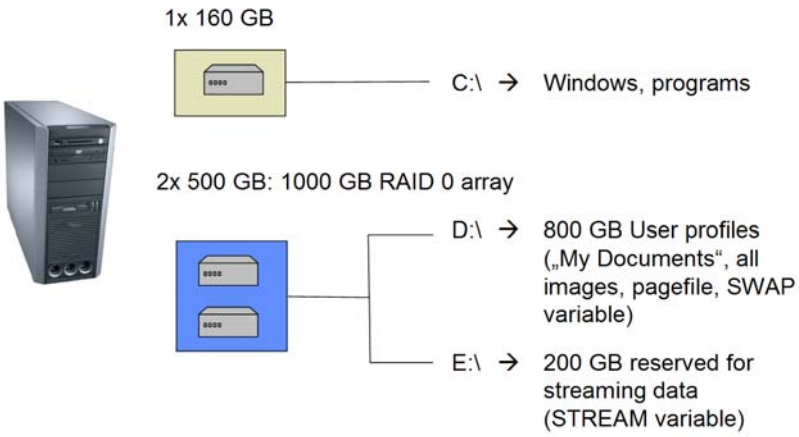
Acquisition principles

The Fast Acquisition module enables you to acquire multidimensional images (**Time Lapse**, **Z-Stack**, **Multichannel Fluorescence**) at a speed that is only restricted by the technical limitations of the devices you are using (camera, light source, focusing unit) and the selected camera settings. To achieve this speed, the Fast Acquisition module automatically uses a "streaming" technique, where the raw camera data is written to a fast hard disk (or a suitable empty partition), which is only used for streaming. This can be defined in **Tools** ⇒ **Options** ⇒ **Storage** ⇒ **Folder for Camera Streaming File**.

The result of acquisition is a streaming document (*.ziAR).

After the data is collected,, the streaming document is converted into a regular ZVI image document. This is required for all further processing steps in AxioVision.

The principle is illustrated in the following schematic (the actual harddisk configuration in your computer may be different):



This method of acquisition greatly depends on the PC equipment and configuration. Please adhere to the technical requirements for fast acquisition systems, which can be found in the "AxioVision Product Suite" installation manual.

The Fast Acquisition menu entry contains three functions for calling up the **Acquisition**, **Cutter** and **Information** dialogs.

Note:

- You can also call up the **Fast Acquisition** entry from the **Workarea**.

Experiment property page

Activating this function you will find the property page **Experiment** as well as property pages for the modules **Multichannel Fluorescence**, **Z-Stack** and **Time Lapse**. In many ways the operation of these functions is comparable to the respective modules of the **Multidimensional Acquisition**.

On the **Experiment** property page you will find an overview of the available functions and also of a range of important control elements. From this property page you can load, save and execute a previously prepared experiment. Here you can also extract the relevant experiment data from a stored image and start a new experiment under identical conditions.

Each dimension function has its own property page.

- **C** = Channels
- **Z** = Z-Stack
- **T** = Time Lapse

From the **Experiment** property page you can specify the dimension that you want included in your experiment. AxioVision requires information such as the definition of channels, z-planes and time series. This collection of information is referred to in AxioVision as an "experiment".

You can save the experiment and execute it by clicking on the 

button. The result of this process is always a new, unsaved image.

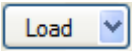
Note:

- Experiments defined for the Fast Acquisition are different from experiments of the **Multidimensional Acquisition** and are therefore not interchangeable. This is reflected by the document extension ZVXF.

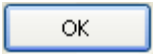
Options

Each dimension function has a check box that allows you to specify whether you want to use the function in question. The status line to the right provides the most important information on the settings you have made on the corresponding property page.

Experiment

In the first line you will see the name of the current experiment. If a default experiment has been defined, this line is empty. You can load a previously saved experiment using the 

button. Another dialog opens in which previously stored experiments present on the system are listed:

Now select an experiment and click either on 

to load the experiment or on 

to close the dialog again.

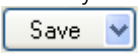
Note:

- Please note that experiments can be recognized automatically by AxioVision in three different places on the system:

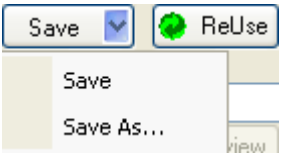
    - In a central location in the program directory (accessible to all users) (Windows XP/VISTA: *C:\Program Files\Carl Zeiss Vision\AxioVision\Data\AcquisitionRecording\FastAcquisition*).

    - In a central location for a work group (accessible to all members of that group) (Windows XP: *C:\Documents and Settings\All Users\Shared Documents\Carl Zeiss\Data\AcquisitionRecording\FastAcquisition; Windows VISTA: D:\User_Data\Public\Documents\Carl Zeiss\Data\AquisitionRecording\FastAquisition*).

- In your personal work folder (for the user "Administrator" (Windows XP: e.g. *C:\Documents and Settings\Administrator\My Documents\Carl Zeiss\Data\AcquisitionRecording\FastAcquisition*) (accessible to you only); Windows VISTA: D:\User_Data\Administrator\Documents\Carl Zeiss\Data\AquisitionRecording\FastAquisition.

A newly created or modified experiment can be saved by clicking on 

. Experiments are always saved with the extension "ZVXF" in your personal document folder (e.g. for the user "Administrator": *C:\Documents and Settings\Administrator\My Documents\Carl Zeiss\Data\AcquisitionRecording\ FastAcquisition*).

You can also use 

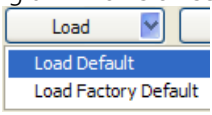
to save an experiment in a different location. This means, for example, that you can make a ZVXF experiment file available to several local Microsoft Windows users by saving it in the work-group folder (*C:\Documents and Settings\All Users\Documents\Carl Zeiss\Data\ AcquisitionRecording\FastAcquisition*).

Working with default experiments

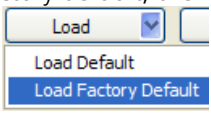
Instead of loading stored experiments it might be useful to store a standard experiment as the default experiment. This is useful when several users are working with the systems. For example, a standard experiment could contain all of the available possible channels on the imaging system for example. Starting with this default set up all channels not needed for the experiment can then simply be deactivated (right mouse click on the channel tab) temporarily.

The experiment which should become the default first has to be configured as desired. Then, to make an experiment the default, the **Save As Default** option has to be selected from the **Save** dialog (accessed by clicking on ). From now on, every time, AxioVision is started, the default experiment will load automatically. Changes made to this experiment during an AxioVision

session are automatically discarded when the program is closed. This ensures that the standard experiment is always available to other users and not accidentally modified.

Changes made to the default experiment during an AxioVision session can be discarded by choosing Load **Default** from the 

button.

In order to reset the experiment to the factory default, the **Load Factory Default** option can be selected from the 

button.

A newly acquired image is given the name **Fast-Acquisition-Stream-**, followed by a serial number. If you want to change the name of the image, you can do this in the **Prefix** input field.

Camera

From the drop-down list field you can select, which camera should be used for fast acquisition. This is important for systems which are set up with two identical cameras in a **Dual Camera** configuration. You can choose either the **ActiveAcquisition** camera, which is the camera currently active in the system or you can specifically specify one camera of your choice (e.g. AxioCamHS_2) which will then be automatically selected for acquisition. Finally, you can choose **Dual Camera**. In this case, two identical cameras can be used simultaneously. Please see the help for **Dual Camera** elsewhere for further details.

Hardware-Settings for Experiment

Here you can enter hardware component settings that you want to be executed at the start of the experiment before image acquisition starts, e.g. switching of light path to camera or desired camera resolution.

- **Before experiment**: Here you can select a suitable hardware setting from the drop-down list box. If you click on the Go button, you can apply the selected hardware setting to the microscope to test the settings.

- **After experiment**: You can use an additional hardware setting for the end of the experiment. If you click on the Go button, you can apply the selected hardware setting to the microscope to test the settings.

C (Channels) property page

Here you can define up to 5 channels to be used for acquisition. Only one channel is displayed per page. To switch between channels, click on the corresponding button 

. The tabs show the channel number and are also displayed in the selected channel color to help you to find the channel you are looking for.

You can temporarily disable a channel by right-mouse clicking on the channel tab. The channel will become inactive 

and will not be used during the next acquisition.

You can also change the order in which the channels are acquired by dragging a channel tab to another place 

. The channel number is reset after release of the mouse button 

.

Settings

- **Dye**: Here you can make a selection from a list of frequently used fluorescent dyes. If you make a selection from the drop-down list box, a suggested color is also inserted into the **Color** field. Values for excitation and/or emission maximums are also assigned to each fluorochrome. This information is important if you are using the **3D Deconvolution** module, for example.

- In the  dialog you can also enter a new fluorochrome into the **Dye** text field. The appropriate wavelength information can be entered in the corresponding text fields (please also see **Extended parameters**).

- **Color**: Here you can select a suitable channel color (often also known as pseudo color, in particular with black/white cameras in fluorescence microscopy).

  Click in the drop-down list box to select from a range of default colors.

  A new color can be selected via the Microsoft Windows color selection dialog by clicking on  . Select a color field from the left-hand area or pick out a color from the color gradients on the right-hand side. Colors you define yourself appear as **Custom** colors in the bottom part of the list.

- **Name**: Here you can assign a name of your choice to the channel. Default channel name is the dye name.

Extended parameters

In addition to the setting options offered on the **C** (Channels) property page, you can also make more advanced channel settings in an expert mode. The options available here are restricted to parameters that cannot reduce the speed of fast image acquisition. For this reason you will not find all the options here that are available in **Multidimensional Acquisition**.

The  button opens the new dialog **Extended Channel Parameters** in the form of a floating window.

This dialog displays all the channels of the current experiment next to each other in tabular form. Some functions are brought together as subgroups. Click on ⊞ to open the group and on ⊟ to close it again.

The channel table also contains all the control elements you need to be able to make the correct settings. The parameters are listed and explained individually below:

Enable

Here you can deactivate the use of a channel temporarily without having to remove the entire channel entry from an experiment.

Name

Give the channel a name of your choice (e.g. "DAPI-Automatic").

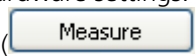
Channel Color

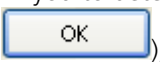
This allows you to select a suitable channel color (often also known as pseudo color, in particular with black/white cameras in fluorescence microscopy).

Click in the drop-down list box to select from a range of default colors.

A new color can be selected via the Microsoft Windows color selection dialog by clicking on  . Select a color rectangle from the left-hand area or pick out a color from the color gradients on the right-hand side. Colors you define yourself appear as "Custom" colors in the bottom part of the list.

Exposure Time [ms]

Here you can enter the exposure time being used for the channel into the value field, either directly or using the  buttons. To determine the exposure time interactively, click on  . The dialog that opens contains a live image, generated using the channel-specific hardware settings. In this live image the main control elements are available (

button) to allow you to determine the correct exposure time. After confirmation (clicking on 

), the dialog is closed and the new exposure time is adopted automatically. Please note that fast image acquisition only allows images to be acquired in rapid succession if suitably short exposure times are used.

Exposure Mode

In the drop-down list box, three modes are available:

1. Camera settings: The value that has just been entered on the camera's **Adjust** property page (called up e.g. via **Acquisition** menu ⇒ **Select Camera**) is used. Any value present in the channel table is ignored.

2. Channel settings: The exposure time entered in the channel table is used. The value on the camera's **Adjust** property page (called up e.g. via **Acquisition** menu ⇒ **Select Camera**) is ignored.

3. Optimum Speed Value: The exposure time is chosen according to the readout time of the camera, which is determined by the used camera parameters (Binning, ROI). With this setting it is possible to maximize the acquisition speed in the case of 2D time lapse imaging. This setting does not make sense in all other cases involving other hardware such as wavelength switching devices or fast piezo focussing devices.

Dye

This information is essential if you have installed the ApoTome 2 or 3D Deconvolution modules. Here you can make a selection from a list of frequently used fluorescent dyes in the drop-down list box. This suggested color is also entered into the **Color** field. The correct values for excitation and/or emission maximums have also already been assigned to each fluorochrome. This information is important if you are using the **3D Deconvolution** module, for example.

You can, however, also enter a fluorochrome into the text field yourself. The corresponding wavelength information can be entered into the following two fields.

Excitation wavelength [nm]

This information is crucial if images are to be used in the **3D Deconvolution** module. If you have entered your own fluorochrome under **Dye**, here you can specify the excitation maximum for that fluorochrome. Please bear in mind

that the value that is relevant here may be dependent on the excitation filter you are using.

Emission wavelength [nm]

This information is crucial if images are to be used in the **3D Deconvolution** module. If you have entered your own fluorochrome under **Dye**, here you can specify the emission maximum for that fluorochrome. Please bear in mind that the value that is relevant here is dependent on the emission filter you are using. If you are using a bandpass emission filter, for example, its maximum should be entered here. If you are using a longpass emission filter, you should enter the actual emission maximum of the fluorochrome.

Display settings

Here you can influence the way in which your images are displayed on the screen. These settings do not influence the data in your images. The settings guarantee that images are always correctly displayed on the screen, irrespective of the dynamic range you are using on the camera. Any settings you make here have exactly the same function as the editing of the display characteristic curve (**Properties** function on the **View** menu). In the drop-down list box, six display modes are available:

1. System default (from Live image): The values currently set for the display curve in the live image properties window are copied to the acquired image. The display curve is normally set to linear by default.

2. Gamma and automatic black/white value: The values entered in the table for **Gamma**, **Auto black value** and **Auto white value** are all used.

3. Gamma only: Only the value entered in the table for gamma is used. Display settings for black and white values are carried over from the **Live image property / Display** window and not from the table values here.

4. Automatic black/white value only: Only the values entered in the table for **Auto black value** and **Auto white value** are used. The **Gamma** value is carried over from the **Live image property / Display** window and not from the table value here.

5. Black/white value autom. to min/max: The black value for the display curve is automatically set to the darkest pixel (=min), the white value to the brightest pixel (=max) in the image. This corresponds to the **Min/Max** function in the image **Properties/Display** window. The gamma value is carried over from the **Live image property / Display** window and not from the table value here.

6. Fixed black/white value and gamma: Sets the display curve to both Gamma and a fixed black and white value based on the available dynamic range of the camera. Values are taken from the table entries for **Gamma**, **Fixed black value** and **Fixed white value**.

Gamma

In this edit field you can enter a Gamma value which is used to emphasize certain intensity ranges within your image when it is displayed on the monitor. A value <1 emphasizes the medium pixel intensities (also called "mid tones"). A value >1 emphasizes pixel intensities of very dark and very bright pixels, and therefore increases the contrast. Recommended settings are 0.8 for fluorescence images, 1.2 for phase contrast or DIC and 0.45 for true color images. Please bear in mind that a "correct" setting of the gamma value is dependent on numerous parameters, such as the monitor setting, ambient brightness etc., and a universal value cannot be prescribed. This value is only used in the modes **Gamma and automatic black/white value**, **Gamma only** and **Fixed black/white value**.

Auto black value [% dark pixels]

Indicate here the percentage of dark pixels in the image that you want to be displayed as 100% black. This reduces the unspecific background of the display. For fluorescence images a value of 5% is a good starting point. This value is used in the modes **Gamma and automatic black/white value** and **Automatic black/white value only**.

Auto white value [% bright pixels]

Indicate here the percentage of bright pixels in the image that you want to be displayed as 100% white. A value of 0.1% for fluorescence images, for example, emphasizes structures that are represented by a small number of very bright pixels and is therefore a good starting point. This value is used in the modes **Gamma and automatic black/white value** and **Automatic black/white value only**.

Fixed black value [% of camera range]

Here you can define a fixed black value below which all pixels will be displayed as 100% black. This value is defined as percentage of the dynamic range of the camera used rather than the image content. This guarantees equal and comparable display settings for all images acquired with this experiment. This value is used only in the mode **Fixed black/white value and gamma**. The following table shows some percentage values and the corresponding gray value of a camera with a given gray value range:

Camera bit depth	8	10	12	14	16
Maximum gray value	*256*	*1024*	*4096*	*16384*	*65536*
Fixed black value	Resulting black value [in gray values]				
1 %	3	10	41	164	655
5 %	13	51	205	819	3277
10 %	26	102	410	1638	6554
20 %	51	205	819	3277	13107

Fixed white value [% of camera range]

Here you can define a fixed white value below which all pixels will be displayed as 100% white. This value is based upon the dynamic range of the camera used rather than the image content. This value is defined as percentage of the dynamic range of the camera used rather than the image content. This value is used only in the mode **Fixed black/white value and gamma**. The following table shows some percentage values and the corresponding gray value of a camera with a given gray value range:

Camera bit depth	8	10	12	14	16
Maximum gray value	*256*	*1024*	*4096*	*16384*	*65536*
Fixed white value	Resulting white value [in gray values]				
80 %	205	819	3277	13107	52429
90 %	230	922	3686	14746	58982
95 %	243	973	3891	15565	62259
99 %	253	1014	4055	16220	64881

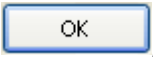
Channel display weight [%]

In the case of a multichannel image you can enter a bias for the contribution of individual channels in the mixed-color display (pseudo color). This function can also be found on the **Colors** property page (**Properties** function on the **View** menu).

Exposure

Here you can set the main acquisition parameters.

- **Time**: Here enter the exposure time being used for the channel into the value field, either directly or using the  buttons.
- **Measure**: To determine the exposure time interactively, click on the 

button. The dialog that opens contains a live image, generated using the channel-specific hardware settings. The exposure time is automatically measured when the dialog is opened. After you click on 

, the dialog is closed and the new exposure time is adopted automatically.

Mode

- **Fixed**: The time (in ms) entered in the **Time** field is used. The value on the camera's **Adjust** property page is ignored.
- **Camera**: The time (in ms) entered on the camera's **Adjust** property page is used.

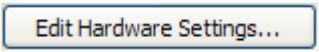
Settings during Acquisition

This field shows icons of those hardware components, which are switched before the selected channel is acquired.

Settings after Acquisition

This field shows icons of those hardware components, which are switched after the selected channel has been acquired.

Edit Hardware Settings ...

Clicking on 

opens the **Fast Acquisition Setting Editor**.

Fast Acquisition Setting Editor

In Fast Acquisition, fast hardware devices as well as a few selected slow components can be controlled during an experiment. Fast hardware devices include fast switchable light sources such as Colibri, Sutter DG4 or Uniblitz shutters. Relevant slow components include the motorized reflector turret or internal shutters in a microscope.

This dialog contains all the channels configured for Fast Acquisition and allows a quick configuration of all the hardware relevant for setting up the channels. The channel name of the currently selected channel is also shown in the title of the **Settings** window. The dialog is subdivided in two horizontal areas. The upper area refers to settings which are activated before the acquisition while the lower area refers to settings activated after acquisition of the selected channel.

Both areas show a list of available components, dependent on the available hardware components set up in the **Microscope Configuration**. As a rule, slow components are controlled through serial commands (via RS232 or USB) while fast devices are controlled directly through electrical signals ("TTL") which are connected to the system using the Analog/Digital Input/Output card installed in the computer.

The listed items show an **Enable** checkbox which enables or disables a hardware component for Fast Acquisition. By selecting an entry in the list, the corresponding control elements are displayed on the right side where the appropriate settings can be made. An entry can be selected (meaning the components line in the list is selected by mouse click) but still be disabled (**Enable** checkbox is off). In this case the control elements are shown in a grayed out manner.

The **Status** shows, if a component belongs to the class of fast or slow devices. After the **Component** name a fourth column **Value** summarizes the status the device will assume during acquisition of the selected channel. This text is also shown on the Fast Acqusition **C (Channel)** page.

Both areas can be further configured for the experiment using a set of control elements situated at the bottom of each list. The order in which the devices are activated during an experiment can be changed by moving an entry upward to the top of the list. This can be done using the up or down arrow buttons  . Slow components should be activated first so that faster components are only switched after the slow components have reached their final position. This prevents unnecessary damage to the sample. By clicking on the  icon all settings are applied to the hardware items. This allows you to test, if the settings would work during acquisition. Clicking on the  button on the other hand puts a selected hardware item in a mode so that changes are immediately applied to the hardware. This helps especially when adjusting laser power for the laser module.

The complete list can be reset to its default by clicking on  .

Settings for Dual Camera

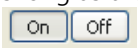
If two identical cameras are available and properly configured in the system, the acquisition speed in **Fast Acquisition** can be considerably increased by using the optional **Dual Camera** module. If the **Dual Camera** option is selected on the **Experiment** page (**Camera**) the system uses both cameras at the same time.

- Please observe the information about dual camera installation in the **Dual Camera** chapter of this document.

The synchronous mode of operation for both cameras further depends on how the Fast Hardware Settings in the **Fast Acquisition Setting Editor** are set up. *Channel #1 may have a hardware settings configured before but not after acquisition while for channel #2 only settings after acquisition may be configured.* Checking the **Synchronize Dual Camera** checkbox will gray out the appropriate before/after acquisition section in the channel Settings fields. This guarantees synchronous operation of both cameras. The checkbox is enabled by default as soon as **Dual Camera** is selected on the **Experiment** page (→ **Camera**).

If the checkbox is unchecked and settings are configured contrary to these instructions, acquisition will commence anyway but both cameras *will be operated **sequentially***, i.e, without synchronicity. This can still make sense for certain applications, if the primary goal for **Dual Camera** is the fact that both cameras can be optically configured to see different spectral signals but there will be a time delay between the two channels.

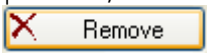
Adjusting the laser intensity and assignment of wavelengths

In contrast to all other hardware items available for Fast Acquisition adjustments to the AOTF module can only be done on the first channel page. Transmission settings for the three possible AOTF channels have to be done on the set up page for the first channel but are used correctly in the following channels as long as the proper AOTF channel has been activated  and turned on 

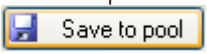
.

Channel Actions

- Using the 

button you can add a channel from the **Channel pool** to the experiment, and then remove it from the experiment again using 

. 

is used to add a copy of the currently selected channel to the experiment. If you want to permanently store a channel setting you have made so that it can be used again in other experiments, you can transfer channels to the **Channel pool** (

button).

Z property page (Z-stack)

Settings

Note:

- Please note that a fast piezo focusing unit must be available and correctly connected to allow the fast acquisition of z-stacks. This will be used automatically during acquisition. The option of direct control of the piezo focusing unit by the user is not available, in order to prevent operating errors. Please use the microscope's internal focus drive to focus on the sample.

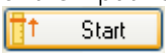
- The desired number of slices can either be entered directly into the **# Slices** value field or via the arrow buttons  . The distance between the individual z-planes (in µm) can also be specified in this way in the **Slice distance** input field.

- If you click on the 

button, the correct z-interval is calculated automatically in accordance with the Nyquist theory (2x oversampling in axial direction). This is a prerequisite for successful use of the Deconvolution module.

- Under **Mode** you can select whether you want to specify a z-stack from a central z-position (**Center**) or interactively specify an upper/lower z-position limit (**Start/Stop**). The input field for **Center** and the

corresponding button 

or the input fields for **Start** and **Stop** and the corresponding buttons 

and 

will become active in accordance with your selection. If you click on the buttons, the current z-position is adopted into the value field.

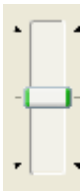
- The total thickness of the stack is indicated in the **Z-Stack height** value field. If a z-stack height is achieved that exceeds the maximum travel range of the fast piezo focusing unit, a warning message is displayed ( Piezo out of range).

Within the context of Fast Acquisition, the **All channels per slice** mode is used automatically, as channel switching can generally be performed faster than focusing. In contrast to **Multidimensional Acquisition**, it is not therefore possible to deactivate this mode here.

Z-Stack navigation

This field allows you to use the focus drive with the z-stack acquisition parameters. Using the  and  buttons, you can move the focus drive in defined increments. If you click on 

, the z-drive moves to the center of the stack.  and  move it to the lower/upper limit.

The slider  allows you to move directly to a certain position. The height of the z-stack is represented by a blue bar. If you go beyond the lower/upper limit using the  or  button, this bar gets smaller and smaller.

T (Time) property page

The time lapse dimension function allows you to acquire time lapse images and create films of moving processes in living cells or tissue and even of certain material applications. A time lapse image can be defined by entering the maximum speed, interval, maximum duration, number of images required or total duration of the experiment. The entries in the fields influence each other. AxioVision can calculate an approximate duration as a function of the time and the number of cycles, or the number of cycles as a function of the total duration and intervals.

Interval Settings

You can choose between **Maximal Speed** and a defined **Interval**. The desired time interval can either be specified directly in the **Interval** input field or via the arrow buttons  . The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds).

Duration Settings

You can choose between **Maximal Duration**, a defined number of cycles or a defined duration. The desired number of cycles can either be entered directly in the # **Cycles** input field or via the arrow buttons  .
The total duration can either be entered directly in the **Duration** input field or via the arrow buttons  . The desired unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds).
If you have changed either of the input values by entering a value directly into the field, click on the 

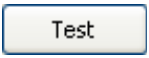
button to automatically calculate the other value, i.e. the possible number of cycles (if you have defined the duration) or the time required (if you have defined the number of cycles). The input field for which the value is to be calculated is inactive. Please note that the values calculated in this way are only approximations. If you want the possible minimum interval times to be calculated precisely, please use the 

button.

Current Size

Once the time settings have been made for your experiment, the memory required for the raw data and the resulting image series appears in this field. Please note that streaming data files should not be stored on the same physical hard disk as the ZVI image data generated from them during conversion. Only in this way can maximum speed be achieved during acquisition and conversion.

Limits

If you have selected **Maximal Speed** or **Maximal Duration**, you can click on the 

button to have the absolute time limit that is possible with the current microscope setting calculated for your experiment. A limited number of images are acquired for this purpose. The limits resulting from this are displayed as the minimum possible interval.

If any of the dimensions you have selected are not possible with the current settings due to insufficient hard-disk capacity, a warning message appears. In addition, details on the storage medium currently being used are displayed.

Starting an experiment

To acquire an experiment, open the **Fast Acquisition** dialog window by clicking on 

.

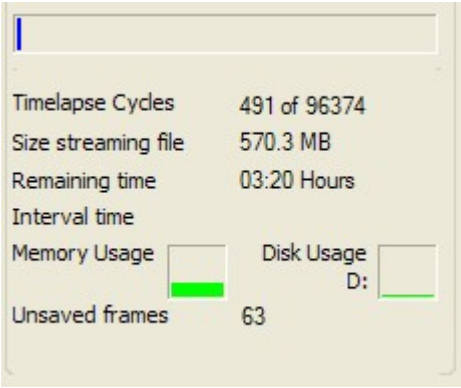
Fast Acquisition dialog

- **Start (F5)**: Click on this button to start acquisition of the image sequence. This procedure can be repeated several times without the need to exit the **Fast Acquisition** dialog. The acquired image sequences are stored as raw data on the defined streaming partition.

- **Stop (F8)**: Click on this button to stop acquisition before the end point defined in the parameters has been reached. Once you have stopped the process, a new image sequence can be initiated using the **Start (F5)** button, although this will then be saved in a separate file.

- **Pause (F6)**: Click on this button to pause the acquisition procedure.

- **Continue (F7)**: Click on this button to continue acquisition in the same image file.



- A progress indicator shows the elapsed time for the selected experiment.

- Indicators for the available main memory and disk space for the raw data provide important information on the status of the overall system. It is also possible to see for how long it will be possible to acquire image data.

- In case the harddisk performance for the Streaming partition being too low some images may not be saved during acquisition. These images are instead stored temporarily in RAM. The number of affected images is shown here.

- To give you an impression of the current acquisition process, the acquired images are shown in a **Preview**. Here you can select a channel () and influence its display settings (), e.g. to improve the display of dark images with a low dynamic range. Due to the special acquisition technique, here you do not have access to all the intervention options that would be available to you when using the multidimensional image acquisition, where a ZVI image is generated immediately.

Further processing of the image

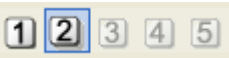
- The **Start Cutter** button closes the acquisition dialog and opens the **Fast Acquisition Document Cutter** dialog.

Fast Acquisition Document Cutter

- 

Describes the name of the input document.

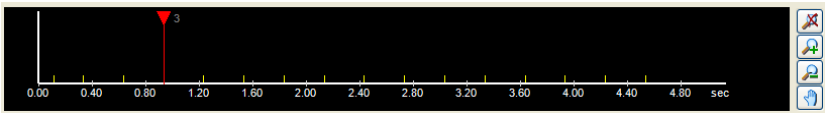
- The  button opens a gallery from which you can also load a document other than the one that was last active. To do this, click on the thumbnail of the desired streaming document.

-  : With these buttons you can select a channel to affect its display settings.

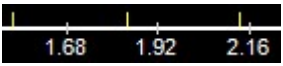
-  : The buttons on this toolbar can be used to adjust the display (brightness, contrast, zoom etc.) of the images.

- Using the  button on the toolbar, it is possible to define a region of interest in the image that you want to be taken into account during the conversion to ZVI. This also allows you to reduce the data volume to the relevant parts of the image.

- **Experiment Overview**: In the bottom area you will see an overview of the entire experiment along a timeline:

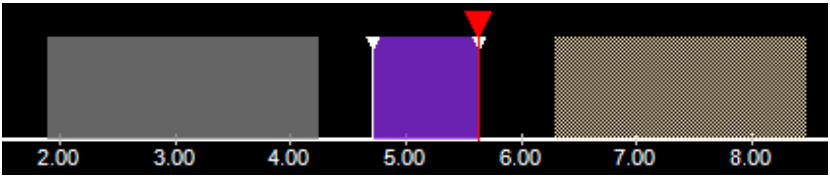


- Each acquisition event is signified by a yellow line allowing easier frame selection

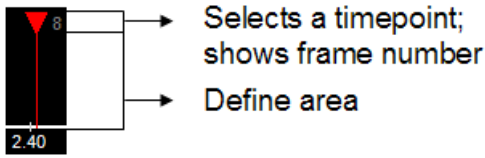


- It is possible to define the areas of the streaming document that you want to be converted to ZVI. When this dialog is called up for the first time, no time points are selected by default. Selection can be done by clicking with the left mouse button and dragging whilst keeping the mouse key depressed. Selection is symbolized by a magenta selection bar. The selection that is currently active is marked in magenta, whilst inactive selections are shown in gray

- Segments within the experiment where the acquisition had been paused are signified through a lighter color. Such paused areas are automatically excluded from conversion.:



- The time point currently displayed is indicated using the following marking line:



  If you grab the upside-down triangle with your mouse, you can select the time point of your choice. In the area at the lower half you can use the left-hand mouse button to define an area to be converted: hold down mouse button, drag, release → defined area is marked in violet. You can mark as many areas as you wish.

- It is possible for selected areas to be moved (left-hand mouse button).

-  Adjusts the zoom factor of the histogram in such a way that the entire experiment is displayed.

-  Allows you to display a part of the experiment in the histogram with a higher zoom factor (**Zoom in**).

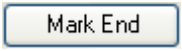
-  Reduces the zoom factor for the displayed histogram area (**Zoom out**).

-  Moves the displayed histogram area (only active with **Zoom in**).

- In the bottom area of the dialog you will find a table containing the selected areas with an indication of the exact start and end time points, the resulting duration and the start-image position and end-image position for the marked segments.

- It may be beneficial, especially if you need to select a time point precisely,, to position the time point marking line 

and set it as the start point for a new area selection. This is done using the 

button. As soon as a start point has been defined, the 

button for defining an end point becomes active.

- You can use the two arrow buttons 

to browse through the streaming file frame by frame. This allows a more precise frame selection.

- To select the entire time series for conversion, click on the 

button.

- To delete selected area markings, you can use the 

button (or alternatively the **Delete** key).

Conversion Information

- Here you will find a display of the file size of the newly generated ZVI image, the number of individual images it contains and the available memory on the target drive.

Executing editing



converts the currently shown frame into a ZVI image and leaves the dialog open



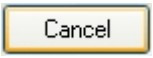
selects the complete streaming file, starts conversion immediately and closes the dialog. This button is selected as default which means that you can confirm this action immediately by hitting **Enter** on your keyboard.



Carries out the conversion to a ZVI image according to the individual selections, after which the dialog is closed automatically.



Carries out the conversion to a ZVI image, but the dialog remains open. This allows you to generate several ZVIs of different areas and regions conveniently one after another, without having to call up the dialog again each time.



Closes the dialog. No ZVI image is generated.

The result of the conversion is a new, unsaved ZVI image. The ZVI images generated in this way can now be subjected to further, more precise analysis in a second step.

Information for Fast Acquisition

This function opens a dialog window, where you can find in tabular form important information in connection with the experiment. The information it contains refer always on the current experiment and will be updated automatically upon changing the experiment.

Show

In the drop-down list you can select, whether only an overview or all available information are shown.

To get more explanations to the existing information, you can select an item. In the bottom area of the dialog you will find more explanation.

9.10 Physiology

Introduction

Many intra- and intercellular processes are accompanied by rapid changes in the local concentration of various ions, such as calcium (Ca^{++}) or magnesium (Mg^{++}). For example, the penetration of an egg cell by a sperm cells sets in motion an intracellular signal cascade that results, within a few seconds, in the egg-cell membrane becoming impermeable for other sperm. The signal transmission between cells, such as neurons, is another example. In these cases the transmission of the signal is also accompanied by the release of calcium ions. It is therefore possible to visualize these processes by measuring the Ca++ concentration via imaging.

The AxioVision **Physiology** module supports all the common methods of calcium measurements as well as many other ion measurements such as pH.

Method 1: excitation at one wavelength, detection at another wavelength.
What is measured here is the increase in the intensity of a calcium-sensitive dye as a function of the Ca++ concentration. Examples of such dyes are Calcium-Green-1, Fluo-3, Fluo-4 or the BAPTA-based Oregon-Green dyes. The fact that only rapid sequential acquisition in one channel is necessary for this method means that it is ideal for measuring relative calcium changes, even with extremely rapid processes. However, it is difficult to perform quantitative measurements using this method. One method by which this can be attempted is to calculate the ratio of F/F0 by which a baseline time point is selected as F0.

Method 2: excitation in two wavelengths, detection of the changes in fluorescence intensity in one wavelength, generation of a ratiometric output image. **("Excitation ratioing")**
This method is based on the fact that certain calcium-sensitive dyes change their excitation spectrum as soon as calcium is bound. The most popular dye from this class is Fura-2. This is particularly well suited to imaging techniques, as it is easy to change the excitation wavelength with the help of fast wavelength changers (e.g. Sutter Lambda DG4). In addition, the ratiometric measurement principle means that the quantification is concentration-independent. One disadvantage of Fura-2 is the need to perform excitation in

the UV range. Measurement of pH values can be performed using the dye BCECF, which changes its excitation maximum upon binding to protons (H+ ions).

This method can also be used to perform calibrated calcium or pH measurements to determine absolute calcium concentrations or pH values. There are two methods for calibration: One involves creating one calibration valid for the whole image, the other method involves measuring minimum and maximum responses on a cell per cell base and using these values to deduce calcium values for each measured ROI. Physiology supports the second calibration method, also known as "in situ" calibration...

Method 3: Excitation in one wavelength, detection of the changes in fluorescence intensity in two wavelengths ("Emission ratioing")
This third method uses dyes with which the calcium binding produces a change in the emission spectrum. This is also a ratiometric method. This requires the simultaneous detection of the signal in two wavelengths, which requires the use of the two camera module **Dual Camera**. With this module two cameras, which have been appropriately configured using a dual camera adapter to each see a different wavelength, can be operated simultaneously producing a two channel image in the same instant. A popular dye used for emission ratioing is Indo-1. Also Calcium sensitive FRET sensors such as the yellow cameleon fluorescent proteins follow a similar acquisition principle.

For an overview of this issue, we recommend that you refer to Takahashi, A., et al., Measurement of Intracellular Calcium. Physiological Reviews 70, No. 4, pp1089 ff, 1999. Another good review appeared in Methods in Cell Biology, Vol. 72, pp 369-387 ("Ratio Imaging: Measuring Intracellular Ca++ and pH in Living Cells" by Randi B. Silver, 1998). For further information on the reagents and techniques used, see also http://www.invitrogen.com/probes.

Acquisition principles

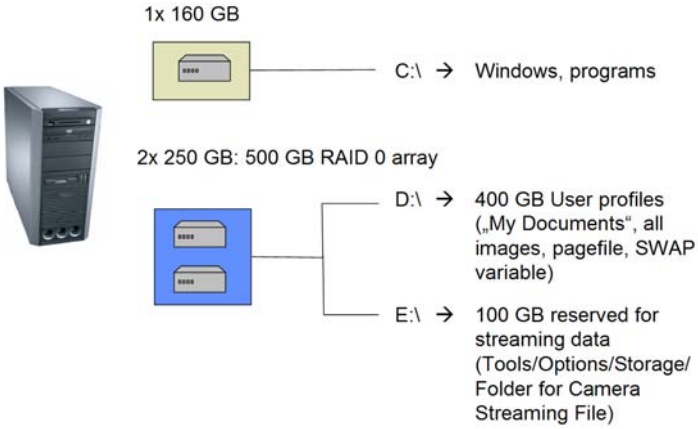
The Physiology module enables you to acquire time lapse images at a speed that is only limited by the technical limitations of the devices you are using (camera, light source) and the selected camera settings. To achieve such speeds, the Physiology module automatically uses a "streaming" technique, which involves the raw camera data being written directly to their own fast **streaming hard disk** (or a suitable empty partition).

The results of the acquisition process are therefore a streaming document (*.ziAR).

At the same time, enough memory is reserved so that the measurement data can also be managed, which are necessary for online display of the charts. **Physiology** calculates the maximum possible number of individual images that can be acquired from the number of measurement regions drawn in and the memory available on the streaming hard disk. As this value is dependent on numerous factors (binning, ROI, number of measurement regions drawn in, demands made on available free memory by other applications etc.), the entire hard-disk space that is theoretically available cannot always be used.

In a second step, which is separate from the acquisition process, the streaming document is converted into a regular ZVI image document. This is required for all further processing steps in AxioVision.

The principle is illustrated in the following schematic (the actual hard disk configuration in your computer may be different):



This acquisition technique depends to a great extent on ensuring that you have the correct PC equipment and the correct configuration. Please observe the minimum technical requirements, which can be found in the "AxioVision Product Suite" installation manual.

The procedure for calcium measurements with the Physiology module can be divided into four steps:

- Preparation of the experiment, setting of the camera and drawing in of measurement regions → **Setup**.
- Acquisition of the time lapse image, including measurement of the changes in concentration during the acquisition process itself, with options for interaction to control the experiment → **Image Acquisition**.
- Conversion of the acquired raw data into ZVI and downstream analysis → **Cutter**
- Measurement of the acquired data with options to select measurement methods, specify the type of background correction or even select custom calculation formulas → **Physiology Measurements**.

These steps are explained below following the individual control elements as they appear in the software.

Acquisition

If you select this function on the **Acquisition** menu ⇒ **Physiology Acquisition** ⇒ **Acquisition**, the **Physiology Acquisition** dialog opens. This dialog can be resized to accommodate the text presented in the **Parameters** field.

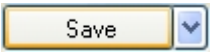
- **Parameters**: An overview of the most important acquisition parameters of the experiment currently active is displayed in list form: acquisition mode, number of ROIs (measurement regions), number of possible acquisition cycles, time interval currently set and maximum possible streaming file size. Please note that these values depend on the settings you entered in the **Setup** dialog.
- 

: Opens the **Physiology Setup** dialog.

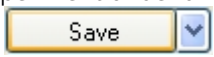
Template

All settings relating to an image acquisition procedure with the Physiology module are managed as an **Experiment**.

- **Name**: Name of the experiment. If the default experiment is loaded, the name field remains empty (*****).

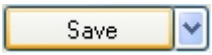
- A newly created or modified Physiology experiment can be saved by clicking on 

. Experiments are always saved with the extension "ziAR" in your personal document folder (e.g. for the user "Administrator": *C:\Documents and Settings\Administrator\My Documents\Carl Zeiss\Data\AcquisitionRecording\PhysiologyAcquisition*).

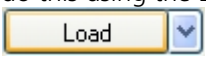
- You can also save an experiment under a new name using **Save As** (click on  at the side of the 

button).

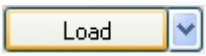
- It is also possible to define a Physiology experiment as a default experiment, instead of constantly reloading experiments.

- To do this, the Physiology experiment must first be configured in the desired way. To make this experiment a default experiment, you then need to select the **Save As Default** option (click on  at the side of the 

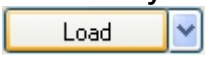
button). This Physiology experiment will now be loaded as a default experiment every time you start AxioVision. Any changes that are made to this experiment while AxioVision is running will be rejected automatically when the program is closed. This ensures that the next user will always find the familiar default experiment.

- If, after making changes, you want to restore the default experiment while AxioVision is running, you can do this using the **Load Default** option (click on  at the side of the 

button).

- To load a saved experiment, click on 

.

- To reset the **Physiology Experiment** to the factory default state, the **Load Factory Default** option is available (click on at the side of the 

button).

- **Prefix**: Here you can enter the basic name for all images acquired using the Physiology module.

Camera

From the drop-down list field you can select, which camera should be used for fast acquisition. This is important for systems which are set up with two identical cameras in a **Dual Camera** configuration. You can choose either the **ActiveAcquisition** camera, which is the camera currently active in the system or you can specifically select one camera of your choice (e.g. AxioCamHS_2) which will then be automatically selected for acquisition irrespective of which camera is the currently active camera. Finally, you can choose **DualCamera**. In this case, two identical cameras can be used simultaneously. Please see the help for **Dual Camera** elsewhere for further details.

Hardware-Settings for Experiment

In this field you can enter hardware settings that prepare the hardware for fast **Physiology Acquisition**, e.g. switching the light path to the camera or preparing the light sources for fast acquisition.

- **Before experiment ...**: Here you can select a suitable hardware setting from the drop-down list box. If you click on the Go button, you can apply the selected hardware setting to the microscope to test the settings.

- **After experiment ...**: You can use an additional hardware setting for the end of the experiment. If you click on the Go button, you can apply the selected hardware setting to the microscope to test the settings.

Physiology Setup

Here you can enter settings for the experiment on the **Regions**, **C (Channel)** and **Markers** property pages.

As soon as more than one channel has been configured, channel 1 is ratiometrically processed with channel 2 (**method 2 or 3**). If only one channel has been configured, the method **Ratio Single Channel** should be used and the analysis performed with the help of an F0 image. It is also possible to configure more than 2 channels (e.g. for a brightfield image). However, it is always only channels 1 and 2 that are analyzed. For method 3 (emission ratioing) Dual Camera has to be selected. Here camera #1 is always assigned to channel #1, camera #2 to channel #2.

The **Physiology Setup** dialog is opened by clicking on 

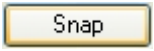
.

Regions property page

On this property page you can prepare the **Physiology Experiment** by defining the method for calculation, acquiring test images, drawing in regions, setting the camera exposure time and specifying how the ratio image should be calculated.

Setup Measurement Regions of Interest (ROI)

This page is divided into 6 areas, containing an image display and the corresponding chart display. First of all, you should acquire a test image. In order to do this, the channels must have been correctly set (on the **C (Channel)** property page of the **Physiology Setup** dialog). The explanations given here relate to method 2 with the acquisition of 2 channels. For other type of experiments (e.g. for the FRET calculations) additional control elements will appear.

- To perform acquisition, click on the 

button. Three images are acquired for each channel using the currently active camera settings and a mean-gray-value image is calculated to suppress background noise. You will now see channel 1, channel 2 and the calculated ratio image, including a rainbow color table ("Rainbow LUT").

- At the lower edge of the dialog you will find a toolbar. A description of the first 11 tools can be found under **Display Panel**.

- Using the other tools it is possible to draw in up to 100 regions, which will be used during the acquisition procedure itself for the online analysis. When you draw in several regions, the next color from the color list is automatically assigned to each new region.

 : Allows you to click on and select measurement regions that have already been drawn in to move them, enlarge them, delete them or change their color.

 : Used to draw in a rectangular measurement region. If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

 : Used to draw in a measurement region with a free form of your choice (spline). If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

 : Used to draw in an ellipse as a measurement region. If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other. If the Shift key is depressed while drawing in the ellipse, a circular form is enforced.

 Draws a circle. Starting point and end point of the drawing defines the diameter making it easy to e.g. define a circle encompassing a complete cell.

If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

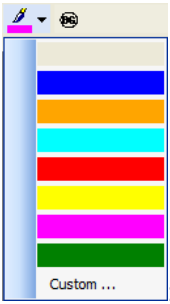
 When clicked, the last shape used for drawing a ROI is "remembered" and drawn into the overlay by simple clicking. This allows the cloning of a shape with ease. It works with all ROI tools except the free form spline tool.

This function can be disabled by selecting  .

 : Deletes all selected measurement regions.

 : Selects all available measurement regions.

 : Allows you to change the color for a measurement region.



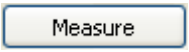
: Via **Custom...** it is possible to add further colors to the list from a color selection dialog.

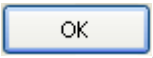
 : Defines a selected measurement region as a background region. The mean gray value from this region can, if desired, be automatically subtracted from the measurement values from other regions. Selecting this option will also include subtraction of the background for the calculation of the ratio image.

Settings Channel 1

Here it is possible to enter the desired exposure time for channel 1.

- **Time**: enter the exposure time to be used for the channel into the value field, either directly or using the arrow buttons  .

- **Measure**: To determine the exposure time interactively, click on the 

button. The dialog that opens contains a live image, generated using the channel-specific hardware settings. The exposure time is automatically measured when the dialog is opened. After you click on 

, the dialog is closed and the new exposure time is adopted automatically.

Note:

- Please note that, in order to achieve high acquisition speeds, the exposure time should not be set too high. Maximum exposure time allowed in Physiology experiments is 800 msec. If resulting images are displayed too dark, adjust the display settings accordingly. Also, consider using camera binning and define a region of interest for the camera to further increase the acquisition speed.

Settings Channel 2

Here it is possible to enter the desired exposure time for channel 2.

- **Time**: Here enter the exposure time being used for the channel into the value field, either directly or using the  buttons.

- **Measure**: To determine the exposure time interactively, click on the Measure button. The dialog that opens contains a live image, generated using the channel-specific hardware settings. The exposure time is automatically measured when the dialog is opened. After you click on OK, the dialog is closed and the new exposure time is adopted automatically.

Sync

In order to ensure identical exposure times for all channels click on the **Sync** button. Running experiments with the same exposure times is highly recommended for most FRET measurements.

Note:

- Settings for channel 2 are only available if more than one channel is set up. Also, for single channel ratioing (method 1), the image area for channel 2 is used to display the so-called F0 reference image instead. An exposure time for channel 2 can be defined nevertheless.

Ratio Image Definition

- **Method**: From the drop down list box you can select which calculation method you want to use for your experiment. 7 Methods are available for analysis during acquisition ("online"):

  - **Ratio Dual Channel**: Classical dual channel ratio imaging using either method 2 (excitation ratioing, e.g. Fura-2) or 3 (emission ratio imaging, e.g. Indo-1). Ratio is always calculated dividing channel #1 by channel #2. Only available with setups containing two or more channels.
  - **Ratio Single Channel**: Single Channel ratio imaging such as Fluo-4; can be done with single or multiple channel setups, online processing always done with channel #1.
  - **Fret Youvan**: Sensitized emission FRET measurements using three images. Channel #1 = Donor, Channel #2 = Acceptor, channel #3 = FRET. Only available with setups containing three or more channels.

- **Fret Gordon**: Derivative of FRET Youvan. Only available with setups containing three or more channels.

- **Fret Xia**: Derivative of FRET Youvan. Only available with setups containing three or more channels.

- **Fret Siegel BL**: Acceptor bleaching method, requires two channel setup (channel #1 = donor, channel #2 = bleached acceptor). Measurement done on channel #1 only.

- **Fret Dual Channel**: Ratiometric FRET method with 2 channels only disregarding the acceptor channel from the FRET calculation. This method can be used for fast FRET measurements using two cameras simultaneously in a **Dual Camera** configuration

Note:

- All the following explanations are based upon the Ratio Dual Channel method. A detailed explanation of the available FRET methods please see Physiology Measurements.

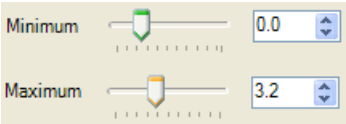
To calculate the ratio image, the gray values from channel 1 are divided by the gray values from channel 2 pixel by pixel. As the resulting ratio values are usually very small, the results are multiplied by an arbitrary factor. This makes it possible to display the ratio values as an image. To enable you to distinguish ratio differences more easily, these values are displayed in color using a rainbow colored look up table.

➢ The ratio image is calculated as outlined in the formula shown here:

$$r(x,y) = \text{Multiplier} * \frac{ch1(x,y)}{ch2(x,y)} + \text{Offset}$$

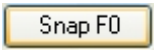
- r (x,y) = ratio image
- Multiplier = factor by which the result of the division is multiplied
- channel 1 (x,y): image from channel 1
- channel 2 (x,y): image from channel 2
- Offset = factor that influences the general brightness of the ratio image.

- In order to make it easier to match ratio image display and ratio charts the display can be influenced directly by changing lower (**Minimum**) and upper (**Maximum**) boundaries of the actual Ratio values. Use the sliders for **Minimum** or **Maximum** or by enter the values directly in the edit fields:



  The corresponding multiplier and offset factors are updated in the displayed formula.

- By increasing the lower limit one can reduce the background of small ratios, by raising the upper limit, also larger ratios can be displayed properly. A minimum of 0 and a maximum of 3 cover the range of ratios commonly occurring in biology.

- In the case of a single channel (**method 1**), a reference image is used as a denominator for the calculation. This so-called F0 image can be acquired using the 

button. Please note, that once an acquired F0 image is available it will remain active as long AxioVision is running. As soon as the conditions of the experiment change, a new F0 image has to be acquired.

- If a ROI has been selected for background correction the ratio image is calculated after background subtraction. This can lead to very small gray values producing very large ratios which are then visible as white noise in the ratio image. This happens e.g. around the edges of cells. This negative effect can later be dealt with during offline measurement by including the **Confidence Mapping** function.

C (Channel) property page

The basic functions available on this property page are identical to those on the **C (Channels)** property page for **Fast Acquisition**.

In **Physiology**, up to 5 channels can be configured for acquisition. However, only the first (for single channel experiments, method 1) or the first and second channel (methods 2&3) will be taken into account for the online analysis. The other channels will nevertheless be acquired and used during the conversion into a ZVI document and will therefore be available for further processing.

Markers property page

Marker

One of the most important functions for ion measurements is the ability to change the conditions of the experiment during acquisition and to log changes in the experiment. Physiology makes two types of markers available for this purpose.

- **Speed Marker**: These markers can be used to change the interval between the individual time points for image acquisition at the touch of a button.

- **Event Marker**: These markers enable you to enter previously defined events, such as a change of media, into the current experiment at the touch of a button. It is also possible to apply hardware settings using **Event Markers**.

Both types of marker are incorporated into the log when they are used. They subsequently enable you to navigate more easily within the acquired data and can also be inserted into the image as annotations.

Speed Marker

- You can define as many markers as you wish. To create a new marker, click on the Add New button and enter the desired settings.

- **Marker Settings** control area: Here you can give the **Speed Marker** a **Name**, enter a **Tool tip text** that will appear as a yellow text box if the mouse is moved over the button, allocate a **Color** to the marker from the  drop-down list box and define a **Shortcut** that enables you to operate the marker rapidly without having to click with the mouse.

- **Interval settings** control area: Here you can define the acquisition frequency of a Speed marker. You can choose between 6 options:

  - **As fast as possible:** Acquisition will happen as fast as the camera allows
  - **Interval:** The desired time interval can either be specified directly in the **Interval** input field or via the arrow buttons  . The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds).
  - **Start for**: Instead of starting the experiment for an undefined period of time this marker setting allows you to specify a time duration for the whole experiment. You have to start the experiment with this marker for the timing constraint to take effect and not the regular **Start (F5)** button.

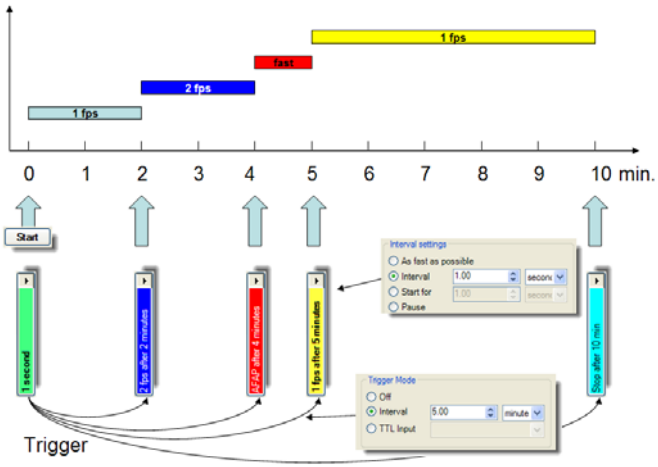
    Note:
    The remaining time of the experiment when using the **Start for** function is determined by calculating the number of remaining frames given the currently active interval setting when clicking the **Start for** Speed marker. This means, that when changing the interval during acquisition after having clicked on a **Start for** speed marker the timing of the experiment will not be as anticipated as the nr. of remaining frames will not be modified.

- **Pause**: Pauses the experiment
- **Continue**: Continues a paused experiment
- **Stop**: Stops the experiment

➢ **Trigger Mode** control area: Here you can set up a marker to activate itself automatically upon an incoming trigger. Triggers can be either a predefined timer starting with the experiment or an electrical TTL signal coming in through the Digital I/O Device/SVB-1.

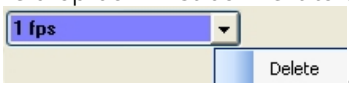
➢ **Off**: Turns off automatic triggering

- **After**: The desired timing for a software trigger event can either be specified directly in the **After** input field or via the arrow buttons . The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds). The marker will be set **once** after starting the experiment when the specified time has elapsed. This feature allows setting up heterogeneous physiology experiments for unattended operation. This is done simply by defining several speed markers with different time intervals and trigger timings according to the following example:



The experiment gets started with an interval setting of 1 frame/s by clicking on the **Start (F5)** button. At the same time the counter for the software trigger events is started. After 2 min. the frequency is increased to 2 frames/s, after 4 minutes to as fast as possible, after 5 minutes the original acquisition frequency of 1 fps is restored and the experiment stopped after 10 minutes.

- **TTL Input**: Alternatively one can also use hardware trigger signals to activate a speed marker. Two TTL input lines are available through the **Digital I/O Device** together with the **SVB-1** (TTL In 7 or TTL In 8). Both can be configured to react upon a change from low to high voltage ("High") or from high to low ("Low") depending on the type of external TTL source.

➢ To delete a marker, click on the drop-down list box next to each button and select the **Delete** option 

.

Event Marker

➢ Here you can define as many event markers as you wish. To create a new marker, click on the 

button and enter the desired settings.

➢ **Marker Settings** control area: Here you can give the **Event Marker** a **Name**, enter a **Tool tip text** that will appear as a yellow text box if the mouse is moved over the button, allocate a **Color** to the marker from the  drop-down list box and define a **Shortcut** that enables you to operate the marker rapidly without having to click with the mouse.

➢ **Hardware Settings** control area: Here it is possible to select an existing hardware setting that you want to be executed if the marker is selected during acquisition. Please note that you should only make changes to devices which make sense to change during fast image acquisition. You should not make any adjustments to, in particular, the camera resolution or the light source, to prevent the sequence of the experiment being

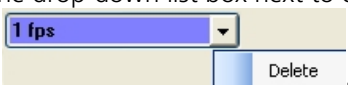
disrupted. Please check the **Enabled** check box in order to activate the execution of the hardware setting.

- **Trigger Mode** control area: Here you can set up an event marker to activate itself automatically upon an incoming trigger. Triggers can be either a predefined timer (counting starts with the start of the experiment) or an electrical TTL signal coming in through the Digital I/O Device. Since Event marker can also be linked with hardware settings it is possible to set up flexible experiment protocols where specific hardware components are switched during the experiment automatically.

  - **Off**: Turns off automatic triggering

  - **After:** The desired software trigger timing can either be specified directly in the **After** input field or via the arrow buttons  . The unit can be selected from a drop-down list box (hours, minutes, seconds, milliseconds). Markers triggered that way will be executed once during the experiment after the specified time has elapsed.

  - **TTL Input**: Alternatively one can also use hardware trigger signals to activate an event marker. Two TTL input lines are available through the **Digital I/O Device** together with the **SVB-1** (TTL In 7 or TTL In 8). Both can be configured to react upon a change from low to high voltage ("High") or from high to low ("Low") depending on the type of external TTL source.

- To delete a marker, click on the drop-down list box next to each button and select the **Delete** option 

.

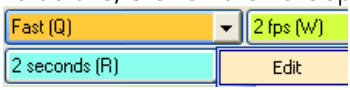
Physiology Acquisition

The dialog for image acquisition is opened using the Start button. This dialog is divided into four areas:

- Area for setting markers.
- Area containing buttons for starting and stopping acquisition as well as the most important status information.
- Area containing charts and the acquired images.
- Overview chart for the entire experiment.

Speed Marker

Here you can activate a speed marker, either by mouseclick, by using a keyboard shortcut (if configured) or by a trigger signal. The acquisition speed then changes accordingly. Each marker if clicked is entered in the marker list for the experiment. It is also possible to use special speed marker settings to automatically change the acquisition speed, start the experiment for only a predefined period of time, pause, continue or stop the experiment.

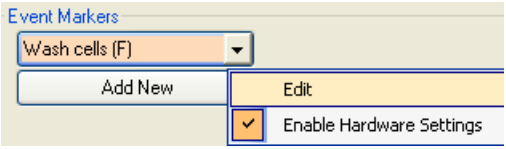
It is also possible to create new markers or modify existing ones during acquisition. To do this, click on the **Edit** option in the drop-down list box for the marker. 

In the dialog that opens you can then make the desired changes.

Event Marker

Here you can activate an event marker, either by mouseclick, by using a keyboard shortcut (if configured) or by a trigger signal. The corresponding coment is then entered in the marker list for the experiment. If an event marker is linked to a hardware setting, the corresponding hardware setting is executed.

It is also possible to create new markers or modify existing ones during acquisition. To do this, click on the **Edit** option in the drop-down list box for the marker.



In the dialog that opens you can then make the desired changes. The Enable Hardware Settings option activates or deactivates the link to the **Event Marker**.

Acquisition area

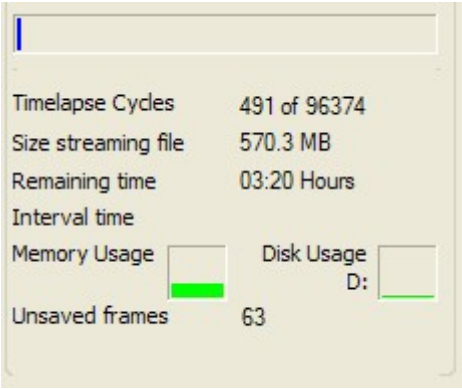
- **Start (F5)**: Clicking on this button (or keyboard shortcut F5) starts acquisition for the experiment. It is possible to repeat this procedure multiple times once acquisition has been completed without leaving the **Physiology Acquisition** dialog. The acquired image sequences are stored as raw data on the defined streaming partition.

- **Stop (F8)**: Clicking on this button (or keyboard shortcut F8) stops acquisition. Once you have stopped the process, a new image sequence can be initiated immediately using the **Start (F5)** button, although this will then be saved in a new streaming file.

- **Pause (F6)**: Clicking on this button (or keyboard shortcut F6) pauses the acquisition procedure.

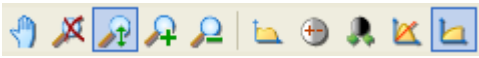
- **Focussing (F9)**: Clicking this button (or using the keyboard shortcut F9) changes the acquisition speed to **As fast as possible** in order to allow focusing the sample. During this time the system continues delivering images but they will not be saved. This function is therefore equivalent to pausing the experiment while still updating the images. This helps avoid gathering unnecessary data. Clicking on **Continue (F7)** will commence acquisition with image storage with the last selected acquisition speed. The focusing time in the final image will be indicated by a focusing marker as well as a **Pause** segment.

- **Continue (F7)**: Clicking on this button (or keyboard shortcut F7) allows you to continue acquisition in the same image file.



- A progress indicator shows the elapsed time for the selected experiment.
- Indicators for the available main memory and disk space for the raw data provide important information on the status of the overall system. It is also possible to see for how long it will be possible to acquire image data.
  - In case the hard disk performance for the Streaming partition being too low some images may not be saved during acquisition. These images are instead stored temporarily in RAM. The number of unsaved images is indicated here as well as the usage of RAM memory (bar on the left).

Charts and image display

- **Markers**: Shows all entries for the current experiment in list form. Every event is entered as a comment and transferred to the ZVI image during subsequent conversion.
-  : These buttons allow you to adjust the display (brightness, contrast, zoom etc.) for the images during acquisition as well as show a display histogram.
- The drop-down list box next to the  icon opens a dialog in which the sliders for influencing the ratio image are available. These allow you to change the ratio display during acquisition.

- For **method 2** (excitation ratioing in two channels) or **method 3 (emission ratioing)**, channel 1, channel 2 and the ratio image are displayed. To the left of these are the corresponding measurement value charts.

- For **method 1** (single-channel experiments), only channel 1 and the acquired reference image F0, as well as the ratio image, are displayed.

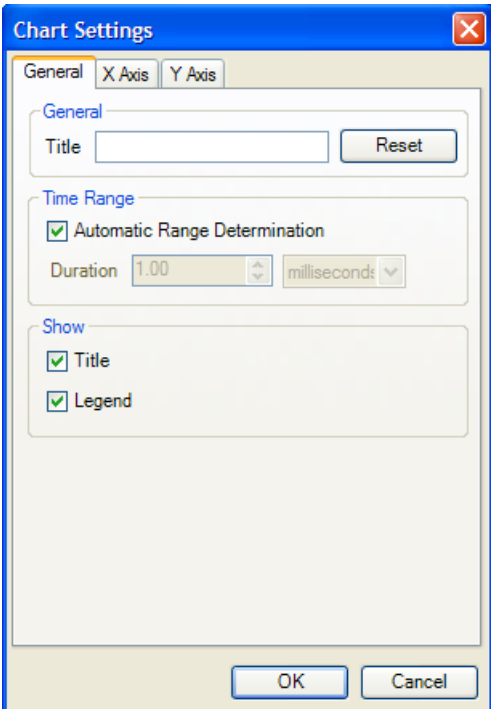
- For **FRET** imaging you will see Donor, Acceptor and the processed FRET image.

- If you double-click into the Ratio image it will magnify and hide the other image displays. Also only the Ratio chart will be displayed. This will allow easier observation of the experiment. Double-clicking once more restores the standard layout.

- The charts can display 6 different measurement values for all drawn-in measurement regions (this setting can be entered via a shortcut menu **Displayed value …**, which is opened by right-clicking):

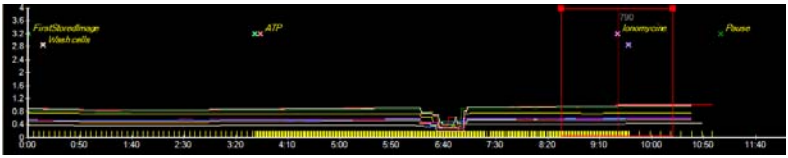
  - Mean gray value channel 1
  - Mean gray value channel 1 minus background
  - Mean gray value channel 2
  - Mean gray value channel 2 minus background
  - Ratio value
  - Ratio value minus background

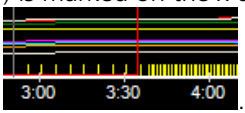
- **Chart settings**: The three charts next to channel 1, 2 and ratio image can be configured by right-clicking on a chart and selecting **Chart Settings** upon which a **Chart Settings** dialog is opened:



The dialog is divided into three tabs which allow the configuration of the chart parameters such as changing the default title, specifying a specific value range for X- or Y-axis, displaying or hiding legend and title or specifying tick marks. A **Reset** button on the X Axis and Y Axis page resets the chart configuration to its default setting.

- **Experiment overview chart**: At the bottom edge of the window you will see an overview of the entire experiment. Here you see the Ratio values displayed as measurement traces, all event markers are shown by name and with a cross marking the time point and a navigation tool with a line marking the current time point.



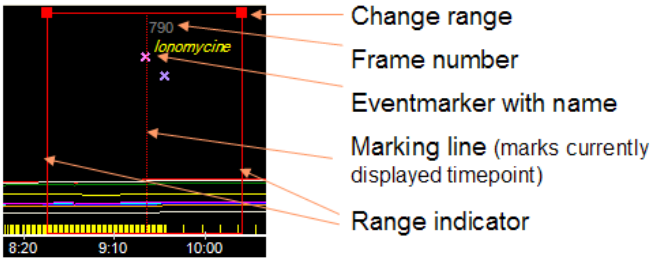
Each camera acquisition ("snap") is marked on the x-axis by a yellow line allowing precise frame selection 

.

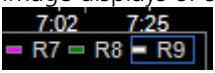
Here too it is possible to select which values you want the chart to display (right-hand mouse button, **Chart settings**). During the experiment, the new images and measurement values of the time lapse image are added continuously. The X-axis of the overview chart is updated once every 10 minutes during an experiment. The broken red line of the navigation tool moves with each acquired image which is always displayed automatically.

However, you can also stop the marking line by deactivating the Follow button.

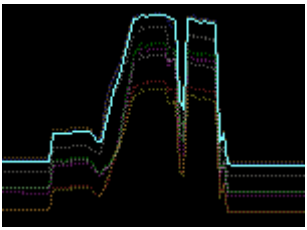
You have the option of using the marking line like a slider to move to an earlier time point and revisit the acquired data. The red, broken marking line has two red, solid peripheral lines. The area within these two lines corresponds to the area that is displayed in the charts next to the images. If you grab the squares at the top edge of the area boundaries, it is possible to change the range shown in the charts.



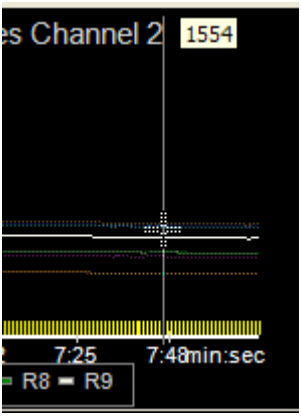
- **ROI and line selection**: During the experiment, especially one with many measurement ROI's drawn in, it may be desirable to e.g. see which cell is especially active. You can therefore click on either an interesting looking measurement line in any of the charts, select a ROI in one of the displayed image displays or select it in the ROI legend at the bottom of each chart



  Either selection will automatically select the corresponding shapes in the other areas. Selected lines show up more brightly on the screen, unselected lines become subdued in appearance.



- If a ROI or measurement line is selected, moving the mouse cursor along the line with no buttons depressed shows a gray line and a pop up text showing the relevant intensity or ratio values.



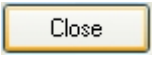
- To move the marking line step by step, you can use the 

and 

buttons.

- After stopping the experiment (Stop or F8), you can directly load the new file in the **Cutter** to allow conversion into ZVI by clicking on the 

button.

- Clicking on the 

button will exit the dialog.

Note:

- No other setting options are accessible in AxioVision while the **Physiology Acquisition** dialog is open. This is designed on purpose to prevent operating errors.

- Once the dialog is closed, all acquired image data are loaded in AxioVision in streaming format, but have not yet been saved. They exist on hard disk but not in a format which AxioVision can read.

- The streaming data can be saved as a *.ziStream document. Streaming files per se cannot be reopened in AxioVision. Upon saving each *.ziStream file will be accompanied by a *.ziAR file containing all relevant meta-information about the experiment and the data, which you will see, when reopening a streaming file. Both files are necessary to open an experiment again.

- Please also note, that streaming files must not remain on the streaming disk in order to prevent disk fragmentation and the resulting loss in hard disk performance. Upon closing AxioVision, you will be therefore asked if you want to save or discard unsaved streaming files.

Physiology Acquisition Document Cutter

To generate a ZVI image from a saved or unsaved *.ziAR streaming document, you will require the **Cutter**. Open the **Cutter** via the corresponding entry in the work area or the **Acquisition** menu ⇒ **Physiology Acquisition** ⇒ **Cutter**.

The principal task of the **Cutter** is to reduce the time-consuming conversion of the raw data into a ZVI image to a sensible level. This makes it possible to reduce the data volume and speed up the further processing of the data.

Note:

- The **Cutter** can only be opened if a suitable streaming file has also been loaded in AxioVision (otherwise the following error message appears: "*No streaming document available to edit. Please first open a streaming document (*.ziAR)*").

Cutter Physiology

The **Cutter Physiology** dialog is structured in a similar way to the **Physiology Acquisition** dialog.

- 

Describes the name of the input document.

- The  button opens a gallery from which you can also load a document other than the one that was last active. To do this, click on the thumbnail of the desired streaming document.

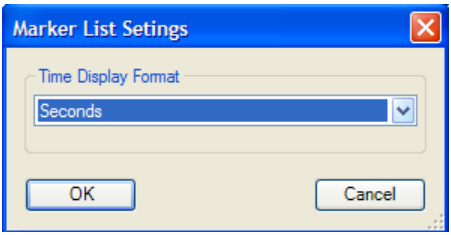
-  : The buttons on this toolbar can be used to adjust the display (brightness, contrast, zoom etc.) of the images.

- Using the  button on the toolbar, it is possible to define a region of interest in the image that you want to be taken into account during the conversion to ZVI. This will dramatically reduce the data volume to the relevant parts of the image.

- The drop-down list box next to the  icon opens a dialog in which the sliders for influencing the ratio image are available.

- **Marker**: Shows all entries for the current experiment in list form to the right of the dialog. Every marker is transferred as a comment to the ZVI image during conversion and can later be inserted into the generated ZVI image as an annotation via **Annotations ⇒ Frequent Annotations ⇒ Event marker / Speed marker**.

- The **time format** for the marker list can be changed by right-mouse click in the header row of the list and selecting the **Properties Dialog**:



  Please select the desired time format (**Seconds**, **Minutes**, **Hour** or the generic format **hh:mm:ss:msec** which is the default setting) from the drop down list. You can also select the frame number.

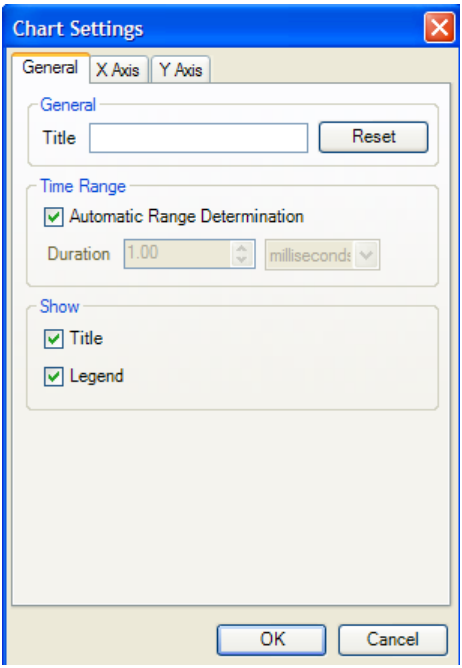
- For **methods 2 or 3** (excitation or emission ratioing in two channels), channel 1, channel 2 and the ratio image are displayed. To the right of these are the corresponding charts displaying the measurement values .

- For **method 1**, only channel 1 and the acquired reference image F0, as well as the ratio image, are displayed.

- The charts can display 6 different measurement values for all drawn-in measurement regions (this setting can be entered via the menu **Displayed value …**, which is opened by right-clicking):

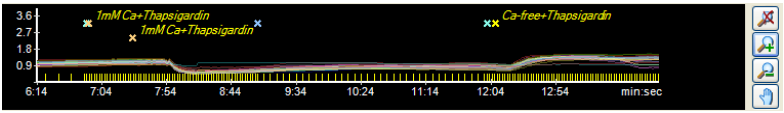
  - Mean gray value channel 1
  - Mean gray value channel 1 minus background
  - Mean gray value channel 2
  - Mean gray value channel 2 minus background
  - Ratio value
  - Ratio value minus backgroundccc

- **Chart settings**: The charts can be configured by right-clicking on a chart and selecting **Properties** upon which a **Chart Settings** dialog is opened:

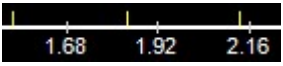


The dialog is divided into three tabs which allow the configuration of the chart parameters such as changing the default title, specifying a specific value range for X- or Y-axis, displaying or hiding legend and title or specifying tick marks. A **Reset** button resets the chart configuration to its default setting.

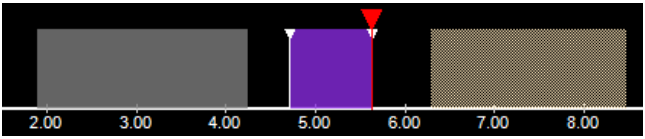
- **Experiment Overview**: In the bottom area you will see an overview of the entire experiment displaying markers and measurement curves:



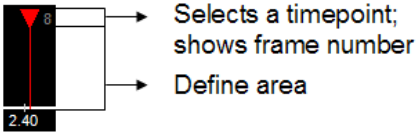
- Each acquisition event is signified by a yellow line allowing easier frame selection



- In this control element it is possible to define the areas of the streaming document that you want to be converted to ZVI. When this dialog is called up for the first time, nothing is selected. Selection can be done by clicking with the left mouse button and dragging whilst keeping the mouse key depressed. Selection is symbolized by a magenta selection bar. Segments within the experiment where the acquisition had been paused are signified through a lighter color. Such paused areas are automatically excluded from conversion.

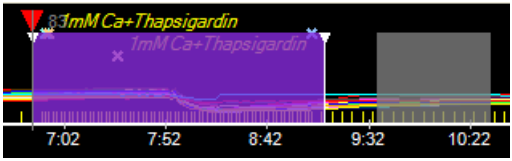


- The time point currently displayed is indicated using the following marking line:



If you grab the upside-down triangle with your mouse, you can select the time point of your choice. In the area at the lower half you can use the left-hand mouse button to define an area to be converted: hold down mouse button, drag, release → defined area is marked in magenta. You can mark as many areas as you wish.

- The area that is currently active is marked in magenta, whilst inactive selections are shown in gray



- It is possible for marked areas to be moved (left-hand mouse button).

 Adjusts the zoom factor of the chart in such a way that the entire experiment is displayed.

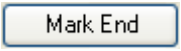
 Allows you to display a part of the experiment in the chart with a higher zoom factor (**Zoom in**).

 Reduces the zoom factor for the displayed chart area (**Zoom out**).

 Moves the displayed chart area (only active with **Zoom in**).

- In the bottom area of the dialog you will find a table containing the selected areas with an indication of the exact start and end time points, the resulting duration and the start-image position and end-image position for the marked segments.

- It may be beneficial, especially if you need to select a time point precisely, to position the time point marking line  and set it as the start point for a new area selection. This is done using the 

button. As soon as a start point has been defined, the 

button for defining an end point becomes active.

- You can use the two arrow buttons 

to browse through the streaming file frame by frame. This allows an even more precise frame selection.

- To select the entire time series for conversion, click on the 

button.

- To delete selected area markings, you can use the 

button (or alternatively the **Delete** key).

Conversion Information

- Here you will find a display of the file size of the newly generated ZVI image, the number of individual images it contains and the available memory on the target drive.

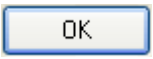
Executing editing



converts the currently shown frame into a ZVI image and leaves the dialog open



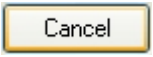
selects the complete streaming file, starts conversion immediately and closes the dialog. This button is selected as default which means that you can confirm this action immediately by hitting **Enter** on your keyboard.



Carries out the conversion to a ZVI image according to the individual selections, after which the dialog is closed automatically.



Carries out the conversion to a ZVI image, but the dialog remains open. This allows you to generate several ZVIs of different areas and regions conveniently one after another, without having to call up the dialog again each time.



Closes the dialog. No ZVI image is generated.

The result of the conversion is a new, unsaved ZVI image. Images generated in this way can now be subjected to post acquisition analysis using the **Measurements** function

Physiology Measurements

During acquisition preliminary measurements are performed based on the drawn in measurement regions. These online measurements mainly feed the charts with data for online display. Measurement regions can also be moved to compensate for moving cells during the experiment. This option as well as other vagaries during the experiment can lead to measurement values inconsistent with the experimental goals. Therefore a detailed analysis of the acquired data must be done after the acquisition. This can be done using the **Measurements** function in the **Physiology** module.

Measurements can read any ZVI time series, also if the series has not been acquired using **Physiology**. ZVI time series can have one or more channels, which can be selected flexibly for measurement. The strict channel order which is required for online measurement does not apply for offline measurement. Streaming files (*.ziAR) must be converted into ZVI first using the **Cutter**.

- You can open the **Physiology Measurements** dialog by clicking on Measurements in the **Acquisition** menu ⇒ **Physiology Acquisition** ⇒ **Measurements**.

- Always the active ZVI image is loaded into the Measurement dialog. By using the  button another ZVI image can be selected from the **Gallery**.

- The left side of the dialog features three tabs for setting up which channels should be measured (**Channel Setup**), for configuring the measurement parameters (**Measurement**) as well as parameters for generation of the ratio image (**Ratio Image**).

Channel Setup

- **Method**: From the drop down list box you can select a calculation method. Default method is Ratio Dual Channel. There are 8 predefined methods but you can also use your own custom formula. The parameters which can be changed depend on the method selected. For the various FRET methods the different formulas used are displayed as well:

- **Ratio Dual Channel**: Classical dual channel ratio imaging using either method 2 (excitation ratioing, e.g. Fura-2) or 3 (emission ratio imaging, e.g. Indo-1), only with two channel setups. Ratio is always calculated dividing channel #1 by channel #2.

- **Ratio Single Channel**: Single Channel ratio imaging such as Fluo-4; can be done with single or multiple channel setups, online processing always done with channel #1.

- **Fret Youvan**: Sensitized emission FRET measurements using three images. Channel #1 = donor, channel #2 = acceptor, channel #3 = FRET.

- **Fret Donor Ratio:** channel #1 = donor, channel #2 = acceptor, channel #3 = FRET.

- **Fret Acceptor Ratio:** channel #1 = donor, channel #2 = acceptor, channel #3 = FRET.

- **Fret Gordon**: Derivative of FRET Youvan.

- **Fret Xia**: Derivative of FRET Youvan.

- **Fret Siegel BL**: Acceptor bleaching method, requires two channel setup.

- **Custom formula**: Using regular algebra terms a customized formula can be edited and applied to the data.

Examples for FRET calculations using the Custom Formula function

Youvan

Fc = (GWfr - BGfr) - CFdon * (GWdon - BGdon) - CFacc * (GWacc - BGacc)
(ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) - 0.09 * (ch2 - 1.275 * bg2)
(ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) - 0.09 * (ch2 - 1.3 * bg2)
(ch3 - bg3) - 0.19 * (ch1 - bg1) - 0.09 * (ch2 - bg2)
(ch3) - 0.19 * (ch1) - 0.09 * (ch2)

Gordon

Fn = Fc / ((GWdon - 1.275 * BGdon) * (GWacc - 1.275 * BGacc))
((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) - 0.09 * (ch2 – 1.275 * bg2)) / ((ch1 - 1.275 * bg1) * (ch2 - 1.275 * bg2))
((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) - 0.09 * (ch2 - 1.3 * bg2)) / ((ch1 - 1.3 * bg1) * (ch2 - 1.3 * bg2))
((ch3 - bg3) - 0.19 * (ch1 - bg1) - 0.09 * (ch2 - bg2)) / ((ch1 - bg1) * (ch2 - bg2))
((ch3) - 0.19 * (ch1) - 0.09 * (ch2)) / ((ch1) * (ch2))

Xia

NF = Fc / SQRT((GWdon - 1.275 * BGdon) * (GWacc - 1.275 * BGacc))
((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) - 0.09 * (ch2 – 1.275 * bg2)) / sqrt((ch1 - 1.275 * bg1) * (ch2 - 1.275 * bg2))
((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) - 0.09 * (ch2 - 1.3 * bg2)) / sqrt((ch1 - 1.3 * bg1) * (ch2 - 1.3 * bg2))
((ch3 - bg3) - 0.19 * (ch1 - bg1) - 0.09 * (ch2 - bg2)) / sqrt((ch1 – bg1) * (ch2 - bg2))
((ch3) - 0.19 * (ch1) - 0.09 * (ch2)) / sqrt((ch1) * (ch2))

Acceptor Ratio

AcR = Fc / (GWacc - 1.275 * BGacc)
((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) - 0.09 * (ch2 – 1.275 * bg2)) / (ch2 - 1.275 * bg2)
((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) - 0.09 * (ch2 - 1.3 * bg2)) / (ch2 - 1.3 * bg2)
((ch3 - bg3) - 0.19 * (ch1 - bg1) - 0.09 * (ch2 - bg2)) / (ch2 - bg2)
((ch3) - 0.19 * (ch1) - 0.09 * (ch2)) / (ch2)

Siegel (Acceptor Bleaching)

BL = 100 * (1 - (GWdonbef - BGdonbef) / (GWdonaft - BGdonaft))
100 * (1 - (f0 - 1.275 * bgf0) / (ch1 - 1.275 * bg1))
100 * (1 - (f0 - 1.3 * bgf0) / (ch1 - 1.3 * bg1))
100 * (1 - (f0 - bgf0) / (ch1 - bg1))
100 * (1 - (f0) / (ch1))

Calculation of correction factors:

Donor correction factor

CFdon = 100 * (GWfr - BGfr) / (GWdon - BGdon)
100 * (ch3 - 1.275 * bg3) / (ch1 - 1.275 * bg1)
100 * (ch3 - 1.3 * bg3) / (ch1 - 1.3 * bg1)
100 * (ch3 - bg3) / (ch1 - bg1)
100 * (ch3) / (ch1)

Acceptor correction factor

CFacc = 100 * (GWfr - BGfr) / (GWacc - BGacc)
100 * (ch3 - 1.275 * bg3) / (ch2 - 1.275 * bg2)
100 * (ch3 - 1.3 * bg3) / (ch2 - 1.3 * bg2)
100 * (ch3 - bg3) / (ch2 - bg2)
100 * (ch3) / ch2)

Neue Formeln:

Donor Ratio 1

DoR = Fc / (GWdon - 1.275 * BGdon)
((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) - 0.09 * (ch2 – 1.275 * bg2)) / (ch1 - 1.275 * bg1)
((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) - 0.09 * (ch2 - 1.3 * bg2)) / (ch1 - 1.3 * bg1)
((ch3 - bg3) - 0.19 * (ch1 - bg1) - 0.09 * (ch2 - bg2)) / (ch1 - bg1)
((ch3) - 0.19 * (ch1) - 0.09 * (ch2)) / (ch1)

New formulas for customer formula (examples):

2-channel methods:

Donor Ratio 2 (Gordon)

FR01 = (GWfr - BGfr) / (GWdon - BGdon)
(ch3 - 1.275 * bg3) / (ch1 - 1.275 * bg1)
(ch3 - 1.3 * bg3) / (ch1 - 1.3 * bg1)
(ch3 - bg3) / (ch1 - bg1)
(ch3) / (ch1)

Donor Ratio 3 (Gordon)

FR02 = (GWacc - BGacc) / (GWdon - BGdon)
(ch2 - 1.275 * bg2) / (ch1 - 1.275 * bg1)
(ch2 - 1.3 * bg2) / (ch1 - 1.3 * bg1)
(ch2 - bg2) / (ch1 - bg1)
(ch2) / (ch1)

Donor Ratio 4

FR03 = ((GWfr - BGfr) - CFdon * (GWdon - BGdon)) / (GWdon - BGdon)
((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1))/(ch1 - 1.275*bg1)
((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1)) / (ch1 - 1.3 * bg1)
((ch3 - bg3) - 0.19 * (ch1 - bg1)) / (ch1 - bg1)
((ch3) - 0.19 * (ch1)) / (ch1)

3-channel methods:

Zal

Eapp = ((GWfr - BGfr) - CFdon * (GWdon - BGdon) - CFacc * (GWacc – BGacc)) / ((GWfr - BGfr) - CFdon * (GWdon - BGdon) + G * (GWdon-BGdon))
((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) - 0.09 * (ch2 – 1.275 * bg2)) / ((ch3 - 1.275 * bg3) - 0.19 * (ch1 - 1.275 * bg1) + 2.0 * (ch1 - 1.275 * bg1))
((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) - 0.09 * (ch2 - 1.3 * bg2)) / ((ch3 - 1.3 * bg3) - 0.19 * (ch1 - 1.3 * bg1) + 2.0 * (ch1 – 1.3 * bg1))
((ch3 - bg3) - 0.19 * (ch1 - bg1) - 0.09 * (ch2 - bg2)) / ((ch3 - bg3)– 0.19 * (ch1 - bg1) + 2.0 * (ch1 - bg1))
((ch3) - 0.19 * (ch1) - 0.09 * (ch2)) / ((ch3) - 0.19 * (ch1) + 2.0 * (ch1))

Note:

- All the following explanations refer prominently to the Ratio Dual Channel method.

➢ **Channel Selection:** The ratiometric measurement can be set up here by selecting **numerator** and **denominator** channel used in the ratio formula. Select the desired channel (channel name as specified in the experiment) from the appropriate drop down list.

➢ **Ratio Image Definition**: In both methods ratio values are displayed by applying a color look up table (LUT) to aid the visual distinction of small ratio changes. The ratio image is calculated as outlined in the formula shown here:

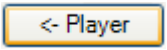
$$r(x,y) = \text{Multiplier} * \frac{ch1(x,y)}{ch2(x,y)} + \text{Offset}$$

- r (x,y) = ratio image
- Multiplier = factor by which the result of the division is multiplied
- channel 1 (x,y): image from channel 1
- channel 2 (x,y): image from channel 2
- Offset = factor that influences the general brightness of the ratio image.

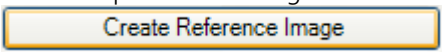
➢ The lower and upper limit of the displayed ratio can be entered using the sliders for **Minimum** or **Maximum** or by entering the values directly in the edit fields. These values are also put in the output ratio image as image tags and are used by the LUT Scale annotation. By raising the lower limit one can reduce the background of small ratios, by raising the upper limit, also larger ratios can be displayed properly. A minimum of 0 and a maximum of 3 cover the range of ratios commonly occurring in biology.

➢ **Look-up Table** (LUT): Clicking on  opens the **Open Table** dialog which allows selection of a desired look up table. Depending on the application or the personal preference various LUTs serve different purposes. For Ratio imaging a rainbow look up table is frequently used (RAINBOW.COL).

- In case of the **Ratio Single Channel** method the **Reference Image (F0)** section becomes active which allows definition of time points for calculation of an averaged F0 reference image.

- In order to generate an appropriate reference image for a single channel measurement a suitable range of time points can be defined from within the same channel. This, e.g., could be a resting phase, where cells do not show any ion fluctuations as well as do not move. You can either enter the desired time points directly in the **From** and **To** edit fields or take over the current time point as selected with the time player by clicking on the 

buttons.

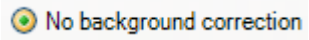
- From this series of individual time points an averaged reference image is calculated after click on 

.

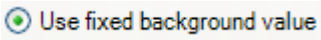
- After the reference has been created, the charts become active and measurements can be performed.

Measurement

This tab contains functions for background correction as well as for in situ calibration should absolute concentrations be determined. Background correction is applied both to the measurement results for each measurement ROI and the corresponding measurement charts as well as to the ratio image.

- 

: If selected, the uncorrected raw gray values are used to calculate the ratio values

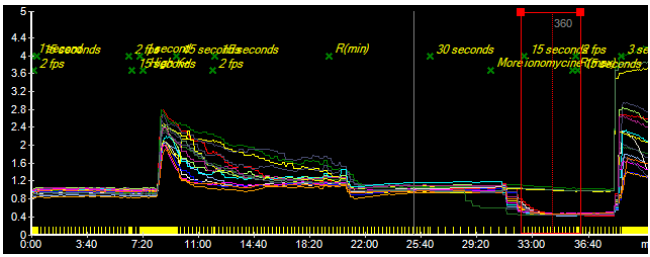
- 

: If selected, a fixed background value is subtracted from all time points. This value is channel specific and can be entered in the edit fields next to the appropriate channel number. In case of single channel images for which an F0 reference image has been defined, the F0 reference is considered as a pseudo channel #2.

- Use background ROI : If a measurement region (ROI) has been selected in the image, this option becomes active. Here a background value for each channel and time point is measured and subtracted from the gray values of the other measurement regions used to calculate the ratio. If either of the two other options is selected, the selected background ROI is converted into a standard measurement region. To again use a background region use the button from the toolbar to convert a ROI into a background ROI.

- **In Situ Calibration**: If enabled, the check box enables a dialog to perform in situ calibration for calibrated Calcium and pH measurements. As a prerequisite for in situ calibration you need to treat your cells with agents who allow you to determine the minimum (R(min) and the maximum R(max) response of the loaded dye. In the case of Calcium measurements using Fura-2, e.g., this is frequently done using Ionomycine, a drug, which permeabilizes the cell membrane followed by treatment with low and high calcium buffer.

  - Move the cursor of the navigator tool to the part in the chart, which shows minimum ratio:



  - click on <- Player to use the current cursor position as the time point used for determining the minimum ratio of each ROI.

  - Repeat the same for R(max).

- Clicking on Show opens a floating dialog **Calibration editor** showing the calibration charts.

- Here you can select between Calcium Calibration or pH Calibration. In the case of Calcium calibration the formula according to Grynkiewicz et al. (Journal of Biological Chemistry, Volume 260: 3440, 1985) is used. Here you can also modify the kD, which has been calculated to be 224 nM at 1 mM Mg^{++} and 37°C.

- A viscosity correction factor typically between 0.7 and 0.85 has been suggested to be used by M. Poenie (Cell Calcium Volume 11, 2-3, pp 85-91, 1990), which can be entered here as well. Setting the value to 1 disables correction.

- In the case of pH calibration a simple linear interpolation is performed using R(min) and R(max).

- OK closes the dialog and activates calibration.

All measurements are done online, i.e., when the dialog first opens, all calculations are done once. The system then tries to update the measurements on the fly upon changes to ROIs etc. For large time series with many time points and/or many measurement regions, calculation of all values for all time points can take several seconds. To prevent the system from responding slowly, the calculation update is interrupted after a few seconds so that not all values may be present. In this case the charts may be incomplete which will be signified by a yellow signal  . Clicking on the Update Data button forces the complete calculation which is symbolized by changing the signal color to  .

Options

You can choose between two methods of calculating the ratio image: **Standard** and **Confidence Mapping**. Standard is active if the check box is unchecked.

The second algorithm is called **Confidence mapping**. This method has been suggested by Tsien and Harootunian (Tsien, R.Y., and Harootunian, A.T. 1990. Practical design criteria for a dynamic ratio imaging system. *Cell Calcium* **11**: 93-109.) to prevent noise from influencing the ratio display negatively.

When dividing two small gray values by each other, fluctuations in the gray value introduced by noise can result in quite large ratio values. With this method each ratio pixel value is put in proportion to the brightness of its original input pixels. This effectively introduces a bias for the display of brighter and therefore more relevant pixels. Consequently dark pixels become underrepresented in the result image reducing the displayed noise in the image.

- The calculation is done in two steps:

  - Calculation of the Ratio

    $$r(x,y) = \text{Multiplier} * \frac{ch1(x,y)}{ch2(x,y)} + \text{Offset}$$

  - Calculation of the confidence:

    $$\text{Confidence} = \frac{ch1(x,y) * ch2(x,y)}{2 * \text{ImageMaxValue}}$$

    while ImageMaxValue is the brightest pixel present in the image.

- The display can be explained by setting the **Hue = LUT (Ratio)** and the **Luminance = Confidence** or as follows:

$$\text{Red} = \text{Lut - red (Ratio)} * \text{Confidence}(x, y)$$
$$\text{Green} = \text{Lut - green (Ratio)} * \text{Confidence}(x, y)$$
$$\text{Blue} = \text{Lut - blue (Ratio)} * \text{Confidence}(x, y)$$

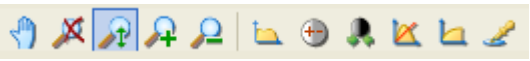
➢ For confidence mapping it is not possible to directly enter a Ratio value as annotation in the resulting ratio output image due to the dual functionality required to calculate the ratio pixel appearance.

➢ In order to influence the displayed value range both **Multiplier** (range between 0 and 10) and **Offset** values (value range of -1 to 1) can be changed on the **Channel Selection** tab using the sliders or by directly entering the values in the edit fields.

Information Color Ratio Image

The size and number of cycles of the resulting ratio image are displayed here.

Tool bar

 : The buttons on this toolbar can be used to adjust the display (brightness, contrast, zoom etc.) of the images or analyze individual pixels using the picker tool.

Using the other tools it is possible to draw in up to 20 regions, which will be used for measurement. When you draw in several regions, the next color from the color list is automatically assigned to each new region.

 Allows you to click on and select measurement regions that have already been drawn in to move them, enlarge them, delete them or change their color.

 Used to draw in a rectangular measurement region. If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

 Used to draw in a measurement region with a free form of your choice (spline). If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other.

 Used to draw in an ellipse as a measurement region. If the  button is not selected, the tool remains active and it is possible to draw in several measurement regions one after the other. If the Shift key is depressed while drawing in the ellipse, a circular form is enforced.

 Draws a circle. Starting point and end point of the drawing defines the diameter making it easy to e.g. define a circle encompassing a complete cell.

 When clicked, the last shape used for drawing a ROI is "remembered" and drawn into the overlay by simple clicking. This allows the cloning of a shape with ease. It works with all ROI tools except the free form spline tool.

This function can be disabled by selecting  .

 : Displays the measurement values instead of the charts. You can click on an entry upon which the corresponding time point is selected in the time series. These values can also be exported as a data list later. The data table is set up as shown here, the "Channel 1" etc. is replaced by the actual channel name as specified in the experiment. Also, the set up shown here is only valid for the **Ratio Dual Channel** case. Especially in FRET experiments, the Ratio column will contain the calculated FRET values.

Index	Row index
TimeStamp	relative time
Markers	Marker
R1_Channel1	mean density of region 1, channel 1
R1_Channel2	mean density of region 1, channel 2
R1_Ratio	ratio value region 1, channel 1 / channel 2
R1_Channel1(-BG)	background corrected mean density of region 1, channel 1
R1_Channel2(-BG)	background corrected mean density of region 1, channel 2
R1_Ratio(-BG)	background corrected ratio value region 1 channel 1 / channel 2
R2_Channel1	mean density of region 2, channel 1
R2_Channel2	mean density of region 2, channel 2
R2_Ratio	ratio value region 2, channel 1 / channel 2
R2_Channel1(-BG)	background corrected mean density of region 2, channel 1
R2_Channel2(-BG)	background corrected mean density of region 2, channel 2
R2_Ratio(-BG)	background corrected ratio value region 2, channel 1 / channel 2
etc…	etc…

If no background correction is done, the background corrected value column is omitted from the table. In the case of calibrated measurements additional value columns will be present.

Clicking again on  hides the table and displays the measurement charts again

 Deletes all selected measurement regions.



Selects all available measurement regions.

 Allows you to change the color for a measurement region.



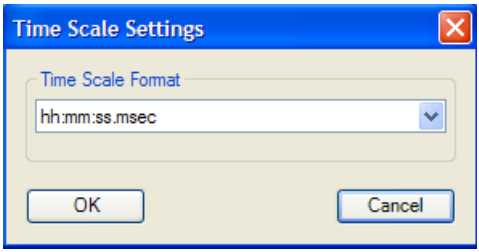
: Via **Custom...** it is possible to add further colors to the list from a color selection dialog.

 Defines a selected measurement region as a background region. The mean gray value from this region can, if desired, be automatically subtracted from the measurement values of all other regions.

Markers

Shows every event which has been transferred as a comment to the ZVI image during conversion. Clicking on an event moves the time marker tool to the corresponding time point.

- The **time format** for the marker list can be changed by right-mouse click in the header row of the list and selecting **Properties**:



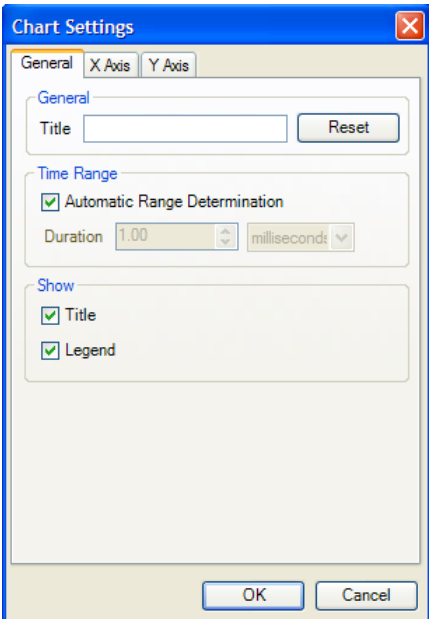
Please select the desired time format (**Seconds**, **Minutes**, **Hours** or the generic format **hh:mm:ss:msec** which is the default setting) from the drop down list. You can also select the frame number.

Charts

The charts can display 6 different measurement values for all drawn-in measurement regions (this setting can be entered via a shortcut menu, which is opened by right-clicking):

- Mean gray value channel 1
- Mean gray value channel 1 minus background
- Mean gray value channel 2
- Mean gray value channel 2 minus background
- Ratio value
- Ratio value minus background

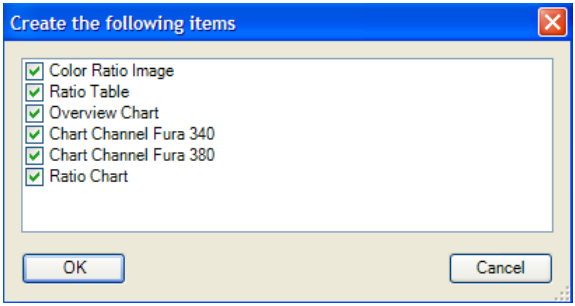
➢ **Chart settings**: The three charts next to channel 1, 2 and ratio image (two in the case of single channel experiments) can be configured by right-clicking on a chart and selecting **Properties** upon which a **Chart Settings** dialog is opened:



The dialog is divided into three tabs which allow the configuration of the chart parameters such as changing the default title, specifying a specific value range for X- or Y-axis, displaying or hiding legend and title or specifying tick marks. A **Reset** button resets the chart configuration to its default setting.

Creation of output documents

When all settings are made, clicking on **OK** will prompt the opening of the following dialog

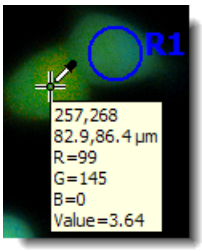


- Check all documents you want to be created as result of the measurement. In most cases at least the **Color Ratio Image** as well as the **Ratio Table** containing all measurement data will make sense to keep.

- The data can be saved in the generic CSV file format or in an XML format, which can be opened directly with Microsoft Excel (2003 edition necessary). AxioVision has basic charting features for all data tables which can be accessed via the **Chart View** button at the bottom of the table.

- The ratio image is a 24 Bit color image including all ROIs as annotation. Several annotations are available to help create informative movies for presentation. The Ratio range can be displayed using the LUT Scale annotation, both speed and event markers can be added as well.

All annotations can be found in the **Annotation menu** ⇒ **Frequent annotations**. The **Show values** picker tool  additionally shows the ratio value of each pixel for ratio images aiding the data evaluation:



- The resulting charts may be useful to quickly put together a presentation of the data. They can be saved as EMF (enhanced meta file) or WMF (Windows meta file) data. Both formats are vector based so that they can be inserted in programs like Microsoft PowerPoint and rescaled without loss of quality.

Clicking on **OK** closes the measurement dialog, the output documents are created and displayed as unsaved documents in AxioVision.

Information for Physiology

This function opens a dialog window, where you can find in tabular form important information in connection with the physiology experiment. The information it contains bear always on the current experiment and will be updated during each sequence of the experiment.

Show

In the drop-down list you can select, whether only an overview or all available information are shown. To get more explanations to the existing information, you can select an item. In the bottom area of the dialog you will find more explanation.

Information

In the **Information Current Physiology Experiment** dialog, various items of information about the currently active Physiology experiment are shown, i.e. expected image size, available hard disk space etc. These values do not relate to open image documents or streaming files.

Show

Using the drop-down list box it is possible to select whether you want an overview only (**Reduced information**) or all information (**All information**) to be displayed. All parameters relating to the current experiment are presented in list form. For further explanations on the meaning of the entries, click on an entry. You will then see an explanation in the text box at the bottom edge of the dialog.

9.11 FRET Analysis in the Physiology Module

Method

The FRET technique allows you to quantify the distance between two molecules conjugated to different fluorochromes.

By combining FRET (Fluorescence Resonance Energy Transfer) with optical microscopy, it is possible to obtain quantitative temporal and spatial information about the binding and interaction of proteins, lipids, enzymes, DNA and RNA in vivo. Usually, these processes lie below the resolution of an ordinary microscope. Thanks to the recent development of numerous variants of green fluorescent proteins (mutant GFPs), it is possible to use FRET to measure the interaction of intracellular molecular groups in intact living cells where the donor and acceptor fluorescent dyes are actually part of the molecules themselves.

However, FRET microscopy measurements currently suffer from many different sources of distortion, which need to be corrected. These include direct excitation of the acceptor at the donor excitation wavelength and the dependence of FRET on the concentration of the acceptor.

Software package

The Carl Zeiss AxioVision FRET software is part of the Physiology module and offers you a choice between several of the methods described in the literature for determining FRET (see section 9.10 Physiology Measurements "Examples of FRET formulae"). Each method includes the acquisition of time lapse image series with the option of determining the FRET amount inside selected regions. These regions are defined using circles with variable sizes or are drawn in freehand. The results of time lapse series can be displayed as time-dependent curves for each individual region. In addition, color-coded images are created for each time point.

Depending on the method selected, these color-coded images are calculated for each pixel individually and either show an intensity value for FRET (Fc – Youvan), a FRET ratio image in relation to donor * acceptor (Fn – Gordon), a FRET ratio image in relation to the square root of donor * acceptor (NF – Xia), or in relation to the acceptor (Acc) or donor (Don) alone.

As an alternative method, FRET can be measured after bleaching of the acceptor by calculating the increase in the donor emission (BL – Siegel). It is also possible to enter your own methods using the formula generator.

Either a motorized Axio Imager or an Axio Observer microscope can be used – these will serve as an outstanding input unit. The method for direct FRET measurement uses a combination of three filter sets: the donor, FRET and acceptor filter set. These filters have been specially developed to isolate and maximize three signals: the donor fluorescence, the acceptor fluorescence dependent on FRET and the directly excited acceptor fluorescence. The Fast Acquisition and Dual Camera modules are available for rapid image acquisition.

FRET determination using correction methods

As donors such as CFP generally show a long-wavelength tail in their emission spectrum, there is significant fluorescence at YFP emission wavelengths from CFP alone. It is therefore difficult to determine FRET accurately using conventional measurement methods without making use of mathematical corrections.

In the AxioVision Physiology module it is possible to choose between five different methods for direct FRET measurement. Youvan's method (Fc) measures the intensity of the FRET image and performs a correction by means of the proportion of donor and acceptor direct excitation. Two additional samples are necessary to determine these correction factors. In the first sample the cells must be labeled with the donor fluorescent dye only, and in the second with just the acceptor fluorescent dye. For these samples the correction factors for crosstalk can be determined very accurately (see 9.10 Physiology Measurements containing user formulae for the "donor correction factor" and "acceptor correction factor").

Youvan's method forms the basis for all 3-channel correction measurement techniques in AxioVision FRET. It results in an intensity value being determined. The directly measured FRET intensity from the FRET filter set is corrected by the crosstalk from the donor alone and from the acceptor alone. Firstly, however, the intensity from each individual filter set is corrected by the relevant background value.

The following formula is used for the calculation:

$Fc = (fret_{gv} – bg_{fret}) – cf_{don} * (don_{gv} – bgd_{on}) – cf_{acc} * (acc_{gv} – bg_{acc})$

Fc = FRET concentration according to Youvan et al. (1997)
- gv = intensity as gray value
- bg = background intensity
- cf = correction factor
- fret = FRET image
- don = donor image
- acc = acceptor image

With Gordon's method (Fn) this result is normalized to the direct donor and acceptor signal. The result is a ratio value. FRET is determined according to Youvan's method, as described above, and the result is divided by the product of the donor and acceptor intensity.

$Fn = Fc / [(don_{gv} – bg_{don}) * (acc_{gv} – bg_{acc})]$

Fn = FRET concentration according to Gordon et al. (1998)
- Fc = FRET concentration according to Youvan et al. (1997)
- gv = intensity as gray value
- bg = background intensity
- don = donor image
- acc = acceptor image

Due to the multiplication of donor and acceptor, however, this method actually calculates the ratio twice, with the effect that, in many regions, relatively low intensities lead to extremely high FRET values.

The third method according to Xia (NF) is similar to Gordon's method, but uses an adapted formula. Here, FRET according to Youvan is divided by the square root of the product of donor and acceptor. In this way the ratio is calculated only once, and FRET is determined independently of the relevant donor and acceptor concentration.

$NF = Fc / sqrt [(don_{gv}–bg_{don}) * (acc_{gv}–bg_{acc})]$

Fn = FRET concentration according to Xia et al. (2001)
Fc = FRET concentration according to Youvan et al. (1997)
sqrt = square root
gv = intensity as gray value
bg = background intensity
don = donor image
acc = acceptor image

The fourth method calculates the FRET ratio in relation to the acceptor alone (Acc). Here, the FRET value according to Youvan is divided by the acceptor intensity only.

$Acc = Fc / (acc_{gv}–bg_{acc})$

Acc = FRET concentration as acceptor ratio according to Malkusch (2004)
Fc = FRET concentration according to Youvan et al. (1997)
gv = intensity as gray value
bg = background intensity
acc = acceptor image

As with Xia's method, here too the ratio is only calculated once, but the FRET amount is dependent on the acceptor concentration. This results in relatively low FRET values with high acceptor intensities and high FRET values with low acceptor concentrations, due to the division.

The fifth method calculates the FRET ratio in relation to the donor alone (Don). Here, the FRET value according to Youvan is divided by the donor intensity only.

$Don = Fc / (don_{gv}–bg_{acc})$

Don = FRET concentration as donor ratio according to Vanderklish et al. (2000)
Fc = FRET concentration according to Youvan et al. (1997)
gv = intensity as gray value
bg = background intensity
don = donor image

As with Xia's method, here too the ratio is only calculated once, but the FRET amount is dependent on the donor concentration. This results in relatively low FRET values with high donor intensities and high FRET values with low donor concentrations, due to the division.

FRET determination using acceptor bleaching method

As an alternative and relatively simple way to determine the absolute efficiency of FRET between the donor (CFP) and the acceptor (YFP), it is possible to measure the donor emission before and after selective bleaching of the acceptor. The increase, or dequenching, of donor emission can be used as a direct measure of the FRET efficiency.

$$BL = [1 - (don_{gv}bef - bg_{don\text{-}bef}) / (don_{gv}aft - bg_{don\text{-}aft})] * 100$$

BL =	FRET concentration by acceptor bleaching according to Siegel et al. (2000)
don =	donor image
gv =	intensity as gray value
bg =	background intensity
bef =	before bleaching
aft =	after bleaching

This method uses each cell or each subregion as its own internal standard and, according to the authors, is therefore far more accurate than comparing cells with unknown FRET with separate cells with very strong FRET or even no FRET at all. Using this method, values of just a few percent can be quantified with reasonable confidence.

The result is a percentage for the donor concentration, as only the donor is directly determined. The authors claim that this is the only genuinely quantitative method. However, it has decisive disadvantages when used in living cells, due to extremely small movements of the cells themselves or of the cell organelles inside the cells.

FRET determination using formula generator

With the help of the formula generator it is possible to make use of new or your own techniques for determining FRET. FRET determination according to Zal is used here as an example:

$$Eapp = Fc / [(fret_{gv} – bg_{fret}) – cf_{don} * (don_{gv} – bg_{don}) + 2.0 * (don_{gv} – bg_{don})]$$

Eapp = FRET concentration according to Zal et al. (2004)
Fc = FRET concentration according to Youvan et al. (1997)
gv = intensity as gray value
bg = background intensity
don = donor image
fret = FRET image

The channel images are entered into the formula by means of the channel numbers used (ch1, ch2, ch3) and the associated background values are entered accordingly as bg1, bg2, bg3. If the various time points are related to an identical reference image (as with the acceptor bleaching method), this calculated reference image can be entered into the formula as f0. The associated background value is available under bgf0.

You would therefore integrate the FRET analysis according to Zal into the formula generator as follows:

((ch3–bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)) /
((ch3–bg3) – 0.19*(ch1-bg1) + 2.0 * (ch1-bg1))

The formula generator can also be used to calculate the correction values for the donor and acceptor crosstalk:

Donor (cf_{don}): (ch3-bg3) / (ch1-bg1)
Acceptor (cf_{acc}): (ch3-bg3) / (ch2-bg2)

Giving the results as a percentage the numbers have to be multiplied by 100.

Examples of FRET formulae for the formula generator

FRET according to Youvan DC et al. (Biotechnology 3, 1997)
(ch3-bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)

FRET donor ratio method (PNAS 97, 2000)
((ch3-bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)) / (ch1-bg1)

FRET acceptor ratio method (Bioscience Technology 01, 2004))
((ch3-bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)) / (ch2-bg2)

FRET according to Gordon GW et al. (Biophysical Journal 74, 1998)
((ch3-bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)) / ((ch1-bg1) * (ch2-bg2))

FRET according to Xia Z et al. (Biophysical Journal 81, 2001)
((ch3-bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)) / sqrt((ch1-bg1) * (ch2-bg2))

FRET via acceptor bleaching (%) according to Siegel RM et al. (Science's stke, 2000)
100 * (1 – (f0-bgf0) / (ch1-bg1))

FRET according to Zal T et al. (Biophysical Journal 86, 2004)
((ch3–bg3) – 0.19*(ch1-bg1) – 0.09*(ch2-bg2)) /
((ch3–bg3) – 0.19*(ch1-bg1) + 2.0 * (ch1-bg1))

Donor crosstalk calculation
(ch3-bg3) / (ch1-bg1)

Acceptor crosstalk calculation
(ch3-bg3) / (ch2-bg2)

FRET (2-channel donor ratio via FRET and donor) according to Gordon (without correction factor)
(ch3-bg3) / (ch1-bg1)

FRET (2-channel donor ratio via acceptor and donor) according to Gordon (without correction factor)

(ch2-bg2) / (ch1-bg1)

FRET (2-channel donor ratio via FRET and donor with correction factor)

((ch3-bg3) – 0.19*(ch1-bg1)) / (ch1-bg1)

Samples

With AxioVision FRET it is possible to use both fixed and living cells for determining FRET. Fixed cells are suitable for analyses on an upright as well as on an inverse microscope. For analyses involving living cells, an inverse microscope is usually the most suitable option, as living cells are generally examined in Petri dishes.

All applications for the correction methods require, for each new experiment (each new preparation series), at least one sample labeled with the donor only and one labeled with the acceptor only – in addition to the actual FRET specimens. These are used to determine the correction factors in order to compensate for crosstalk. The precise method for measuring the correction factors is described under the formula generator.

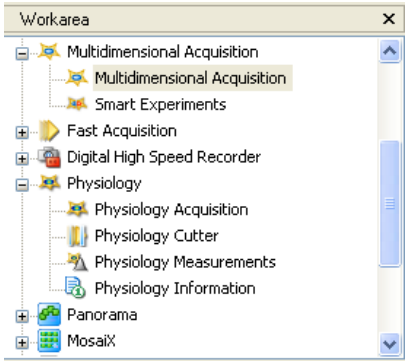
Basic settings for image acquisition

Multichannel time lapse images are always required for FRET analyses. The settings for image acquisition are defined in the Multichannel Setup function. For precise instructions, please refer to the description there.

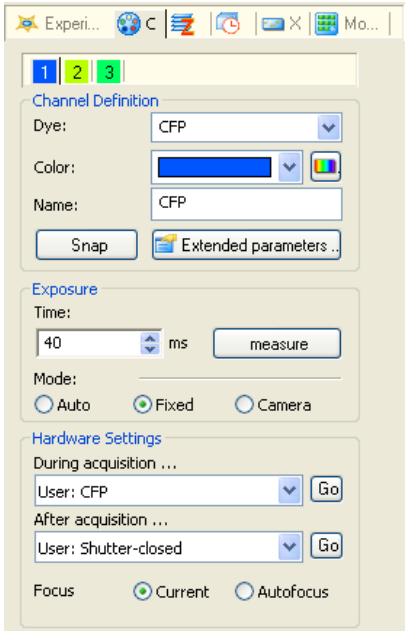
The correction methods always require 3-channel images (donor, acceptor and FRET image), or two image groups (donor and acceptor before and after bleaching) for the acceptor bleaching method. There is also a range of methods that perform FRET determination with just 2 channels (FRET – Donor, FRET – Acceptor, Donor – Acceptor; see 9.10 Physiology Measurements: "Example formulae of FRET calculations"). Longer time lapse images are needed if histograms or time lapse movies are desired.

Image acquisition for AxioVision FRET analyses

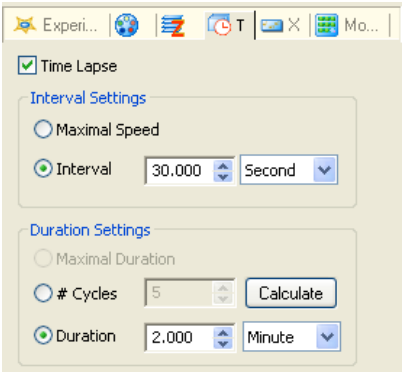
The settings for the multidimensional image series for offline FRET analyses are made using the Multidimensional Acquisition function. Detailed explanations can be found in the "Multidimensional Acquisition" section.



- All the settings for each channel are made on the **C** (Channel) property page.
- When you select the fluorochrome under **Dye,** the color and channel name are determined automatically. You can change these default settings.
- If you click on the **measure** button in the **Exposure** field, a window for determining the appropriate exposure time is opened automatically. All predefined hardware settings are set at the same time.
- All the specific settings for all channels can be checked and adjusted in the overview under **Extended Parameters**.

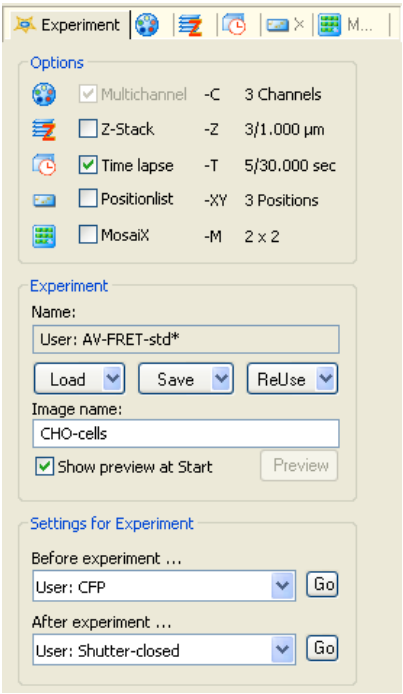


- For Physiology FRET measurements, time lapse images are required. The intervals for the acquisition of the multichannel images can be entered on the **T** (Time Lapse) property page.



- The **Time Lapse** check box must be selected for these to be used in the experiment. The intervals can be defined as required, in any unit of time. It is possible to predefine the length of the experiment by specifying the number of cycles or the duration.

- On the **Experiment** property page, under an experiment name, you can save all settings from all property pages, together with the hardware settings before and after the experiment, so that you can use them again.



- The options that you have selected for the experiment are also shown here, to allow you to check them.

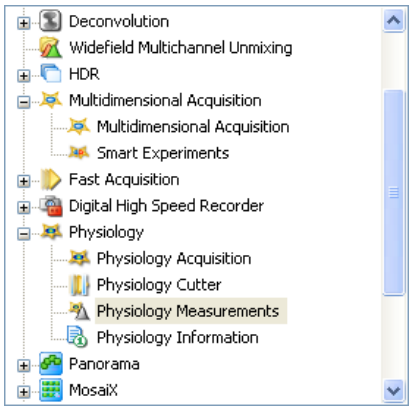
- Using the **ReUse** button you can read out the settings of a saved image and reuse them as an experiment, provided that the settings were saved with the image.

- Start image acquisition by clicking on the **Start** button.

FRET measurement in the AxioVision Physiology module

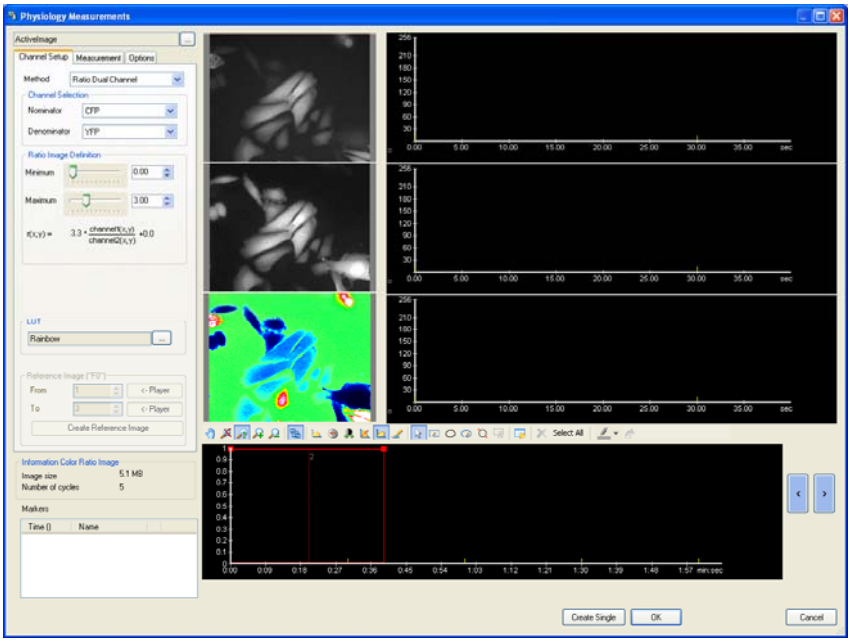
- Before you start the FRET measurement, you need to open a multichannel time lapse image that is suitable for the FRET measurement in the AxioVision image window.

- Clicking on the **Physiology Measurements** function in the **Physiology** module gives you access to the ratio and FRET measurements.



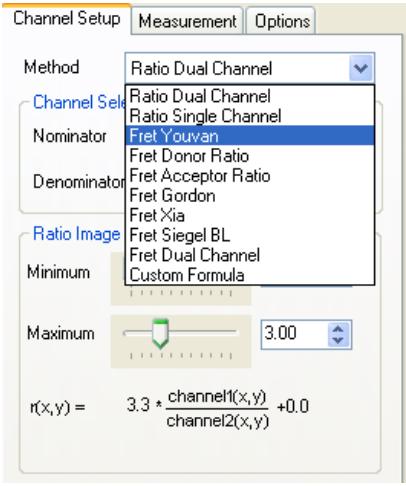
The **Physiology Measurements** window is opened with the last settings that were used.

The meanings of the various windows on the property pages differ according to the method selected.



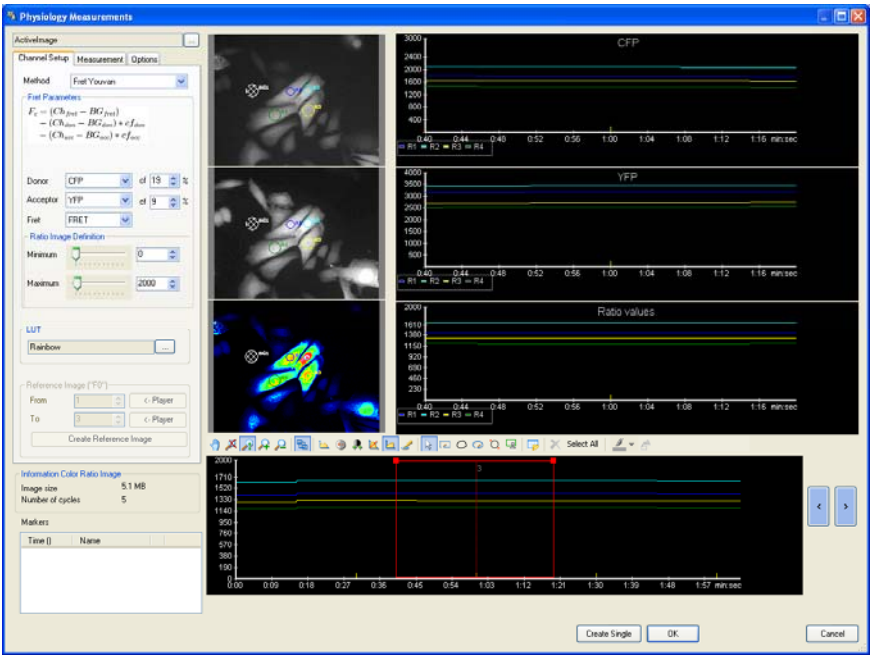
- Select the desired method using the **Method** drop-down list box.

- The FRET functions are initially explained below using the example of the correction method according to Youvan. For the methods that follow this one, reference is only made to any differences that apply.



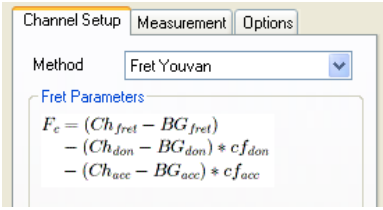
FRET measurement according to Youvan

The **Physiology Measurements** dialog window displays the property pages containing the corresponding settings, the two raw images for the donor and acceptor channel and also the color-coded FRET ratio image. Next to each image is a corresponding chart window in which the results display for the signals is shown. The overview chart presents the results for the entire experiment. All functions for working with the images and charts are arranged on a toolbar.



Functions of the Physiology window for FRET measurements

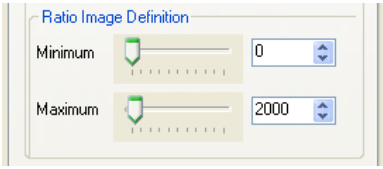
- The **Fret Parameters** window on the **Channel Setup** property page first shows the formula used for the selected method of performing FRET measurements (Ch = channel, BG = background, cf = correction factor, fret = FRET signal, don = donor signal, acc = acceptor signal).



- In the **Donor**, **Acceptor** and **Fret** drop-down list boxes you can select the filter sets used in each case. Using **cf** you can define the correction factor for the crosstalk in percent (%).



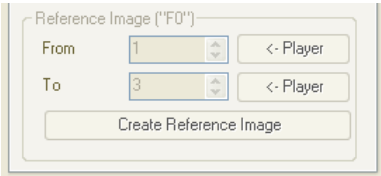
- In the **Ratio Image Definition** field you set the range for the FRET color coding. For Youvan the value ranges depend on the bit depth of the camera used. In the example an AxioCam MRm with 12 bits was used. Initially, half of the bit depth can be entered as a maximum starting value.



- Various lookup tables are available for the color coding. The **Rainbow** LUT is usually used for the FRET color coding.

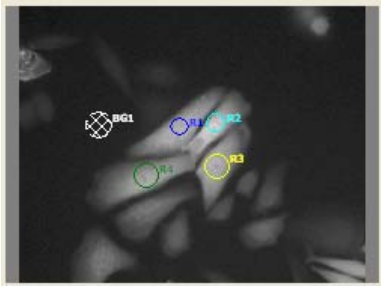


- The **Reference Image ("F0")** has no significance for the FRET correction methods. It is always used if the ratio calculation is related to a starting image. For FRET measurements this is the case with the acceptor bleaching method.



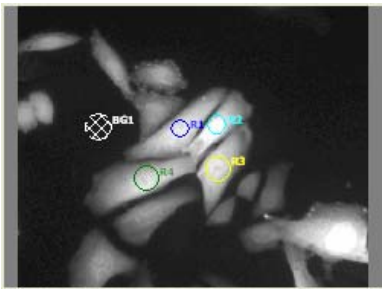
Donor image

Using the function keys it is possible to define regions for the measurement in each image. Function key 23 allows you to redefine a region as the region for measuring the background.



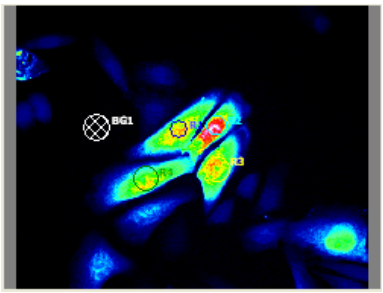
Acceptor image

The regions are generated in parallel in all three image windows. To allow more precise positioning, it is also possible to increase the zoom factor of the images in parallel (function keys 2 - 5) and adjust their contrast (function keys 7 - 11).

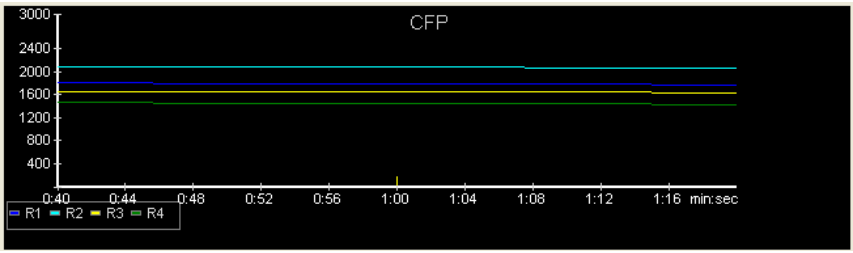


FRET color-coded image

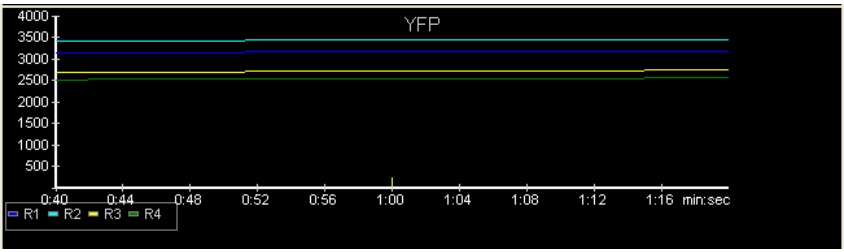
The color coding is carried out in accordance with the LUT selected using the range set under Ratio Image Definition.



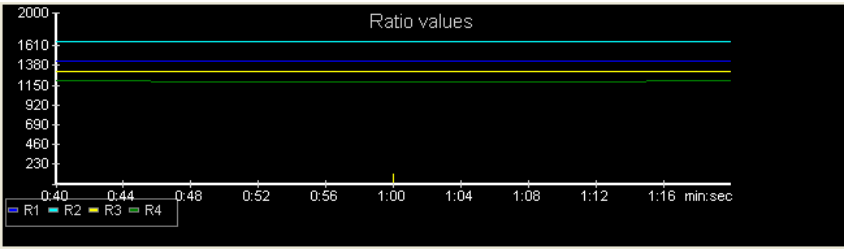
Part of the **Donor** signal curves. The part in question is defined in the overview image:



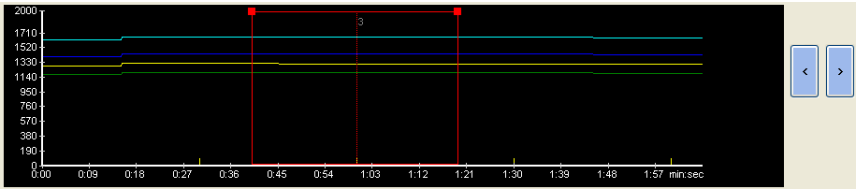
Part of the **Acceptor** signal curves. To change the colors of the curves, it is possible to select the corresponding regions using the left-hand mouse button. The color can then be changed using the color selection function key (22):



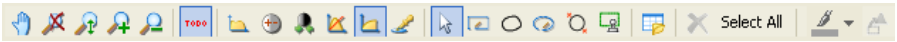
For the FRET calculation according to Youvan the **ratio values** represent gray values. The scale range of the Y-axis therefore depends on the bit depth of the input image (8, 12, 14 or 16 bits).



Overview image of the entire duration of the FRET experiment. The size of the window defining the part of the curves displayed can be changed by using the cursor to adjust the corner points. Its position can be moved using the two arrow keys or the left/right keys on the keyboard.



Function keys for FRET measurement





1 Opens the pan mode with the cursor



2 Displays the images with no zoom factor



3 Displays the image so that it fills the image windows



4 Increases the zoom factor (F7)



5 Decreases the zoom factor (F8)



6 Shows/hides the measurement regions



7 Shows/hides the gray value histogram in the donor and acceptor image



8 Best fit distribution of the gray value images between Min and Max, minus tolerances



9 Min/Max distribution of the gray value images



10 Linear distribution of the gray values over the entire range


11 Gamma setting of gray value curve to 0.45


12 Display of pixel values at cursor position


13 Object selection mode (Alt F1)


14 Draws a rectangle at the position defined using the cursor and with the properties set (Alt F2)


15 Draws a rounded external contour


16 Draws an ellipse at the position defined using the cursor and with the properties set


17 Draws a circle at the position defined using the cursor and with the properties set


18 Copies a region with the same size and shape


19 Shows the measurement value table


20 Deletes all regions (Del)



21 Activates all regions (Ctrl+A)

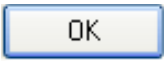

22 Assigns a color to a region and line in a chart


23 Defines the selected region for the background measurement

Buttons



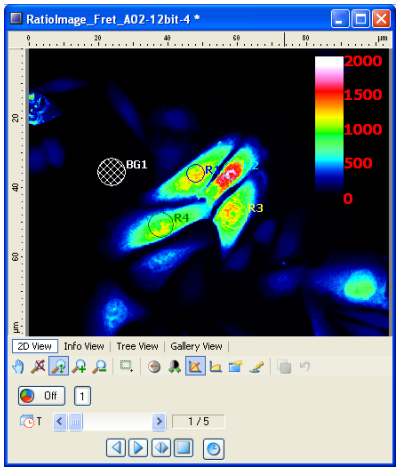
transfers the color ratio image to AxioVision as a ZVI image and leaves the Physiology window open.



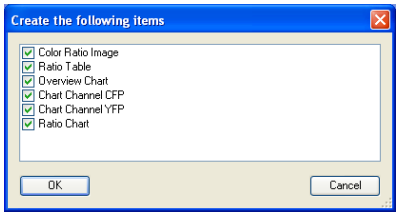
transfers all images, charts and tables selected during the confirmation stage to AxioVision in separate windows and closes the Physiology window **Physiology Measurements**.

Cancel closes the Physiology window and takes you back to AxioVision.

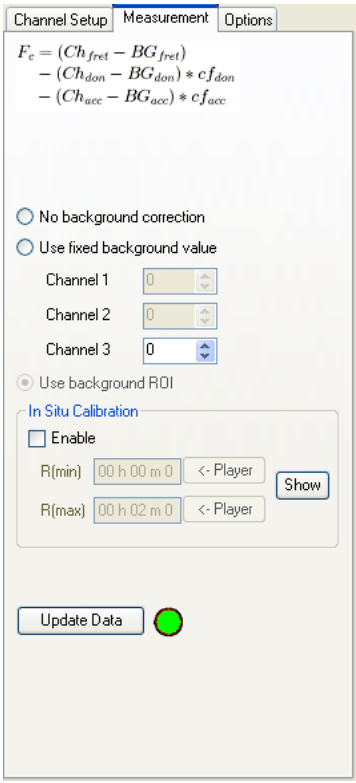
- The color-coded FRET image transferred to AxioVision displays all regions for the FRET measurements and the background. If you click on **Create Single**, only the color image with the regions is transferred. If you carry out the transfer using the **OK** button, a color scale with value indications is added to the left of the image. You can use the cursor to activate this, change its size and move it to the desired position.



- Click on the **OK** button. All the required tables, charts or images can then be selected in a selection window.



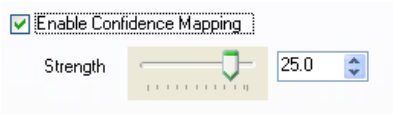
- After changes are made to the settings of the Min/Max values under **Ratio Image Definition,** to the correction factors or to the channel selection, the calculation must be performed again in order to update the FRET values in the table. This is done on the **Measurement** property page by clicking on the **Update Data** button.



- Once the new calculation has been performed successfully, the color of the status circle changes to green.

- Such an **update** is always required if the color of the circle is yellow and the results are to be transferred to AxioVision.

- Some FRET calculations may result in very high FRET values, particularly in the area of the background. These can become visible in the form of white noise against the dark background. You can suppress this effect by activating the **Confidence Mapping** option on the **Options** property page (according to Tsien RY, 1990).

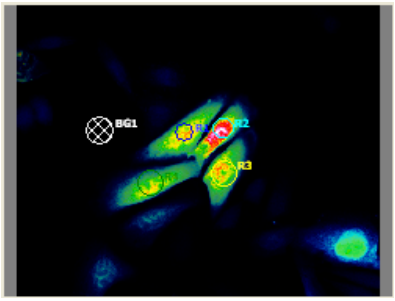


- The extent of the background elimination can be influenced by moving the **Strength** slider.

The **Confidence** value is calculated according to the following formula:

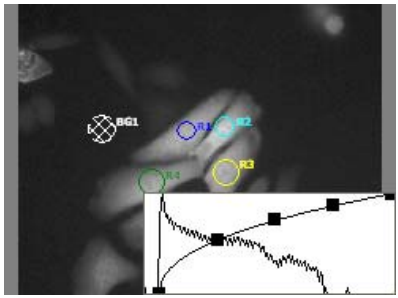
$$\frac{\text{channel1} * \text{channel2}}{2 * \text{intensity maximum}}$$

This confidence value is multiplied by each red, green and blue value for the color display.



- By showing the **gray value histogram** (function key 7) in the donor or acceptor image you can also modify the gray value distribution manually by using your mouse to adjust the five points.

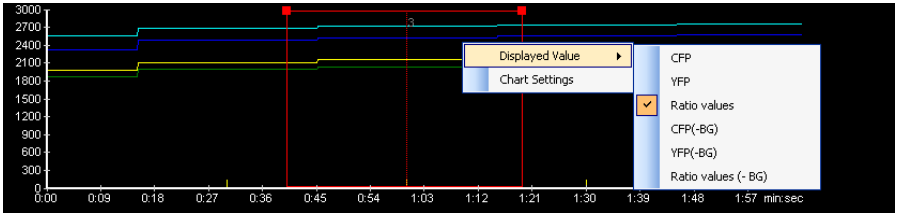
- The middle point influences the brightness, the outer points the contrast and the two intermediate points the gamma setting.



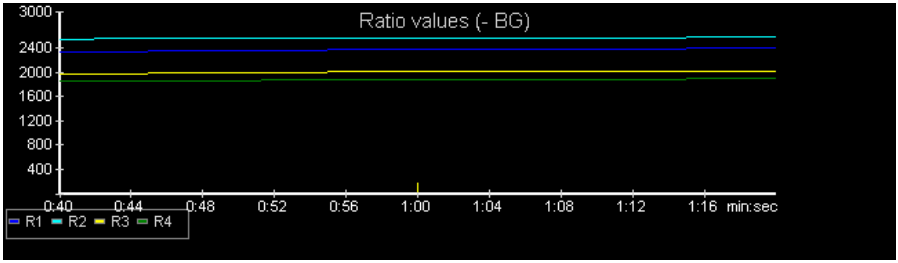
In the **data table** (function key 19) the donor, acceptor and ratio values are listed without and with background correction (**-BG**) for all time points and for each region.

#	TimeStamp	R1_CFP	R1_YFP	R1_FRET	R1_Ratio values	R1_CFP(-BG)
0	00:00:00.000	1745.40	2838.11	1954.84	1367.78	1454.35
1	00:00:30.000	1768.66	3005.90	2002.29	1395.71	1476.43
2	00:01:00.000	1748.71	3059.88	2000.16	1392.52	1456.91
3	00:01:30.000	1728.34	3079.52	2001.31	1395.77	1436.83
4	00:02:00.000	1704.94	3088.68	1986.04	1384.12	1414.11

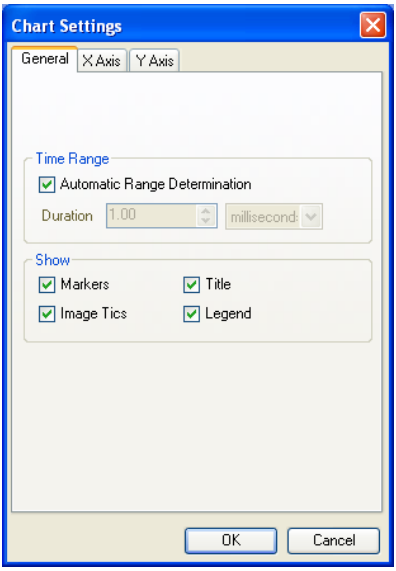
In the charts the curves are initially displayed without background correction.



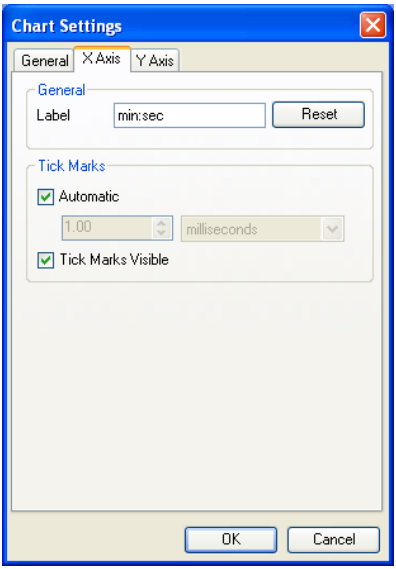
You can call up the display with background correction in each window via the right-hand mouse button.



The appearance of the charts can be adapted via the **Chart Settings**, which can be called up for each chart using the right-hand mouse button.

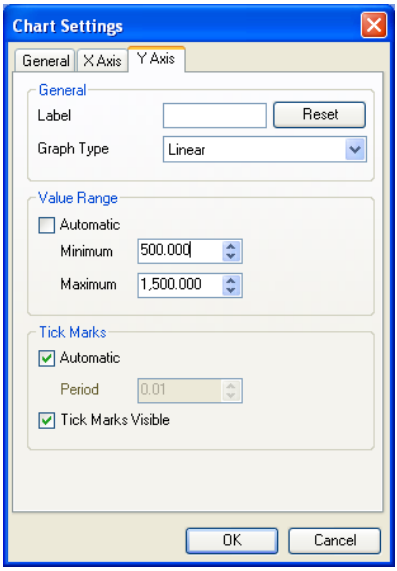


- In the **Time Range** field you can determine the range automatically or manually.
- In the **Show** field on the **General** property page you can show and hide the display of the markers, image-acquisition time points, chart title and legend.



- In the **General** field on the **X Axis** property page you can enter the **Label** for the X-axis. This entry can be made in text form via the keyboard. The **Reset** button resets the label to the original text.
- In the **Tick Marks** field you can also define the time periods for the marks manually. These marks can also be hidden here.

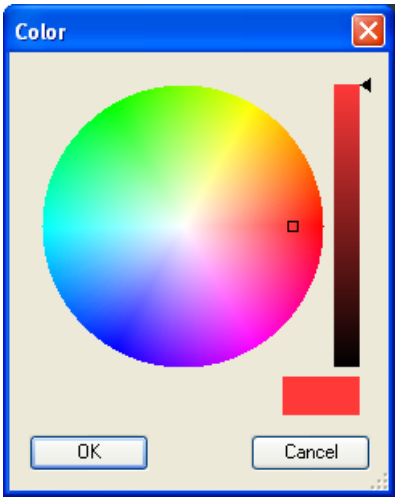
- On the **Y Axis** property page, again in the **General** field, you can enter a **Label** for the Y-axis in text form via the keyboard. It is also possible to choose between **Linear** and **Logarithmisch**.



- The value range can be determined automatically or manually in the **Value Range** field.

- In the **Tick Marks** field you can determine the periods for the marks automatically or manually and/or show or hide them.

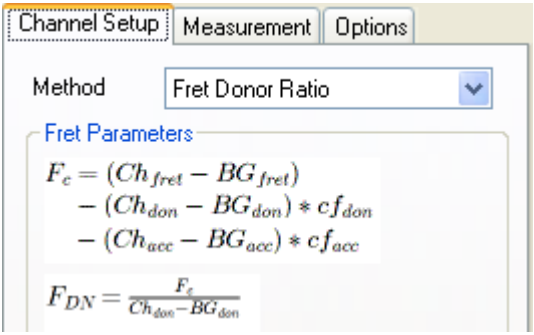
- When you select a region in object selection mode (function key 13), you can use the color selection function (function key 22) to assign a predefined color from a color list to the region and the corresponding curve, or you can select a color freely from the color circle in the **Color** window.



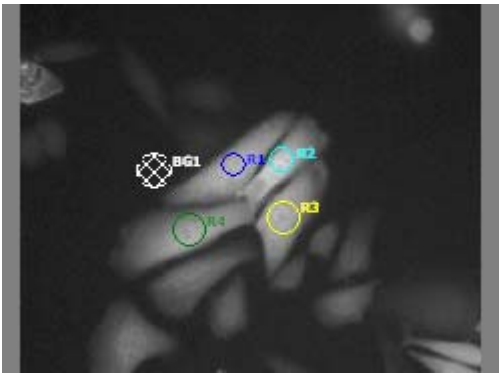
Other pre-programmed FRET methods

Donor Ratio

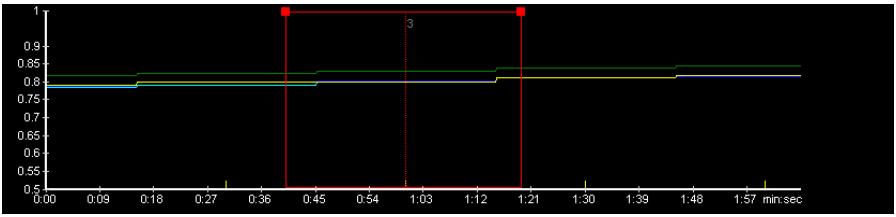
Selection from the **Method** drop-down list box. The formula used is displayed in the **Fret Parameters** field:



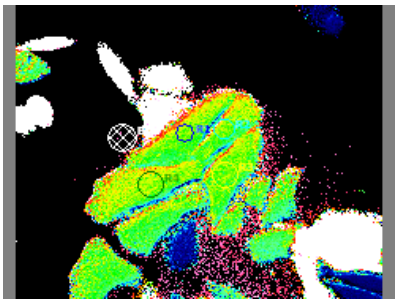
The measurement regions (R1 – R4) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23:



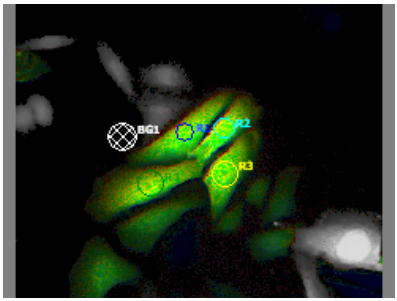
Results of the Donor Ratio FRET measurement. Genuine FRET values are usually between 0.0 and 1.0:



- Color-coded FRET ratio image for the Donor Ratio calculation. In regions where donor values are very low, the division can result in extremely high FRET values, which appear as white points of noise in the color-coded image.

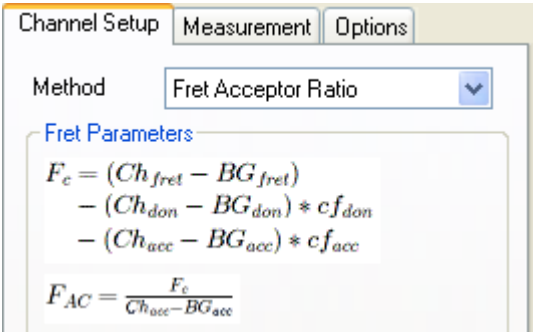


- If each pixel is converted using **Confidence Mapping** (according to Tsien RY, 1990), these points are displayed as very dark in the resulting image. The color coding is confined to the regions with a genuine FRET portion.

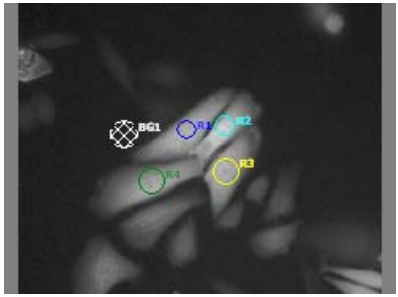


Acceptor Ratio

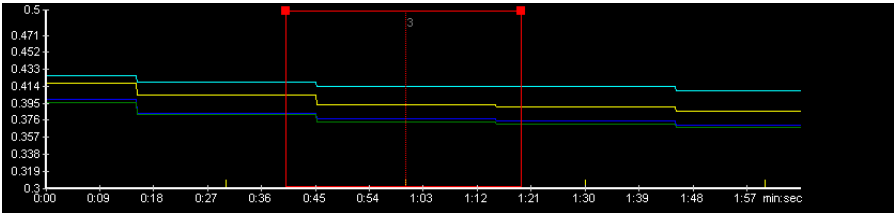
Selection from the **Method** drop-down list box. The formula used is displayed in the **Fret Parameters** field:



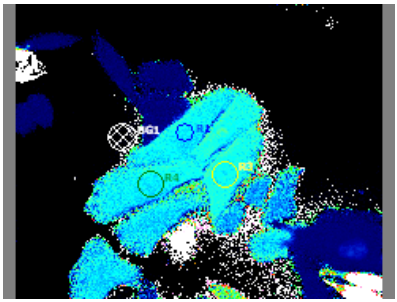
- The measurement regions (R1 – R4) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23.



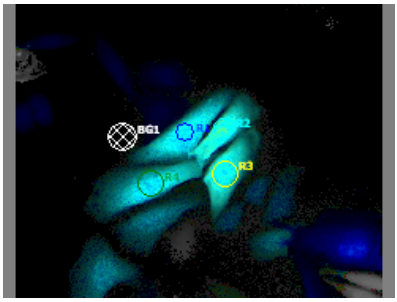
Results of the Acceptor Ratio FRET measurement. Genuine FRET values are usually between 0.0 and 1.0:



- Color-coded FRET ratio image for the Acceptor Ratio calculation. In regions where acceptor values are very low, the division can result in extremely high FRET values, which appear as white points of noise in the color-coded image.

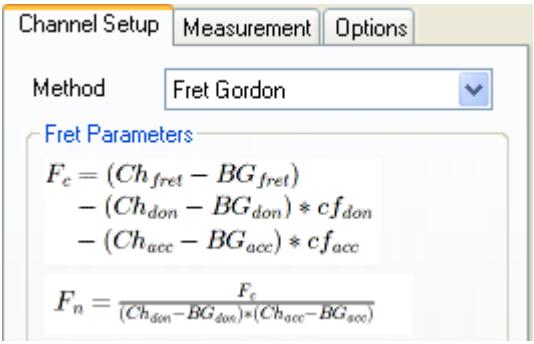


- If each pixel is converted using **Confidence Mapping** (according to Tsien RY, 1990), these points are displayed as very dark in the resulting image. The color coding is confined to the regions with a genuine FRET portion.

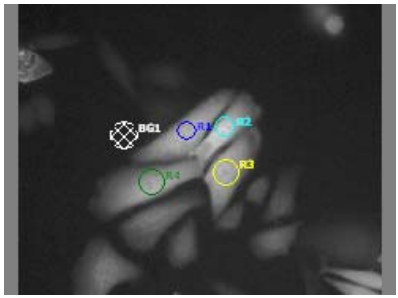


Gordon

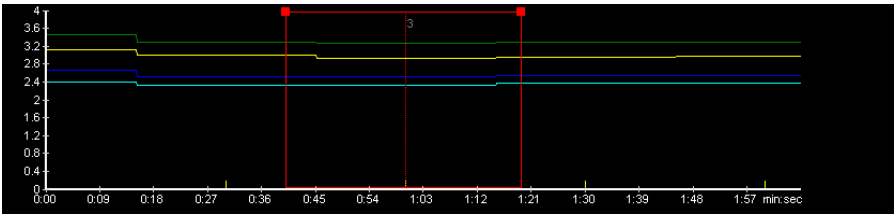
Selection from the **Method** drop-down list box. The formula used is displayed in the **Fret Parameters** field:



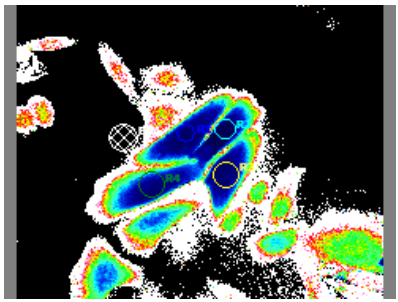
- The measurement regions (R1 – R4) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23.



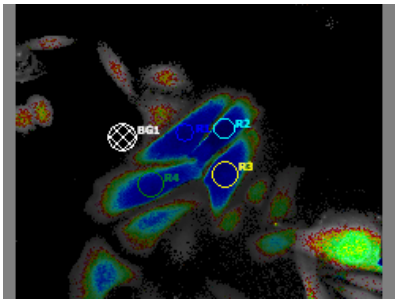
Results of the Gordon FRET measurement. In this case genuine FRET values are extremely low and depend on the bit depth of the acquired image. The Gordon values are therefore always multiplied by 10,000. For a 12-bit image, genuine FRET values are usually between 0.0 and 10.0:



- Color-coded FRET ratio image for the Gordon Ratio calculation. In regions where values are very low, the double division can result in extremely high FRET values, which appear as white points of noise in the color-coded image.

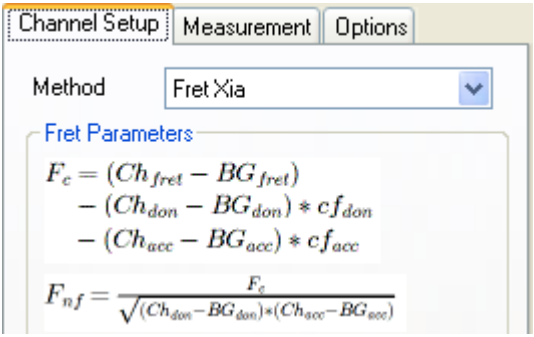


- If each pixel is converted using **Confidence Mapping** (according to Tsien RY, 1990), these points are displayed as very dark in the resulting image. The color coding is confined to the regions with a genuine FRET portion.

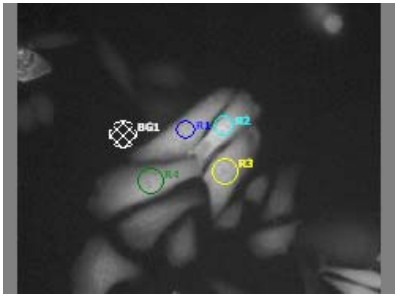


Xia

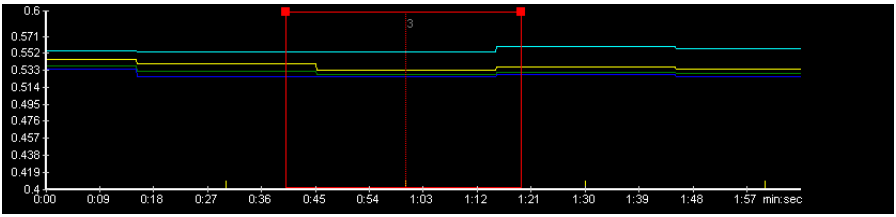
Selection from the **Method** drop-down list box. The formula used is displayed in the **Fret Parameters** field:



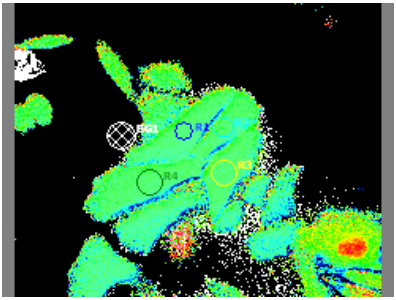
- The measurement regions (R1 – R4) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23.



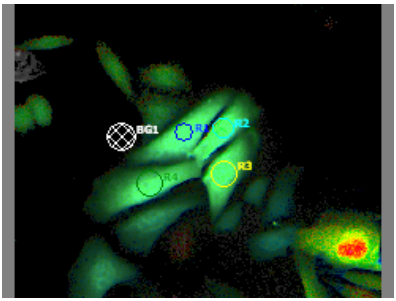
Results of the Xia Ratio FRET measurement. Genuine FRET values are usually between 0.0 and 1.0:



- Color-coded **FRET ratio image** for the Xia Ratio calculation. In regions where values are very low, the division can result in extremely high FRET values, which appear as white points of noise in the color-coded image.



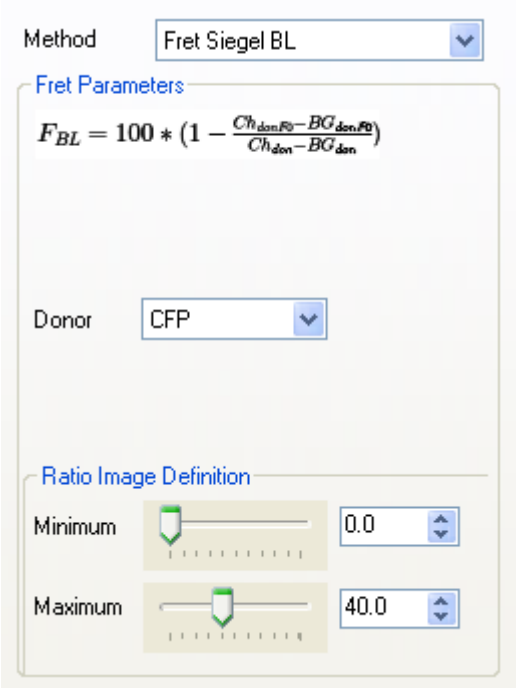
- If each pixel is converted using **Confidence Mapping** (according to Tsien RY, 1990), these points are displayed as very dark in the resulting image. The color coding is confined to the regions with a genuine FRET portion.



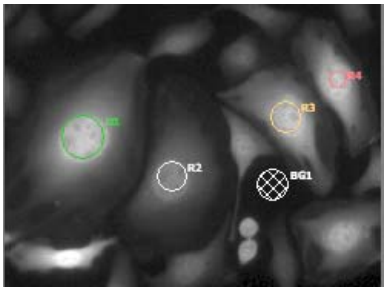
Siegel (FRET calculation via acceptor bleaching)

Selection from the **Method** drop-down list box. The formula used is displayed in the **Fret Parameters** field.

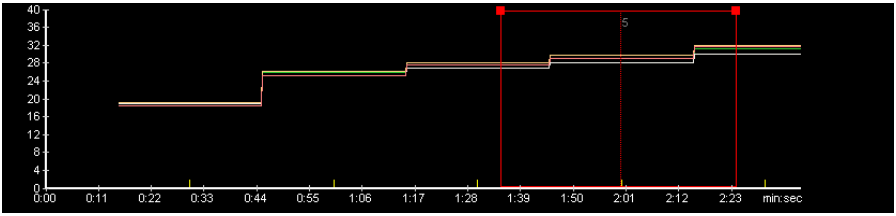
The FRET calculation is performed via the donor channel only. The acceptor channel is only acquired too so that the bleaching process can be checked.



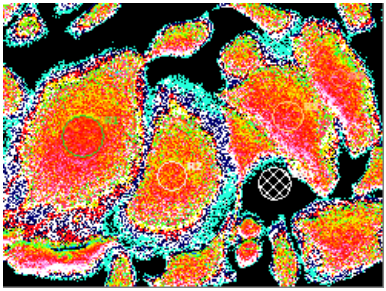
- The measurement regions (R1 – R3) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23.



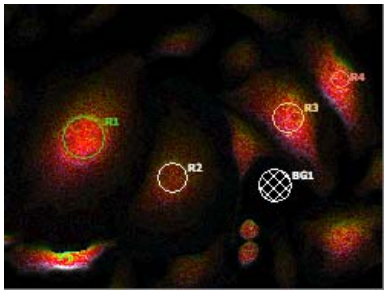
Results of the FRET measurement in percent. Genuine FRET values are therefore between 0.0 and 100.0:



- Color-coded **FRET efficiency image** of the calculation according to Siegel. In regions where values are very low, the division can result in very high FRET values, which appear as white points of noise in the color-coded image.

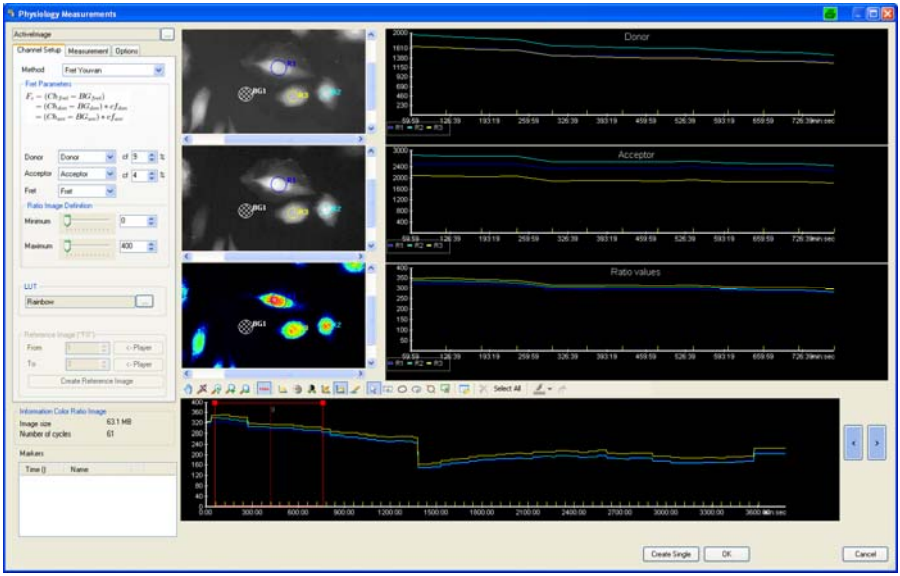


- If each pixel is converted using **Confidence Mapping** (according to Tsien RY, 1990), these points are displayed as very dark in the resulting image. The color coding is confined to the regions with a genuine FRET portion.



Analysis of long time lapse images

Example of an offline FRET analysis of a time lapse image (donor, acceptor, FRET) over 60 hours. The calculation was performed using the method according to Youvan:



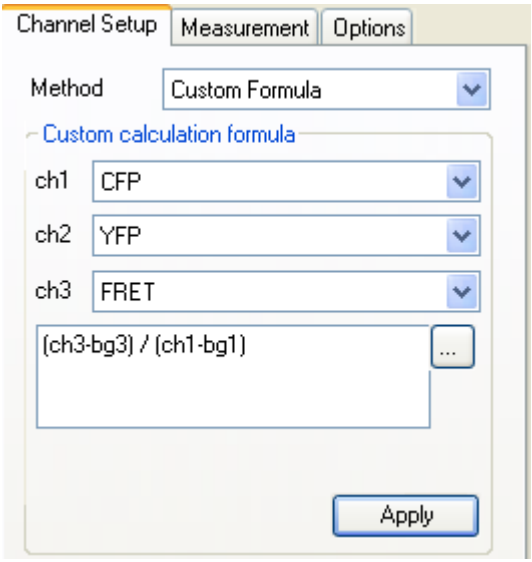
User-defined FRET measurements (formula generator)

Example of a 2-channel measurement

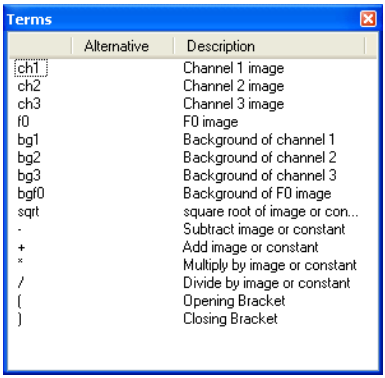
User-defined FRET analyses are possible with the help of the formula generator. This option is selected on the **Channel Setup** property page using the **Custom Formula** function.

In the **Custom calculation formula** field the corresponding channels (**ch1**, **ch2**, **ch3**) of the open image are selected from the drop-down list boxes. It is possible to define 2-channel, 3-channel or f0 analyses here.

The formula is entered into the formula field using the keyboard.

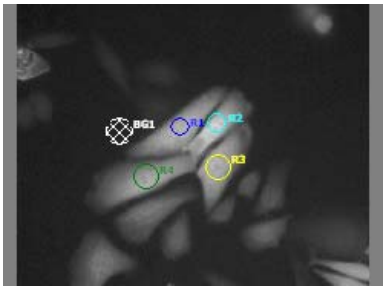


- The variables and operators for the formulae can be entered freely via the keyboard. Alternatively, they can be selected from the **Terms** list and inserted into the formula by double-clicking. The **Terms** list is called up using the ... button.

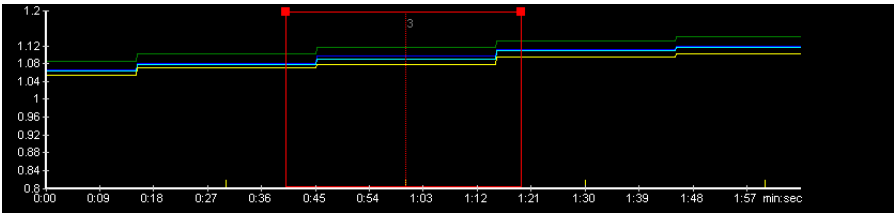


- Examples of user formulae with different correction methods are provided in 9.10 Physiology Measurements "Examples of FRET formulae " and can be copied into the input field via the online help.

- As with the pre-programmed methods, the measurement regions (R1 – R4) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23.

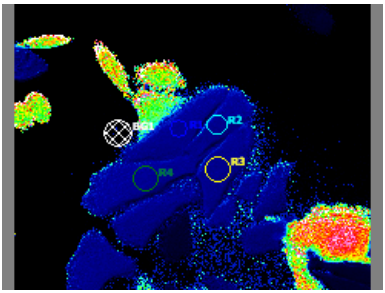


The range of the FRET values depends on the formulae used. In the example FRET was calculated by means of FRET / Donor with background correction:



- Color-coded **FRET ratio image** for the Donor Ratio calculation.

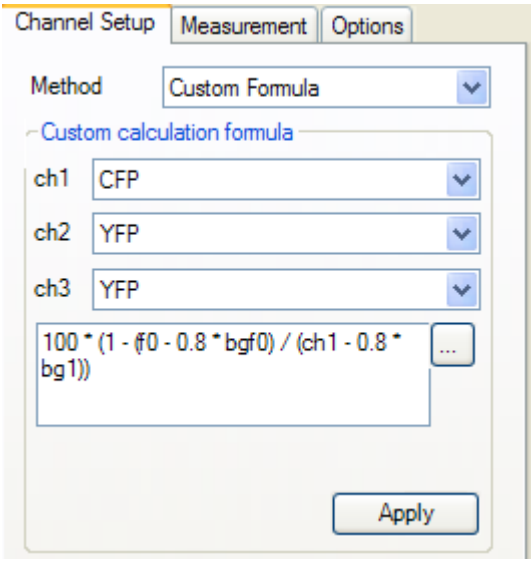
  (fehlerhaft)



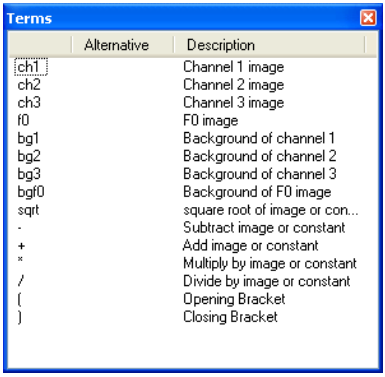
Example of an fO measurement

User-defined FRET analyses according to the acceptor bleaching method with constant correction to minimize the background. This option is selected on the **Channel Setup** property page using the **Custom Formula** function.

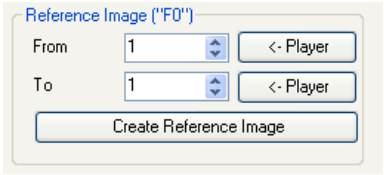
The formula is entered into the formula field using the keyboard (it can also be copied from the collection of formulae in 9.10 Physiology Measurements "Examples of FRET formulae for the formula generator"):



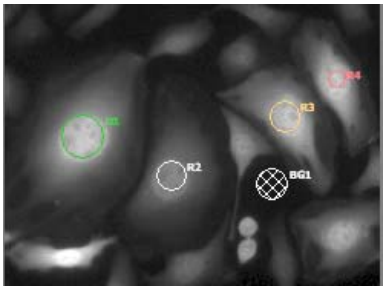
- The variables and operators for the formulae can be entered freely via the keyboard. Alternatively, they can be selected from the **Terms** list and inserted into the formula by double-clicking. The **Terms** list is called up using the ... button.



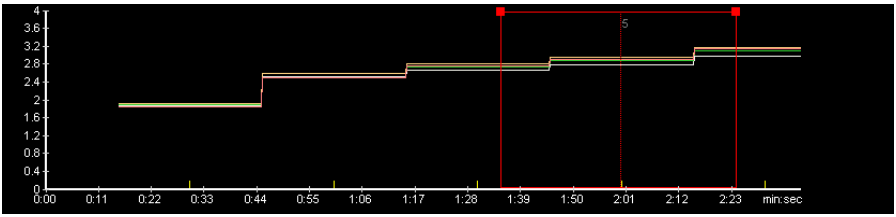
- With this method FRET is calculated in relation to the starting image (see section "Siegel FRET calculation via acceptor bleaching" on the acceptor bleaching method). The reference (f0) image can be calculated from the first 3 images of the time sequence using the **Create Reference Image** button on the **Channel Setup** property page.



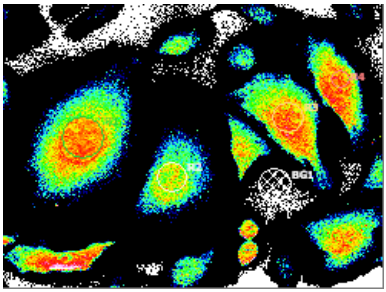
- As with the pre-programmed methods, the measurement regions (R1 – R3) are defined in the image windows via the mouse, using function keys 14 - 18. A region can be defined as the region for measuring the background value (BG1) using function key 23.



The range of the FRET values depends on the formulae used. In the example FRET was calculated as a percentage of the reference image f0:

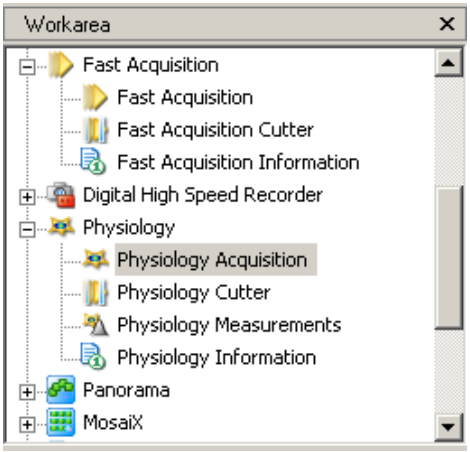


➢ Color-coded FRET ratio image for the Acceptor Bleaching calculation.



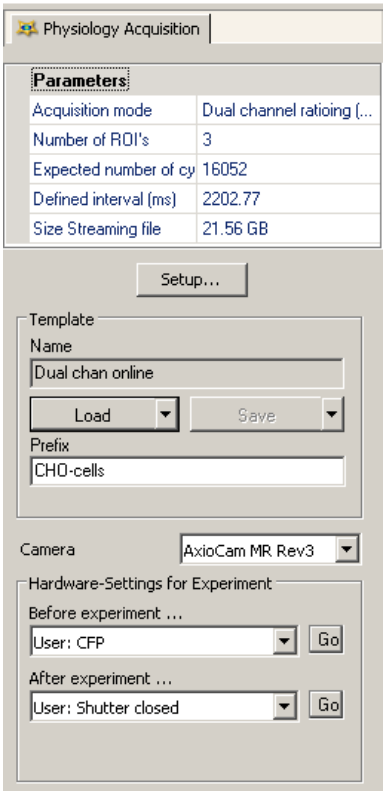
FRET online measurements

In the AxioVision Physiology module, **FRET online measurements** are accessed in the work area via **Physiology** and **Physiology Acquisition**.

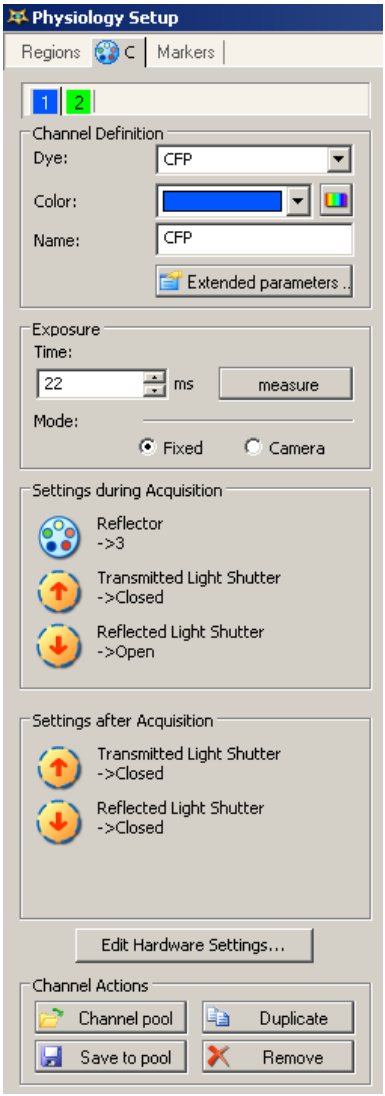


Other options for fast acquisition and fast online FRET analysis are described under the **Fast Acquisition** and **Dual Camera** modules.

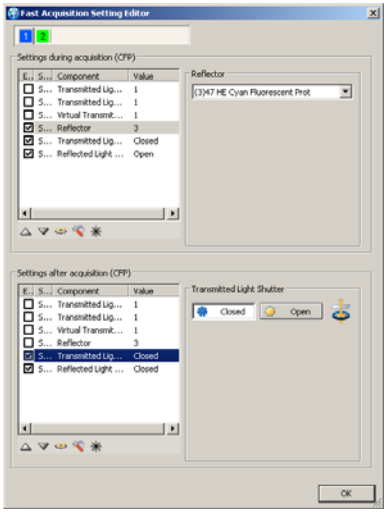
- The **Physiology Acquisition** page that now opens displays all important system information under the parameters.
- The settings for acquisition are defined using the **Setup** button.
- In the **Template** field you can load existing settings (**Load**) and save new ones (**Save**). The prefix defines the basic name for the multidimensional images to be acquired.
- In the **Camera** drop-down list box you can select the camera type from the installed cameras.



- Under **Physiology Setup** you define the channel properties according to the multichannel settings.
- In the **Dye** drop-down list box you select the fluorochrome.
- The channel name can be entered freely via the keyboard in the **Name** input field. The suggested channel color can also be changed, if required.
- Using the **measure** button you can determine the exposure time for the channel automatically or interactively.
- If you click on the **Edit Hardware Settings** button, the dialog window opens for changing the **Settings during Acquisition** and **Settings after Acquisition** parameters.
- A separate property page is created for each channel.

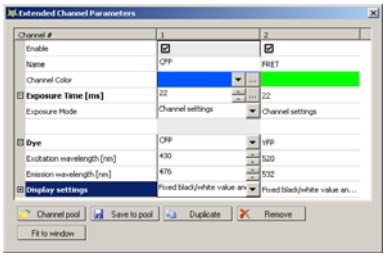


- Clicking on the **Edit Hardware Settings** button opens the **Fast Acquisition Setting Editor** dialog window. Here, for each channel, you can enter the settings during and after acquisition separately for all components concerned.



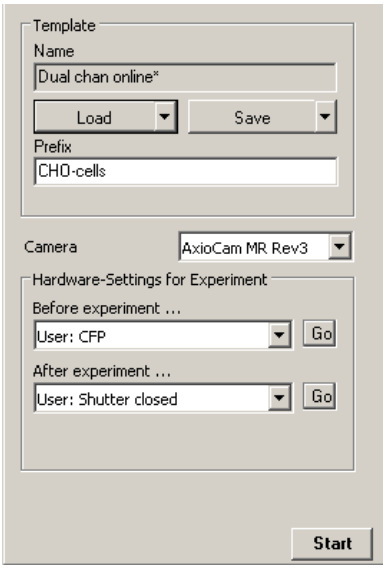
- All parameter settings are summarized in the **Extended Channel Parameters** dialog window.

- These settings can be called up using the **Extended Parameters** button in the **Channel Definition** field.



- Once all settings have been made, you can save the experiment with a **Name** in the **Template** field (**Save as** via the ▼ key). This means that the settings will be available again for later use.

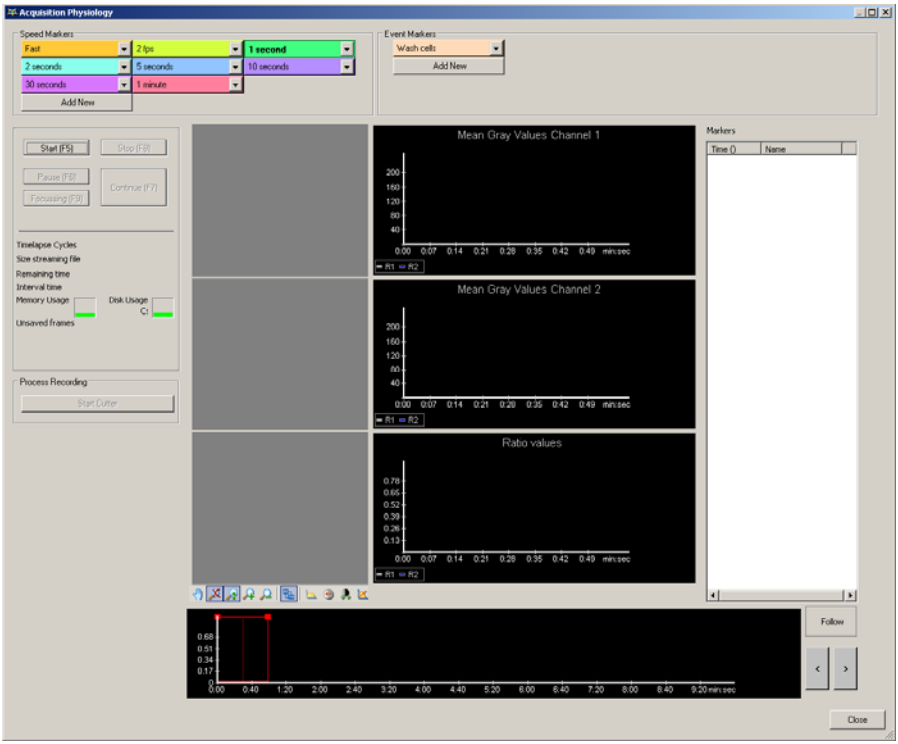
- Image acquisition is started by clicking on the **Start** button.



- The window for Physiology acquisition (**Acquisition Physiology**) is opened by clicking on the **Start** button.

- Before acquisition begins you can define additional **speed markers** and **event markers**.

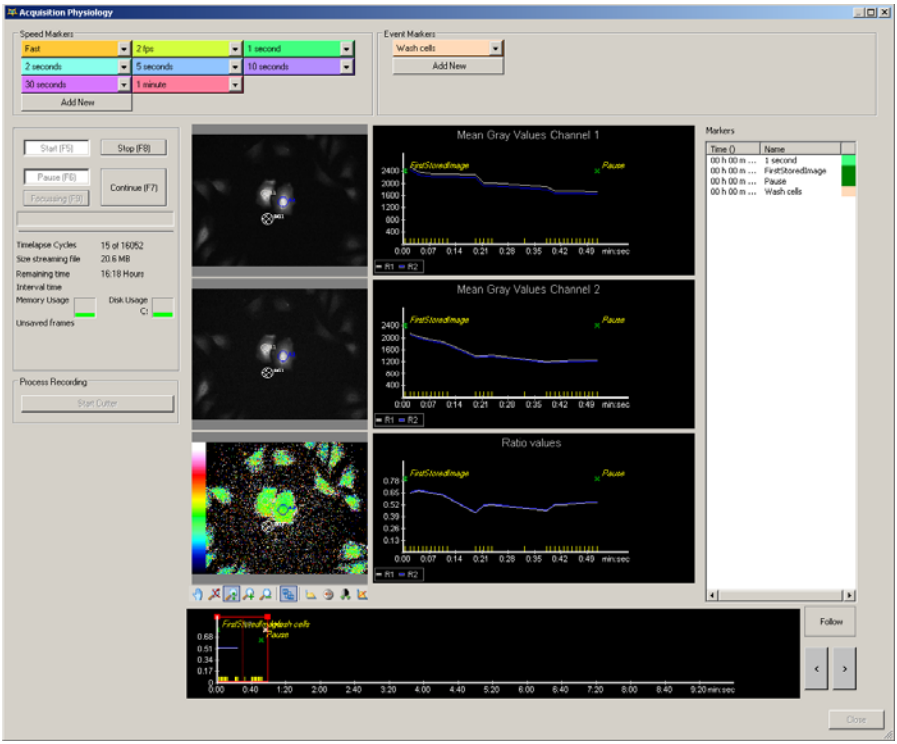
- The acquisition sequence is started by clicking on the **Start** button.



During the acquisition sequence the current gray value images of the donor and acceptor channel and the calculated ratio FRET color image containing the defined measurement regions are permanently displayed in the **image windows**.

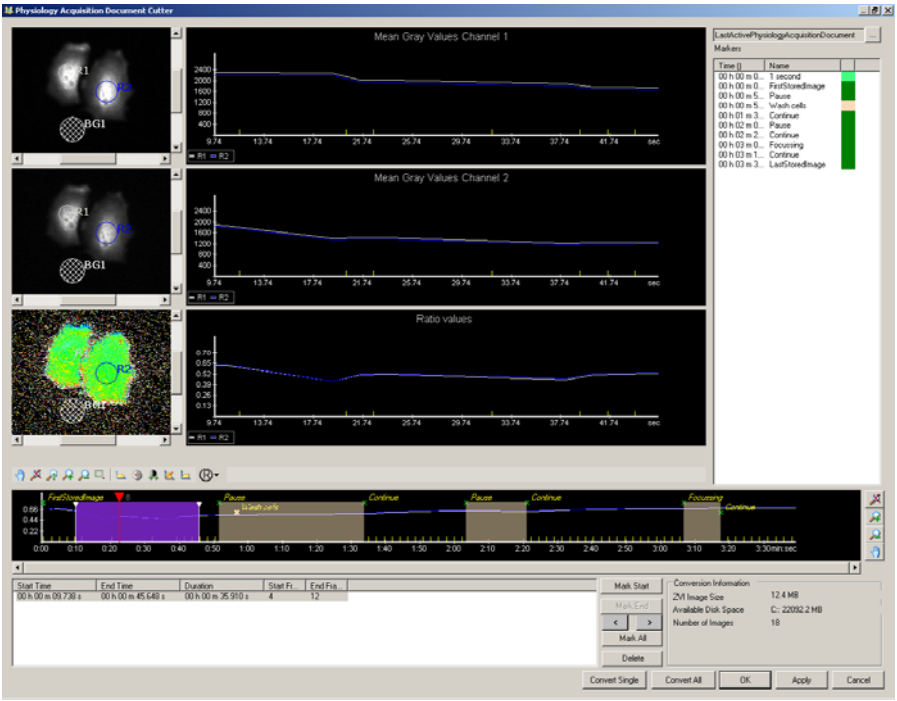
In the **overview window** the progression of the measurement results over time is displayed, whilst the **chart windows** contain the results in the region window selected in the overview.

In the event of interventions during the experiment, the corresponding **markers** can be adopted into the chart at any time with a click of the mouse.



After the experiment has ended the results can be reopened for further editing from the work area using the **Cutter** function.

Detailed descriptions of all the functions relating to **Physiology Acquisition** and the **Cutter** can be found in the description of the Physiology module.


Physiology Acquisition Document Cutter
Mean Gray Values Channel 1
Mean Gray Values Channel 2
Ratio values
R1
R2
BG1
Markers
Time ()
Name
00 h 00 m 0... 1 second
00 h 00 m 0... FirstStoredImage
00 h 00 m 5... Pause
00 h 00 m 5... Wash cells
00 h 01 m 3... Continue
00 h 02 m 0... Pause
00 h 02 m 2... Continue
00 h 03 m 0... Focusing
00 h 03 m 1... Continue
00 h 03 m 3... LastStoredImage
FirstStoredImage
Pause
Wash cells
Continue
Pause
Continue
Focusing
Continue
Start Time
End Time
Duration
Start Fr...
End Fra...
00 h 00 m 09.738 s
00 h 00 m 45.648 s
00 h 00 m 35.910 s
4
12
Mark Start
Mark End
Mark All
Delete
Conversion Information
ZVI Image Size
12.4 MB
Available Disk Space
C:: 22092.2 MB
Number of Images
18
Convert Single
Convert All
OK
Apply
Cancel

Literature

Bastiaens P I H, Majoul I V, Verveer P J, Söling H-D, Jovin T M: Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. The EMBO Journal 15 (1996) 4246-4253.

Förster T: Zwischenmolekulare Energiewanderung und Fluoreszenz. Annalen der Physik 2 (1948) 55-75.

Gordon GW, Berry G, Lang XH, Levine B, Herman B: Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy. Biophysical Journal 74 (1998) 2702-2713.

Malkusch W: Quantitative Measurements of FRET Using Standard Fluorescence Microscopy. Bioscience Technology 01 (2004) 32-34.

Siegel RM, Chan FK, Zacharias DA, Swofford R, Holmes KL, Tsien RY, Lenardo MJ: Measurement of Molecular Interactions in Living Cells by Fluorescence Resonance Energy Transfer Between Variants of Green Fluorescent Protein. Science's stke (2000) 1-6.

Tsien RY, Harootunian AT: Practical design criteria for a dynamic ratio imaging system. Cell Calcium 11 (1990) 93-109.

Vanderklish PW, Krushel LA, Holst BH, Gally JA, Crossin KL, Edelman GM: Marking synaptic activity in dendritic spines with a calpain substrate exhibiting fluorescence resonance energy transfer. Proceedings of the National Academy of Sciences USA 97 (2000) 2253-2258.

Xia Z, Liu Y: Reliable and Global Measurement of Fluorescence Resonance Energy Transfer Using Fluorescence Microscopes. Biophysical Journal 81 (2001) 2395-2402.

Youvan DC, Silva CM, Bylina EJ, Coleman WJ, Dilworth MR, Yang MM: Calibration of Fluorescence Resonance Energy Transfer in Microscopy Using Genetically Engineered GFP Derivates on Nickel Chelating Beads. Biotechnology 3 (1997) 1-18.

Zal T, Gascoigne RJ: Photobleaching-Corrected FRET Efficiency Imaging of Live Cells. Biophysical Journal 86 (2004) 3923-3939.

9.12 Dual Camera

Dual Camera is a module which allows the simultaneous viewing and acquisition of images from two identical AxioCam cameras. Major advantages of using two cameras simultaneously are:

- Acquisition is performed simultaneously, i.e., objects with dual color labeling which move very fast can be acquired without delay between channel 1 and channel 2
- Speed of acquisition is increased
- Observation of two spectrally variant signals such as infrared transmitted light techniques and fluorescence at the same time, useful for neurobiology and electrophysiology
- Ability to independently adjust exposure times for each camera

The module primarily enhances the acquisition functionality of the modules **Fast Acquisition** and **Physiology**. But Dual Camera also has its own dialog which produces two dual camera live images for simultaneous observation as well as auxiliary images which facilitate easy alignment of both cameras using a suitable calibration sample.

A suitable calibration slide is the so called Home slide (order number 453583), which features suitable markings, For a finer calibration beads stained with two fluorescent colors can be used, which are sold through Invitrogen/Molecular Probes e.g.

Dual Camera setup hints

Dual Camera looks for two identical cameras and, if finding such cameras, controls them simultaneously. The order in which both cameras are recognized in the system is determined by the cameras serial number.

Unfortunately it is not possible to easily derive this information by looking up the serial number at the label on the camera due to the way, the operating system interprets this number. Please follow the suggested steps in order to install two cameras for proper use with the Dual Camera module before configuring the cameras in the MTP2004 configuration program.

- Connect both cameras to separate Fire Wire channels on the computer (the current Zeiss workstations are equipped with the appropriate dual channel fire wire card) and start AxioVision. Select the first camera via **Acquisition** ⇒ **Select Camera** as the active camera. This should be the camera without a numeral in its name.

- Open the live image. The camera which is now active can be recognized by the red blinking LED. Label this camera as camera #1 and the other one as camera #2.

- If your computer is equipped with the **Digital I/O Board** as well as the **signal distribution box SVB-1** both cameras must be connected to it. Connect the camera trigger cable with the appropriate port on the **SVB-1**. Please see the Peripheral Devices Reference Guide which came with the installation for further details.

- Close AxioVision and open the configuration program MTB2004. Select the camera port to which the dual camera adapter has been attached and configure the correct magnification camera adapter (e.g. 1x Camera Adapter).

- From the list of possible cameras on the right select the camera without numbering in its name, e.g. AxioCam MR R3 and add it to the camera adapter.

- As soon as a camera name is selected in the configuration on the left, a text field **Camera Name** becomes active on the right. In the case of an AxioCam the default name here is the same as in the list of possible cameras. You can now change the name in any way you like, e.g. in order to add the proper emission filter and the port orientation (e.g. CFP-Camera-top). This name will be shown in several of the camera related dialogs in AxioVision, e.g. in the Live image window and also stored together with the image. Repeat this for the second camera.

- In order to use both cameras as dual camera select the check box **Use Dual Camera option (with 2 identical cameras only)**.

- In the second drop-down list box which should now be active you can now assign a camera to a channel in **Fast Acquisition**. The camera

selected for channel #1 will be used for channels #3 and #5, camera assigned to channel #2 will also be used for channel #4.

- Please remember, that this assignment determines, in which channel order the information from both cameras is being acquired. This is important in applications such as dual channel ratio imaging with the Physiology module, where during online measurement the ratio is always determined as channel #1 over channel #2. (See **Fast Acquisition ⇒ C (Channels)** property page.)

The following AxioCam models are supported:

- AxioCam HSm/c
- AxioCam MRm/c rev. 3 (FireWire version)
- AxioCam HRm/c rev. 3, (FireWire version)
- PVCam Roper QuantEM (FireWire version only)

Other cameras such as the AxioCam MRc5, the AxioCam ICc models or other third party cameras are not supported currently.

Open **Dual Camera Live** from the **Acquisition** menu (see the following description).

Layout

Dual Camera Live is divided into 6 areas. Live images for Camera #1 and Camera #2 are shown next to each other, while a color merge image and a subtract image are shown beneath the live images. Both can be turned off and are mostly useful for alignment purposes. To the left of the dialog two property pages are available to adjust both cameras. Finally at the bottom edge of the dialog a toolbar contains the most important tools for adjusting exposure and display curves and a status bar shows the performance of both cameras.

Live images

By right mouse click you can access a context menu which allows turning all live images on or off. This way, you can configure the Dual Camera Live dialog in any way you want.

Color Merge Image

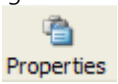
The Live images of both cameras are pseudocolored and shown as a merged overlay. The image of camera #1 is shown in red and the one from camera #2 in cyan to enhance visual differentiation. The colors are fixed and cannot be changed.

Subtract Image

The subtract image mainly serves the purpose of aligning both cameras. The goal is to achieve an image as homogenously gray and with as little contrast as possible. Even smallest differences will be displayed in an enhanced fashion.

The calculation occurs as follows: Images from both cameras are converted into 8 bits, then normalized to each other and then subtracted from each other. A fixed value of 128 is added to the resulting image which will then have intensities somewhere between 0 and 255. Those data are then spread out to fill the full 8 bit range. A perfectly aligned image should be of a homogeneous gray.

Dual Camera property pages

You can access the property pages either via the context menu (right mouse click) or via the toolbar button 

. In the **Dual Camera Live** dialog you can only adjust the most important parameters such as exposure time and camera resolution. All other adjustments such as shading correction or camera image orientation etc. must be done using the standard camera property pages and will subsequently be used by **Dual Camera Live**.

Adjust property page

- **Camera #1: Exposure**: Here you can adjust the exposure time for the first camera. Normally it is recommended to define fixed exposure times for fast acquisition with dual camera. If the **Dual Camera Live** dialog is used for observation only it is also possible to use **Auto Live** for automatic adjustment of the exposure time. You can also define, which

percentage of the dynamic range of your cameras sensor should be filled by the exposure measurement algorithm. For fluorescence images this value can be as low as 20 percent without compromising image quality.

- **Camera #2 Exposure**: identical to Camera #1

- **Dual Camera Frame Shift**: Here it is possible to correct small shifts between both cameras. The upper slider controls lateral shift while the lower slider controls vertical shifts. Maximum shift range is -128 to 128 pixels. This is done by shifting the region of interest on the camera sensor. When using binning, the minimum step size which can be influenced using these sliders will become coarser with increases binning steps and may not give the best results.

- **Automatic**: This button tries to determine the optimal shift between the two cameras. It works best in cases, where structures are clearly seen as identical by both cameras. Automatic only corrects lateral and vertical shifts but not rotational shifts

Frame property page

- Here you can adjust the binning as well as select a ROI to be used for acquisition. Please see help for the camera you are using for more details.

Toolbar

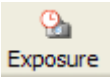
A Toolbar is available with the following functions:



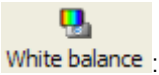
: Opens and closes the live images.



: Creates a new, unsaved 2-channel ZVI image.



: Performs an exposure measurement for both cameras at the same time.



: In the case of color cameras performs an automatic **White Balance**.



: Adjusts the display line with the **Best Fit** function for a stronger contrast with 0.1% value settings (see help for **Best Fit**).



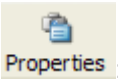
: Performs a **Min/Max** adjustment of the display line.



: Makes a linear adjustment of the display line.



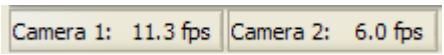
: Creates a gamma value of 0.45.



: Opens the property pages on the left side of the dialog.

Status bar

The status bar displays the performance of each camera in frames per second (fps):



10 Image Processing Modules

10.1 Imaging Plus

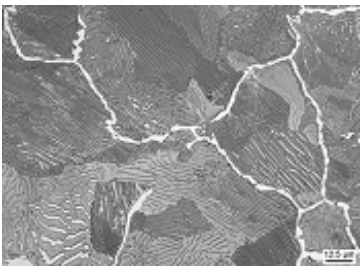
General

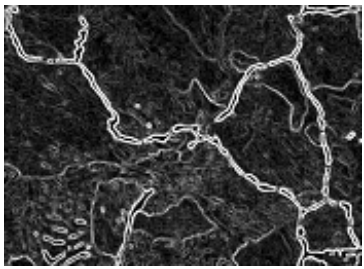
As already described in chapter 5 "Image Processing", image processing functions have a range of different tasks. Besides the "traditional" areas of application of correction and optimization, which have already been mentioned, there is also a third area: data extraction. Here, for example, structures are highlighted in relation to the image background to make them more visible, or objects are purposefully detected so that they can be measured later.

The Imaging Plus module expands the image processing functions of the basic program with the following functions and functional groups.

Image enhancement

Additional functions to enhance contrast, brightness and color, plus filters for smoothing, sharpening and edge detection with user-definable filter operators.

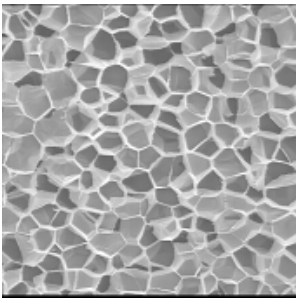


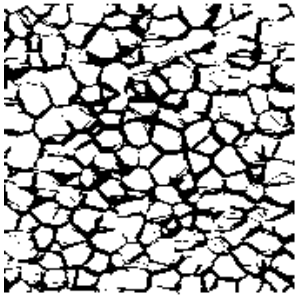


Edge detection with Sobel filter

Gray morphology

The gray morphology functions enable you to reconstruct the boundaries of connected objects.





Through the reconstruction of grain boundaries images are prepared for automatic measurement.

Image arithmetics

AxioVision enables you to combine and compare images, as well as extract information, by means of the image arithmetics functions in Imaging Plus.

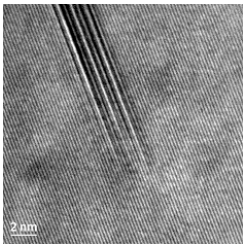


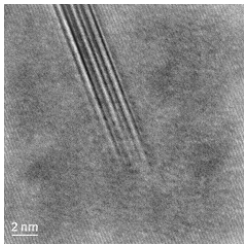




Fourier transformation

Fourier transformation allows you to restore images by purposefully filtering out defined frequencies.

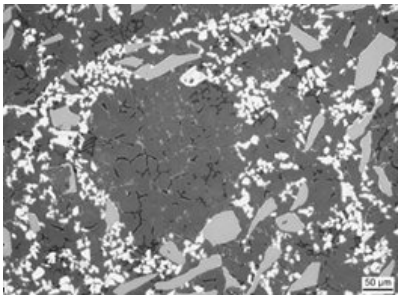




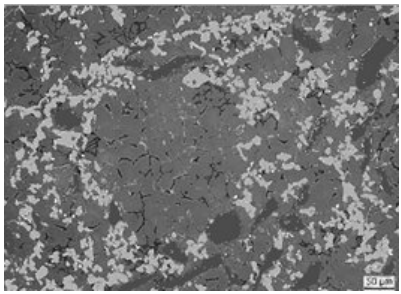
With Fourier transformation it is possible to purposefully remove (right) sinusoidal super positioning (left), for example.

Color space transformation

Color discrimination enables you to separate object and image background more effectively on the basis of color. This makes it possible e.g. to distinguish better between individual classes of object.



Bright field image of a composite material, contrasted with PtOx



Heterochromatic super positioning of two different phases

Working with image processing functions

The principal working method for the functions of the Imaging Plus module is described in chapter 5 "Image Processing".

The chapter also contains background information on the image processing functions and storage, etc. We recommend that you work through this chapter as a basis for applying the functions of the Imaging Plus module. A description of the individual functions and their parameters can be found in the online help.

The individual steps in the following examples can also be practiced using the original images that were employed. The sample images can be found in the folder *Demo Images and Scripts\AxioVision Images* of the Product DVD.

Reducing or enlarging images or image regions

The **Resample** function can be used to reduce or enlarge an entire image or a freely selectable region of an image. The reduction or enlargement is defined using a zoom factor (**Scale**). If only a region is being reduced or enlarged, the center of the region (**Shift**) must be indicated.

The size of the resulting image can be adapted (**ForceSizes** = **On**), depending on the scaling factors (**Scale**). You can, however, also generate the resulting image in the size of the original image (**ForceSizes** = **Off**).

It is also possible to take the influence of the scaled neighboring pixels into account (**Interpolation**).

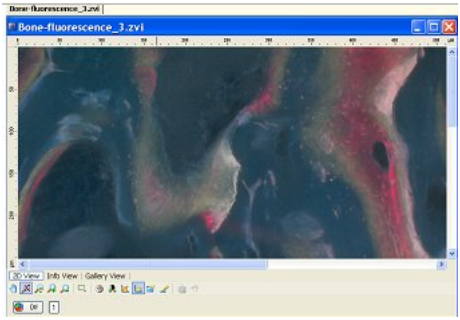
Note:

- If you simply want the entire image to be displayed in reduced or enlarged form without a new image being generated, this is also possible using the **+ Zoom** and **- Zoom** functions from the image window's shortcut menu.

The following example explains step by step how to reduce an image or enlarge a region of an image.

- Load the image "Bone-fluorescence_3.zvi" via the function **File** ⇒ **Open Image**, or acquire an image with the camera.

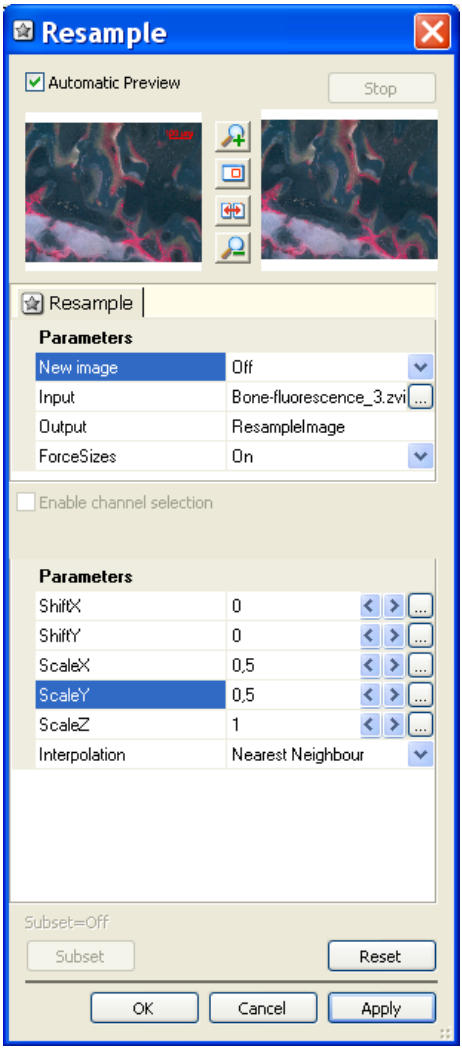
- The image is displayed in AxioVision's image window.



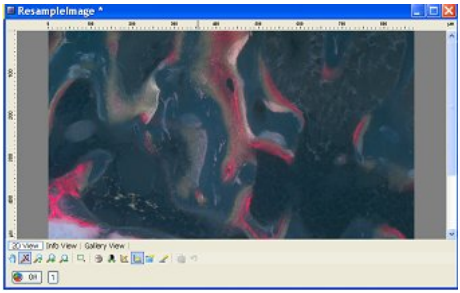
In the first example, the loaded image is to be reduced by the factor 0.5. In the second example it is to be enlarged by the factor 2.

Now select from the **Processing** menu the **Utilities** functional group and then the **Resample** function.

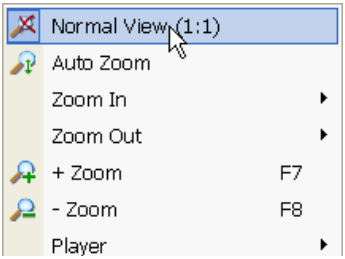
- You will now see the dialog window of the **Resample** function.
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.
- Set **CreateNew** to **Off** so that you can select the images freely.
- Set the following values by clicking on the arrow keys or by entering the values directly: **ForceSizes**=On, **ShiftX**=0, **ShiftY**=0, **ScaleX**=0.5, **ScaleY**=0.5, **ScaleZ**=1, **Interpolation**= NearestNeighbour.
- Click on **OK** to close the dialog.



- The resulting image shows all the structures of the original image, but has been reduced by the factor 0.5.
- The image size has been adjusted accordingly, as **ForceSizes** was set to **On**.



- Position the cursor over the resulting image, press the right-hand mouse button and select **Normal View (1:1)** shortcut menu.

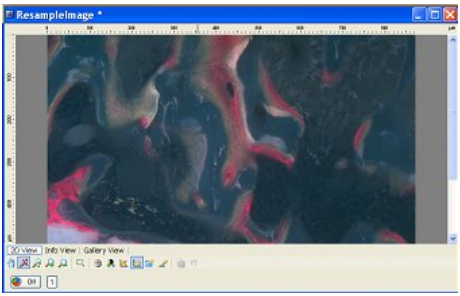


- Move the scroll bar to adjust the desired area.
- Do the same with the original image.
- Compare the two images. In the normal view of the image the difference can be clearly seen.

Original image

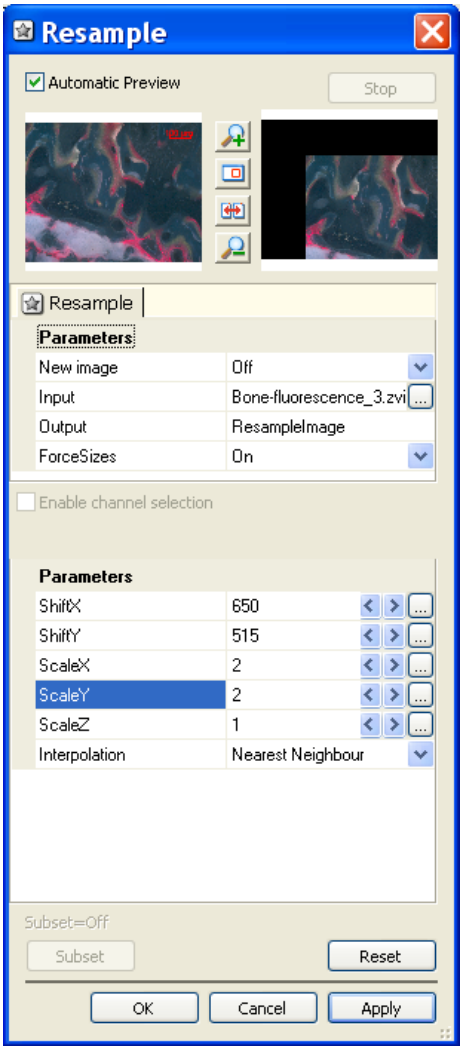


Resulting image



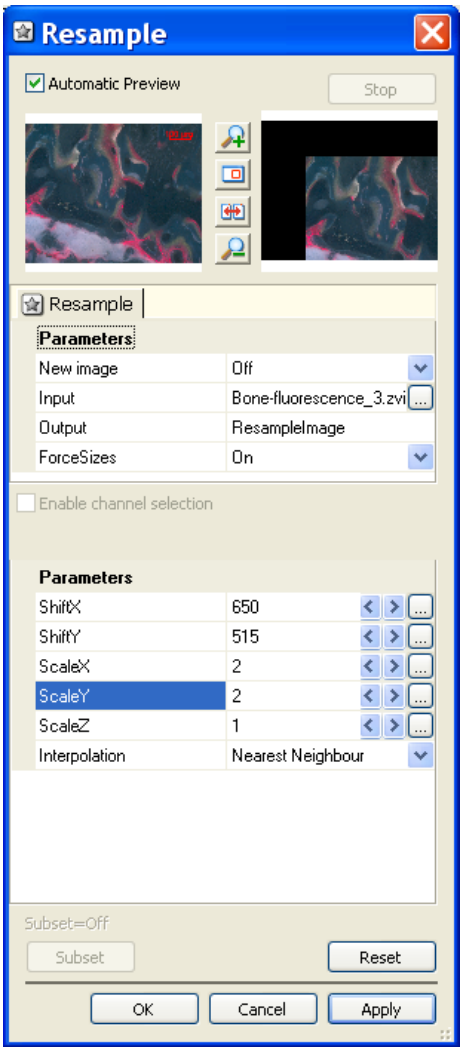
For the second example, again select from the **Processing** menu the **Utilities** functional group and then the **Resample** function.

- You will now see the dialog window of the **Resample** function.
- If the **Automatic Preview** check box is activated, the reduced input image appears.
- Under **Input** select the original image again and allocate a new image name under **Output**.
- Set the following values by clicking on the arrow keys or by entering the values directly: **ForceSizes**=On, **ShiftX**=650, **ShiftY**=515, **ScaleX**=2, **ScaleY**=2, **ScaleZ**=1, **Interpolation**= NearestNeighbour.
- Click on **Apply** to view the resulting image in the preview.
- Click on **OK** to close the dialog.

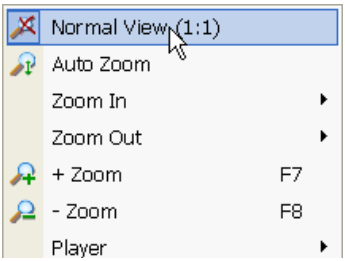


The resulting image shows only the top left quarter of the structures in the original image. This region was defined by **ShiftX** and **ShiftY**.

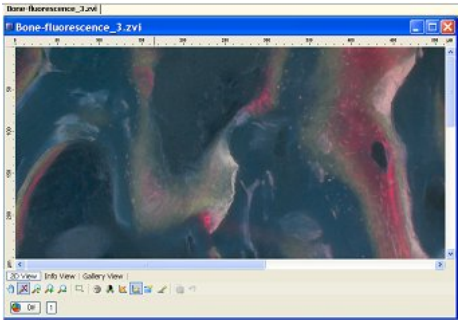
- The region is now larger than the original image by the factor 2.
- The image size has been retained, as **ForceSizes** was set to **Off**.



➢ Position the cursor over the resulting image, press the right-hand mouse button and select **Normal View (1:1)** from the shortcut menu. Do the same with the original image.

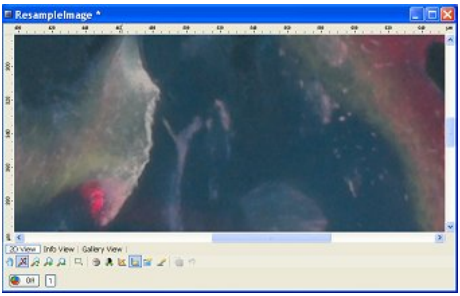


➢ Compare the two images. In the normal view of the image the difference can be clearly seen.



Note:

- Adjust the display characteristic curve in the resulting image if necessary.



Copying various regions from an image and generating a new image

Often you will only want to further process a particular region (ROI) of an image, or generate a composite image from various images or from regions of various images.

Using the **Copy Region** function, you can copy a freely selectable region from the original image into a new image. The starting coordinates in the original and the resulting image (**SourceStart**, **DestinationStart**) and the size of the region to be copied (**Size**) must be specified. If you are copying one region only, the size of the resulting image can be adapted to the region (**ForceSizes** = **Set region size**). You can, however, also generate an image in the size of the original (**ForceSizes** = **Keep input image size**).

To create a composite image from several images or image regions, you need to define the overall image size (**Size**, **ForceSizes** = KeepMaxSize). The individual images can then be copied into this image step by step. During the copying of an individual image, the area outside the region to be copied can be retained (**ClearOutside** = **Off**) or deleted (**ClearOutside** = **On**) in the resulting image.

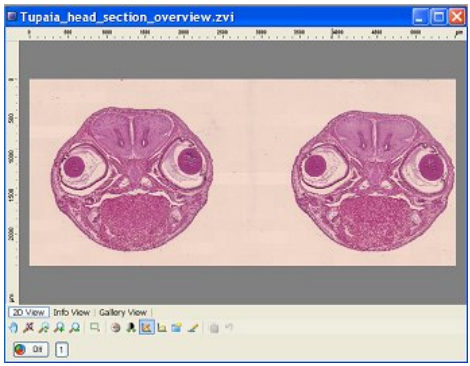
Note:

- If you simply want a region of an image to be copied, and a new image to be generated from it, this process can also be performed using the **Select ROI/Copy ROI** and **Paste** functions on the **Edit** menu.

The following example explains step by step how to copy various regions from one image into a new image.

- Load the image that you want Load the image "Tupaia_head_section_overview.zvi" via **File** ⇒ **Open Image**, or acquire an image with the camera.

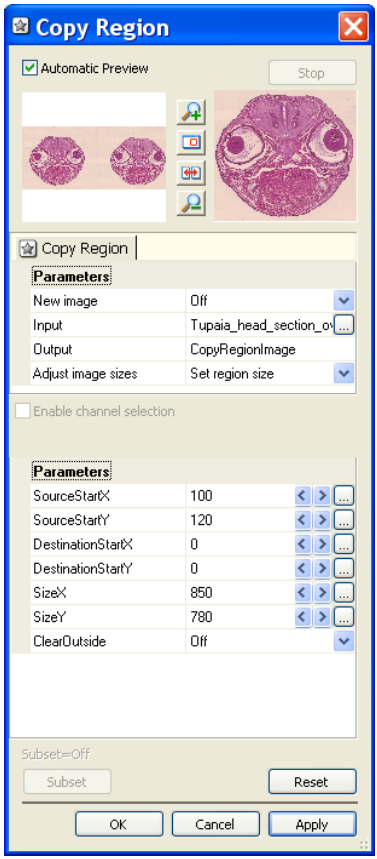
- The image is displayed in AxioVision's image window.



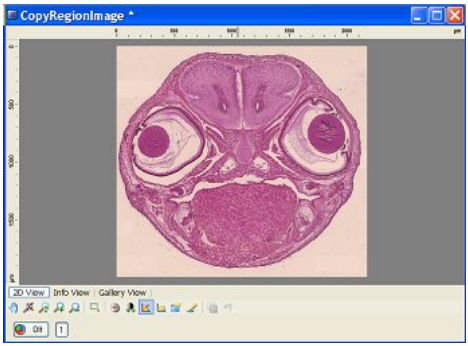
The image contains two histological sections through embryo heads. In the first example, only one embryo head is to be copied into a new image. In the second example, both embryo heads will be copied into a new image, with as little background as possible.

Select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

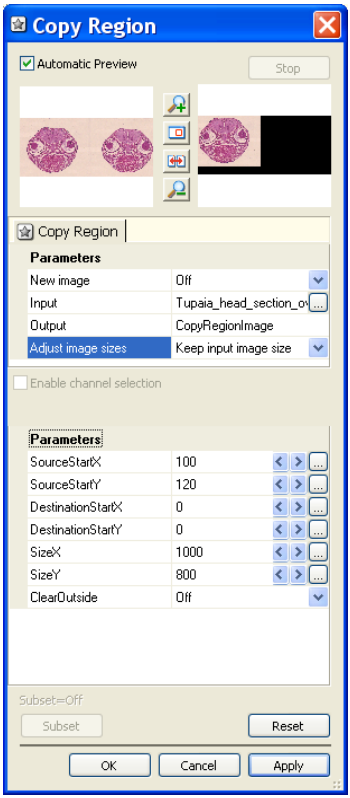
- You will now see the dialog window of the **Copy Region** function.
- If the **Automatic Preview** check box is activated, the reduced input image appears.
- Set **CreateNew** to **Off**, so that you can select the images freely.
- Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=Set region size,
  SourceStartX =100,
  SourceStartY=120,
  DestinationStartX=0,
  DestinationStartY=0, **SizeX**=850, **SizeY**=780, **ClearOutside**=Off.
- Click on **OK** to close the dialog.



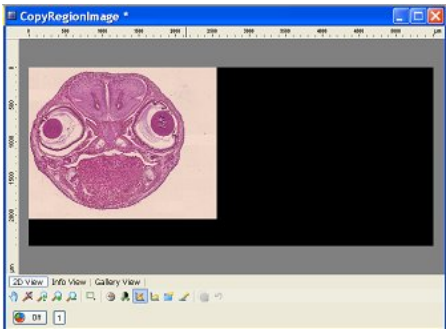
- In this example, only one region (ROI) has been copied into a new image. The resulting image therefore shows the first embryo head only.
- The image size has been adapted to the specified size of 850 x 780 pixels, as **ForceSizes** was set to **On**.



For the second example, again select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

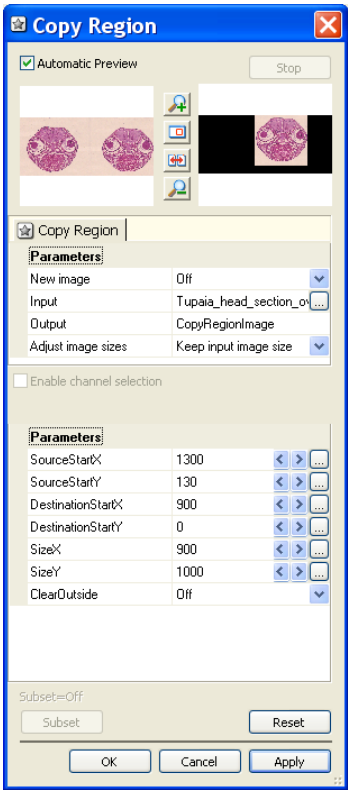


- You will now see the dialog window of the **Copy Region** function.
- If the **Automatic Preview** check box is activated, the reduced input image appears.
- Select the original image again under **Input**.
- Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=Keep input image size, **SourceStartX** =100, **SourceStartY**=120, **DestinationStartX**=0, **DestinationStartY**=0, **SizeX**=1000, **SizeY**=800, **ClearOutside**=Off.
- Click on **OK** to close the dialog.

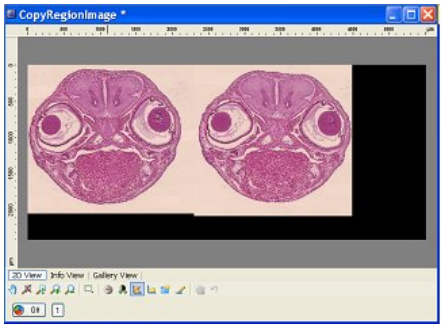


- The resulting image then shows the first embryo head in the top left-hand corner.
- As **ForceSizes** was set to **Off**, the image size corresponds to that of the original. The rest of the image is black.

Again select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.



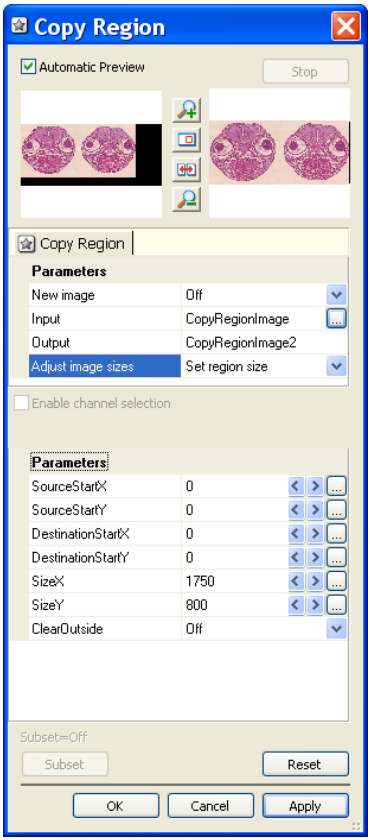
- You will now see the dialog window of the **Copy Region** function.
- If the **Automatic Preview** check box is activated, the reduced input image appears.
- Select the original image again under **Input**.
- Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=Keep omput image size, **SourceStartX** =1300, **SourceStartY**=130, **DestinationStartX**=900, **DestinationStartY**=0, **SizeX**=900, **SizeY**=1000, **ClearOutside**=Off.
- Click on **OK** to close the dialog.



- Both embryo heads are now visible in the resulting image.
- As the image size has been retained, the image is not completely filled, and contains some black regions. The next step therefore involves cutting out both embryo heads again in full.

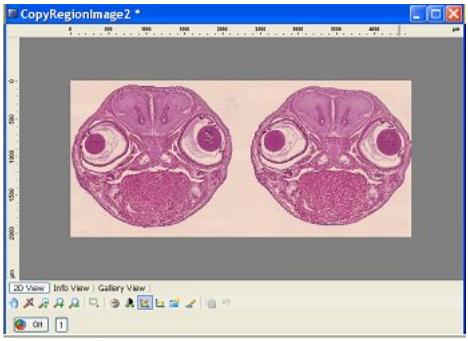
Again select from the **Processing** menu the **Utilities** functional group, and then the **Copy Region** function.

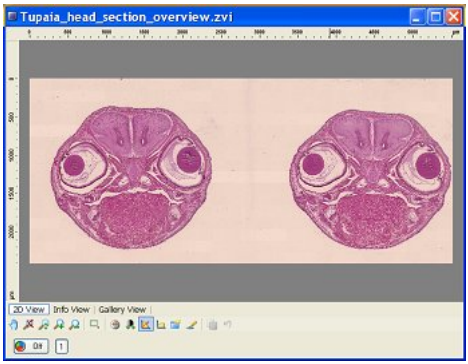
- You will now see the dialog window of the **Copy Region** function.
- If the **Automatic Preview** check box is activated, the reduced input image appears.
- Under **Input** select the resulting image that has been generated, and allocate a new image name under **Output**.
- Set the following values by clicking on the arrow keys, or by entering the values directly: **ForceSizes**=On, **SourceStartX** =0, **SourceStartY**=0, **SourceStartZ**=0, **DestinationStartX**=0, **DestinationStartY**=0, **DestinationStartZ**=0, **SizeX**=1750, **SizeY**=800, **SizeZ**=1, **ClearOutside**=Off.
- Click on **OK** to close the dialog



- The resulting image now shows both embryo heads on an area smaller than that of the original image.

- The image size has been adapted in accordance with the values for **SizeX** and **SizeY**, as **ForceSizes** was set to **Set region size**.





Note:

- Adjust the display characteristic curve in the resulting image if necessary.

Working with individual color channels

Color images consist of the three superimposed black/white images of the individual color channels red, green and blue. In many cases you will only want to use an image processing function for a specific color channel. This means that you have to split the image up into its individual channels. These individual color channels appear as black/white images, and allow you to carry out image processing procedures for the channels independently of each other. A color image is created again by merging the individual channels that have been processed.

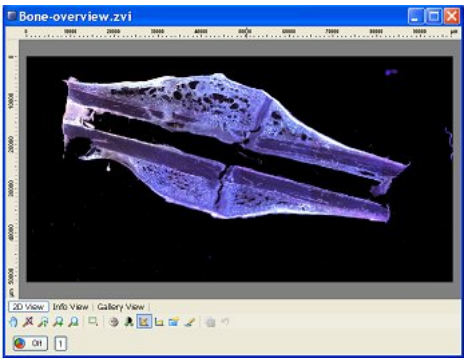
Note:

- The HLS color space (Hue, Lightness, Saturation) is more suitable for many image processing requirements. In the HLS model, the image can also be split up into its individual channels. To do this, you must first perform a conversion into the HLS color space using the **Color Model** function. Once you have split the image up into its individual channels using the **Split RGB Extractions** function, each channel can be processed separately. The individual channels are merged again using the **Combine RGB Extractions** function. The combined image must then be converted back into the RGB color space using the **Color Model** function.

The following example explains step by step how to split up a color image into its individual color channels, and merge it again after processing a channel.

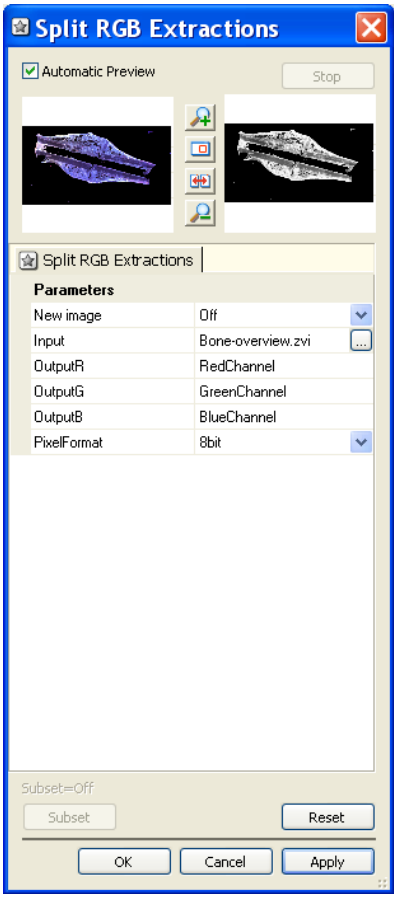
➢ Load the image "Bone-overview.zvi" via the function **File** ⇒ **Open Image**, or acquire an image with the camera.

➢ The image is displayed in AxioVision's image window.

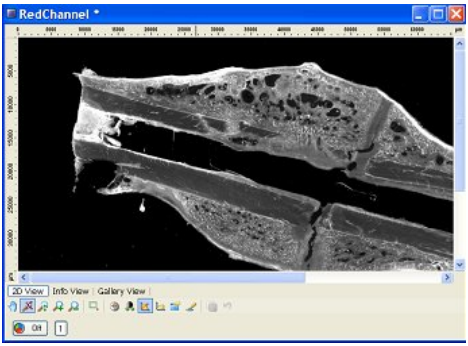


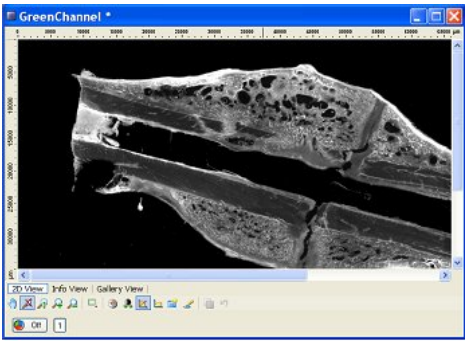
Select from the **Processing** menu the **Utilities** functional group, and then the **Split RGB Extractions** function.

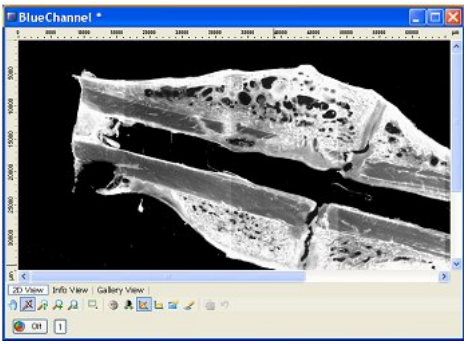
- You will now see the dialog window of the **Split RGB Extractions** function.
- If the **Automatic Preview** check box is activated, you will see the reduced input image.
- Set **CreateNew** to **Off**, so that you will be able to select the input image (**Input**) freely later.
- Under **PixelFormat** select "8bit".
- Click on **OK** to close the dialog.



- The **Split RGB Extractions** function splits up the color image into the individual color channels red, green and blue. The result is a black/white image for each channel.

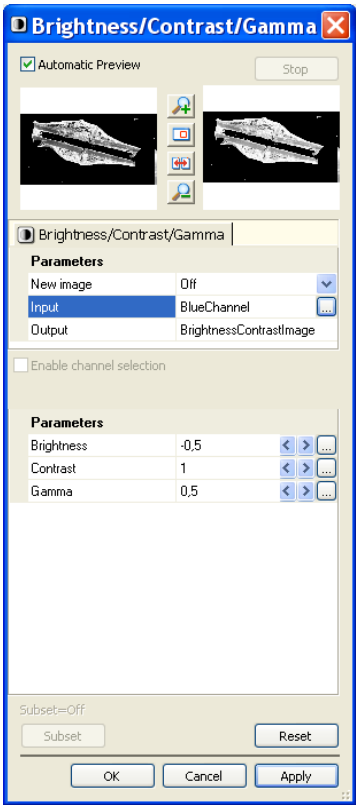




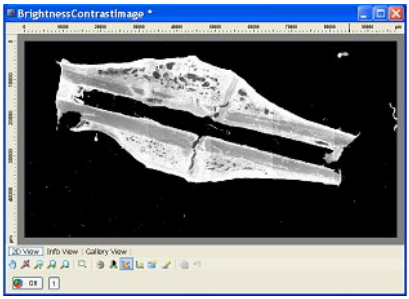


Select from the **Processing** menu the **Adjust** functional group, and then the **Brightness/Contrast/Gamma** function.

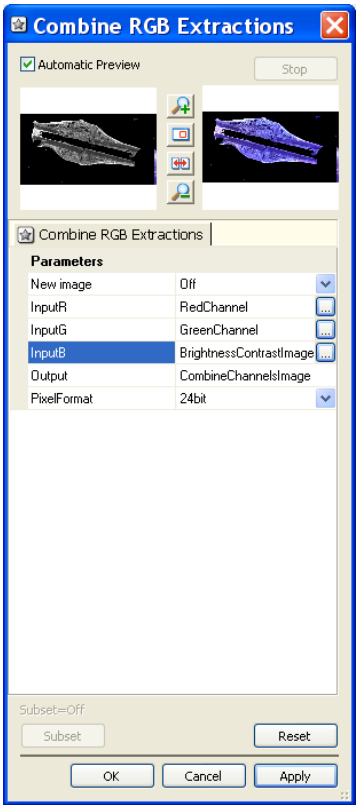
- You will now see the dialog window of the **Brightness/Contrast** function.
- If the **Automatic Preview** check box is activated, you will see the reduced input image.
- If you click on the  button in the **Input** field, the image gallery appears. Select the black/white image of the blue channel by clicking on it.
- Set the value -0.5 for **Brightness**, the value 1 for **Contrast** and the value 0.5 for **Gamma** by clicking on the arrow keys, or by entering the values directly.
- Click on **OK** to close the dialog.



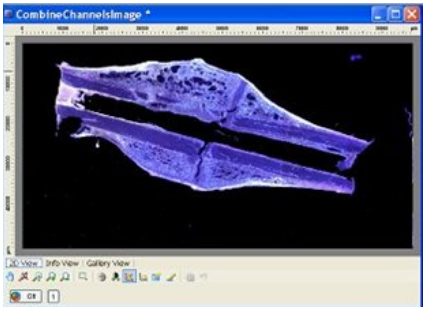
- This increases the brightness in the blue channel image.



Select from the **Processing** menu the **Utilities** functional group, and then the **Combine RGB Extractions** function.



- You will now see the dialog window of the **Combine RGB Extractions** function.
- If the **Automatic Preview** check box is activated, you will see the reduced input image.
- If you click on the button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **InputR** select the black/white image of the red channel, for **InputG** the black/white image of the green channel, and for **InputB** the enhanced image of the blue channel.
- Under **PixelFormat** select "24bit".
- Click on **OK** to close the dialog.
- In the resulting image the brightness has been greatly enhanced.



Note:

- Adjust the display characteristic curve in the resulting image if necessary.

Converting a color image into a black/white image

Note:

- More information about image format and bit depth can be found In chapter 5 "Imae Processing"- 5.1 "General".

If images are acquired using a digital camera, they will take the form, depending on the camera, of 10 bit images (or 3x10 = 30 bit color images), 12 bit images (or 3x12 = 36 bit color images) or 14 bit images (or 3x14 = 42 bit color images). An analog camera, on the other hand, only produces images with 8 bits (or 3x8 = 24 bit color images).

The gray value distribution of the image is displayed in the display characteristic curve (**View** menu ⇒ **Properties** function ⇒ **Display** property page). The number of bits that an image has can be seen from the maximum gray value.

Bits	Maximum gray value
8	256
10	1024
12	4096
14	16384
16	65536

As many programs work with 8 bit images only, or only distinguish between 8 and 16 bits, it is useful to be able to convert images into other bit formats. The **Convert Pixel Format** function therefore also only distinguishes between 8 and 16 bit black/white and 24 or 48 bit color images.

As they have three color channels, color images always require three times as much storage space as black/white images. A 16 bit image requires greater storage space due to the increased gray value information it contains. Often the color information is not required, or 8 bit gray value information is sufficient. For this reason the **Convert Pixel Format** function is suitable for converting a color image into a black/white image, or a 16 bit image into an 8 bit image.

If you want the same image to be converted into various pixel formats, we recommend that you work with the setting **CreateNew** = **Off**. This allows you not only to select the input image, but also to overwrite or enter a new name for the resulting image. Conversely, with **CreateNew** = **On**, a new resulting image is always created automatically, starting from the input image.

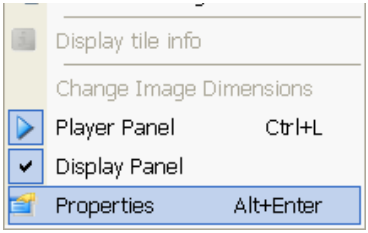
The following example explains step by step how to convert an image into a different pixel format whilst continuing to use the same input image.

- Load the image "Cut-Cerebellum(1300).zvi" via the function **File** ⇒ **Open Image**, or acquire an image with the camera.

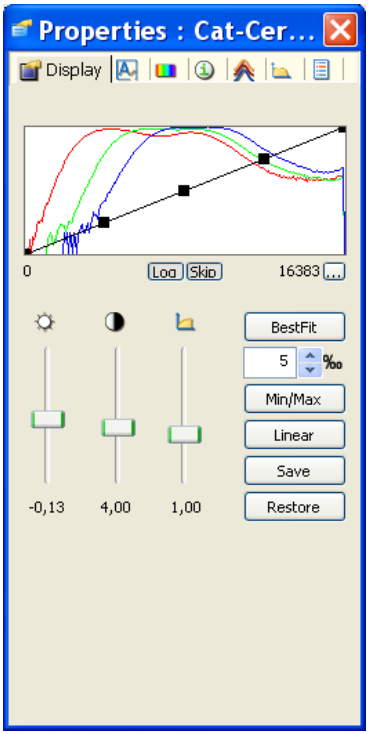


- The image is displayed in AxioVision's image window.

- Position the cursor over the image, press the right-hand mouse button, and select the **Properties** function from the shortcut menu.

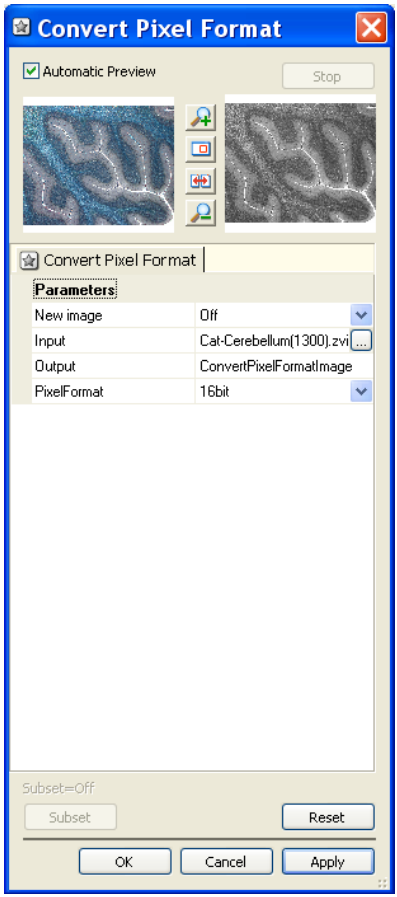


- Click on the **Display** tab sheet, where the image's display characteristic curve is shown. This shows a gray value distribution of 3 x 14 bits for the red, green and blue color channels.



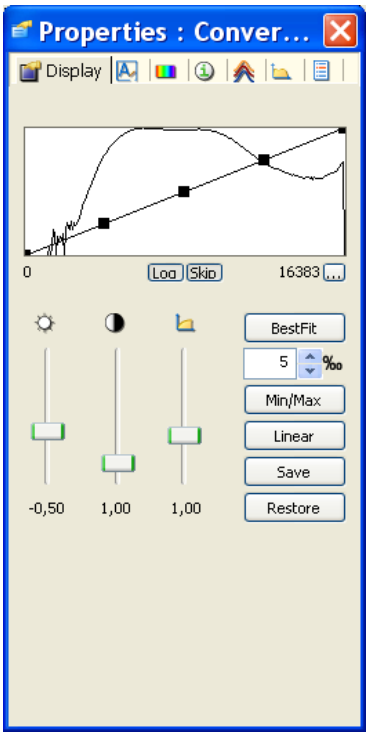
Now select from the **Processing** menu the **Utilities** functional group, and then the **Convert Pixel Format** function.

- You will now see the dialog window of the **Convert Pixel Format** function.
- If the **Automatic Preview** check box is activated, the reduced input image appears.
- Set **CreateNew** to **Off**, so that a different conversion can be executed using the same image later.
- Under **PixelFormat** select 16bit.
- Click on **OK** to close the dialog.



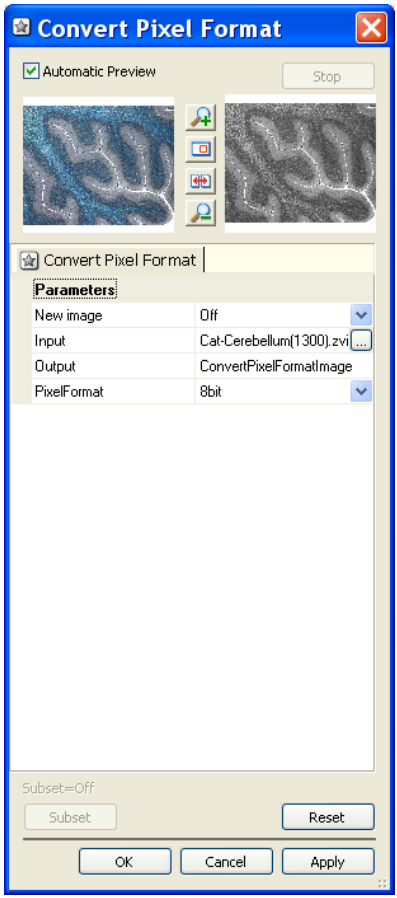
➢ The original image has been converted into a 16 bit black/white image. The display characteristic curve shows the distribution of the gray values.



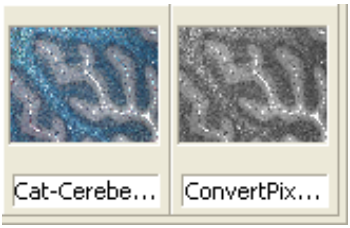


Again select from the **Processing** menu the **Utilities** functional group, and then the **Convert Pixel Format** function.

- You will again see the dialog window of the **Convert Pixel Format** function.

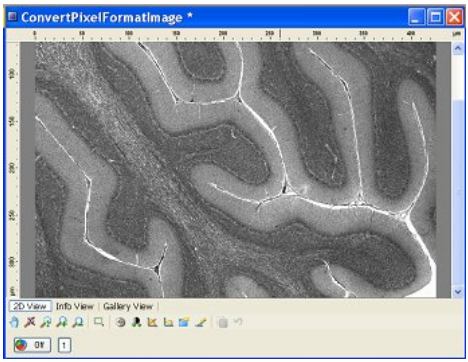


- If you click on the  button in the **Input** field, the image gallery appears. Select the original image by clicking on it.

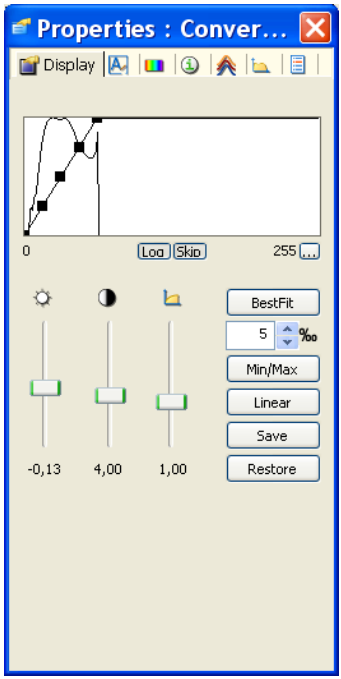


- Under **PixelFormat** select "8bit".
- The name of the resulting image remains unchanged. The image is overwritten.
- Click on **OK** to close the dialog.

- The resulting image is an 8 bit black/white image.

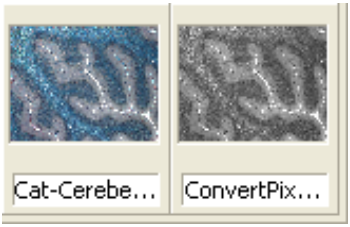


- The display characteristic curve of the resulting image now shows the gray value distribution of an 8 bit black/white image.

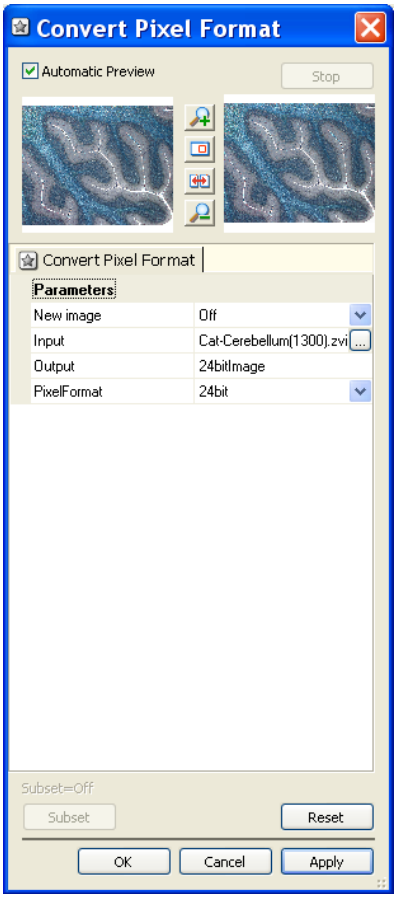


Again select from the **Processing** menu the **Utilities** functional group, and then the **Convert Pixel Format** function.

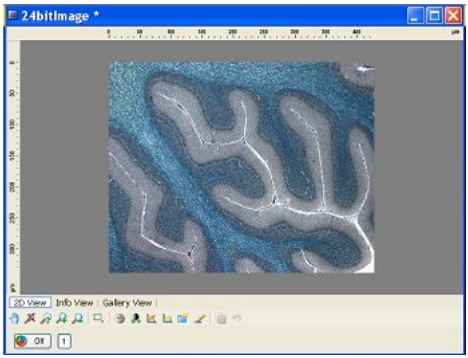
- Select the original image again in the **Input** field.
- For **Output**, allocate a new name, so that a new resulting image is generated.
- If you click on the ... button in the **Input** field, the image gallery appears. Select the original image by clicking on it.



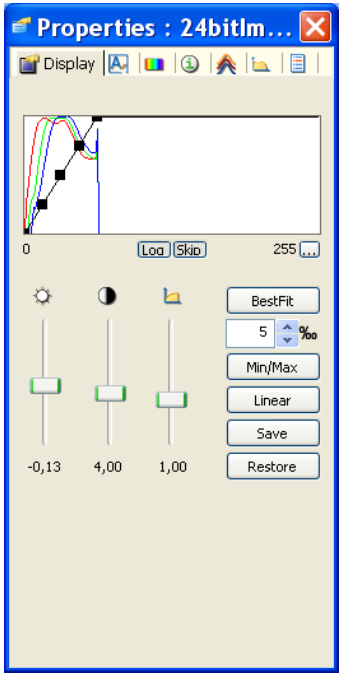
- Under **PixelFormat** select "24bit".
- Click on **OK** to close the dialog.



➢ The resulting image is a 24 (3x8) bit color image.



➢ The display characteristic curve of the resulting image shows the gray value distribution of the individual color channels.



10.2 Inside4D (3D View) / 3D Measure

With Inside4D you can display the Z-stack fluorescence images you have generated using AxioVision in three dimensions. The Z-stack images have to be black & white (acquired with a monochrome camera), can consist of up to 32 channels and can also be a time lapse series.

To display the third dimension on a two-dimensional medium such as a monitor, the data are projected into a virtual space. The impression of three dimensions can be created either by setting reference points (e.g. a shadow) or by animating the view. A three-dimensional display can be particularly effective for image stacks that have been processed using the **Deconvolution** module, acquired using the **ApoTome 2** module or acquired using a Confocal Laser Scanning Microscope (LSM).

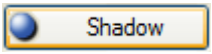
It is also possible to include time (from time lapse images) in each display as a fourth dimension (simultaneous animation of 3D projection and playback of time sequence).

3D Measure allows you to perform automatic measurements in Z-stack images.

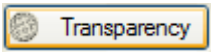
Note:

- Switching between 2D and 3D View is best done using the buttons 2D View and 3D View at the lower edge of the image.

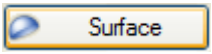
Additional buttons



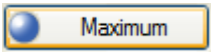
activates the **Shadow** rendering mode.



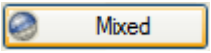
activates the **Transparency** rendering mode.



activates the **Surface** rendering mode.



activates the **Maximum** rendering mode.



activates the **Mixed** rendering mode.



activates the **Measure 3D** mode. This button is only available when the **3D Measure** module is activated.



creates a binary mask image of the displayed channel based on the current threshold settings. You can create a binary mask image of all channels whne you press the **Ctrl** key while clicking the **Create Lable Image** button. This mask image can e.g. be processed by the **3D Binary Image Editor** function. This button is only available when the **3D Measure** module is activated.

- 

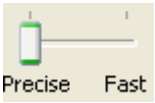
displays the 3D objects that are selected interactively in the **Measure 3D** mode. This check box is only available when the **3D Measure** module is activated.

- Clicking on the 

button opens the **Inside4D Settings** dialog. Here detailed settings for the selected rendering modes can be specified. Also creation of a rendered animation series is started from here (see **Series** property page).

- The 

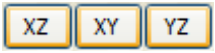
button creates a new unsaved 2D image of the currently displayed rendering.

- Using the 

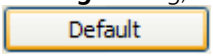
slider, you can reduce the complexity of the image data used for the calculation. The speed of the calculation increases as the complexity of the data decreases. This applies to all display modes, although the slider has different effects (number of pixels, textures, triangles etc.) depending on the mode in question. Even more flexible settings can be made on the **View** property page.

- A progress bar appears during the rendering procedure. If the calculation is taking too long, you can interrupt the procedure by clicking on the 

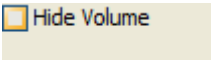
button.

- **Clipping** buttons: 

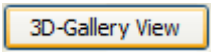
: With these buttons one can introduce three so called **Clipping planes** into the volume. Clipping planes can cut the volume open to unveil structures inside the volume. The labeling of the buttons indicates the clipping planes default orientation.

- if the orientation of a clipping plane has been changed (see detailed description of the clipping plane options in the **Clipping** tab of the **Settings** dialog) the default orientation can be regained by using the 

button

- 

if checked this option hides the volume data. This feature enhances the effect of displaying textures mapped onto **Clipping** planes.

- 

: This button is active in **Surface** mode only (method **Variable detail (graphics card)** and can be used for multichannel z-stacks to display the rendering of individual channels as well as the merge of all channels. All manipulation options for the volume are available as in normal 3D view.

Control elements in the window

The standard view when you first switch into **3D view** is a front view in the **Transparency** mode (graphics card accelerated mode).

- You can use the wheels  to zoom in and out of the view  and/or to rotate the view around the x  , y  and z axes  .

- The mouse function for direct interaction with the image volume can be configured using the five buttons at the center of the left-hand image edge

  - The  button allows the selection of measurement tools present in the volume. As long as the mouse cursor is not in the vicinity of an endpoint of a measurement tool the cursor symbol  is shown. If the cursor moves into the vicinity of an endpoint the cursor shape changes into  . The now selected endpoint is displayed in yellow and can be moved by keeping the left mouse button clicked down and moving the mouse to a new position within the volume. This action also updates the measurement values. If in this mode it is possible to temporarily switch into the default mode  by keeping the **Ctrl+Shift** keys depressed. This permits the quick reorientation of the volume during interactive measurements. If released the cursor resumes the selection shape.

  - If the  button is selected, you can rotate the image volume freely by depressing the left mouse button

  - The  button allows you, with the left mouse button depressed, to zoom into the image by moving the mouse in an upward direction, or to zoom out of the image by moving the mouse in a downward direction

- If the  button is depressed, you can move the image volume laterally, e.g. to make certain structures visible when in zoom mode. You can also activate this function temporarily by pressing the Ctrl key followed by the left mouse button

- With the  button selected changes the function of the left mouse button into a fly mode. By depressing the left mouse button you can fly into and through the sample. Moving the mouse at the same time changes the flight path. Alternatively the fly mode can also be invoked by pressing the mouse wheel key.

Note:

- The  and  button and the associated functionality is only available for graphics card accelerated rendering modes.

There are six additional buttons at the lower edge of the image

- You can use the  button at the lower edge of the image to apply a frame to your image stack,

- ... the  button to show the x, y and z axes (x=red, y=blue, z=green)

  or …

- … in the case of scaled data, the  button to add a scale. These functions help you to get oriented inside the virtual space. If you move the mouse pointer above one of these buttons, a tool tip will be displayed explaining the function.

- The  button hides measurement shapes drawn into the volume (Only available in graphics card accelerated rendering modes)

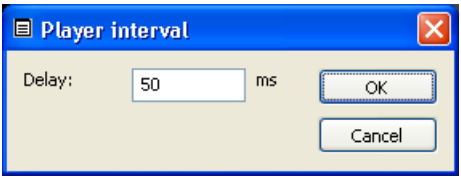
- The  button returns you to the default front view at any time from within the selected viewing mode. This resets all positions including Shift, Zoom or Flight so that the complete image content is visible in the image frame.

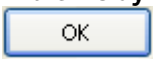
- The  button allows you to touch the object using your mouse and set it into continuous motion ("animation'). This significantly improves the impression of three dimensions.

Note:

- In animation mode a view is re-rendered for each projection. In the case of large image stacks (more than 20 MB) a powerful computer with a good graphics card is required to guarantee smooth animation.

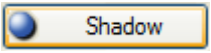
If you have loaded a time lapse image the player bar appears at the bottom edge of the window. You can then play back the time lapse sequence. However, the player does not wait for the rendering of the scene to be completed before moving on to the next instant. That's why you should select a slower time setting for rendering using the  button. This opens the **Player interval** dialog window:



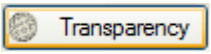
In the **Delay** field enter the desired time in milliseconds and click 

.

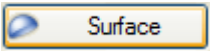
The viewing modes Shadow, Transparency, Surface, Mixed and Maximum



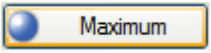
: In the **Shadow** mode the structures in the image are illuminated by a virtual light source. The volume is viewed as if looking through the eyepieces of the microscope and the light source projects a shadow onto a background plane. This gives the data a reference in space, making visualization much easier. The rendering effect itself consists of a combination of light reflection and opacity (degree of transparency). The display in this mode is only calculated by the main processor (CPU) of the computer (Voxel mode).



: In the **Transparency** mode a three-dimensional image with a transparent effect is calculated. At least two two-dimensional texture stacks (computed from two different angles) are used depending on the volume orientation (only for the graphics card accelerated rendering mode). In contrast to the Shadow mode the scene here is illuminated by diffuse white light from the rear. By changing the available parameters one can mix different channels and reveal relationships between information in those channels. This view is particularly useful for visualizing the three-dimensional relationships between structures within the volume. This mode can be displayed both in **CPU** based **Voxel** mode and also (with the help of a suitable graphics card) with accelerated calculations in so-called OpenGL mode.

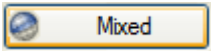


: In the **Surface** mode grey values in the image are computed as solid surfaces (also known as **Isosurfaces**) instead of soft transparent shades. This display mode is useful to focus the attention on certain structures (e.g plant cell walls, nucleoli, intracellular vesicles) while potentially hiding additional structures deeper within. In this mode you also can select between Voxel- (CPU) and OpenGL- (graphics card) display.



: Clicking on this button switches to **Maximum** projection mode, which calculates a three-dimensional view of the data by only showing pixels of the highest intensity along the projection axis. This view is particularly useful for displaying three-dimensional images in two dimensions, e.g. in publications, and guarantees a maximum possible degree of transparency. The

display in this mode can be calculated by the main processor (CPU) of the computer (Voxel mode) or the graphics card in OpenGL mode.



: In **Mixed** mode both transparency and surface modes can be mixed in one volume display. Using this mode one can render small structures within cells such as vesicles or speckles (e.g. FISH signals) as surfaces in one channel of a multichannel image while rendering the surrounding cytoplasm from another channel in transparency mode. This way one can also visualize more complex relationships convincingly.

Measure 3D Mode

To measure 3D objects the surface of the objects has to be determined first. You can specify this surface on the **Data** tab in the **Inside4D Settings** dialog. Here you can modify the **Threshold** value to specify the gray value which define the objects to be measured.

In **Measure 3D** mode the spcified 3D objects are measured and displayedin individual colors. Activate the To select an object activate the standard cursor mode by clicking the  button. When the mouse cursor has detected a 3D object the shape changes from  to  . No click the detected object. The color of the object and the circumscribing cuboid are now displayed in a higher brightness. Keep the **Ctrl** key pressed to select more objects. Use the wheels  to rotate the view around the various axes in order to select hidden 3D objects.

The measurement data for **all** measured objects are displayed on the **3D Measurement** tab of the **Properties** window.

The Measure 3D mode can not be combined with the following modes:
- Shadow
- Transparency - Rendering mode **Voxel (computer)**
- Surface - Rendering mode **Voxel (computer)** and **Maximum detail (graphics card)**
- Maximum - Rendering mode **Voxel (computer)**

Settings for the viewing modes

In the **Inside4D Settings** dialog you can adjust settings, which influence the 3D view, on the property pages **Data, Illumination, Series, View, Info, Clipping, Measure, Stereo** and **Special**. You can also create animations as image series (required for movie export) as well as perform simple interactive measurements.

Settings you have made and want to keep can be saved independent of the image and applied to other image stacks. On the **Data** property page click on 

button in the **Settings** field. Now you can name the settings file, to which a *.zv3 ending will be attached. In this setting file all settings you can change in Inside4D will be included. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

Depending on the chosen Rendering method the settings you can change will be different for the **Data**, **Illumination** and **View** property pages. In the following chapters each property page will be explained separately for each rendering mode.

Data property page

On the **Data** property page, select the threshold starting at which individual pixels will be used for the calculation. All pixel values that are not being used are hidden and interpreted as background. Once you have specified the pixels to be used, define the surface composition of the objects. Different parameters are available for this purpose in the individual modes.

Settings

These functions are identical for all render modes.

The 

button loads a previously saved *.zv3 settings and applies it to the z stacks.

Save saves the settings you made, independently from the image, so they can be used for other z-stacks. Now you can name the settings file, to which a *.zv3 ending will be attached. All parameters are included in the setting file. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

The Save As Default button saves the current setting as default setting, which will be used by all followed images.

The Reset button restores the default state.

Note:

- You can create a new image from any current view by clicking on the Create Image button on the right side of your image.

Shadow mode

Data

- **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g. 1).
- **Threshold** specifies a lower threshold as a percentage of the gray values displayed. This decides which data enter the rendered image. If one fluorescence channel contains a homogeneous fluorescence signal with high signal strength, one would set the threshold higher in order to "protect" signals from weaker channels from being quenched.
- **Ramp** controls the degree of the transition from completely transparent to completely opaque.
- **Maximum** influences the degree of opacity.

- The settings are combined into a histogram  . The x axis corresponds to the gray values and the y axis to the degree of opacity. You can also use the mouse to interact with the histogram curve.

- **Roughness**: You can also influence the roughness used to display the surface of the image structures. This changes the plasticity of the display (relative scale between 0 and 1). This setting quickly can produce artifacts if pixels reach saturation. The degree of roughness should be reduced accordingly.

Transparency mode

Data

- **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g. ).

- **Threshold** specifies a lower threshold as a percentage of the gray values displayed (relative scale between 0 and 1). This decides which data enter the rendered image.

- **Ramp** controls the degree of the transition from completely transparent to completely opaque (relative scale between 0 and 1).

- **Maximum** influences the degree of opacity (relative scale between 0 and 1).

- The parameters are schematically illustrated in a histogram  . The x axis represents the gray values and the y axis the transparency. You can also use the mouse to interact with the histogram curve.

- **Gamma**: With this slider the transparency effect can be further enhanced in a channel specific manner (relative scale between 0 and 1). This

parameter influences all pixel gray values in the same way in contrast to the **Ramp** and **Maximum** settings.

Global

- **Opacity**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the degree of opacity in the scene anywhere between 0.00 and 1.00. Use the slider or enter the value into the input field. A value of 0.00 will make all objects fully transparent.

- **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 0.1° and 89.9°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

- If the **Voxel (computer)** method is selected in the **Special** property page, this adjustment cannot be done.

Surface mode

Data

- **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g. 

).

- **Threshold**: Sets the gray value threshold on a relative scale between 0 and 1 at which a pixel will belong to the object.

- **Ambient**: describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 1).

- **Specular**: sets a directional brightness (values on a scale between 0 and 1). Influences the differences between dark and bright structures, which are strongest at a value of 0.

- **Shininess** changes the surface gloss (on a scale between 0 and 1).

Global

- **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

- If the **Voxel (computer)** method is selected in the **Special** property page, this adjustment cannot be done.

Note:

- If the render method **Maximum** detail (graphics card) is selected on the **View** property page AND on the **Special** property page the option Transparent surfaces is selected, the **Data** property page shows the slider **Transparency**. Using this slider one can assign an additional transparency effect to the surfaces of a channel.

Maximum mode

Data

- **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field. To select a channel click on the corresponding button (labeled with the channel color, e.g. 

).

- **Threshold**: In the **Threshold** area, you can use the slider or value field to set an upper and a lower threshold. Values smaller than the lower threshold value, or greater than the upper threshold value, are not

considered in the rendered stack. This setting option is particularly useful for the rendering of multichannel image stacks.

Note:

- Please note, that the setting for upper threshold is only available for the CPU method Voxel (computer) (**View** property page → Mode **Maximum** → Method **Voxel**).

Global

- **Opacity**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the degree of opacity in the scene anywhere between 0.00 and 1.00. Use the slider or enter the value into the input field. Please note that visibility of opacity adjustments in maximum mode depends on background color and/or presence of colored clipping planes in the image.

- **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

- If the **Voxel (computer)** method is selected in the **Special** property page, this adjustment cannot be done.

Mixed mode

Data

In the **Mixed mode** a volume can be displayed both in surface and transparency mode. For this is it necessary to define settings for both methods separately for each channel. To accommodate this the Data page has been subdivided in two halves.

- **Channels**: The settings are entered separately for each channel using sliders or by entering a numerical value in the corresponding input field.

To select a channel click on the corresponding button (labeled with the channel color, e.g. ). All changes you make are only applied to the selected channel.

- **Transparency**: if checked, the selected channel is shown in **Transparency** mode.

  - **Threshold** specifies a lower threshold as a percentage of the gray values displayed (relative scale between 0 and 1). This decides which data enter the rendered image.

  - **Ramp** controls the degree of the transition from completely transparent to completely opaque (relative scale between 0 and 1).

  - **Maximum** influences the degree of opacity (relative scale between 0 and 1).

  - The parameters are schematically illustrated in a histogram  . The x axis represents the gray values and the y axis the transparency. You can also use the mouse to interact with the histogram curve.

  - **Gamma**: With this slider the transparency effect can be further enhanced in a channel specific manner (relative scale between 0 and 1). This parameter influences all pixel gray values in the same way in contrast to the Ramp and Maximum settings.

- **Surface**: if checked, the selected channel is shown in **Surface** mode.

  - **Threshold**: Sets the gray value threshold on a relative scale between 0 and 1 at which a pixel will belong to the object.

  - **Ambient**: describes a diffuse, non-directed light source, which lets both bright and dark structures appear darker or brighter in an equal fashion (values on a scale between 0 and 1).

- **Specular**: sets a directional brightness (values on a scale between 0 and 1). Influences the differences between dark and bright structures, which are strongest at a value of 0.

- **Shininess** changes the surface gloss (on a scale between 0 and 1).

Global

- **Opacity**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the degree of opacity of all transparent objects in the scene anywhere between 0.00 and 1.00. Use the slider or enter the value into the input field. A value of 0.00 will make all transparent rendered parts in the scene become fully transparent. Please note that opacity adjustments will not influence the rendering of surface portions in the scene.

- **Angle**: If you select the **Texture (graphics card)** method in the **Special** property page, you can change the projection angle used to view the scene anywhere between 10° and 80°. Use the slider or enter the **Angle** into the input field. The effect of this on the display perspective is similar as if you were viewing the 3D image through a telephoto or wide-angle lens.

Note:
- The **Mixed** Mode is only available as a graphics card accelerated mode and not as CPU only mode.

Illumination property page

With the settings made on this property page you influence the light sources, that illuminate the volume as well as the background color. The settings available on **Illumination** are different for each render mode and are therefore explained separately. In contrast to the settings on **Data** property page all illumination settings act on all channels at the same time.

Settings

These functions are identical for all render modes.

The Load... button loads a previously saved *.zv3 settings and applies it to the z stacks.

Save saves the settings you made, independently from the image, so they can be used for other z-stacks. Now you can name the settings file, to which a *.zv3 ending will be attached. All setting options, with the exception of the lateral position (), are taken into consideration. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

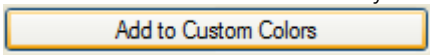
The Save As Default button saves the current setting as default setting, which will be used by all followed images.

The Reset button restores the default state.

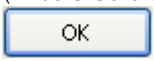
Background

Functions for changing the background color are available in the same fashion for all render modes.

- Clicking on the Choose color... button opens a window that allows you to select the background color.
  - You can either select the background color from 8 standard colors or create your own colors. In the drop down list field click on the More Colors... option and select colors from the palette

- You can also enter color values manually in the value fields. Clicking on the 

button adds the newly selected color to the selected field for user-defined colors.

- To use a user-defined color click on the corresponding color field ("Basic Colors" or "Custom Colors") and confirm by clicking 

.

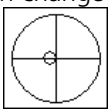
Shadow mode

- **Brightness** can be controlled via input field or slider bar (value range 0.2 – 4). Fluorescence data usually profit from brightness values > 1.6 when viewed in pseudo color mode (

button in the Player panel).

- **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range 0°-90°)

- **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range -180° to +180°)

- You can change the settings also by moving the small circle with the mouse 
. The position of the light source is represented by the small circle inside the large circle.

Transparency mode

- **Brightness** can be controlled via input field or slider bar (value range 0.2 to 4). Fluorescence data usually profit from brightness values > 1.6 when viewed in pseudo color mode (

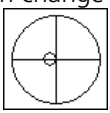
button in the **Player Panel**).

Surface mode

- **Distance** (value range 0.1 – 9-5) determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").

- **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range 0°-90°)

- **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range -180° to +180°)

- You can change the settings also by moving the small circle with the mouse  . The position of the light source is represented by the small circle inside the large circle.

Maximum mode

- **Brightness**: Control the brightness of the display using the slider or input field (value range 0.1 to 4). With multichannel images, screen saturation quickly occurs in mixed color mode. If this happens, reduce the overall brightness.

Mixed mode

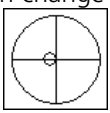
- **Brightness** can be controlled via input field or slider bar (value range 0.2 to 4). Fluorescence data usually profit from brightness values > 1.6 when viewed in pseudo color mode (

button in the **Player Panel**).

- **Distance** (value range 0.1 – 9-5) determines the distance between light source and volume. At a value of 0.1 the light source is close by, shadow effects etc. are minimal. The further away the light source, the more prominent the shadow effects become ("evening shadows are longer").

- **Azimuth** describes the angle of the light source above the virtual horizon and can be set by entering values directly or by slider (value range 0°-90°)

- **Elongation** describes the horizontal direction of lighting and can be set by entering values directly or by slider (value range -180° to +180°)

- You can change the settings also by moving the small circle with the mouse  . The position of the light source is represented by the small circle inside the large circle.

Series property page

Once you have decided on a viewing mode and entered your settings, you can have your view calculated as a series of individual images.

Note:
- This property page is always identical for the **Shadow**, **Transparency**, **Surface**, **Maximum** and **Mixed** modes.

Whereas the  button (see **Control elements in the window**) produces a live animation, which requires each image to be re-calculated for display, here you can have a series of individual images calculated to represent the animation you would like.

Depending on the image size, the display of an image series like this can be much faster and smoother than a "live" animation, as the views no longer have to be re-rendered. This type of image series can also be exported as a film in Video for Windows (AVI) or QuickTime -format (MOV) with excellent results. However, you can also export an image series into other file formats as a series of individual images.

The settings you make here define the parameters to be used to perform the animation. A minimum required entry is the definition of a start angle as well as an end angle.

Series

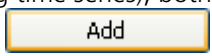
- **Series**: In this drop-down list box you have the choice between five different options: **X-Axis**, **Y-Axis**, **Z-Axis**. With these first three options you can create the series using a fixed axis (x,y,z), around which the animation turns. If you select one of these options, three sliders appear.

- **Views**: This slider specifies the number of images you want to be included in the animation. The greater the number of images you enter, the smoother the display will be. However, the resulting image will also increase in size accordingly. The value range of the slider extends from 1-1000 images. However, you may also enter a higher value in the value field (up to 9999).

- **Start Angle** / **End Angle**: These two sliders can be used to specify the start angle and end angle. Here you can make a selection between -360° and +360°. Frequently entering a small range such as -35° to +35° will already give impressive results, smooth "tumbling" without the need for large number of views.

- Alternatively, you can also save any views of the object in the space as positions in a position list and generate an animation with changing rotation axes and/or zoom factors. To do this, select in the drop-down list box **Series** the **Position List** option. This opens another drop-down list box.

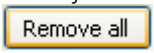
  - Clicking on the 

button adds the current position with the values for x, y, z and d (for zoom factor). A position also incorporates a lateral shift as well as the position of the time player (when rendering time series), both parameters are not shown in the list though. The 

button is also available on the right hand side directly next to the rendering window.

  - Now change the orientation of the object in the image window as required and save the new position.

- You can delete one or more positions by clicking 

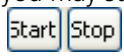
or delete all positions by clicking on 

. A double click on a position entry will invoke this setting on the image.

➢ If you have created a time lapse image, you can generate a time lapse series of the 3D view you have just selected by choosing the **Time Series** option from the drop down list. Please be aware, that it might be better in many cases to use the position list option instead in order to combine both spatial and temporal changes in the render series. This will enhance the 3D effect on 4D movies.

➢ If you want to generate a series using the settings you have made on the **Data** and **Illumination** property pages, select the **Use Settings** or **Without Settings** function from the drop-down list box at the bottom edge of the position list. Using this feature you can make one channel disappear by setting the threshold to 100%. Using this technique you can demonstrate structures otherwise hidden behind dominant signals from other channels.

➢ You always generate the series by clicking on 

. The result is a new image that corresponds to a time lapse image in terms of its operation (player bar). Additionally, you may start and stop a preview of your render series by clicking on the 

buttons on the right hand side directly next to the rendering window. This preview uses the number of views that are adjusted by the views slider but excludes some options and details of the final render series (e.g. clipping planes) to enable a fast preview.

The new image series document can be saved as a ZVI-image or exported in another format, such as Video for Windows (AVI).

Notes:

- It can take up to several minutes to calculate complex image series with large numbers of images and/or positions.

- In uncompressed form, complex series can become extremely large. This can make it difficult to import them into e.g. Microsoft PowerPoint™ for a presentation. It is therefore recommended that you compress the AVI or QuickTime files during export (for help on this subject, see the **Export** help topic).

Settings

The 

button loads a previously saved *.zv3 settings including stored position lists and applies it to the z stacks.



saves the settings including positions you made independent from the image, so they can be used for other z-stacks. Now you can name the settings file, to which an *.zv3 extension will be attached. All setting options are applied. This function is particularly important if you want to observe images of samples under identical conditions, to allow comparison.

The 

button saves the current setting as default setting, which will be used by all followed images.

The 

button restores the default state.

View property page

On this page, you can choose amongst the following viewing parameters: geometric parameters, volume resolution as well as settings influencing the rendering method.

Shadow mode

Geometry

- **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

- **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the  tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0

- **Zoom**: defines the magnification factor (value range 0-1000).

Note:
- Stretch Z is not active in Shadow mode.

Resolution
- **Reduction XY** / **Reduction Z**: In this field, for shadow mode you can reduce the number of pixels used for the volume rendering step by step in both the lateral (x,y) and axial (z) direction (subsampling). In some cases this can significantly reduce the time for the calculation.

  - To do this, move the corresponding slider, or enter the desired value in the edit field. A reduction value of 2 (x,y) combines 2 pixels in the x and y directions, reducing the data volume by a factor of four. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

  - The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

  - To get the optimum effect you can also adjust these settings on the **Info** property page. There the performance measurements give a direct feedback on the speed improvement.

Rendering Mode

- Here you can also switch between the different modes (using the drop-down list box). In the OpenGL accelerated modes, here you can also change the rendering method being used (using the drop-down list box).

- **Method**: As in shadow mode only the computer's processor ("CPU method") is used, the **Method** drop-down list box remains inactive.
- Shadow ☑: hides the shadow if unchecked
- **Distance:** regulates the distance between shadow and volume using the slider or entering the desired value in the edit field (value range 0.5 to 3)
- **Use Depth Coding:** activates a depth coding in the z-dimension that ranges from blue over green, orange and yellow to red. **Depth Coding** effects only the rendering modes Maximum, Transparency and transparent parts of the Mixed mode.
- **Draw Depth Coding Scale:** draws a color coded scaling below the rendered image. This option is only available for the Transparency rendering mode of the CPU method Voxel (computer)

Note:
- Stretch Z is not active in Shadow mode.

Measure

- ☑ Show Measure3D Regions: You can specify whether the measured 3D objects shall be displayed.
- ☑ Show Surface: With this option you specify whether the surface of the measured objects shall be displayed.
- ☑ Show Boundingbox: This option specifies whether the circumscribing cuboid shall be displayed.
- ☐ Show Standard Surface: Check this option to combine the color-coded display of the measured objects with the display of their surface.

Transparency mode

Geometry

- **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

- **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the  tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0.

- **Zoom**: defines the magnification factor (value range 0-1000).

- **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Note:
- Stretch Z is only active for the graphic card method.

Resolution

- **Reduction (xy)** / **Reduction (z)**: For Transparency mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

    - To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

- The ability of the graphics card to hold all texture planes in its memory is a key factor in determining whether the image stack can be rendered smoothly (animation mode!) with the **Texture (graphics card)** method. You should therefore set the sliders in such a way that in the info field on the **Info** property page, the value `"Nr. Texture planes = all"` is displayed.

Rendering Mode

- Here you can also switch between the different modes (using the drop-down list box). In the OpenGL accelerated modes, here you can also change the rendering method being used (using the drop-down list box).

- **Method**: In the Transparency mode, the two options **Voxel (computer)** and **Texture (graphics card)** are available.

  - With the **Voxel (computer)** method, rendering is performed using the main processor only, without OpenGL support; the model that forms the basis for the calculation is also less complex. As a result, this method is usually faster on slow computers with small graphics cards. The results, however, are also of reduced quality.

  - The more complex method **Texture (graphics card)** gives the 3D images surface structures, so-called textures, which are in turn constructed from individual elements (texels). The fewer elements required, the faster the rendering speed (images/s).

- **Use Depth Coding:** activates a depth coding in the z-dimension that ranges from blue over green, orange and yellow to red. Depth Coding effects only the rendering modes Maximum, Transparency and transparent parts of the Mixed mode.

- **Draw Depth Coding Scale:** draws a color coded scaling below the rendered image. This option is only available for the Transparency rendering mode of the CPU method Voxel (computer)

Measure

- ☑ Show Measure3D Regions: You can specify whether the measured 3D objects shall be displayed.
- ☑ Show Surface: With this option you specify whether the surface of the measured objects shall be displayed.
- ☑ Show Boundingbox: This option specifies whether the circumscribing cuboid shall be displayed.
- ☐ Show Standard Surface: Check this option to combine the color-coded display of the measured objects with the display of their surface.

Surface mode

Geometry

- **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.
- **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the  tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0
- **Zoom**: defines the magnification factor (value range 0-1000).
- **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Note:
- Stretch Z is only active for the graphic card method.

Resolution

- **Reduction (xy)** / **Reduction (z)**: For Surface mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

  - To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

  - The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

  - You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

  - In Surface mode, here you can also determine the maximum number of triangles used for calculation. The fewer triangles used by the model, the higher the rendering speed. At the same time, this also reduces the level of detail.

  - The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 in the x,y and z directions is sufficient for a smooth display. The number of triangles should not be less than 5000, as otherwise the quality of the resolution is insufficient. To achieve the same quality in the **Variable detail (graphics card)** mode as in the **Maximum detail (graphics card)** mode, the maximum number of triangles must be set, depending on the image data, to several million (entry of figures in the value field).

Rendering Mode

- Using the drop-down list box you can switch between the modes. In the Open GL accelerated modes you can in addition change the render method used (drop down list box).

- **Method**: Three methods are available, which you can select in the drop-down list box: The method **Voxel (computer)**, the graphics-card-accelerated method **Variable detail (graphics card)** and the method **Maximum detail (graphics card)**.

  - With the **Voxel (computer)** method, rendering is performed using the main processor only, without OpenGL support; the model that forms the basis for the calculation is also less complex. As a result, this method is faster on slow computers with small graphics cards. The results, however, are also of reduced quality.

  - Depending on the size of the data, the method **Variable detail (graphics card)** can be accelerated by the OpenGL-compatible graphics card.

  - The **Maximum detail (graphics card)** method calculates the surface model layer by layer. Here the available channels are added to the model one after the other. This process takes significantly longer than with the **Variable detail (graphics card)** method, which attempts to keep all image data in the graphics card's memory. On the other hand, this method usually produces the best results.

- **Use Depth Coding**: activates a depth coding in the z-dimension that ranges from blue over green, orange and yellow to red. Depth Coding effects only the rendering modes Maximum, Transparency and transparent parts of the Mixed mode.

- **Draw Depth Coding Scale**: draws a color coded scaling below the rendered image. This option is only available for the Transparency rendering mode of the CPU method Voxel (computer)

Measure

- ☑ Show Measure3D Regions : You can specify whether the measured 3D objects shall be displayed.
- ☑ Show Surface : With this option you specify whether the surface of the measured objects shall be displayed.
- ☑ Show Boundingbox : This option specifies whether the circumscribing cuboid shall be displayed.
- ☐ Show Standard Surface : Check this option to combine the color-coded display of the measured objects with the display of their surface.

Maximum mode

Geometry

- **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.
- **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the  tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0
- **Zoom**: defines the magnification factor (value range 0-1000).
- **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Note:
- Stretch Z is only active for the graphic card method

Resolution

- **Reduction (xy)** / **Reduction (z)**: For Maximum mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

  - To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

  - The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

  - You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

  - In contrast to the **Transparency** and **Surface** modes **Maximum** rendering is based upon a much simpler calculation model. As a result the rendering speed increase by data reduction is not as pronounced as with the other modes. It is for this reason, that the Maximum mode is very suitable for the rendering of large volume stacks.

  - To get the optimum effect you can also adjust these settings on the **Info** tab. There the performance measurements give a direct feedback on the speed improvement.

Rendering Mode

- Here you can also switch between the different modes (using the drop-down list box). In the OpenGL accelerated modes, here you can also change the rendering method being used (using the drop-down list box).

- **Method**: In the Transparency mode, the two options **Voxel (computer)** and **Maximum (graphics card)** are available.

- With the **Voxel (computer)** method, rendering is performed using the main processor only, without OpenGL support; the model that forms the basis for the calculation is also less complex. As a result, this method is usually faster on slow computers with small graphics cards. The results, however, are also of reduced quality.

- The more complex method **Maximum (graphics card)** performs the maximum projection calculation on the graphics card and produces faster and better rendering results.

➢ **Use Depth Coding:** activates a depth coding in the z-dimension that ranges from blue over green, orange and yellow to red. Depth Coding effects only the rendering modes Maximum, Transparency and transparent parts of the Mixed mode.

➢ **Draw Depth Coding Scale:** draws a color coded scaling below the rendered image. This option is only available for the Transparency rendering mode of the CPU method Voxel (computer)

Measure

➢ ☑ Show Measure3D Regions : You can specify whether the measured 3D objects shall be displayed.

➢ ☑ Show Surface : With this option you specify whether the surface of the measured objects shall be displayed.

➢ ☑ Show Boundingbox : This option specifies whether the circumscribing cuboid shall be displayed.

➢ ☐ Show Standard Surface : Check this option to combine the color-coded display of the measured objects with the display of their surface.

Mixed Mode

Geometry

- **Angles (x,y,z):** shows the current orientation of the volume in degrees (-180 to 180) with respect to the main axis (x, y, z). You can also define an angle by entering a desired value in the edit fields.

- **Center (x,y,z):** describes the relative position of the rotation center to the center of the 3D stack. This translates into a lateral shift of the volume and is best defined by the  tool. The values are given in units of volume diagonals (x, y, z): the diagonal is set to 1. For the home position all values equal 0.

- **Zoom**: defines the magnification factor (value range 0-1000).

- **Stretch Z** stretches the volume display in axial (z) direction. Entering values >1 (value range 0-20) increased the height of flat structures.

Resolution

- **Reduction (xy)** / **Reduction (z)**: For Transparency mode you can reduce the number of pixels used for the calculation step by step in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

  - To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 for x,y, for example, combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

  - The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- You can also adjust the resolution on the **Info** property page where you can see the performance values of the rendering directly.

- The parameter **Triangles** as available for **Surface** mode is not available for **Mixed** mode.

Rendering Mode

- Here you can also switch between the different modes (using the drop-down list box). For Mixed mode only the OpenGL accelerated method calculated with the graphics card is available.

- **Use Depth Coding:** activates a depth coding in the z-dimension that ranges from blue over green, orange and yellow to red. Depth Coding effects only the rendering modes Maximum, Transparency and transparent parts of the Mixed mode.

- **Draw Depth Coding Scale:** draws a color coded scaling below the rendered image. This option is only available for the Transparency rendering mode of the CPU method Voxel (computer)

Measure

- ☑ Show Measure3D Regions: You can specify whether the measured 3D objects shall be displayed.

- ☑ Show Surface: With this option you specify whether the surface of the measured objects shall be displayed.

- ☑ Show Boundingbox: This option specifies whether the circumscribing cuboid shall be displayed.

- ☐ Show Standard Surface: Check this option to combine the color-coded display of the measured objects with the display of their surface.

Info property page

This property page shows performance values for the different render modes. With the settings in the **Resolution** field, you can accelerate the rendering speed significantly for large volumes.

Info

The **Info** field on this property page shows performance values that are designed to help you achieve a realistic estimate of the rendering speed you can expect. Partially different parameters will be displayed for the different render modes.

For all render modes:

- **Performance**: You are given a time value (in msec) for each image calculated resulting in a Frames/second value.
- **Angles**: You can also still see the angle positions that are currently set. This can prove useful for the precise orientation of image stacks in space. The angle positions can be changed with the control elements of the image or on the **View** property page.
- **Center**: You can change the center of the image with the control elements of the image or on the **View** property page.
- **Zoom**: The current zoom factor of the display. You can change the zoom factor with the control elements of the image or on the **View** property page.
- Additional parameters for **Transparency**:
  - Number of calculated Texture elements per second
  - Number of calculated pixels per second

- Number of loaded texture planes: Maximum rendering speed is only obtainable, if all texture planes can be loaded in the graphic card's memory (`Nr. Texture planes = all`)

- Number of rendered textures as well as texels, bytes and pixels (all in millions)

- **Nr. of loaded …** shows number and size of textures during the process of loading them into the memory of the graphics card

➢ Additional parameters for **Surface**

- Number of calculated triangles / second

- Number of calculated vertices (= points where the line segments of two triangles meet each other)

- Total number of triangles

- Total number of vertices

Resolution

➢ **Reduction XY** / **Reduction Z**: In this field you can reduce the number of pixels used for the calculation in both the lateral (x,y) and axial (z) directions (subsampling). In some cases this can significantly reduce the duration of the calculation.

- To do this, move the corresponding slider, or enter the desired value in the input field. A reduction value of 2 (x,y) combines 2 pixels in the x and y directions, reducing the data volume by a factor of 4. A value of 2 for z combines two z-planes in one single plane, reducing the data volume by half.

- The more you reduce the data, however, the smaller the benefits in terms of speed. As a rule, a reduction of 2-3 is sufficient for a smooth display.

- **Triangles**: In **Surface** mode, here you can also determine the maximum number of triangles used. The fewer triangles used by the model, the higher the rendering speed. At the same time, this also reduces the level of detail.

  - The number of triangles should not be less than 5000, as otherwise the quality of the resolution is insufficient.

  - To achieve the same quality in the **Variable detail (graphics card)** mode as in the **Maximum detail (graphics card)** mode, the maximum number of triangles must be set, depending on the image data, to several million (entry of figures in the value field).

Clipping property page

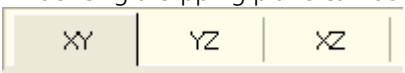
Inside4D features up to three so called **Clipping planes**. These planes can be used to cut the volume open in order to visualize structures which are within the volume. Clipping planes make most sense when used in the **Surface** mode but can also be used with limited functionality with the rendering modes **Maximum** and **Transparency**.

In **Surface** mode clipping planes can also be used in a channel specific manner. This means, that in a multichannel image one can selectively cut away portions of the volume in one channel in order to unveil other structures from other channels. This technique also works in **Mixed** mode.

A further possibility is, to use clipping planes in order to display only the gray values of the volume which touch the clipping plane.

All these functions can be used for creating a render series in order to make spectacular movies for presentation.

There are three clipping planes, which are named according to their default orientation with respect to the volume axis: **XY**, **YZ**, **XZ**. All the parameters influencing a clipping plane can be found on the corresponding tab:



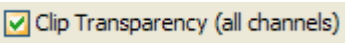
.

When set, each plane is placed into the center of the volume and oriented in orthogonal fashion according to its name (XY, XZ, YZ).

Clipping

- **Clip mode**: Here you can select between 4 clipping modes:
  - **None**: Plane has no clipping function.
  - **Back**: Plane cuts away the background.
  - **Front**: Plane cuts away the foreground.
  - **Both**: Plane cuts away both fore- and background. This mode is similar to the check box **Hide Volume** on the right side bar of the render window.
- 

: Here you can choose which channel should be clipped. Simply disable the channel you do not want to clip by clicking on the appropriate channel button: 

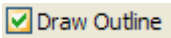
→ channel 1 and 3 will be clipped, channel 2 not. This function is only available for the modes **Surface** and **Mixed**.
- 

: This check box is available for the modes Surface, Maximum and Mixed. This option clips all transparently rendered channels in a global fashion. For technical reasons it is not possible to correctly clip transparent volumes in a channel specific manner.

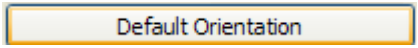
Plane Parameters

- **Draw Mode**: From this drop down box one of 6 options can be selected, which influence the way, the clipping plane is drawn:
  - **None**: plane is transparent

- **Colored**: clipping plane is colored in a semitransparent fashion with a green hue
- **Binary**: Gray levels above the defined threshold, which are touched by the plane are displayed binary white, black pixels are opaque
- **Transparent (linear)**: Data, which are touched by the plane, are displayed as in the render mode **Transparent** but in a two-dimensional projection. The ramp for the transparency in this case is always linear. Black pixels are transparent.
- **Transparent (with settings)**: Data, which are touched by the plane, are displayed as in the render mode **Transparent** but in a two-dimensional projection. The ramp for the transparency is as defined on the **Data** property page. Black pixels are transparent.
- **Opaque**: as with **Transparent (with settings)** but in this case black Pixels are non-transparent, rendered volume data in the background do not shine through.

➢ 

: draws a green frame outlining the plane. Especially useful, it plane is in **draw mode None**.

➢ 

: With this button the plane can be reset to its default orientation

➢ **Horizontal**: allows the rotation of the clipping plane along the horizontal volume axis (oblique orientation)

➢ **Axial**: allows defining an oblique orientation of the plane along the z-axis of the volume.

➢ **Position**: shifts the plan along the planes perpendicular.

Stereo property page

Sometimes the display of three-dimensional structures on a two dimensional display can be difficult. In this case it can be helpful to display volumes as **Anaglyphs**. The volume is separated into two separate views which are calculated as if viewed through two different cameras. Both cameras are positioned in a defined distance from each other. Each view is colored differently (red and green) so that when viewed through red-green glasses, a 3 dimensional impression is created for the observer.

The Stereo view can be invoked for all graphic card accelerated rendering modes (**Transparency**, **Surface**, **Maximum** and **Mixed**).

Stereo

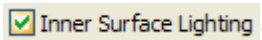
- **Stereo Mode**: enables (**Anaglyph Stereo)** or disables (**None**) the stereo mode.

- **Channel**: The drop down box contains the options **Both**, **Left** and **Right**. Only in **Both** is the analglyph stereo effect visible. The other modes each turn off the other channel.

- **Camera**: Defines the distance of both cameras from each other (Units in meters).

- **Parallax Shift**: Determines the degree of shift which is necessary to correlate both camera views. The slider should be adjusted to maximize the stereo effect. Please note, that this setting will be different for each observer.

Special property page

- 
: If checked, illuminates the inside of a surface rendered volume. If unchecked, the inside is dark. If one views the inside of a volume by flying or zooming into it, the inside illumination is helpful. This option is only available for the **Surface** mode.

- Front and Back Side Lit Differently: with activated **Inner surface lighting**, this checkbox colors the inside lighting with a bluish hue. This makes distinguishing inside from outside easier. This option is only available for the **Surface** mode.

- Draw Border Triangles: This option specifies to render plain surfaces. If this option is not activated these surfaces look like „windows" you can look through into the rendered volume.

- Transparent Surfaces ('Max.Detail' only: This option allows transparency to be assigned to surface rendered volumes in a channel specific manner. If checked, the slider **Transparency** will be available on the **Data** property page. This option is only available for the **Surface** mode if the method **Maximum Detail** is chosen on the **View** property page. Please be aware, that with multichannel volumens the transparency effect depends on the order in which the channels are rendered. This technical limitation does not always lead to satisfactory results.

- Gallery - Draw combined image If checked, this option displays the merge image including all channels of a multichannel volume in **3D-Gallery View**. This option is only available for the **Surface** mode.

- **Texture-Switch**: This parameter influences the volume angle, upon which the next set of 2D-Textures will be loaded into the graphics card (value range 1-100 degree). Depending on the object orientation, the number of z-slices and the chosen render settings this loading process can be visible and may be disturbing especially when rendering a series for movie export. Changing this angle can postpone or even avoid this switch during a series rendering. This Option is only available for **Transparency** and **Maximum** modes.

- Fly-Speed: Influences the fly speed (value range 0.0001 – 0.9999) when flying through a volume.

- **Mouse Sensit.:** Influences the mouse sensitivity (value range 1-720). Small values make the mouse actions precise but slow, large values less precise but faster.

- **VRML Export:** This button opens a standard Microsoft Windows dialog to save a surface mode in the VRML file format. VRML (Virtual Reality Markup Language) is a standard file format for representing 3D graphics. It has been designed particularly with the world wide web (WWW) in mind. VRML files are commonly called "worlds" and have the *.wrl file extension. To open and view these files a suitable Web Browser with a VRML PlugIn is required.

10.3 Deconvolution

A prerequisite for 2D deconvolution is a 2-dimensional image with 8-16 bit gray levels. All functions that are not necessary for 2D deconvolution are inactive.

A prerequisite for 3D deconvolution is an 8-16 bit gray level image in the form of a z-stack. To acquire a z-stack using AxioVision, the optional Z-stack module is required. If you wish to acquire z-stacks in several fluorescence channels, you will also require the Multichannel Fluorescence module.

The following processing methods can be used for the relevant image types in deconvolution:

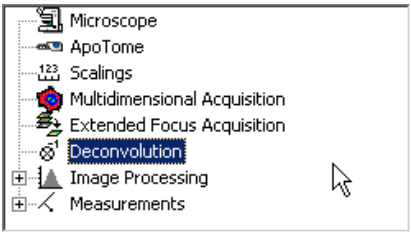
Frame rate	2D Deconvolution		3D Deconvolution	
2D Fluorescence	NN		NN	**x**
	RIF	**x**	RIF	**x**
	FIT	**x**	FIT	**x**
	CIT	**x**	CIT	**x**
Z-Stack Fluorescence	NN		NN	**x**
	RIF	**x**	RIF	**x**
	FIT	**x**	FIT	**x**
	CIT	**x**	CIT	**x**
3D ApoTome SIM	NN		NN	
	RIF		RIF	**x**
	FIT		FIT	
	CIT		CIT	

NN: Nearest Neighbor
RIF: Regularized Inverse Filter
FIT: Fast Iterativ
CIT: Constrained Iterativ

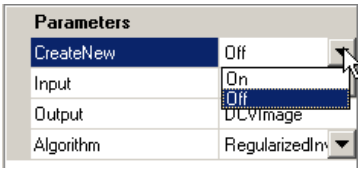
How do I perform deconvolution using the Regularized Inverse Filter?

- Load a suitable image. On the Product DVD, you will find the image "FluoCells_DCV-Teststack.zvi" in the folder *Demo Images and Scripts\AxioVision Images*. This is a 3-channel z-stack image, and is a good image to use here.

- In the work area, switch to the **Deconvolution** entry.



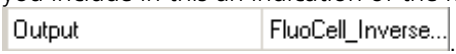
- This opens the **Deconvolution** property page. First of all, deactivate the **CreateNew** function. This setting ensures that only one resulting image is generated, and that the input image remains selected. This allows you to change parameters as often as you like, without a new resulting image being generated each time.



- If the desired image was in the foreground when **Deconvolution** was selected, it is now loaded as the input image. Otherwise load it by clicking on the ... button and selecting the image in the gallery:

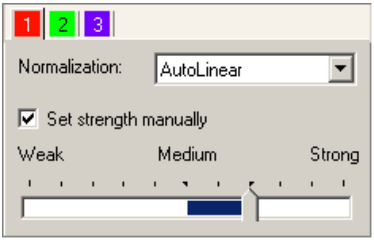


- The name of the resulting image can be changed. We recommend that you include in this an indication of the method used:



- The **Regularized Inverse Filter** method is already selected.

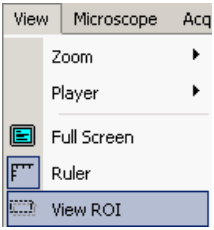
- In the **Channel specific settings** section, you can enter settings that may differ from channel to channel, e.g. the desired reconstruction strength. For the second channel (green), select a manual strength setting:



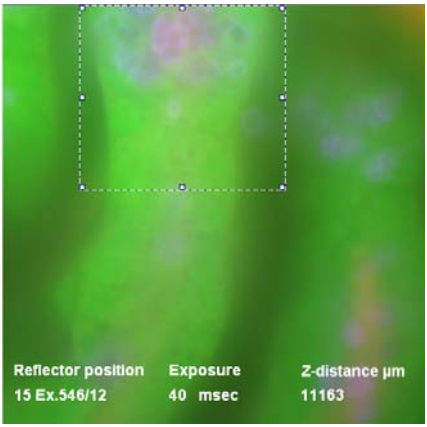
- Leave the other channels on the automatic strength setting.

- Leave the **AutoLinear** setting for normalization.

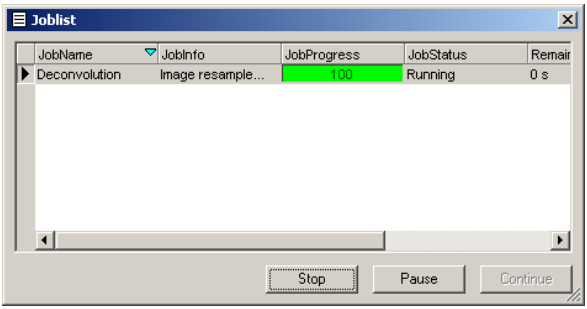
- Define a sub-region using the **Select ROI** menu command:



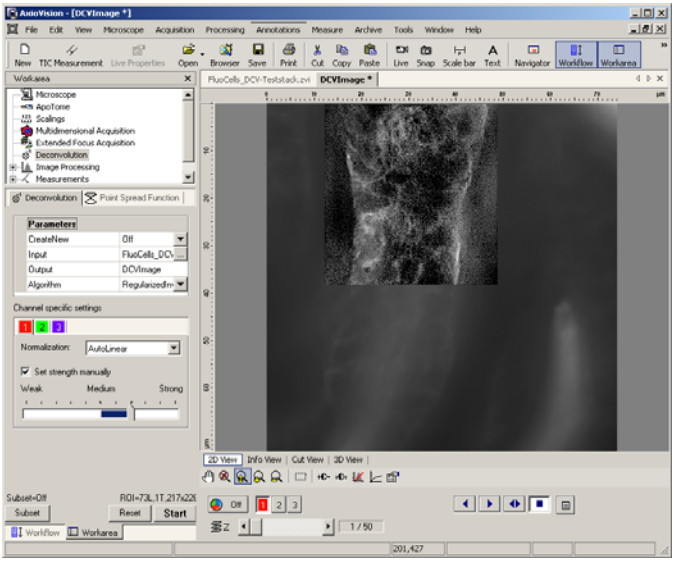
➢ In the image, define a small region to which you want deconvolution to be applied:



➢ Start the procedure by clicking on **Start**. Follow the display in the job list:



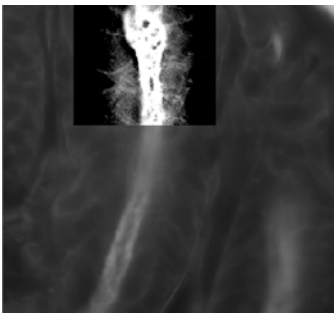
The resulting image should now look like this:



The first channel is displayed, and you can see the effect of the inverse filter applied to the ROI.

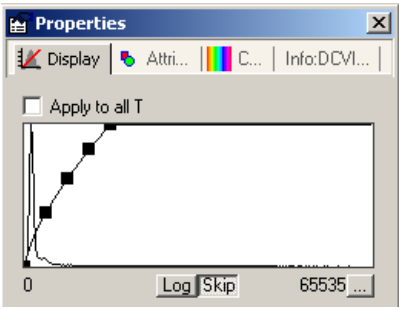
- Adjust the slider for Z until you arrive more or less at the center of the z-stack 

. You should now see an image that looks something like this:

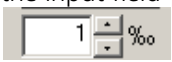


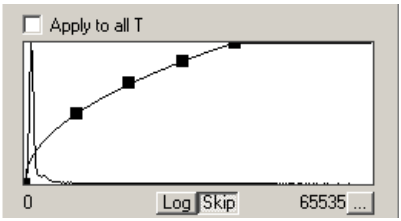
The bright regions in the acting structure of the fluocell sample appear over-saturated. This is the effect of automatic normalization. To compensate for this, open the **Properties** window using the  button in the image window's display field (or **Alt+Enter**).

In the image histogram, you can now see that the setting of the display characteristic curve is still the same as it was for the original image:

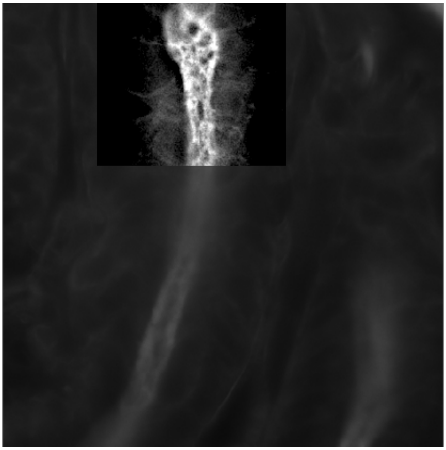


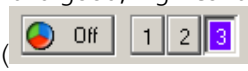
As the 16-bit dynamic range (= 65536 gray levels) is now being used in full, the display characteristic curve needs to be adapted to the new data range.

- To do this, click on the  button. The value in the input field underneath this button should then be one per thousand:  . The display characteristic curve is now set in such a way that 0.1% of the brightest pixels in the image are shown as completely white, and 0.1% of the dark pixels in the image as completely black. These settings only influence the display on the monitor, not the pixel values in your image. The histogram should now look something like this:



- The contrast setting of the image is now more or less correct. This means that you are able to properly assess the image components that have been enhanced using deconvolution:

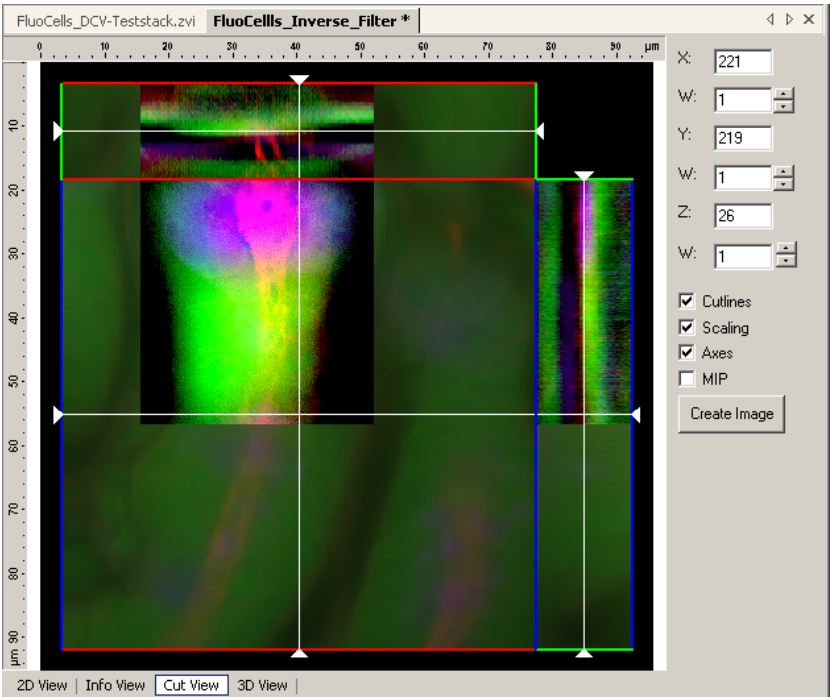


- Repeat these steps for channels 2 and 3 until a good, high-contrast image is also visible in superimposed color mode (

button).

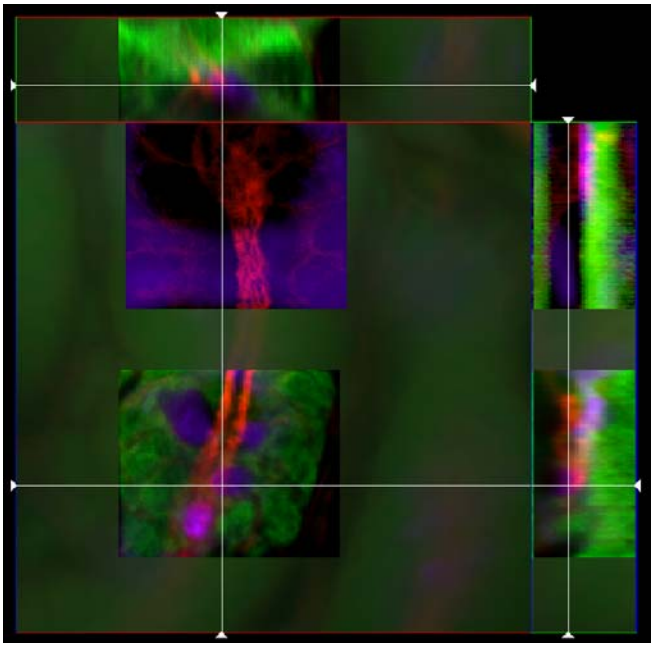
- The orthogonal slice view is particularly useful for assessing the result. Switch to the slice view by clicking on the **Cut View** tab 

. Position the slice lines in the deconvoluted region (for example):



➢ You can now clearly see the effect of the contrast enhancement. In particular, the separation of the green channel (which represents the cell nuclei of the intestinal cells in the fluocell sample) from the red actin elements is only clearly visible after deconvolution.

You can now experiment with the various parameters and deconvolution methods. Provided you do not make any changes to the name of the resulting image, the resulting images are overwritten every time you click on the **Start** button. This procedure also enables you to display various deconvolution methods simultaneously, in a single resulting image, by moving the ROI (region of interest) in the input image after selecting a different method.



Save the desired resulting image. These images are ideal for use with the 3D rendering module Inside4D.

10.4 Widefield Multichannel Unmixing

Background

Widefield Multichannel Unmixing is a new function for the removal of crosstalk between fluorescent dyes in multichannel images with up to eight fluorescence channels using a traditional epifluorescence microscope. Crosstalk is a phenomenon which occurs, whenever dyes are excited by excitation light of more than one filter combination. This problem occurs for example, when dyes with broadly overlapping excitation spectra are used concurrently in one sample. Good examples are the spectral variants of fluorescent proteins, e.g. BFP, CFP, GFP and YFP. With traditional reflector filter sets it has been often difficult to achieve 100% signal separation for such dyes.

In essence this means, that a certain proportion of a signal in one channel is actually derived from another dye spilling over into the channel.

A parameter based separation is now available with the **Widefield Multichannel Unmixing** module. The procedure is separated into three steps:

- Measurement of the amount of crosstalk in 2 alternative procedures:

  With appropriate reference samples
  by automatic component extraction (ACE) from the sample to be unmixed

- Creation of the unmixing matrix file.

- Unmixing of the image to be corrected by means of using the unmixing matrix file generated in step 2.

Note:

- Non-fluorescent channels such as DIC or Phase contrast are automatically detected and disregarded by all unmixing functions. Such channels are automatically copied into the unmixed result images to facilitate merged views with fluorescent channels.

- The module Widefield Multichannel Unmixing is designed to work with images created using the AxioVision Multichannel Fluorescence module. For the creation of correct reference images the use of the AxioVision Multichannel module is required.

- The images used in these examples you will find on the Product DVD in the folder *Demo Images and Scripts\AxioVision Images.*

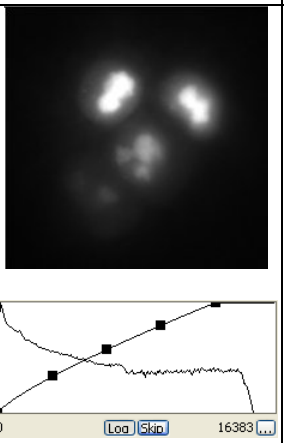
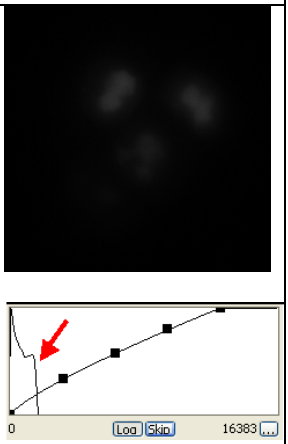
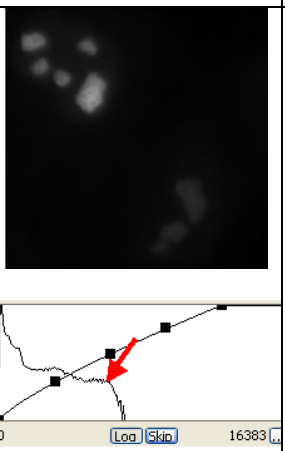
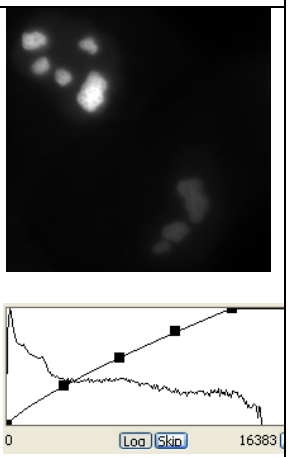
The Principle of unmixing

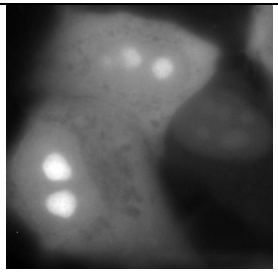
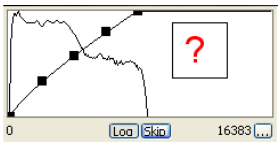
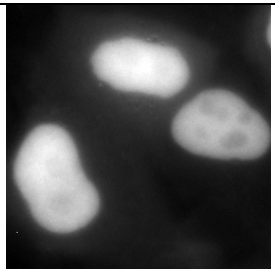
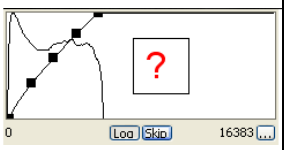
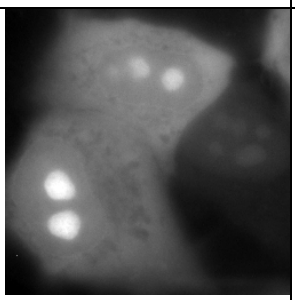
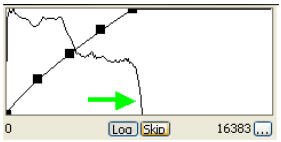
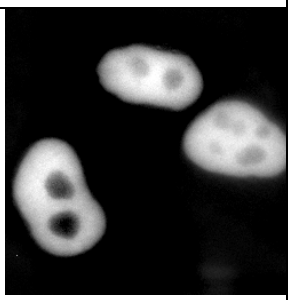
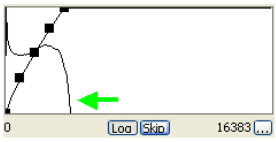
The following example tries to demonstrate the problem of cross talk as well as show the potential of unmixing such images. In this case, determination of cross talk is done using reference samples and not ACE.

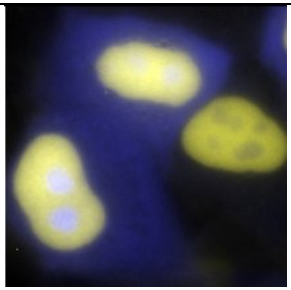
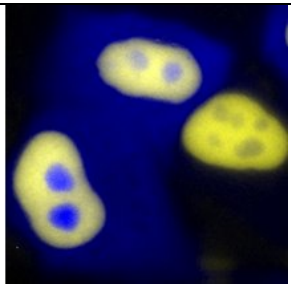
Reference sample A (CFP-Reference1.zvi): HeLa cells containing a virus proteins coupled to cyan fluorescent protein (CFP). This protein accumulates especially in the nucleolar regions of the cell nucleus.

Reference sample B (GFP-Reference1.zvi): HeLa cells containing a virus proteins coupled to green fluorescent protein (GFP). This protein accumulates in the nucleolar regions of the cell nucleus too.

Sample C: (RevCFP-H2-GFP_3.zvi): HeLa cells containing two proteins with different fluorescence: coupled to CFP is the same viral protein as in reference sample A (nucleoli); coupled to GFP is a histone protein, which stains the chromosomes and thus the entire nucleus a lesser degree of staining of the nucleoli.

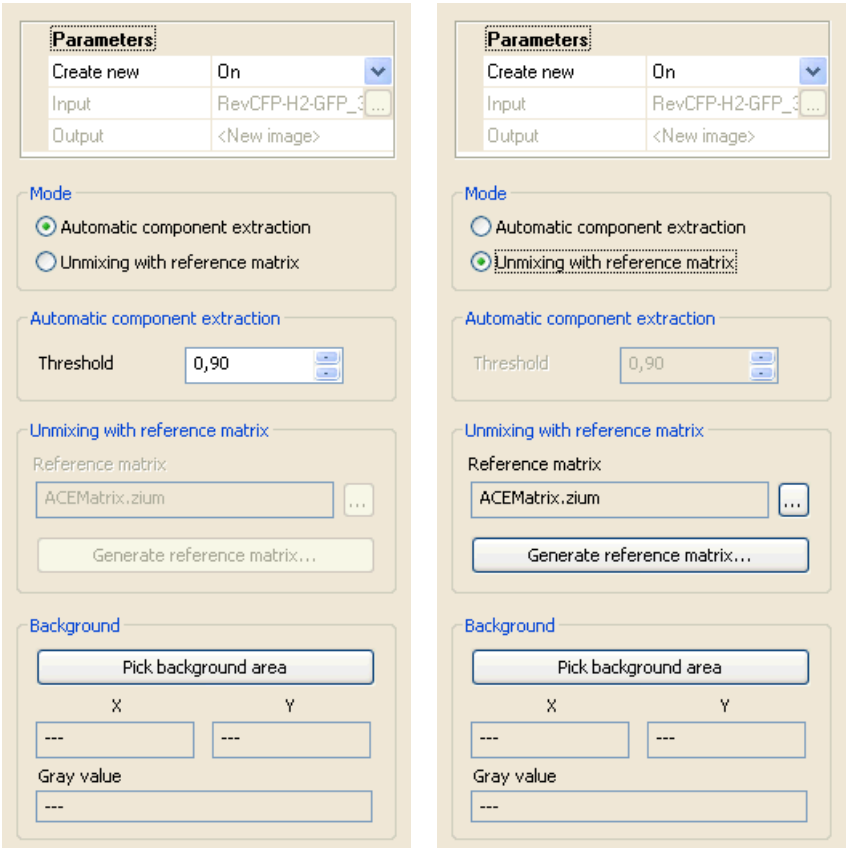
Sample	Channel 1(CFP)	Channel 2 (GFP)
A. Reference sample **CFP only**: A small degree of crosstalk is visible in channel 2 coming from channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		
B. Reference sample **GFP only**: A moderate degree of cross talk from channel 2 is visible in channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		

Sample	Channel 1(CFP)	Channel 2 (GFP)
C. 2-channel fluorescence sample (CFP and GFP): Before Unmixing: How much signal from channel 2 is visible in channel 1 (cross talk) and vice versa? **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.	 	 
D. 2-channel fluorescence sample (CFP and GFP): After Unmixing: A net percentage of pixel intensity values from channel 2 are added to the signal in channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.	 	 

	CFP&GFP-sample original pseudocolor mode	CFP&GFP-sample unmixed pseudocolor mode
E. 2-channel fluorescence sample (CFP and GFP): Comparison "before" and "after" unmixing: shows a markedly improved signal separation (shown at identical display settings.		

The Widefield Multichannel Unmixing function

The **Widefield Multichannel Unmixing** function is the central function for unmixing samples:



Using the function dialog, you can select whether the unmixing should take place via "automatic component extraction" (left-hand image) or using a reference matrix (right-hand image). If you choose unmixing using a reference matrix, you will need a reference matrix file containing the corresponding information for the unmixing.

If such a file does not yet exist, you can start a wizard via the 

button, which will lead you through the process of generating a reference matrix file.

Working with the basic functions

The basic functions in the menu **Processing** ⇒ **Widefield Multichannel Unmixing** are the functions for unmixing that will already be familiar to you from the previous version of AxioVision. These functions are still available, for compatibility reasons and for use in Commander scripts. The following two examples describe how you can use these functions for unmixing.

Unmixing with reference samples

This example shows you how to measure cross talk using reference samples and use this information to unmix a 2 channel fluorescence image.

The same images are used as in example 1.

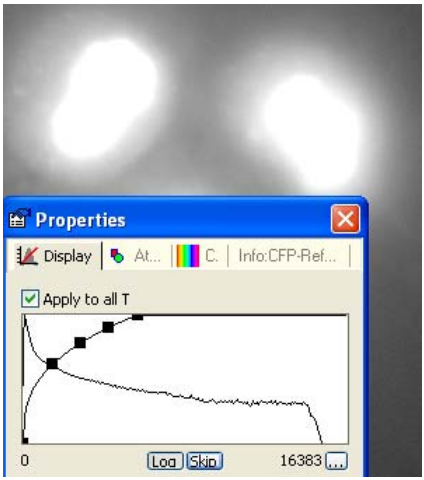
- Open the first reference image "CFP-Reference1.zvi".
  It is a 3 channel image. The first channel contains transmitted light information (DIC).
  The CFP signal is visible strongest in channel 2 (Zeiss filter set #47). Change into b/w mode (

) and channel 

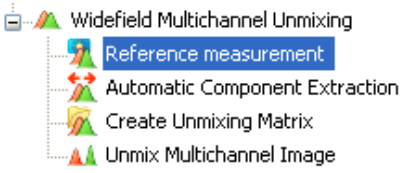
.



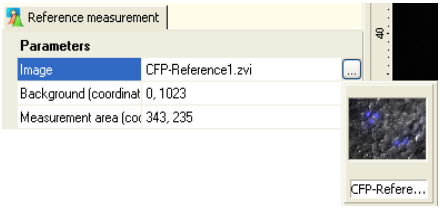
- Open the properties dialog (menu **View ⇒ Properties**) and adjust the display line in a way to enhance the gray value visibility (e.g. **Best Fit: Gamma value** ~ 0.25).
  Now it is easier to distinguish true image background from signal derived from the fluorescent dye.



- In the workarea select Image Processing/Widefield Multichannel Unmixing and then **Reference measurement**.

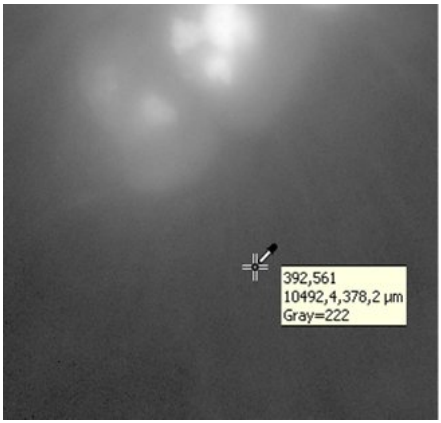


- The image "CFP-Reference1.zvi" is selected automatically as **Input** image because it the active image in the foreground.



- If it is not selected, select the image from the pop up gallery.

- In the edit field Background (coordinates) click on the ... button.



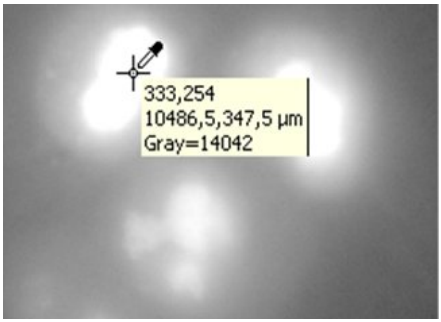
- Select a suitable region in the image for background correction (ideally without any signal coming from the dye). The pixel coordinates (x/y) as well as the gray value of the selected pixel are shown. If a scaling is available, the stage coordinates are shown.

- Click at an suitable position once in the image. The x and y coordinates are shown in the input field.



- Click the ... button in the input field Measure area (coordinates).

- Search for a suitable region in the image for measurement. Ideally search for a region with an intensity as high as possible.



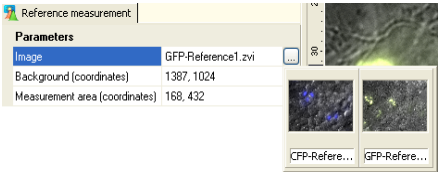
- Click the Start button to carry out measurement.



- The regions taken for measurement are shown in the image. Save the image, because it's used later for generation of the unmixing matrix.

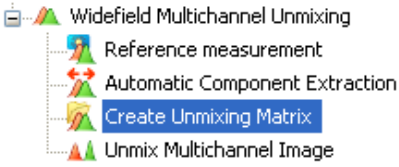


- Open the second reference image **GFP-Reference1.zvi**. The third channel contains the GFP fluorescence (fluorescence filter set # 44).



- Repeat the corresponding steps also for this image.

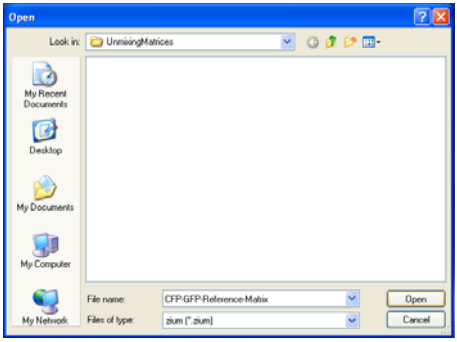
- In the workarea select the function **Create Unmixing Matrix** from the function group Image Processing/Widefield Multichannel Unmixing.



- 10. Select the input images accordingly: CFP-Reference1 as **ReferenceImage1**, GFP-Reference1.zvi as **ReferenceImage2**. Please take care to enter channels and reference images in the same order. If you use images with more than two channels, the function is extended accordingly.

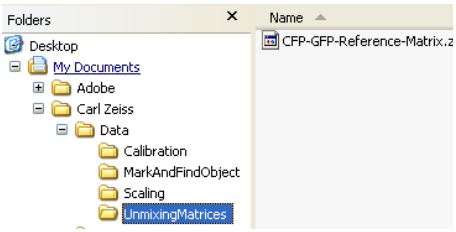


- To define a name for the unmixing matrix file, click the ... button in the input field MatrixFile. In the following dialog you can enter a name for the matrix file.

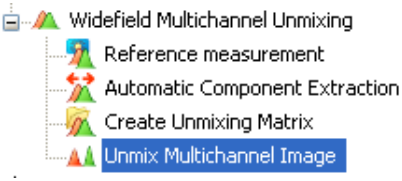


- Click the Open button to accept the filename.

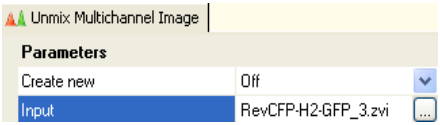
- Click the Start button to generate the unmixing matrix file. The file is saved – like other AxioVision files – in the user folder.



- In the workarea select the function **Unmix Multichannel Image** from the Image Processing/Widefield Multichannel Unmixing function group.



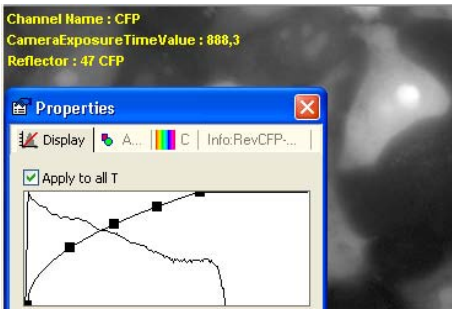
- Open the image to unmix and select it as **Input** image. In this example, the image "RevCFP-H2-GFP_3.zvi" is used.



- Switch to the black and white view (

) and activate channel 2 (

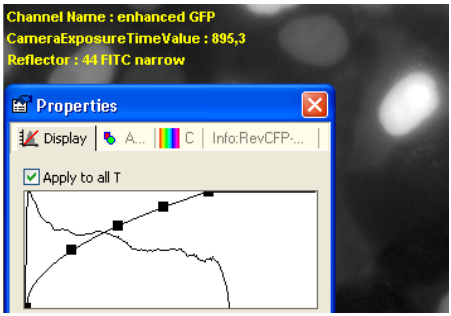
).

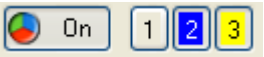


- Right click in the image and select **Properties**. Set the display characteristic line in such a way, that the gray values are displayed extremely amplified. So you can easily detect, where the background is not caused by the sample fluorescence.

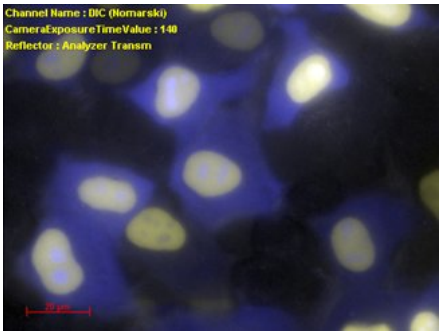
- Switch to channel 3 

and repeat the setting of the display characteristic line like in previous step.



- Switch back to the color view and switch off channel 1 

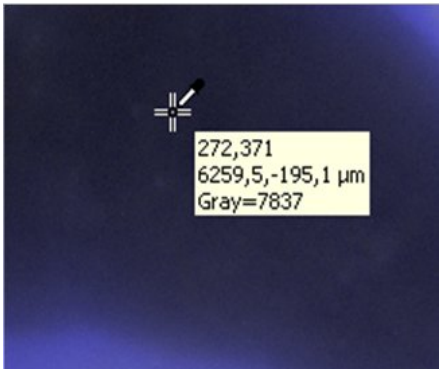
.



- Now you can separate the two fluorescence colors clearly from the background signal.

- Click the  button in the input field 

. In the image search for a suitable region for the background measurement (preferably without a signal caused by the fluorescence dye).

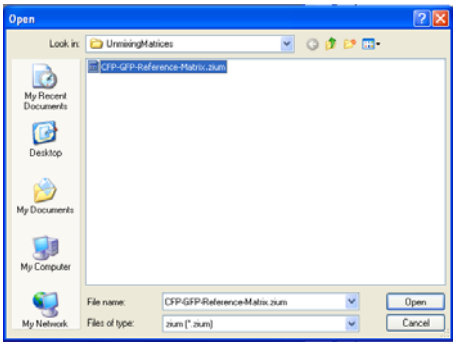


- The pixel coordinates (x/y) as well as the gray value of the selected pixel and stage positions are shown. Click at a suitable position once in the image. The x and y coordinates are shown in the input field.

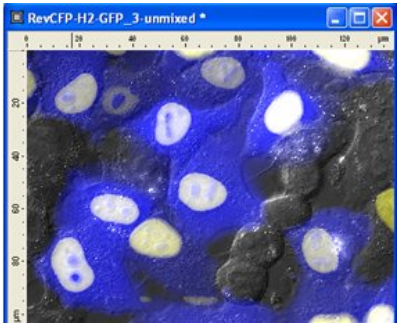
- Enter a name for the **Output** image.



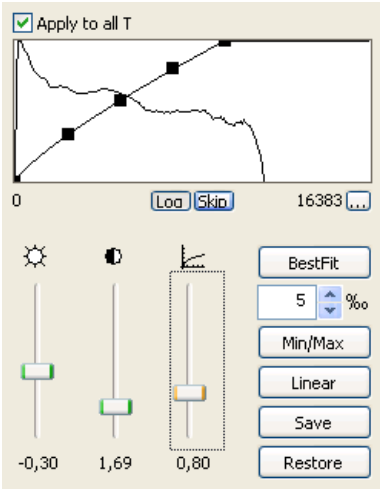
- Click the ... button in the input field MatrixFile to select the unmixing matrix file. Click the Open button to load the file. The dialog is closed.



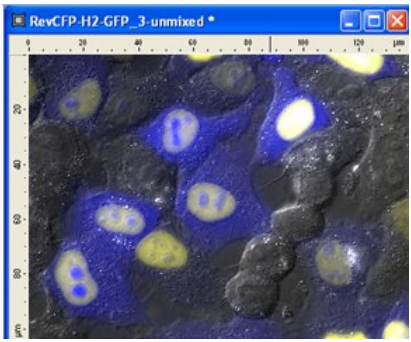
- Click the Start button to start the **unmixing**. The result image shows a clear separation of the fluorescence channels 2 (CFP) and 3 (GFP). Channel 1 without fluorescence (DIC) is copied to the output images unchanged.



- To optimize the display, you should set the display characteristic line for channel 2 and 3 accordingly:



- Save the output image.

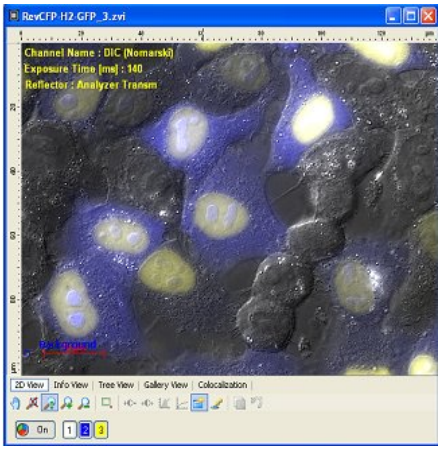


Unmixing of a multichannel image using ACE

This example shows you how to measure cross talk directly an image for unmixing with Automatic Component Extraction (ACE) and how to use it for unmixing of samples.

The same images are used as in example 1.

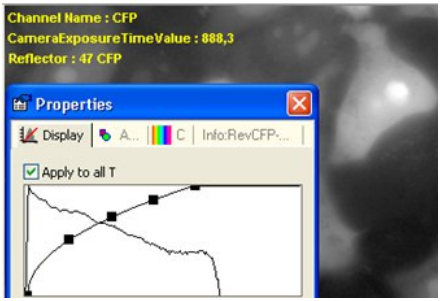
- Open the image to unmix. In this example the image "RevCFP-H2-GFP_3.zvi" is used.



- Switch to the black and white view (

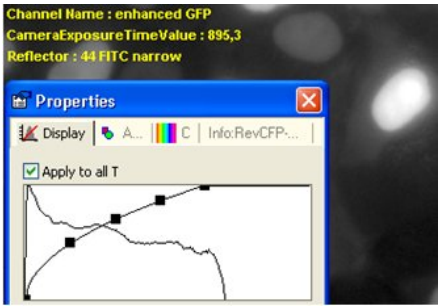
) and activate channel 2 (

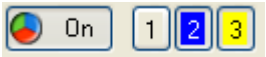
). Right click in the image and select **Properties**. Set the display characteristic line in such a way, that the gray values are displayed extremely amplified. So you can easily detect, where the background is not caused by the sample.



- Switch to channel 3 

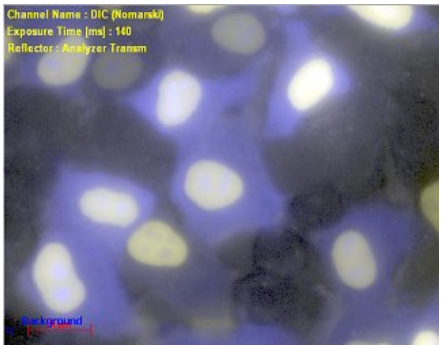
and repeat the setting of the display characteristic line like in previous step.



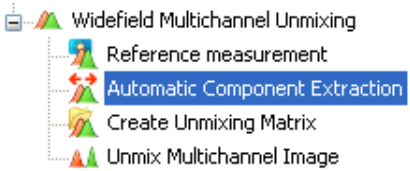
- Switch back to the color view and switch off channel 1 

.

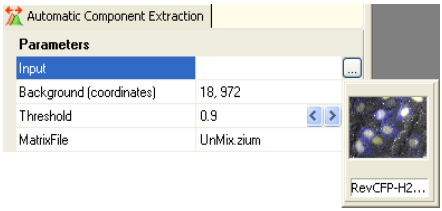
- Now you can separate the two fluorescence colors clearly from the background signal.



- In the workarea select the function **Automatic Component Extraction** from the Image Processing/Widefield Multichannel Unmixing function group.

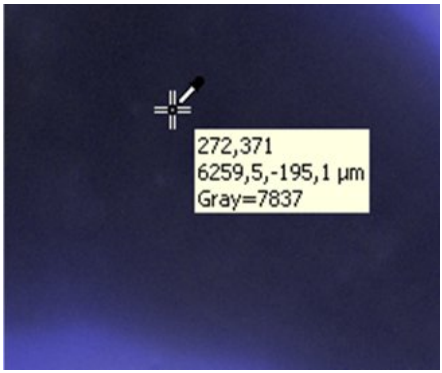


- The reference image "RevCFP-H2-GFP_3.zvi" is selected automatically as Input image because it the active image in the foreground.



- If it is not selected, select the image from the pop up gallery.

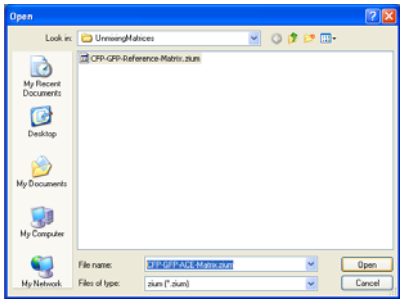
- Click the button in the input field Background (coordinates). In the image search for a suitable region for the background measurement (preferably without a signal caused by the fluorescence dye). The pixel coordinates (x/y) as well as the gray value and the stage position of the selected pixel are shown.



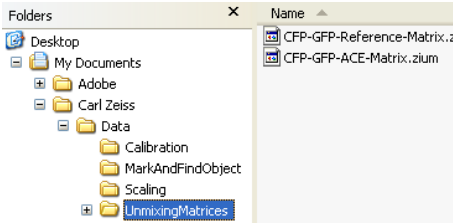
- Click at an suitable position once in the image. The x coordinate is shown in the input field.

- The predefined threshold in the input field **Threshold** can be used unchanged Threshold 0,9. For further information about this parameter, please read in the online help.

- To define a name for the unmixing matrix file, click the ... button in the input field MatrixFile. In the following dialog you can enter a name for the matrix file. Click the Open button to accept the filename.

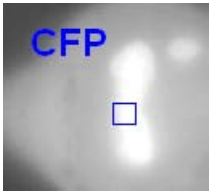


- Click the Start button to generate the unmixing matrix file. The file is saved – like other AxioVision files – in the user folder.



- The measurement regions defined by ACE are drawn to the image using the channel color.

Channel 2:

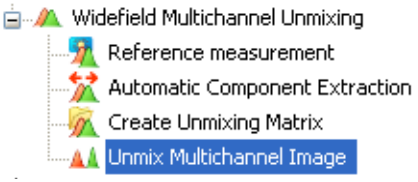


- Switch to the black and white view (Off) and activate the accordant channel.

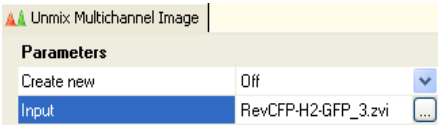
Channel 3:



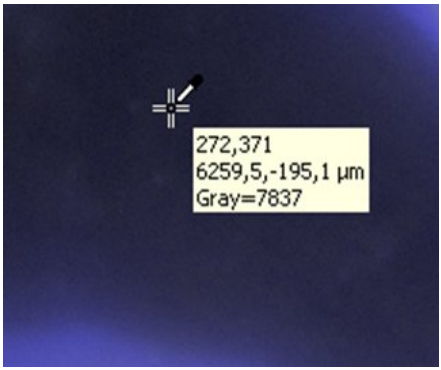
- In the workarea select the function **Unmix Multichannel Image** from the Image Processing/Widefield Multichannel Unmixing function group.



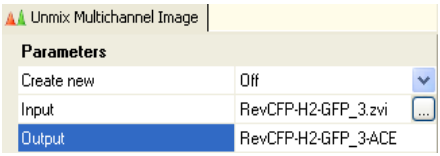
- Open the image to unmix and select it as **Input** image. In this example, the image "RevCFP-H2-GFP_3.zvi" is used.



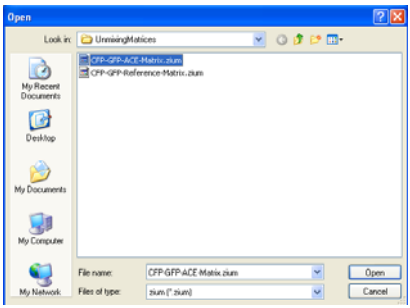
- Now repeat the background measurement:
- Click the ... button in the input field Hintergrund (Koordinaten) and then click in a background region.



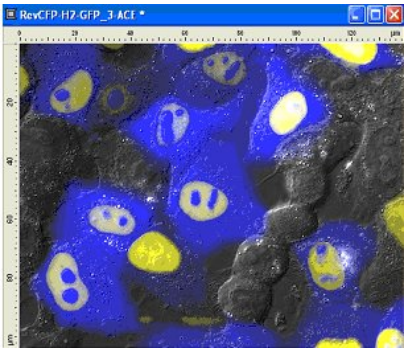
- Enter a name for the **Output** image.



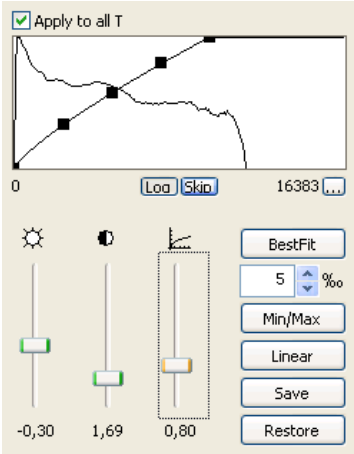
- Click the ... button in the input field MatrixFile to select the **unmixing matrix file**. Click the Open button to load the file. The dialog is closed.



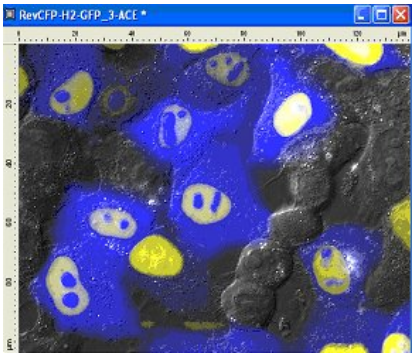
- Click the Start button to start the unmixing. The result image shows a clear separation of the fluorescence channels 2 (CFP) and 3 (GFP). Channel 1 without fluorescence (DIC) is copied to the output images unchanged.



- To optimize the display, you should set the display characteristic line for channel 2 and 3 accordingly:



- Save the output image.



10.5 Colocalization

Background

The acquisition of fluorescence images in several channels makes it possible to visualize the relationship between biological structures. Displaying two channels together in color merge mode makes it easier to establish whether the components are "colocalized", i.e. are occupying the same position in space.

The classic display is the color-coded overlaying of two fluorescence channels. The most common form is red/green overlaying. Regions in which both fluorescent dyes occupy the same position in space are therefore shown as yellow. However, it is not possible to use this display as a basis for quantitative statements about the extent of colocalization. At best, a qualitative statement can be made on whether or not two dyes are colocalizing. The **Colocalization** module can fill this gap and provides you with a tool that enables you to determine colocalization quantitatively.

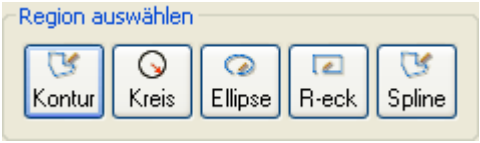
Principle:

- The colocalization of two channels is always analyzed. For images with more than 2 channels, the user is required to make a selection. Colocalization results from the pixel-by-pixel comparison of intensities per channel.

Control elements

The colocalization display consists of three elements: the scatter plot, the image (2 channels are shown) and the table of measurement values. On the right-hand side of the display is a control field for the colocalization settings. The colocalization display can be activated in the **View** ⇒ **Views** ⇒ **Colocalization** menu or by clicking on the **Colocalization** button at the bottom edge of the image.

Regionn Select



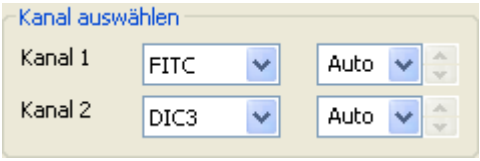
: Using these buttons you can select up to 32 regions in the input image or the scatter plot. Five different forms can be used by clicking on the buttons: contour, circle, ellipse, rectangle, contour (spline). If you hold the button down, the form of the mouse pointer changes to the drawing symbol. Once you have finished drawing, the mouse pointer changes back to the original arrow symbol and the button is no longer shown as depressed.

Once a region has been drawn, it is automatically treated as an active region. The scatter plot shows the pixel-value frequencies of these regions. The colocalization table shows the data for the entire image and for the selected region. If you wish to select several regions, you can do so by holding down the Shift key and clicking on the regions in question. You can also deselect them in the same way.

Note:

- The regions are only visible in the **Colocalization** view and are not adopted if you switch to a different type of display.

Channel Select



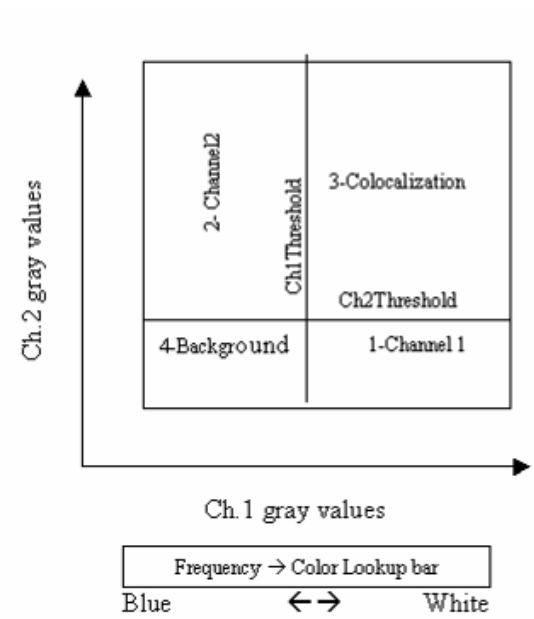
: Here you can select the two channels that you want to view.

In the drop-down list boxes on the right you can set a gray value range for each channel. This allows you to make optimum use of the available gray value range. You can have the range set automatically, choose between predefined fixed values or enter a value of your choice interactively. The scaling of the scatter plot is adapted according to the selection you make.

Threshold



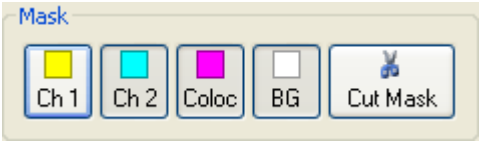
: In these list boxes you can set the threshold value for both channels in accordance with the graphic below.



- 

: This button becomes active as soon as a region is inserted into the scatter plot. It remains active as long as regions are being selected or moved there. By activating and deactivating this button, you can switch between the selection of threshold values using the mouse and the selection/shifting of selected regions in the scatter plot image. If regions have been defined in the scatter plot, the corresponding data appear in the table, in addition to the entire image.

Mask



: Using these buttons you can mask the four quadrants described in the **Threshold** section and assign user-defined colors to the channels of the original image (**Ch1** and **Ch2**). The same process can also be carried out for the colocalized pixels (**Coloc**) and the background (**BG**). The color selection menu is opened by right-clicking with the mouse.

- 

: This button becomes active as soon as a quadrant has been masked. If you press the button by clicking on it with the mouse, a new image containing exclusively the masked pixels is created.



: Using these buttons you can select the scatter plot and/or the value table for the image currently being displayed and edited for saving in a separate file. This enables you, for example, to edit the data in another program (e.g. Microsoft Excel).

Graphical Display

The **Scatter Plot** is a graphical display of the pixel-value frequencies of channels 1 and 2. These frequencies are shown using a rainbow lookup table (units between 0 and 255). Channel 1 is distributed along the x-axis and channel 2 along the y-axis. The threshold value can be changed directly in the graphic by left-clicking with the mouse. It is also possible to move the threshold value lines interactively using the mouse.

The **Colocalization table** contains 17 columns. These are used to present the following details and calculations:

1. **Image Region**: When a region has been selected, it is assigned a number. This number appears in the image and the table. Data from the scatter plot can either relate to the entire plot or to the selected region. For this reason no numbering for the selected region appears here in the data table.

2. **Scatter Quadrant**: As described under the **Threshold** field, the scatter plot display is divided into four quadrants, which are numbered 1,2,3 and 4. These numbers correspond to channel 1, channel 2, colocalization and background. All data can be obtained individually for these four quadrants. In addition, the threshold value set in the scatter plot is displayed for each channel.

3. **Number of Pixels**: Shows the total number of pixels in each quadrant. The sum of all the pixels in this column for all 4 quadrants corresponds to the product of the height and width of the original image.

4. **Area (µm²)**:
   Area = number of pixels x X-scale x Y-scale
   If no scaling is available for the original image, the following applies:
   1 pixel = 1 µm.

5. **Relative Area (%)**:
   Relative area = area of quadrant x / total area
   x = 1, 2, 3, 4; total area = area of quadrant 1 + area of quadrant 2 + area of quadrant 3 + area of quadrant 4.

6. **Mean Intensity Channel 1**: The sum of all the gray values of channel 1 divided by the total number of pixels in this channel.

$$\frac{\sum \text{GrayValuesChannel1}}{\text{AreaChannel 1}}$$

7. **Mean Intensity Channel 2**:

$$\frac{\sum \text{GrayValueChannel}\,2}{\text{AreaChannel}\ \ 2}$$

8. **Standard Deviation Channel 1**:

$$\sqrt{\frac{\sum (\text{GrayValuesChannel}1 - \text{MeanIntensityChannel}1)^2}{\text{AreaChannel}\,1 - 1}}$$

9. **Standard Deviation Channel 2**:

$$\sqrt{\frac{\sum (\text{GrayValueChannel}2 - \text{MeanIntensityChannel}\ \ 2)^2}{\text{AreaChannel}\,2 - 1}}$$

10. **Colocalization Coefficient Channel 1**: This coefficient indicates the relative number of colocalized pixels in channel 1 or 2 in relation to the total number of pixels above the threshold value:

$$\frac{\sum \text{Pixels Channel}1_{,\text{colocalized}}}{\sum \text{Pixels Channel}1_{,\text{total}}}$$

The values vary between 0 and 1, where 0 indicates no colocalization and 1 complete colocalization.
Numerator = number of pixels in quadrant 3
Denominator = number of pixels in quadrant 3 + number of pixels in quadrant 1

11. **Colocalization Coefficient Channel 2**:

$$\frac{\sum \text{Pixels Channel 2}_{,\text{colocalized}}}{\sum \text{Pixels Channel 2}_{,\text{total}}}$$

Numerator = number of pixels in quadrant 3
Denominator = number of pixels in quadrant 3 + number of pixels in quadrant 2

12. **Weighted Colocalization Coefficient Channel 1 (Pearson)**:
Provides information on the intensity distribution within the colocalization region.
Value range: -1 to 1.

1: All pixels lie in a straight line in the scatter plot (if you have used the same channel twice for colocalization, you will obtain the value 1 in this column).
0: The pixels in the scatter plot are distributed in the form of a cloud with no preferred direction.

$$\frac{\sum((\text{GVC1}-\text{MVC1})\times(\text{GVC2}-\text{MVC2}))}{\sqrt{\sum(\text{GVC1}-\text{MVC1})^2\times\sum(\text{GVC2}-\text{MVC2})^2}}$$

GV: gray value; MV: mean value; C: channel

13. **Overlap Colocalization Coefficient (Manders)**:
Insensitive to differences in signal intensity between the two channels and to bleaching.
Value range: 0 to 1

$$\frac{\sum \text{GrayValueC1}\times\text{GrayValueC2}}{\sqrt{\sum \text{GrayValueC1}^2\times\sum \text{GrayValueC 2}^2}}$$

C: channel

14. **Index Z**: Indicates the z-index for z-stack images.

15. **Index T**: Indicates the time index for time lapse images.

16. **Acquisition Time**: Indicates the time of acquisition for all dimensions of a multidimensional image.

17. **Focus Position**: Indicates the focus position at which an image was acquired.

11 Image Analysis Modules

11.1 Interactive Measurement

General

The Interactive Measurement module builds on the standard image analysis functions in the basic program. It offers the following two additional features:

- A clearly extended list of measurement parameters: besides the standard parameters there are, among other things, several tools for distance measurement and multiple distance measurement, two additional options for cyclometry (including clicking on contour points), and much more besides.
- The possibility for generating measurement programs for interactive measurements.

Notes:

- For general background information and basic details on how to operate the measurement function, please read through chapter 6 "Image Analysis" first.

- A complete list, plus a description, of all measurement parameters can be found in the online help.

Working with interactive Measurement Programs

Measurement programs allow you to put together parameters of your choice, which the user must then run through in the defined sequence. This creates a kind of electronic work instruction that ensures that a measurement sequence is run through properly and completely. The programs can be saved so that they can be used in routine operation.

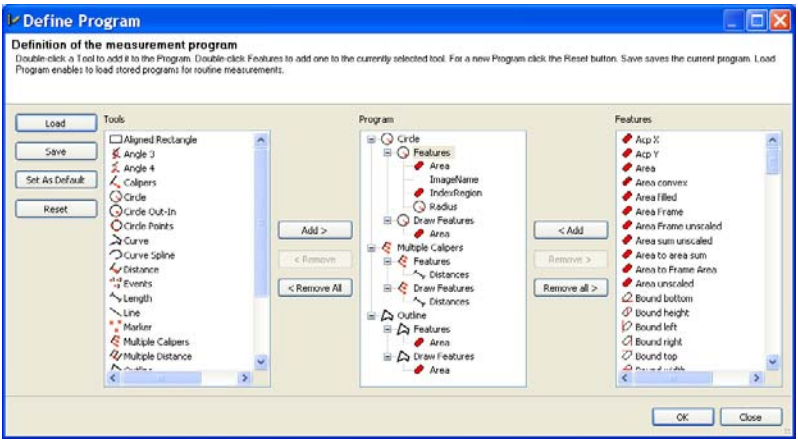
Working with interactive measurement programs usually involves two steps: 1. loading and 2. executing a measurement program. However, before a measurement program can be used for routine measurements, it must first be configured.

Note:

- Further explanations on all the functions described can be found in the online help.

Generating measurement programs

Using the **Measure** ⇒ **Interactive Measurement Programs** ⇒ **Define** function, you can load and modify existing measurement programs or generate new ones.



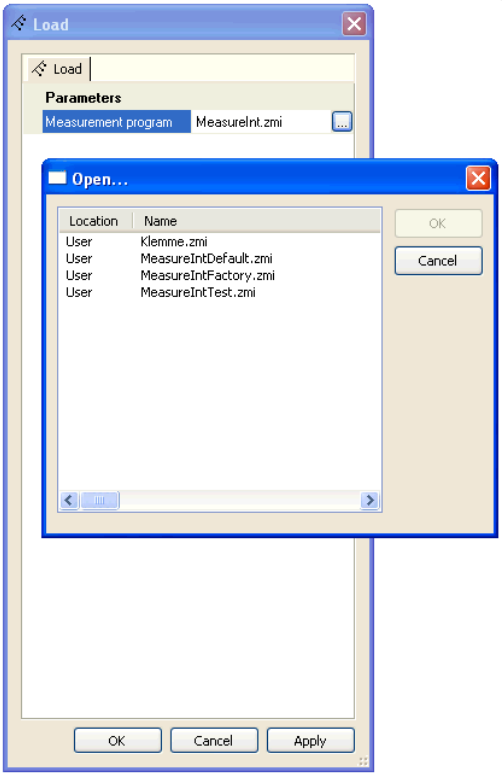
Generating measurement programs: selecting measurement tools and parameters

The **Interactive Measurement Programs**⇒ **Define** function is also suitable for loading measurement programs, as the relevant program is also visible here. There is an independent function for loading a program. This is particularly useful for automatic execution within a Commander script.

Loading and executing measurement programs

In order to execute an existing measurement program, it must first be "loaded". This means that one program is selected from a list of existing measurement programs. This program then becomes active. When the execution function is started, the program that is currently active is always the one that is used.

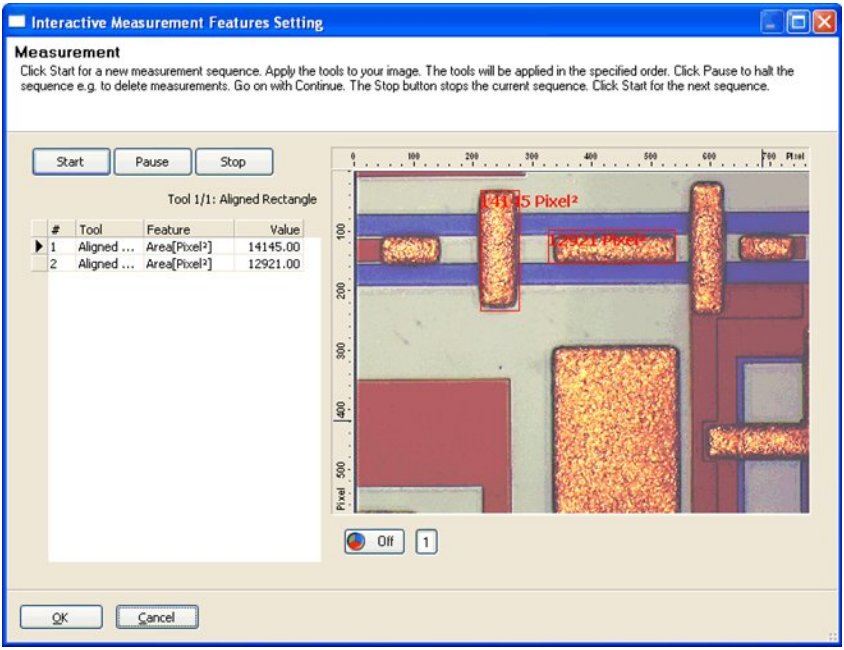
To load a measurement program, please select the **Measure ⇒ Interactive Measurement Programs ⇒ Load** function:



Loading a measurement program

The desired program can be selected from a list of existing programs. You can also use the **Measure ⇒ Interactive Measurement Programs ⇒ Define** function to load a measurement program. The advantage of this is that a loaded program is also displayed in this function. However, if a measurement program is to be automatically loaded and executed within the context of a Commander script, the function for generating programs is not suitable.

When the measurement program has been loaded, you can start it via the **Measure ⇒ Interactive Measurement Programs ⇒ Run** function:



Executing a measurement program

11.2 AutoMeasure (Automatic Measurement)

General

The process of measuring interesting objects in an image automatically is closely linked to the rapid development of the hardware sector. Without this development it would take a huge amount of time to perform these measurements (which can involve several hundred images in some cases).

Thanks to the performance of modern PCs, it is now possible to use increasingly complex functions for image analysis, and to perform an automatic evaluation.

The term automatic measurement is understood to mean, in particular, the **automatic recognition** of interesting objects. In contrast to interactive measurement, it is no longer necessary to use the mouse to outline the objects to be measured in the image. The objects can be recognized automatically on the basis of their brightness and/or color and other descriptive parameters (shape, size etc.).

An automatic measurement usually consists of the six steps briefly listed below. These steps represent the general sequence of an image analysis procedure. They are in no way specific to AxioVision, and should always be carried out in this or a similar form.

1. Image acquisition

Image acquisition is – besides segmentation – the most important step within image analysis. It is only during image acquisition that you have all the original data available. Image information that is poor or, worst of all, incorrect prevents you from achieving accurate measurement results. This can even lead to a situation in which a sample that should be simple to measure from an image analysis perspective is no longer analyzable, as the system can no longer recognize the objects to be measured. In such cases you have to revert to interactive measurement.

Information that is not acquired during image acquisition is very difficult to reconstruct during image enhancement. In many cases this is not possible at all. You *must* therefore take advantage of all of the technical possibilities offered by your microscope and camera during the preparation of the sample.

Often there is room for improvement with regard to sample preparation. You need to be self-critical, and check whether the quality of your samples could be improved.

2. Image enhancement

In general terms, it is possible to distinguish between two different application situations:

The **optical enhancement** of an image helps the user to correctly interpret the accuracy of the solution suggested by the measurement system. This includes enhancement of the brightness and contrast, and also gamma correction. Enhancing the quality of the image from an optical perspective, however, has no influence on the quality of the recognition results.

Functions to enhance recognition performance (segmentation) allow you under certain circumstances, that images can be measured automatically even when the image quality would lead you to assume otherwise. These functions include, for example, the elimination of illumination errors (shading correction).

3. Segmentation

Segmentation is the key to a correctly functioning automatic measurement. This process involves having the interesting objects determined automatically by the measurement system, and separated from the regions that are not to be measured (the image background). Initially this takes place on the basis of brightness or color information. Reference objects are outlined or highlighted in the image using the mouse. AxioVision then recognizes all other objects independently. It is also possible to directly select the thresholds for the brightness or color ranges that represent the objects using the distribution of the brightness or color information (histograms). However, this requires a certain amount of experience.

The result of segmentation is a "measurement mask", which is represented internally by a so-called binary image. This mask conceals the background regions using black pixels, and shows the objects to be measured as white. You will only come into contact with this measurement mask indirectly. AxioVision shows it as a color image superimposed over the original image.

4. Processing the measurement mask

A measurement cannot be performed until the objects to be measured, and only those objects, have been correctly recognized. Sometimes, however, this is not possible. Artifacts that you do not want to measure may also be acquired, or objects may be touching and may not therefore be recognized as individual objects by the system. These must then be separated before proceeding.

Typical processing steps therefore involve the deletion of artifacts, the filling of gaps in objects and the automatic separation of objects. In difficult cases, subsequent interactive processing (deletion, separation, additional drawing in of objects etc.) may also be required.

5. Measurement

During the measurement, the selected parameters are determined by AxioVision and saved in a results file. Two types of file are distinguished: files for so-called **region specific** and **field specific** image data.

- **Region specific** image data are measurement parameters that are determined for every individual object (an image ***region***). These are, for example, the area, circumference and shape of the object in question, or even its densitometric values, such as the average gray value. The measurement results are written within the results file in the form of **one line per region** or object.

- **Field specific** data are measurement parameters that are determined for the entire image ***field***. These are, for example, the number of objects, or the percentage of the entire image area represented by all the objects present, etc. Field-specific measurement results lead to **one line per image** in the results file.

6. Evaluation

Before you can actually make a statement about the image using the data for the images or objects in which you are interested, this data needs to be evaluated. As a wide range of special evaluation programs, such as Microsoft Excel, are now available, AxioVision itself does not feature any evaluation functions. The data are made available in Excel format, and can therefore be evaluated in the environment you are familiar with.

In addition to the pure measurement data, image documentation should also always be used to make clear which objects were used to determine the measurement results. AxioVision gives you the option of displaying the objects, e.g. by outlining their contours, and the measurement results directly in the image.

The concept of the AutoMeasure module

The concept of the AutoMeasure module is based on Carl Zeiss' many years of experience in the field of automated image analysis. It has become clear that in most cases only a small proportion of the range of available functions are actually required and employed. The aim was to arrange these functions in a way that was simple to operate and would also allow less experienced users to perform fast and accurate measurements.

As with automated analyses you are usually not just measuring an individual image, but in some cases are analyzing a very large number of images, the interactive execution of individual steps via menu functions or a workflow is often not a realistic option. Nevertheless, with the AutoMeasure module you have the option of measuring "just" one image quickly and efficiently (further information on this will be provided later).

The two points touched upon – "Complex technology, simply packaged" and "Analysis of large numbers of images" – are reflected by the structure of the AutoMeasure module.

The module therefore consists of two parts:

- The **Automatic Measurement Program Wizard** allows you to generate automatic measurement programs quickly and simply.

- The function for the **Run Automatic Measurement Program** allows you to execute a program you have generated for any number of images. It makes no difference whether these images are acquired via a camera or are already present as files.

Notes:

- At this point, we should emphasize that the AutoMeasure module is not suitable for resolving every conceivable measurement task. It is intended more as a tool to simplify your everyday tasks.

- More complex applications are possible using the AutoMeasure Plus module. In this module the full range of image analysis functions are available via the AxioVision menus, and can be freely combined. You can therefore even realize exotic applications, although this requires the person generating the program (*not the actual user of the program*) to have some knowledge of the background to digital image analysis.

The wizard for generating measurement programs

The Automatic Measurement Program Wizard leads you step by step through the individual stages of digital image analysis (please also read the "General" section earlier in this chapter).

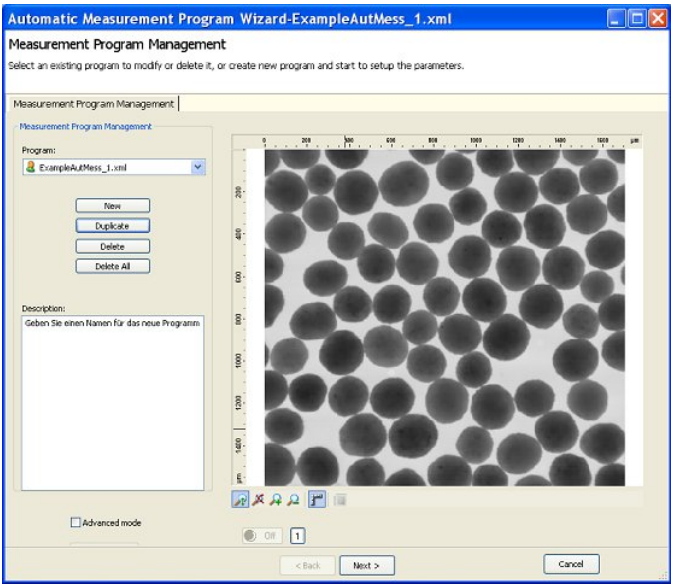
➢ From the Product DVD (folder *AxioVision Images\AutoMeasure*) load the image "round.zvi".

➢ Start the wizard in the menu **Measure** ⇒ **Automatic Measurement Programs** ⇒ **Program Wizard**.

Further information on the individual steps, and the functions and functional parameters used, can be found in the online help by pressing the **F1** key.

Program management

Program management gives you an overview of the measurement programs available, and a brief description on them individually:



Program overview with brief description on the selected program

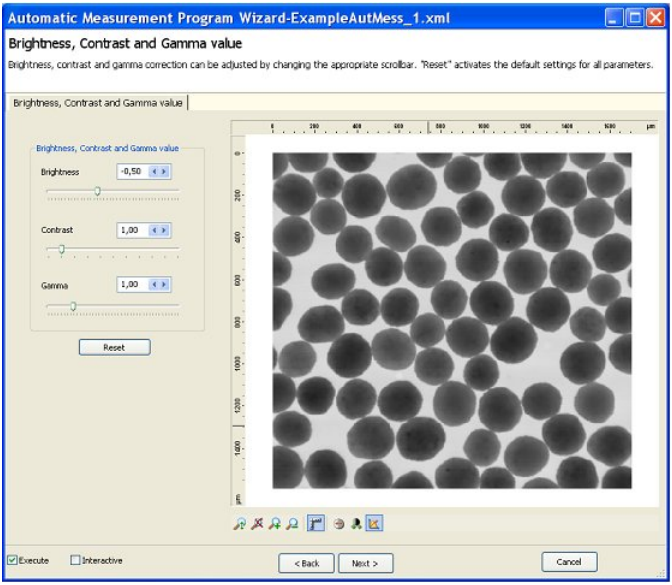
Click on the **New** button and enter a name for your measurement program. It will be saved in the folder *My Documents\Carl Zeiss\Data\Automatic Measurement Projects*.

Note:

- The measurement programs are saved by default in the folder *"My Documents\Carl Zeiss\Data\Automatic Measurement Projects"*.

Image enhancement

For optical image enhancement, functions are available for brightness adjustment, contrast enhancement and gamma correction.

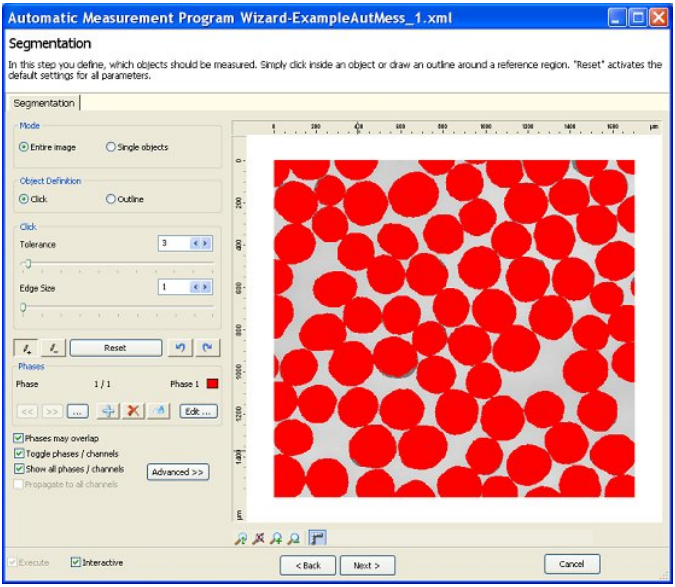


Enhancement of brightness, contrast and gamma value

To improve detection, images can be smoothed (Sigma filter), illumination errors corrected (Shading correction), and object edges sharpened (Delineation).

Segmentation

Segmentation is the most important function of the AutoMeasure module.



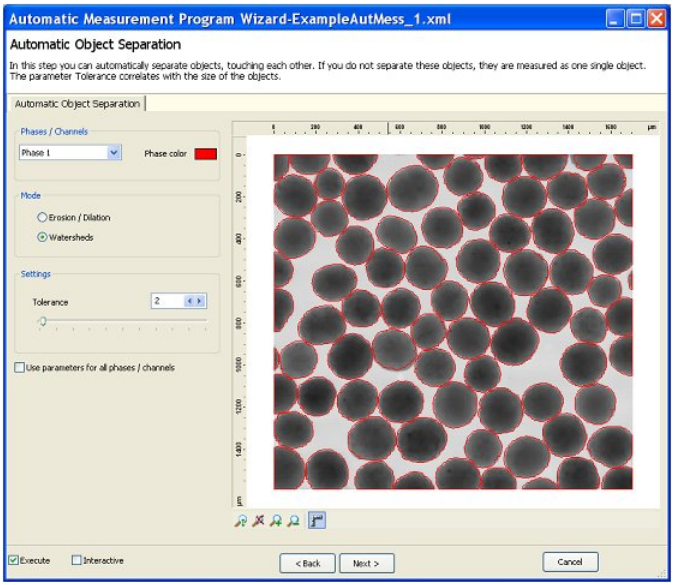
Dialog for image segmentation

Here you can separate the objects to be measured from the background simply by clicking on or outlining reference objects. Select whether you want individual objects only, or the entire image, to be segmented.

With multidimensional images, you also have the option of performing segmentation separately for each channel of a multichannel fluorescence image. If two-dimensional gray level or color images are used, up to 32 different phases can be defined (in place of channels).

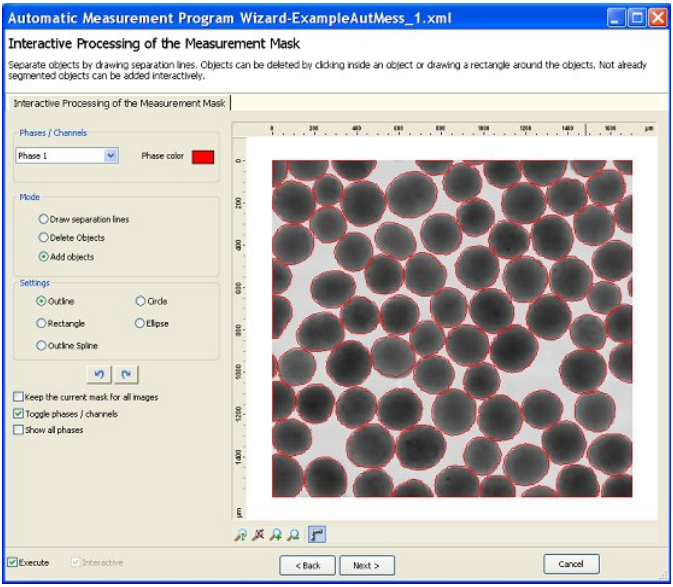
Object Separation

To separate connected objects automatically, you can use the functions **Erosion/Dilatation** (for round objects) or **Wasserscheiden** (for objects with no distinct shape). The extent of the separation is set via the tolerance.



Processing the measurement mask (binary image editor)

In most cases, the measurement mask generated during segmentation has to be processed subsequently to ensure that only the objects you actually want to measure are highlighted. With the interactive correction of the measurement mask you can draw-in separating lines, delete and add objects).

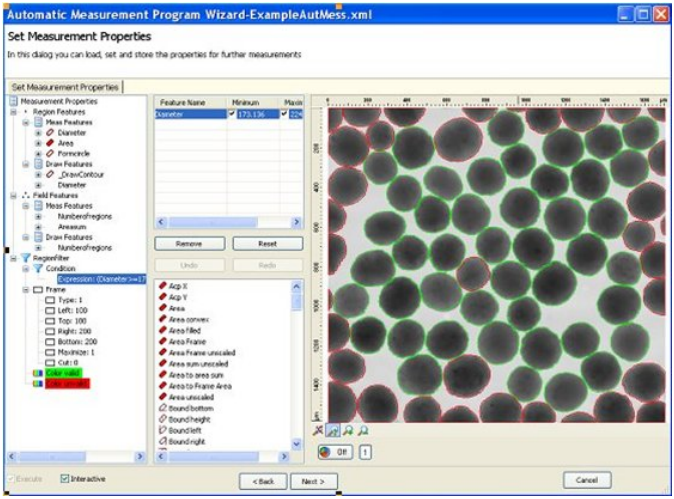


Step for interactive correction of the measurement mask

The functions for the automatic separation of touching objects (Erosion/Dilation, Watersheds) and for the subsequent interactive processing of the mask start with a function for eliminating artifacts and for filling gaps ("cleaning" of the measurement mask).

Set Measurement Propeeties

The actual measurement procedure consists of several steps. These are used to define how the measurement should be carried out in detail.



Definition of a measurement condition ("object filter")

The individual steps

- Determination of region-specific (individual objects) and field-specific (entire image) measurement parameters.

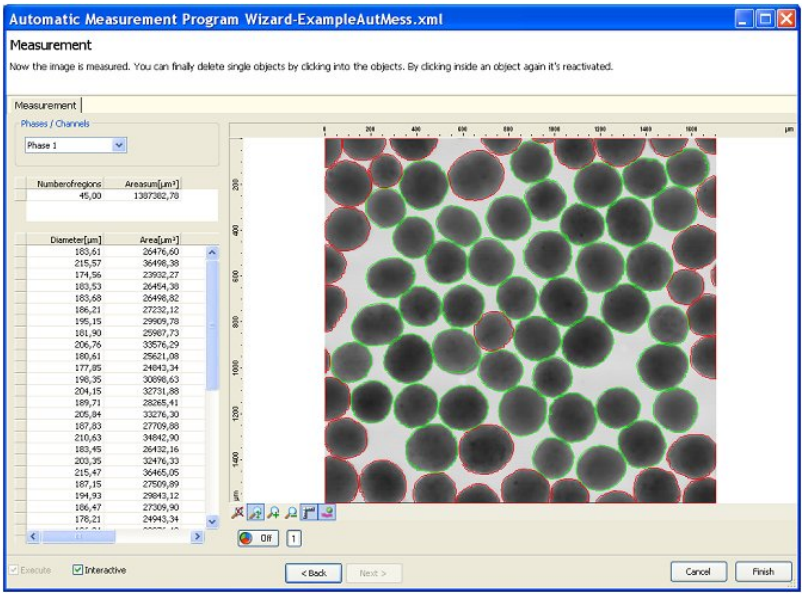
- Definition of the way in which the measured objects should be highlighted in the resulting image at the end of the measurement (contour, measurement parameters etc.).

- Generation of a measurement condition. This defines the conditions that an object must satisfy in order to be measured. This is a kind of "object filter", which can be used, for example, to specify that only objects within a certain size category should be counted.

- Definition of a measurement frame, which is used to specify how objects that are cut off by the image edge, and are therefore likely to distort the measurement result, should be handled.

Evaluation

In the final step, AxioVision shows you the image with the objects selected for measurement again. Here you can make any final changes, e.g. deleting individual objects by clicking on them.



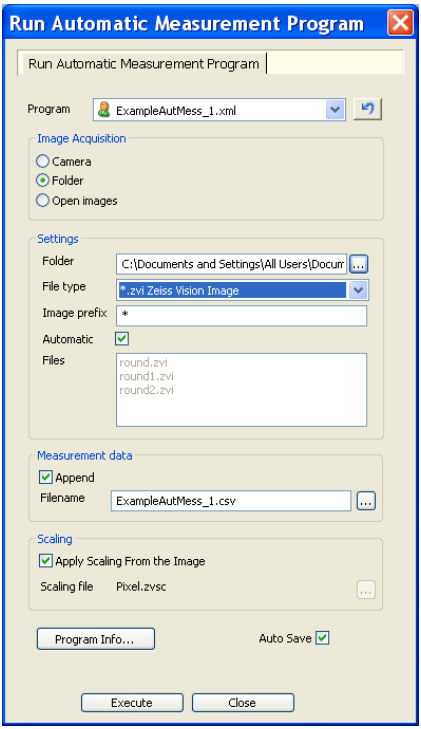
The measurement data are saved at the end of the measurement in so-called CSV format (**C**omma **S**eparated **V**alues).

This is a pure text format that separates the individual values from one another using special text symbols. The specific country settings that have been set in the Microsoft Windows control panel apply.

Files in CSV format offer two advantages: on the one hand, they are a special Microsoft Excel format, and can be opened and processed directly by that program. On the other hand, as the files contain pure text data, you can import the data into practically any other program.

Executing measurement programs

Once measurement programs have been generated, they can be applied to any number of images for series analyses. To do this, start from the menu **Measure ⇒ Automatic Measurement Programs** the function **Run Program**. Further information can be found in the online help by pressing the **F1** key.



As the tables of measurement results are available at the end of the measurement wizard, the wizard can also be used to measure an individual image. A useful feature is that rather than a new measurement program being generated each time for this purpose, an "auxiliary program", which always has the same name (e.g. "individual image measurement"), is used.

11.3 AutoMeasure Plus (Advanced Automatic Measurement)

General

While the **AutoMeasure** module has been designed primarily with ease of operation in mind (which naturally means that concessions have to be made in terms of flexibility), the **AutoMeasure Plus** module aims to offer unlimited possibilities for use.

A certain amount of knowledge of the procedure used for image analysis measurements is required in order to use these functions. You should therefore make sure that you also read the "General" section under 12.2 "AutoMeasure (Automatic Measurement)" as an introduction, before you start working with the individual functions of the AutoMeasure Plus module. There you will find important background information on the principal procedure used for measurements.

The concept of the AutoMeasure Plus module

To offer you as much flexibility as possible, all of the functions available within the context of measurements can be accessed individually via the **Processing** and **Measure** menus. By way of comparison, the respective functions in the AutoMeasure module can only be activated via the wizard.

In general, there are two ways in which you can automate measurement procedures:

The first step involves specifying the operations that you want to be performed during the actual measurement. This means that you need to define the measurement properties (see also the section “Working with the functions for automatic measurement”). In this step you need to answer questions including:

- Which parameters do I want to be measured?
- How do I want objects that are no longer fully visible in the image to be handled?
- Do I only want objects that satisfy special conditions to be measured (e.g. objects with an area between 50 and 100 µm²)?

During the definition of these settings you are supported by a wizard. Once you have defined properties, it is then possible to save them for routine measurement and simply reload them as required. This also takes place via the wizard. A separate function allows you to test the measurement properties before performing the measurement.

The second step simply involves performing the measurement on the basis of the properties you have defined.

- In general, there are three ways in which you can automate measurement procedures:
- Call up the individual functions via workflows or toolbars (see also section 9.4 "Adapting the User Interface").
- Create scripts with the Commander modul (please read also Chapter 13.1 "Commander").
- Generate applications via the **VBA** module (see also section 13.2 "VBA").

Working with the segmentation functions

The individual steps in the following examples can also be practiced using the original images that were employed. The sample images can be found in the folder *Demo Images and Scripts\AxioVision Images* on the Product DVD.

Dynamic segmentation

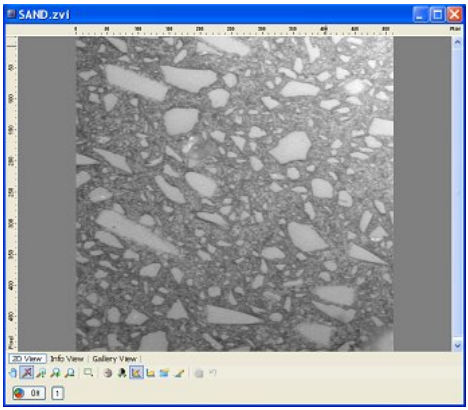
Dynamic segmentation is particularly useful for detecting fine structures against a varying background in cases where an optimum result cannot be achieved using threshold value segmentation.

It is, however, also suitable for images with inhomogeneous illumination. The **Dynamic** function employs a smoothing filter internally. This is used to balance out uneven brightness, or gray value fluctuations, in the background.

The following example explains step by step how to perform dynamic segmentation.

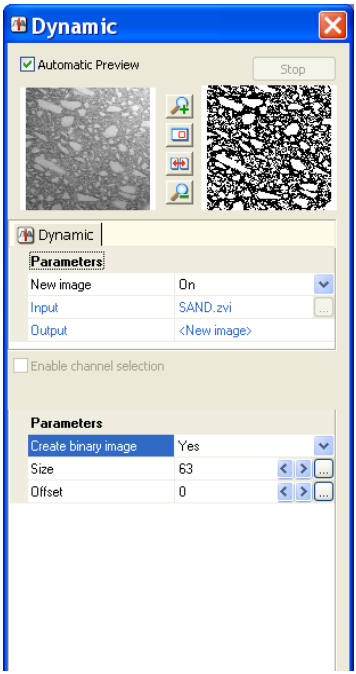
- From the Product-DVD load the image " sand.tif" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**.

- The image is displayed in AxioVision's image window.

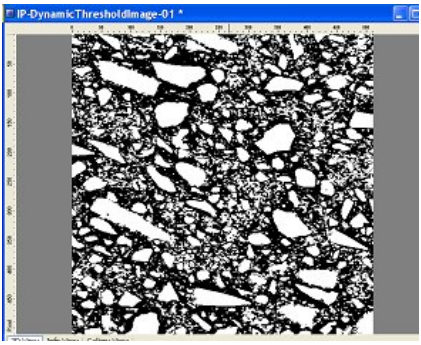


Select from the **Processing** menu the **Segment** functional group, and then the **Dynamic** function.

- You will now see the dialog window of the **Dynamic** function.
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.
- Set the value 63 for **Size** and the value 0 for **Offset** by clicking on the arrow keys, or by entering the values directly. Set **Binary** to **Yes**.
- Click on **OK** to close the dialog.



- The resulting image provides a satisfactory segmentation result.
- Although the original image exhibited inhomogeneous illumination, no shading correction was performed in advance.
- The uneven brightness in the image is compensated for by the dynamic segmentation.



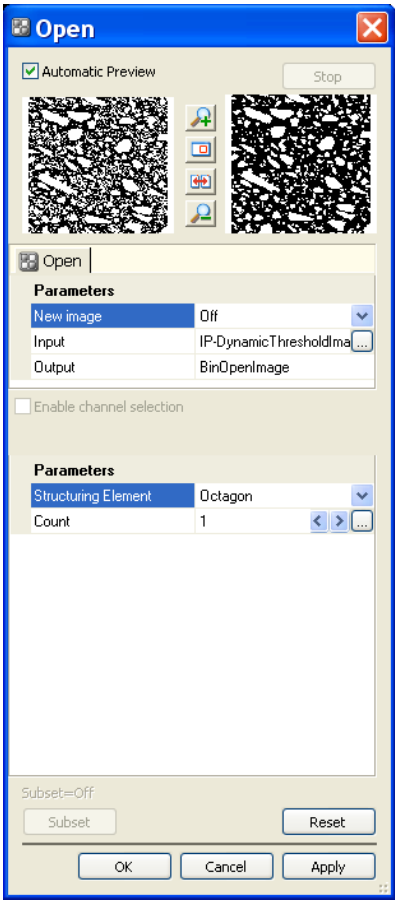
- You are now able to process the binary image further. Now select from the **Processing** menu the **Binary** functional group, and then the **Open** function.

- You will now see the dialog window of the **Open** function.

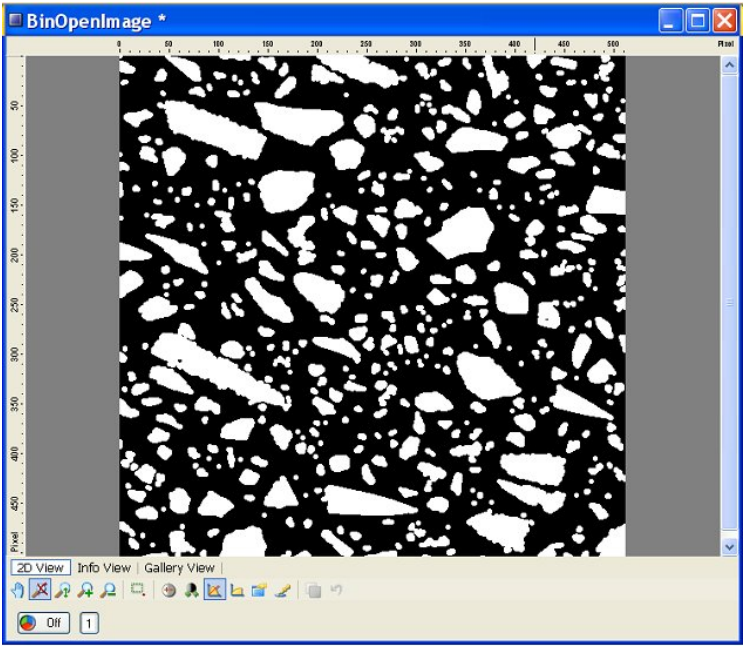
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- Set "Octagon" for **Structuring Element** and the value "1" for **Count**.

- Click on **OK** to close the dialog.



In the resulting image, the detected objects have been smoothed, and small particles removed.



Automatic segmentation of a fluorescence channel in a multichannel z-stack image

If you want to detect structures in a multichannel z-stack image, functions are available that take the entire z-stack into account, or all of the channels in full or on a selective basis.

As the individual channels clearly differ in terms of colors and structures, here it is possible to make settings on a channel-by-channel basis. The specific settings for the z-stack do not have to be entered separately for each individual image. A setting made for an individual image is applied to all the images in the stack, and therefore provides a very fast detection result.

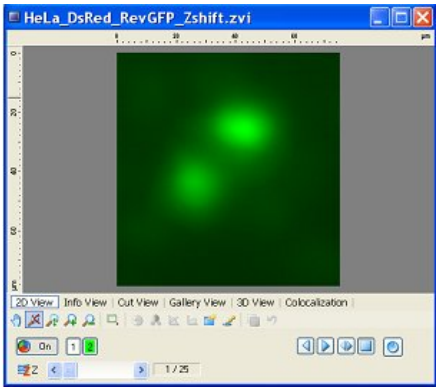
The automatic segmentation of an image is based on the automatic detection of local minimums in the image's gray value distribution. The **Automatic** function uses these minimums as thresholds in order to separate the structures to be measured from the background. This means that entering thresholds manually, as in the **Threshold** function, is not necessary here.

Often only certain structures, present in just one fluorescence channel, are of interest (e.g. cell nuclei). In a multichannel z-stack image, each channel's z-stack always has the same number of sections. In some cases the structure to be measured may not extend over the entire stack.

Setting the **Subset** therefore allows you, in the case of a multichannel z-stack, to choose selectively between individual channels or sections.

The following example explains step by step how to segment selected sections of a fluorescence channel in a multichannel z-stack.

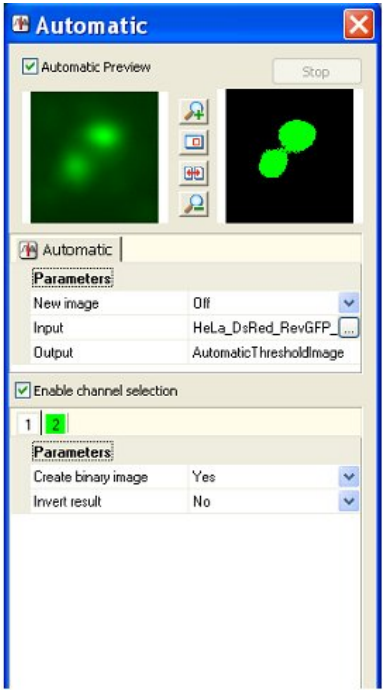
- From the Product-DVD load the image "Hela_DsRed_RevGFP.zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**.



- The image is displayed in AxioVision's image window.

- Now select from the **Processing** menu the **Segment** functional group, and then the **Automatic** function.

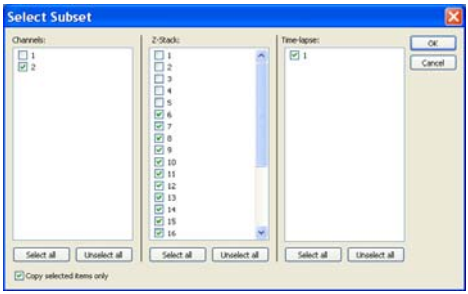
- You will now see the dialog window of the **Automatic** function.



- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

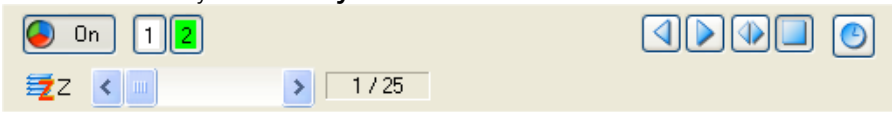
- Click on **Subset** to enter the specific settings in the **Select Subset** window.

- Activate only the channel that you want to be segmented (channel 2 in the example).

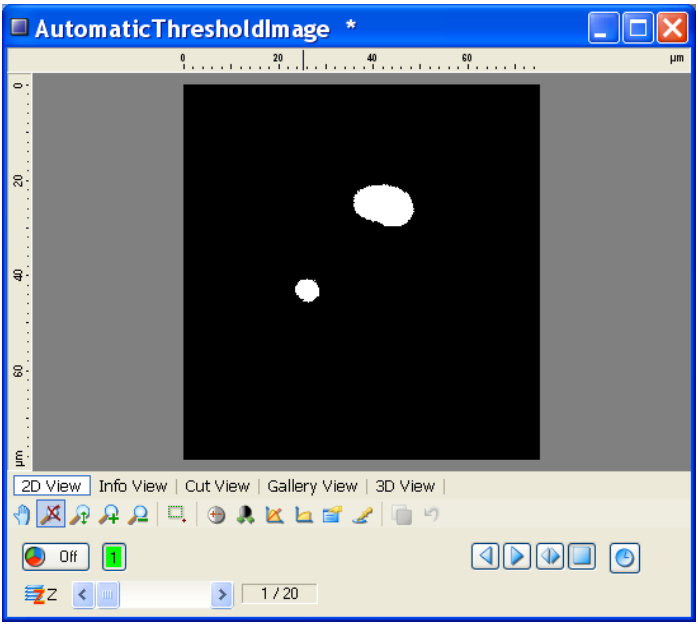


- The first 5 sections contain hardly any structures, and should not be taken into account during segmentation. Deactivate these sections by clicking on them.
- Activate the **Copy selected items only** check box, so that only the selected channels and sections are segmented.
- Click on **OK**.
- In the **Automatic** dialog window, click on **OK** to close the dialog.

The resulting image is a binary image that contains only the selected sections of channel 2. This image can be used as a mask for automatic measurement. Use the arrow keys in the **Player Field**



to display the segmentation in single steps.



Edge detection

In many images there is no clear distinction between the objects and the background. The objects lie very close together, and are separated from each other by brighter or darker contours. In such cases, threshold value segmentation does not usually produce a satisfactory result, as the contours exhibit significant gray value fluctuations. For these kinds of images, edge detection offers a possible solution.

Imagine an image as a gray value mountain range, where the valleys are formed by dark gray values, and the peaks by bright gray values. In the following example, the **Valleys** function is used. This detects dark edges (gray value valleys) between bright regions.

Before the edges are detected, smoothing is performed. This is determined by the **Sigma** parameter. A high value for **Sigma** means that strong smoothing is performed.

As a rule, the edges are not uniformly dark, and brighter edges (weakly pronounced valleys) and darker edges (strongly pronounced valleys) are present. The detection of the edges can be controlled using the **Threshold** parameter. A low value for **Threshold** excludes bright edges. The higher the value for **Threshold**, the lower the number of edges detected. The value is normally set at a low level, to ensure that any artifacts that are also detected are removed.

Note:

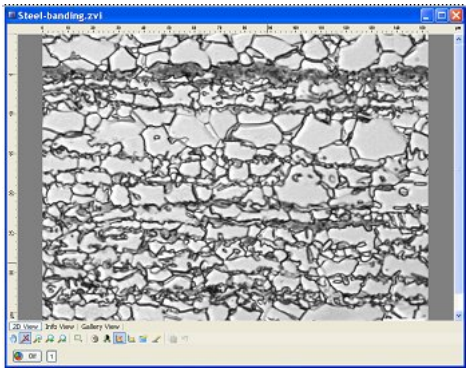
- Please note that the setting of the value for **Threshold** depends on the image's pixel type. A value of 0.00001 for **Threshold** corresponds to a gray value in a 16 bit image (1/65536). In the case of an 8 bit image, a gray value corresponds to a value of 0.03 (1/255). This means that with 16 bit images, very low values are sufficient, whilst with 8 bit images, the value has to be set correspondingly higher.

If you have images in which dark objects are separated from each other by bright contours (edges), we recommend that you invert the image before using the **Valleys** function. To do this, use either the **Invert** function from the **Adjust** functional group, or the **Not** function from the **Binary** functional group.

If a color image is used as an input image, you should split the image up into its individual color channels before or after executing the **Valleys** function (**Utilities** functional group, **Split RGB Extractions** function), process these channels separately, and combine them as an individual binary image again, before the measurement (**Binary** functional group, **Or** function) is performed.

The following example explains step by step how to detect these contours (edges) in an image that contains bright structures separated by dark contours.

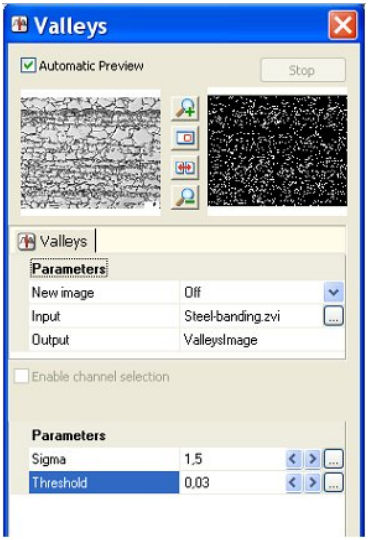
- From the Product-DVD load the image "Steel-banding.zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**.



- The image is displayed in AxioVision's image window.

- Select from the **Processing** menu the **Segmentation** functional group, and then the **Valleys** function.

- You will now see the dialog window of the **Valleys** function.



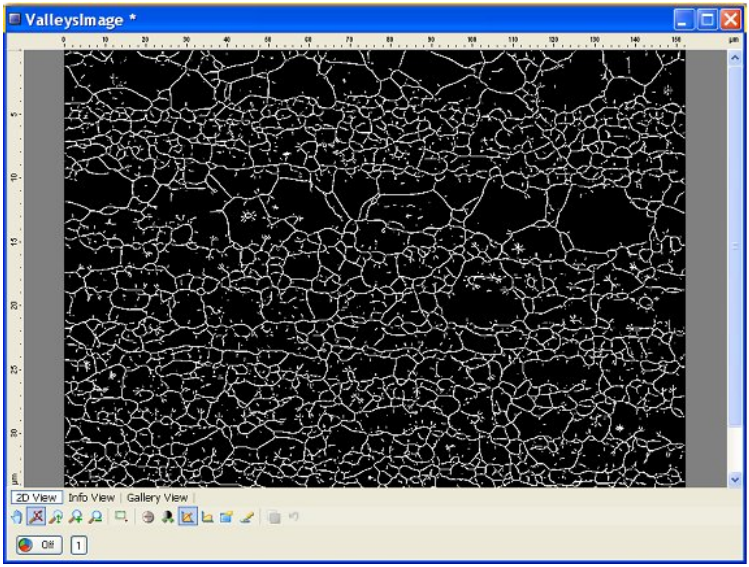
- If the **Automatic Preview** check box is activated, you will see the reduced input and output images.

- Set the value 1.5 for the parameter **Sigma** by clicking on the arrow keys, or by entering the value directly.

- Set the value 0.03 for **Threshold** by entering the value directly.

- Click on **OK** to close the dialog.

The resulting image shows the detected edges, but also numerous small structures (artifacts) that have also been detected.



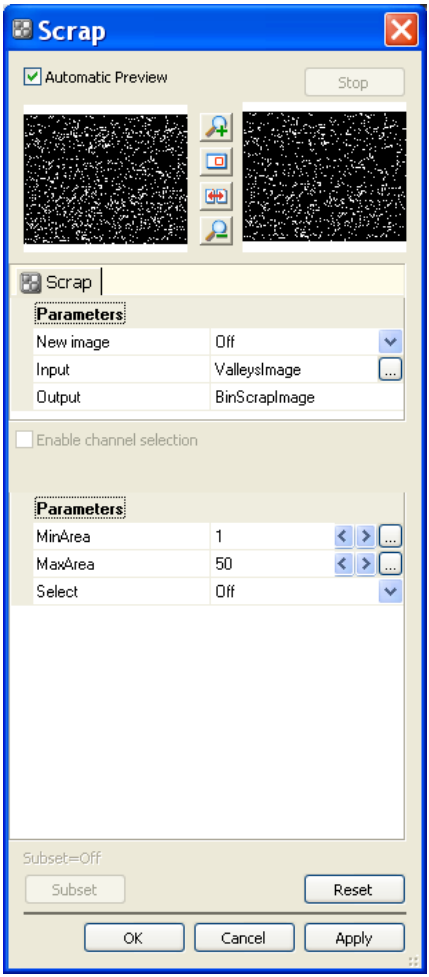
- Select from the **Processing** menu the **Binary** functional group, and then the **Bin Scrap** function.

- You will now see the dialog window of the **Scrap** function.

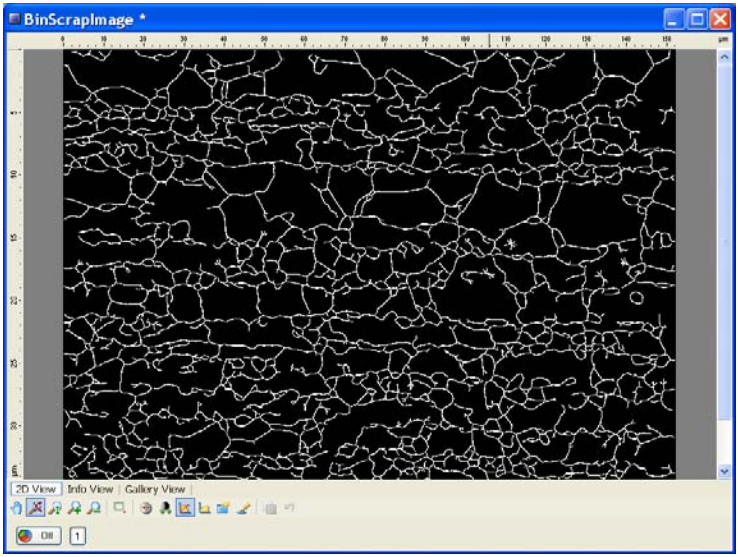
- If the **Automatic Preview** check box is activated, you will see the reduced input and output images.

- Set the value 1 for the parameter **MinArea** and the value 50 for **MaxArea** by clicking on the arrow keys, or by entering the values directly.

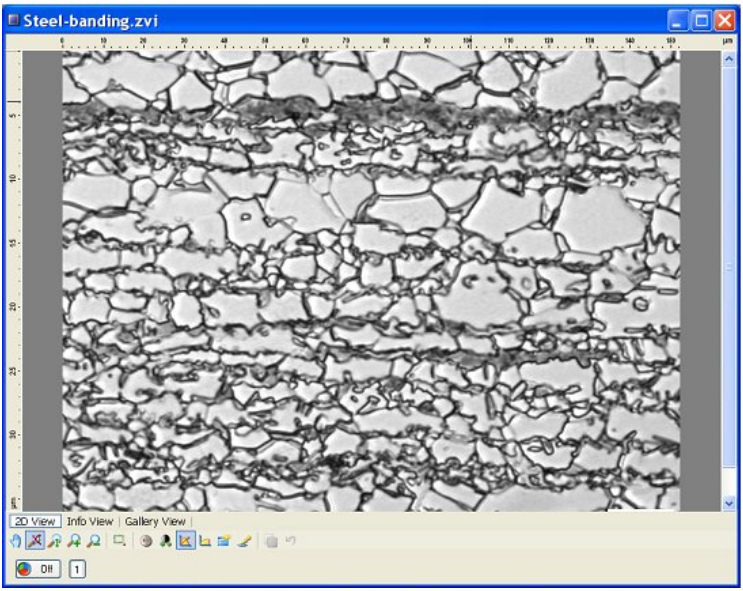
- Click on **OK** to close the dialog.



In the resulting image, the artifacts have now disappeared. A comparison with the original image, however, shows that some of the detected edges contain gaps.



In order to measure the image correctly, the edges of the objects (regions) to be measured must be closed. Using the watersheds function, it is possible to reconstruct them, so that they form complete contours (see chapter *"Separation and/or reconstruction of structures using watersheds"*).



Performing binary image processing

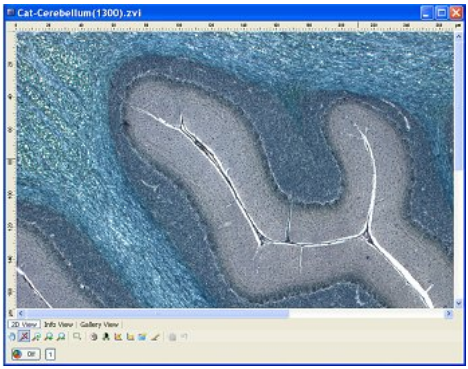
Processing a binary image (smoothing, removing artifacts, filling gaps)

If you want an image to be measured automatically, you must first perform segmentation. This produces a so-called mask (binary image). Frequently the binary image is not perfect, and can only be used for measurement following further processing. During segmentation, incomplete or superfluous structures (gaps, peripheral regions or other structures) are often detected. Special functions are available for processing such structures.

The following example explains step by step how to process a segmented image morphologically, and thereby optimize it.

- From the Product-DVD load the image "Cat-Cerebellum(1300).zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**.

- The image is displayed in AxioVision's image window.



- Now select from the **Processing** menu the **Smooth** functional group, and then the **Gauss binomial** function.

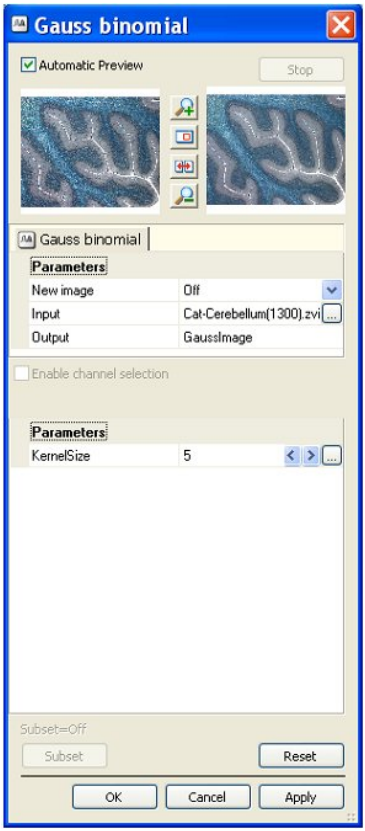
- You will now see the dialog window of the **Gauss binomial** function.

- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- In places the image contains inhomogeneous structures, which make segmentation more difficult. For this reason the image is smoothed in advance using a Gauss filter.

- Set the value 5 for the parameter **KernelSize** by clicking on the arrow keys, or by entering the value directly.

- Click on **OK** to close the dialog.

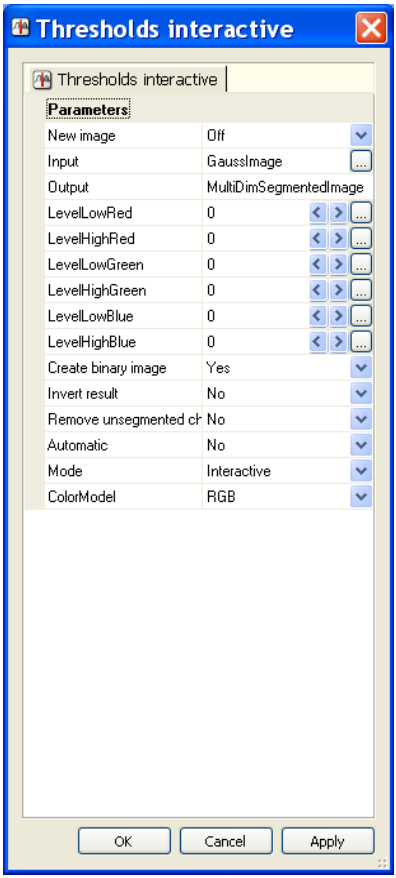


- Now select from the **Processing** menu the **Segment** functional group, and then the **Threshold interactive** function.

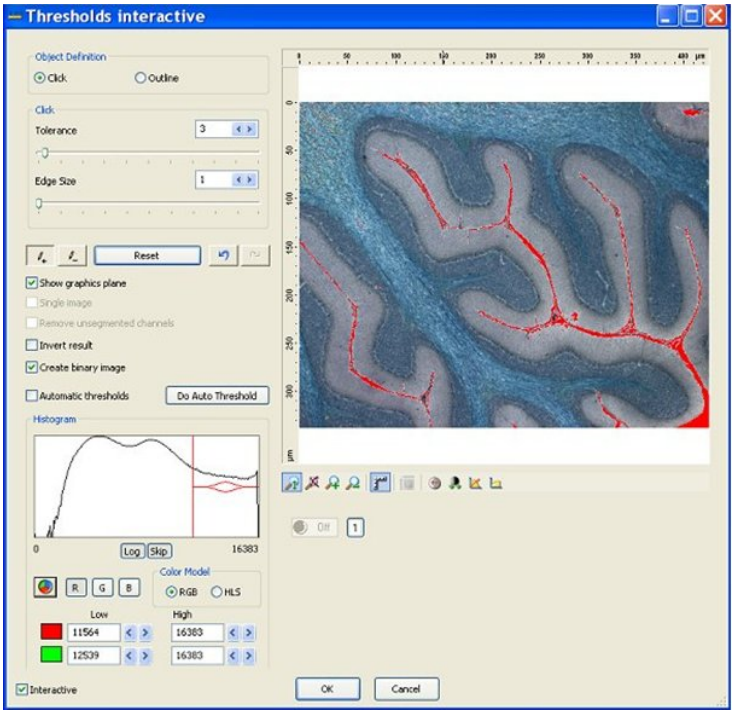
- You will now see the dialog window of the **Threshold interactive** function.

- Set **New image** to **Off**. Under Input select **GaussImage**.

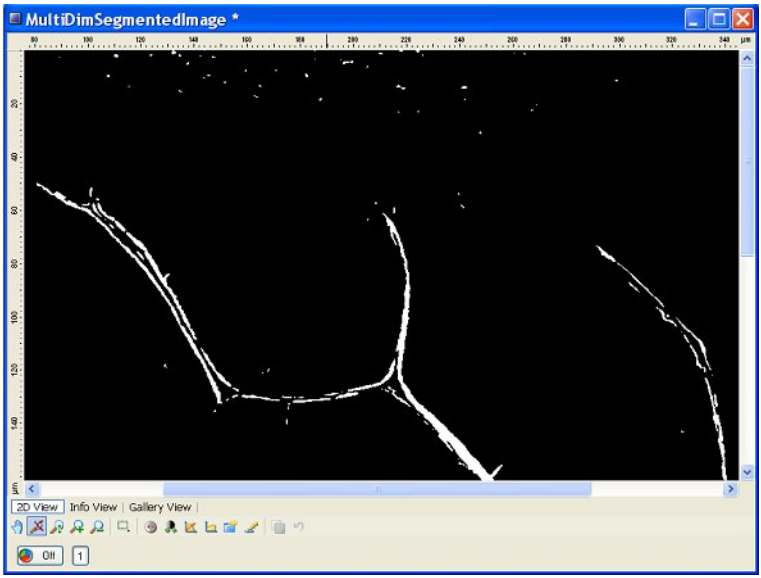
- Click on **OK**.



➢ You will now see the dialog window with the segmentation function.



➢ Click on **OK**. The resulting image is displayed in the image window.



Separation and/or reconstruction of structures using watersheds

Often the image structures that you want to measure are present in the form of agglomerates, or are positioned very close together, which means that separation is required following segmentation. If only the object contours have been detected, these are frequently incomplete, and need to be reconstructed to form complete contours.

If conventional separating functions (combination of **Bin Erode** and **Bin Dilate** or **Exoskeleton**) fail to produce a usable result, the watersheds function can be employed.

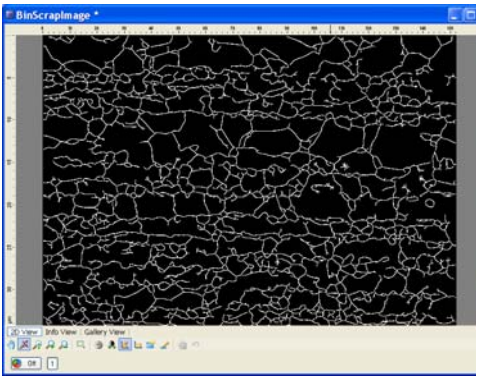
In the segmented image it is necessary, first of all, to perform smoothing (using **Lowpass**, **Gauss** or **Median**), in order to eliminate excess structures and structures that are too small. Smoothing can, however, also take place prior to segmentation.

Distance transformation (**Bin Euclidean Distance**) is then used to generate a distance map from the smoothed binary image. The distances of the white regions from the black background are coded as gray values in the resulting image. Bright gray values in the distance map indicate a large distance from the object.

An image can be regarded as a gray-value landscape, with valleys (dark gray values) and hills (bright gray values). In the distance-transformed image, the regions have to be the valleys, in order for the watersheds algorithm to function. The **Watersheds** function floods the valleys, and marks the places where the water masses flow together. These are then identifiable as separating lines or reconstruction lines.

The following example explains step by step how to generate separating lines using watersheds.

- Use the resulting image from the **Edge detection** example in the previous section, or generate a similar image.



- Select from the **Processing** menu the **Smooth** functional group, and then the **Lowpass** function.

- You will now see the dialog window of the **Lowpass** function.

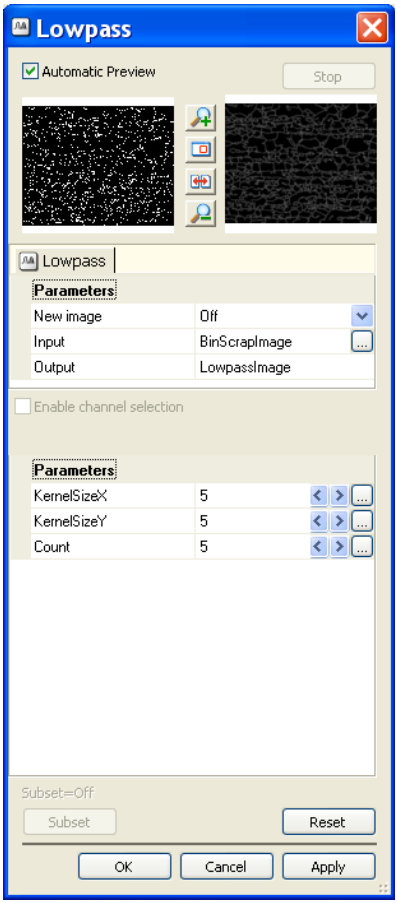
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- Set **New image** to **Off**, so that you can select the images freely.

- If you click on the  button in the **Input** field, the image gallery appears. Select the original image by clicking on it.

- Set the value 5 for **KernelSizeX**, **KernelSizeY** and **Count** by clicking on the arrow keys, or by entering the value directly.

- Click on **OK** to close the dialog.

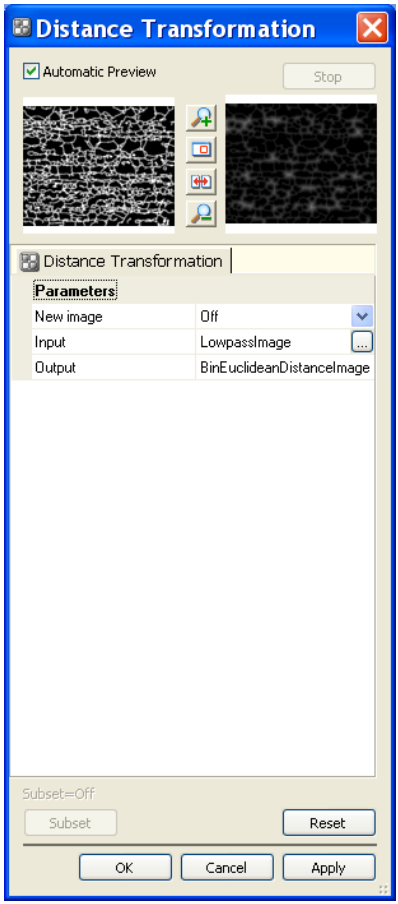


- Select from the **Processing** menu the **Binary** functional group, and then the **Distance Transformation** function.

- You will now see the dialog window of the **Distance Transformation** function.

- If the **Automatic Preview** check box is activated, the reduced inputand output images appear.

- Click on **OK** to close the dialog.



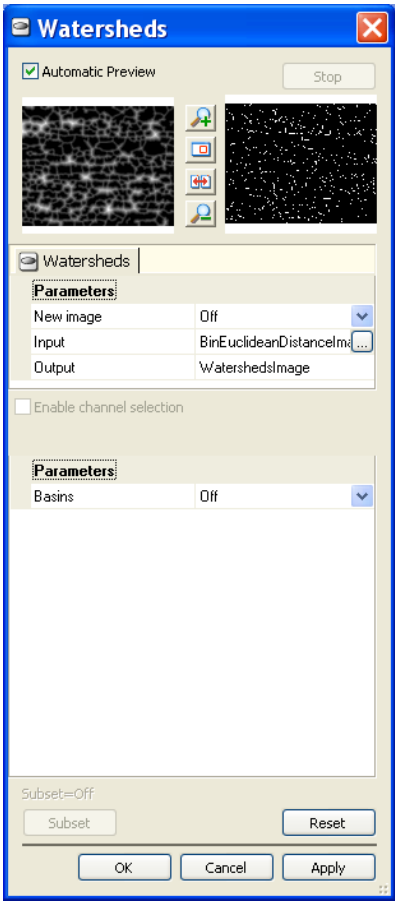
- Now select from the **Processing** menu the **Morphology** functional group, and then the **Watersheds** function.

- You will now see the dialog window of the **Watersheds** function.

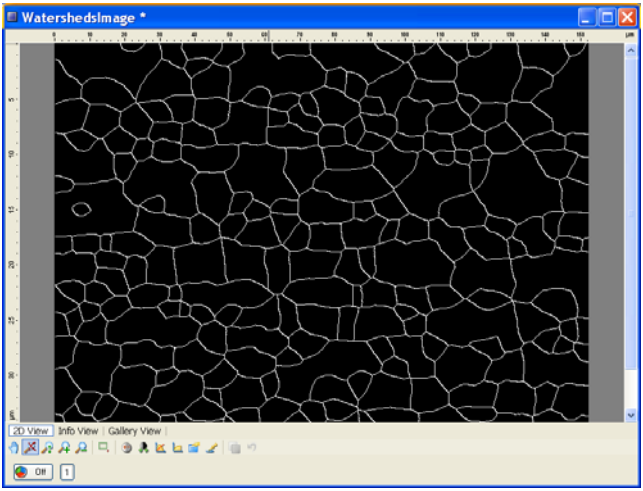
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- Select under **Basins** the **Off** setting.

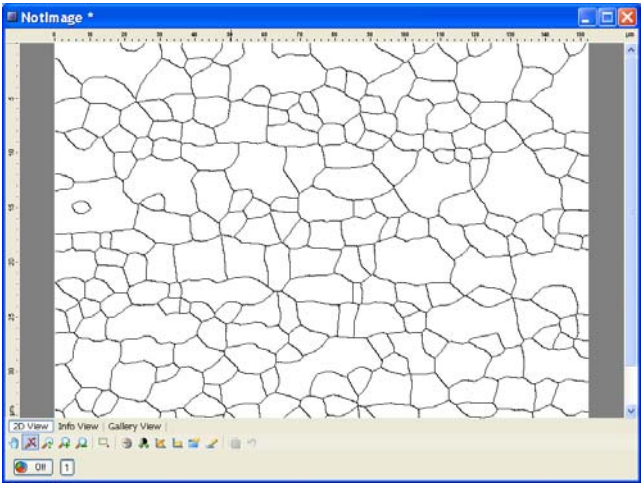
- Click on **OK** to close the dialog.



The resulting image now shows the reconstructed separating lines.



Incomplete separating lines have been completed, and superfluous structures removed. If the resulting image is to be used as a mask for measuring, it must first be inverted using the **Not** function (menu **Processing** ⇒ **Binary**).

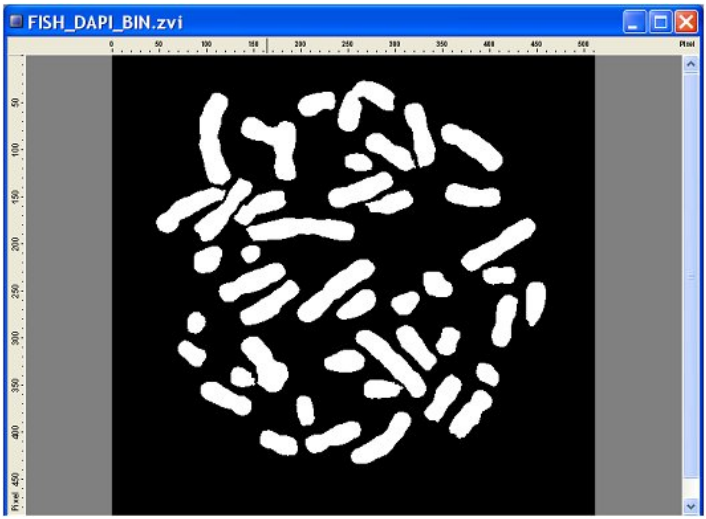


Masking structures/Using Boolean functions

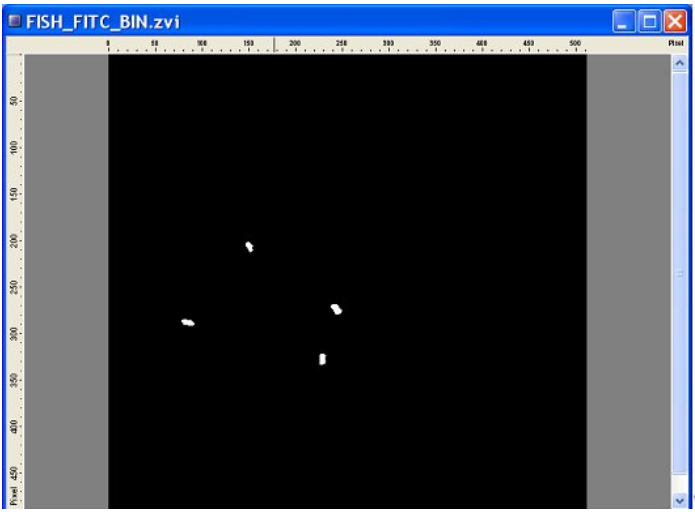
Images often contain structures with varying brightnesses or colors, which are detected over several segmentation steps. In some cases, the binary images produced have to be combined in order to join together or mask structures. Boolean operations are available for this purpose. The **And** function can be used to mask structures, whilst the **Or** and **Xor** functions are better suited to combining structures. Frequently an inverted image is required, which can be generated using the **Not** function.

The following example explains step by step how to mask or combine structures from various binary images. The example uses a metaphase image, from which the DAPI channel, the FITC channel and the Rhodamine channel have been detected in three separate steps.

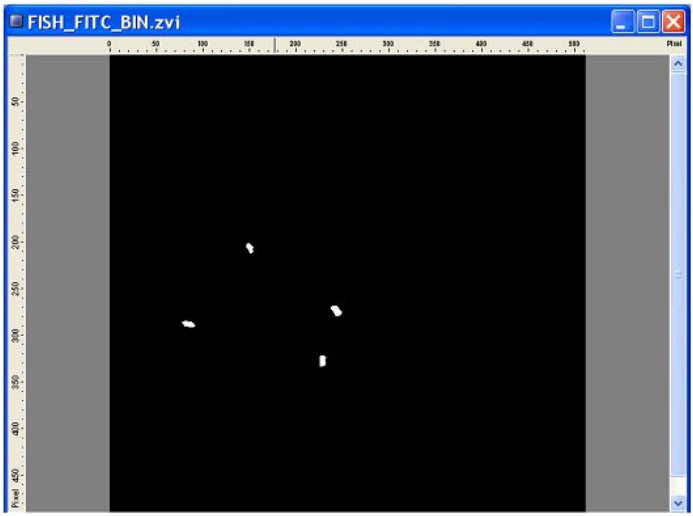
- 使用二值化函数生成三幅二进制图像，每幅均有不同的结构。
- 或者：从AxioVision产品安装光盘的目录*Demo Images and Scripts\AxioVision Images*中找到并加载下面的三个图像文件："FISH_DAPI_BIN.zvi"，"FISH_FITC_BIN.zvi"• ""FISH_RHO_BIN.zvi"。



FISH_DAPI_BIN.zvi



"FISH_FITC_BIN.zvi



FISH_RHO_BIN.zvi

- Now select from the **Processing** menu the **Binary** functional group, and then the **And** function.

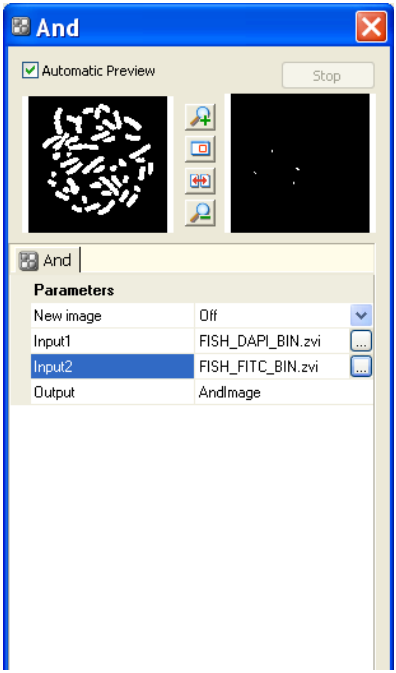
- You will now see the dialog window of the **And** function.

- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- Set **CreateNew** to **Off** so that you will be able to select the images freely later.

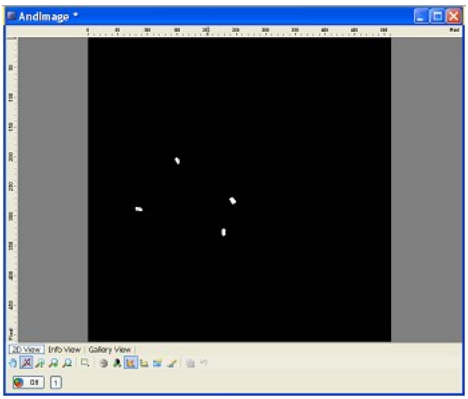
- If you click on the  button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. Select different binary images for **Input1** and **Input2**.

- Click on **OK** to close the dialog.



- The **And** function only masks structures that are present in both images.

- As, in the sample image, the FITC structures lie directly on top of the DAPI structures, the resulting image is identical to the FITC binary image.



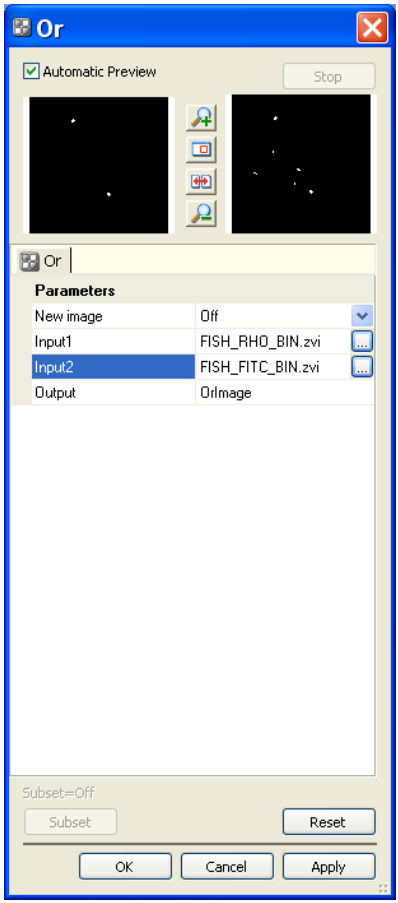
- Now select from the **Processing** menu the **Binary** functional group, and then he **Or** function.

- You will now see the dialog window of the **Or** function.

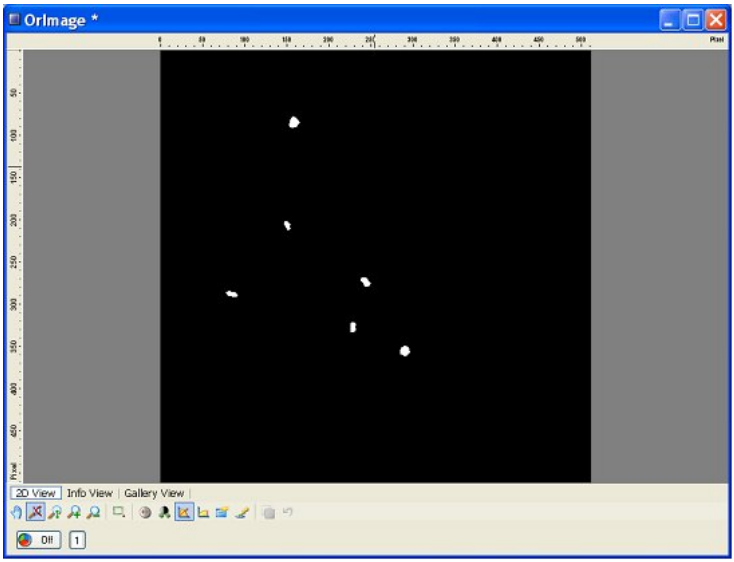
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- If you click on the ... button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. Select different binary images for **Input1** and **Input2**.

- Click on **OK** to close the dialog.



The **Or** function masks structures that are present in either one image or the other.In the sample image, the FITC structures and the Rhodamine structures are in different positions. Both types of structure appear in the resulting image.



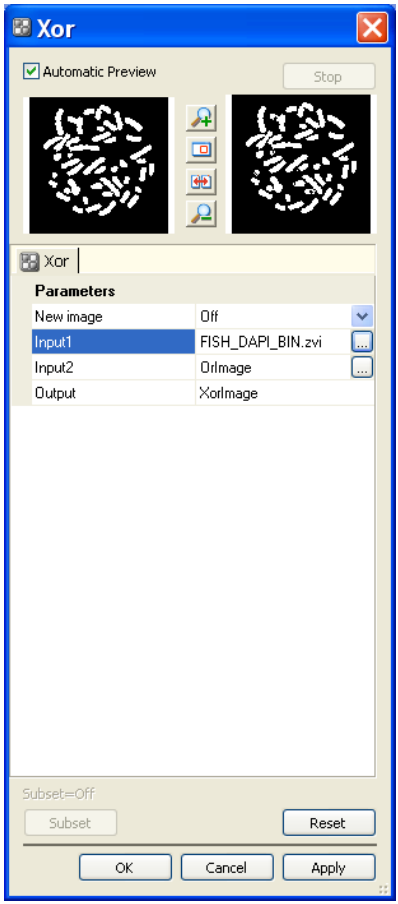
- Now select from the **Processing** menu the **Binary** functional group, and then the **Xor** function.

- You will now see the dialog window of the **Xor** function.

- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

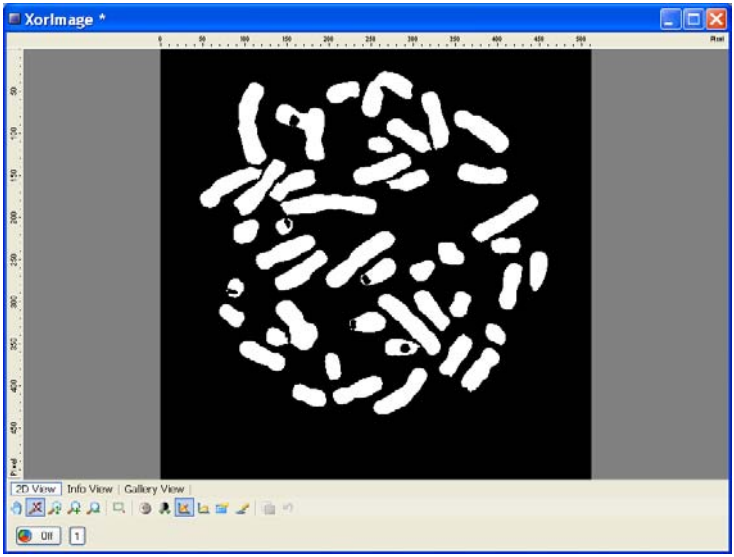
- If you click on the button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **Input2** select the resulting image of the **Or** function, and for **Input1** another binary image.

- Click on **OK** to close the dialog.



The **Xor** function masks as black any structures present in both images. Any white structures that do not overlap also appear as white in the resulting image.

In the sample image, the FITC and Rhodamine structures lie directly on top of the DAPI structures. Structures in the resulting image therefore appear as gaps in the DAPI image.



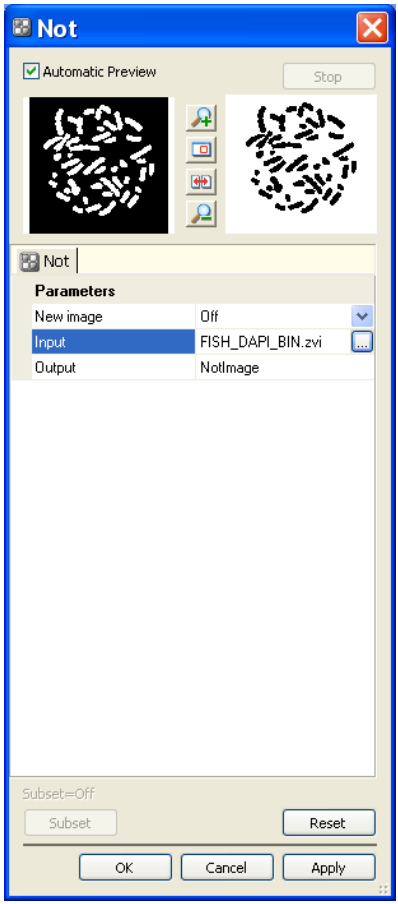
- Now select from the **Processing** menu the **Binary** functional group, and then the **Not** function.

- You will now see the dialog window of the **Not** function.

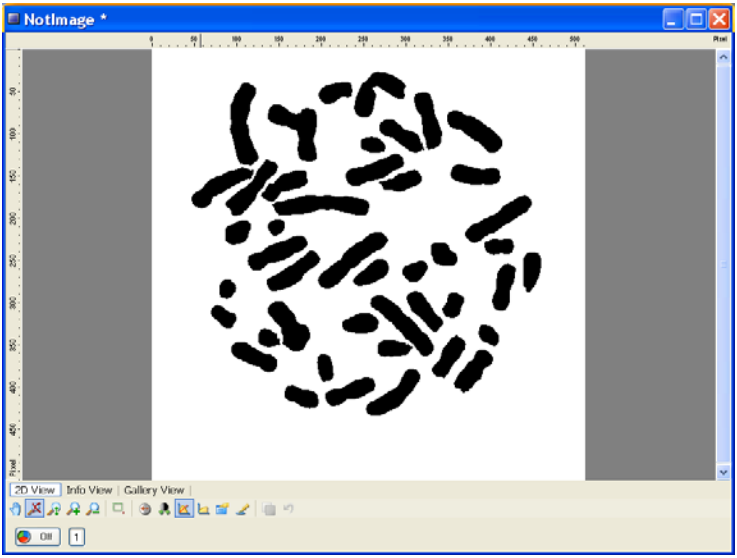
- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- If you click on the  button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **Input1**, select any binary image.

- Click on **OK** to close the dialog.



The resulting image now shows all white structures as black, and vice versa.

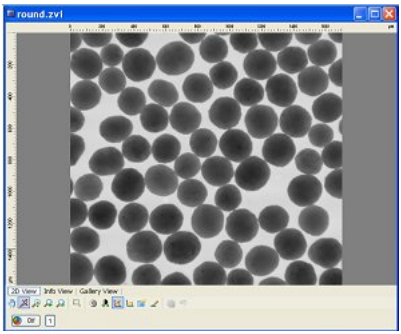


Working with the Functions for automatic Measurement

Generation of Measurement Properties Files and Measurement

- From the Product-DVD load the image "round.zvi" from the folder *Demo Images and Scripts\AxioVision Images* via the function **File** ⇒ **Open Image**.

- The image is displayed in AxioVision's image window.



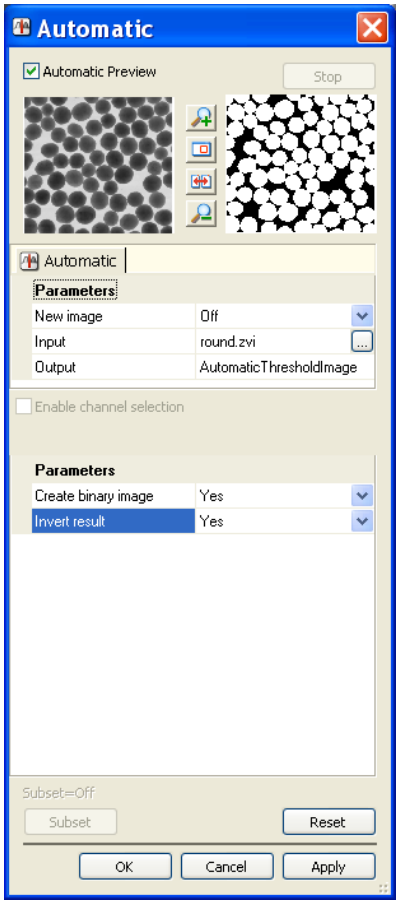
- Now select from the **Processing** menu the **Segmentation** functional group, and then the **Automatic** function.

- You will now see the dialog window of the **Automatic** function.

- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- If you click on the … button in the **Input** field, the image gallery appears. Here select the image in question by clicking on it. For **Invert reult**, select **Yes**.

- Click on **OK** to close the dialog.



- Now select from the **Processing** menu the **Segmentation** functional group, and then the **Separatrion** function.

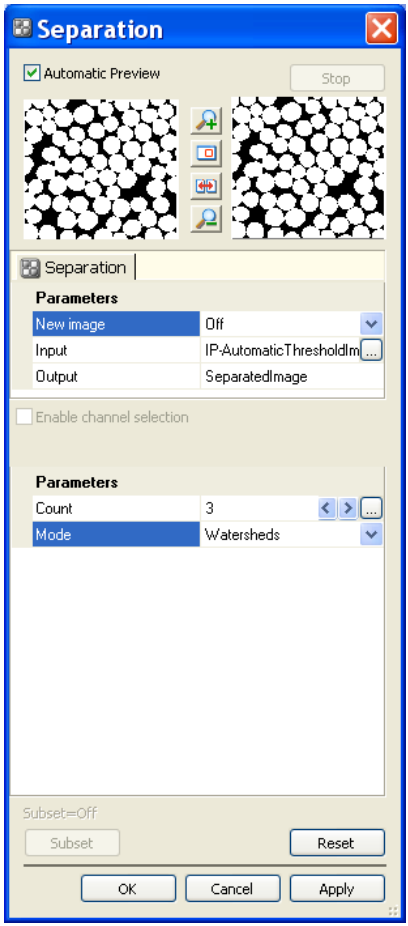
- You will now see the dialog window of the **Separatrion** function.

- If the **Automatic Preview** check box is activated, the reduced input and output images appear.

- Set the value 3 for **Count** by clicking on the arrow keys, or by entering the value directly.

- For the parameter **Mode** select **Watersheds**.

- Click on **OK** to close the dialog.

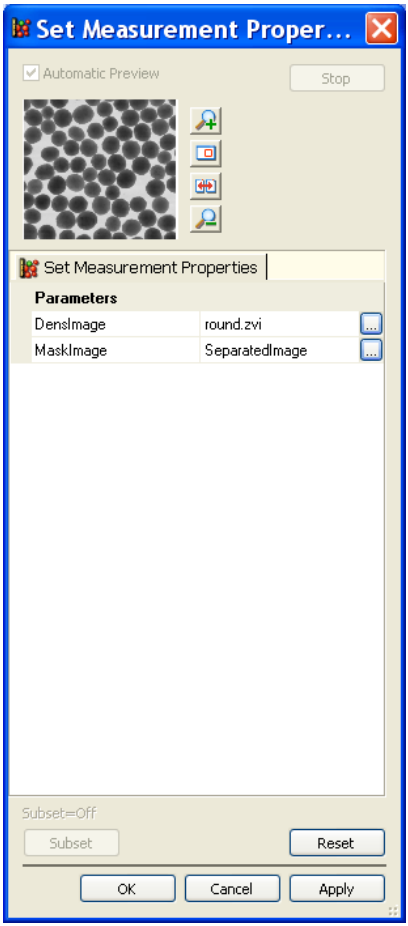


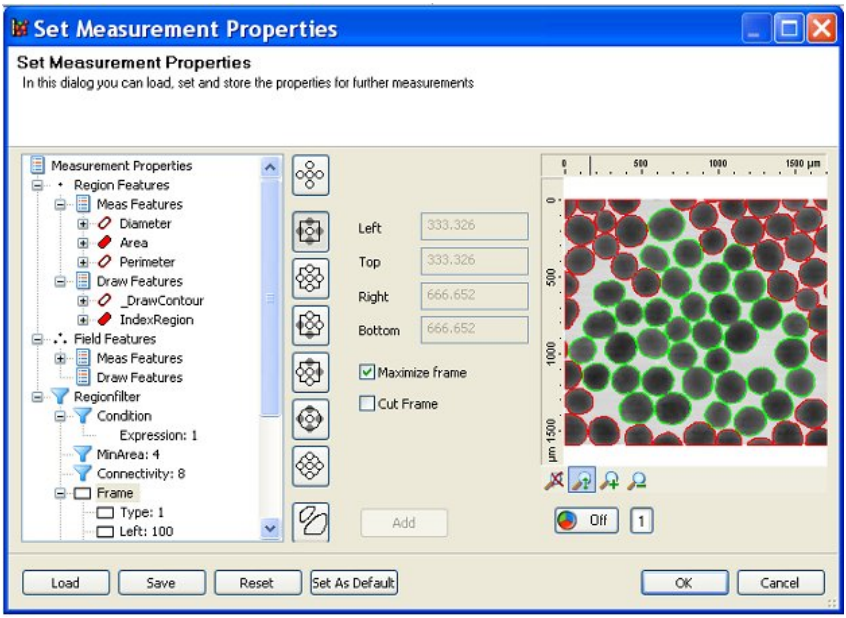
- Now select from the menu **Measure** ⇒ **Automatic Measurement** the function **Set Measurement Properties**.

- You will now see the dialog window of the **Set Measurement Properties** function.

- From the image gallery  for the parameter **DensImage** the image "round.zvi" and for parameter **MaskImage** select "SeparatedImage".

- Click on **OK** to close the dialog.





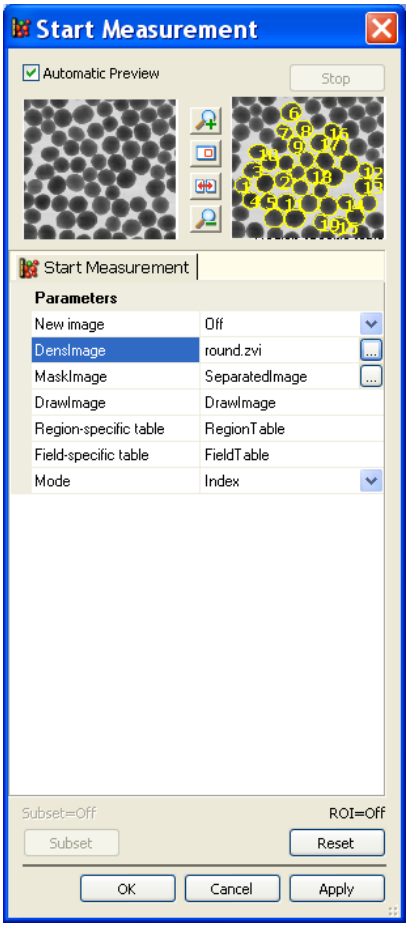
Set the selected measurement features, draw features as well as the measurement frame and activate the check bos **Maximiz frame**. Save the settings and click **OK** to close the dialog window.

- Now select from the menu **Measure** ⇒ **Automatic Measurement** the function **Start Measurement**.

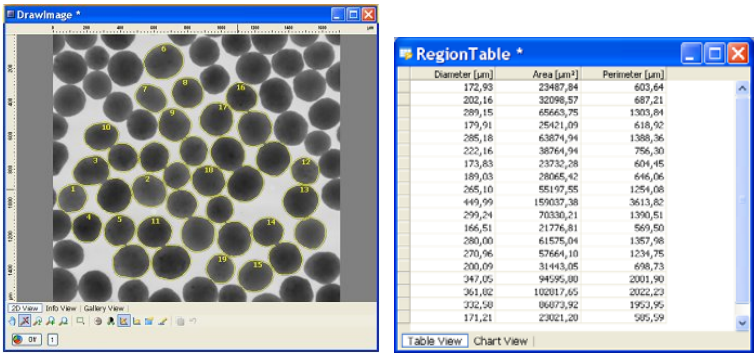
- You will now see the dialog window of the **Start Measurement** function.

- Select the parameters as shown in the screenshot of the dialog window.

- Click on **OK** to close the dialog.



In the AxioVision's image window the result image **DrawImage** and the result table **RegionTable** will be displayed.



The results will be displayed in the graphics plane of the resulting image. With the function Set Mesurement Properties you can define the way in which the Region Features, Draw Features resp. Field Features Draw Features will be displayed.
The measurement values are displayed in region specific resp. field specific tables.

The measurement data are saved at the end of the measurement in so-called CSV format (**C**omma **S**eparated **V**alues).

This is a pure text format that separates the individual values from one another using special text symbols. The specific country settings that have been set in the Microsoft Windows control panel apply.

Files in CSV format offer two advantages: on the one hand, they are a special Microsoft Excel format, and can be opened and processed directly by that program. On the other hand, as the files contain pure text data, you can import the data into practically any other program.

11.4 Particle Analyzer Projects

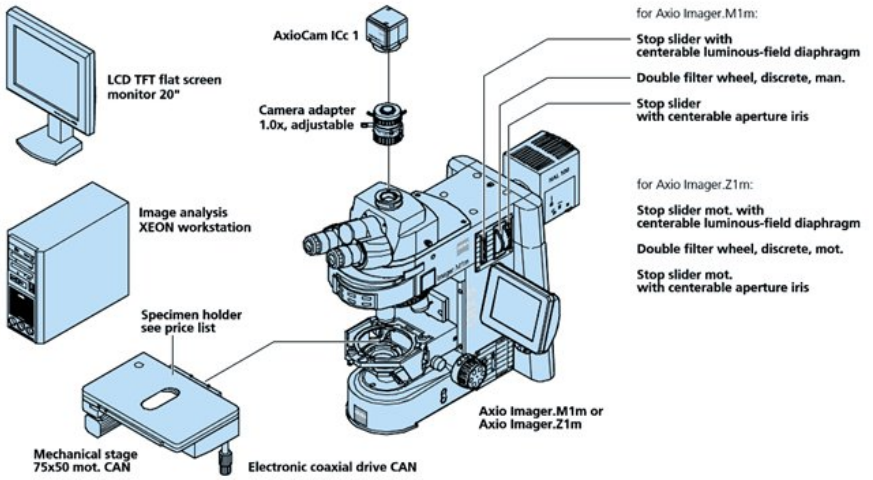
General

The system variants

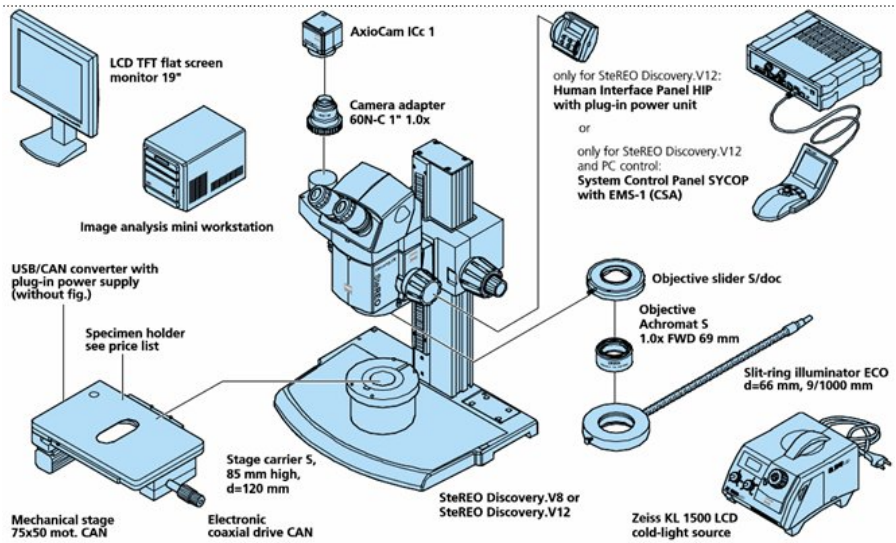
The Particle Analyzer Projects module forms the basis for the **Particle Analyzer** system on the software side. The Particle Analyzer from Carl Zeiss is a complete system for analyzing particles and is currently based on two microscope platforms:

- platform 1: Upright light microscope Axio Imager
- platform 2: SteREO Discovery.V12 stereo microscope

For either platform, manual and partially or fully motorized system versions are available. See the system overviews below:



Particle Analyzer with Axio Imager.M1m or Axio Imager.Z1m



Particle Analyzer with SteREO Discovery.V8 or SteREO Discovery.V12

In addition to the microscopes, the following components are essential for a complete Particle Analyzer system:

- Digital camera (integrated into AxioVision, e.g. AxioCam family).
- PC with monitor.
- AxioVision basic package and the Particle Analyzer Projects module.

Depending on the microscopy platform used and the requirements of your application, you may need the following components in addition:

- For scanning large areas: a motorized stage and the AxioVision MosaiX module. For automatic focusing during image acquisition, the AxioVision Autofocus module will be helpful.
- For the individual modification of analysis scripts: the Commander module.

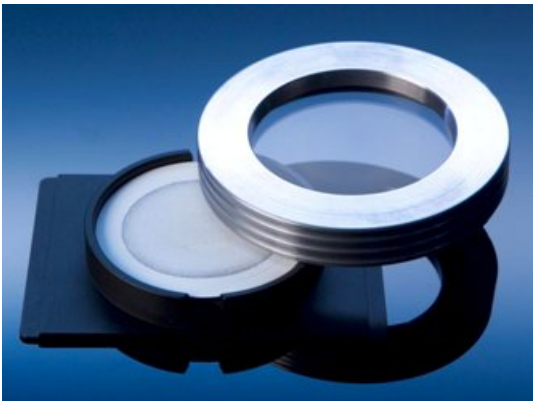
For details on setting up and using the hardware components, such as the microscope, motorized stage and camera, please refer to the manuals supplied with the corresponding devices.

Note:

- Before using the Particle Analyzer, familiarize yourself with the basic functions of the AxioVision software. You should be familiar with the operation of the camera and microscope components in particular.

Accessories for particle analysis

Specimen holder for round particle filters

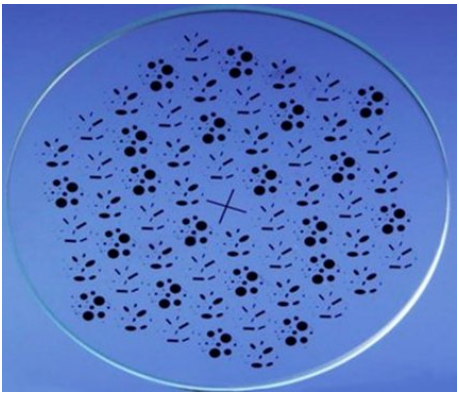


The specimen holder for round particle filters is used to hold round filters up to 50 mm in diameter. It can be used for the following tasks/has the following features:

- Reproducible positioning of filters on a mechanical stage or scanning stage.
- Plane-parallel glass plate with retaining ring for flat insertion of a filter. This means that autofocusing is no longer necessary. Automatic focusing would make the acquisition process many times longer.
- Suitable for objectives up to Epiplan 10x.
- Marking to ensure correct positioning of filters with a 25 mm diameter.

Depending on the type of stage used (mechanical or scanning stage), special specimen holders or inserts may also be required in addition to the specimen holder for particle filters to ensure that the latter is correctly positioned on the stage in question.

Particle analysis calibration standard



Using the calibration standard, you can check whether measurements made using the Particle Analyzer lead to reproducible results. The calibration standard has the following features:

- Depiction of size classes of guideline VDA volume 19 and ISO 16232
- 10 size classes: 10, 20, 35, 75, 125, 175, 300, 500, 800, 1200 µm
- Total number of particles: 6960
- Three different shapes: circles, rectangles, ellipses
- Diameter: 47 mm
- Diameter containing particles: 44 mm
- Anti-reflective on both sides

Note:

- The calibration standard is also available in a certified version.

The following table provides an overview of the particle distribution for the calibration standard:

VDA 19, ISO 16232		Calibration standard	
Class	**Class boundaries (µm)**	**Particle size (µm)**	**Number of particles**
B	5 ≤ x < 15	10	1920
C	15 ≤ x < 25	20	1920
D	25 ≤ x < 50	35	960
E	50 ≤ x < 100	75	960
F	100 ≤ x < 150	125	480
G	150 ≤ x < 200	175	240
H	200 ≤ x < 400	300	120
I	400 ≤ x < 600	500	120
J	600 ≤ x < 1000	800	120
K	1000 ≤ x	1200	120
		Total number of particles	**6960**

Cleaning of sample holder and calibration standard

You will need to differentiate between dust particles (e.g. glass particles from slides, skin particles from the microscope user, fluff from textiles, etc.) and other kinds of dirt (e.g. cleaning residues, finger prints and grease). Dust particles may lie loosely on optical surfaces or become more or less stuck to them. Other dirt is either water-soluble or only completely removable using organic solvents.

Depending on the type of soiling, different methods can be used to clean the sample holder or the calibration standard. You will find further information in the brochure "The clean microscope" by Carl Zeiss. This brochure can be found on the Internet at www.zeiss.de.

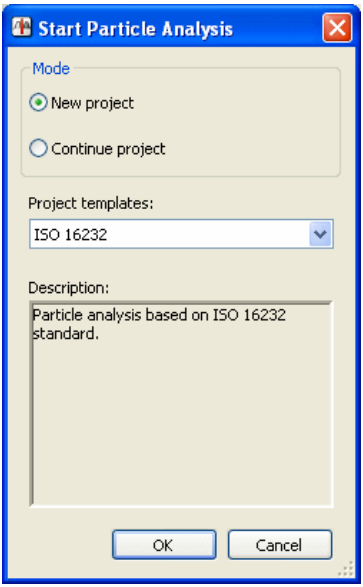
The concept

Operation and configuration

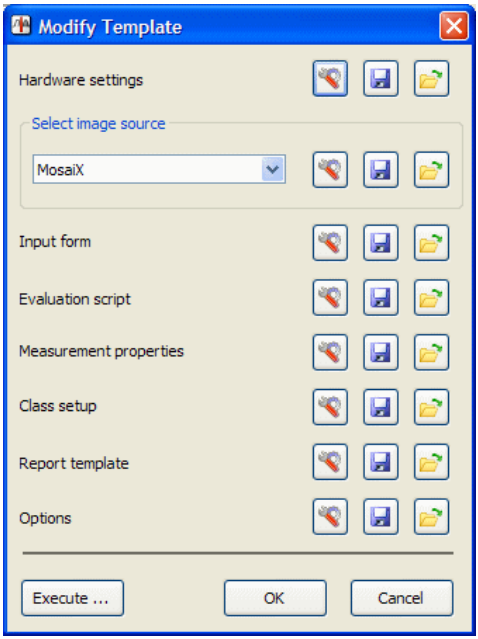
The operating concept of the Particle Analyzer is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement.

The performance of a measurement is always based on what is called test specifications. All important settings are saved in a test specification. A test specification cannot be created or edited by a user. This is reserved to Power Users resp. Administrators. Access to the respective functions is governed by the access rights of the operating system:



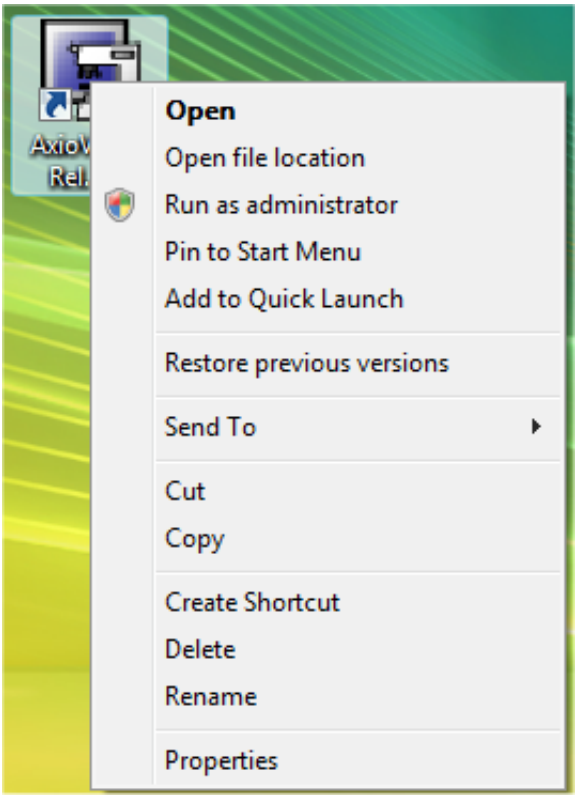
Dialog for starting a particle measurement (for standard users and administrators)



Dialog for editing a test specification (only for administrators, not available to standard users)

Under Microsoft Windows Vista the administrator must actively run AxioVision as an administrator. To do this, after the administrator has right-clicked on the AxioVision 

start icon, the **Run as administrator** option must be activated.



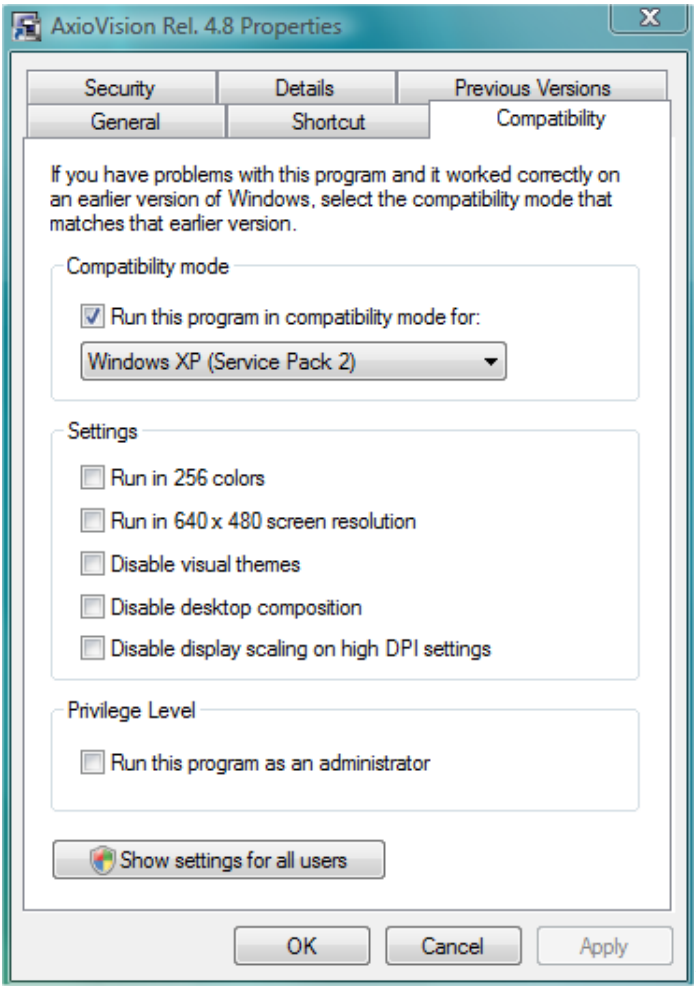
Note:

- Under Microsoft Windows Vista you can also set this option permanently. Please read the following chapter.

➢ Right-mouse click on the AxioVision icon 

and select the **Properties** function.

➢ The **AxioVision Properties** dialog window will be displayed. In the **Privilege Level** field activate the option **Run this program as an administrator**.



*

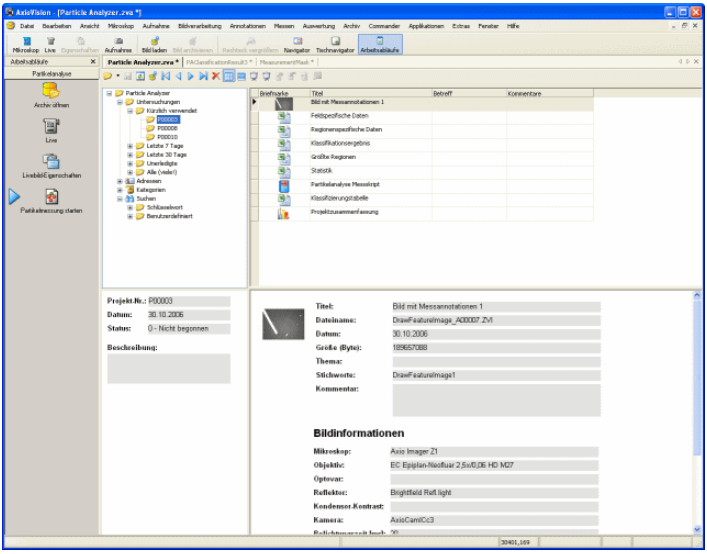
From now on the program will be executed with administrator rights when starting AxioVision.

Projects and test specifications

The configuration data of a measurement are saved to so-called test specifications. A test specification contains all the information on the performance of a particle measurement in detail: from the configuration of the device and the type of image acquisition through to the determination of the size of the original data to be saved and how the results should be displayed at the end of a measurement.

The test specifications are saved to the archive, together with the measurement projects, but they are not visible in the archive window. They can only be modified via the **Manage Test Specifications** function.

Data are saved and managed in a project-based archive. All the data generated (images, data tables, reports etc.) are arranged by project and saved centrally:



Archive view of the Particle Analyzer software

Preparing the system

Introduction

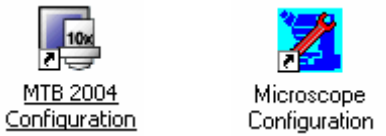
General

The functionality of the Particle Analyzer Projects module is based on the basic functionality of AxioVision. Before you can work with the Particle Analyzer, you must, therefore, configure the basic settings for AxioVision. You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

To be able to use your microscope together with AxioVision, you need to define once your individual system configuration via the separate **MTB2004 Configuration** program. Should you be retrofitting the Particle Analyzer to your existing microscope system, you may have to do this configuration via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:

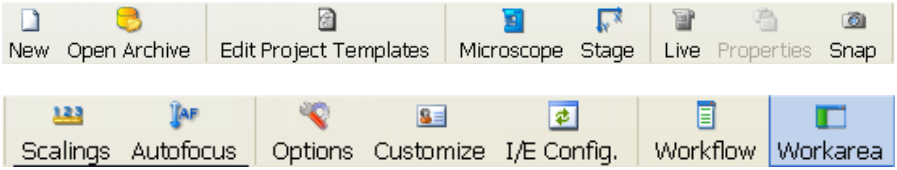


Note:

- For detailed information, see Chapter 4 "Microscope Control".

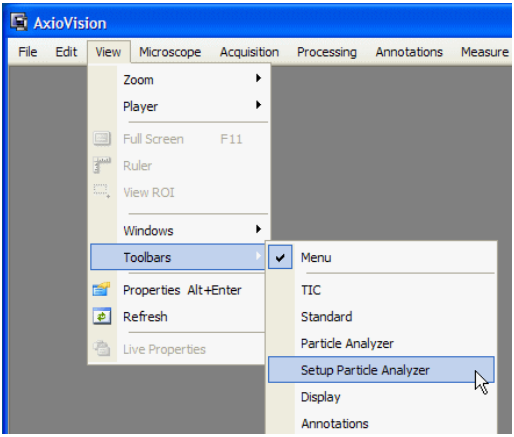
The Particle Analyzer Setup toolbar

The **Particle Analyzer Setup** toolbar is specially intended for setting and configuring the Particle Analyzer:



In the following it is assumed that the toolbar is open. If the toolbar is not displayed on your monitor, select Toolbars in the View menu:

Hereinafter it is assumed that the toolbar is open. If it should not be displayed on your system, open it via the menu **View** ⇒ **Toolbars**:



General AxioVision Settings

Please make sure that the following settings have been made:

Alignment of camera and motorized stage

If you wish to acquire images of large areas using the MosaiX module, you must ensure that the camera and stage are exactly aligned with the camera horizontal.

To check this, in the live image move a clearly recognizable object point as close as possible to the upper image edge. When the stage is moved in the X direction, this point in the image must remain at exactly the same distance from the upper image edge. If this is not the case, the camera must be rotated until the direction of movement and the image edge are completely parallel. Otherwise the individual component images will not be put together properly. The aligning and setting process may take quite some time with conventional camera adapters; therefore use of the adjustable Carl Zeiss camera adapter is recommended:



Note:

- For detailed information, see Chapter 9.7 "MosaiX" – "Setting up the camera and scanning stage").

Scaling

Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:
- For detailed information, see Chapter 8.4 "Scalings".

Autofocus calibration

To be able to use the autofocus feature, you need to calibrate it for the magnifications used. The autofocus feature can then be used for defining interpolation nodes for the focal plane, and for acquiring mosaic images.

Notes:
- For detailed information, see Chapter 9.2 "Autofocus".

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:
- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the **Particle Analysis** workflow and, perhaps, the **Particle Analyzer** toolbar. The **Particle Analyzer Setup** toolbar is not required for routine measurements. All toolbars, as well as the main menu, can be displayed and hidden via the **View** ⇒ **Toolbars** menu.

Notes:

- To show the main menu again, click the right mouse button on the title bar of the AxioVision window, or on a toolbar. This opens a context menu in which you can display everything again.

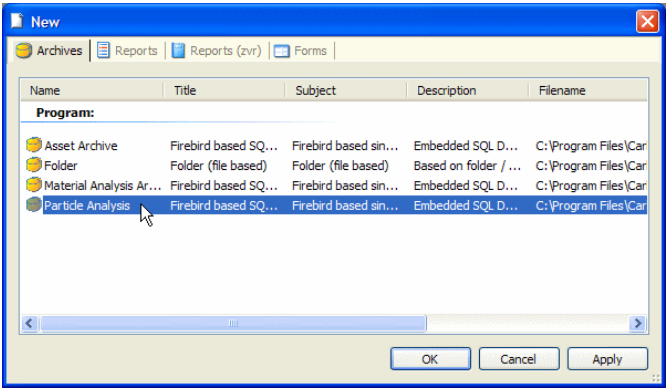
- For detailed information, see Chapter 8.5 "Adapting the user interface".

Special settings for particle analysis

Creating an archive

The database of the Particle Analyzer constitutes an archive. All data (images, measurement data tables, reports, etc.) are saved to this archive in a project-related order. To be able to work with the Particle Analyzer Projects module, therefore, an archive must exists and be opened in AxioVision.

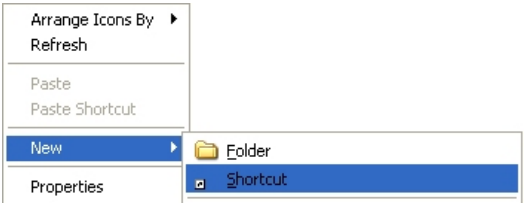
In the **Particle Analyzer Setup** toolbar, click on the **New** button. On the **Archive** tab, select the **Particle Analysis** template:



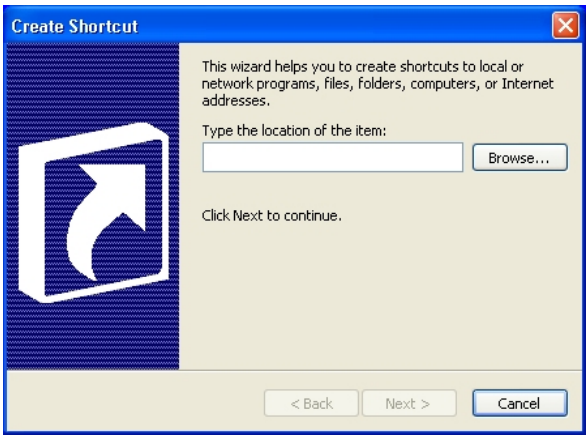
Opening the particle analysis archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

- Click the right mouse button on a free area on the desktop, and select **New ⇒ Shortcut**



- This opens the dialog **Create Shortcut**. Click on **Browse...** and select the Particle Analysis Archive (file extension *.zva):



- Click on **Next** and, in the next step, enter a name for the link.

- To create the link, click on **Finish**.

Note:

- Via the properties of the shortcut you can also adapt the icon. Suitable icons can be found in the "ZiResrc.dll" file in the *"C:\Program Files\Common Files\Carl Zeiss Vision\System\Resource"* folder.

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:

- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Separation of Particle Analysis and standard AxioVision

It is a strong point of the Particle Analyzer that the system is completely based on the standard AxioVision software. Therefore, it can also be used for simple documentation jobs or other applications such as, e.g., analysis of non metallic inclusions in steel. To start AxioVision always with a special, application-specific configuration (e.g., specific toolbars, workflows, etc.), you can establish any number of links with separate icons on the Microsoft Windows Desktop.

Note:

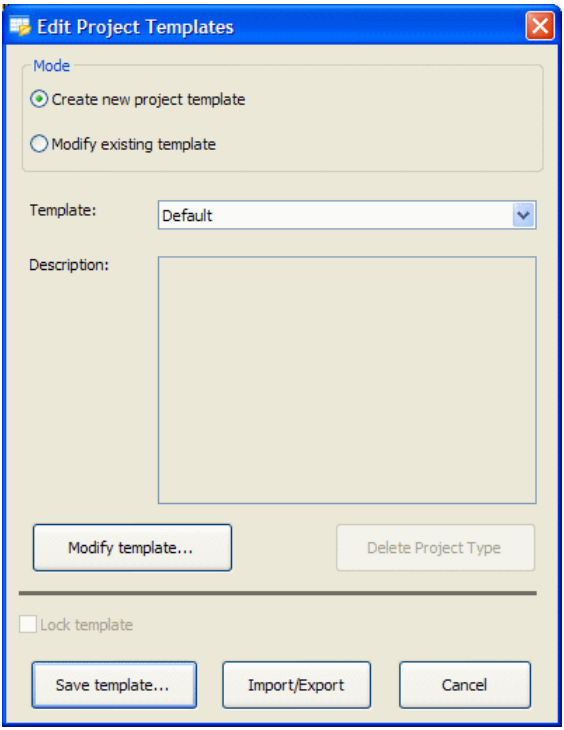
- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

Creating test specifications

General

In test specifications, all settings and files for performing a particle measurement are saved. All subsequent particle measurements always are based on a test specification. When you start a measurement you are prompted to select the desired test specification first before you can actually start the measurement.

The test specifications are managed via the **Edit Test specifications** dialog:



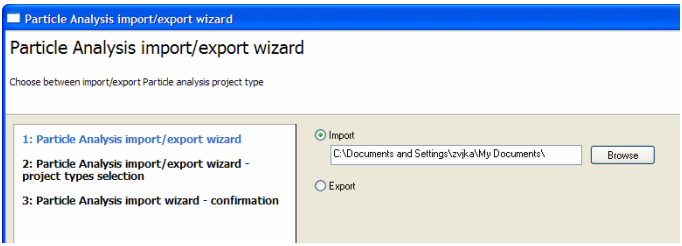
In the **Edit Test specifications** dialog you can edit, create and delete test specifications. Further, you can import and export test specifications.

Importing and exporting test specifications

To make the preparation and setup for Particle Analysis as easy as possible, you can simply import existing test specifications for special applications and use them right away.

This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:

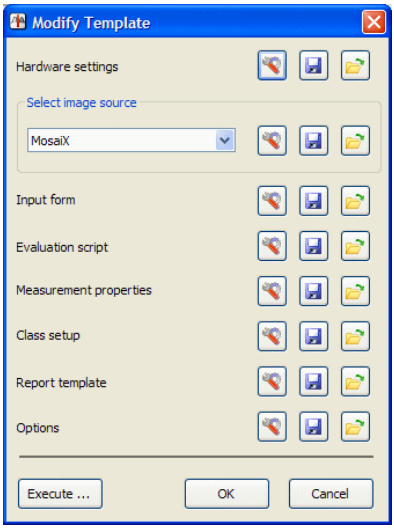


Note:

- Test specifications available at Carl Zeiss can be downloaded from the Particle Analyzer Internet website **www.zeiss.de/particle** and then imported. Test specifications are available, e.g., for data analysis conforming to ISO 16232, and for measuring the Carl Zeiss particle standard.

The Change Test Specification dialog

Test specifications are created via the **Change Test Specification** function.



Here again you can, for each single item required for creating a test specification, load settings already existing in another template. For the management of template files, use the buttons on the right of the respective creation items:  .

Thus, you can directly copy device settings, settings of the image source, input forms, etc. from existing templates into the current test specification you are creating. Further, you can save your own settings to a new template in order to use them in further test specifications.

And finally, you can set every step separately. To open the respective dialog, click on the **Edit...** button: 

The following chapters describe the procedure step by step.

Testing test specifications

If you want to test whether the settings of a test specification really produce the desired results, you can click on the **Execute...** button to run a complete measurement without having to create a project in the archive. It is important that you perform this step with a real sample, because all settings made in the functional dialogs during the test run are adopted by the test specification.

Settings that are particularly important in this context are, e.g., those concerning the segmentation thresholds for particle detection, or the size and position of the measurement frame.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a Particle Analysis. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Example: Setting the camera mode (color, gray levels)

The camera setting has a decisive influence on the size of your files. Given the memory capacity of modern computers, the use of color images is considered a standard feature. However, color images require three times as much memory space as gray scale images. In most cases, though, color information

is not required for particle detection; therefore, images should be acquired as gray scale images. This setting can be made in the camera control dialog, and it can be changed there by every user:



By including the camera mode in the hardware settings you can ensure that you are always acquiring gray level images for a measurement, irrespective of the actual settings of the camera:

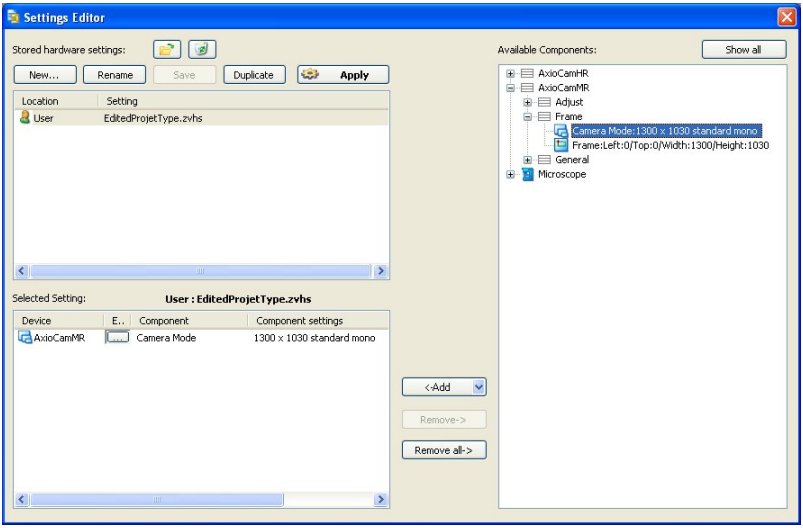


Image Source

The image source defines the way images are acquired. When large areas are acquired by the mosaic technique, the size and position of the acquired area are defined in addition via the **Setup** function of the MosaiX module. Then it is important, in routine work, that the samples are actually located in the places previously defined. This can be ensured through the use of holders for

different slide types. A special holder is available for measuring round particle filters:

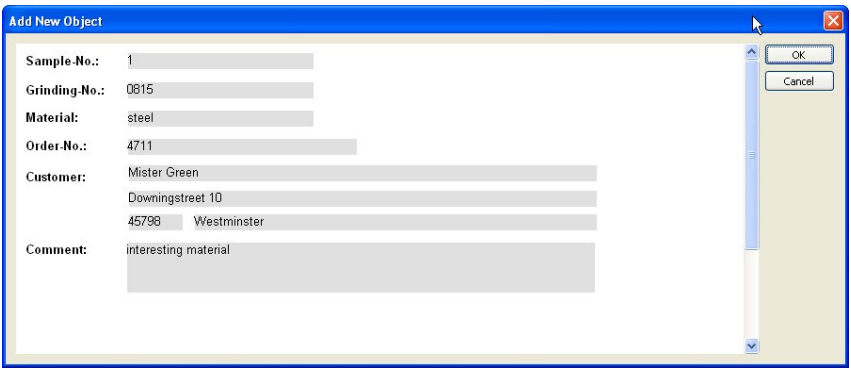


For details, see Chapter "Accessories for particle analysis".

To ensure unmistakable repositioning in the acquisition of MosaiX images, the z focus and the x and y settings of the motorized stage are calibrated after every restart of AxioVision.

Input form

During a measurement, the user enters the required project data into input forms. You can freely define which data to enter. The input fields required and their linking to the archive are defined in what is called the Layout mode. The Layout mode is active immediately when you start editing.



Standard input form for project data

Note:

- For detailed information on editing form templates, see Chapter 7.4 "Templates for forms and reports".

Evaluation scripts

General

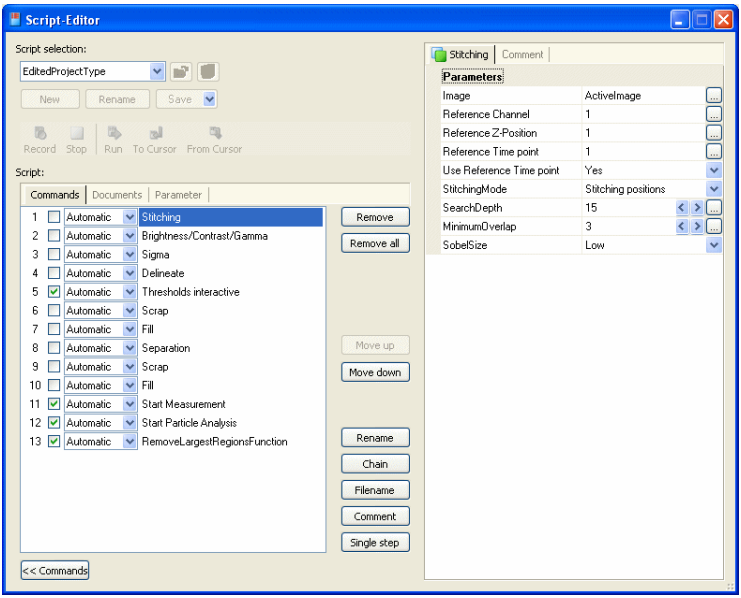
The analysis script determines the way in which the particle analysis is to be carried out. Consequently, this script is the key to the entire analysis. Besides the sequence of the commands, here you can also define the functions that you want to be executed interactively, i.e. which functions should be opened during the performance of a measurement to allow the user to enter parameter settings.

Notes:

- In order to display and modify the analysis script, you will need the Commander module. The Commander module however is NOT required for executing a particle measurement.

- Since modification of the analysis script has dramatic consequences with regard to the quality of the analysis, this should only be performed by experienced users. It is essential that you read the "Commander" section and the information in the online help before making any modifications.

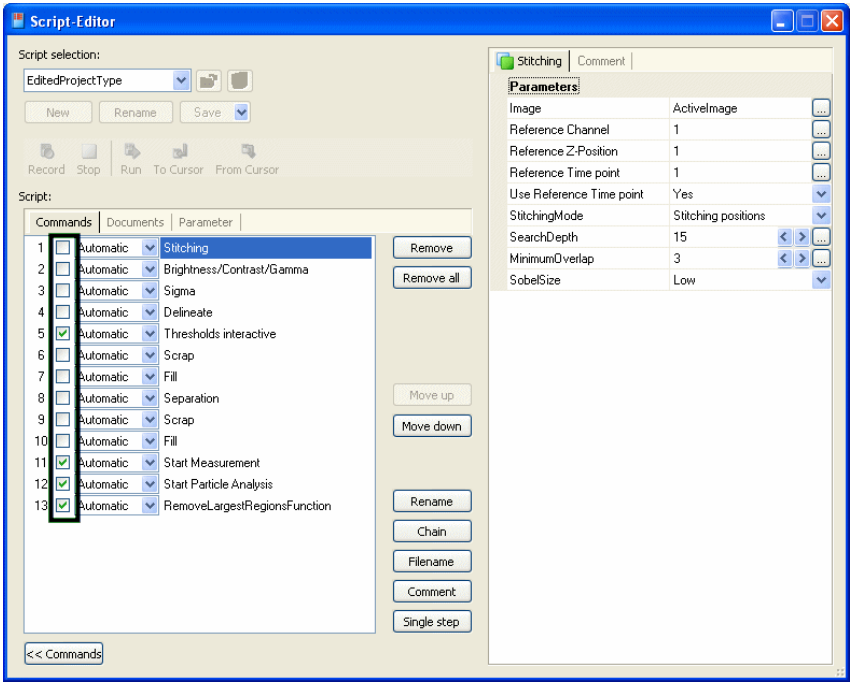
- The analysis scripts are not managed in the usual standard AxioVision folders. The **New** and **Rename** buttons are therefore inactive. The **Save** button only becomes active once you have made changes to the script.



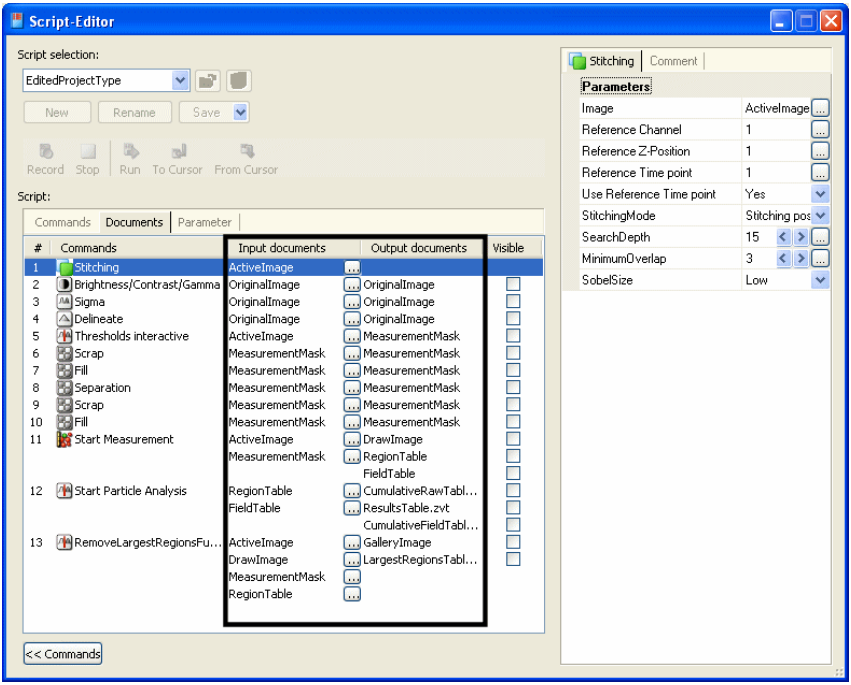
Analysis script in the Commander script editor

Adapting the evaluation script

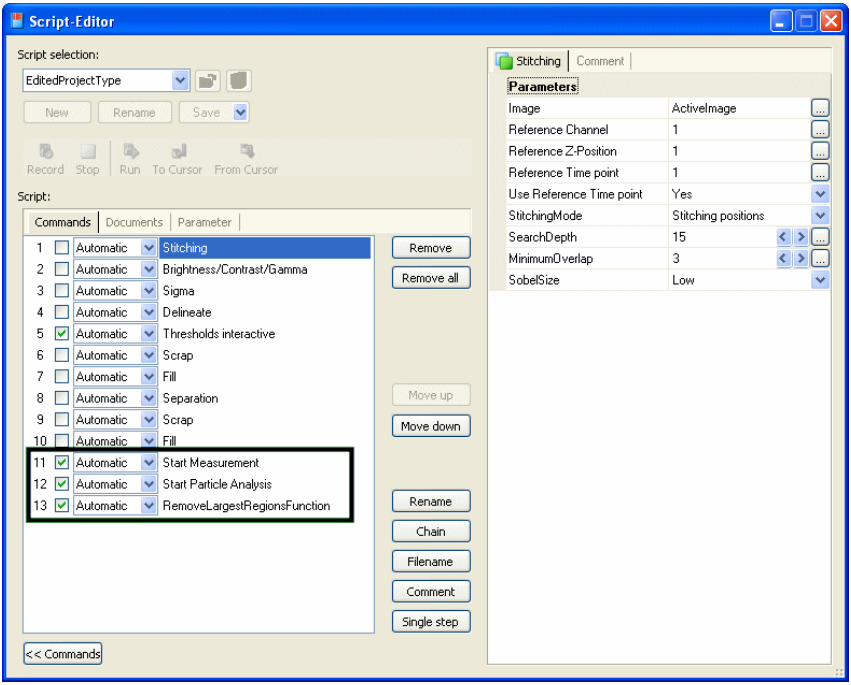
As long as you do not insert or delete any commands, you can modify the behavior of the script simply by switching the commands on the **Commands** tab on and off.



You can any functions, available in AxioVision, incorporate into the script. After doing so, however, you must ensure that the input and output documents are correctly linked.



The three command lines at the end of the script must not be deleted under any circumstances. These commands calculate the actual classification and generate the results document.



Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of a test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:

- For detailed information, see Chapter 6.4 "Creating data reports".

Optional settings

General

With optional settings you define essentially the following items:

- Selection of the data to be saved (above all, image data).
- Behavior during image acquisition.
- Presentation of result data after the measurement.

It is imperative that you consult the Online Help function for information on details of the parameters that can be set.

Memory space required

The quantity of data obtained in particle analyses is sometimes enormous, especially when you acquire large images using the MosaiX module. The table below shows the memory requirements for different conditions:

Pixel scaling used	Image size in MB*	
	Gray scale image	Color image
1.0 µm / pixel	1450	4350
2.5 µm / pixel	232	696
5.0 µm / pixel	58	174
10.0 µm / pixel	15	44

** Area approx. 1500mm², 8 bit gray levels*

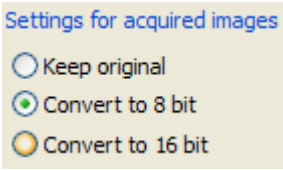
You should carefully consider which original data should actually be saved. Among others, the two factors outlined below may be a basis for decision:

- If you want to ensure that a measurement can be reproduced at any time, you need to save the original data as they are. It is not permissible in this case to scale down the original image or to save it in a compressed format.

- If you want to save the original image for documentation purposes, you should consider whether a scaled down version could be saved in a compressed format (e.g., ZVI or JPG). This will considerably reduce the amount of image data, but they will not be suitable for subsequent measurements.

Number of gray levels or color values of the images acquired

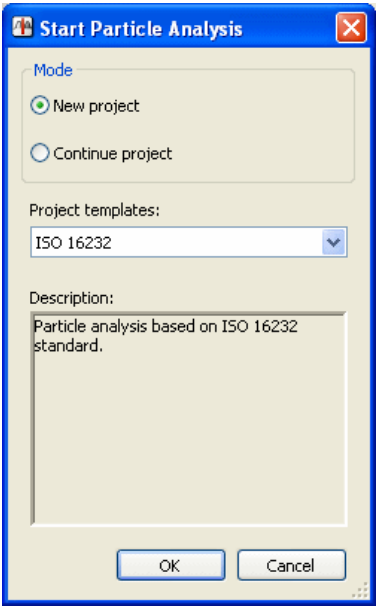
Another important consideration is the bit depth, i.e. the number of gray levels or color values used for image acquisition. ***In the past***, the entire brightness or color range was captured 256 different gray levels (8 bit). Most modern digital cameras have more than 256 gray levels. But here, too, this means that the resulting image data files are exceedingly large; the great number of gray levels (possibly more than 65,000) is not needed for a measurement. It is possible, therefore, to enforce reduction to 256 gray levels:



Conversion of acquired images to 8 bit

Performing measurements

A measurement is performed via the **Start Particle Analysis** function in the **Particle Analysis** workflow:



Dialog for starting a particle measurement

A particle measurement is always based on a previously defined test specification. This must have been created beforehand by a qualified user. It is not possible to perform a measurement if no test specification has been created.

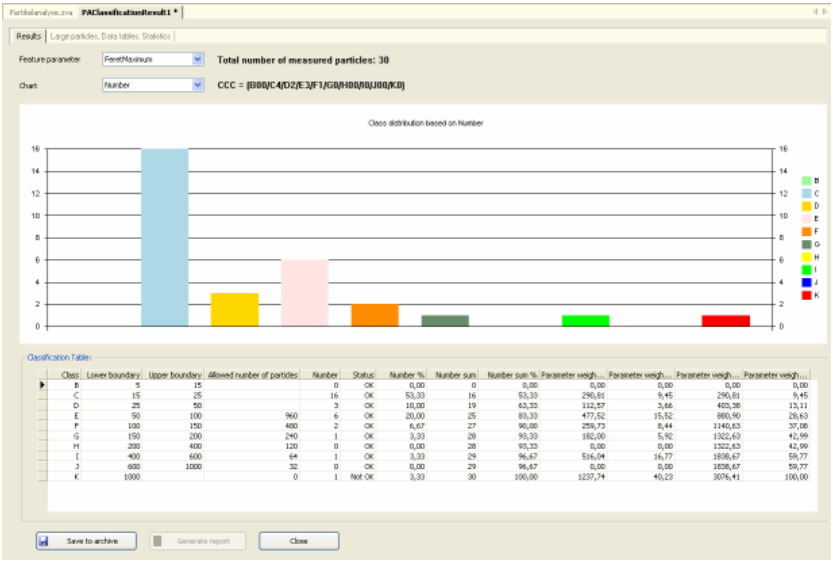
During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the analysis script will be displayed. All other functions run automatically in the background.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question.

The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

Once the measurement is complete, the results window will be displayed or, alternatively, the report can be displayed immediately:



Results document

Note:

- Whether it is the results document or report that is displayed depends on the settings for the test specification.

11.5 NMI (Non-Metallic Inclusions)

General

Note:

- For detailed information on the individual functions and commands, please refer to the online help (F1 key).

The system variants

The NMI module is the software for the NMI system. The NMI (non-metallic inclusions) system from Carl Zeiss is a complete system for analyzing the purity of steels in accordance with current standards. This system is currently based on three fully motorized microscopy platforms:

- Platform 1: upright light microscope Axio Imager.Z2m for multiple sample analysis
- Platform 2: inverse microscope Axio Observer.M2m individual sample analysis
- Platform 3: inverse microscope Axio Observer.Z1m

In addition to the microscopes, the following components are essential for a complete NMI system:

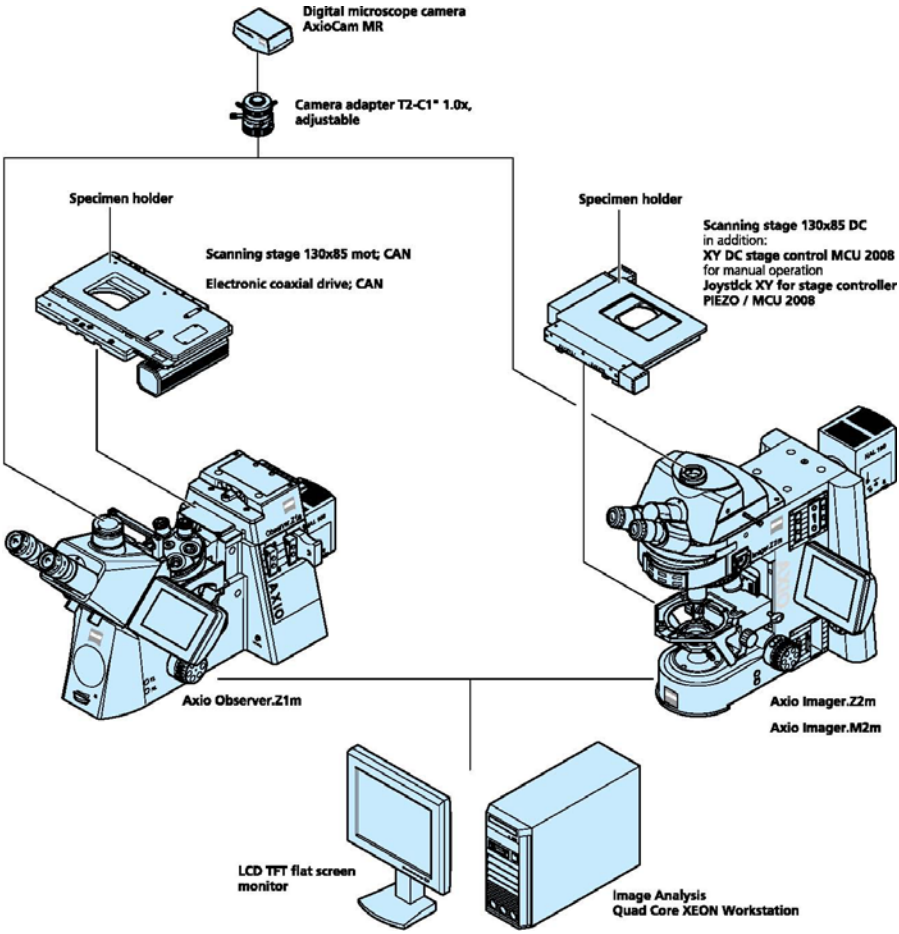
- Digital camera AxioCam MRc
- High-performance PC with monitor
- AxioVision basic package and the NMI and MosaiX modules

Depending on the microscopy platform used and the application-related requirements, the following components may also be necessary:

- A multiple sample holder for up to six round samples up to 40 mm in diameter
- For the individual modification of evaluation scripts: Commander module

For details on setting up and using the hardware components, such as the microscope, scanning stage and camera, please refer to the manuals supplied with the corresponding devices.

An overview of the system:



Note:

- Before using NMI, familiarize yourself with the basic functions of the AxioVision software. You should be familiar with the operation of the camera and microscope components in particular.

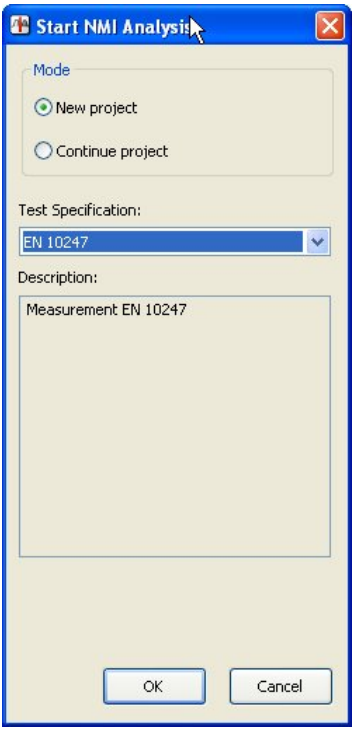
The concept

Operation and configuration

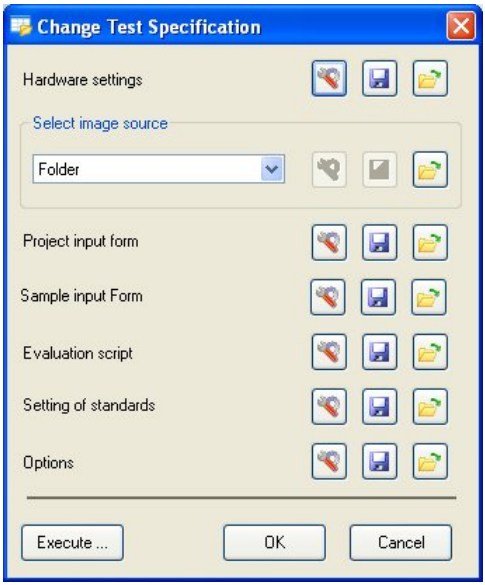
The operating concept of NMI is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement.

The performance of a measurement is always based on what is called test specification. All important settings are saved in a test specification. A test specification cannot be created or edited by a user. This is reserved to Power Users or Administrators. Access to the respective functions is governed by the access rights of the operating system:



Dialog for starting an NMI Analysis



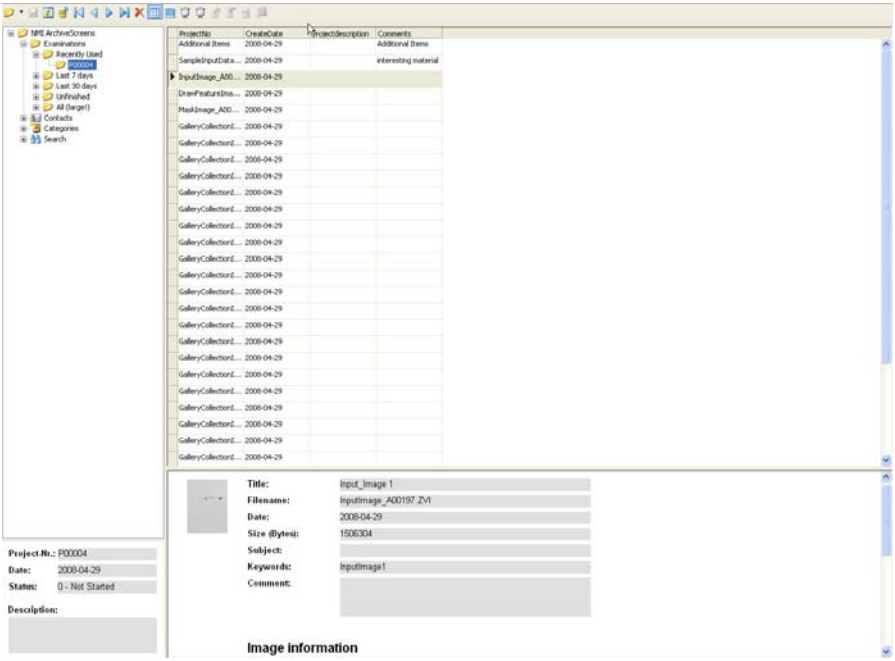
Dialog for editing a test specification (not available to standard users)

Projects and test specifications

The configuration data of a measurement are saved to so-called test specification. A test specification contains all the information on the performance of an NMI analysis in detail: from the configuration of the device and the type of image acquisition through to the determination of the size of the original data to be saved.

The test specifications are saved to the archive, together with the measurement projects, but they are not visible in the archive window. They can only be modified via the **Manage Test Specification** function.

Data are saved and managed in a project-based archive. All the data generated (images, data tables, reports etc.) are saved by project:



Archive View

Preparing the system

Introduction

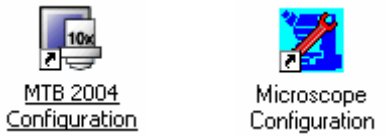
General

The functionality of the NMI module is based on the basic functionality of AxioVision. Before you can work with NMI, you must, therefore, configure the basic settings for AxioVision. You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

To be able to use your microscope together with AxioVision, you need to define once your individual system configuration via the separate **MTB2004 Configuration** program. Should you be retrofitting NMI to your existing microscope system, you may have to do this configuration via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:



Note:

- For detailed information, see Chapter 4 "Microscope Control".

General AxioVision Settings

Please make sure that the following settings have been made:

Alignment of camera and motorized stage

If you wish to acquire images of large areas using the MosaiX module, you must ensure that the camera and stage are aligned correctly with one another and that the stage's direction of movement has been set correctly. Otherwise the individual component images will not be put together properly. The aligning and setting process may take quite some time with conventional camera adapters; therefore it is recommendable to use the adjustable Carl Zeiss camera adapter:



Note:

- For detailed information, see Chapter 9.7 "MosaiX" – "Setting up the camera and scanning stage").

➢ **Scalings**: Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:

- For detailed information, see Chapter 8.4 "Scalings".

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:
- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the **NMI** workflow.

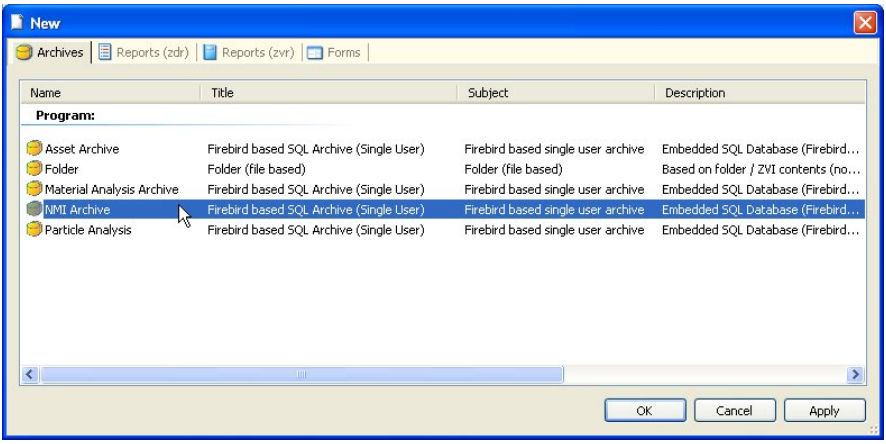
Notes:
- To show the main menu again, click the right mouse button on the title bar of the AxioVision window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

Special settings for NMI

Creating an archive

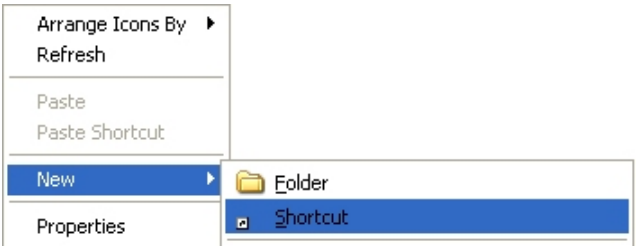
The database of NMI constitutes an archive. All data (images, measurement data tables, reports, etc.) are saved to this archive in a project-related order. To be able to work with the NMI module, therefore, an archive must exists and be opened in AxioVision.



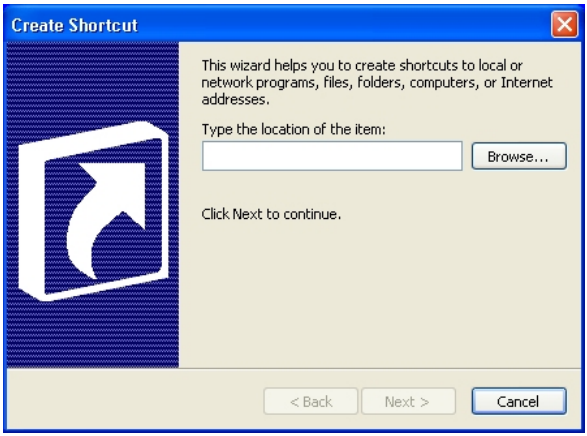
Opening the NMI archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

- Click the right mouse button on a free area on the desktop, and select **New** ⇒ **Shortcut**:



- This opens the dialog **Create Shortcut**. Click on **Browse...** and select the NMI Archive (file extension *.zva):



➢ Click on **Next** and, in the next step, enter a name for the link.

➢ To create the link, click on **Finish**.

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:

- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Separation of Particle Analysis and standard AxioVision

It is a strong point of NMI that the system is completely based on the standard AxioVision software. Therefore, it can also be used for simple documentation jobs or other applications such as, e.g., particle analysis or analysis of non metallic inclusions in steel. To start AxioVision always with a special, application-specific configuration (e.g., specific toolbars, workflows, etc.), you can establish any number of links with separate icons on the Microsoft Windows Desktop.

Note:

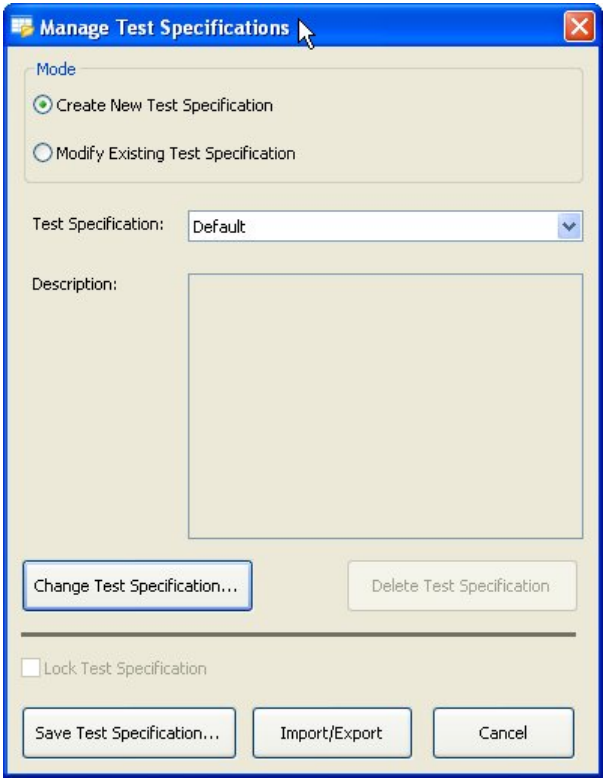
- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

Creating test specifications

General

In test specification, all settings and files for performing an NMI analysis are saved. All subsequent NMI analysis always are based on a test specification. When you start a measurement you are prompted to select the desired test specification first before you can actually start the measurement.

The test specifications are managed via the **Manage Test Specification** dialog:



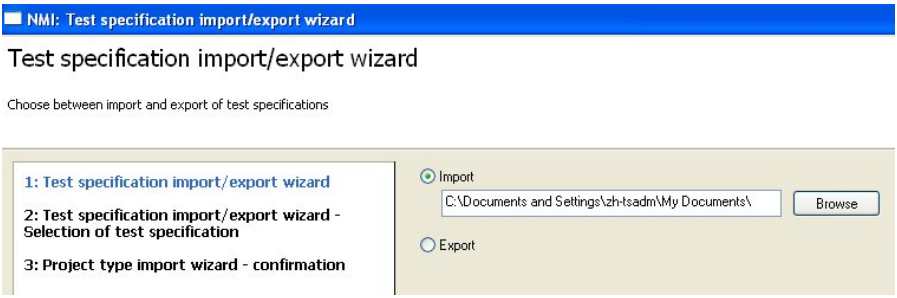
In the **Manage Test Specifications** dialog you can edit, create and delete test specifications. Further, you can import and export test specifications.

Importing and exporting test specifications

To make the preparation and setup for NMI Analysis as easy as possible, you can simply import existing test specifications for special applications and use them right away.

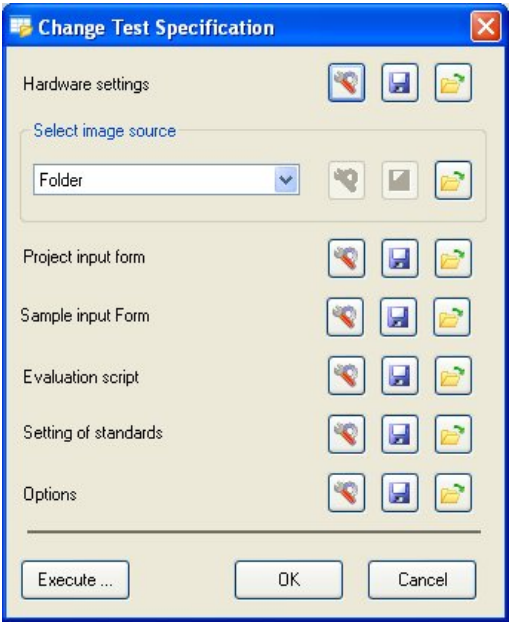
This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:



The Change Test Specification dialog

Test specifications are created via the **Change Test Specification** function. All the dialogs used to set up an NMI analysis are called up via this dialog:



Dialog for changing a test specification

Each individual step can be modified entirely. The relevant dialog can be opened using the button  . Here again you can, for each single item required for creating a test specification, load settings already existing in another template. For the management of template files, use the buttons on the right of the respective creation items:  .

Thus, you can directly copy device settings, settings of the image source, input forms, etc. from existing test specifications into the current test specification you are creating. Further, you can save your own settings to a new test specification in order to use them in further test specification.

The following chapters describe the procedure step by step.

Testing test specifications

If you want to test whether the settings of a test specification really produce the desired results, you can click on the **Execute...** button to run a complete measurement without having to create a project in the archive. It is important that you perform this step with a real sample, because all settings made in the functional dialogs during the test run are adopted by the test specification.

Segmentation threshold settings for detecting inclusions, in particular the threshold values for sulfides, oxides and titanium nitrides, are especially important here, for example.

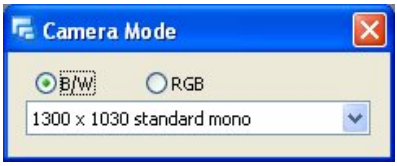
Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start NMI Analysis. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

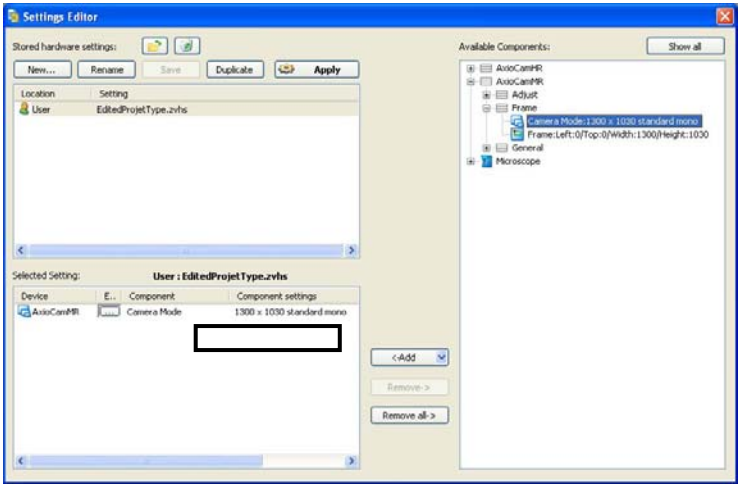
The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Example: Setting the camera mode (color, gray levels)

The camera setting has a decisive influence on the size of your files. Given the memory capacity of modern computers, the use of color images is considered a standard feature. However, color images require three times as much memory space as gray scale images. In most cases, though, color information is not required for the detection of inclusions; therefore, images should be acquired as gray scale images. This setting can be made in the camera control dialog, and it can be changed there by every user:



By including the camera mode in the hardware settings you can ensure that you are always acquiring gray level images for a measurement, irrespective of the actual settings of the camera:



If hardware settings have been activated, these can be checked immediately before the measurement on the sample to be measured.

Image source

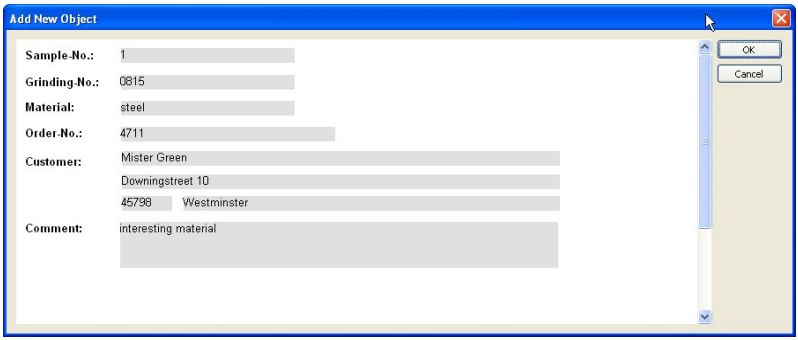
The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select folder or gallery as the image source. In most cases, however, an image is acquired and analyzed immediately. When you are acquiring images of large surfaces using the mosaic technique, the size and position of the acquisition region are defined using the MosaiX module's **Setup** function. To do so select the **Acquisition** option.
To ensure unmistakable repositioning in the acquisition of MosaiX images, the z focus and the x and y settings of the motorized stage are calibrated after every restart of AxioVision.

Input forms

Input forms allow the user to enter the necessary project and sample data during the measurement. You can freely define the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.
The module includes two input forms: the project-specific input form and the sample-specific input form. The project-specific input form is displayed immediately after the measurement. It is used for entering general project data. The sample-specific input form, on the other hand, is not displayed until the data are saved in the archive. The number of times that it is opened corresponds to the number of samples you have measured. In this form you enter data relating to the individual sample, e.g. sample name or material.



Standard input form for project data



Standard input form for sample data

Note:

- For detailed information on editing form templates, see Chapter 7.4 "Templates for forms and reports".

Evaluation script

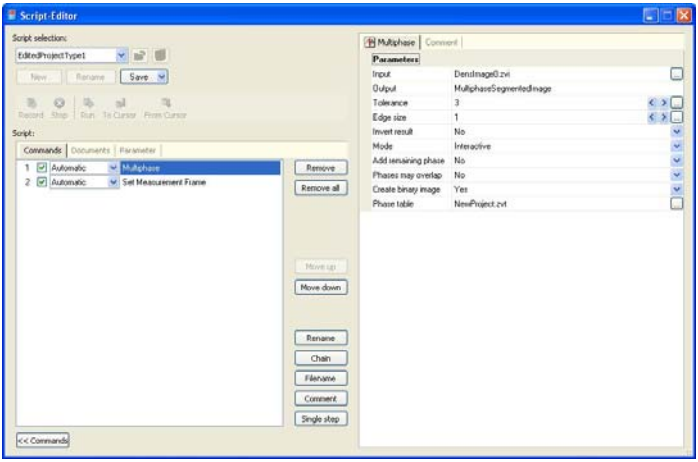
General

An evaluation script defines which image-processing steps will be performed in which sequence. It is not normally necessary to change the evaluation script. You can choose which functions will be executed interactively, i.e. which functions will be opened while a measurement is being performed to allow the user to adjust parameters.

Notes:

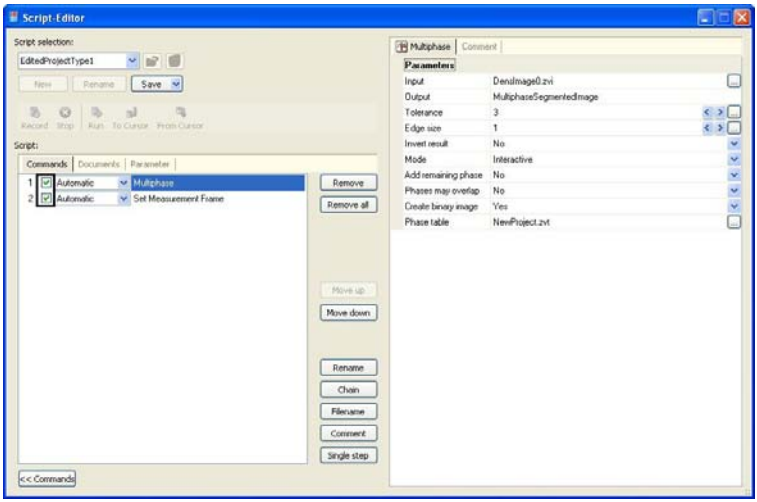
- In order to display and modify the evaluation script, you will need the Commander module. The Commander module however is NOT required for executing NMI Analysis.
- Since modification of the evaluation script has dramatic consequences with regard to the quality of the analysis, this should only be performed by experienced users. It is essential that you read the "Commander" section and the information in the online help before making any modifications.

- The evaluation scripts are not managed in the usual standard AxioVision folders. The **New** and **Rename** buttons are therefore inactive. The **Save** button only becomes active once you have made changes to the script.

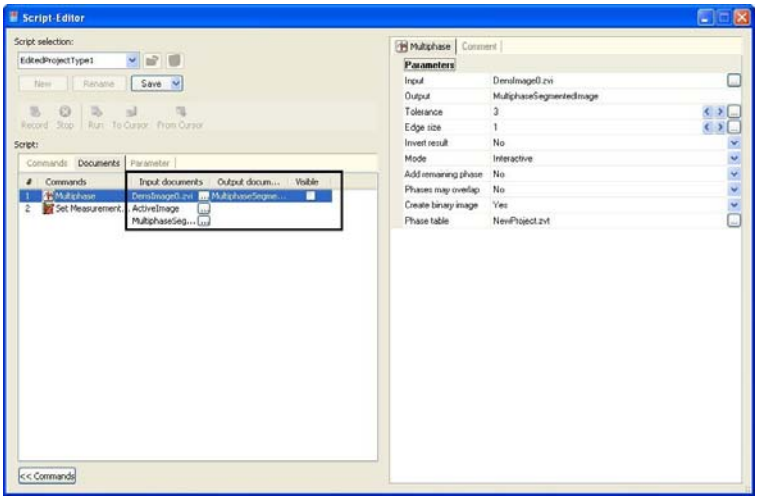


Adapting the evaluation script

As long as you do not insert or delete any commands, you can modify the behavior of the script simply by switching the commands on the **Commands** tab on and off.



You can incorporate any functions available in AxioVision into the script. After doing so, however, you must ensure that the input and output documents are correctly linked.



The multiphase segmentation and its settings should not, however, be deleted or changed under any circumstances.

Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:
- For detailed information, see Chapter 6.4 "Creating data reports".

Optional settings

General

With optional settings you define essentially the following items:

- Selection of the data to be saved (above all, image data)
- Behavior during image acquisition
- Presentation of result data after the measurement

It is imperative that you consult the Online Help function for information on details of the parameters that can be set.

Memory space required

The quantity of data obtained in NMI is sometimes enormous, especially when you acquire large images using the MosaiX module. The table below shows the memory requirements for different conditions:

Pixel scaling used	Image size in MB*	
	Gray scale image	Color image
1.0 µm / pixel	1450	4350
2.5 µm / pixel	232	696
5.0 µm / pixel	58	174
10.0 µm / pixel	15	44

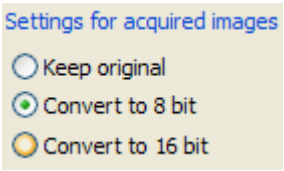
You should carefully consider which original data should actually be saved. Among others, the two factors outlined below may be a basis for decision:

- If you want to ensure that a measurement can be reproduced at any time, you need to save the original data as they are. It is not permissible in this case to scale down the original image or to save it in a compressed format.

- If you want to save the original image for documentation purposes, you should consider whether a scaled down version could be saved in a compressed format (e.g., ZVI or JPG). This will considerably reduce the amount of image data, but they will not be suitable for subsequent measurements.

Number of gray levels or color values of the images acquired

Another important consideration is the bit depth, i.e. the number of gray levels or color values used for image acquisition. ***In the past***, the entire brightness or color range was captured 256 different gray levels (8 bit). Most modern digital cameras have more than 256 gray levels. But here, too, this means that the resulting image data files are exceedingly large; the great number of gray levels (possibly more than 65,000) is not needed for a measurement. It is possible, therefore, to enforce reduction to 256 gray levels:



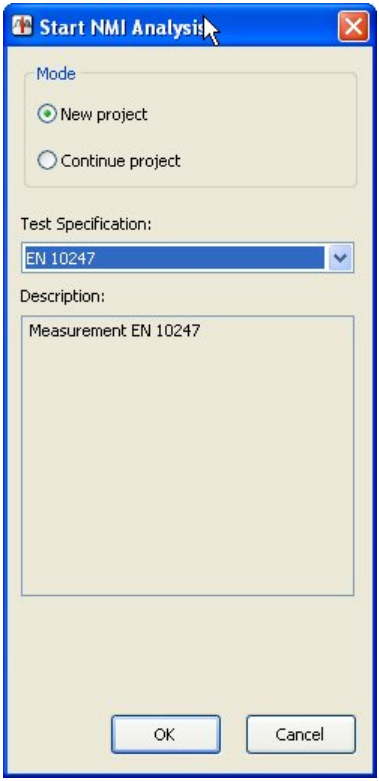
Conversion of acquired images to 8 bit

Note:

- If only sulfides and/or oxides are analyzed, a gray scale image is normally perfectly sufficient. In order to distinguish between sulfides and titanium nitrides, however, color images are required.

Performing measurements

A measurement is performed via the **Start NMI Analysis** function in the **NMI** workflow:



An NMI analysis is always based on a previously defined test specification. This must have been created by a qualified user. No measurement can be started until a test specification has been created.

During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the evaluation script will be displayed. All other functions run automatically in the background.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question.

The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

The results document

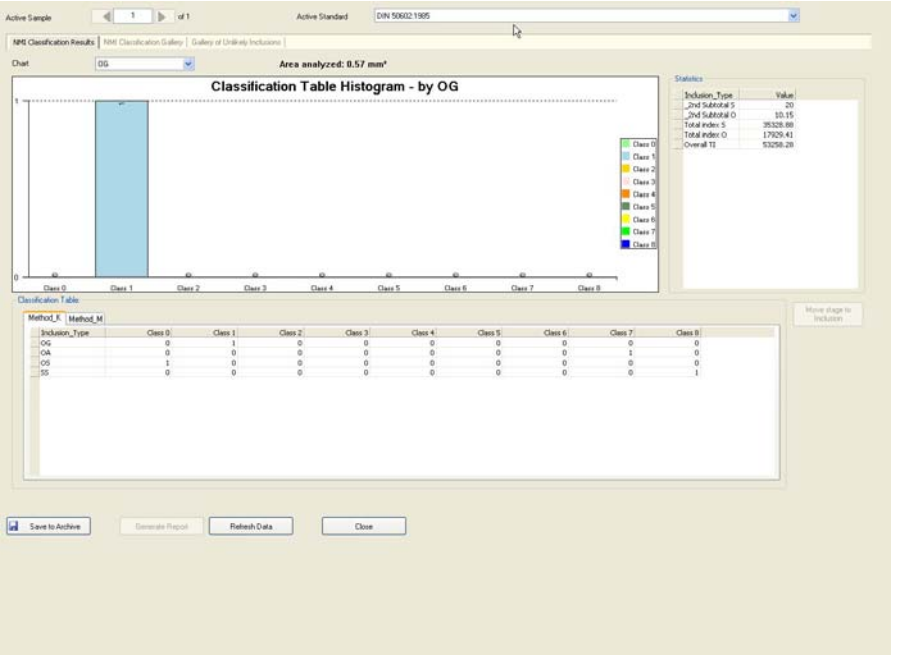
At the end of the measurement the results window is displayed. This is divided into three areas:

- Results view
- Gallery of images of the largest inclusions
- Gallery of unlikely inclusions

In this document the measurement results for each sample and each standard are displayed separately. It is possible to view the results for a different standard or sample here.

The results window is divided into different property pages. The **Results, Data** property page shows the numerical values with a classification table, statistical values and an overview chart. The **Gallery of largest inclusions** property page shows the gallery containing images of the largest inclusions and allows the repositioning of objects and artifacts to be deleted. The **Gallery of unlikely inclusions** property page shows objects with properties, which are unlikely for inclusions.

The results view



Results document with results view

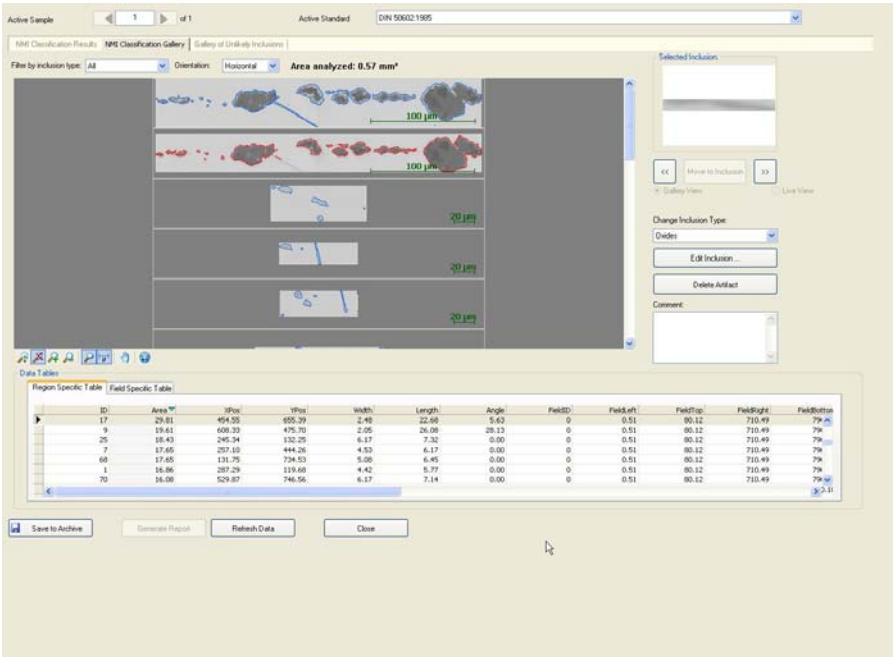
The document is essentially divided into three areas:

1. Chart
2. Classification tables
3. Statistics

The gallery of images of the largest inclusions

This gallery shows the largest inclusions. It is possible to filter the gallery according to inclusion type (sulfides, oxides) or display it for all inclusion types. In addition, in all standards (except EN 10247) it is possible to display the worst field for each inclusion type.

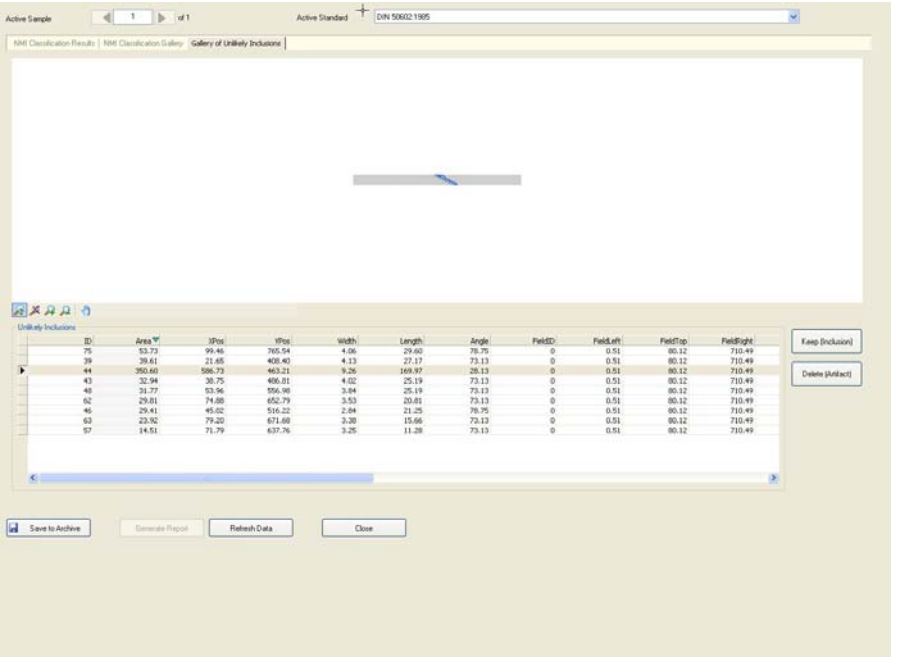
If the sample is still under the microscope and has not been changed, the inclusion can be repositioned. This means that, if you click on the **Edit Inclusion** button, the microscope stage is moved in such a way that you are able to view the currently highlighted inclusion directly in the live image or through the eyepiece. If you establish that this object is an artifact, you are also able to delete it.



Results document with gallery of the largest inclusions

The gallery of unlikely inclusions

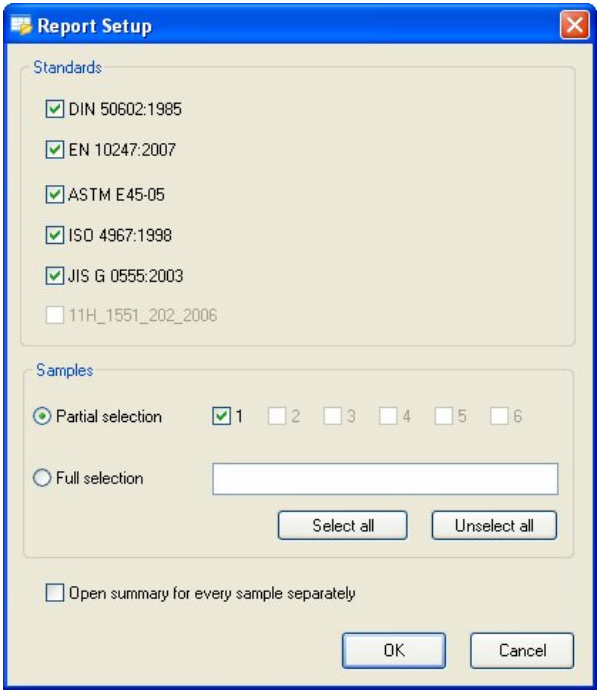
This gallery shows objects that are "unusual". Scratches, for example, are filtered out on the basis of their angle. However, as it is also possible for inclusions to have unusual forms or angles, automatic filtering of artifacts is not sensible. Instead, all objects to which the parameters apply are collected in a gallery and presented to the user following the measurement. The user is then able to decide whether they are inclusions or artifacts. The parameters can be changed under **Change Test Specification**.



Results document with gallery of unlikely inclusions

Generating reports

Reports are generated separately for each standard. It is also possible to combine several samples in a single report on the basis of a particular standard. There are no restrictions with regard to either the number of reports or the combination options. This means that you can measure the samples of several customers or from several casts using a single measurement but nevertheless generate a separate report for each case.

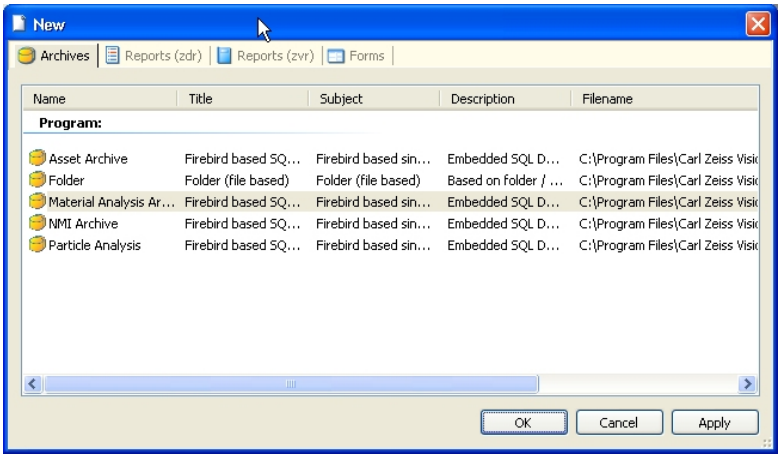


How do I create an NMI archive?

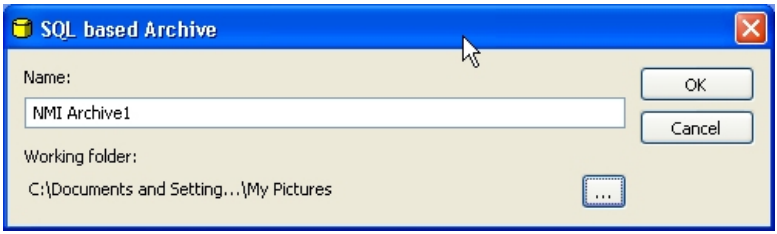
The NMI Archive forms the basis for managing the data resulting from NMI analysis. All the data relating to the performance of a measurement are managed here. In order to perform a measurement, it is necessary for an archive to be opened in AxioVision. The creation and opening of an archive are therefore shown first.

- Select the **New** function in the **File** menu.

- On the **Archives** tab sheet, select the **Material Analysis Archive** and then click **OK**.



- The **SQL based Archive** dialog window will appear. Enter a name for the archive in the **Name** input field and confirm by clicking on **OK**.



Notes:

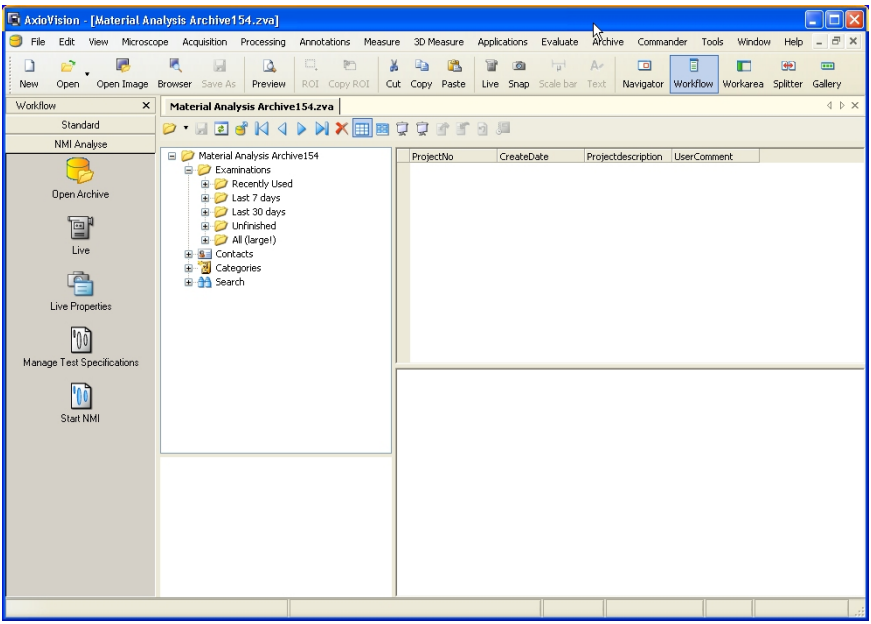
- An archive is created in the *My Documents\My Pictures* folder by default.

  If you wish to save the archive elsewhere, click on the ... button. Select the location where you wish the archive to be saved in the Microsoft Windows standard dialog that then opens.

- In the case of large data volumes in particular, we recommend that you save the archive to a local hard drive.

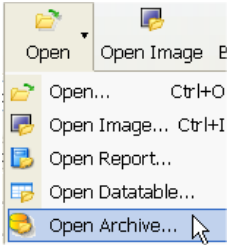
- Only one archive can be opened at a time in AxioVision.

The newly created archive is now displayed in AxioVision.



Opening an archive

➢ Click on the **Open** icon in the menu bar and then on the **Open Archive** function:

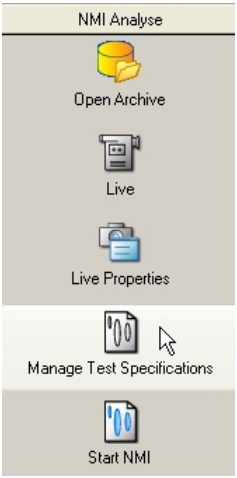


How do I create a test specification?

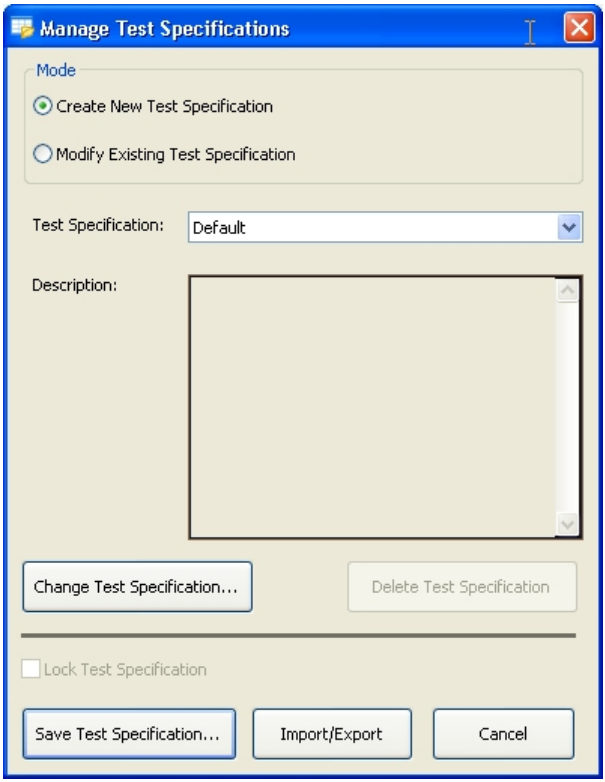
The test specification contains all the settings required for performing a measurement.

➢ From the workflows, select the **NMI** workflow.

➢ Click on the **Manage Test Specifications** function.



This opens the **Manage Test Specifications** dialog.

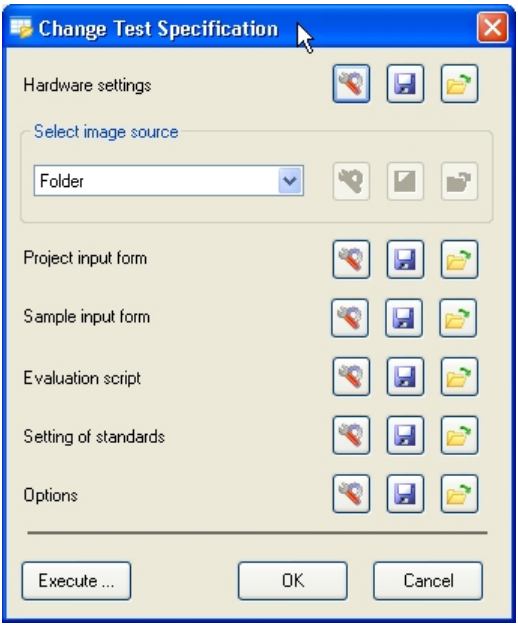


➢ Select the test specification that you wish to use as a basis for the new test specification. If you have not yet created a test specification, you can select one of the standard test specifications.

Note:

- In the **Create New Test Specification** mode, a copy is generated of the selected test specification, which you can then adapt. The original test specification that is used is not changed. If you only wish to make a modification to the test specification, change to the **Modify Existing Test Specification** mode. The standard templates of NMI analysis cannot be changed, but only used as the basis for a new test specification.

➢ Click on the **Change Test Specification** button. This opens the **Change Test Specification** dialog.



➢ You can now save the hardware settings, select the image source, set options or edit the input form and report template. It is recommended, however, that you modify the report template after running the program, because measurement data are then available. The report editor is displayed automatically after running the program.

Notes:

- You will find more detailed information about the hardware settings in Chapter 4.5 "Settings Editor" of this manual.
- You will find more detailed information on modifying form templates in Chapter 7.4 " Templates for forms and reports" of this manual.
- You will find more detailed information on editing a report template in Chapter 6.4 " Creating Data Reports" of this manual.

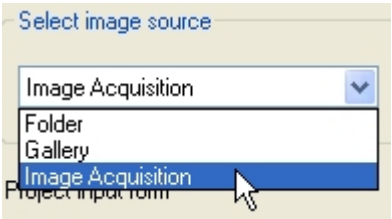
- Once you have made all the necessary settings, click on the Run ... button. The individual steps of the evaluation script are now run through, followed by an NMI measurement. Here you can test When you have made all the necessary settings, leave the **Change Test Specifications** dialog by clicking on the OK button.

- Save the test specification by clicking on the Save button. This opens the **Save Test Specification** dialog. You can enter a name and a description of the test specification here. A description helps the user when starting the measurement, as he is unable to open the **Manage Test Specifications** area and does not, therefore, see the settings that have been made there.

How do I set up image acquisition for several samples?

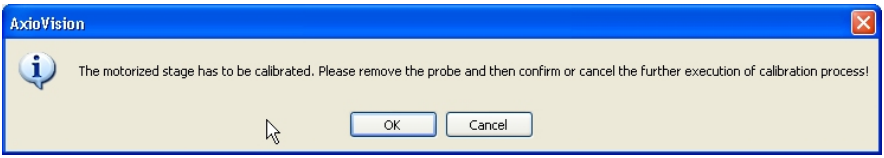
Create a test specification and open the **Change Test Specification** dialog.

Select **Image Acquisition** as the image source.

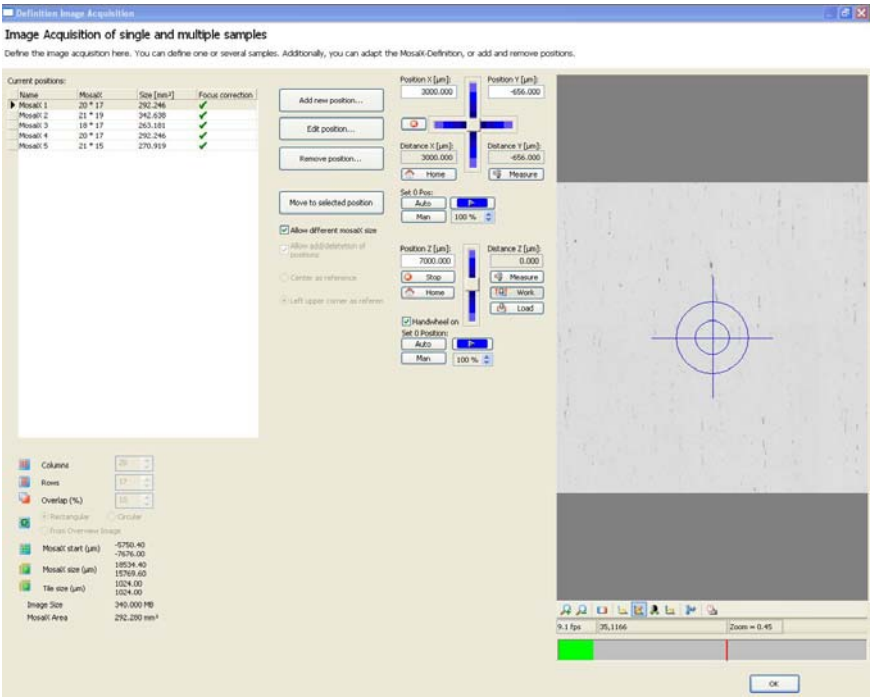


To set up image acquisition, now click on the  button.

First of all, calibration is performed. This is required in order for exact repositioning to be guaranteed. During this process the z-focus and the scanning stage are each calibrated once in the x and y direction. This calibration is only performed if necessary (e.g. if the microscope is restarted).

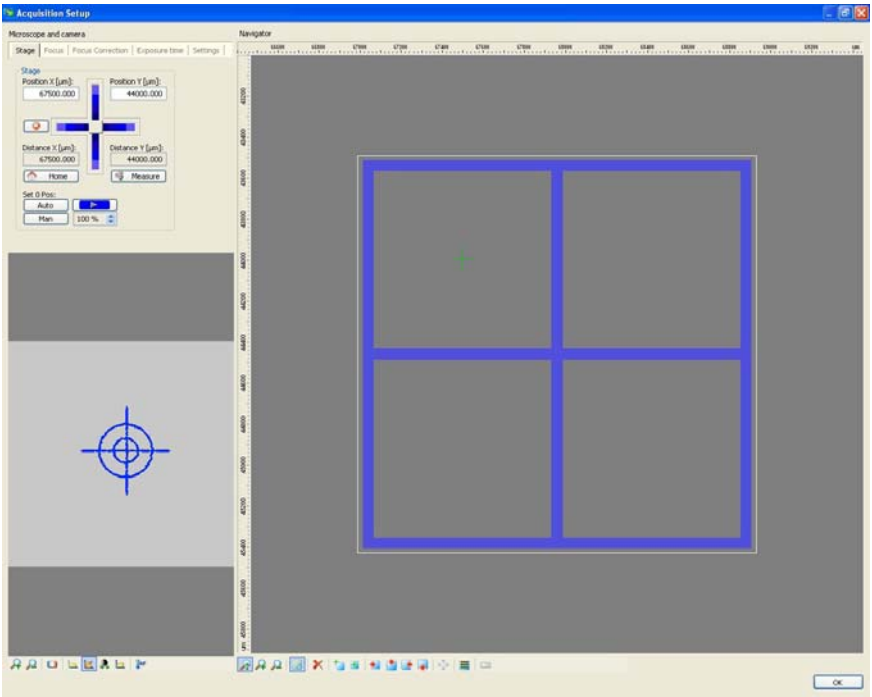


The **Definition Image Acquisition** dialog opens.



To add a sample, move to the top left-hand corner of your sample using the scanning stage and click on the **Add New Position** button.

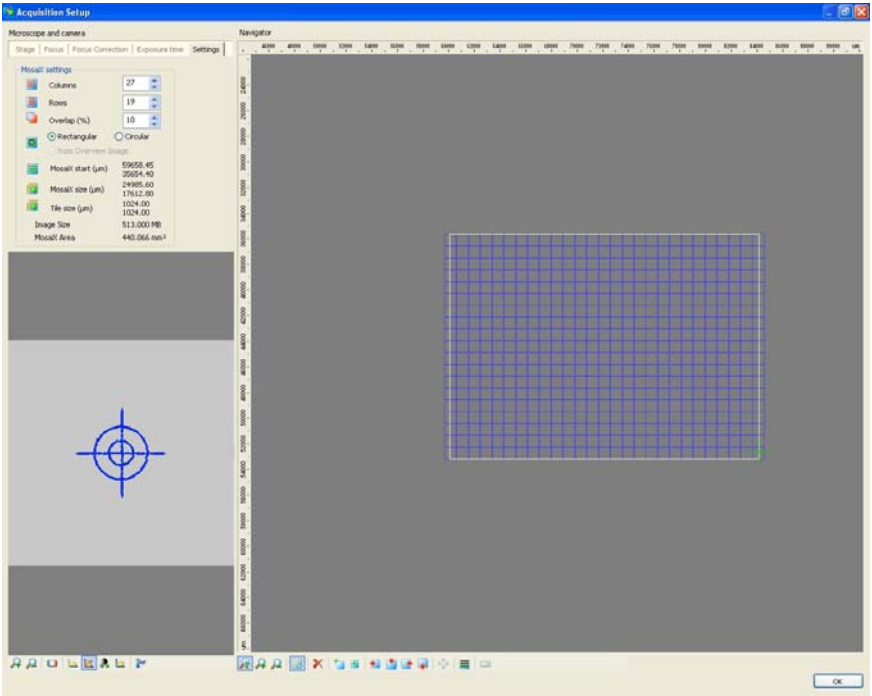
The **MosaiX Acquisition - Setup** dialog is opened. This contains a MosaiX grid with 2x2 tiles. The top left-hand tile is the section of the image that was visible in the live image in the previous dialog.



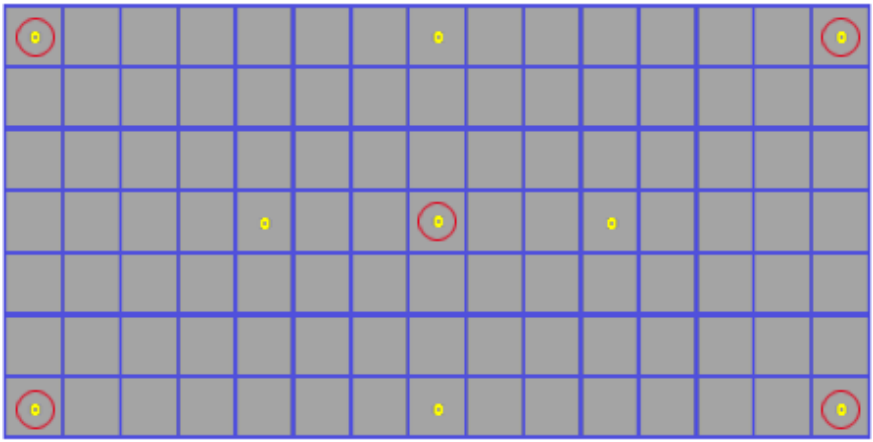
Note:

- You can obtain detailed information on the individual functions of the dialog in the online help (F1 key).

Move to the bottom right-hand corner of the sample using the scanning stage and click on the  button. This extends the size of the MosaiX image to the selected position.



Now define the focus correction points. To do this, first select the **Correct Focus Position** property page. In the case of samples that have been prepared using modern preparation machines, the following positions are recommended for the focus correction points:



Schematic image of the recommended focus position. The red bordered positions are the default positions which should be selected in any case. The other points should be selected if the default positions are not sufficient for focusing.

Confirm the settings by clicking on **OK**. The **Definition Imagr Acquisition** dialog then becomes active again.

In order to define another sample, move to the top left-hand corner of this sample. Clicking on the **Add New Position** button moves the MosaiX grid to the new position. The focus correction points are also moved relative to this. The current focus position is adopted as the z-position.

Check the MosaiX definition by locating all corners of the MosaiX with the help of a double-click on the corner points. If the sample is the same size as the first one, you can adopt the settings using the **OK** button. If it is not the same size, you can adjust the MosaiX definition using the following buttons: , , , , and . You must then also adjust the focus correction points, if necessary.

Note:

- In order to acquire MosaiX images of different sizes, the **Allow different MosaiX size** option must be activated. If this is not active, the image size of the last position will be adopted for all other positions.

Once you have defined all the positions, exit the **Definition Image Acquisition** dialog by clicking on the **OK** button, close the **Change Test Specification** dialog, also by clicking **OK**, and save the test specification by clicking on the **Save** button.

11.6 Grains

In the Grains module, the grain size is determined in accordance with current international standards. Three methods are available for determining grain sizes:

- **Auto**: automatic reconstruction of the grain boundaries
- **Chord**: determination of the grain size using the intercept method
- **Compare**: comparison of micrographs with comparative diagrams

These methods differ in terms of their accuracy and in the level of interaction with the user. Starting from Compare, a purely interactive method, the accuracy increases with the semi-automatic Chord method and increases still further with the Auto method, but the duration of the analysis can increase too. The visibility of the grain boundaries also plays a role in the selection of the method.

The Compare method

The micrograph is compared with the comparative diagrams. This method is primarily suited to steels, as the comparative diagrams have been created on the basis of steels. This is a purely interactive method. The following comparative diagrams are available:

- **SEP151061**: microscopical grain size analysis of steel samples with help of tables using different Grain sizes (1-8) and stretch factors (1,2,4).
- **ASTMIA**: is for coarse grains, grain size 0-2.5.
- **ASTMIB**: is for finer grains, grain sizes 3-10.
- **ASTMII**: is used in cases when twin grains are seen in grains (flat etch), grain size 1-8.
- **ASTMIII**: is the alternative to ASTMII (contrast etch versa flat etch), grain sizes 0.005-0.2 mm
- **ASTMIV**: is used to evaluate austenitic grain structures in steel, grain sizes 1-8.
- **BS4990**: this image series is used for fine grain sizes (grain sizes 1 to 8).

The Chord method

The micrograph is overlaid with a pattern. It is possible to choose between different patterns here. The size of the pattern and distance between the lines are then adjusted to the image. The intercept points with the grain boundaries are marked. Additional intercept points can be added or removed interactively. As the length of the pattern of lines is known, it is possible to determine the average grain size. This procedure, in particular the adjustment of the pattern of lines to the size of the image and the setting of the distance between the lines, corresponds to the requirements of the standard and the method is suitable for all sample types.

The analysis takes place in accordance with the following standards:

- ASTM E 112
- ASTM E 1382
- DIN EN ISO 643

The Auto method

The grain boundaries are reconstructed automatically and the individual grain sizes determined. Using this method the grain size distribution is also available in addition to the average grain size. This method is most suitable for samples with grain boundaries that are comparatively easy to detect.

The analysis takes place in accordance with the following standards:

- ASTM E 112
- ASTM E 1382
- DIN EN ISO 643
  Other, regional names are also available for DIN EN ISO 643.

All data are saved and managed in one place – the archive – which has search and filter functions. This ensures that the data can be relocated easily.

The operating concept of Grains is described below. This is followed by a practical section that provides step-by-step instructions in relation to the individual points.

Note:

- For detailed information on the individual functions and commands, please refer to the online help (F1 key).

The concept

The operating concept of Grains is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement. For this reason, the operation of Grains is essentially divided into two functional areas:

- administrator mode (definition of a measurement)
- user mode (performance of a measurement)

Administrator mode

A measurement is always based on a test specification, which is created in the administrator mode. All the key settings required for a grains analysis are saved here. This test specification may only be created or modified by principal users or administrators. Here, the administrator can also specify whether or not certain parameters may be changed by the user. A standard user has no access to the management of test specifications. Access to the corresponding functions is controlled by means of the access rights of the operating system.

User mode

In the user mode, measurements can be performed on the basis of a test specification. The administrator determines to what extent the user may change the measurement parameters.

Operation and configuration

Preparing the system

General

The functionality of the Grains module is based on the basic functionality of AxioVision. Before you can work with Grains, you must, therefore, configure the basic settings for AxioVision.

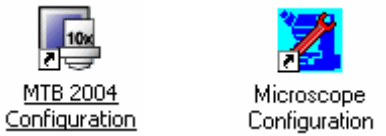
Note:

- You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

The microscope is controlled with the help of the separate **MTB 2004 Configuration** program. In order to use your microscope together with AxioVision, your individual system configuration must first be defined via this separate program. If you upgrade a pre-existing system for grains analysis, the configuration may take place via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:



Note:

- For detailed information, see Chapter 4 "Microscope Control".

Scalings

Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:

- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the Grains workflow.

Notes:

- To show the main menu again, click the right mouse button on the title bar of the AxioVision image window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:

- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Grains and other AxioVision applications

One of AxioVision's particular strengths is its modular structure. Each module is added directly onto the basic system. This means that the system can also be used for simple documentation tasks or other applications such as particle analysis or multiphase analysis. To ensure that AxioVision always starts with a particular configuration specific to the application (e.g. specific toolbars, workflows, etc.), any number of configurations can be started via individual icons on the Microsoft Windows desktop.

Note:

- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

The Material Analysis archive

Data are saved and managed in an archive. There, all data (images, measurement data tables, reports, etc.) are saved by project.

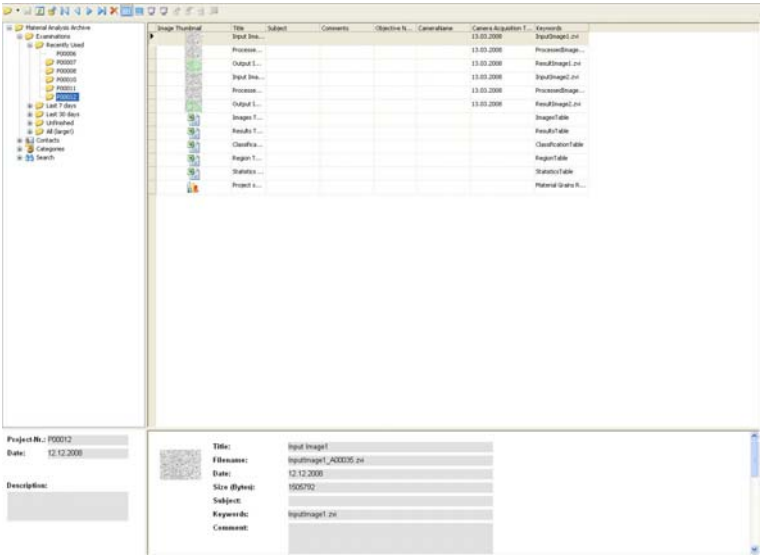
The test specifications are also saved in the archive together with the measurement projects. These are not visible in the archive window, and can only be modified using the **Manage Test Specifications** function.

There are several types of archive in AxioVision. Besides the folder and asset archive, there are also specific archives for certain modules. The Material Analysis archive is intended for Grains. The measurements of the AxioVision Multiphase, Graphite and/or Comparative Diagrams modules can also be stored in this archive.

In order to work with the Grains module, an archive must therefore exist and be opened in AxioVision.

Note:

- The grains analysis can **only** be performed in the Material Analysis archive. No other types of archive are suitable for this!

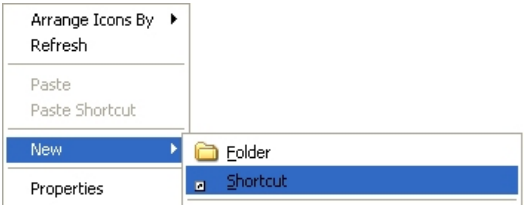


Archive View

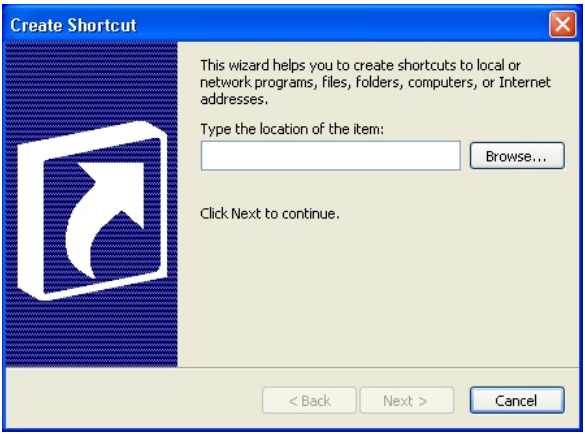
Opening the Material Analysis Archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

- Click the right mouse button on a free area on the desktop, and select **New** ⇒ **Shortcut**:



- In the **Create Shortcut** dialog that follows, select the Material Analysis archive (extension *.zva) by clicking on the **Browse...** button:



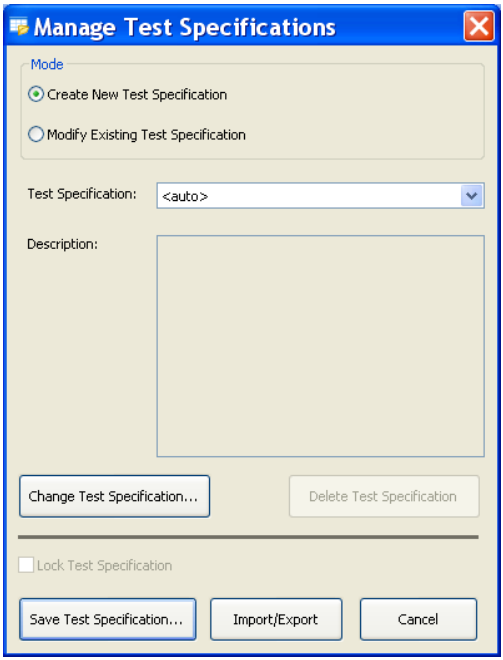
- Click on **Next** and, in the next step, enter a name for the shortcut.

- To create the shortcut, click on **Finish**.

Projects and test specifications

All settings and configuration data are saved in test specifications. These therefore contain all the information required for performing a grains analysis: from the configuration of the hardware and the origin of the images through to the definition of the report. All measurements performed at a later date are based on a test specification. When starting a measurement, the user is prompted to select the desired test specification. Only then can the measurement be started.

Via the **Manage Test Specifications** dialog, test specifications can be modified, created or deleted. It is also possible to import and export test specifications. This dialog is only available to users who are logged on to Microsoft Windows as principal users or administrators.



Importing and exporting test specifications

To make the preparation and setup for grains analysis as easy as possible, you can simply import existing test specifications for special applications and use them right away.

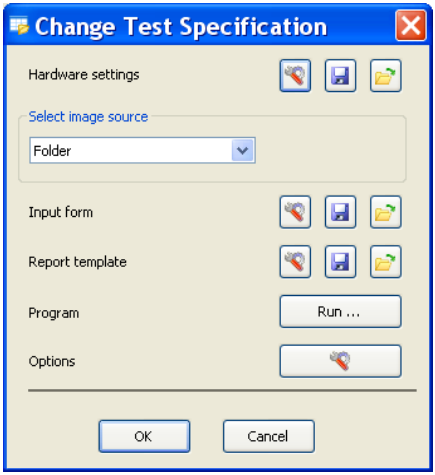
This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:



Modify Test Specifications

Test specifications are created via the **Change Test Specification** function. All the dialogs used to set up a grains analysis are called up via this dialog:



Dialog for adapting and changing a test specification

Each individual step can be modified. The relevant dialog can be opened using the button  .

Here again you can, for each single item required for creating a test specification, load  and save  settings already existing in another template.

Thus, you can directly copy hardware settings, settings of the image source, input forms, etc. from existing templates into the current test specification you are creating. Further, you can save your own settings to a new template in order to use them in further test specification.

The following chapters describe the procedure step by step.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a measurement. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Note:

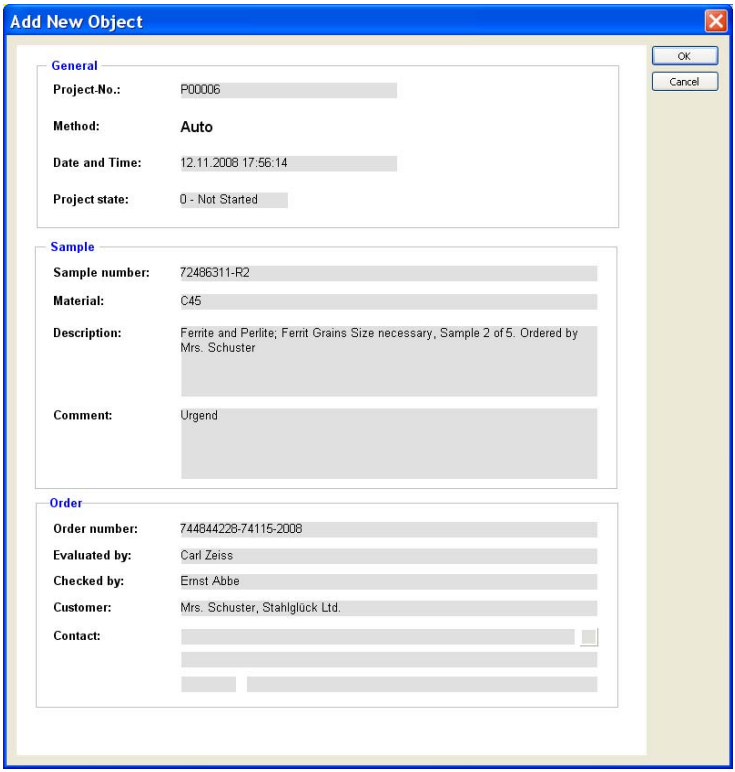
- For detailed information on hardware settings, see Chapter 4.5 "The Settings Editor".

Image source

The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select **Folder** or **Gallery** as the image source. In most cases, however, when an image is acquired and analyzed immediately, use the **Camera** setting as the image source.

Input forms

Input forms allow the user to enter the necessary project and sample data during the measurement. You can freely define or adapt the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.



Standard input form for project data

Note:

- For detailed information on editing form templates, see Chapter 7.4 "Templates for forms and reports".

Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:

- For detailed information, see Chapter 6.4 "Creating data reports".

Options – Auto

In the options, you specify the settings relating to the standard and the classification. It is possible to specify a lower limit for the classification. A more refined classification in half steps is also possible.

Options – Chord

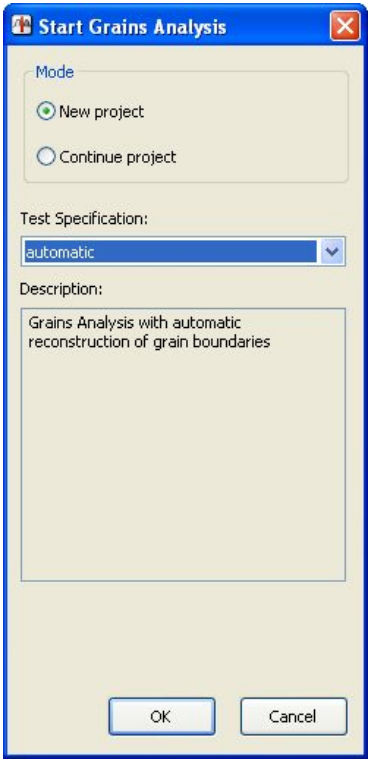
Here you mainly select the standard on which the measurement should be based. It is also possible to preselect the other parameters here. However, these can still be adjusted during the measurement procedure.

The measurement procedure

You can specify the measurement procedure by clicking on the **Run** button. This opens a wizard that guides you through the individual steps. All possible steps relating to image preparation, segmentation and classification are displayed here.

Performing measurements

A measurement is performed via the **Start Grains Analysis** function in the **Grains** workflow:



A live image is then shown which you can use to help check the hardware settings.

A grains analysis is always based on a previously defined test specification. This must have been created by a qualified user. No measurement can be started until a test specification has been created.

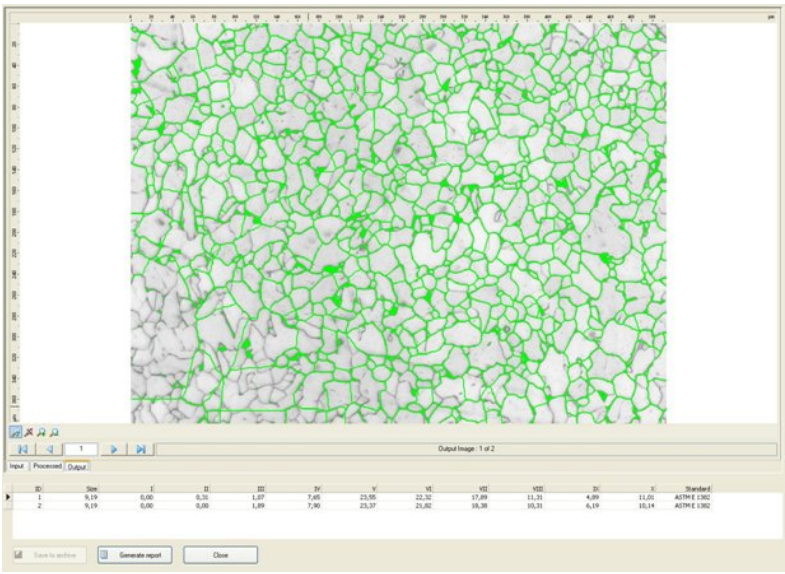
During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the evaluation script will be displayed. All other functions run automatically in the background, if the check box **Execute** is selected.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question. The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

The results document

At the end of the measurement the results window is displayed. Here, the results are displayed in the form of a table for all the images measured, as well as the image of the sample currently selected. Below the images are three tabs which you can use to switch between the input, output and processed image. If the image has been processed using image enhancement methods (e.g. sigma filter), you can therefore see the effect if you switch between the input and processed image.



Generating reports

The data are not saved directly in a report, but are only displayed there. This avoids data being saved twice. As a result, the data must first be saved in the archive. Then you can generate the report, which, immediately upon being generated, is also saved automatically in the archive. Provided you do not delete any of the tables or images from the archive, the data are also displayed in the report. In order to display the report on other PCs too, you can export the report into PDF or RTF format (**File** menu ⇒ **Save as PDF** or **Save as RTF**).

Creating and performing a grains analysis

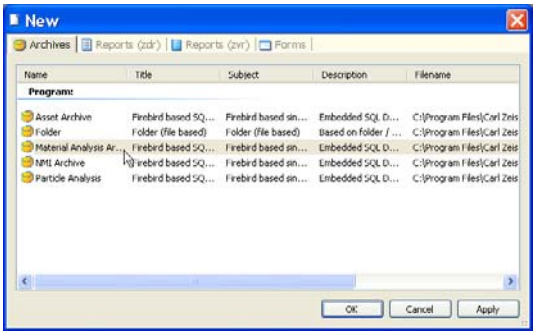
Now that the theoretical principles have been explained above, below you will find step-by-step instructions for creating and performing a grains analysis.

How do I create an archive?

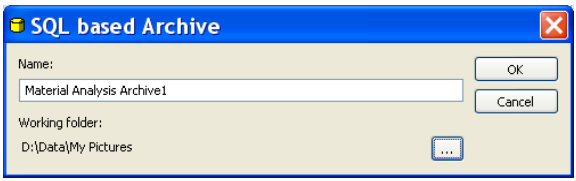
The Material Analysis Archive forms the basis for managing the data resulting from grains analysis. All the data relating to the performance of a measurement are managed here. In order to perform a measurement, it is necessary for an archive to be opened in AxioVision. The creation and opening of an archive are therefore shown first.

- Select the **New** function in the **File** menu.

- On the **Archives** tab sheet, select the **Material Analysis Archive** and then click **OK**.



The **SQL based Archive** dialog window will appear. Enter a name for the archive in the **Name** input field and confirm by clicking on **OK**.



Notes:

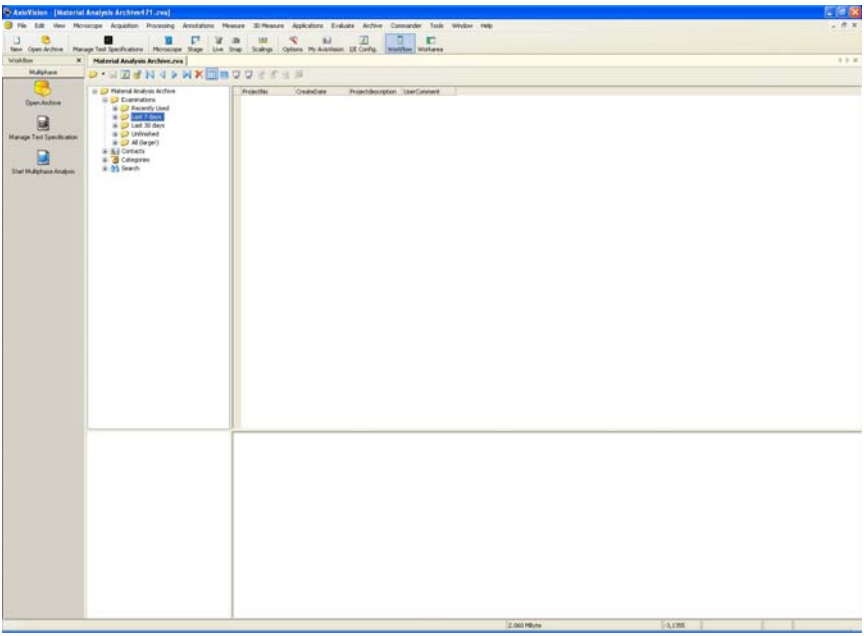
- An archive is created in the *My Documents\My Pictures* folder by default.

  If you wish to save the archive elsewhere, click on the ... button. Select the location where you wish the archive to be saved in the Microsoft Windows standard dialog that then opens.

- In the case of large data volumes in particular, we recommend that you save the archive to a local hard drive.

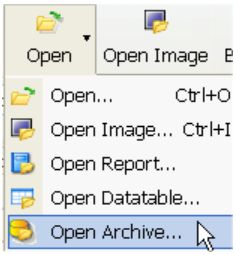
- Only one archive can be opened at a time in AxioVision.

The newly created archive is now displayed in AxioVision.



Opening an archive

➢ Click on the **Open** icon in the menu bar and then on the **Open Archive** function:

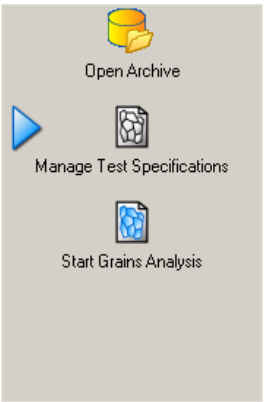


How do I create a test specification?

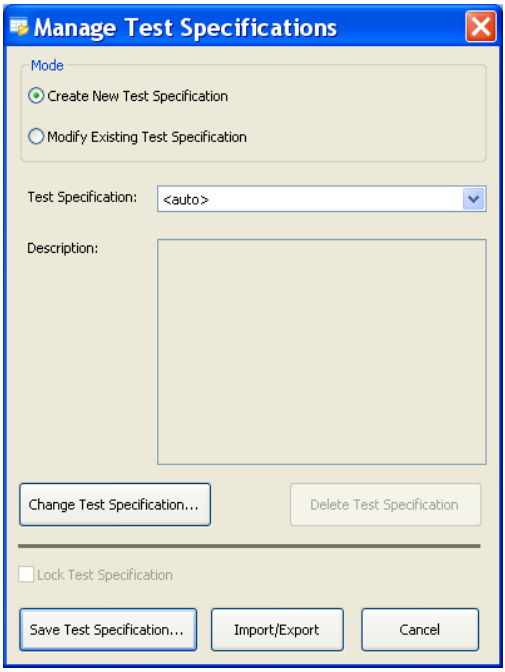
The test specification contains all the settings required for performing a measurement.

➢ From the workflows, select the **Grains** workflow.

➢ Click on the **Manage Test Specifications** function.



This opens the **Manage Test Specifications** dialog.

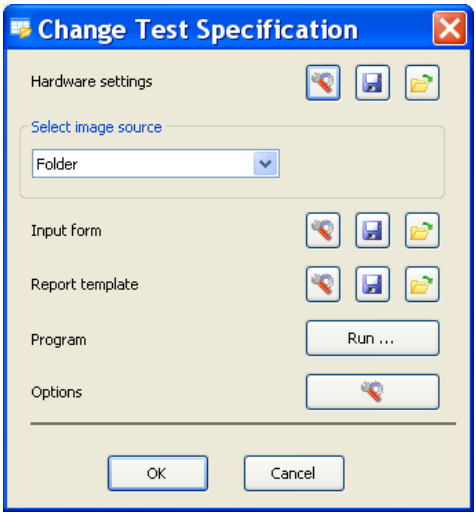


➢ Select the test specification that you wish to use as a basis for the new test specification. If you have not yet created a test specification, you can select one of the standard test specifications "Auto", "Chord" or "Compare", depending on which measurement method you require.

Note:

- In the **Create New Test Specification** mode, a copy is generated of the selected test specification, which you can then adapt. The original test specification that is used is not changed. If you only wish to make a modification to the test specification, change to the **Modify Existing Test Specification** mode. The standard templates of Grains cannot be changed, but only used as the basis for a new test specification.

➢ Click on the **Change Test Specification** button. This opens the **Change Test Specification** dialog.



➢ You can now save the hardware settings, select the image source, set options or edit the input form and report template. It is recommended, however, that you modify the report template after running the program, because measurement data are then available. The report editor is displayed automatically after running the program.

Notes:

- You will find more detailed information about the hardware settings in Chapter 4.5 "Settings Editor" of this manual.
- You will find more detailed information on modifying form templates in Chapter 7.4 " Templates for forms and reports" of this manual.
- You will find more detailed information on editing a report template in Chapter 6.4 " Creating Data Reports" of this manual.

- Once you have made all the necessary settings, click on the Run ... button. This opens a wizard that guides you through the program. Using the **Execute** option, you can determine whether or not the processing step is carried out. If you activate the **Interactive** option, this step will also be displayed during the measurement. The user can also change the parameters here.

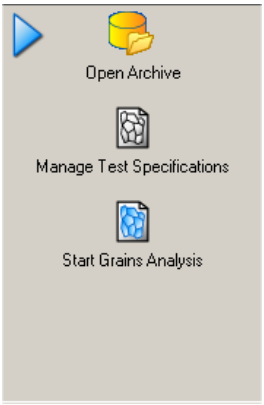
- When you have made all the necessary settings, leave the **Change Test Specifications** dialog by clicking on the OK button.

- Save the test specification by clicking on the Save button. This opens the **Save Test Specification** dialog. You can enter a name and a description of the test specification here. A description helps the user when starting the measurement, as he is unable to open the **Manage Test Specifications** area and does not, therefore, see the settings that have been made there.

How do I perform a measurement?

- From the workflows, select the **Grains** workflow.

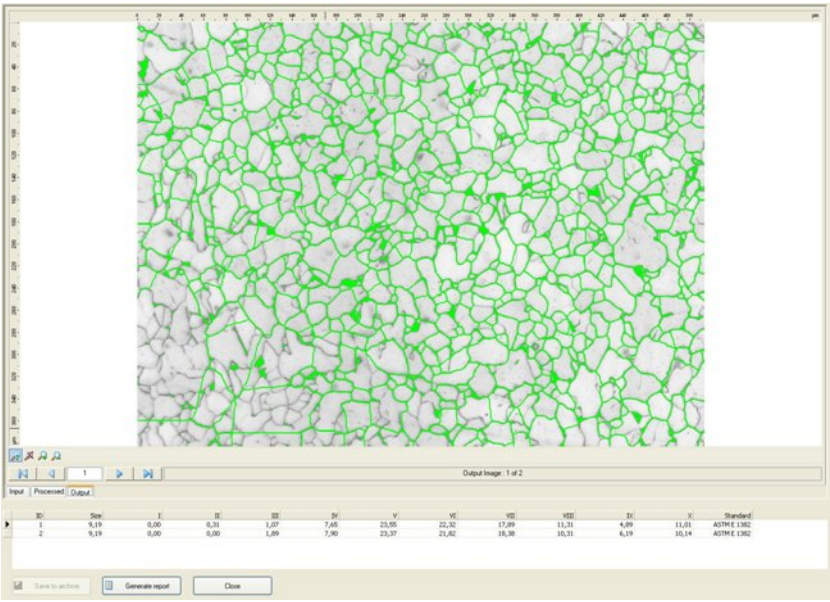
- First of all you will need an open archive. To open one, click on the **Open Archive** function.

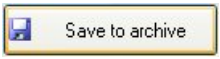


- This opens the standard dialog for opening a file in Microsoft Windows. Select a Material Analysis Archive.

- Then click on the **Start Grains Analysis** function in the workflow.

This opens a wizard that guides you through the measurement. At the end of the measurement, you will be shown the **Results View**:



- To generate a report, first click on the 

button.

- Then click on the 

button that is now active and the report will be generated and displayed. It will already have been saved in the archive.

11.7 Multiphase

In the Multiphase module, phases are determined based on both the size of the individual particles of a phase as well as the percentage of the area they represent. It is possible to determine up to 32 phases.

When classifying the particles of a phase, you can determine a measurement parameter and set the upper and lower limit for the classification. The range is then divided into ten classes. Both linear and logarithmic classification are available. The following parameters may be selected for the classification:

- Area
- Densitometric mean
- Diameter
- Feret Maximum
- Ellipse major
- Ellipse minor
- Perimeter convex
- Form circle
- Volume Ellipsoid
- Volume Sphere

In addition, the number of particles and the percentage of the area they represent in relation to the image are displayed for each phase.

All data are saved and managed in one place – the archive – which has search and filter functions. This ensures that the data can be relocated easily.

The operating concept of Multiphase is described below. This is followed by a practical section that provides step-by-step instructions in relation to the individual points.

Note:

- For detailed information on the individual functions and commands, please refer to the online help (F1 key).

The concept

The operating concept of Multiphase is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement. For this reason, the operation of Multiphase is essentially divided into two functional areas:

- administrator mode (definition of a measurement)
- user mode (performance of a measurement)

Administrator mode

A measurement is always based on a test specification, which is created in the administrator mode. All the key settings required for a multiphase analysis are saved here. This test specification may only be created or modified by principal users or administrators. Here, the administrator can also specify whether or not certain parameters may be changed by the user. A standard user has no access to the management of test specifications. Access to the corresponding functions is controlled by means of the access rights of the operating system.

User mode

In the user mode, measurements can be performed on the basis of a test specification. The administrator determines to what extent the user may change the measurement parameters.

Operation and configuration

Preparing the system

General

The functionality of the Multiphase module is based on the basic functionality of AxioVision. Before you can work with Multiphase, you must, therefore, configure the basic settings for AxioVision.

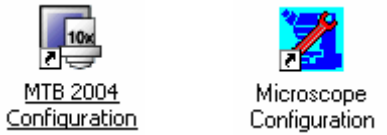
Note:

- You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

The microscope is controlled with the help of the separate **MTB 2004 Configuration** program. In order to use your microscope together with AxioVision, your individual system configuration must first be defined via this separate program. If you upgrade a pre-existing system for multiphase analysis, the configuration may take place via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:



Note:

- For detailed information, see Chapter 4 "Microscope Control".

Scalings

Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:

- For detailed information, see Chapter 8.4 "Scalings".

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:

- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the Multiphase workflow.

Notes:

- To show the main menu again, click the right mouse button on the title bar of the AxioVision image window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:

- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Multiphase and other AxioVision applications

One of AxioVision's particular strengths is its modular structure. Each module is added directly onto the basic system. This means that the system can also be used for simple documentation tasks or other applications such as particle analysis or grain size analysis. To ensure that AxioVision always starts with a particular configuration specific to the application (e.g. specific toolbars, workflows, etc.), any number of configurations can be started via individual icons on the Microsoft Windows desktop.

Note:

- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

The Material Analysis archive

Data are saved and managed in an archive. There, all data (images, measurement data tables, reports, etc.) are stored/saved by project.

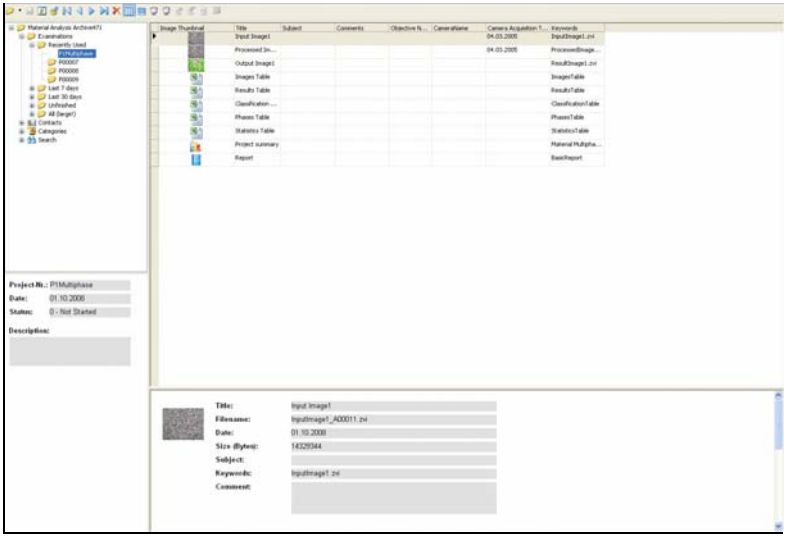
The test specifications are also saved in the archive together with the measurement projects. These are not visible in the archive window, and can only be modified using the **Manage Test Specifications** function.

There are several types of archive in AxioVision. Besides the folder and asset archive, there are also specific archives for certain modules. The Material Analysis archive is intended for Multiphase. The measurements of the AxioVision Grains, Graphite and/or Comparative Diagrams modules can also be stored in this archive.

In order to work with the Multiphase module, an archive must therefore exist and be opened in AxioVision.

Note:

- The multiphase analysis can **only** be performed in the Material Analysis archive. No other types of archive are suitable for this!

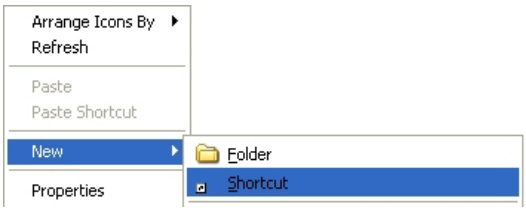


Archive View

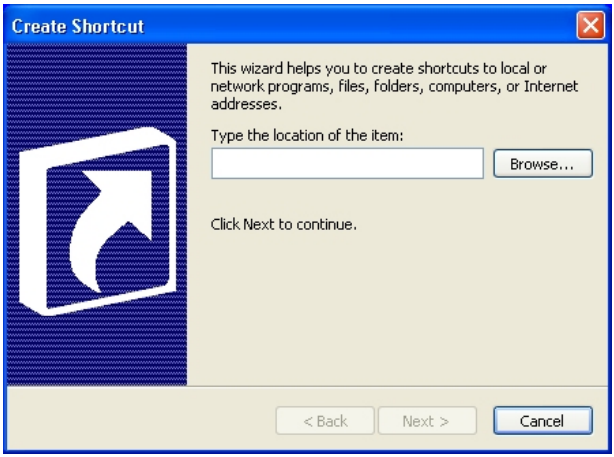
Opening the Material Analysis Archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

- Click the right mouse button on a free area on the desktop, and select **New** ⇒ **Shortcut**:



- In the **Create Shortcut** dialog that follows, select the Material Analysis archive (extension *.zva) by clicking on the **Browse...** button:



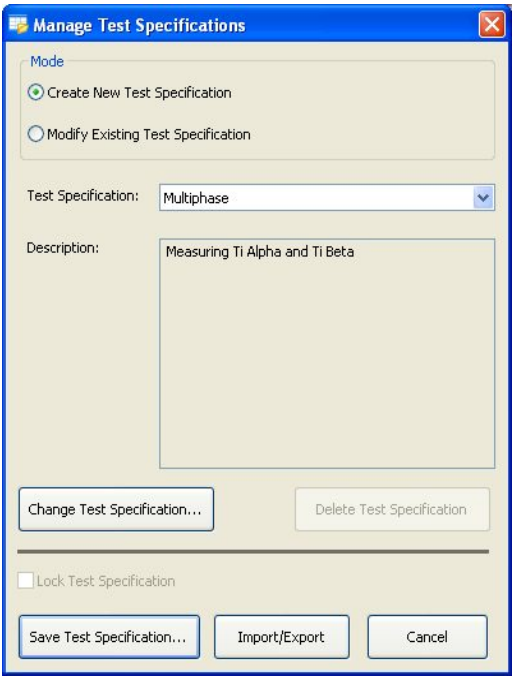
- Click on **Next** and, in the next step, enter a name for the shortcut.

- To create the shortcut, click on **Finish**.

Projects and test specifications

All settings and configuration data are saved in test specifications. These therefore contain all the information required for performing a multiphase analysis: from the configuration of the hardware and the origin of the images through to the definition of the report. All measurements performed at a later date are based on a test specification. When starting a measurement, the user is prompted to select the desired test specification. Only then can the measurement be started.

Via the **Manage Test Specifications** dialog, test specifications can be modified, created or deleted. It is also possible to import and export test specifications. This dialog is only available to users who are logged on to Microsoft Windows as principal users or administrators.

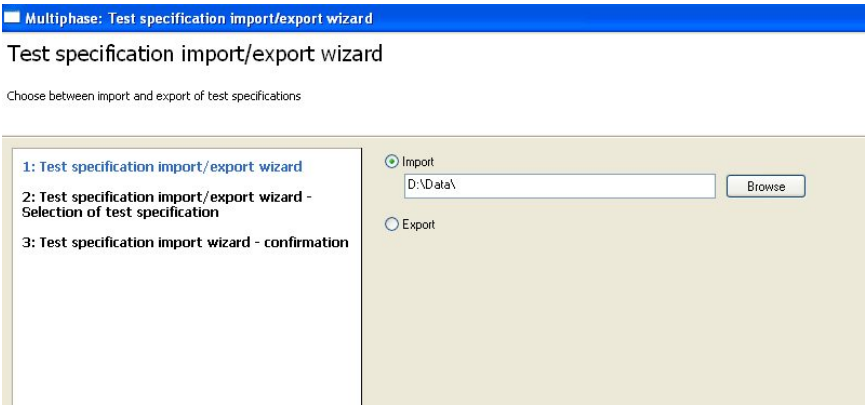


Importing and exporting test specifications

To make the preparation and setup for Multiphase Analysis as easy as possible, you can simply import existing test specifications for special applications and use them right away.

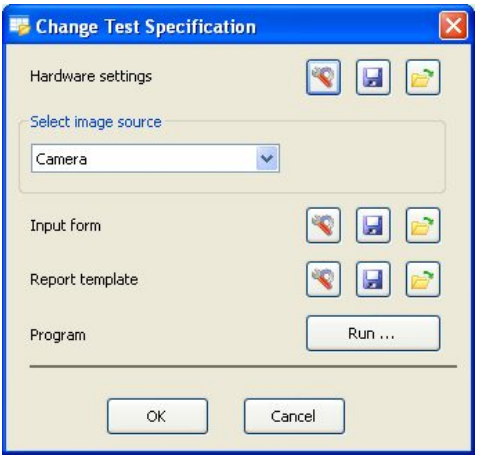
This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:



The Change Test Specification dialog

Test specifications are created via the **Change Test Specification** function. All the dialogs used to set up an Multiphase Analysis are called up via this dialog:



Dialog for adapting and changing a test specification

Each individual step can be modified. The relevant dialog can be opened using the button  .

Here again you can, for each single item required for creating a test specification, load  and save  settings already existing in another template.

Thus, you can directly copy hardware settings, settings of the image source, input forms, etc. from existing templates into the current test specification you are creating. Further, you can save your own settings to a new template in order to use them in further test specifications.

The following chapters describe the procedure step by step.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a measurement. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Note:

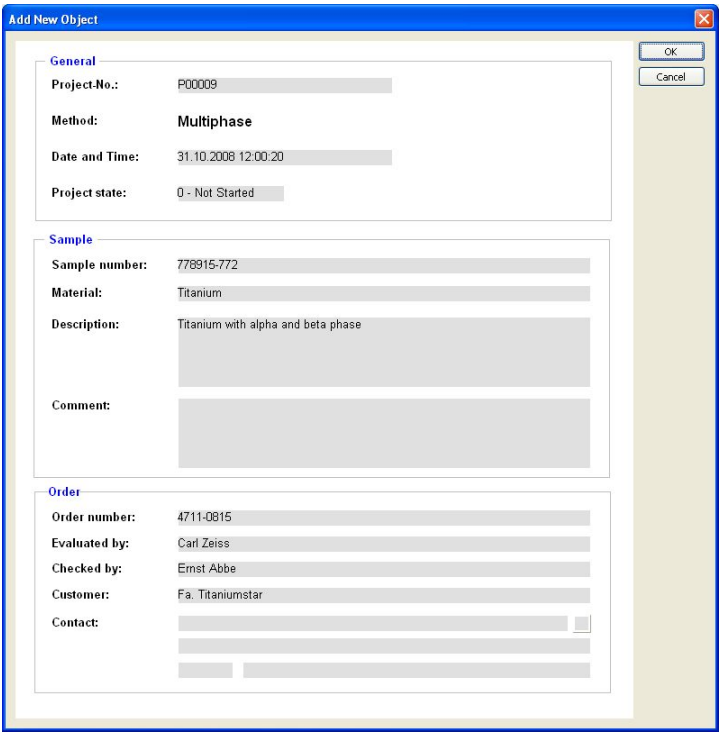
- For detailed information on hardware settings, see Chapter 4.5 "The Settings Editor".

Image source

The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select **Folder** or **Gallery** as the image source. In most cases, however, when an image is acquired and analyzed immediately, use the **Camera** setting as the image source.

Input forms

Input forms allow the user to enter the necessary project and sample data during the measurement. You can freely define or adapt the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.



Standard input form for project data

Note:

- For detailed information on editing form templates, see Chapter 7.4 "Templates for forms and reports".

Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:

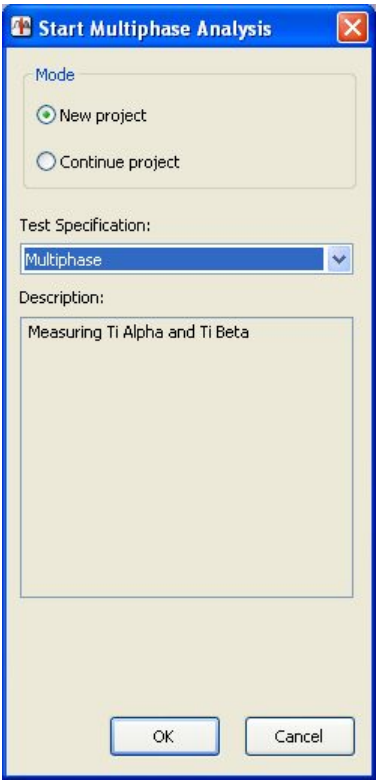
- For detailed information, see Chapter 6.4 "Creating data reports".

The measurement procedure

You can specify the measurement procedure by clicking on the **Run** button. This opens a wizard that guides you through the individual steps. All possible steps relating to image preparation, segmentation and classification are displayed here.

Performing measurements

A measurement is performed via the **Start Multiphase Analysis** function in the **Multiphase** workflow:



Starting dialog for multiphase analysis

A multiphase analysis is always based on a previously defined test specification. This must have been created by a qualified user. No measurement can be started until a test specification has been created.

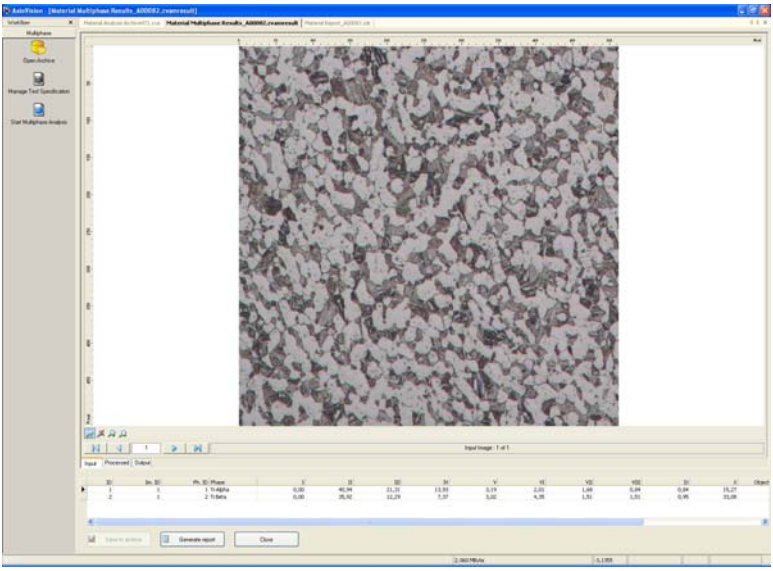
During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the evaluation script will be displayed. All other functions run automatically in the background, if the check box **Execute** is selected.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question. The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

The results document

At the end of the measurement the results window is displayed. Here, the results are displayed in the form of a table for all the images measured, as well as the image of the sample currently selected. Below the images are three tabs which you can use to switch between the input, output and processed image. If the image has been processed using image enhancement methods (e.g. Sigma filter), you can therefore see the effect if you switch between the input and processed image.



Generating reports

The data are not saved directly in a report, but are only displayed there. This avoids data being saved twice. As a result, the data must first be saved in the archive. Then you can generate the report, which, immediately upon being generated, is also saved automatically in the archive. Provided you do not delete any of the tables or images from the archive, the data are also displayed in the report. In order to display the report on other PCs too, you can export the report into PDF or RTF format (**File** menu ⇒ **Save as PDF** or **Save as RTF**).

Creating and performing a multiphase analysis

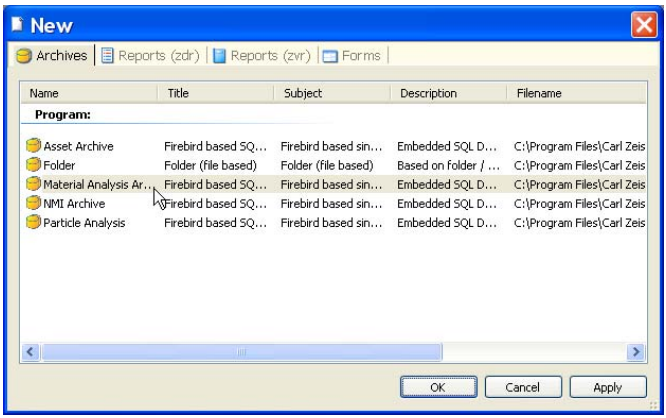
Now that the theoretical principles have been explained above, below you will find step-by-step instructions for creating and performing a multiphase analysis.

How do I create an archive?

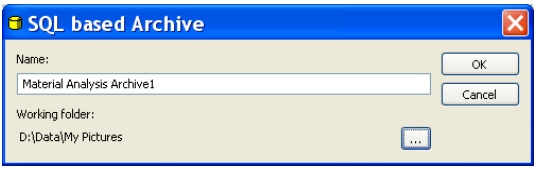
The Material Analysis Archive forms the basis for managing the data resulting from multiphase analysis. All the data relating to the performance of a measurement are managed here. In order to perform a measurement, it is necessary for an archive to be opened in AxioVision. The creation and opening of an archive are therefore shown first.

- Select the **New** function in the **File** menu.

- On the **Archives** tab sheet, select the **Material Analysis Archive** and then click **OK**.



The **SQL based Archive** dialog window will appear. Enter a name for the archive in the **Name** input field and confirm by clicking on **OK**.



Notes:

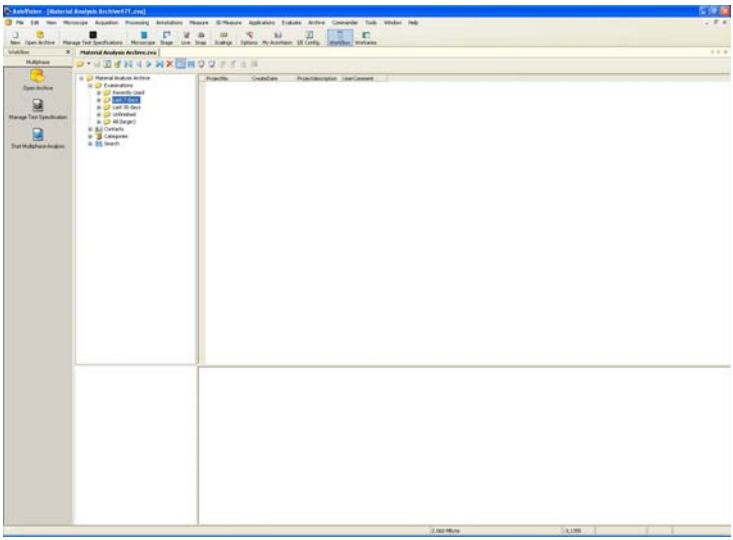
- An archive is created in the *My Documents**My Pictures* folder by default.

  If you wish to save the archive elsewhere, click on the ... button. Select the location where you wish the archive to be saved in the Microsoft Windows standard dialog that then opens.

- In the case of large data volumes in particular, we recommend that you save the archive to a local hard drive.

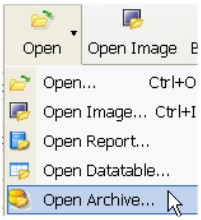
- Only one archive can be opened at a time in AxioVision.

The newly created archive is now displayed in AxioVision.



Opening an archive

➢ Click on the **Open** icon in the menu bar and then on the **Open Archive** function:

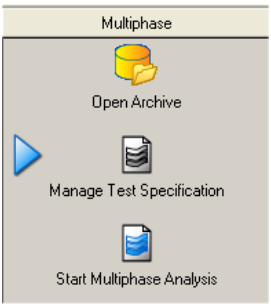


How do I create a test specification?

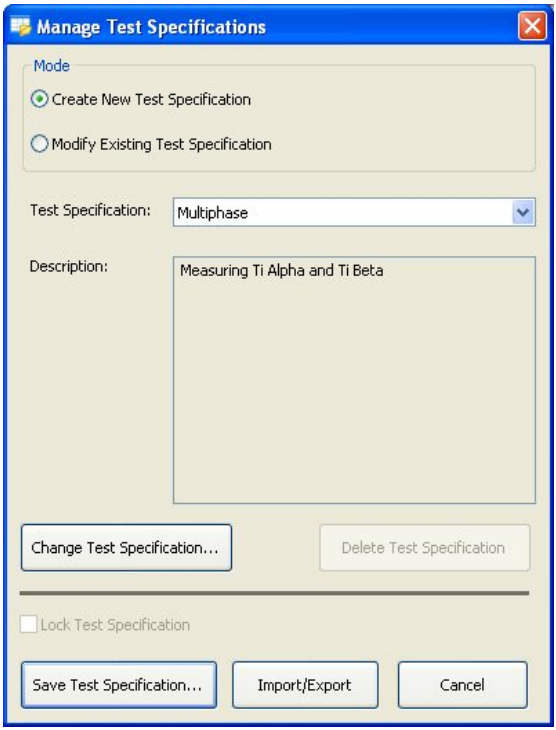
The test specification contains all the settings required for performing a measurement.

➢ From the workflows, select the **Multiphase** workflow.

➢ Click on the **Manage Test Specification** function.



➢ This opens the **Manage Test Specifications** dialog.



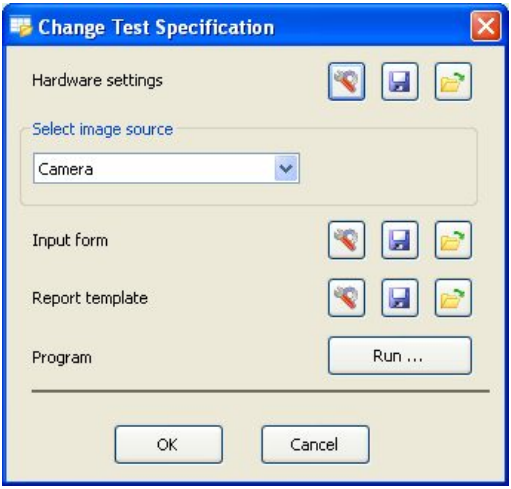
➢ Select the test specification that you wish to use as a basis for the new test specification. If you have not yet created a test specification, select the standard "Multiphase" test specification.

Note:

- In the **Create New Test Specification** mode, a copy is generated of the selected test specification, which you can then adapt. The original test specification that is used is not changed. If you only wish to make a modification to the test specification, change to the **Modify Existing Test Specification** mode.
- The standard Multiphase test specification cannot be changed, but only used as the basis for a new test specification.

➢ Click on the **Change Test Specification** button.

➢ This opens the **Change Test Specification** dialog.



➢ You can now save the hardware settings, select the image source or edit the input form and report template. It is recommended, however, that you modify the report template after **running** the programme, because measurement data are then available. The report editor is displayed automatically after **running** the programme.

Notes:

- You will find more detailed information about the hardware settings in Chapter 4.5 "Settings Editor" of this manual.

- You will find more detailed information on modifying form templates in Chapter 7.4 " Templates for forms and reports" of this manual.

- You will find more detailed information on editing a report template in Chapter 6.4 " Creating Data Reports" of this manual.

- Once you have made all the necessary settings, click on the Run ... button. This opens a wizard that guides you through the program. Using the **Execute** option, you can determine whether or not the processing step is carried out. If you activate the **Interactive** option, this step will also be displayed during the measurement. The user can also change the parameters here.

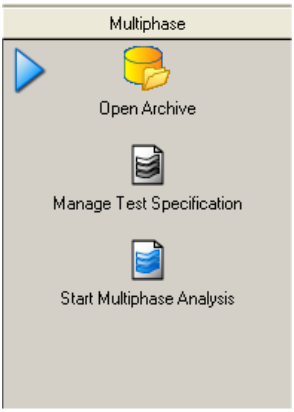
- When you have made all the necessary settings, leave the **Change Test Specifications** dialog by clicking on the OK button.

- Save the test specification by clicking on the Save button. This opens the **Save Test Specification** dialog. You can enter a name and a description of the test specification here. A description helps the user when starting the measurement, as he is unable to open the **Manage Test Specifications** area and does not, therefore, see the settings that have been made there.

How do I perform a measurement?

- From the workflows, select the **Multiphase** workflow.

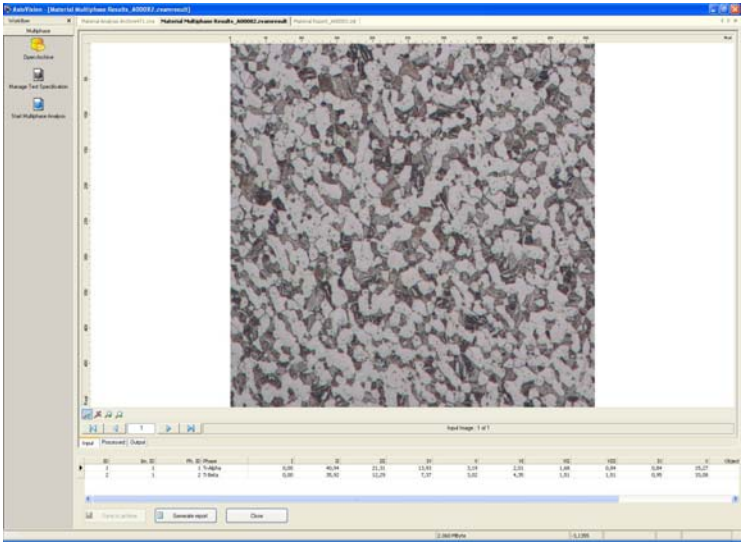
- First of all you will need an open archive. To open one, click on the **Open Archive** function.

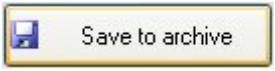


- This opens the standard dialog for opening a file in Microsoft Windows. Select a Material Analysis Archive.

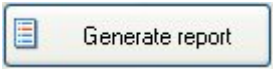
- Then click on the **Start Multiphase Analysis** function in the workflow.

- This opens a wizard that guides you through the measurement. At the end of the measurement, you will be shown the results view:



- To generate a report, first click on the 

button.

- Then click on the 

button that is now active and the report will be generated and displayed. It will already have been saved in the archive.

11.8 Comparative Diagrams

The Comparative Diagrams module allows the comparision of a micrograph with comparative diagrams interactively.

The result is a table containing the comparative diagram numbers relating to each image and a table containing statistical analyses. You can also have a micrograph displayed with an overlaid comparative diagram.

The following standards are available:

- ASTM E 112
- ASTM E 1382
- BS 4990
- DIN 50602
- EN 10247
- EN ISO 643
- SEP 151061

All data are saved and managed in one place – the archive – which has search and filter functions. This ensures that the data can be relocated easily.

The operating concept of Multiphase is described below. This is followed by a practical section that provides step-by-step instructions in relation to the individual points.

Notes:

- The license for the comparative diagrams taken from the standards does not form part of the AxioVision Comparative Diagrams module. Before you can install these, you must confirm that you have purchased the standard. For this reason, each standard must also be installed separately.

- For detailed information on the individual functions and commands, please refer to the online help (F1 key).

The concept

To set up a comparative-diagram comparison, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement. For this reason, the operation of Comparative Diagrams is essentially divided into two functional areas:

- Administrator mode (definition of a measurement)
- User mode (performance of a measurement)

Administrator mode

A measurement is always based on a test specification, which is created in the administrator mode. All the key settings required for comparative-diagram comparisons are saved here. This test specification may only be created or modified by principal users or administrators.

A standard user has no access to the management of test specifications. Access to the corresponding functions is controlled by means of the access rights of the operating system.

User mode

In the user mode, measurements can be performed on the basis of a test specification. The administrator determines to what extent the user may change the measurement parameters.

Operation and configuration

Preparing the system

General

The functionality of the Comparative Diagrams module is based on the basic functionality of AxioVision. Before you can work with Comparative Diagrams, you must, therefore, configure the basic settings for AxioVision.

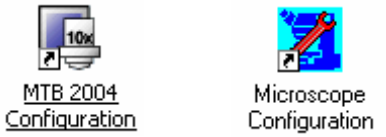
Note:

- You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

The microscope is controlled with the help of the separate **MTB 2004 Configuration** program. In order to use your microscope together with AxioVision, your individual system configuration must first be defined via this separate program. If you upgrade a pre-existing system for comparative-diagram comparisons, the configuration may take place via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:



Note:

- For detailed information, see Chapter 4 "Microscope Control".

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:
- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommended that you hide all control elements that are not required, as far as possible. For a comparative-diagram comparison, you will usually only need the Comparative Diagrams workflow.

- To show the main menu again, click the right mouse button on the title bar of the AxioVision image window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:
- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Comparative Diagrams and other AxioVision applications

One of AxioVision's particular strengths is its modular structure. Each module is added directly onto the basic system. This means that the system can also be used for simple documentation tasks or other applications such as particle analysis or grain size analysis. To ensure that AxioVision always starts with a particular configuration specific to the application (e.g. specific toolbars, workflows, etc.), any number of configurations can be started via individual icons on the Microsoft Windows desktop.

Note:

- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

The Material Analysis archive

Data are saved and managed in an archive. There, all data (images, measurement data tables, reports, etc.) are saved by project.

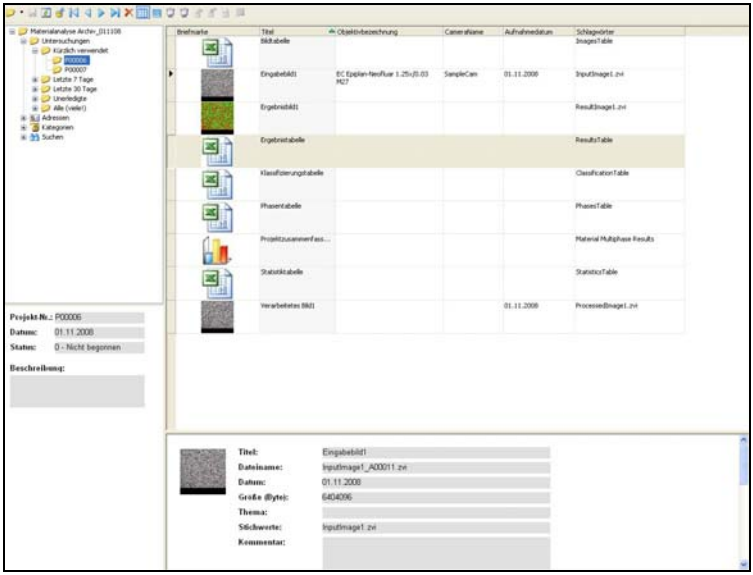
The test specifications are also saved in the archive together with the measurement projects. These are not visible in the archive window, and can only be modified using the **Manage Test Specifications** function.

There are several types of archive in AxioVision. Besides the folder and asset archive, there are also specific archives for certain modules. The Material Analysis archive is intended for Comparative Diagrams. The measurements of the AxioVision Grains, Graphite and/or Multiphase modules can also be stored in this archive.

In order to work with the Comparative Diagrams module, an archive must therefore exist and be opened in AxioVision.

Note:

- A comparative-diagram comparison can **only** be performed in the Material Analysis archive. No other types of archive are suitable for this!

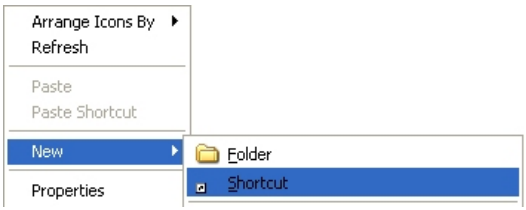


Archive View

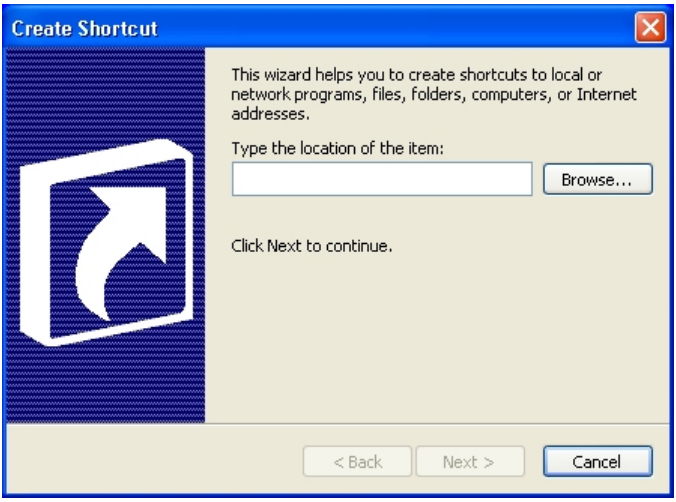
Opening the Material Analysis Archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

➢ Click the right mouse button on a free area on the desktop, and select **New** ⇒ **Shortcut**:



- In the **Create Shortcut** dialog that follows, select the Material Analysis archive (extension *.zva) by clicking on the **Browse...** button:



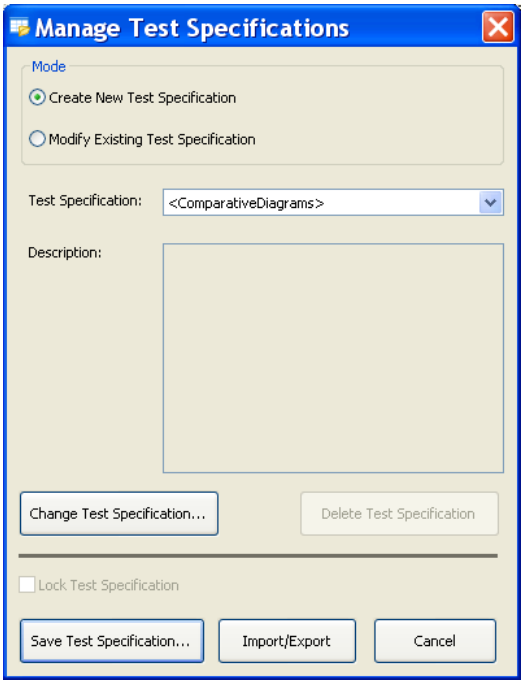
- Click on **Next** and, in the next step, enter a name for the shortcut.

- To create the shortcut, click on **Finish**.

Projects and test specifications

All settings and configuration data are saved in test specifications. These therefore contain all the information required for performing a comparative-diagram comparison: from the configuration of the hardware and the origin of the images through to the definition of the report. All measurements performed at a later date are based on a test specification. When starting a measurement, the user is prompted to select the desired test specification. Only then can the measurement be started.

Via the **Manage Test Specifications** dialog, test specifications can be modified, created or deleted. It is also possible to import and export test specifications. This dialog is only available to users who are logged on to Microsoft Windows as principal users or administrators.



Importing and exporting test specifications

To make the preparation and setup for Comparative Diagrams as easy as possible, you can simply import existing test specifications for special applications and use them right away.

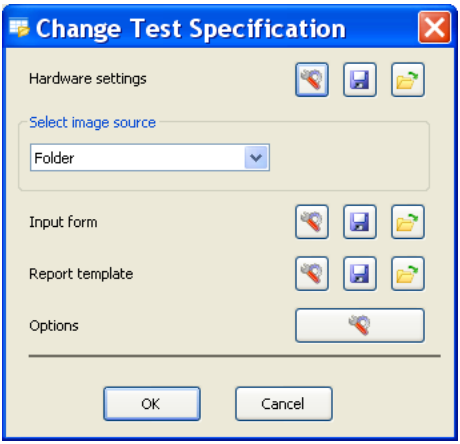
This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:



The Change Test Specification dialog

Test specifications are created via the **Change Test Specification** function. All the dialogs used to set up Comparative Diagrams are called up via this dialog:



Dialog for adapting and changing a test specification

Each individual step can be modified entirely. The relevant dialog can be opened using this  button.

Here again you can, for each single item required for creating a test specification, load  and save  settings already existing in another template.

Thus, you can directly copy hardware settings, settings of the image source, input forms, etc. from existing templates into the current test specification you are creating. Further, you can save your own settings to a new template in order to use them in further test specification.

The following chapters describe the procedure step by step.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a measurement. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Note:

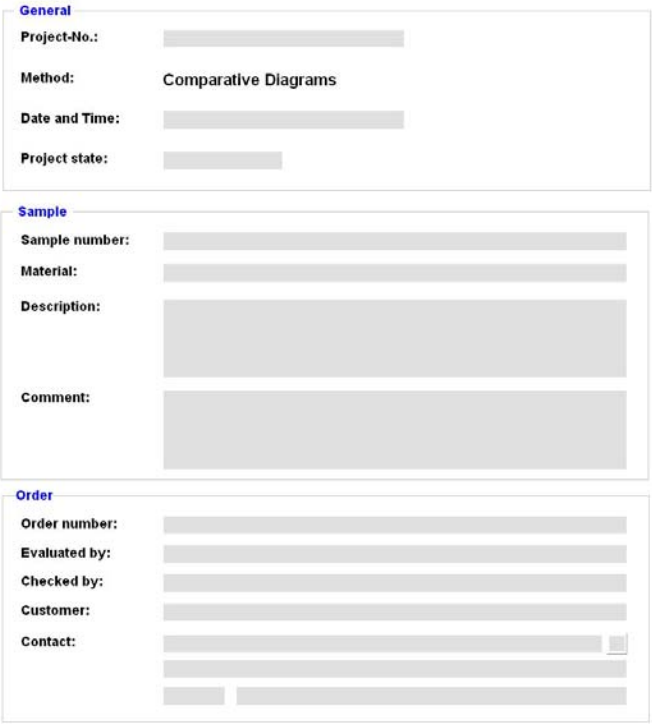
- For detailed information on hardware settings, see Chapter 4.5 "The Settings Editor".

Image source

The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select **Folder** or **Gallery** as the image source. In most cases, however, when an image is acquired and analyzed immediately, use the **Camera** setting as the image source.

Input forms

Input forms allow the user to enter the necessary project and sample data during the measurement. You can freely define or adapt the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.



Standard input form for project data

Note:

- For detailed information on editing form templates, see Chapter 7.3 "Templates for forms and reports".

Modifying report templates

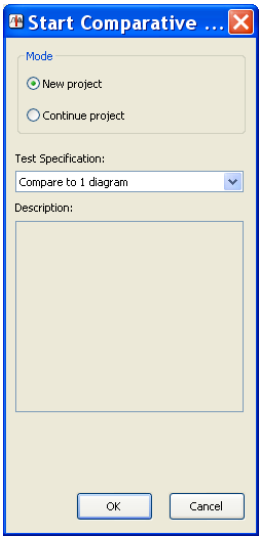
In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:

- For detailed information, see Chapter 6.4 "Creating data reports".

Performing a comparative-diagram comparison

The comparative-diagram comparison is performed via the **Start Comparative Diagrams** function in the **Comparative Diagrams** workflow.



Starting dialog for Comparative Diagrams

A comparative-diagram comparison is always based on a previously defined test specification. This must have been created by a qualified user. No comparison can be started until a test specification has been created.

Generating reports

The data are not saved directly in a report, but are only displayed there. This avoids data being saved twice. As a result, the data must first be saved in the archive. Then you can generate the report, which, immediately upon being generated, is also saved automatically in the archive. Provided you do not delete any of the tables or images from the archive, the data are also displayed in the report. In order to display the report on other PCs too, you can export the report into PDF or RTF format (**Datei** menu -> **Speichern als PDF** or **Speichern als RTF**).

Creating and performing a comparative-diagram comparison

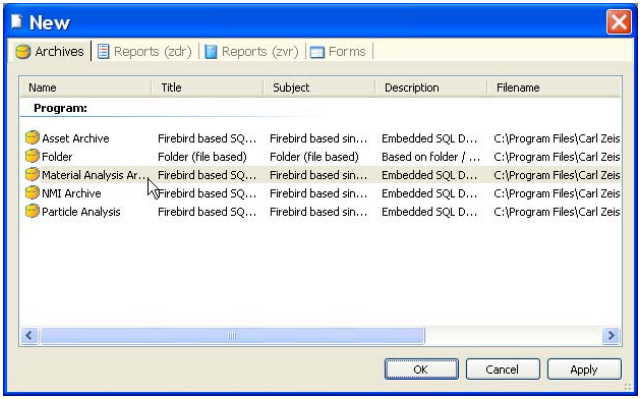
Now that the theoretical principles have been explained above, below you will find step-by-step instructions for creating and performing a comparative-diagram comparison.

How do I create an archive?

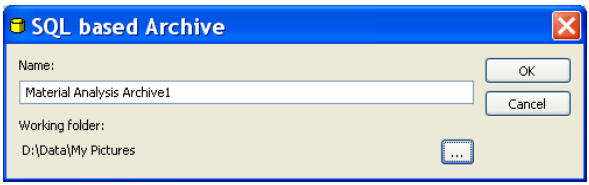
The Material Analysis Archive forms the basis for managing the data resulting from the Comparative Diagrams module. All the data relating to the performance of a measurement are managed here. In order to perform a measurement, it is necessary for an archive to be opened in AxioVision. The creation and opening of an archive are therefore shown first.

- Select the **Neu** function in the **Datei** menu.

➢ On the **Archives** tab sheet, select the **Material Analysis Archive** and then click **OK**.



➢ The **SQL based Archive** dialog window will appear. Enter a name for the archive in the **Name** input field and confirm by clicking on **OK**.



Notes:

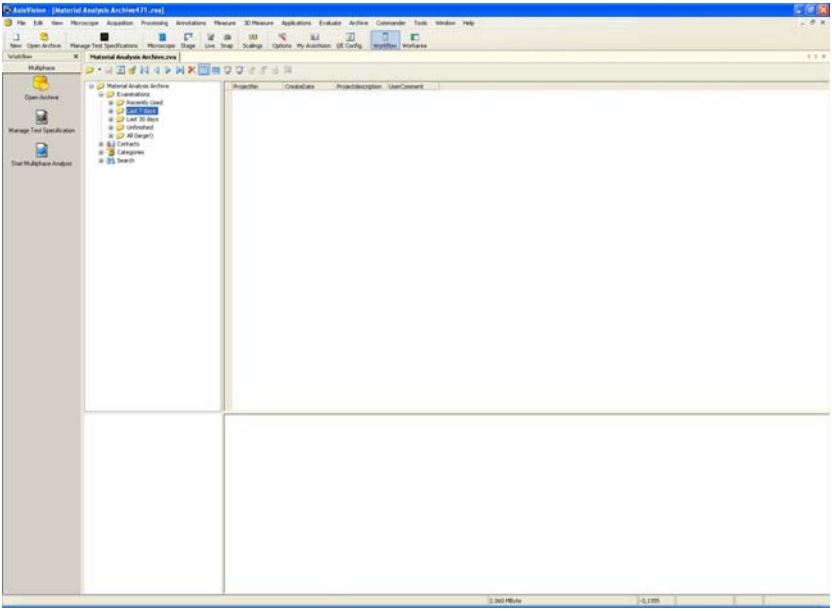
- An archive is created in the *My Documents\My Pictures* folder by default.

  If you wish to save the archive elsewhere, click on the ... button. Select the location where you wish the archive to be saved in the Microsoft Windows standard dialog that then opens.

- In the case of large data volumes in particular, we recommend that you save the archive to a local hard drive.

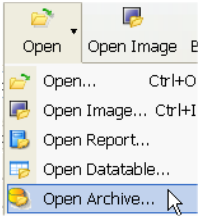
- Only one archive can be opened at a time in AxioVision.

The newly created archive is now displayed in AxioVision.



Opening an archive

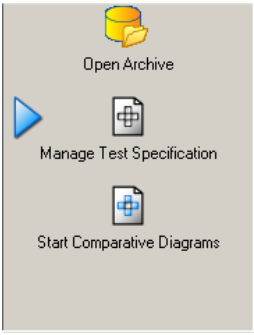
➢ Click on the **Open** icon in the menu bar and then on the **Open Archive** function:



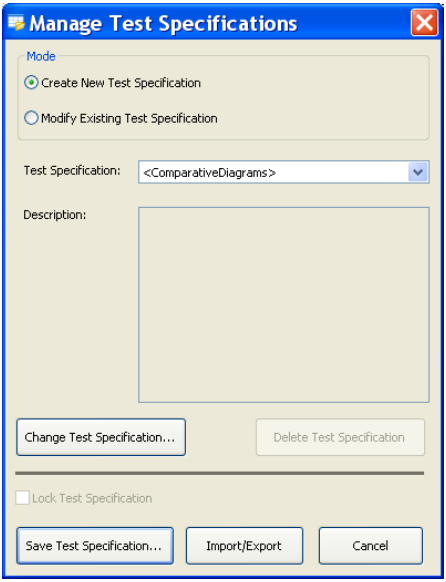
How do I create a test specification?

The test specification contains all the settings required for performing a measurement.

- From the workflows, select the **Comparative Diagrams** workflow.
- Click on the **Manage Test Specification** function.



- This opens the **Manage Test Specifications** dialog.



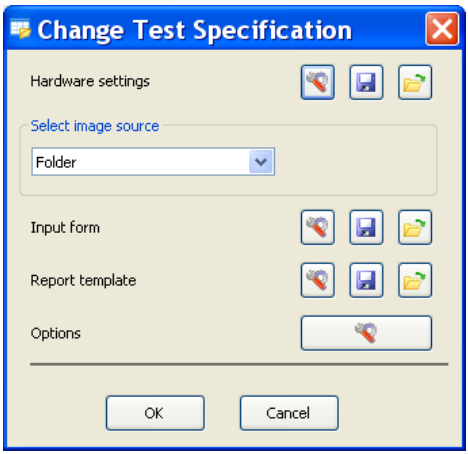
➢ Select the test specification that you wish to use as a basis for the new test specification. If you have not yet created a test specification, select the standard "Comparative Diagrams" test specification.

Note:

- In the **Create New Test Specification** mode, a copy is generated of the selected test specification, which you can then adapt. The original test specification that is used is not changed. If you only wish to make a modification to the test specification, change to the **Modify Existing Test Specification** mode. The standard "Comparative Diagrams" test specification cannot be changed, but only used as the basis for a new test specification.

➢ Click on the **Change Test Specification** button.

➢ This opens the **Change Test Specification** dialog.



➢ You can now save the hardware settings, select the image source or edit the input form and report template. It is recommended, however, that you modify the report template after **running the program**, because measurement data are then available. The report editor is displayed automatically after **running the program**.

Notes:

- You will find more detailed information about the hardware settings in Chapter 4.5 "Settings Editor" of this manual.
- You will find more detailed information on modifying form templates in Chapter 7.4 " Templates for forms and reports" of this manual.
- You will find more detailed information on editing a report template in Chapter 6.4 " Creating Data Reports" of this manual.

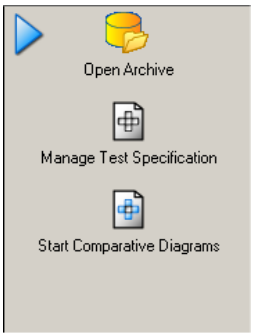
➢ When you have made all the necessary settings, leave the **Change Test Specification** dialog by clicking on the OK button.

➢ Save the test specification by clicking on the Save button. This opens the **Save Test Specification** dialog. You can enter a name and a description of the test specification here. A description helps the user when starting the measurement, as he is unable to open the **Manage Test Specifications** area and does not, therefore, see the settings that have been made there.

How do I perform a comparative-diagram comparison?

➢ From the workflows, select the **Comparative Diagrams** workflow.

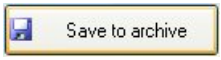
➢ First of all you will need an open archive. To open one, click on the **Open Archive** function.



- This opens the standard dialog for opening a file in Microsoft Windows. Select a Material Analysis Archive.

- Then click on the **Start Comparative Diagrams** function in the workflow.

- Once you have allocated a comparative diagram to every image, click on the **Close** and **Generate Report** button.

- To generate a report, first click on the 

button.

- Then click on the 

button that is now active and the report will be generated and displayed. It will already have been saved in the archive.

Note:

- For detailed information on the individual functions and commands, please refer to the online help (F1 key).

11.9 Graphite

In the Graphite module, the size of graphite particles in cast iron is determined in accordance with current international standards. Various methods are available for analyzing the graphite in cast iron:

Shape and Size: for determining the shape and size distribution in accordance with
EN ISO 945-1.

SinterCast: for determining nodularity – analysis of vermicular and spheroidal graphite in accordance with SAE J 1887.

Spherolite: for determining the size of spheroidal graphite in accordance with EN ISO 945:1994 (graphite size).

Lamellar: for determining the size of lamellar graphite in accordance with EN ISO 945:1994 (graphite size).

The Shape and Size method

The spheroidal graphite particles are detected by means of a gray-value and shape-based segmentation. Then the particles are classified on the basis of their shape and size. In addition, the spheroid number is also calculated. This classification is based on the EN ISO 945-1 standard.

The SinterCast method

The analysis performed with the SinterCast method follows the SAE J 1887 standard. This determines the "nodularity", i.e. the proportion of spheroidal graphite to vermicular graphite.

The Spherolyte method

The spheroidal graphite particles are detected by means of a gray-value and form-based segmentation. The particles are then classified by size (i.e. diameter). This classification is based on the EN ISO 945:1994 standard.

The Lamellar method

Using this measurement method, the size of individual lamellar graphite particles is measured in each image and the distribution of these particles is shown. In this case too the particles are detected by means of a gray-value and form-based segmentation. The classification follows the requirements of EN ISO 945:1994, which means that the length of the lamellar graphite particles is taken as a basis for the size.

The Concept

The operating concept of Graphite is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement. For this reason, the operation of Graphite is essentially divided into two functional areas:

- Administrator mode (definition of a measurement)
- User mode (performance of a measurement)

Administrator mode

A measurement is always based on a test specification, which is created in the administrator mode. All the key settings required for a graphite analysis are saved here. This test specification may only be created or modified by principal users or administrators. Here, the administrator can also specify whether or not certain parameters may be changed by the user. A standard user has no access to the management of test specifications. Access to the corresponding functions is controlled by means of the access rights of the operating system.

User mode

In the user mode, measurements can be performed on the basis of a test specification. The administrator determines to what extent the user may change the measurement parameters.

Operation and configuration

Preparing the system

General

The functionality of the Graphite module is based on the basic functionality of AxioVision. Before you can work with Graphite, you must, therefore, configure the basic settings for AxioVision.

Note:

- You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

The microscope is controlled with the help of the separate **MTB 2004 Configuration** program. In order to use your microscope together with AxioVision, your individual system configuration must first be defined via this separate program. If you upgrade a pre-existing system for graphite analysis, the configuration may take place via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:





Note:
- For detailed information, see Chapter 4 "Microscope Control".

Scalings

Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:
- For detailed information, see Chapter 8.4 "Scalings".

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:
- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily. It is recommendable to hide all controls not used for the purpose. For routine measurements, you usually need only the Graphite workflow.

Notes:
- To show the main menu again, click the right mouse button on the title bar of the AxioVision image window, or on a toolbar. This opens a context menu in which you can display everything again.
- For detailed information, see Chapter 8.5 "Adapting the user interface".

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:

- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Graphite and other AxioVision applications

One of AxioVision's particular strengths is its modular structure. Each module is added directly onto the basic system. This means that the system can also be used for simple documentation tasks or other applications such as particle analysis or multiphase analysis. To ensure that AxioVision always starts with a particular configuration specific to the application (e.g. specific toolbars, workflows, etc.), any number of configurations can be started via individual icons on the Microsoft Windows desktop.

Note:

- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

The Material Analysis archive

Data are saved and managed in an archive. There, all data (images, measurement data tables, reports, etc.) are saved by project.

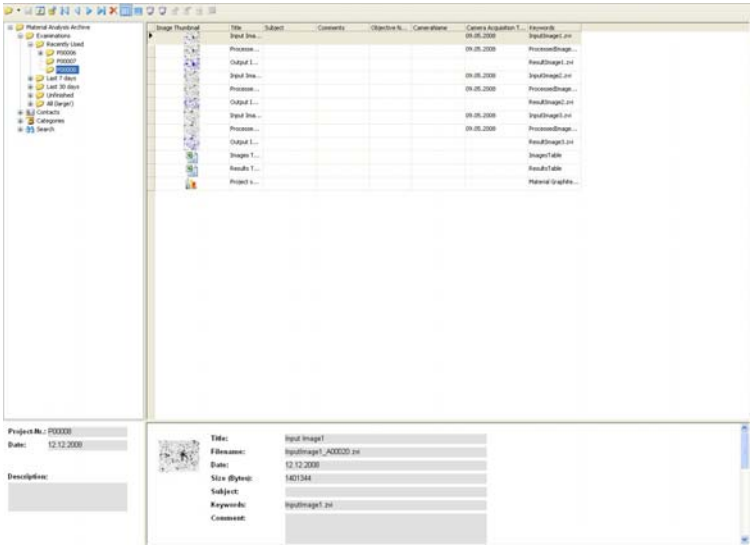
The test specifications are also saved in the archive together with the measurement projects. These are not visible in the archive window, and can only be modified using the **Manage Test Specifications** function.

There are several types of archive in AxioVision. Besides the folder and asset archive, there are also specific archives for certain modules. The Material Analysis archive is intended for Graphite. The measurements of the AxioVision Multiphase, Grains and/or Comparative Diagrams modules can also be stored in this archive.

In order to work with the Graphite module, an archive must therefore exist and be opened in AxioVision.

Note:

- The graphite analysis can **only** be performed in the Material Analysis archive. No other types of archive are suitable for this!

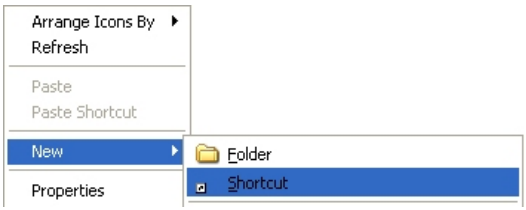


Archive View

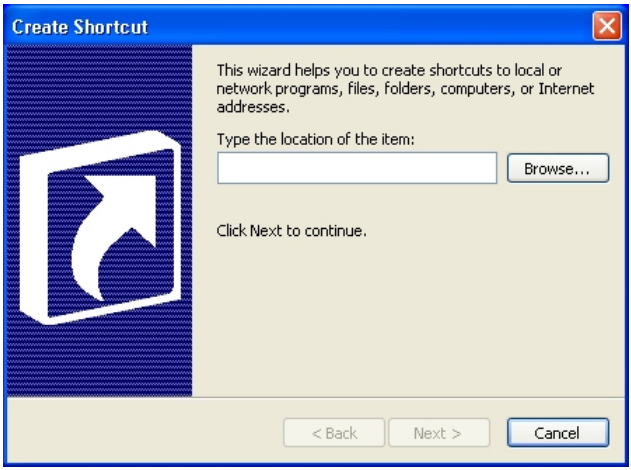
Opening the Material Analysis Archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

- Click the right mouse button on a free area on the desktop, and select **New** ⇒ **Shortcut**:



- In the **Create Shortcut** dialog that follows, select the Material Analysis archive (extension *.zva) by clicking on the **Browse...** button:



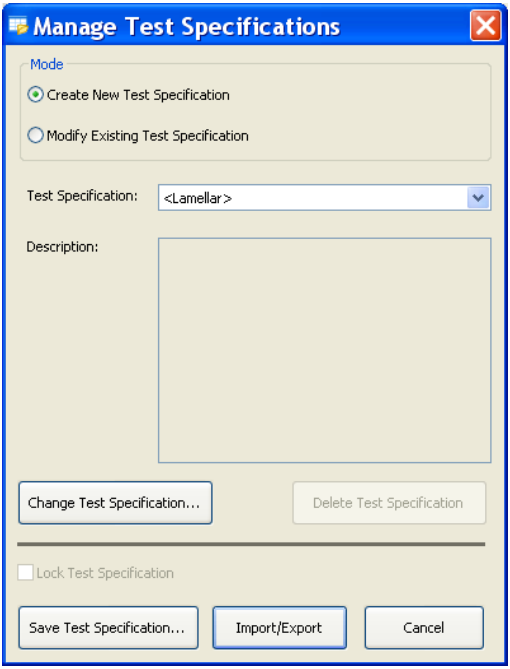
- Click on **Next** and, in the next step, enter a name for the shortcut.

- To create the shortcut, click on **Finish**.

Projects and test specifications

All settings and configuration data are saved in test specifications. These therefore contain all the information required for performing a graphite analysis: from the configuration of the hardware and the origin of the images through to the definition of the report. All measurements performed at a later date are based on a test specification. When starting a measurement, the user is prompted to select the desired test specification. Only then can the measurement be started.

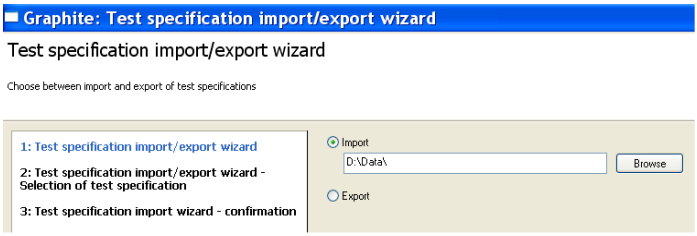
Via the **Manage Test Specifications** dialog, test specifications can be modified, created or deleted. It is also possible to import and export test specifications. This dialog is only available to users who are logged on to Microsoft Windows as principal users or administrators.



Importing and exporting test specifications

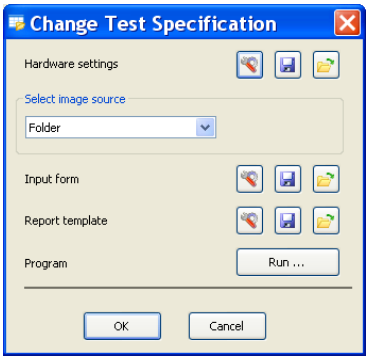
To make the preparation and setup for graphite analysis as easy as possible, you can simply import existing test specifications for special applications and use them right away. This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:



Modify Test Specifications

Test specifications are created via the **Change Test Specification** function. All the dialogs used to set up a graphite analysis are called up via this dialog:



Dialog for adapting and changing a test specification

Each individual step can be modified. The relevant dialog can be opened using the button  .
Here again you can, for each single item required for creating a test specification, load  and save  settings already existing in another template.

Thus, you can directly copy hardware settings, settings of the image source, input forms, etc. from existing templates into the current test specification you are creating. Further, you can save your own settings to a new template in order to use them in further test specification.

The following chapters describe the procedure step by step.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a measurement. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Note:

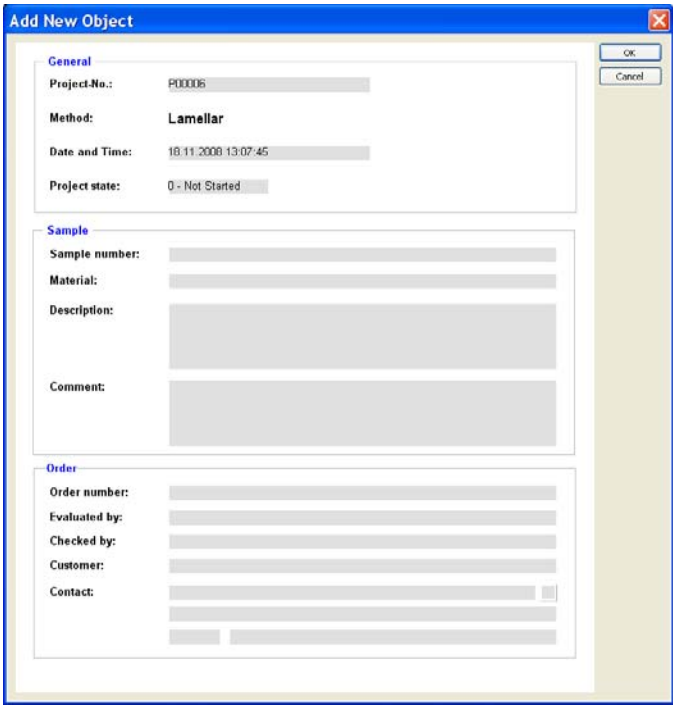
- For detailed information on hardware settings, see Chapter 4.5 "The Settings Editor".

Image source

The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select **Folder** or **Gallery** as the image source. In most cases, however, when an image is acquired and analyzed immediately, use the **Camera** setting as the image source.

Input forms

Input forms allow the user to enter the necessary project and sample data during the measurement. You can freely define or adapt the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.



Standard input form for project data

Note:

- For detailed information on editing form templates, see Chapter 7.4 "Templates for forms and reports".

Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report individually. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:

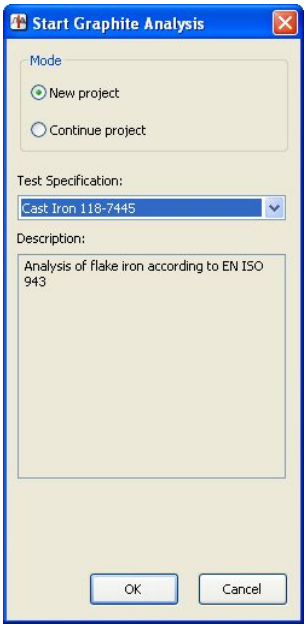
- For detailed information, see Chapter 6.4 "Creating data reports".

The measurement procedure

You can specify the measurement procedure by clicking on the **Run** button. This opens a wizard that guides you through the individual steps. All possible steps relating to image preparation, segmentation and classification are displayed here.

Performing measurements

A measurement is performed via the **Start Graphite Analysis** function in the **Graphite** workflow:



Starting dialog for graphite analysis

A graphite analysis is always based on a previously defined test specification. This must have been created by a qualified user. No measurement can be started until a test specification has been created.

During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the evaluation script will be displayed. All other functions run automatically in the background, if the check box **Execute** is selected.

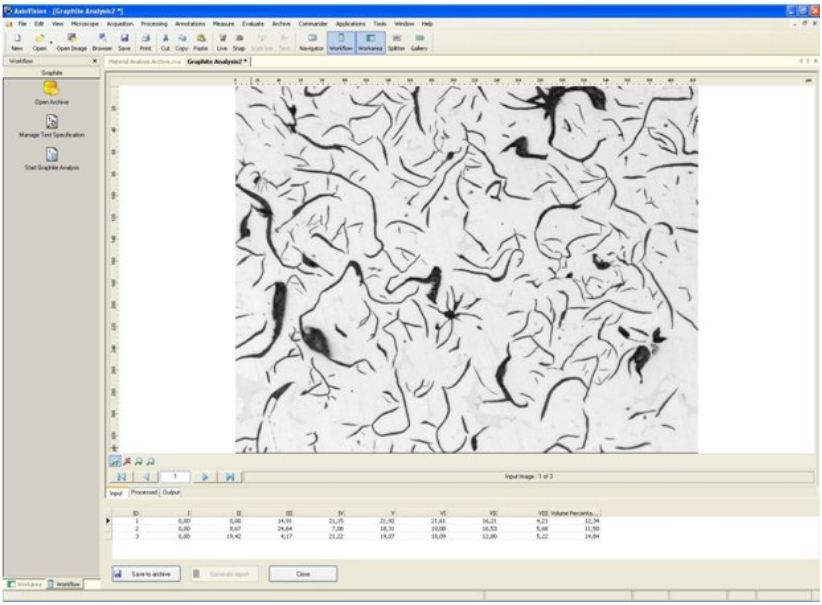
The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question. The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for

example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program.

The results document

At the end of the measurement the results window is displayed. Here, the results are displayed in the form of a table for all the images measured, as well as the image of the sample currently selected. Below the images are three tabs which you can use to switch between the input, output and processed image. If the image has been processed using image enhancement methods (e.g. sigma filter), you can therefore see the effect if you switch between the input and processed image.



Generating reports

The data are not saved directly in a report, but are only displayed there. This avoids data being saved twice. As a result, the data must first be saved in the archive. Then you can generate the report, which, immediately upon being generated, is also saved automatically in the archive. Provided you do not delete any of the tables or images from the archive, the data are also displayed in the report. In order to display the report on other PCs too, you can export the report into PDF or RTF format (**File** menu -> **Save as PDF** or **Save as RTF**).

Creating and performing a graphite analysis

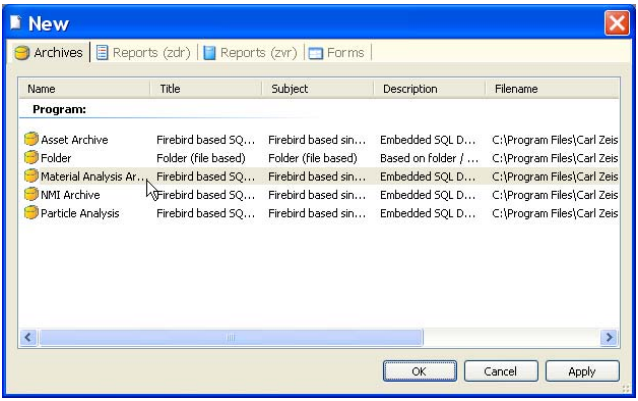
Now that the theoretical principles have been explained above, below you will find step-by-step instructions for creating and performing a graphite analysis.

How do I create an archive?

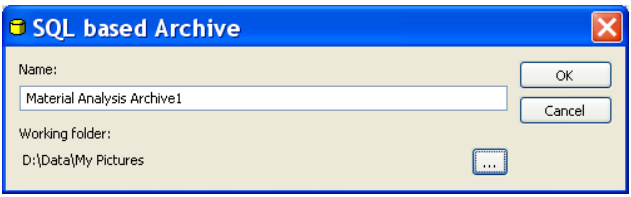
The Material Analysis Archive forms the basis for managing the data resulting from graphite analysis. All the data relating to the performance of a measurement are managed here. In order to perform a measurement, it is necessary for an archive to be opened in AxioVision. The creation and opening of an archive are therefore shown first.

- Select the **New** function in the **File** menu.

- On the **Archives** tab sheet, select the **Material Analysis Archive** and then click **OK**.



➢ The **SQL based Archive** dialog window will appear. Enter a name for the archive in the **Name** input field and confirm by clicking on **OK**.



Notes:

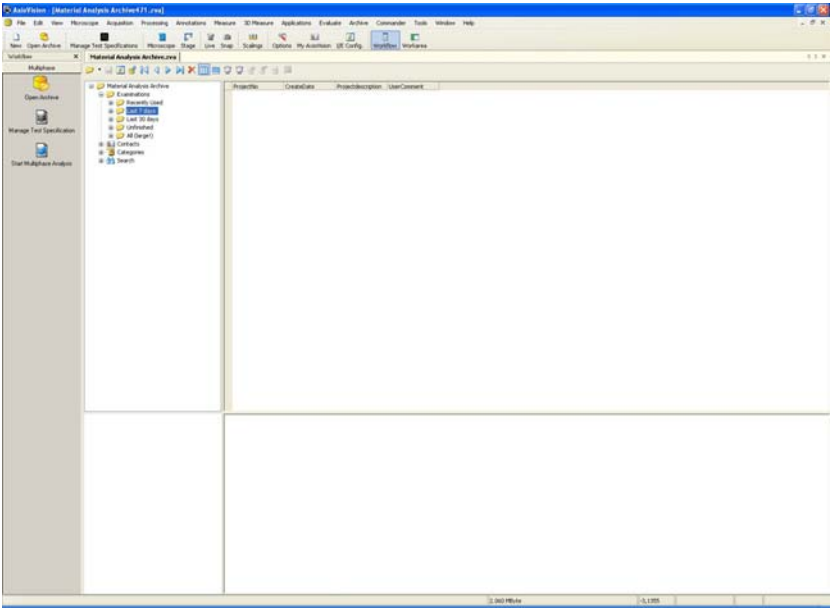
- An archive is created in the *My Documents**My Pictures* folder by default.

  If you wish to save the archive elsewhere, click on the ... button. Select the location where you wish the archive to be saved in the Microsoft Windows standard dialog that then opens.

- In the case of large data volumes in particular, we recommend that you save the archive to a local hard drive.

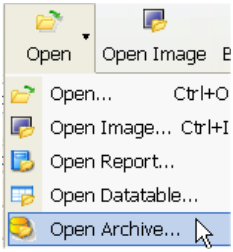
- Only one archive can be opened at a time in AxioVision.

The newly created archive is now displayed in AxioVision.



Opening an archive

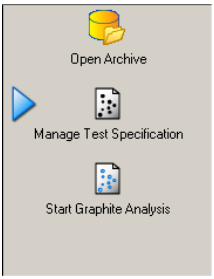
- Click on the **Open** icon in the menu bar and then on the **Open Archive** function:



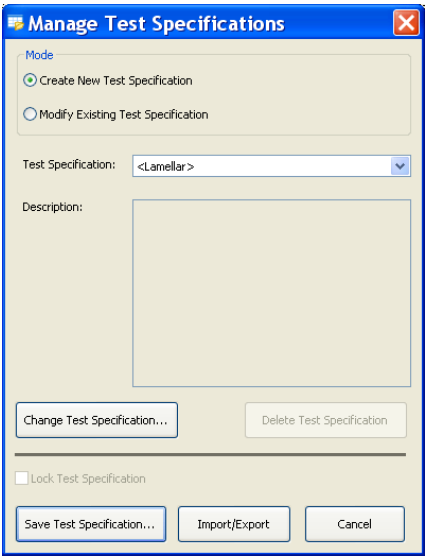
How do I create a test specification?

The test specification contains all the settings required for performing a measurement.

- From the workflows, select the **Graphite** workflow.
- Click on the **Manage Test Specification** function.



This opens the **Manage Test Specifications** dialog.

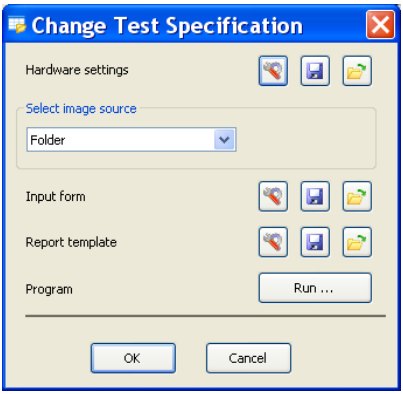


➢ Select the test specification that you wish to use as a basis for the new test specification. If you have not yet created a test specification, you can select one of the standard test specifications "Sperolyte", "Lamellar" or "SinterCast", depending on which measurement method you require.

Note:

- In the **Create New Test Specification** mode, a copy is generated of the selected test specification, which you can then adapt. The original test specification that is used is not changed. If you only wish to make a modification to the test specification, change to the **Modify Existing Test Specification** mode. The standard test specifications of Graphite cannot be changed, but only used as the basis for a new test specification.

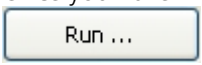
➢ Click on the **Change Test Specification** button. This opens the **Change Test Specification** dialog.



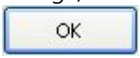
➢ You can now save the hardware settings, select the image source, set options or edit the input form and report template. It is recommended, however, that you modify the report template after running the program, because measurement data are then available. The report editor is displayed automatically after running the program.

Notes:

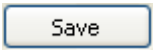
- You will find more detailed information about the hardware settings in Chapter 4.5 "The Settings Editor" of this manual.
- You will find more detailed information on modifying form templates in Chapter 7.4 "Templates for forms and reports" of this manual.
- You will find more detailed information on editing a report template in Chapter 6.4 "Creating Data Reports" of this manual.

➢ Once you have made all the necessary settings, click on the 

button. This opens a wizard that guides you through the program. Using the **Execute** option, you can determine whether or not the processing step is carried out. If you activate the **Interactive** option, this step will also be displayed during the measurement. The user can also change the parameters here.

➢ When you have made all the necessary settings, leave the **Change Test Specifications** dialog by clicking on the 

button.

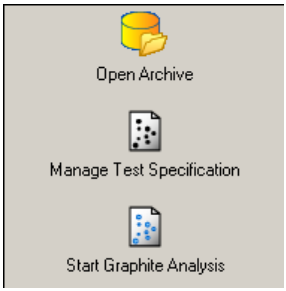
➢ Save the test specification by clicking on the 

button. This opens the **Save Test Specification** dialog. You can enter a name and a description of the test specification here. A description helps the user when starting the measurement, as he is unable to open the **Manage Test Specifications** area and does not, therefore, see the settings that have been made there.

How do I perform a measurement?

➢ From the workflows, select the **Graphite** workflow.

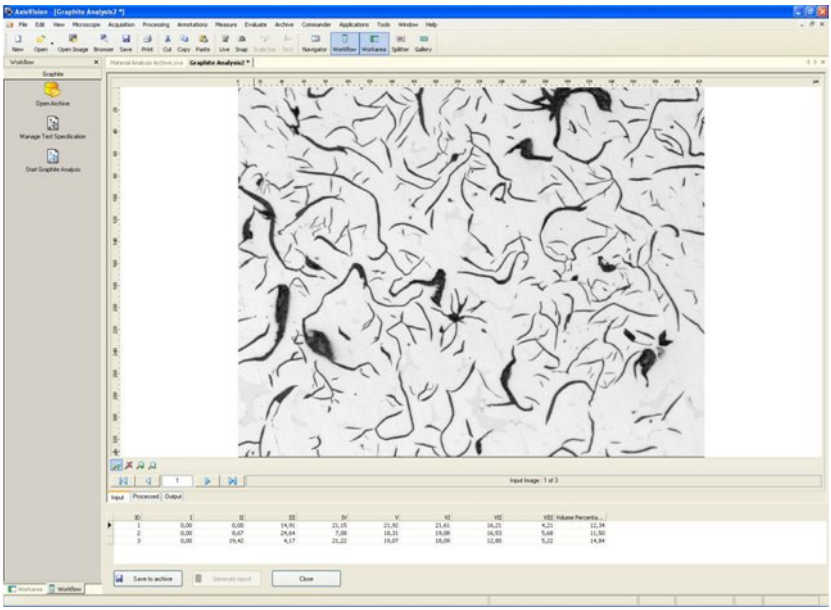
➢ First of all you will need an open archive. To open one, click on the **Open Archive** function.

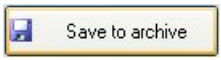


- This opens the standard dialog for opening a file in Microsoft Windows. Select a Material Analysis Archive.

- Then click on the **Start Graphite Analysis** function in the workflow.

This opens a wizard that guides you through the measurement. At the end of the measurement, you will be shown the results view:



- To generate a report, first click on the 

button.

- Then click on the 

button that is now active and the report will be generated and displayed. It will already have been saved in the archive.

11.10 Layer Thickness Measurement

The Layer Thickness Measurement module is used to measure simple and complex layers in individual or MosaiX images. Layers to be measured are detected using image-analysis techniques and measured with the help of chords. The result of this measurement is a clearly structured report containing images and measurement data. In the text that follows, the concept of Layer Thickness Measurement and its operation are described.

Note:

- Further explanations on all the functions described can be found in the online help.

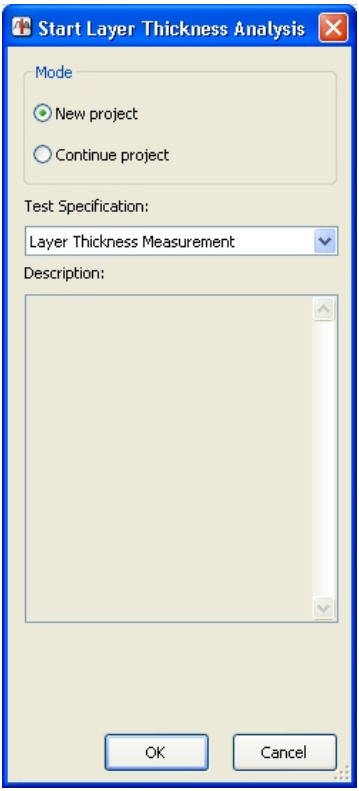
The concept

Operation and configuration

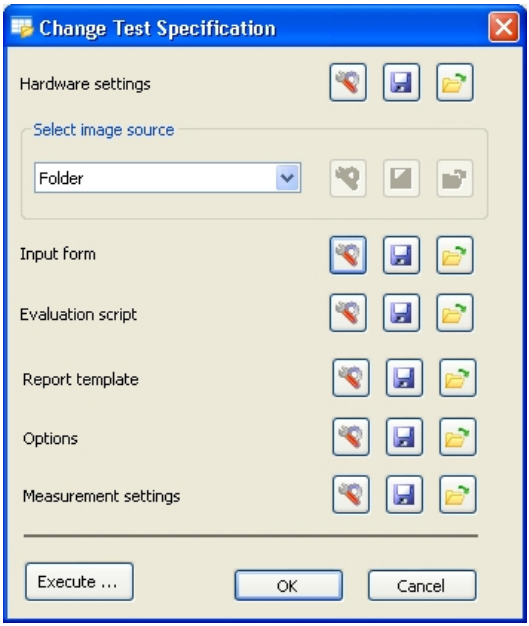
The operating concept the Layer Thickness Measurement is designed to make it possible to achieve a reproducible result with as little interaction as possible. The performance of a measurement can be automated to such an extent that only the project data need to be entered and the entire analysis process can run automatically.

To automate a measurement completely, you need extensive knowledge of systems engineering and the respective application. Carelessly made alterations of a setting can promptly lead to faulty measurement results. It is important, therefore, to prevent users not having such knowledge from changing the basic settings. This is achieved by dividing up the tasks involved into the definition of a measurement and the performance of a measurement.

The performance of a measurement is always based on what is called test specification. All important settings are saved in a test specification. A test specification cannot be created or edited by a user. This is reserved to Power Users or Administrators. Access to the respective functions is governed by the access rights of the operating system:



Dialog for starting a Layer Thickness Measurement



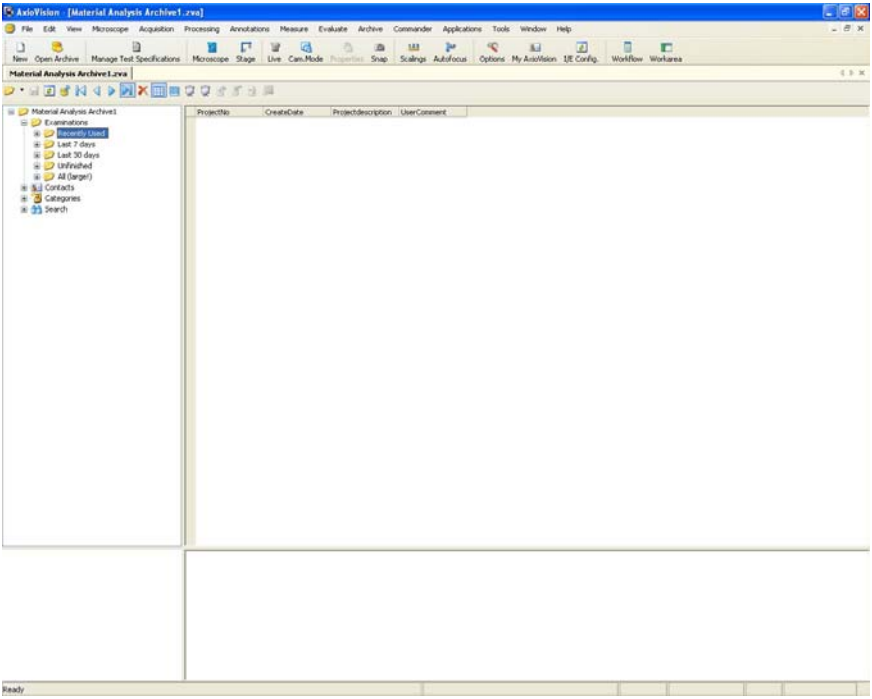
Dialog for editing a test specification (not available to standard users)

Projects and test specifications

The configuration data of a measurement are saved to so-called test specification. A test specification contains all the information on the performance of a Layer Thickness Measurement in detail: from the configuration of the device and the type of image acquisition through to the determination of the size of the original data to be saved.

The test specifications are saved to the archive, together with the measurement projects, but they are not visible in the archive window. They can only be modified via the **Manage Test Specification** function.

Data are saved and managed in a project-based archive. All the data generated (images, data tables, reports etc.) are saved by project:



Archive View

Preparing the system

Introduction

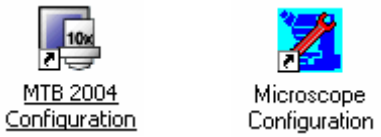
General

The functionality of the Layer Thickness Measurement module is based on the basic functionality of AxioVision. Before you can work with Layer Thickness Measurement, you must, therefore, configure the basic settings for AxioVision. You will find further information you require in Section 8 "Configuration" of this manual.

Microscope configuration

To be able to use your microscope together with AxioVision, you need to define once your individual system configuration via the separate **MTB2004 Configuration** program. Should you be retrofitting Layer Thickness Measurement to your existing microscope system, you may have to do this configuration via the **Microscope Configuration** program.

The configuration programs have their own icons and buttons on your Microsoft Windows Desktop:



Note:

- For detailed information, see Chapter 4 "Microscope Control".

General AxioVision Settings

Please make sure that the following settings have been made:

Alignment of camera and motorized stage

If you wish to acquire images of large areas using the MosaiX module, you must ensure that the camera and stage are aligned correctly with one another and that the stage's direction of movement has been set correctly. Otherwise the individual component images will not be put together properly. The aligning and setting process may take quite some time with conventional camera adapters; therefore it is recommendable to use the adjustable Carl Zeiss camera adapter:



Note:

- For detailed information, see Chapter 9.7 "MosaiX" – "Setting up the camera and scanning stage").

➢ **Scalings**: Measurement in real units (e.g., µm) is only possible if there exist scaling files. Although AxioVision has a capability to compute a theoretical scaling on the basis of the current system configuration, it is urgently recommended to create, for measurement tasks, an individual scaling file by means of a stage micrometer.

Note:

- For detailed information, see Chapter 8.4 "Scalings".

User settings

Select the **User** tab in the **Tools** ⇒ **Options** menu. Enter the information about the respective user and select your corporate logo. This information is

administrated separately for each user. It is saved to the archive for each project or each measurement and, by way of default, used for the reports.

Note:
- For detailed information, see Chapter 8.6 "General settings".

Configuration of the user interface

AxioVision offers an extremely wide range of options with regard to user-specific configuration. In particular, the fact that the interface is structured as simply as possible means that even inexperienced users are able to use the system to achieve accurate results both quickly and easily.

It is recommendable to hide all controls not used for the purpose.

Notes:
- To show the main menu again, click the right mouse button on the title bar of the AxioVision window, or on a toolbar. This opens a context menu in which you can display everything again.

- For detailed information, see Chapter 8.5 "Adapting the user interface".

The Material Analysis archive

Data are saved and managed in an archive. There, all data (images, measurement data tables, reports, etc.) are saved by project.

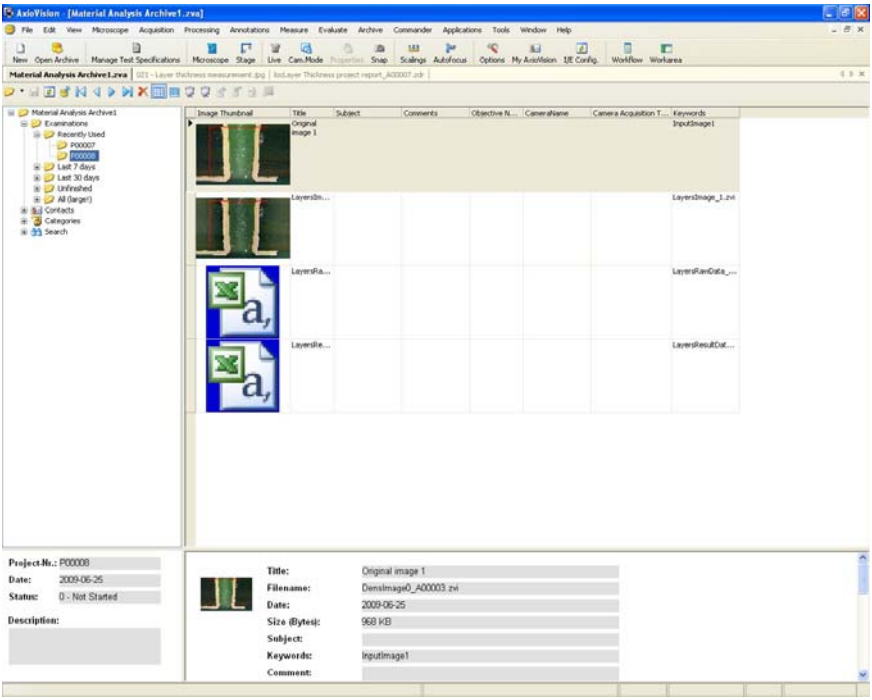
The test specifications are also saved in the archive together with the measurement projects. These are not visible in the archive window, and can only be modified using the **Manage Test Specifications** function.

There are several types of archive in AxioVision. Besides the folder and asset archive, there are also specific archives for certain modules. The Material Analysis archive is intended for Grains. The measurements of the AxioVision Multiphase, Graphite and/or Comparative Diagrams modules can also be stored in this archive.

In order to work with the Layer Thickness Measurement module, an archive must therefore exist and be opened in AxioVision.

Note:

- The Layer Thickness Measurement can **only** be performed in the Material Analysis archive. No other types of archive are suitable for this!

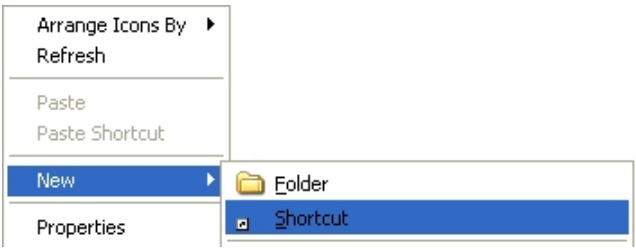


Archive View

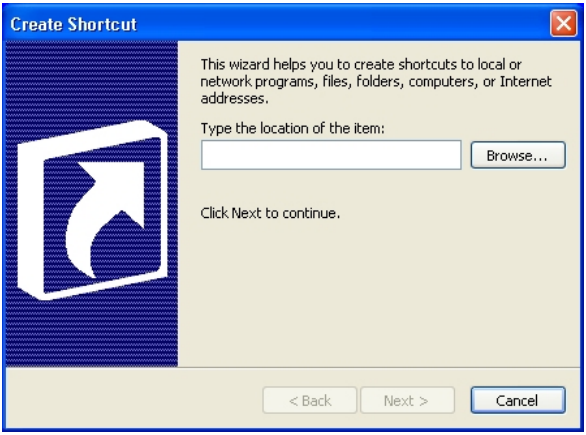
Opening the Material Analysis archive when starting AxioVision

To start AxioVision always with the desired archive, you should establish a shortcut to the archive on the Microsoft Windows desktop:

- Click the right mouse button on a free area on the desktop, and select **New** ⇒ **Shortcut**:



- This opens the dialog **Create Shortcut**. Click on **Browse...** and select the Material Analysis Archive (file extension *.zva):



- Click on **Next** and, in the next step, enter a name for the link.

- To create the link, click on **Finish**.

Administrating several users

The user management of AxioVision is directly based on that of the operating system. Each user with access rights works under a different AxioVision configuration. This means that, e.g., the configuration of the user interface, the entry of user data, etc., are managed separately for each user.

Notes:

- For detailed information on user and data management, see Chapter 8.3 "User and data administration".

- If you use a common account for all users but wish to use different configurations nevertheless, proceed as described in the following section.

Separation of Layer Thickness Measurement and standard AxioVision

It is a strong point of Layer Thickness Measurement that the system is completely based on the standard AxioVision software. Therefore, it can also be used for simple documentation jobs or other applications such as, e.g., particle analysis or analysis of non metallic inclusions in steel. To start AxioVision always with a special, application-specific configuration (e.g., specific toolbars, workflows, etc.), you can establish any number of links with separate icons on the Microsoft Windows Desktop.

Note:

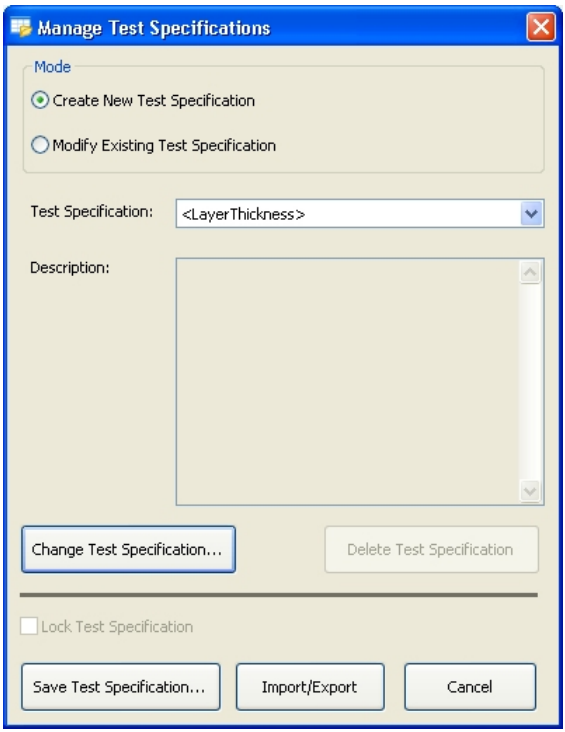
- For detailed information on the creation of configurations, see Chapter 8.8 "Creating various AxioVision configurations for a user".

Creating test specifications

General

In test specification, all settings and files for performing a Layer Thickness Measurement are saved. All subsequent Layer Thickness Measurements always are based on a test specification. When you start a measurement you are prompted to select the desired test specification first before you can actually start the measurement.

The test specifications are managed via the **Manage Test Specification** dialog:



In the **Manage Test Specifications** dialog you can edit, create and delete test specifications. Further, you can import and export test specifications.

Importing and exporting test specifications

To make the preparation and setup for Layer Thickness Measurement as easy as possible, you can simply import existing test specifications for special applications and use them right away.

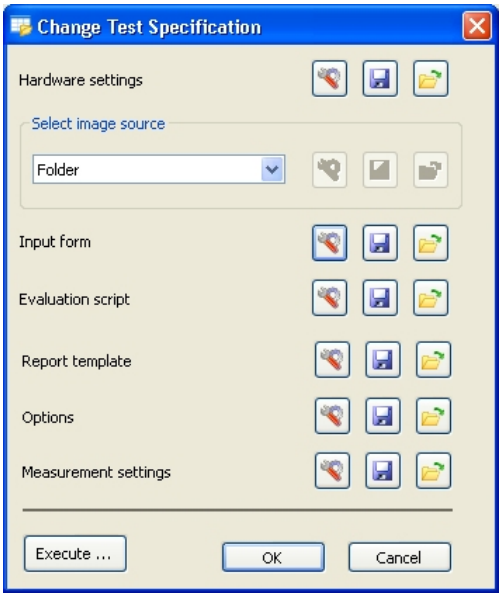
This is especially helpful if measurements are to be made in different places but with exactly the same specifications. Test specifications once created centrally can then be imported on any number of systems installed.

Clicking the **Import/Export...** button starts the wizard that guides you through all the necessary steps:



The Change Test Specification dialog

Test specifications are created via the **Change Test Specification** function. All the dialogs used to set up a Layer Thickness Measurement are called up via this dialog:



Dialog for changing a test specification

Each individual step can be modified entirely. The relevant dialog can be opened using the button  . Here again you can, for each single item required for creating a test specification, load settings already existing in another template. For the management of template files, use the buttons on the right of the respective creation items:  .

Thus, you can directly copy device settings, settings of the image source, input forms, etc. from existing test specifications into the current test specification you are creating. Further, you can save your own settings to a new test specification in order to use them in further test specification.

The following chapters describe the procedure step by step.

Testing test specifications

If you want to test whether the settings of a test specification really produce the desired results, you can click on the **Execute...** button to run a complete measurement without having to create a project in the archive. It is important that you perform this step with a real sample, because all settings made in the functional dialogs during the test run are adopted by the test specification.

Hardware settings

The settings of microscope and camera are decisive for the reproducibility of measurements. Measurements will yield the same results only if they are always made with the same parameters. With the hardware settings you can ensure that you are always using the same hardware configuration before you start a measurement. This is conditional on the use of motorized microscopes or microscope components, and digital cameras. These can be completely controlled via the software.

In case of manual microscopes you need to do the correct settings manually. So-called coded components require a mixed approach. Their current statuses can be checked by the software, but the correct setting has to be done manually as required. If you are using coded components, the system will warn you if a setting is faulty and prompt you to establish the correct configuration.

The most important settings include, e.g., the objective to be used, the brightness adjustment, the used contrasting method, camera resolution, exposure time, camera mode, etc.

Note:

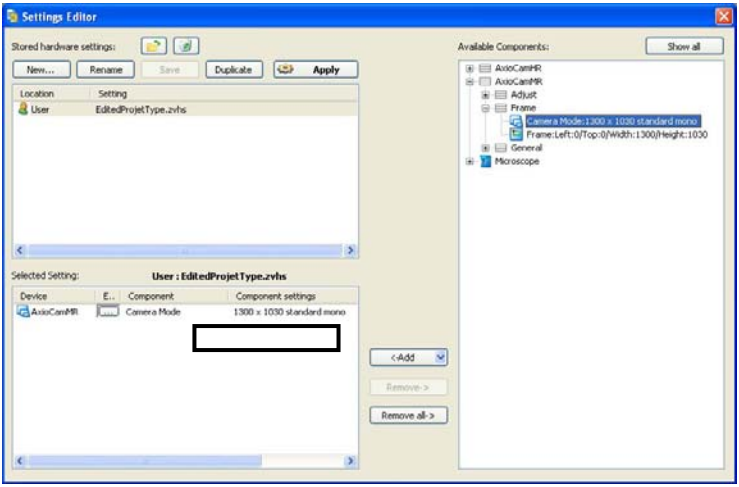
- For detailed information on hardware settings, see Chapter 4.5 "The Settings Editor".

Example: Setting the camera mode (color, gray levels)

The camera setting has a decisive influence on the size of your files. Given the memory capacity of modern computers, the use of color images is considered a standard feature. However, color images require three times as much memory space as gray scale images. In most cases, though, color information is not required for the detection of layers; therefore, images should be acquired as gray scale images. This setting can be made in the camera control dialog, and it can be changed there by every user:



By including the camera mode in the hardware settings you can ensure that you are always acquiring gray level images for a measurement, irrespective of the actual settings of the camera:



If hardware settings have been activated, these can be checked immediately before the measurement on the sample to be measured.

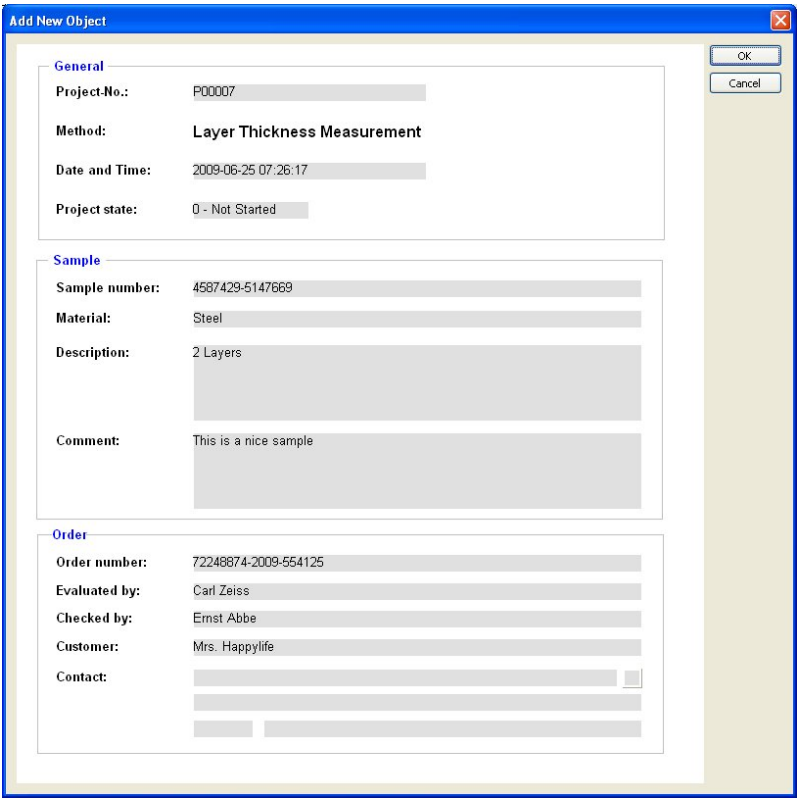
Image source

The image source defines where the image comes from. If you have already acquired images that you wish to analyze, select folder or gallery as the image source. In most cases, however, an image is acquired and analyzed immediately. Select the **Camera** option to execute the analysis with single images. When you are acquiring images of large surfaces using the mosaic technique, the size and position of the acquisition region are defined using the **MosaiX Acquisition** dialog. To do so select the **MosaiX** option.

To ensure unmistakable repositioning in the acquisition of MosaiX images, the z focus and the x and y settings of the motorized stage are calibrated after every restart of AxioVision.

Input forms

The Input form allows the user to enter the necessary project and sample data during the measurement. You can freely define the data that you want to be entered. The necessary fields and their link to the archive are defined in layout mode. Layout mode is active as soon as editing is started.



Standard input form for project data

Note:

- For detailed information on editing form templates, see Chapter 7.4 "Templates for forms and reports".

Preprocessing script

General

A preprocessing script defines which image processing steps will be performed in which sequence. You can choose which functions will be executed interactively, i.e. which functions will be opened while a measurement is being performed to allow the user to adjust parameters.

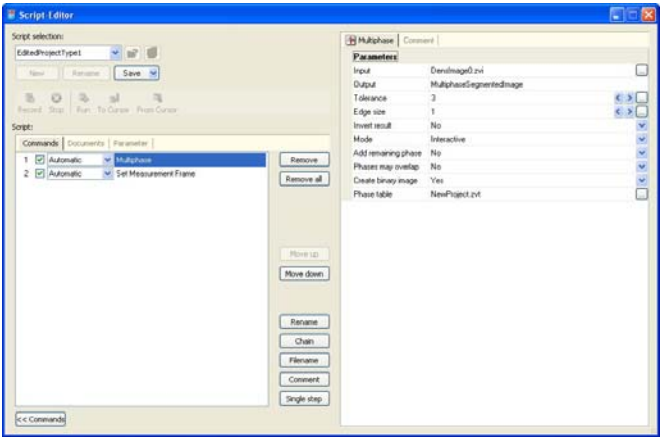
Notes:

- In order to display and modify the preprocessing script, you will need the Commander module. The Commander module however is NOT required for executing a Layer Thickness Measurement.
- Since modification of the preprocessing script has dramatic consequences with regard to the quality of the analysis, this should only be performed by experienced users. It is essential that you read the chapter 13.1 "Commander" section and the information in the online help before making any modifications.
- The preprocessing scripts are not managed in the usual standard AxioVision folders. The **New** and **Rename** buttons are therefore inactive. The **Save** button only becomes active once you have made changes to the script.

The following scripts are supplied when Layer Thickness Measurement is installed:

- **LT_Processing_Wizard**: This script is activated by default. The image processing is set up in such a way that the edges of the layers are detected. It should be used for images in which the edges of the layers can be clearly detected. Name of the output document: "ScriptOutputContour".
- **LT_Multiphase_Segmentation**: This script opens the standard **Multiphase** dialog. One phase is envisaged here for each layer. The result is a multi-level binary image with the name "ScriptOutputMask".

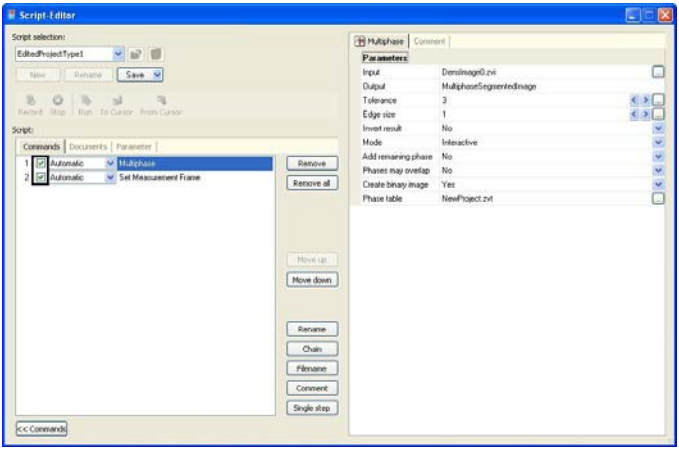
- **LT_Draw_Contour**: This script opens the **Draw Layers** dialog. It has been designed for cases in which automatic detection of the layers by means of segmentation, the **Processing Wizard** or other image processing techniques does not work. In this dialog, the layers are drawn in. There does not have to be a closed contour. Layer Thickness Measurement detects all regions between a minimum of two lines as a layer. In this script, the output document is called "ScriptOutputContour".



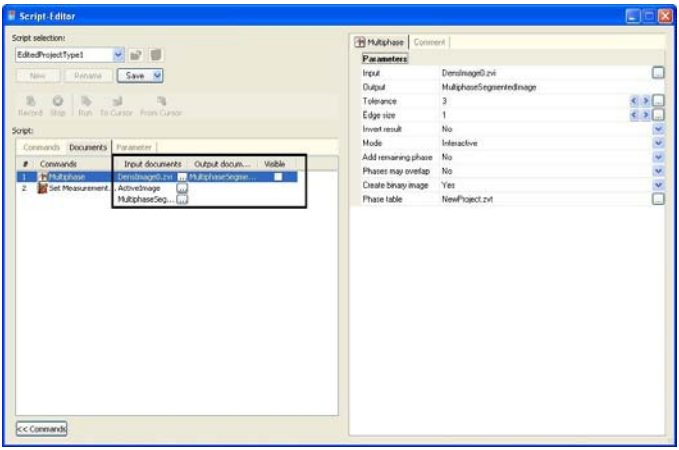
Preprocessing script in the Commander script editor

Adapting the preprocessing script

As long as you do not insert or delete any commands, you can modify the behavior of the script simply by switching the commands on the **Commands** tab on and off.



You can incorporate any functions available in AxioVision into the script. After doing so, however, you must ensure that the input and output documents are correctly linked and labeled.



Modifying report templates

In order to print the results of a measurement clearly, it is possible to structure the appearance of the results report. A report template may only be modified during the creation/editing of each test specification and is, therefore, not accessible for standard users. The predefined template is always used when you perform a measurement.

Note:
- For detailed information, see Chapter 6.4 "Creating data reports".

Optional settings

General

With optional settings you define essentially the following items:

- Selection of the data to be saved (above all, image data)
- Behavior during image acquisition

It is imperative that you consult the Online Help function for information on *details of the parameters that can be set.

Memory space required

The quantity of data obtained in Layer Thickness Measurement is sometimes enormous, especially when you analyze several samples. The table below shows the memory requirements for different conditions:

Pixel scaling used	Image size in MB*	
	Gray scale image	Color image
1.0 µm / pixel	1450	4350
2.5 µm / pixel	232	696
5.0 µm / pixel	58	174
10.0 µm / pixel	15	44

** Area approx. 1500mm², 8 bit gray levels*

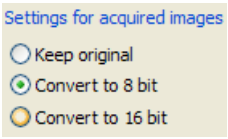
You should carefully consider which original data should actually be saved. Among others, the two factors outlined below may be a basis for decision:

- If you want to ensure that a measurement can be reproduced at any time, you need to save the original data as they are. It is not permissible in this case to scale down the original image or to save it in a compressed format.

- If you want to save the original image for documentation purposes, you should consider whether a scaled down version could be saved in a compressed format (e.g., ZVI or JPG). This will considerably reduce the amount of image data, but they will not be suitable for subsequent measurements.

Number of gray levels or color values of the images acquired

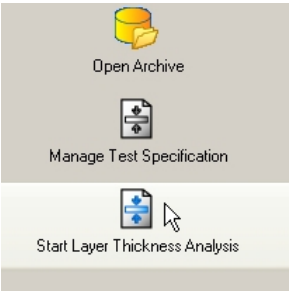
Another important consideration is the bit depth, i.e. the number of gray levels or color values used for image acquisition. ***In the past***, the entire brightness or color range was captured 256 different gray levels (8 bit). Most modern digital cameras have more than 256 gray levels. But here, too, this means that the resulting image data files are exceedingly large; the great number of gray levels (possibly more than 65,000) is not needed for a measurement. It is possible, therefore, to enforce reduction to 256 gray levels:



Conversion of acquired images to 8 bit

Performing measurements

A measurement is performed via the **Start Layer Thickness Measurement** function in the Layer Thickness Measurement workflow:



An Layer Thickness Measurement is always based on a previously defined test specification. This must have been created by a qualified user. No measurement can be started until a test specification has been created.

During the course of the measurement, the function dialog for which the **Interactive** mode has been selected in the preprocessing script will be displayed. All other functions run automatically in the background.

The program will stop at each function for which **Interactive** has been selected and the corresponding dialog will open. The predefined parameters can then be modified and optimally adapted to the image in question.

The functions that are carried out interactively feature the additional parameter **Always interactive** during the execution of the program. This allows the user to decide whether or not the dialog should be opened again for the next image. This is particularly useful if you have set segmentation thresholds, for example, and do not need to modify them again for subsequent images. The thresholds can therefore be set using one or more images and then applied automatically.

This option is useful if you are measuring several images immediately after each other. Otherwise, this setting does not have any impact on the running of the program. After the measurement the data are saved automatically in the archive, a report will be created and displayed.

11.11 Calotte Grinding Measurement

General description

During the process of coating materials, quality is controlled by measuring the thickness of layers in accordance with the DIN V ENV 1071 standard. The Calotte Grinding Measurement module allows you to determine the thickness of calotte section layers. To prepare the calotte section samples, a grinding ball with a defined diameter generates a calotte-shaped hollow in the coating of the sample. This results in circular or elliptical indentations, the diameters of which are determined by drawing in measurement circles. The layer thickness is calculated automatically from the diameters of the measurement circles and the diameter of the grinding ball.

Both single and multilayer samples can be measured.

The measurement results and sample information from the calotte grinding measurement are displayed in an image and stored. A report containing the image, measurement results and information about the sample can also be produced.

Performing a calotte grinding measurement

- Place the sample you want to measure on the microscope stage.
- Turn the nosepiece to the position with the desired objective.
- Now check that the calotte section is positioned correctly, adjust its position if necessary.
- Start AxioVision.

Clicking on the **Calotte Grinding Mesurement** icon opens the wizard. This will guide you through the application.

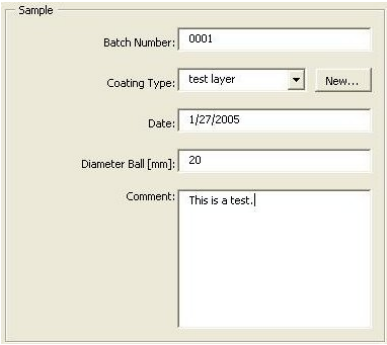


You can also call up the wizard from the **Applications** menu ⇒ **Calotte Grinding Mesurement** ⇒ **Start Assistent**.

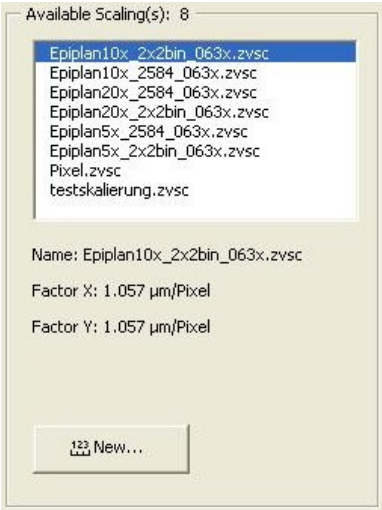
Describing the sample

The first page of the wizard is displayed: **Sample description (Step 1 of 4)**. In the upper area, you will find a brief description of what you should do in this step.

- Enter the sample data in the **Batch Number** field.
- New layer types can be entered using the **New** button. The new layer type will then appear automatically in the list of layer types.



- Select one of the available scalings from the list in the **Available Scaling(s)** field.
- New scalings can be generated using the **New** button. The new scaling will then appear automatically in the scalings list.



- Then click on the **Next** button in the bottom left-hand area of the wizard.

Opening an image

The second page of the wizard is displayed on the screen. Under the title **Open image (Step 2 of 4)** you will find a brief description of what you should do in this step.

- Click in the field **Source: Camera** the button **Live-Start** to open the live image window of the active camera. Center the calotte section in the live window and focus it.



- Click the corresponding camera button (in this case AxioCamMR5) to open the camera dialogue. Set the exposure time by clicking the **Measure** button.



- The click the **Snap** button to acquire the active image.



- If you would like to measure already saved images, click the **Open Image** button. You can open an image saved on the hard disk or any other storage medium.



- Then click on the **Next** button in the bottom left-hand area of the wizard.

Drawing circles

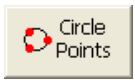
The third page of the wizard is displayed on the screen. Under the title **Draw Circles (Step 3 of 4)** you will find a brief description of what you should do in this step.

- In the **Method** field, select the **Single Layer** function (if the sample only has one layer) or the **Multi Layer** function (if the sample has several layers).



- Click on the tool that you wish to use to perform the measurement:

- Use the **Circle Points** or **Circle** tools for circular indentations:





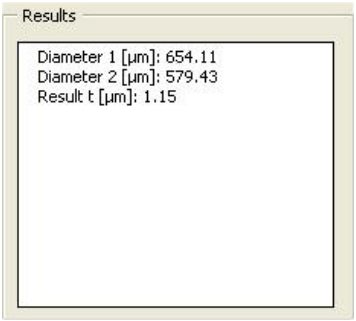
  If you use **Circle Points**, left-click on several points on the edge of the circle. Right-click to display the circle calculated from these points.

  If you use **Circle**, start from the edge of the circle and move inwards, holding down the left-hand mouse button, to drag out a circle the same size as the indentation.

- Use the **Ellipse** tool for elliptical indentations. Start from the edge of the ellipse and move inwards to drag out a circle the same size as the indentation.



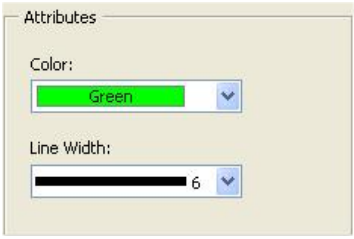
- The results of the measurement (diameter and layer thickness) are displayed in the **Results** field.



- Click on the **Remove circles** button if you want to delete the circles that you have drawn in. If necessary, start drawing in the circles again.



- In the **Attributes** field, you can change the **Color** and **Line Width** of the drawn-in circles. To do this, select the desired color or line thickness from the relevant drop-down list box.



- Click on the **Magnifier** button to magnify individual areas of the image. To do this, position the cursor on the area of the image that you wish to magnify. When you are dragging out the circle, the magnifying glass can help you to position the start point of the circle extremely precisely.



- Press the **Ctrl** key during dragging out the circle to position the border of the circle precisely.

- Then click on the **Next** button in the bottom left-hand area of the wizard.

Evaluation

The fourth page of the wizard is displayed on the screen. Under the title **Evaluation (Step 4 of 4)** you will find a brief description of what you should do in this step.

The results of the measurement are displayed together with the sample data in the **Results** field.

- Click on the **Show Results in Image** button in the **Annotations** field to display sample information and measurement results in the image.

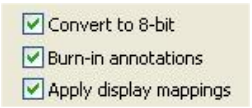


- Select the desired color and font size for the **Annotations** from the appropriate list.

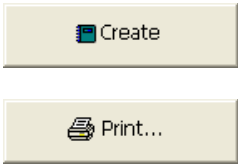
- Then click on the **Save Image As** button to save the image in any directory on the hard drive or another storage medium.



- The annotations are automatically burnt into the image when it is saved. To ensure that this takes place, please make sure that the **Burn-in annotoations** check box in the **Save Image As** dialog window is activated if the image is not being saved in the Carl Zeiss ZVI image format.



- Click on the **Create** button in the **Report** field to see a preview of the report. You can print out the report by clicking on the **Print** button.



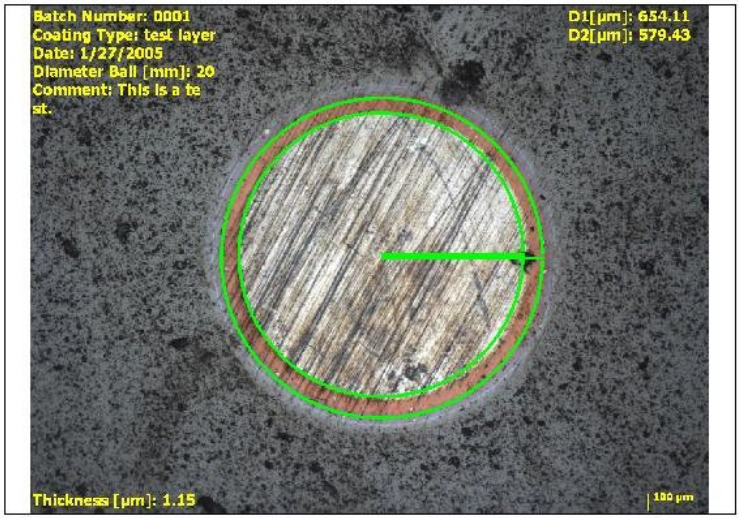
- Then click on the **Finish** button in the bottom right-hand area of the wizard, to leave the wizard.

Appendix A – Example of a Report

AxioVision Calotte Grinding Measurement

Evaluation

Batch Number: 0001
Coating Type: test layer
Date: 1/27/2005
Diameter Ball [mm]: 20
Scaling [µm/Pixel]: Epiplan10x_2x2bin_063x.zvsc



Results (Single Layer):
Diameter 1 [µm]: 654.11
Diameter 2 [µm]: 579.43
Result t [µm]: 1.15
Comment: This is a test.

You can save the report using the function **Save As** in the **File** menu.

11.12 Microhardness Measurement

General

The Microhardness Measurement module allows you to control an MHT (microhardness tester), and measurements of the microhardness of a sample. Detailed information on the MHT and its use and functions can be found in the operating instructions for the MHT from Anton Paar GmbH.

To perform a measurement, you require (in addition to the Microhardness Measurement module) a reflected light microscope with MHT controller and MHT sensor.

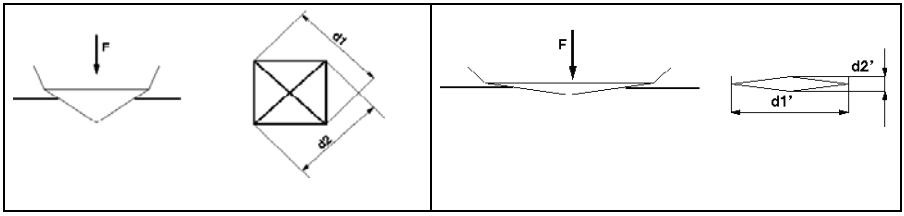
Microhardness measurement is a method used to characterize materials and their surfaces. An indenter (a sensor with a diamond tip) is pressed into the sample with a certain force. The Microhardness Measurement module requires the following three entries:

- **Force in Newton (F)**.
- **Dwell time in seconds**: To avoid plastic deformation or shrinkage of the material due to the sensor being removed too quickly, it is possible for the diamond tip to remain in the sample for a specified period of time.
- **Slope in Newton/second**: The specified slope determines how quickly the applied force reaches the maximum value F from the moment of its first contact with the sample surface. With fragile samples, low slopes should be used to avoid microcracks.

The size of the indent is then measured, and the hardness calculated from this:

$$Hardness = \frac{Force\ applied}{Indent\ size}$$

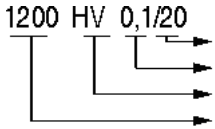
A force of a few Newtons is applied, producing indents of a few micrometers. The form of the indent depends on the type of indenter used.



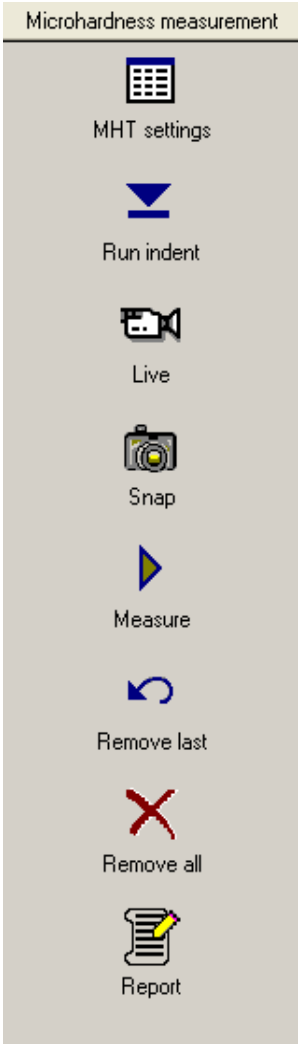
Vickers indenter *Knoop indenter*

In both cases, the size of the indent is determined on the basis of the diagonals of the rectangle produced. The formulae for calculating Vickers hardness and Knoop hardness are different, but produce the same microhardness results.

The microhardness measurement results are presented in the following form:

	Dwell time in seconds Force applied in Newtons Method indicator (HV - Vickers, HK - Knoop) Hardness value

Workflow



MHT settings sets the parameters for the microhardness tester.

Run indent starts the procedure for creating an indent.

Live opens and closes the live window.

Snap acquires an image using the active camera.

Measure allows you to interactively measure the indent and display the results of the measurement in the image.

Remove last deletes the last measurement performed.

Remove all deletes all microhardness measurements in the image.

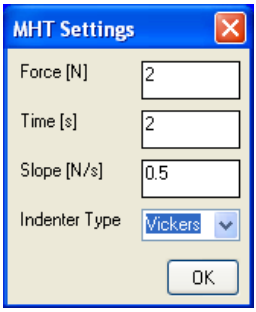
Report generates a report consisting of the measured image and the results of the measurement.

Performing a microhardness measurement

- Place the sample you want to measure on the microscope stage.
- Turn the nosepiece to the position with the MHT sensor.
- Now check that the sample is positioned correctly for the indent, and correct its position if necessary.

If you click on **MHT settings**, the **MHT Settings** dialog window is opened:





Insert the data for the following values:

- **Force [N]**: The force is limited to 2N for MHT4 or 4N for MHT10.
- **Time [s]**: The dwell time should be 10s – 20s.
- **Slope [N/s]**: The slope is limited to 0.999 N/s for MHT4 or 9.99 for MHT10.

➢ If you click on **Run indent**, a flashing window appears:



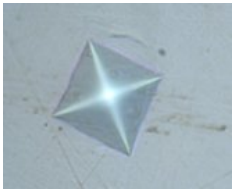


➢ The red light on the MHT control device flashes. Wait until the flashing window has disappeared. If you want to interrupt the process, click on the **ABORT** button.

- Turn the nosepiece to the objective with the desired magnification (normally 20x or 50x).
- Click on **Live**. The live window is opened.



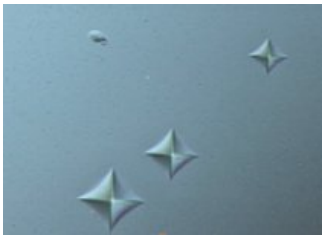
- Position the indent at the center of the live window, and bring it into focus:



- Adjust the brightness and exposure time etc. via the toolbar of the live window.
- Select the scaling for the set objective from the **List of available scalings** under the live window.



- You can make several indents at various positions on the sample in a single workflow.



- Click on the camera icon to acquire the image.



- Use the **Name** and **Acquisition** property pages (**Tools** menu ⇒ **Options** function) if you would like to save images automatically under a predefined name. A detailed description can be found in the online help (**F1**).

- Click on 

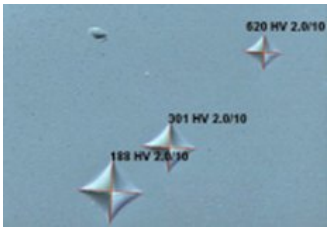
on the standard toolbar, and zoom to change the region you want to be measured:

- Click on **Measure**, and draw in the two diagonals by clicking on the start and end points. The result of the measurement is displayed automatically.



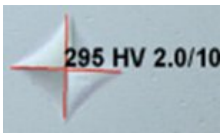


- Repeat this step for all indents in the image. A maximum of four indents per image can be measured.

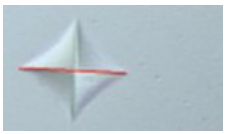


- Click on **Remove last** if you want to delete the diagonals you have drawn in step by step.





before



after

- Click on **Remove all** to delete all the indent measurements you have made up to this point, and start again by clicking on **Measure**.



- If you click on **Report**, a report is generated (see Appendix A).

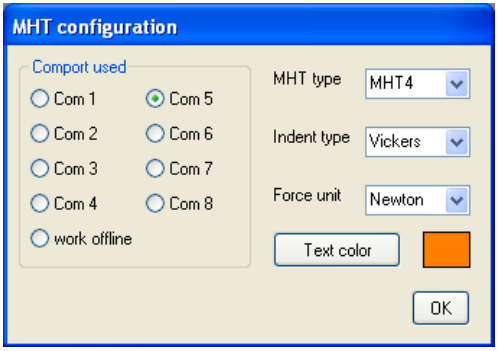




You can also use the **Microhardness Measurement** tool bar to perform the microhardness measurement.

Additionally you will find another button 

to configure the MHT control device. If you click on this button, the following dialog window opens:



Select here the corresponding COM port for the MHT control device.

Select further the type of the MHT device (MHT4 or MHT10), the indent type (Vickers or Knoop), the desired force unit (Newton, Pond, gram force) and the text color for displaying the measurement values in the image.

Glossary

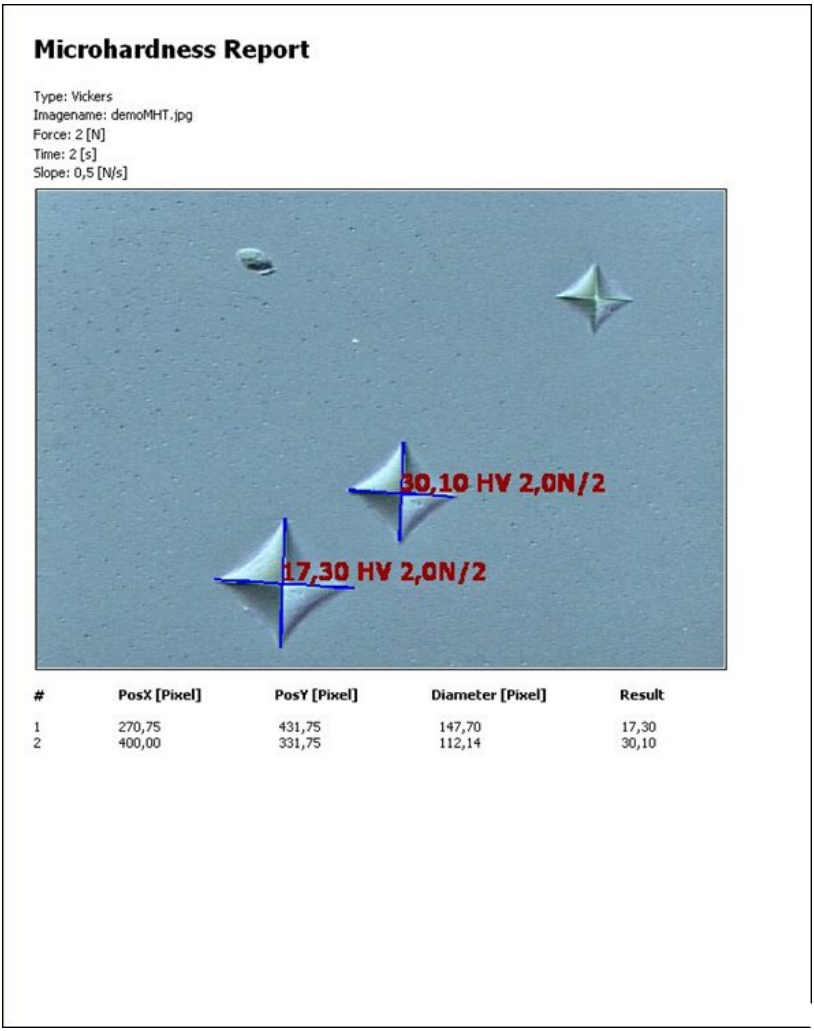
Microhardness: Hardness of the sample at the microscopic level.

Indent: The impression resulting from the microhardness tester being pressed into the surface of the sample.

MHT: Microhardness Tester – the name of the device used for the microhardness measurement. This can be obtained via Carl Zeiss. It is manufactured by Anton Paar GmbH, Graz, Austria.

Sensor: For the microhardness measurement, a tiny diamond tip is pressed into the surface of the sample with a very small, defined force. The sensor is screwed into the nosepiece instead of an objective, and controls and moves the diamond tip.

Appendix A – Example of a report



Microhardness Report

Type: Vickers
Imagename: demoMHT.jpg
Force: 2 [N]
Time: 2 [s]
Slope: 0,5 [N/s]

#	PosX [Pixel]	PosY [Pixel]	Diameter [Pixel]	Result
1	270,75	431,75	147,70	17,30
2	400,00	331,75	112,14	30,10

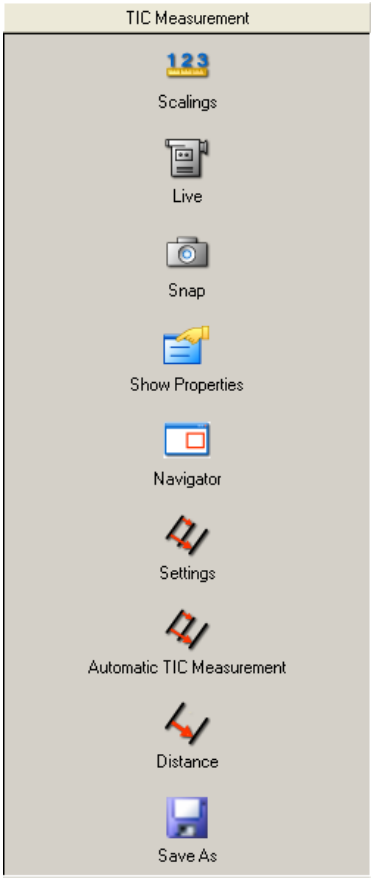
You can save the report using the function **Save As** in the **File** menu.

11.13 TIC Measurement

TIC (**T**otal **I**nterference **C**ontrast) is a method of measuring the heights of thin layers using optical interference.

To perform TIC measurement, you require a reflected light microscope with a C-DIC reflector and a TIC slider, in addition to the AxioVision TIC Measurement module. The operating instructions of the Axioskop 2 MAT mot microscope contain a detailed description of the TIC method.

Automatic TIC measurement



Scalings offers the calibration for the TIC measurement.

Live opens and closes the Live window.

Snap acquires an image using the active camera.

Show Properties opens a dialog window with additional informations and settings for the current image.

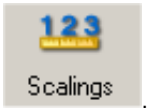
Navigator opens the window for zooming.

Settings allows the input of values for the phase shift object and phase shift substrate.

Automatic TIC Measurement starts the TIC measurement.

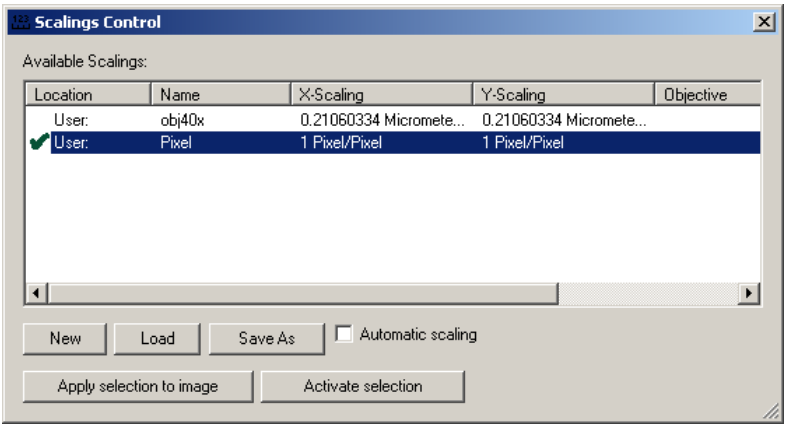
Distance measures the distance between two parallel lines.

Save As saves the measured image in a folder.

➢ Click on 

.

➢ Activate the TIC scaling that corresponds to the selected objective and the selected camera resolution.



Please note that TIC Measurement requires TIC calibrations, and not the usual geometric calibrations. A description of how to generate a scaling can be found under "TIC scaling" at the end of section 7.2.

If you do not intend to change the selected microscope and camera settings, you can now close the calibration window.

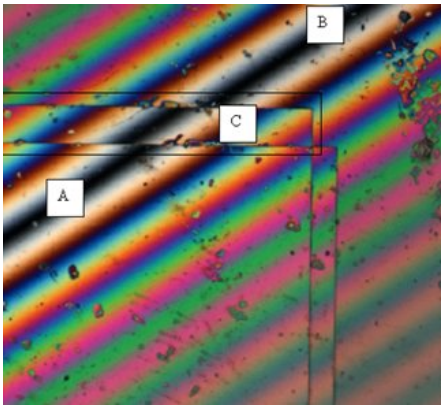
- Click on 

. Set the illumination and contrast such that the image you can see is optimally illuminated.

  Check the camera resolution. This must correspond to the scaling you have loaded.

- Set the image field to be measured on the sample and the direction of the interference fringes.

The image should look something like the sample image below:



The thin-layer sample contains three regions:

A: the top left-hand corner of the structure, which contains the height that you want to measure
B: the surface underneath this
C: the region with displaced interference fringes

The fringe displacement is due to the difference in height between the surface of the structure to be measured (bottom left in image) and the surface underneath this (top right in image).

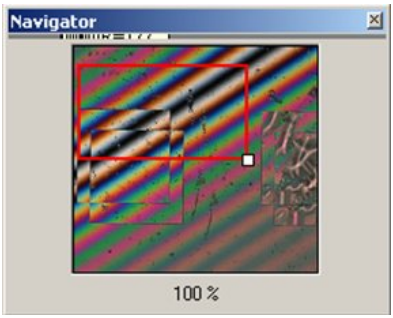
The displacement of the zero-order interference fringe in the overlap region is now to be measured, i.e. the distance between **A, B** (outside the overlap region) and **C** (inside the overlap region).

Please note that the color of the fringes changes symmetrically around the zero-order interference fringe, both inside and outside region **C**. The zero-order interference fringe can be easily identified as the darkest fringe.

- Click on the camera icon 

to acquire the image.

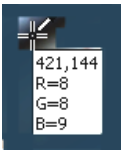
- Click on **Navigator**, and zoom to change the region you want to be measured:



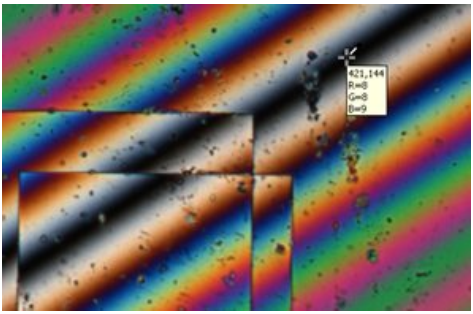
- Click on 

.

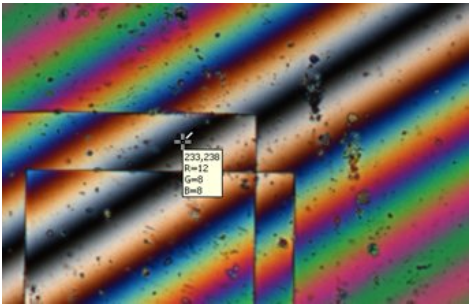
- If you move the mouse inside the image, the cursor symbol changes (from an arrow to a pipette) and indicates the x/y coordinates and color values of the pixel:



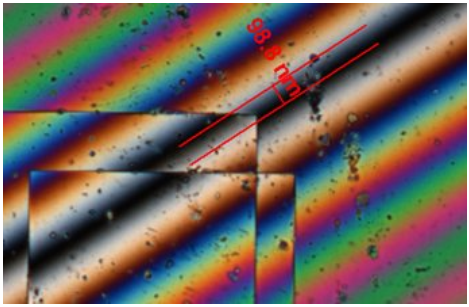
- Move the mouse to the center of the zero-order interference fringe on the underlying surface of the sample and left-click.



➢ Move the mouse into the overlap region (containing the displaced interference fringes), and left-click again at the center of the zero-order interference fringe.



The measured value is displayed:



➢ Click on 

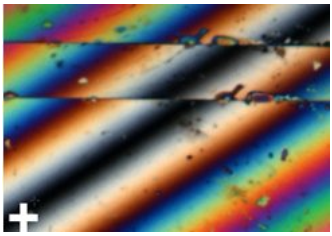
to save the image and the measurement generated.

Interactive TIC measurement

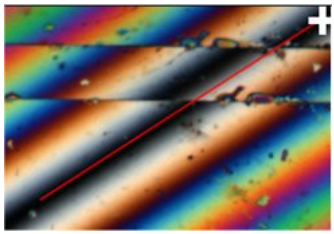
➢ On the **Measure** menu click on the **Distance** function, or click on 

in the menu toolbar.

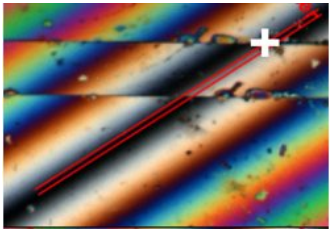
- Position the cursor (shown here as a cross) at the starting point of the line. Left-click.



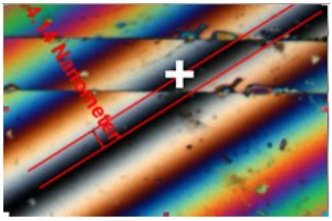
- Move the cursor to the end point of the line. Left-click. You will now see the first line in the image.
- Make sure that the line you have drawn corresponds to the center of the fringe.



- If you move the cursor, you will see the second line and the current measurement result, which changes as you move the cursor.



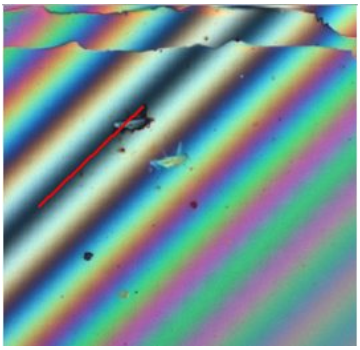
- Move the cursor until the second line corresponds to the center of the zero-order interference fringe. Left-click to end the measurement. The measurement value will be displayed.



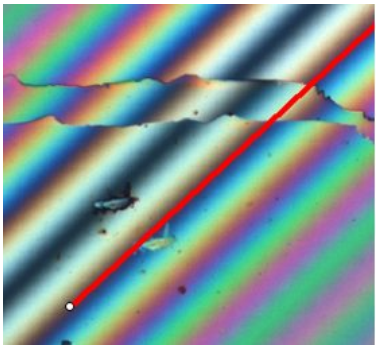
Causes of errors during interactive measurement

The longer the line, the more precise the measurement. Set the starting point of the line at the center of the zero-order interference fringe, and the end point as far as possible from it.

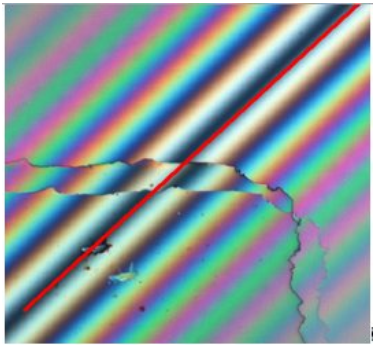
Below you will find four examples of errors that can occur while you are drawing in the line.



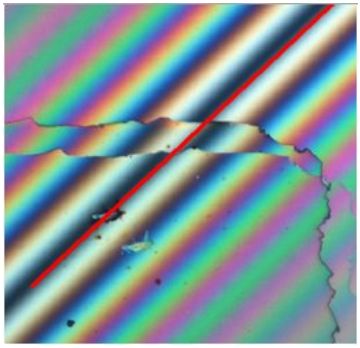
The line is too short.



The zero-order interference fringe has not been selected.

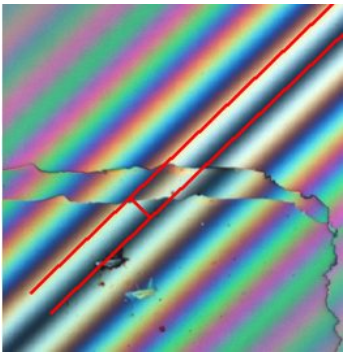


The line is not parallel to the interference fringe.

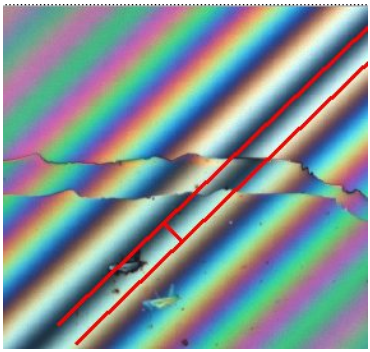


The line is not at the center of the interference fringe.

Below you will find two examples of errors that can occur while you are determining the position of the displaced zero-order interference fringe.



The first-order interference fringe has been selected.



The line is not at the center of the first-order interference fringe.

TIC scaling

In order to perform the TIC measurement, you require a set of TIC scalings (for each magnification and camera combination).

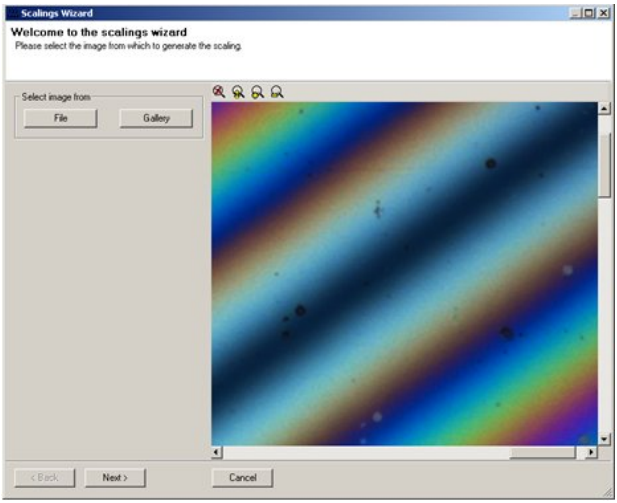
Start with a frequently used combination, such as the 10x objective and the AxioCam at the standard resolution.

Just like any other scaling procedure, TIC requires a reference object. Any reflective surface is suitable for TIC. Focus on the surface, optimize the exposure time and acquire the image by clicking on 

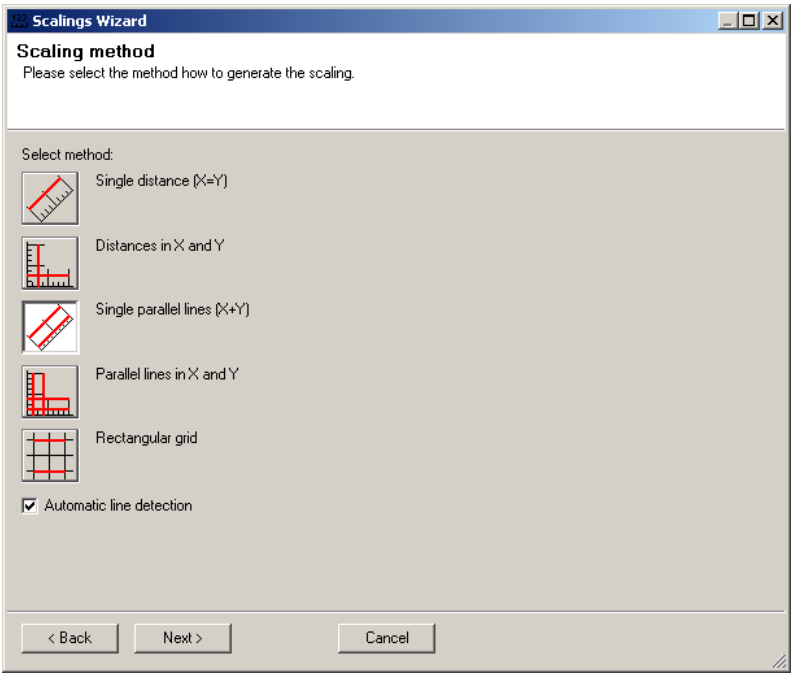
.

- From the **Measure** menu, select the **Scalings** function.
- In the **Scalings Control** window click on **New**. The scaling wizard is started.
- Right-click in the image. The image's shortcut menu is displayed. Select the **Normal View (1:1)**.
- Magnify the window by clicking on  .

- Move the scrollbars  at the bottom and right-hand edges of the image to set the desired frame.

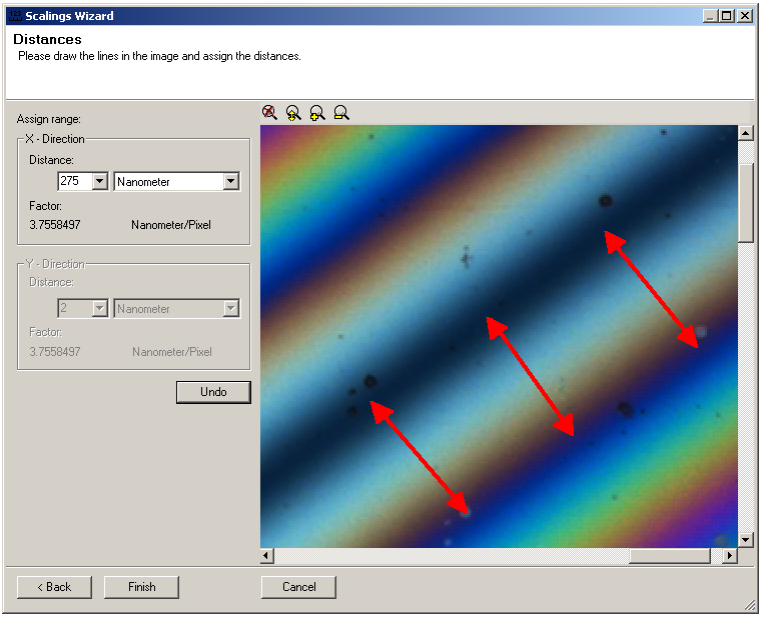


- Click on **Next**.
- Activate **Automatic line-detection**.
- Select the method **Single parallel lines (X+Y)**.



- Click on **Next**.
- Enter the value 275 into the **Distance** text field, and select the unit **Nanometer** (275 nm corresponds to half the wavelength of visible light).
- Now determine the distance between two interference fringes by drawing in two lines between two dark fringes. To do this, click on the starting and end points at the center of the interference fringe.

- The distances are marked by red lines. The correct distance is determined automatically and displayed as a third line between the two lines you have drawn.



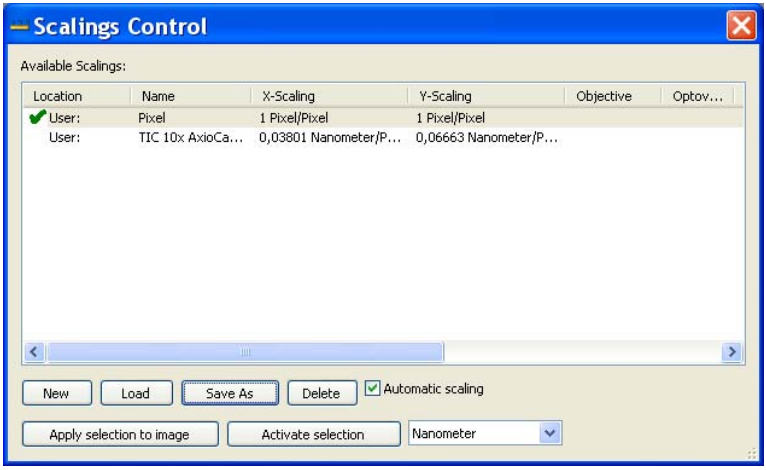
- Click on **Finish**.

- Enter a name for the scaling and click on **OK**.



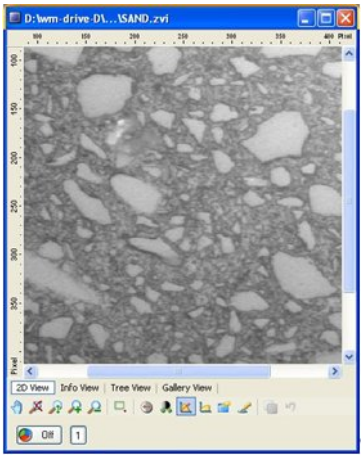
To avoid confusing this scaling with other scalings, you should use TIC as a prefix.

The new scaling now appears in the **Scalings Control** window, and is activated automatically. It is used for all images subsequently acquired.



11.14 SFM (Scanning Fluorescence Microscope)

Any AxioVision ZVI multichannel image and AxioVision CSV data table can be entered into the **SFM Evaluation** in order to generate histograms, scatter plots, image galleries or gated data tables.

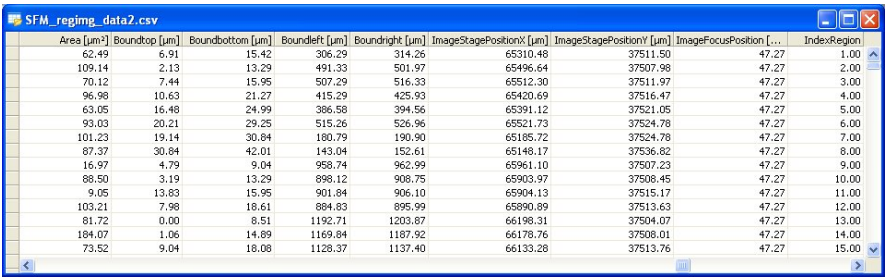


SandTable.csv

Area [Pixel²]	Boundtop [Pixel]	Boundbottom [Pi...	Boundleft [Pixel]	Boundright [Pixel]	De
101.00	7.00	16.00	0.00	14.00	
75.00	0.00	2.00	102.00	131.00	
127.00	1.00	13.00	141.00	156.00	
67.00	7.00	19.00	162.00	170.00	
49.00	17.00	22.00	96.00	107.00	
372.00	1.00	24.00	48.00	81.00	
82.00	13.00	25.00	36.00	46.00	
11.00	36.00	39.00	42.00	46.00	
16.00	28.00	33.00	89.00	92.00	
109.00	31.00	41.00	68.00	85.00	
17.00	35.00	38.00	126.00	131.00	
63.00	35.00	41.00	109.00	123.00	
244.00	55.00	70.00	4.00	32.00	
52.00	81.00	87.00	21.00	31.00	
17.00	80.00	84.00	51.00	56.00	
34.00	84.00	89.00	36.00	43.00	
169.00	91.00	106.00	22.00	38.00	
43.00	107.00	113.00	0.00	8.00	
7.00	89.00	91.00	85.00	88.00	
10.00	92.00	95.00	64.00	68.00	
6.00	102.00	103.00	122.00	125.00	
56.00	48.00	53.00	85.00	101.00	

To cut out objects as component images for the gallery view, the box coordinates of the objects must also be measured. In addition, the index region must be measured and entered in the data table.

To reposition the microscope stage, the x and y coordinates and the focus position must be measured. In order to ensure precise positioning, the stage must be calibrated prior to image acquisition in the x and y direction. The focus position (z) must be set to 0 (zero) in the focus area.

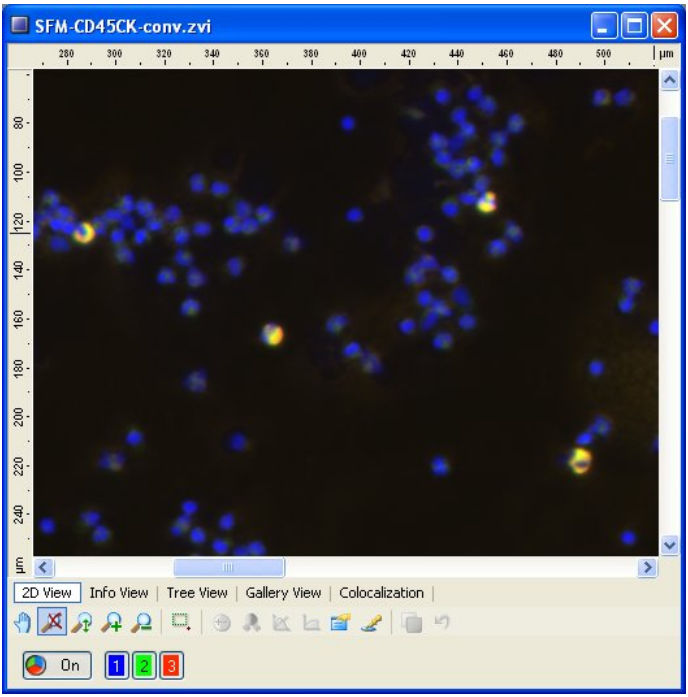


SFM_regimg_data2.csv

Area [µm²]	Boundtop [µm]	Boundbottom [µm]	Boundleft [µm]	Boundright [µm]	ImageStagePositionX [µm]	ImageStagePositionY [µm]	ImageFocusPosition […	IndexRegion
62.49	6.91	15.42	306.29	314.26	65310.48	37511.50	47.27	1.00
109.14	2.13	13.29	491.33	501.97	65496.64	37507.98	47.27	2.00
70.12	7.44	15.95	507.29	516.33	65512.30	37511.97	47.27	3.00
96.98	10.63	21.27	415.29	425.93	65420.69	37516.47	47.27	4.00
63.05	16.48	24.99	386.58	394.56	65391.12	37521.05	47.27	5.00
93.03	20.21	29.25	515.26	526.96	65521.73	37524.78	47.27	6.00
101.23	19.14	30.84	180.79	190.90	65185.72	37524.78	47.27	7.00
87.37	30.84	42.01	143.04	152.61	65148.17	37536.82	47.27	8.00
16.97	4.79	9.04	958.74	962.99	65961.10	37507.23	47.27	9.00
88.50	3.19	13.29	898.12	908.75	65903.97	37508.45	47.27	10.00
9.05	13.83	15.95	901.84	906.10	65904.13	37515.17	47.27	11.00
103.21	7.98	18.61	884.83	895.99	65890.89	37513.63	47.27	12.00
81.72	0.00	8.51	1192.71	1203.87	66198.31	37504.07	47.27	13.00
184.07	1.06	14.89	1169.84	1187.92	66178.76	37508.01	47.27	14.00
73.52	9.04	18.08	1128.37	1137.40	66133.28	37513.76	47.27	15.00

Scripts and macros are available for the special **SFM Evaluation** which combine corresponding multichannel data from identical regions for each channel with the relevant box and stage coordinates in a single file.

Image acquisition takes place using the usual settings for multichannel MosaiX imaging. In the sample scripts and macros, the evaluation is limited to three channels.

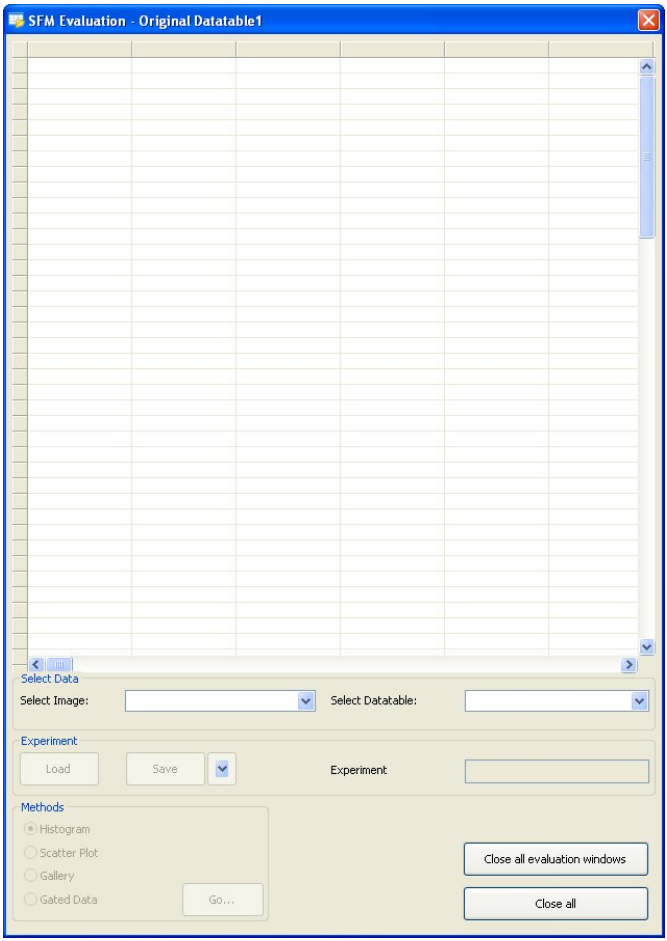


Prerequisites

For SFM evaluations, an image (AxioVision ZVI multichannel image) and the associated data table (AxioVision CSV table) containing the measured values must be available and loaded.

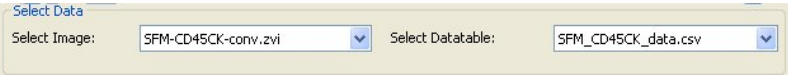
If the selected image and the data table are not suitable for **SFM Evaluation**, the **Methods** cannot be selected and access to the **SFM Results Presentation** remains blocked (the functions under **Methods** are displayed in gray). If the files are suitable, then the **Methods** are accessible.

SFM Evaluation - Original Datatable



- **Select Image**: Selection of the image file. All image files opened in AxioVision are available in the drop-down list box.

- **Select Datatable**: Selection of the relevant data table. All results files opened in AxioVision are available in the drop-down list box.

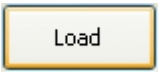


Note:

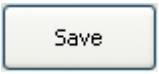
- Make sure that the image and the data table belong together. Otherwise, the cut-out parts of the image in the **SFM Gallery** will not correspond.

- Once both files have been selected, the data will appear in the table field of the window and the functions under **Methods** will become available.

Experiment

- 

: A series of evaluations (series of histograms, scatter plots, etc.) can be loaded from the **Load settings** dialog window that opens. These evaluations are executed immediately.

- 

: This function becomes active as soon as the next evaluations are defined (histogram, scatter plot, etc.). The specified sequence can then be saved as an experiment. The experiments are stored as "INI" files with the path: *"C:\Documents and Settings\All Users\Shared Documents\Carl Zeiss\Data\SFM Evaluation\Settings\Experiment"*.

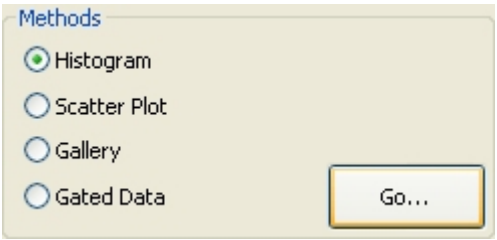
  In this case, the **Save** function overwrites an experiment file that has already been opened.



- 
  Save as: Saves the settings under a new name.

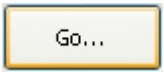
- **Experiment**: Displays the experiment file that has been opened.

Methods



In this field, the evaluation method that is to be used in the next step (**Histogram, Scatter Plot, Gallery, Gated Data**) is selected. The selection is made by clicking the selection box on the left.

Buttons



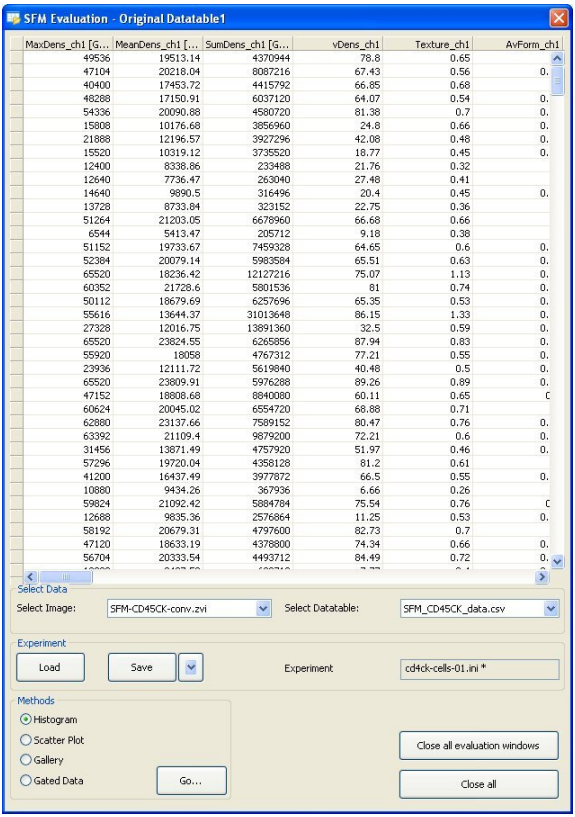
: If you click on this button, the selected method is called up and executed using the default settings.



: Closes all open evaluation windows. If any of the results have not yet been saved, you will be asked beforehand whether these should be saved first.



: Ends the entire SFM evaluation and returns to AxioVision. All SFM-specific windows are closed. You will be asked whether you want to save your data if there are any windows that have not yet been saved.

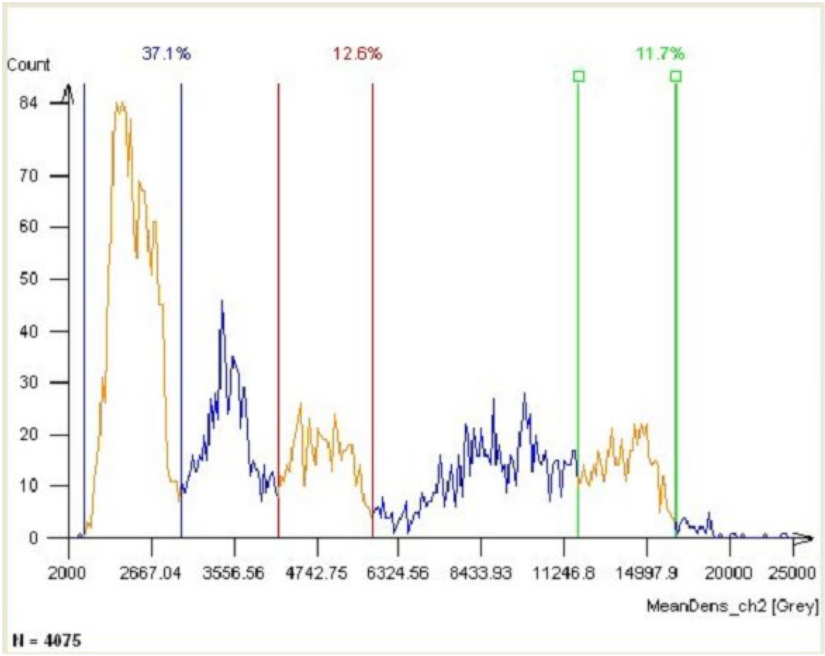


SFM Histogram



Input

The headers display the names of the image file (**Selected Image**) and data table (**Selected Datatable**) being used. The evaluation path up to this point is displayed under **History**.



Graphics

The Graphics field first shows the numerical distribution of the standard parameter:

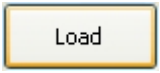
X axis = parameter
Y axis = number
N = total number of objects available for this distribution

Gates: The gates show the delimited areas. The active gate is highlighted by two squares at the top end. The numbers indicate the percentage of objects within the gate in relation to the total number of objects present (N).



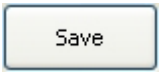
Experiment

Opportunity to save data and call up existing settings again. The file is located under the path: *"C:\Documents and Settings\All Users\Shared Documents\Carl Zeiss\Data\SFM Evaluation\Settings\Histogram "*.

- 

: Opens the **Load settings** dialog window containing the list of settings files available for histograms. Following selection, the settings are loaded and executed immediately.

- 
  - **Load default**: The default setting can be adjusted to the user's requirements under the **Save** function.
  - **Load factory default**: This setting always contains the system's basic setting and may not be changed by the user.

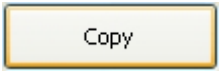
- 

: This function becomes active as soon as changes have been made to the settings. In this case, the active file is overwritten with the changes to the settings.

- 
  - **Save as**: Saves the settings under a new name.

- **Save as default**: The new settings can also be saved as the default setting. These can then be reloaded directly via the **Load default** function.

- **Name**: Displays the name of the settings file currently being used for the histogram. The asterisk (*) after the name indicates that any changes made have not yet been saved.

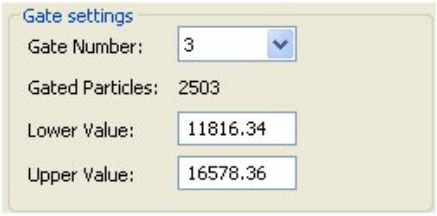
- 

: Copies the histogram as an image to the clipboard.

- **Auto refresh**: By activating this function, every change made to the setting is executed directly in the histogram.

- **Refresh**: This button becomes active if changes have been made to the setting and **Auto refresh** is not active. The changes are only executed by clicking on the button.

Gate settings



- **Gate Number**: Selection of the number of possible gates from the drop-down list (0...3). If no gates are selected, all the data of the histogram are transferred to the next method. If gates are selected, only those objects that are located within the defined gates will be selected.

- **Gated Particles**: Number of objects within the defined gates.

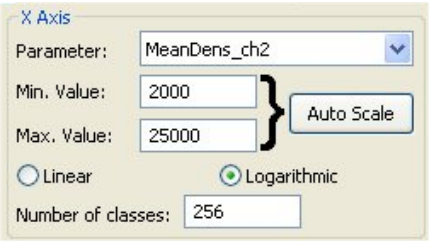
- **Lower Value**: Lower threshold value of the active gate.

- **Upper Value**: Upper threshold value of the active gate.

The values may be entered using the keyboard or by moving the gate boundaries in the graph (clicking the left-hand mouse button). The gate boundaries are moved individually using the squares at the top end. If a gate boundary is selected, it is possible to move the entire gate.

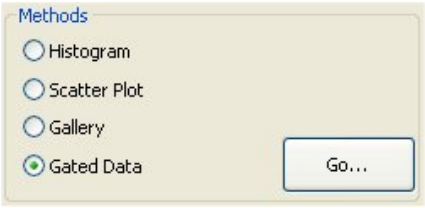
X Axis

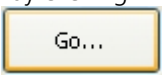
Settings for the measurement parameter.



- **Parameter**: Selection of the measurement parameter from the drop-down list. All the measured parameters of the data table are available.
- **Min. Value**: Lower threshold value of the x axis.
- **Max. Value**: Upper threshold value of the x axis.
- Auto Scale: Automatic setting of the x-axis range using the smallest and largest parameter value in the table.
- **Linear / Logarithmic**: Selection of the method for calculating the class width (linear or logarithmic).
- **Number of classes**: Number of classes for the distribution. The maximum depends on the pixel count available on the x axis (approx. 450).

Methods



Selection of the evaluation method to be used in the next step (**Histogram, Scatter Plot, Gallery, Gated Data**). The selection is made by clicking in the selection box using the left-hand mouse button. Using the 

button, the selected method is called up and executed using the default settings.

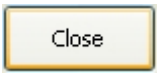
Buttons



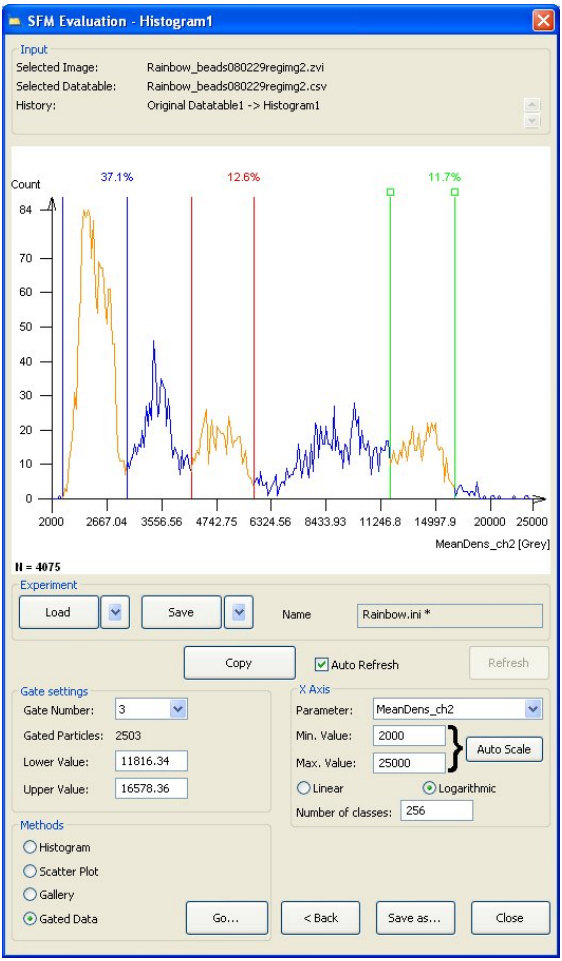
: Closes the histogram window and returns to the last method used (you will first be asked the question **Should it be saved before closing?**).



: Saves the image of the histogram window via the Microsoft Windows **Save Image File As** dialog window.



: Closes the histogram window (you will first be asked the question **Should it be saved before closing?**).

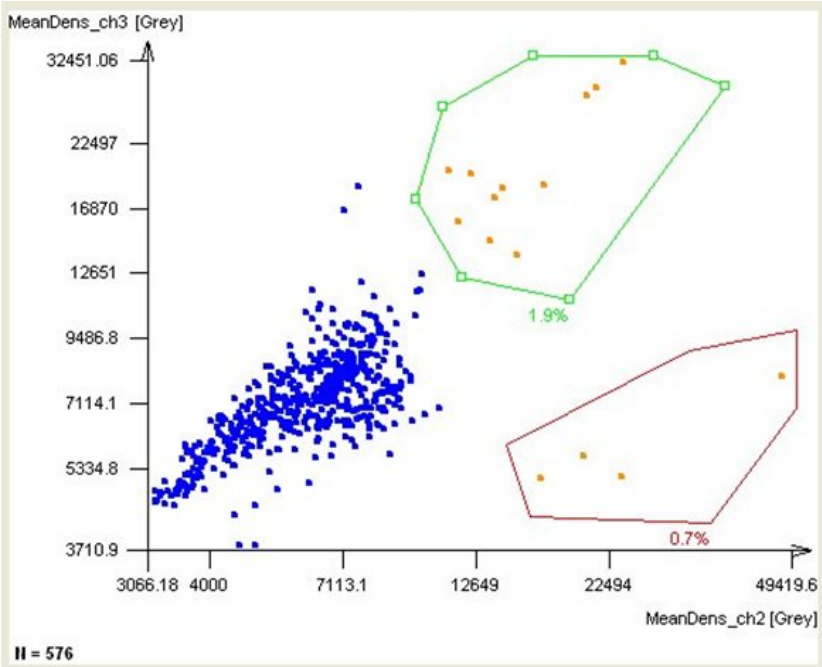


SFM Scatter Plot



Input

The headers display the names of the image file (**Selected Image**) and data table (**Selected Datatable**) being used. The evaluation path up to this point is displayed under **History**.



Graphics

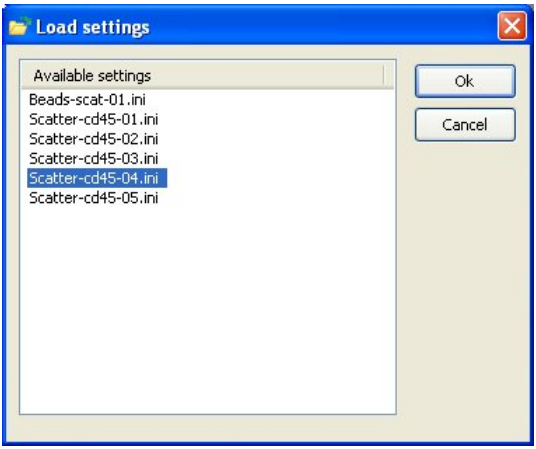
Initially, the Graphics field shows the scatter plot with the standard parameters that correspond to the default settings:

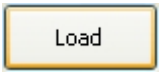
X axis = parameter 1
Y axis = parameter 2
N = total number of objects available for this distribution

Gates: The gates show the delimited areas. The active gate is highlighted by squares at the corner. The numbers indicate the percentage of objects within the gate in relation to the total number of objects present (N).

Experiment

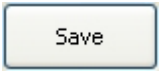
Opportunity to save data and call up existing settings again. The file is located under the path: *"C:\Documents and Settings\All Users\Shared Documents\Carl Zeiss\Data\SFM Evaluation\Settings\Scatterplot"*.



- 

: Opens the **Load settings** dialog window containing the list of settings files available for scatter plots. Following selection, the settings are loaded and executed immediately.

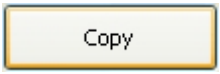
- 
  - **Load default**: The default setting can be adjusted to the user's requirements under the **Save** function.
  - **Load factory default**: This setting always contains the system's basic setting and may not be changed by the user.

- 

: This function becomes active as soon as changes have been made to the settings. In this case, the active file is overwritten with the changes to the settings.

- 
  - **Save as**: Saves the settings under a new name.
  - **Save as default**: The new settings can also be saved as the default setting. These can then be reloaded directly via the **Load default** function.

- **Name**: Displays the name of the settings file currently being used for the scatter plot. The asterisk (✱) after the name indicates that any changes made have not yet been saved.

- 

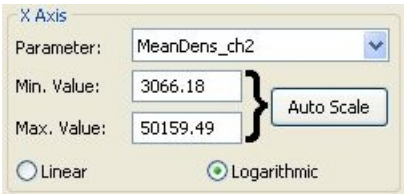
: Copies the scatter plot as an image to the clipboard.

- **Auto Refresh**: By activating this function, every change made to the setting is executed directly in the scatter plot.

- **Refresh**: This button becomes active if changes have been made to the setting and **Auto Refresh** is not active. The changes are only executed by clicking on the button.

X Axis

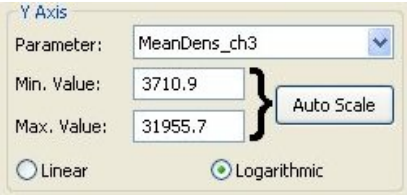
Settings for the measurement parameter.



- **Parameter**: Selection of the measurement parameter from the drop-down list. All the measured parameters of the data table are available.
- **Min. Value**: Lower threshold value of the x axis.
- **Max. Value**: Upper threshold value of the x axis.
- Auto Scale: Automatic setting of the x-axis range using the smallest and largest parameter value in the table.
- **Linear / Logarithmic**: Selection of the method for subdividing the x axis (linear or logarithmic).

Y Axis

Settings for the measurement parameter.



- **Parameter**: Selection of the measurement parameter from the drop-down list. All the measured parameters of the data table are available.
- **Min. Value**: Lower threshold value of the y axis.
- **Max. Value**: Upper threshold value of the y axis.
- Auto Scale: Automatic setting of the y-axis range using the smallest and largest parameter value in the table.
- **Linear / Logarithmic**: Selection of the method for subdividing the y axis (linear or logarithmic).

Gate settings

 (Selection): Using the cursor, an existing gate may be selected so that changes can be made to it. Changes to the size are made by using the cursor to move the corner points displayed; the gate can be moved using the Drag&Drop method by clicking with the cursor within the area of the gate.

 (Rectangle): Allows a rectangular gate to be generated using the cursor.

 (Ellipse): Allows an elliptical gate to be generated using the cursor.

 (Polygon): Allows a polygonal gate to be generated using the cursor.

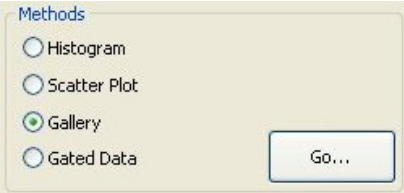
Color 

: Assigns a color to the active gate.



: Deletes the active gate from the scatter plot.

Methods



Selection of the evaluation method to be used in the next step (**Histogram**, **Scatter Plot**, **Gallery**, **Gated Data**). The selection is made by clicking in the selection box using the left-hand mouse button. Using the 

button, the selected method is called up and executed using the default settings.

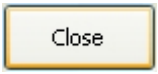
Buttons



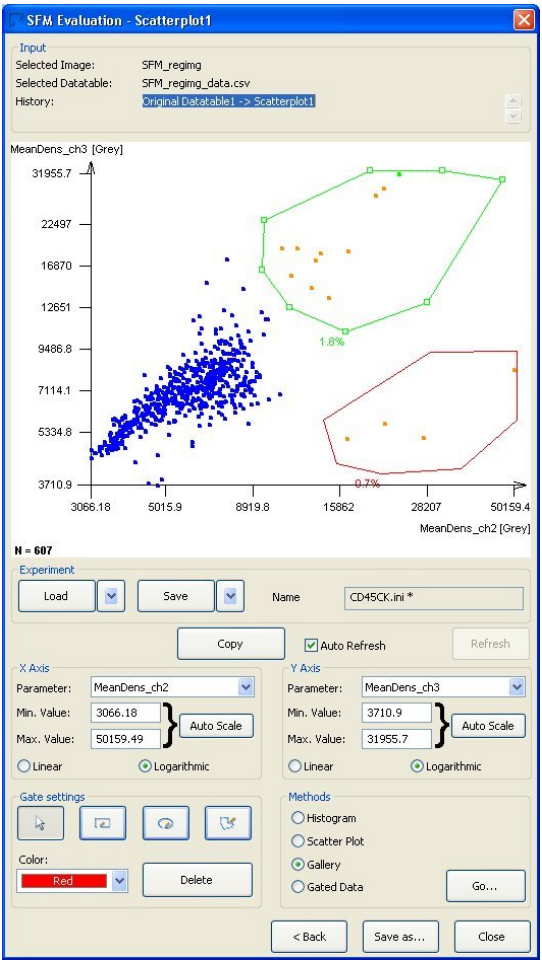
: Closes the scatter plot window and returns to the last method used (you will first be asked the question **Should it be saved before closing?**).



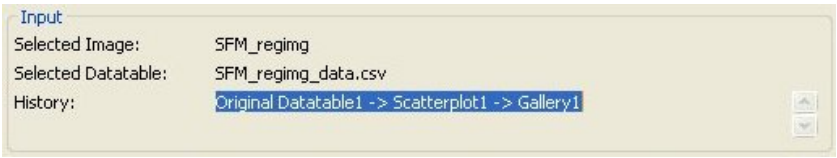
: Saves the image of the scatter plot window via the Microsoft Windows **Save Image File As** dialog window.



: Closes the scatter plot window (you will first be asked the question **Should it be saved before closing?**).

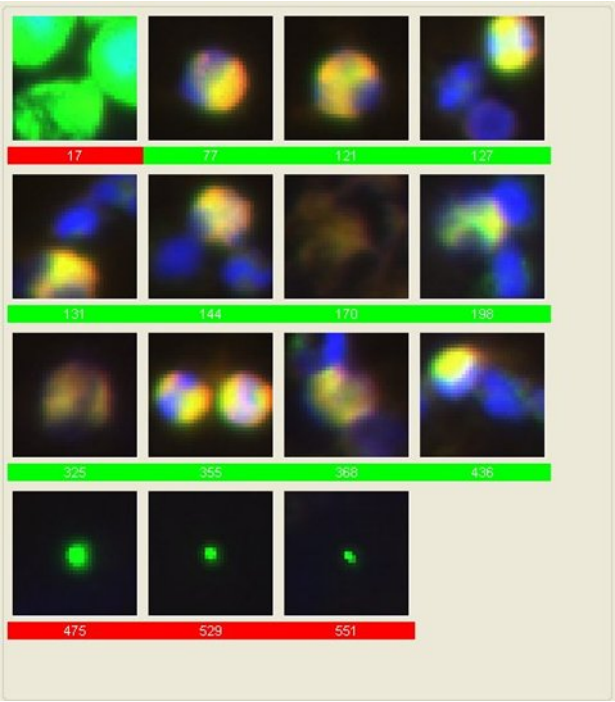

SFM Evaluation - Scatterplot1
Input
Selected Image: SFM_regimg
Selected Datatable: SFM_regimg_data.csv
History: Original Datatable1 -> Scatterplot1
MeanDens_ch3 [Grey]
31955.7
22497
16870
12651
9486.8
7114.1
5334.8
3710.9
1.8%
0.7%
3066.18
5015.9
8919.8
15862
28207
50159.4
MeanDens_ch2 [Grey]
N = 607
Experiment
Load
Save
Name
CD45CK.ini *
Copy
Auto Refresh
Refresh
X Axis
Parameter: MeanDens_ch2
Min. Value: 3066.18
Max. Value: 50159.49
Auto Scale
Linear
Logarithmic
Y Axis
Parameter: MeanDens_ch3
Min. Value: 3710.9
Max. Value: 31955.7
Auto Scale
Linear
Logarithmic
Gate settings
Color:
Red
Delete
Methods
Histogram
Scatter Plot
Gallery
Gated Data
Go...
< Back
Save as...
Close

SFM Gallery


Input
Selected Image: SFM_regimg
Selected Datatable: SFM_regimg_data.csv
History: Original Datatable1 -> Scatterplot1 -> Gallery1

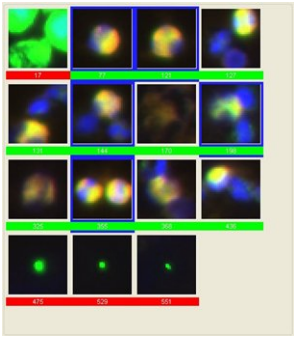
Input

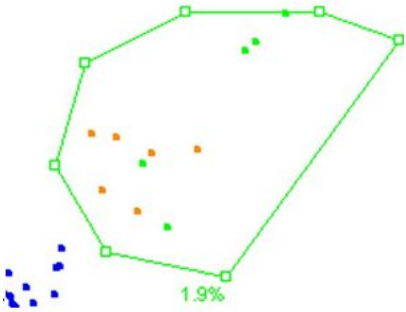
The headers display the names of the image file (**Selected Image**) and data table (**Selected Datatable**) being used. The evaluation path up to this point is displayed under **History**.



Graphics

The Graphics field shows all the objects that have been selected in the last gating step. The colored bar below each cell image corresponds to the relevant gate color for the last method used.





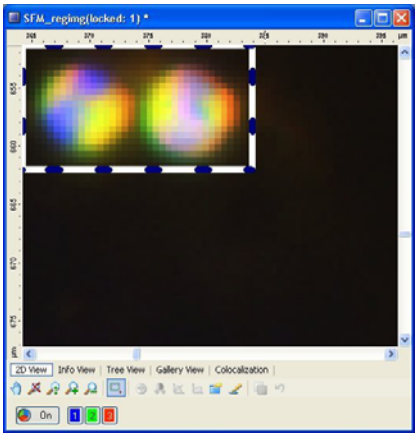


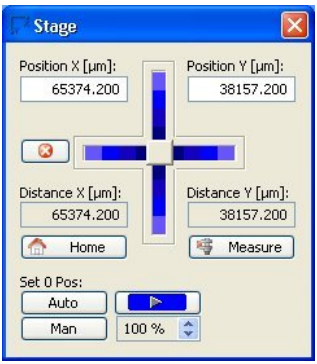
Image selection

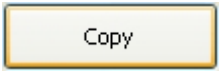
In the gallery, individual images or several images may be selected for further evaluation via **Methods** by using the left-hand mouse button in combination with the **Shift** or **Ctrl** key. The selection is indicated with a blue frame. All selected objects are highlighted in the scatter plot with a different color. In addition, individual objects can be labeled in the AxioVision MosaiX image by clicking with the right-hand mouse button on the gallery image. If a high zoom factor has been applied to the image, the object will appear with the selection frame in the upper left-hand corner of the image frame.

- **Display controls**: In general, the color display for the channels can be switched **On** or **Off** via the color key.

  The contrast settings are only activated for the individual channels. To allow this, the color display must be switched off. Further explanations on the functions can be found in the AV help file menu **View** ⇒ **Properties**.

- **Move stage to**: If this check box has been activated, the stage travels to the position of the object on the sample when you click on a gallery image using the right-hand mouse button. To ensure correct repositioning, the stage must have been calibrated in the x and y direction prior to image acquisition (**Microscope** menu ⇒ **Stage**). Furthermore, the z position at the focus position of the sample should be set to 0 (zero) (**Microscope** menu ⇒ **Stage** ⇒ **Focus**).



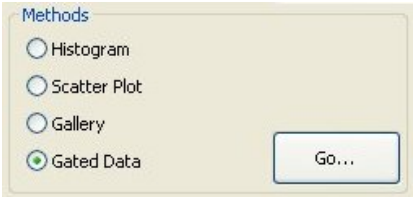
- 

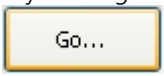
: Copies the content of the gallery as an image to the clipboard.

- **Auto Refresh**: By activating this function, every change made to the setting is executed directly in the gallery.

- **Refresh**: This button becomes active if changes have been made to the setting and **Auto Refresh** is not active. The changes are only executed by clicking on the button.

Methods



Selection of the evaluation method to be used in the next step (**Histogram, Scatter Plot, Gallery, Gated Data**). The selection is made by clicking in the selection box using the left-hand mouse button. Using the 

button, the selected method is called up and executed using the default settings.

- **Zoom Factor**: The zoom factor makes it possible to display the objects in the gallery windows in enlarged or reduced form (1:4 to 4:1). The selection is made via the drop-down list box.

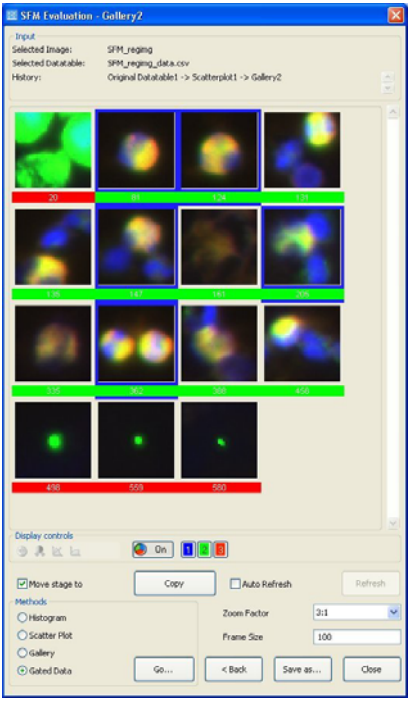
- **Frame Size**: The frame size can be adjusted to your requirements. This is entered into the input field in pixels using the keyboard.

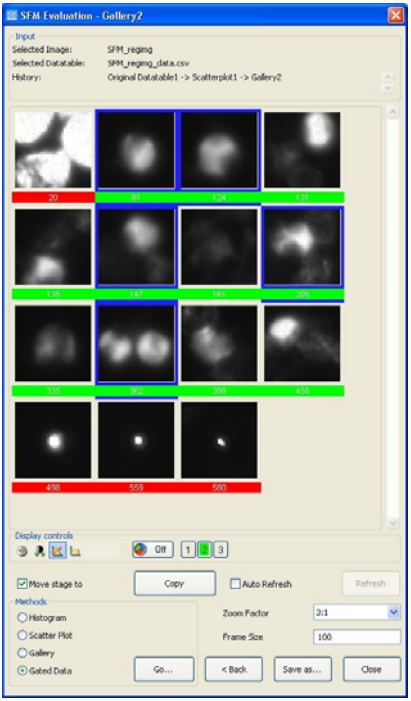
Buttons

< Back: Closes the gallery and returns to the last method used (you will first be asked the question **Should it be saved before closing?**).

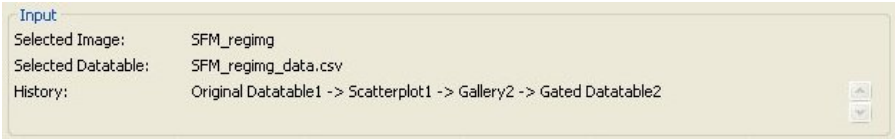
Save as...: Saves the image of the Graphics window via the Microsoft Windows **Save Image File As** dialog window.

Close: Closes the Gallery window (you will first be asked the question **Should it be saved before closing?**).



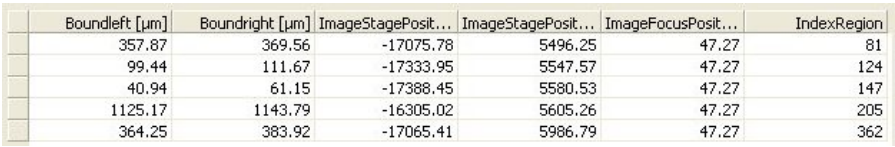


SFM Gated Datatable



Input

The headers display the names of the image file (**Selected Image**) and data table (**Selected Datatable**) being used. The evaluation path up to this point is displayed under **History**.

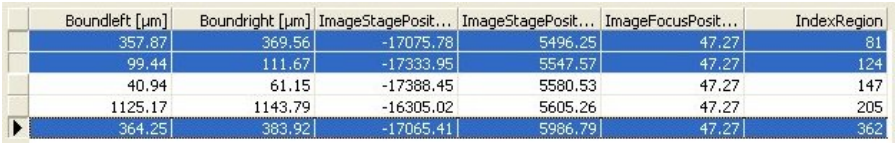


	Boundleft [µm]	Boundright [µm]	ImageStagePosit...	ImageStagePosit...	ImageFocusPosit...	IndexRegion
	357.87	369.56	-17075.78	5496.25	47.27	81
	99.44	111.67	-17333.95	5547.57	47.27	124
	40.94	61.15	-17388.45	5580.53	47.27	147
	1125.17	1143.79	-16305.02	5605.26	47.27	205
	364.25	383.92	-17065.41	5986.79	47.27	362

Data Table

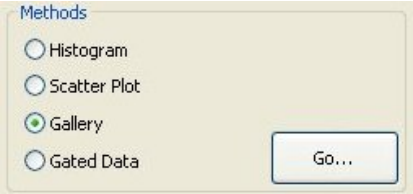
The table shows the list of all parameter values for the objects that have survived all gating processes and selection in the gallery.

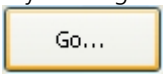
The data may be delimited further in the table by clicking on individual lines or several lines of the list using the cursor (left-hand mouse button) in combination with the **Shift** or **Ctrl** key.



	Boundleft [µm]	Boundright [µm]	ImageStagePosit...	ImageStagePosit...	ImageFocusPosit...	IndexRegion
	357.87	369.56	-17075.78	5496.25	47.27	81
	99.44	111.67	-17333.95	5547.57	47.27	124
	40.94	61.15	-17388.45	5580.53	47.27	147
	1125.17	1143.79	-16305.02	5605.26	47.27	205
▶	364.25	383.92	-17065.41	5986.79	47.27	362

Methods



Selection of the evaluation method to be used in the next step (**Histogram, Scatter Plot, Gallery, Gated Data**). The selection is made by clicking in the selection box using the left-hand mouse button. Using the 

button, the selected method is called up and executed using the default settings.

- **Auto Refresh**: By activating this function, every change made to the setting is executed directly in the table.

- **Refresh**: This button becomes active if changes have been made to the setting and **Auto Refresh** is not active. The changes are only executed by clicking on the button.

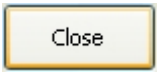
Buttons



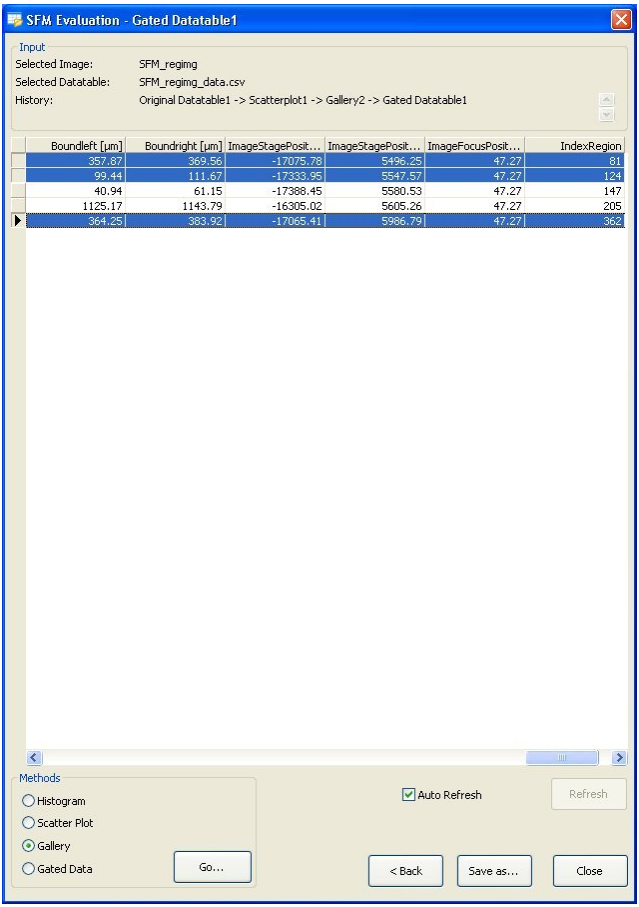
: Closes the Gated Data window and returns to the last method used (you will first be asked the question **Should it be saved before closing?**).

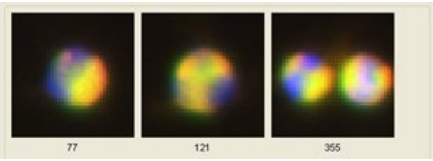


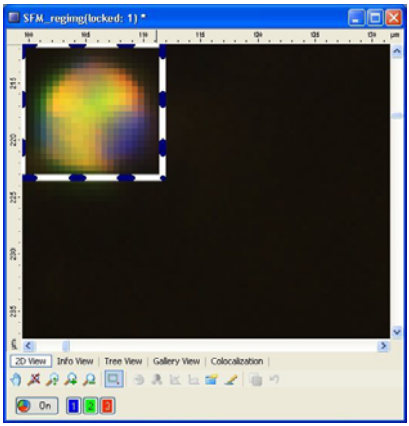
: Saves the Gated Data list as a new data table via the Microsoft Windows **Save Datatable As** dialog window.

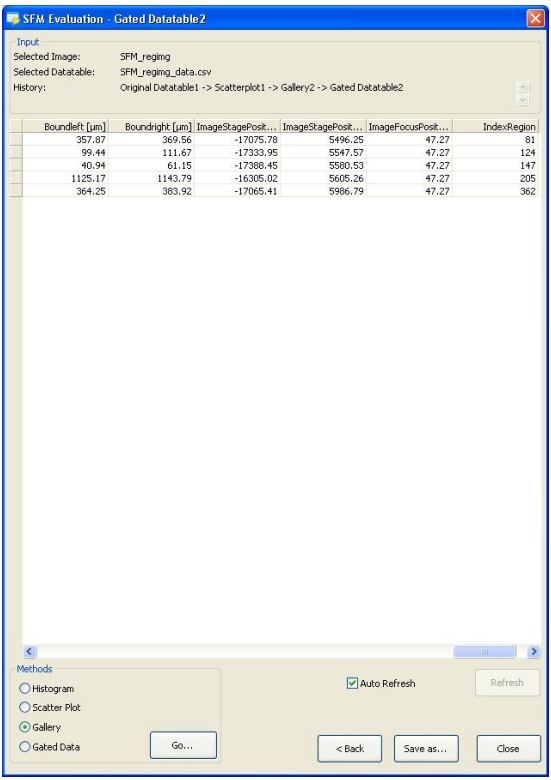


: Closes the Gated Data window (you will first be asked the question **Should it be saved before closing?**).


SFM Evaluation - Gated Datatable1
Input
Selected Image: SFM_regimg
Selected Datatable: SFM_regimg_data.csv
History: Original Datatable1 -> Scatterplot1 -> Gallery2 -> Gated Datatable1
Boundleft [µm] Boundright [µm] ImageStagePosit... ImageStagePosit... ImageFocusPosit... IndexRegion
357.87 369.56 -17075.78 5496.25 47.27 81
99.44 111.67 -17333.95 5547.57 47.27 124
40.94 61.15 -17388.45 5580.53 47.27 147
1125.17 1143.79 -16305.02 5605.26 47.27 205
364.25 383.92 -17065.41 5986.79 47.27 362
Methods
Histogram
Scatter Plot
Gallery
Gated Data
Go...
Auto Refresh
Refresh
< Back
Save as...
Close


77
121
355


SFM_regimg(locked: 1) *
2D View
Info View
Tree View
Gallery View
Colocalization
On


SFM Evaluation - Gated Datatable2
Input
Selected Image: SFM_regimg
Selected Datatable: SFM_regimg_data.csv
History: Original Datatable1 -> Scatterplot1 -> Gallery2 -> Gated Datatable2
Boundleft [µm] Boundright [µm] ImageStagePosit... ImageStagePosit... ImageFocusPosit... IndexRegion
357.87 369.56 -17075.78 5496.25 47.27 81
99.44 111.67 -17333.95 5547.57 47.27 124
40.94 61.15 -17388.45 5580.53 47.27 147
1125.17 1143.79 -16305.02 5605.26 47.27 205
364.25 383.92 -17065.41 5986.79 47.27 362
Methods
Histogram
Scatter Plot
Gallery
Gated Data
Go...
Auto Refresh
Refresh
< Back
Save as...
Close

11.15 Shuttle&Find

Overview

Shuttle&Find is an AxioVision module and uses its own license.

The Shuttle&Find module (S&F) is used to relocate identical positions and defined regions when samples are transferred between different microscopes, particularly from light to scanning electron microscopes and vice versa. Images of the same position on the sample from different microscopes can then be precisely overlaid.

With the LE version of AxioVision (available free of charge), you can make use of the function for loading SEM tiff images.

Important Notes:

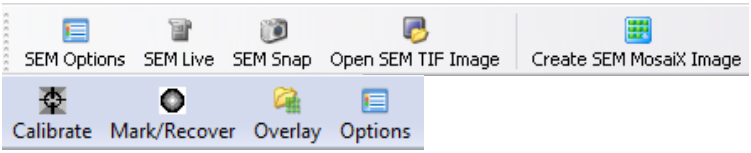
- On light microscopes Shuttle&Find controls both the microscope stage and the focus drive. If used incorrectly, damage can therefore be caused to objectives and/or specimens lying in the stage's traveling range.

- On electron microscopes Shuttle&Find controls the microscope stage. If used incorrectly, damage can therefore be caused to detectors and/or specimens if the height of the stage or sample is too close to the detectors.

- You can call up the online help for a Shuttle&Find dialog at any time using the F1 key.

Setting up Shuttle&FindSetting up

This is the Shuttle&Find toolbar:

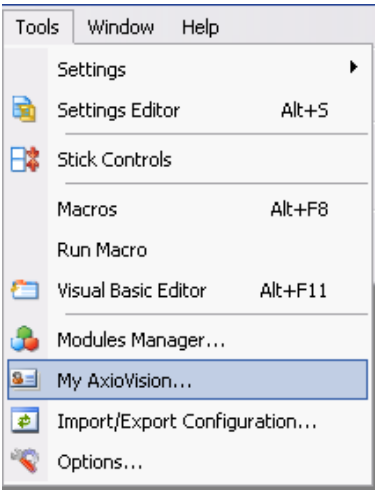


After installing Shuttle&Find, you should define your settings when you use the module for the first time. The settings of Shuttle&Find are user-dependent and therefore only relate to the user currently logged on to the PC.

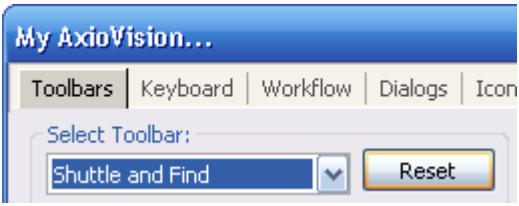
1. Activating the toolbar

Activate the toolbar by right-clicking on the existing toolbars and selecting **Shuttle and Find** from the shortcut menu. All functions can also be called up via the **Applications** menu ⇒ **Shuttle and Find**.

If you have previously installed an earlier version of Shuttle&Find, update the toolbar via the **Tools** menu ⇒ **My AxioVision**.



➢ Select the **Shuttle and Find** entry from the **Select Toolbar** drop-down list box and click on the Reset button.



2. Setting the stage and camera on a light microscope

- Define the settings for the stage and the camera in the same way as for the MosaiX module. When the stage is moved using the stage control the objects in the live image must move in the opposite direction to the one that has been set with the control, i.e.

  a) if you move the joystick forwards or move the slider upwards, the objects in the live image will move downwards.

  b) if you move the joystick or the slider to the left, the objects in the live image will move to the right.

  For further information on this, please refer to section 9.7 "MosaiX" in the AxioVision user manual.

- Set the camera rotation in AxioVision in such a way that the marker in the live image on the monitor is oriented in the same way as when it is viewed through the eyepiece. To do this, insert a ZEISS holder with markers into the microscope stage and locate a marker. Set the light path to the camera.

- Accurate orientation of camera image and eyepiece image can be achieved when rotating the camera together with the camera adapter. Alternatively:

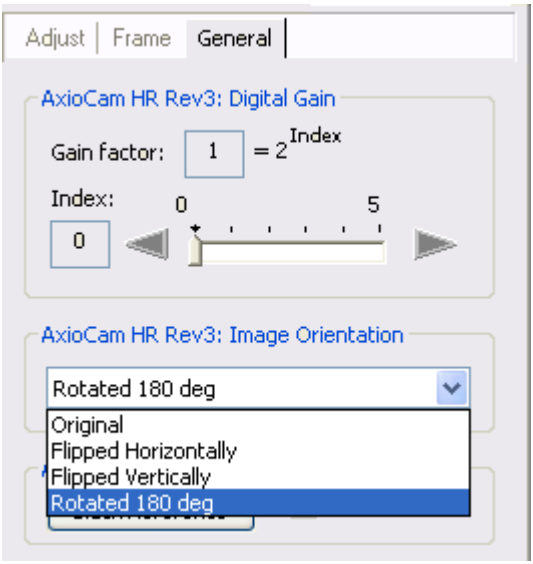
  - Open the live window by clicking on the 

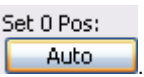
icon. Alternatively, select the **Live** function from the **Acquisition** menu. Observe the marker in the live image and compare the view with the one through the eyepiece.

- To change the image orientation, click on the **Live Properties** button. Alternatively, select the **Live Properties** function from the **View** menu.



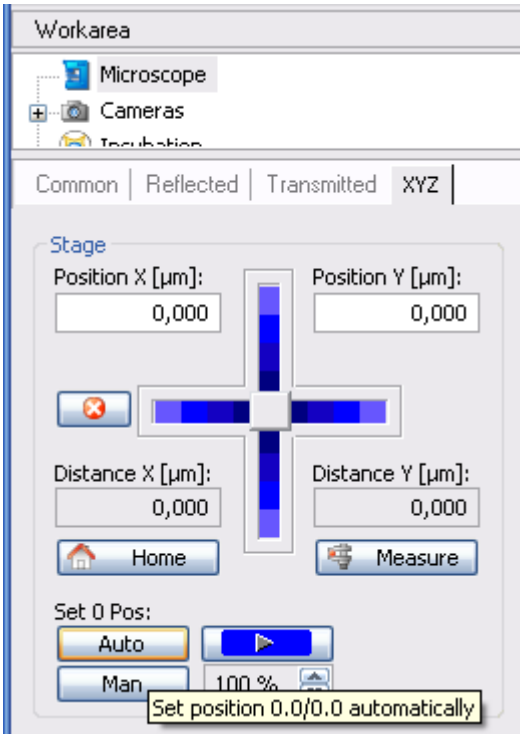
- In the **Live Properties** dialog, select the **General** property page.
- Here, select the appropriate **Image Orientation** from the drop-down list box, so that the live image and eyepiece image correspond.



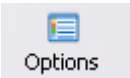
➢ If, when calibrating the coordinate system, you want to use the **To Position 1** function in the normal workflow of Shuttle&Find, always calibrate the stage beforehand using 

.

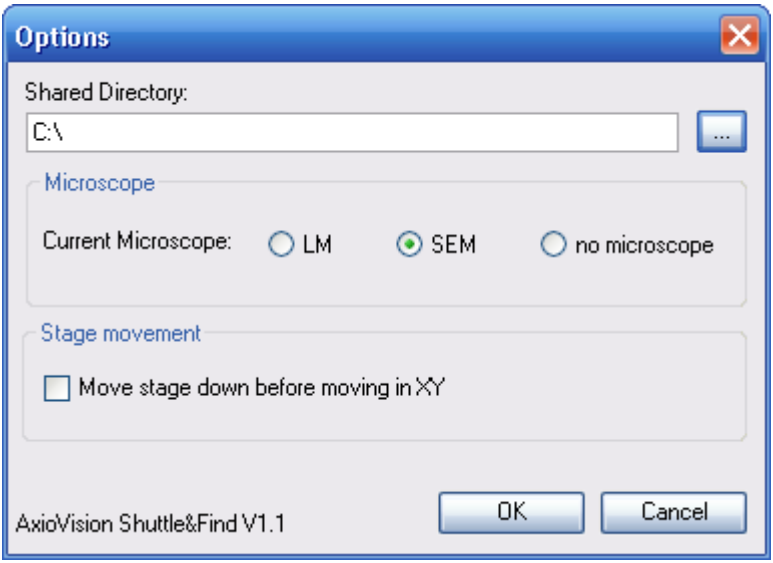
➢ To do this, select the microscope in the work area and go to the **XYZ** property page. Click on the **Auto** button to set the 0 position.



3. Setting options

These options are general settings for the Shuttle&Find module. To call up the Shuttle&Find options, click on the 

icon.



- Click on the ... button to find a shared directory. With Shuttle&Find you will typically be transferring data from one PC to another. It is best if you have a network drive that both PCs can access. Alternatively, you can transfer your data to the other PC using a USB stick, for example.

- Set the microscope type to correspond with the connected microscope: **LM** for a light microscope or **SEM** for an electron microscope. If you have not connected a microscope and, for example, want to define ROIs/points offline, select **no microscope**.

- To avoid collisions, you can activate the Move stage down before moving in XY check box on microscopes with a motorized Z-drive. If this option has been selected, the stage will move downwards first before every movement controlled by Shuttle&Find. On inverse microscopes, the nosepiece will be lowered accordingly before the stage moves.

  - On a light microscope the stage or nosepiece travels to the loading position and then, after the movement, back to the working position.

- On an electron microscope, the stage drops down to its lowest point and then, after the movement, returns to the original position.

Important Note:

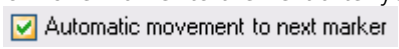
- When moving to the loading position, some microscope stages can travel in the xy direction in addition to the z direction. Make sure that the stage moves only in the z direction. You can define this setting via the TFT display panel on the light microscope:

  - Starting from the main menu select **Settings**, then **Components**.
  - Change to the **Stage** property page.
  - Switch **Stage in load position together with Focus load** to **Off**.

4. Setting the calibration options

➢ Open the calibration dialog by clicking on the 

icon. Select the **Options** property page.

➢ If you want the stage to move from one marker to the next after you have set a position, activate the 

check box. The distances between the markers are specified in the holder files.

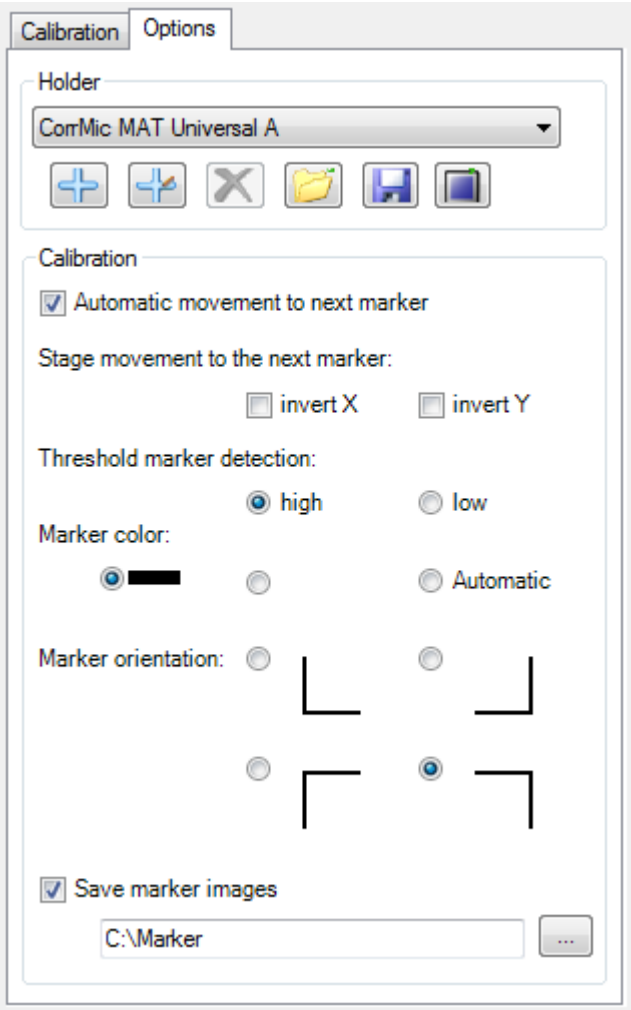
➢ Set the inversions for the movement to the next marker.

On light microscopes Shuttle&Find's XY inversion setting for the movement of the stage to the next marker depends on the stage inversion set in MTB2004 or MTB2004 SE64 and is the same as in the MTB for many stages. This means that it is not usually necessary to make an adjustment, although a one-off check should be performed to ensure that the direction of travel is correct when the next marker is automatically located. An inversion in X or Y results in the stage traveling in the opposite direction when moving to the next marker, e.g. right rather than left.

In the case of an electron microscope, it is usually only Y that is inverted. Inversion on an electron microscope also depends on the rotation of the stage. It is simplest to perform the calibration using automatic movement to the next marker. Correct the movement of the stage during this movement

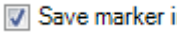
using the inversion settings. It is essential to ensure that the detectors are far enough away from the stage during this check.

You only need to recheck and/or, if necessary, reset the inversion settings if you insert a different type of holder or insert the holder with a different orientation.



Shuttle&Find offers the option of having the markers detected automatically and therefore speeding up the calibration process considerably. The following settings relate to this:

You can leave the threshold value for marker detection at **high**. For damaged markers with an L structure that has an aspect ratio that is not 1:1 we recommend that you specify the low setting.

- Set the **Marker color** and **Marker orientation** to correspond with the appearance of the markers in the camera's live window or, in the case of an electron microscope, in SmartSEM. After the marker has been automatically detected, you will be shown a control image of the marker with a red reticle, from which you can see whether the marker has been detected correctly. If desired, this control image can be stored permanently using 

.

- On a light microscope, define the scaling for all objectives used in micrometers (in no case pixel). Alternatively, you can set the scaling for an objective and activate the automatic scaling. You will find instructions on scaling in section 8.4 "Scalings" of the AxioVision User's Guide.

Procedure for using Shuttle&Find

There are various ways of using Shuttle&Find. You can start on a light microscope and relocate the position on the same instrument, switch to a different light microscope or to an electron microscope or even start from an electron microscope.

In the case of an electron microscope, start the SmartSEM user interface before opening the AxioVision software.

In principle, the basic procedure is the same in all cases:

1. Start with the calibration on a microscope on which Shuttle&Find has been set up correctly (to do this, read the section "Setting up Shuttle&Find").

2. Acquire images of the sample positions that you want to relocate and save them. If necessary, switch to another microscope on which Shuttle&Find has been set up correctly and perform calibration again. In principle, calibration must be performed again each time the holder is changed or inserted.
3. Acquire images of the sample positions that you want to relocate. Save these using the **Mark/Recover** function.

After locating the position(s) it is possible to lay two images, from different microscopes for example, over one another and add them to AxioVision as a new image. To do this, use the **Overlay dialog window**.

1. Setting up Shuttle&Find

If you have never used Shuttle&Find before or if you wish to use a new holder, you must configure the settings for the module. Please also read the previous section "Setting up Shuttle&Find".

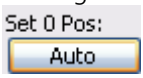
2. Inserting a holder containing a sample

Insert a holder containing your sample into the microscope. Please observe the instructions for use enclosed with the relevant holder.

3. Calibrating holders

Calibration is required in order to define the positions of the markers on the holder. There are three markers on each ZEISS holder. These 3 positions are used for calculating the target position during relocation. It is also possible to define your own holders (please refer to the section "Creating new holders"). There must, however, be 3 markers on such holders.

Calibration is always performed twice (unless you stay on the same microscope with the holder): once at the beginning of the process and again after you have switched to another microscope.

When using the **to Position 1** function on a light microscope, make sure that the stage has been calibrated using the 

function. The **to Position 1** function moves the stage to the first defined marker position.

Open the calibration dialog window by clicking on the 

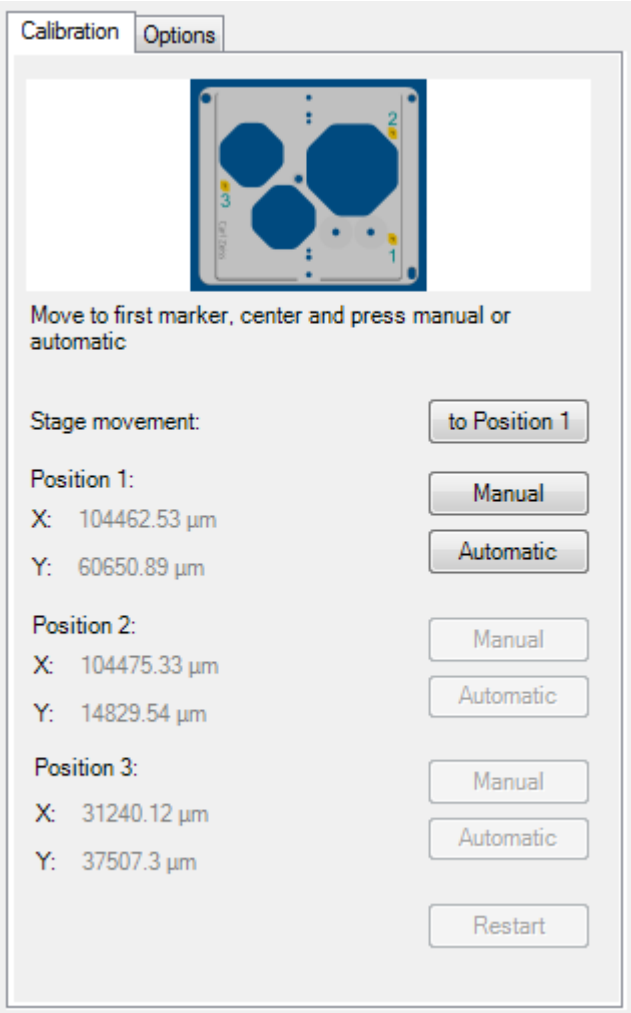
icon. Depending on the microscope that has been set up, the dialog window is called either **SEM Calibration** or **LM Calibration.**

The calibration process on a light microscope is described below. For information on this process in the case of electron microscopes, please see point 6 " Calibrating holders" below.

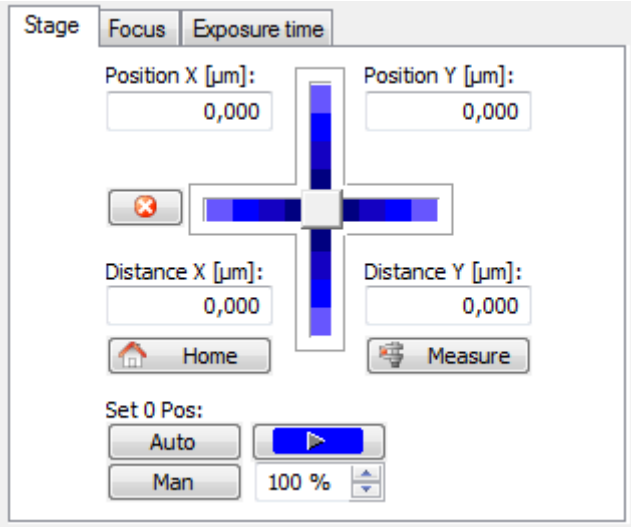
If the **Calibration** tab sheet is active, the window consists of three elements:

- Control menu for controlling the calibration process
- Menu for controlling the stage, focus and camera, as required
- Live image for the convenient monitoring of the calibration process

The control menu:



Menu for controlling the stage, focus and camera:



- Select the holder used on the **Options** property page. It is also possible to use a holder that you have defined yourself (for more information on this, please refer to the "Creating new holders" section).

- Move the stage to the first marker and adjust the microscope to ensure that the small marker can be seen clearly and in full.

It is generally recommended that you use the 20x or possibly also the 10x objective.
It is important, however, that the same objective is used for calibration and image acquisition to increase relocation accuracy during the shuttle process!
Each of the markers is comprised of a large L and a small L. The large L can be identified with the naked eye and is used to locate the small L, which is used to perform the calibration. It is located within the large L. The structure of a marker is shown in the figure on the right.



The sequence of the markers is displayed by means of numbers in the holder image. The numbers themselves can also be found on the holder. In the screenshot above the markers have a yellow background.

- The 3 positions of the markers are set one after the other. Observing the live image and using the control menu for the stage, focus and camera as required, first locate marker no. 1. For the rough positioning of the large L we recommend that you use an objective with low magnification initially and only switch to the objective you want to use for calibration and subsequent image acquisition after this.

- Focus the objective. This can either be done manually or, if your system offers this, using the autofocus function.

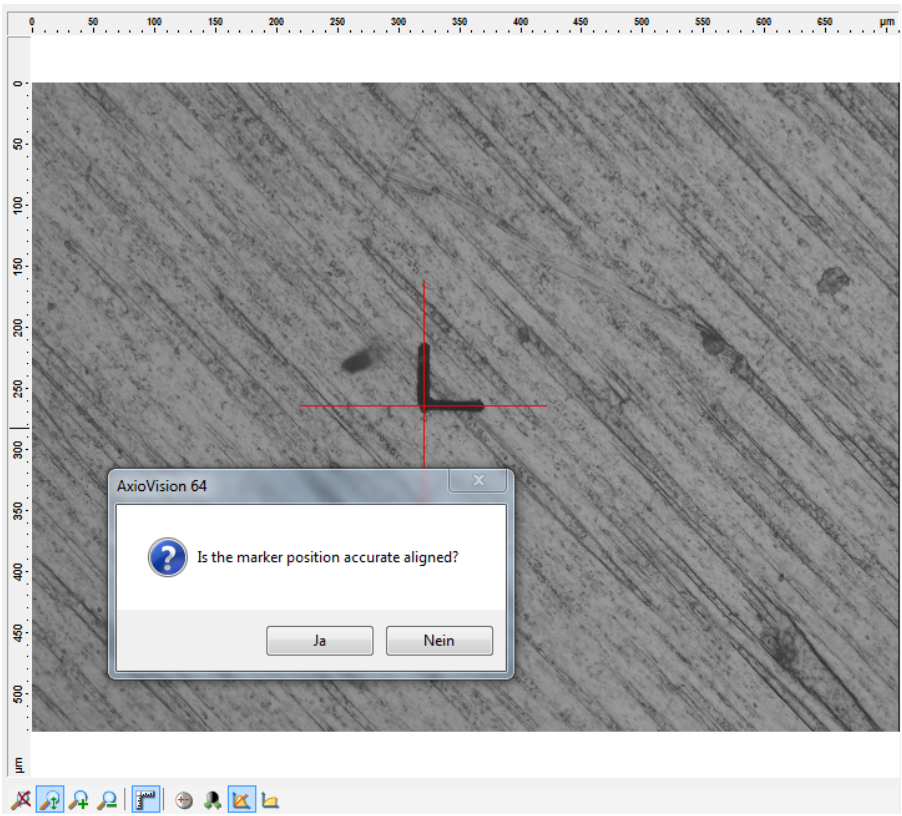
- Next you can decide whether you want to use the automatic detection function for the marker or if you prefer the manual procedure. Experience has shown that our automatic marker detection is very reliable. We therefore recommend that you choose this option. The manual version is mainly useful if the automatic function fails or if you are using markers that are not L-shaped.

- Then click on the 

button to adopt the current stage position as the marker position 1.

The corner of the small marker is used for the marker position.

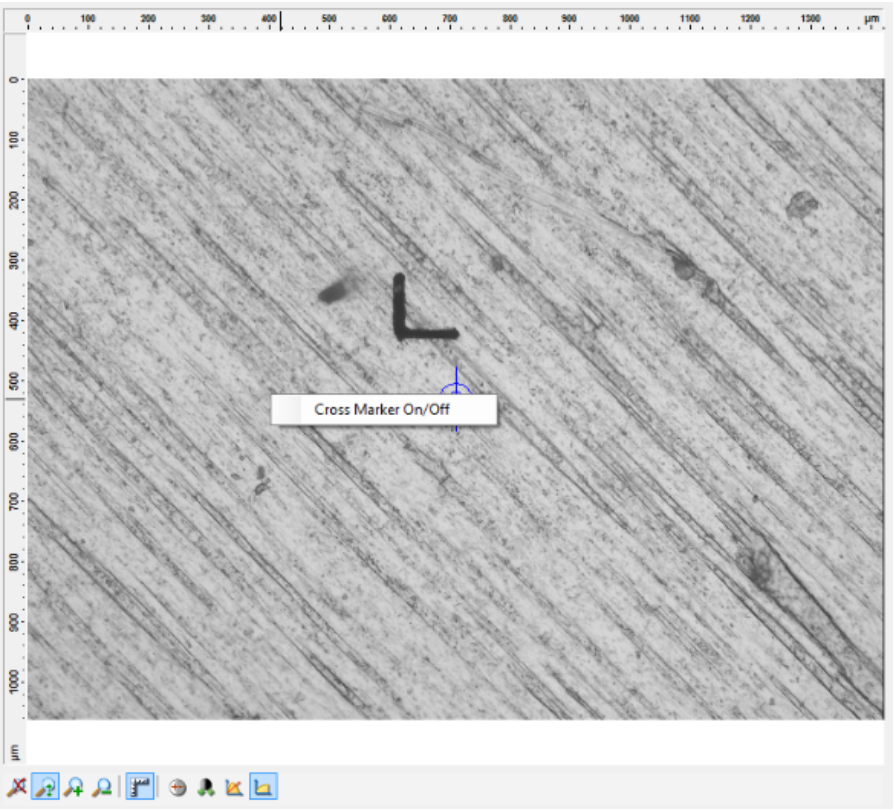




AxioVision now displays a control image of the acquired marker together with a red reticle. The image above shows an example of correct marker detection. Selecting **Yes** in the **Is the marker position accurate aligned?** window prompts the system to locate the next marker position 2. Repeat this process until all three marker positions have been correctly detected. Close the **LM Calibration** window by clicking on **OK**.

Manual marker calibration:

If the automatic marker detection function fails or if you are using different markers that are not L-shaped, please use manual marker detection. Right-clicking in the live image brings up a reticle:



By moving the stage in the XY direction, position the reticle precisely over the marker. Clicking on Manuell results in the marker position being adopted into the system. The procedure continues in the same way as for the automatic calibration process.

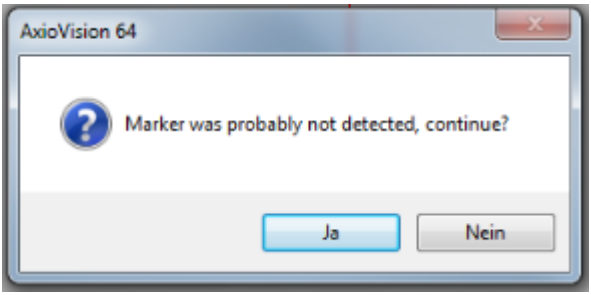
General information on the calibration process:

- If you set the position manually, center the stage as precisely as possible on this point.

- With the **Automatic** function it is sufficient for the marker to be located in the image field. It is detected automatically and its position is calculated.

- If Save marker images has been selected under **Options**, AxioVision saves the 3 control images of the last calibration process in the selected folder.

- If the marker has not been detected or has not been detected properly, a dialog window will open:



  Yes results in the marker coordinates being adopted, **No** gives you the option of now performing the calibration manually.

- After the first and second marker positions have been set, the stage moves to the next position. You can activate and deactivate this on the **Options** property page by means of the Automatic movement to next marker check box. The route is indicated in the holder file.

- It is not possible to select any other holders under the options during calibration.

- As soon as the position of the first marker has been set you can relocate it. To do this, click on the to Position 1 button.

- If you want to restart the calibration, click on the 

button to reset the values.

- Focus each marker and make sure that the small marker can be seen fully in the image.

- If the stage moves in the opposite direction to a marker, you will need to configure the settings for stage inversion again.

- Click on the 

button to move to the first position again and reset the values by clicking on the 

button.

- Go to the **Options** property page. If the stage has moved incorrectly when moving to the second marker, invert Y. If it has moved incorrectly when moving to the third marker, invert X. These settings only apply for the following ZEISS holders: "S&F Cover slip holder", "S&F Grid holder" and "S&F Materials universal holder".
- Once you have set all 3 positions click on the 

button to save the calibration in the current holder file. You can view the calibration again at any time by opening the calibration dialog window. The positions are displayed again in gray.

- **Important note:**
  Due to potential center deviations between individual objectives, we recommend that you perform calibration and acquire images with the same objective wherever possible!

4. Acquiring images

Acquire images of positions that you will want to relocate under the same or a different microscope. How to acquire an image with the LM is described in the AxioVision manual in chapter 3.3 "Step by step to the first image".

In Shuttle&Find you can also use light microscope images that you have created using the MosaiX module. You can load the tile images in exactly the same way as individual images in the **Mark/Recover** dialog window. If you

use the automatic stitching function for the individual tiles, however, precision is reduced when the positions are relocated.

Then save the images using the 

function. The calibration is also saved in 4 user fields in the image. Only images that have been saved after a calibration process can be used for relocation. You can view the calibration in the image in the image's property window on the **Info** property page under user fields 1-4.

5. Switching to a different microscope

Remove the holder from the microscope and switch to another microscope.

In the case of a light microscope you will need an extra frame for the sample holder.

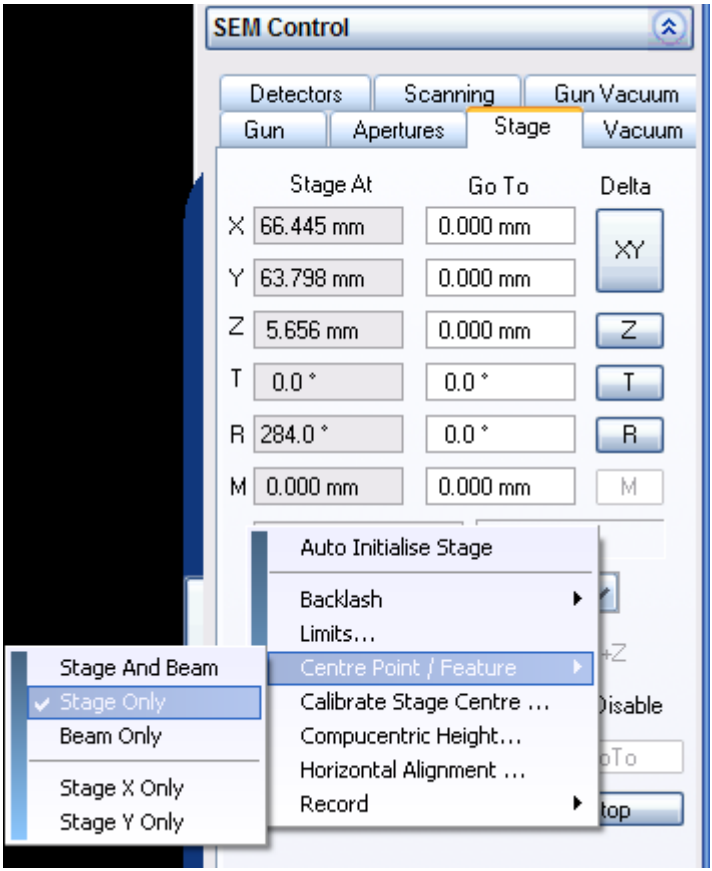
With electron microscopes the adapter for the stage differs depending on the holder. What they all have in common is the dovetail on the bottom. Attach the adapter for the SEM and insert the holder. Make sure the orientation is correct: the flat side of the dovetail is positioned first.

6. Calibrating holders

Perform the calibration described in the point above again on the other microscope.

There are a couple of points to bear in mind with regard to Shuttle&Find on an electron microscope:

- The stage rotation is not taken into account.
- If you are using a ZEISS holder, rotate one-time the stage mechanically to ensure that the L marker is straight in the image.
- With Shuttle&Find the beam shift must be switched off.
- If you use **Center Point / Feature**, deactivate the use of the beam shift. Call up the shortcut menu by right-clicking on the **Stage** property page. Select **Center Point / Feature** ⇒ **Stage Only**.

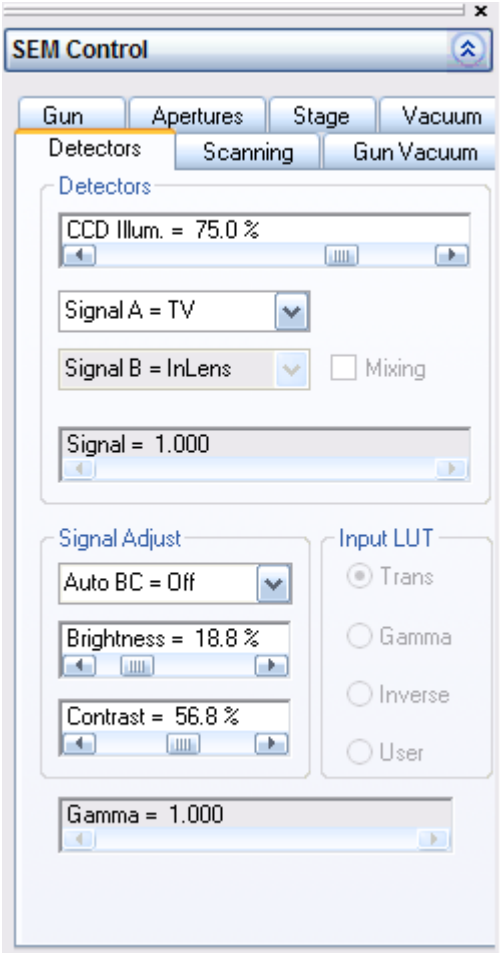


Do not use scan rotation.

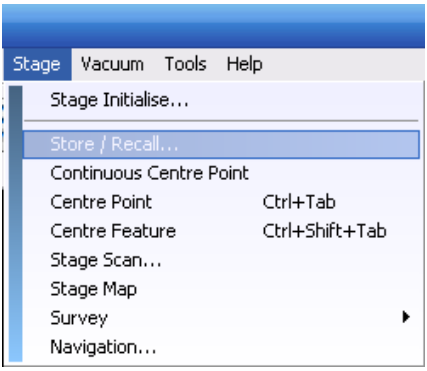
It may be the case that the stage on an SEM moves left/right to the next marker rather than forwards/backwards. In such cases rotate the stage by 90°.

- Each time before you click on the **Automatic** button, set the SEM parameters so that the small L marker can clearly be seen in the image. **Focus** and **Contrast/Brightness** are most important. You can either adjust the focus manually or by means of the autofocus function, using the **Ctrl+F** key combination.

- The **Contrast/Brightness** setting can also be adjusted manually or you can set **Auto BC** correction on the **Detectors** property page in the **Signal Adjust** field.



If you have located the position of the first marker and the stage is correctly oriented, save this position in SmartSEM in the **Stage Points List**. To do this, select the **Store / Recall...** function in the **Stage** menu.



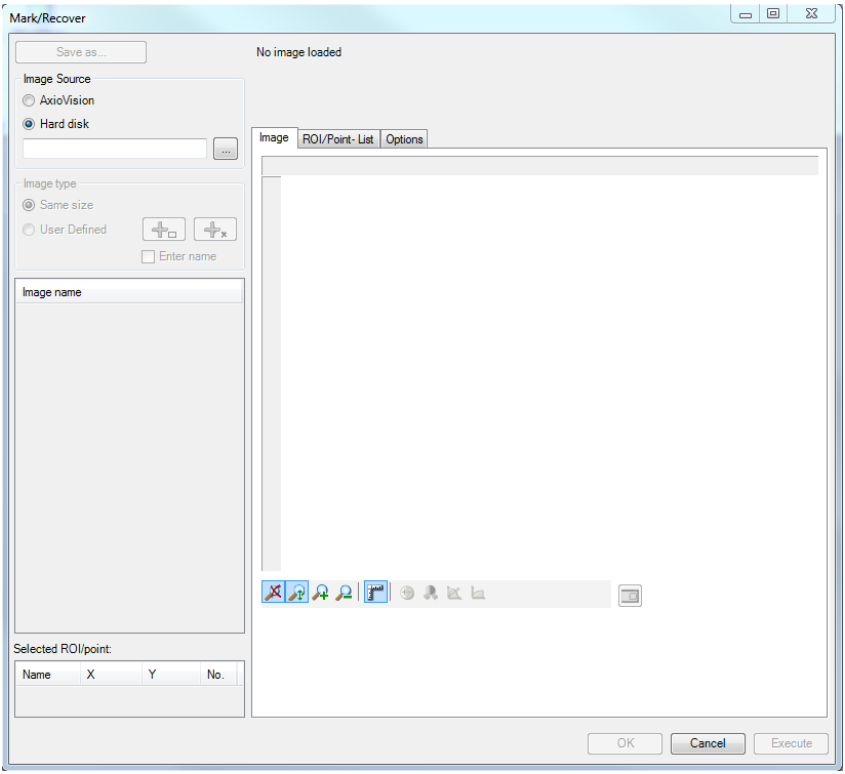
➢ This opens the **Stage Points List** dialog window. Click on the **Add** button here to save the current stage position. You can locate the saved position by double-clicking on an existing entry in the list.



7. Mark/Recover

➢ Open the **Mark/Recover** dialog window by clicking on the 

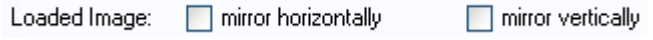
icon.



By selecting the **AxioVision** or **Hard disk** image source you have two options for loading images into the **Mark/Recover** window:

- **AxioVision**: The images from the current AxioVision work area are displayed in the **Image Source** list. If you click on the name of an image, the corresponding image is loaded into the viewing window.

- **Hard disk**: If you select a folder path, images saved there are loaded into the viewing window. Other images saved in the folder will then appear in the **Image Source** list and can also be selected by clicking on them.

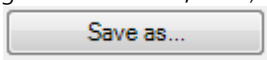
You can flip the current image horizontally or vertically using the 

settings on the **Options** property page. Images from an inverse microscope may have a different orientation compared with images on an electron microscope or upright microscope.

- Activate the **Same size** mode in the **Image type** field. Click on **Execute** to relocate the center point of the loaded image. On an electron microscope the magnification is set in such a way after the position is located that it corresponds to the frame of the loaded image. Depending on the setting, the working distance (*focusing correction)* is also set. Focusing correction can be activated and deactivated on the **Options** property page.

- Using the **User Defined** mode in the **Image type** field you can draw regions of interest (ROIs) or points that you want to locate into the image. You can define a maximum of 100 ROIs/points. Using the mouse you can also subsequently amend ROIs/points that have already been defined.

- Activate the **Enter name** check box if you want to allocate a name to each ROI/point. The **ROI/Point-List** property page also allows you to perform subsequent editing.

- Regions (regions of interest, ROIs) or points in the image can be saved by clicking on 

.

- Other settings can be configured on the **Options** property page. You will find additional information on this in the online help (F1).

To navigate quickly to a position in the image, you can use the mouse to double-click on a position in the loaded image. The microscope then travels to this position. You can activate this double-click function on the **Options** property page 

.

- To close the dialog window, click on the cross in the top right-hand corner or on the **Cancel** button.

8. Acquiring images

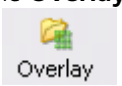
On a light microscope, click on the 

icon to acquire an image from the camera. In the case of an electron microscope, click on the 

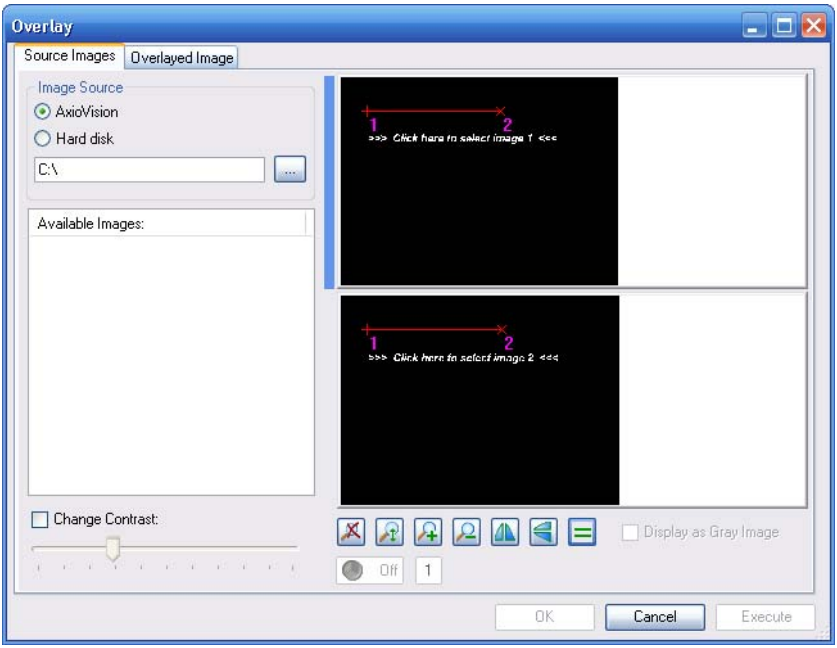
icon.

Overlaying images

It is possible to combine images from different microscopes into one image. To do this, use the **Overlay** dialog window.

- Click on the 

icon to open the **Overlay** dialog window.



- In the **Image Source** field on the **Source Images** property page select where you want to load images from.

  - **AxioVision**: All the images open in AxioVision are listed under **Available Images**.
  - **Hard disk**: Click on the ... button to select a folder.

- Left-click on an image under **Available Images** to load the image into the selected window. The current window can be identified from the blue bar. In the screenshot above the top window is selected.

- You can change the contrast for each image to make regions more clearly visible. Activate the Change Contrast: check box**.** Move the slider using your mouse to change the contrast. If you deactivate the check box again, the contrast is reset; in the case of images from AxioVision, the contrast is adopted from the AxioVision image.

- In both images a line is inserted that determines which points in the two images are assigned to one another. Take two points that are as far apart from each other as possible and can be identified in the two images as belonging to each other. Set the lines by clicking on the end points and moving the mouse with the left mouse button held down. To increase accuracy, it may be beneficial to optimize the size of the images displayed using the  tools.

- Click on the **Overlayed Image** property page. The overlaid image is displayed on this property page and you can set the type of overlay.

- Click on the **Execute** button to adopt the overlaid image into AxioVision as a new image, or on the **OK** button if you also want the window to be closed afterwards.

  Important note: Before you can continue working with AxioVision, the overlay window must be closed.

Creating new holders

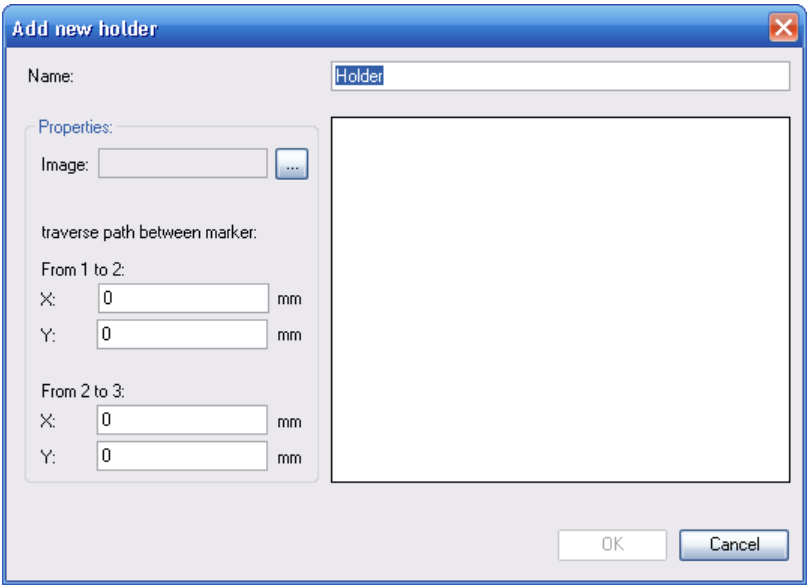
In the calibration dialog it is possible to define a holder yourself in addition to the existing holder files.

- Click on the 

icon to open the calibration dialog window. Go to the **Options** property page.

Under the **Current holder** drop-down list box you will find the buttons for holder management.

- Click on the  icon to open the dialog window for a new holder.



- Enter the name of your holder in the **Name** input field. Once you have created your holder, you can no longer edit the name.

- It is recommended that you also specify an image of your holder to obtain a better overview. Click on the ... button to open an image. You can use an image in the formats .bmp, .gif, .exif, .jpg, .png or .tif.

It is always assumed that there are 3 markers on the holder. In the holder file define the distances in millimeters between the first and second markers and between the second and third markers. The values for the distances in X and Y may not both be 0.

You can define traverse paths using the shown stage positions in AxioVision.

- Observe the camera's live window on the right-hand side of the calibration window.

- Switch on the cross marker. To do this, right-click on the live image, and in the context menu click on the **Cross Marker On/Off** function.

- Move the stage to a marker and center the stage on this marker using the cross marker in the live image.

- Note the current stage position on the stage menu of the calibration window. Move and center the stage to another marker and read out the second position.

- Subtract the two positions from each other to specify the distances in the x- and y-direction. You can refer to the predefined ZEISS holders to determine the signs.

- AxioVision shows the stage positions in µm. Divide the stage positions by 1000 to convert to mm. Afterwards you can enter them into the **Add new holder** dialog window.

- Click on the 

button to create the holder file and close the window.

The new holder is selected as the current holder in the drop-down list box. You can also export or import your holder.

- Click on the  icon to import a holder file.

- Click on the  icon to export a holder file. Any calibration saved in a holder is deleted during export.

Editing holders

The marker distances and the image can be subsequently edited in a previously created holder file.

- Click on the 

icon to open the calibration dialog window. Go to the **Options** property page.

- Under the **Current holder** drop-down list box you will find the buttons for holder management. Click on the  icon to edit the current holder.

The predefined holders are write-protected. These holders can be opened and viewed by clicking on the  icon. They cannot be edited, however.

Fine calibration

To achieve even better accuracy in locating positions, you can set a fine calibration value for a loaded image. This value only applies to the current image and is also not saved. It remains available for as long as the image is loaded. As soon as you load another image or close the dialog window, it is discarded again.

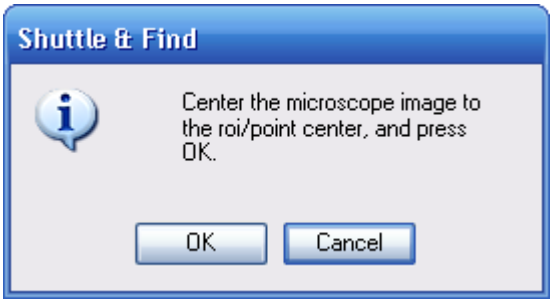
Once you have located your position on a microscope (please read the section "Procedure for using "Shuttle&Find" or the section "Example 2"), you can define a point for determining the fine calibration value.

- Click on the  icon and then left-click on a prominent position in your loaded image.

- Go to the **ROI/Point-List** property page and select your drawn-in point.

- Click on the  icon. Fine calibration starts. The point is located and the dialog window switches to the **Image** property page.

Now compare the position in the image and the position located on the microscope, and correct the microscope stage so that the microscope position corresponds as precisely as possible with the point in the image.



➢ Click on the **OK** button to end fine calibration.

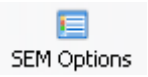
The check box for the fine calibration value is now activated under the options: ☑ Use fine calibration value . Uncheck it if you do not want to use the value. If you load another image or close the dialog window, the value is discarded and the check box is deactivated again.

The SEM image

You can acquire an SEM image on an electron microscope by clicking on the SEM Snap icon. It is always the current image that is acquired. If you use a slow scanning speed, make sure that the SEM image is frozen using the SmartSEM software before acquisition. Otherwise, it is possible that the image will not have been completely updated when it is acquired.

The acquisition settings on the SEM are contained in the images. These can be displayed in the image's property window on the **Info** property page by selecting the **SEM Parameter** or **All** display.

In the dialog window of the **SEM Options** function you can set whether and what information from the SEM image should also be displayed as annotations following the acquisition of an image. These settings relate to the **SEM Snap** function and the **Open SEM TIF-Image** function.

- Open the **SEM Options** dialog window by clicking on the 

icon.

In the online help you will find further information about the dialog window **SEM Options**.

Application examples for Shuttle&Find

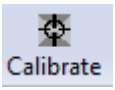
The following two examples explain two possible ways of using the Shuttle&Find module.

Example 1: from light microscope (LM) to electron microscope (SEM)

In the following example an image is acquired on an Axio Observer light microscope and a region of interest (ROI) is located on an electron microscope. The magnification is set in such a way on an SEM that the SEM image corresponds to the selected frame. In addition, an SEM image is acquired and overlaid with the LM image.

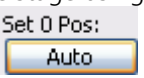
- Set up Shuttle&Find on both PCs, if the settings have not yet been defined (see the section "Setting up Shuttle&Find").

- Start with the Axio Observer light microscope.

- Open the calibration dialog window by clicking on the 

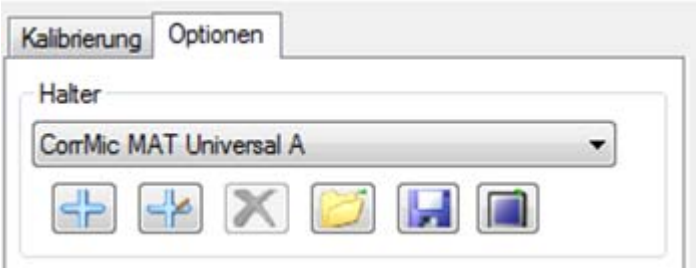
icon. Select the correct holder on the **Options** property page. This selection will also be retained after you exit AxioVision.

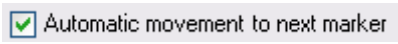
- Open the **Calibration** property page.

- Calibrate the stage using the automatic calibration function. To do this, click on the 

button in the **Stage** menu of the calibration dialog window.

- Insert your holder containing your sample(s) into the light microscope and locate the first marker manually. Set the magnification in such a way that the small marker is clearly visible and completely within the image, and adjust the focus.



- In the **Holder** field activate the 

check box.

- Go to the **Calibration** property page to set the marker positions.

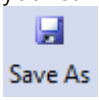
- Click on the 

button to set the position using automatic marker detection. Once the marker position has been determined, the stage moves to the next marker. Check that detection has been performed correctly in the live image and travel to the next marker position by clicking on the **OK** button.

- Repeat the last step for the second and third markers.

- Click on the **OK** button to save the calibration and close the window.

- Move to an interesting position on your sample and acquire an image.

  Save your image by clicking on the 

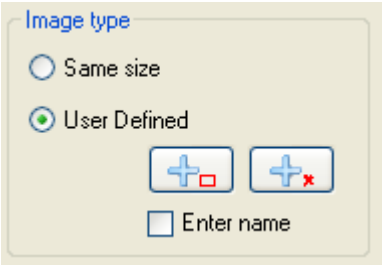
  icon.

  - Alternatively open the **Mark/Recover** dialog window by clicking on the 

    icon.

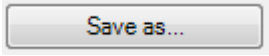
- Select AxioVision as the image source: the images from the current AxioVision work area are displayed in the **Image Source** list. This therefore also includes the last image acquired. Select this by clicking on it.

  - If you wish, you can define a region. To do this, select the **User Definded** image type and click on the  icon.



- Draw a region into your loaded image. Left-click on the top left-hand corner of your region and, with the mouse button held down, drag out a rectangle. Release the mouse button to define the rectangle.

- The rectangles that have been dragged out are automatically adjusted to the correct height-width ratio. Each region or point that you draw in is given a number and is shown in the list on the **ROI/Point-List** property page. You can also subsequently edit or delete a defined region (region of interest, ROI) in the image.

- Click on 

  to save the last image acquired, including any POIs/ROIs and the calibration values.

- Now switch to the SEM. Remove the holder containing the sample from the light microscope and mount it on the SEM. Please observe the instructions for use relating to the holder.

- Slide the holder onto the stage of the electron microscope, with the flat side of the dovetail at the front. Restore the vacuum in the chamber. Start the SEM. Then start AxioVision and re-open the calibration dialog window by clicking on the 

icon.

- Select the correct holder once again on the **Options** property page.

- Move to the first marker manually, or using a position for the first marker that has been saved in SmartSEM. If you are inserting the holder for the first time or a position has not yet been saved, please read the section "Procedure for using Shuttle&Find".

- Calibrate the holder again. The procedure is the same as for calibration for the LM. If a message appears indicating that it is not possible to move in a certain direction, you have presumably not yet set the stage inversions under the options (see the section "Setting up Shuttle&Find").

- Open the **Mark/Recover** dialog window by clicking on the 

neu Kha icon.

- Select **Hard disk** as the image source: if you select a folder path, images saved there are loaded into the viewing window.

  Note: We recommend that the light microscope and electron microscope are connected via a network and that a shared folder is used.

  The images saved in the folder will then appear in the **Image Source** list and can be selected by clicking on them.

In some cases your light microscope image may have a different orientation from the SEM image. To compensate for these differences, you can flip your image horizontally and/or vertically in the **Mark/Recover** dialog window. To do this, go to the **Options** property page and activate the relevant check boxes: 

. When you save your settings, the flipping of the image is not taken into account. This means that you always retain the original orientation and do not need to change any of your settings relating to the flipping of the image.

- Select either **Same size** or **User Definded** as the **Image type** depending on whether or not you have defined ROIs/POIs in the previously saved image. If **Same size** is selected, the electron microscope will automatically adjust the zoom to the image size of the image acquired under the light microscope.

- Click on the 

button to locate the region.

You can display the current microscope position in the loaded image by clicking on the  icon. The size of the green square adjusts to the magnification set.

- Click on the 

icon to open the current SEM image in AxioVision.

You can then combine the two images using the **Overlay** function. Further details on the overlaying of images can be found in the section "Overlaying images".

Example 2: from electron microscope (SEM) to light microscope (LM)

In the following example an image is acquired on an electron microscope, ROIs and points are defined and saved and we then switch to a light microscope. On the light microscope the settings are reloaded and located. Finally, following location of the positions, fine calibration is performed on the light microscope.

- Set up Shuttle&Find on both PCs, if the settings have not yet been defined (see the section "Setting up Shuttle&Find").

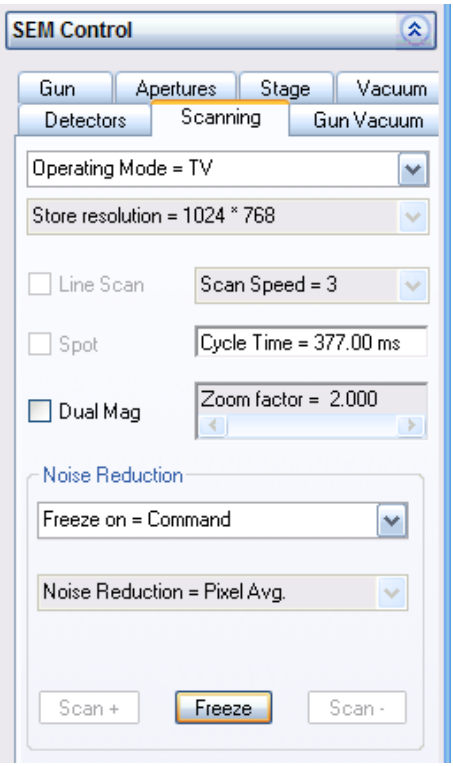
- Insert your holder containing the sample into the electron microscope. Slide the holder onto the stage of the electron microscope, with the flat side of the dovetail at the front. Restore the vacuum in the chamber. Then start AxioVision and open the calibration dialog window by clicking on the 

icon.

- Select the holder used on the **Options** property page. Then go to the **Calibration** property page.

- Move to the first marker manually and calibrate the holder as in example 1.

- Acquire an image of your sample. To do this, freeze the SEM image in SmartSEM by clicking on the **Freeze** button on the **Scanning** property page.



- Then click on the 

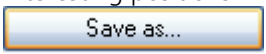
icon. Once the image has been frozen, the **Freeze** button changes to **Unfreeze**. Click on the **Unfreeze** button to unfreeze the image again.

- Save the SEM image by clicking on the 

icon.

- If you want to save additional ROIs, open the **Mark/Recover** dialog window by clicking on the 

icon. Load your previously saved SEM image.

- Select AxioVision as the image source: the images from the current AxioVision work area are displayed in the **Image Source** list. This therefore also includes the last image acquired. Select this by clicking on it. Use the two icons  to define ROIs and/or points at interesting positions in the image. Save your settings by clicking on the 

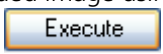
button.

- Now remove the holder from the SEM again. Move to the first marker manually and calibrate the holder as in example 1.

- Open the **Mark/Recover** dialog window by clicking on the 

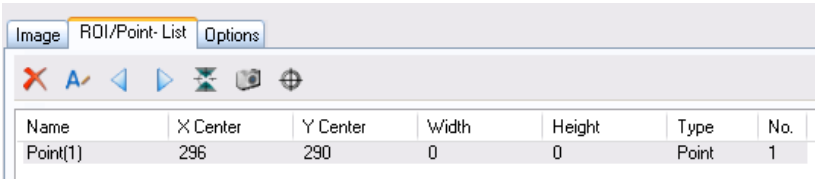
icon.

- Select **Hard disk** as the image source: if you select a folder path, images saved there are loaded into the viewing window.

- You can now relocate your saved ROIs/points. Either select them in the loaded image using the mouse or via the list of ROIs/points. Then click on the 

button.

- Finally, perform a fine calibration. The best way to do this is to select a point from the list of ROIs/points and click on the  icon above the list.



The point is located and you are then asked to correct the position on the microscope. For further details on fine calibration, see the "Fine calibration" section.

Notes on operation

- Make sure that the holder and, in particular, the markers do not get dirty. Work in an environment that is clean and as free of dust as possible. Make sure that air is not blown vertically onto the holder and consequently onto the markers. This could cause dust particles to accumulate, which would result in the accuracy of the marker detection being reduced.

- It is recommended that on the electron microscope the position of the first marker is saved in the SmartSEM software. During calibration you will then be able to locate the first position again quickly.

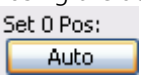
- Once you have calibrated, the most recent calibration that you have set always remains in the holder file. This means that you do not need to perform calibration again, provided that the position of the holder does not change relative to the stage. Generally speaking, however, accuracy is reduced if a calibration is re-used.

- Make sure that the correct holder is always set under the calibration options. As the calibration is saved in the holder file, the calibration from the currently selected holder is always used. The holder files are not user-specific. Your calibration data will be lost if another user performs his or her own calibration using the same holder file.

- If the stage moves in the wrong direction during calibration, check the settings for XY inversion on the **Options** property page.

Tips to ensure accuracy

On the light microscope

- To ensure good calibration, the objectives must be correctly geometrically calibrated. We strongly recommend that you do not use the **Automatic Scaling** function under the AxioVision **Measure** menu, but instead scale the objectives manually using a stage micrometer.
- We recommend 20x magnification for the automatic marker detection.
- Due to potential objective shift, accuracy will be higher if you acquire images using the same objective that you used for calibration.
- After starting AxioVision, calibrate the stage using the automatic calibration function. To do this, click on the 

button.

On the electron microscope

- The SE2 detector is well suited to marker detection.
- Switch off the beam shift and scan rotation.
- The "Stage only" setting should also be activated under **Center Point / Feature**.
- Rotate the stage mechanically before the calibration so that the markers are as straight as possible in the SEM image. Do not otherwise rotate the stage.
- Use the stage backlash to increase the stage's accuracy in locating positions.

General points

- For images on a SEM use conductible samples.
- Initialize the stages before using Shuttle&Find.
- For marker detection, center the corner of the small L marker as well as possible at the center of the image.
- Calibrate again each time the holder has been inserted into a microscope.
- If the marker is a little dirty, it can be helpful for detection purposes to defocus it slightly.
- In the case of stitched MosaiX images accuracy is reduced.
- You can increase the accuracy by performing a fine calibration.

General Information on Accuracy

Influence on Accuracy

There are various factors that can influence the accuracy of S&F:

- The initial accuracy of the light and electron microscopes' XY-stages
- The centering and scaling of the light microscope's objectives
- The accuracy of the calibrations
- The transfer to other microscopes, in case the specimen shifts in its holder during transfer.

Shuttle&Find has an accuracy of 10 µm. You can increase the accuracy by means of a fine calibration or a more precise stage, for example. Below you will find tips on how you can obtain maximum accuracy; additional tips on accuracy may be found in the Shuttle&Find Operating Manual, under *Tips to ensure accuracy*.

How one achieves maximum accuracy:

1. Make sure that S&F is correctly set up (see S&F User's Manual under *Setting up Shuttle&Find*).

2. No objective on LM if possible.

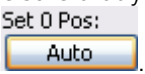
3. The objectives on the light microscope should be calibrated as accurately as possible with respect to scaling.

4. Calibrate as accurately as possible. Check the marker detection by relocating the positions or set the positions manually.



   The position of the small marker at which the positions are set has been drawn into the diagram.

5. Check the saved images on the light microscope by relocating them straight away.

6. Make sure that you have calibrated the stage on the light microscope with 

.

7. In the case of automatic marker detection, check the positions that have been drawn in at which the marker has been detected. Recalibrate if necessary.

8. Allow as little time as possible to pass between calibration and image acquisition, as well as the saving of the images using 

.

9. When switching to a different microscope, do not move the sample in the holder and make sure that it is fixed securely.

Troubleshooting

Calibration

- The window always says **SEM** or **LM calibration**, even though it is an LM or SEM calibration respectively
  - Enter the correct microscope type in the Options. Open this window by clicking the 

button.
- I cannot set the marker position
  - On a light microscope, ensure that the connection to the XY-stage is operating correctly. AxioVision has to be able to control the XY stage. In order to set up the controls in AxioVision, click in the Microscope menu on XYZ and then on Stage.
  - Did you set the correct microscope type in the Options? Open this window by clicking the 

button.
  - Turn the light microscope on first before starting AxioVision.
  - Have you set the inversion settings correctly in the Options window?
- The XY-stage does not move to the next marker
  - In the options window, configure the system to move the XY stage automatically. Switch to the Options tab and activate the checkbox 

- Check the settings for the stage inversion. The XY stage will not move any further on an electron microscope if the settings are not correct and the value would extend beyond the motion limits of the stage. In order to orient using x and y, the holder files in the Options tab can be seen by clicking  .

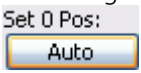
- On a light microscope, check the connection to the motorized stage.

- If you clicked Automatic, it is possible that in some cases, the marker will not be recognized correctly or not at all. A dialogue window will appear in which you can either confirm or end continuation.

- Check the holder you have chosen. Have you chosen the correct holder file or, in a user-defined holder: Are the marker distances correct?

➢ The "to Position 1" function does not work

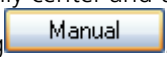
- Has a Position 1 been set? If none has been set, it is impossible to move there.

- Is the stage on the light microscope calibrated? If not, click on 

in order to calibrate it automatically.

- Was the stage on the light microscope calibrated when you set the first marker position?

➢ Automatic marker recognition during calibration takes a long time

- Manually center and use the manual setting of the marker position by clicking 

.

➢ The marker is not recognized automatically

- Set the marker orientation in the Options tab

- Are the scaling of the objectives on the light microscope and therefore the pixel size correct?

- Have you set the colors of the marker correctly? When in doubt, set it to automatic

- Are you also using the small marker for marker recognition?

- Is the small marker completely and clearly visible in the image? Set contrast and brightness.

- Try changing the threshold value in Options from high to low. High is standard.

- Can reflections or charges be seen? These could be affecting recognition. Try to eliminate these.

- Is the marker contaminated and is the L hard to see? Try to achieve recognition with a lightly defocused marker or clean the marker (when doing this, take note of the corresponding supplements to the holders).

- If the measures described above do not help, use the manual setting of the marker position; then, the current stage position will be used – in other words, the center of the image in the Live Image (select "Cross Hair" in the live image options). Previously, the marker had to be manually centered, i.e. the intersection of the centerline of both L legs has had to be brought into the center of the image.

➢ There exists no calibration

- Have you already calibrated? Calibration is only complete if you have set all 3 positions and clicked OK.

- Have you chosen the holder with which you calibrated?

- If you imported the holder, the calibration was not imported.

- Have you reinstalled S&F? When uninstalling, the holder files and with them, the calibration are deleted.

➢ I cannot access my holder

- Have you deleted the holder? Perhaps another user deleted the holder. The holder files can be used by all users.

- Have you applied the holder? Click on OK in the Add new holder dialogue window in order to store a new holder.

Recovery/Find

➢ The recovery function in my saved images does not work

- Was the holder calibrated before the images were saved?

- Was the right holder chosen from the calibration options before the images were saved?

- Have you chosen the right holder for the current microscope?

- Have you calibrated the holder before approaching?

- Have you left the sample in the holder since the first photograph?

➢ Under defined ROIs/points, the stage moves in the opposite direction

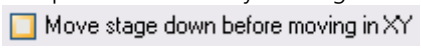
- Are the loaded image and the current microscope image incorrectly oriented/mirrored with respect to one another? Set the mirroring in the Options tab.

- Were the camera orientation and the stage axes set correctly when the pictures were taken? (see S&F Users Guide under Setting up Shuttle&Find or Chapter 9.7, MosaiX, Setting up the camera and scanning stage in the AxioVision Handbook). On systems with installed MosaiX module, it is recommended to check the correct stage direction and camera orientation settings by running a small

MosaiX (3 x 3 e.g.). After that check the correct settings in the S&F options.

- The loaded image is a mirror image of the microscope image
  - Set the mirror settings in the Options tab.
- Using MosaiX images, I always get a warning message.
  - With stitched images or images for which the function for automatic stitching is used, the accuracy when approaching positions is reduced. However, they can be used anyway.
- The accuracy upon recovery is not very high
  - Did you calibrate the holder after inserting it? You can indeed use the prior calibration; however, the approach accuracy will be reduced as a result.
  - Did you consider the tips on accuracy? See the section, "How to achieve maximum accuracy" in this document or Tips to ensure accuracy in the Shuttle&Find Operating Manual
  - Did the sample shift inside the holder during transfer into another microscope? As a result, the accuracy is reduced
  - You can improve the accuracy with a fine calibration; please see the "Fine calibration" section of the Shuttle&Find Operating Manual.
  - Are you using stitched MosaiX images or images for which the function for automatic stitching is being used? This affects the accuracy.
- The stage moves down before every movement.

- You have set the stage to move down before every XY movement in the Options window. Open the Options window by clicking 

and deactivate the checkbox 

Overlay

➢ The loaded image is shown in shades of gray

- Have you activated the checkbox, Display as Gray Image?
- Save the image in TIF format and reload the TIF image.
- Set the AxioVision imaging options to 8 bit.

➢ The loaded images are not in the correct orientation to one another

- Click on the image you want to mirror and then on  for horizontal mirroring or  vertical mirroring.

SEM

➢ The connection to the SEM does not seem to be working

- Close AxioVision and start the SmartSEM interface. Log in. Then start AxioVision again.
- Have you closed SmartSEM? Proceed as in the instructions above. In order to activate the SEM, the SmartSEM interface must be started.
- Have you started SmartSEM but not logged in? Log into SmartSEM.
- Do you have sufficient user rights in your SmartSEM account?

Shuttle&Find Checklist

Make sure that the device is fully functional and configured. S&F (like other modules from AxioVision) is affected in whole or in part by incorrect settings, calibration, and defective components; (it is not the fault of the module if, for example, the lenses are not correctly centered and as a result, the accuracy drops).

Checklist

LM = light microscope, EM = electron microscope

Point	**Device**	**X**
Are all objectives centered?	LM	
Is a motorized XY stage available and fully functional?	LM	
Is S&F configured?		
Is the S&F toolbar active and are all (10) icons visible?	LM, EM	
Have settings for the stage inversion in the MTB as well as for the MosaiX module been made (see Chapter 9.7 *MosaiX, Setting up the camera and motorized stage* in the AxioVision User Guide)?	LM	
Is the display in the eyepiece identical with the display in the Live Image? If not, set the camera image orientation.	LM	
In the Options window, has the current microscope been selected as the microscope type?	LM, EM	
Have you set a common directory in the Options window?	LM, EM	
In the Options within the calibrate dialogue window, has the check mark next to "*Automatic movement to next marker*" been set?	LM, EM	
During calibration, does the XY stage move in the correct X direction? If not, set the x inversion in the calibration options.	LM, EM	
During calibration, does the XY stage move in the correct Y direction? If not, set the y inversion in the calibration options.	LM, EM	
Does the color of the marker in the calibration options match with that of the marker in the microscope image?	LM, EM	
Is the marker orientation in the calibration options the same as the marker in the Live Image window?	LM, EM	

Have the scalings for all lenses been defined and are the defined scalings correct?	LM	
Are the scalings for the lenses in micrometers (never pixels)?	LM	
Is the position of the first marker in SmartSEM stored in the *Stage points list* (Recommendation, so that the first marker can be quickly approached)?	EM	
Have you initialized the stage?	EM	
Is the Beam Shift turned off?	EM	
Is Scan Rotation turned off?	EM	
Is the setting for the Center Point Feature set to "Stage only"?	EM	
Is the marker in the image aligned to be straight? If not, turn the stage mechanically	EM	
Are the scaling messages deactivated (see above)	EM	
If desired, turn on Stage backlash for improved approach accuracy	EM	
Is S&F working?		
During calibration, does the XY stage move in the correct directions?		
4. Calibrate using a Zeiss holder and automatic marker recognition		
5. Take an image of the specimen in the holder or from a position on the holder and save it with *SF Save As*		
6. Move the stage away from the location where you took the picture		
7. Load the image in the *Recovery* window and click *Execute* in order to approach the location again.		
8. Define a point and an ROI and approach it again; save your definition.		
9. If possible, switch to another microscope, calibrate the holder and approach the locations and ROIs/points again with the other microscope.		

From Readme.txt

Calibration notes

The "to position 1" function only works properly on a light microscope if the stage has been previously calibrated.

Always make sure that you have selected the right holder in the calibration options.
The calibration is saved in the file of the current holder.

When you export a holder, the calibration in the holder file is deleted.

---
user manual

When moving to the loading position, some microscope stages can travel in the xy direction in addition to the z direction.
Make sure that the stage only moves in the z direction. You can define this setting via the TFT display panel on the light microscope:
- Starting from the main menu select Settings, then Components.
- Change to the Stage property page.
- Switch Stage in load position together with Focus load set to **Off**.

The first time you use S&F, set the current microscope in the S&F options and set the stage inversion for calibration.

To perform relocation, each image must have been previously saved using the function "SF save as".

Known bugs and limitations.

The refocus in the relocation window does not work on light microscopes with images from electron microscopes.
In the case of light microscope images it only works on the same microscopes and if the focus difference is not greater than 100 micrometers.

The stage movement in electron microscopes disregards the stage rotation. The scan rotation must be deactivated for the automatic marker detection.. The stage movement to the next marker and the movement for relocation only takes place in the x and y direction.

It may not be possible to load images greater than approx. 200MB in the overlay dialog, depending on the amount of working memory on your PC.

11.16 Correlative Particle Analysis (CAPA)

Overview

The Correlative Particle Analysis module uses its own license.

Correlative Particle Analysis is used to quickly relocate individual reflective/metallic particles on an electron microscope which have previously been analyzed using a light microscope. The aim is to perform an automatic EDS (Energy-Dispersive X-ray Spectroscopy) analysis of selected particles. For this Correlative Particle Analysis refers back to the results data of the light microscopy analysis by means of the Particle Analyzer Projects module. These data are used as the basis for further materials analysis on the electron microscope. The images of the same particle from different microscopes can then be precisely overlaid.

First perform the steps on the light microscope as part of a guided workflow in AxioVision using Particle Analyzer Projects/Correlative Particle Analysis as follows:

(1) Start the particle analysis using the **Correlative Particle Analyzer** test specification.

(2) Complete the input mask as far as necessary.

(3) Perform a 3-point calibration to define the reference coordinate system for the light microscopy analysis.

(4) Structure the rest of the workflow for particle analysis using Particle Analyzer Projects. Answers to questions on particle analysis using the Particle Analyzer Projects module can be looked up in the online help or in section 11.4 of the AxioVision manual.

Remove the correlative holder with the filter sample from the motorized stage of the light microscope and attach it to the motorized stage of the electron microscope. A description of how to use the correlative holder can be found in document 432335-9110-701 "Instruction Manual Specimen holder CorrMic for Particle Analysis".

Now perform the steps on the electron microscope as part of a guided workflow using Correlative Particle Analysis and SmartPI as follows:

(1) Import the particle results data from the light microscopy analysis using Correlative Particle Analysis.

(2) Perform a 3-point calibration to define the reference coordinate system for the electron microscopy analysis.

(3) Select the desired **reflective** particles for the EDS analysis from the particle results data table.

(4) Start the automatic EDS analysis of the selected reflective particles. Answers to questions on the SEM/EDS analysis can be looked up in the SmartPI online help or in the SmartPI manual.

Depending on the selected presetting, you will either directly obtain the results report containing the combined results from the light and electron microscopy analyses or an interactive view that allows you to examine the analysis results and gives you the option of performing a new EDS analysis. It is also possible to generate a combined results report from this view.

Please note:

- On light microscopes Correlative Particle Analysis controls both the microscope stage and the focus drive. If used incorrectly, damage can therefore be caused to objectives and/or specimens lying in the stage's traveling range.

- On electron microscopes Correlative Particle Analysis controls the microscope stage. If used incorrectly, damage can therefore be caused to detectors and/or specimens if the height of the stage or sample is too close to the detectors.

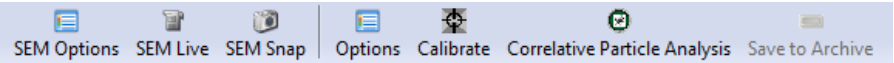
- You can call up the online help for a **Correlative Particle Analysis** dialog at any time using the F1 key.

- To extend the analysis to non-reflective particles, you will also need the Shuttle&Find module.

With the LE version of AxioVision (available free of charge), you can make use of the function for loading SEM TIFF images.

Setting up Correlative Particle Analysis (CAPA)

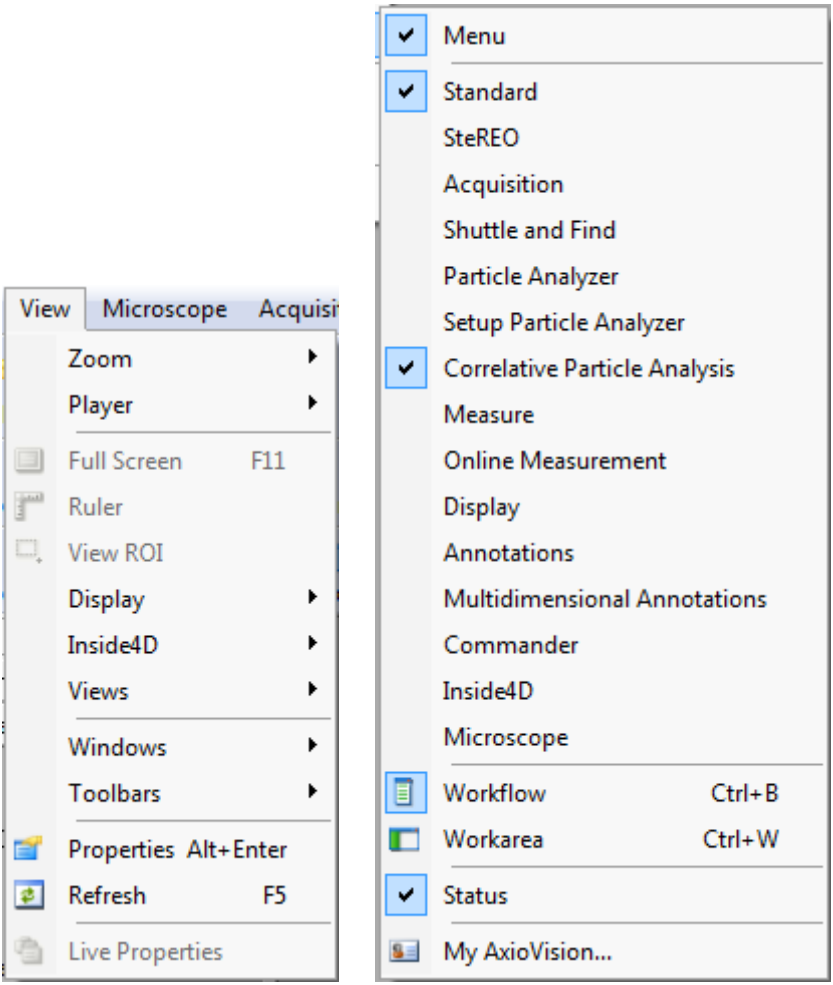
CAPA toolbar:



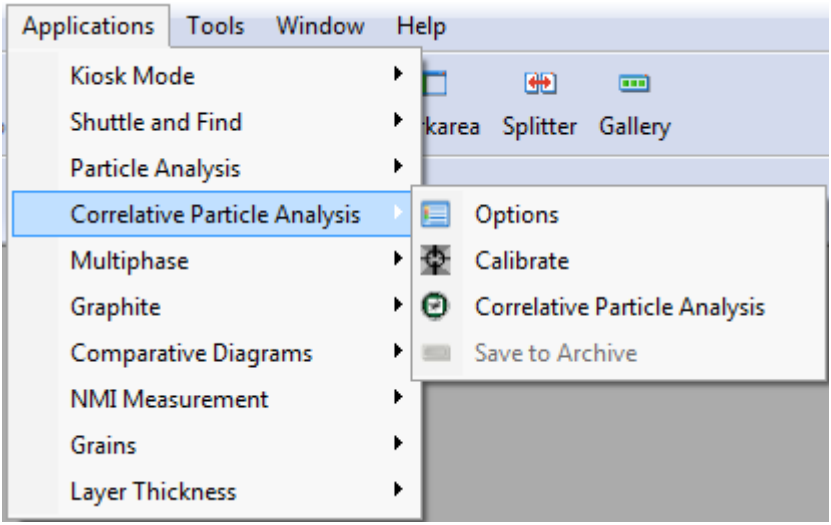
After installing Correlative Particle Analysis, you should define your settings when you use it for the first time. The settings of Correlative Particle Analysis are user-dependent and therefore only relate to the user currently logged on to the PC.

Activating the toolbar

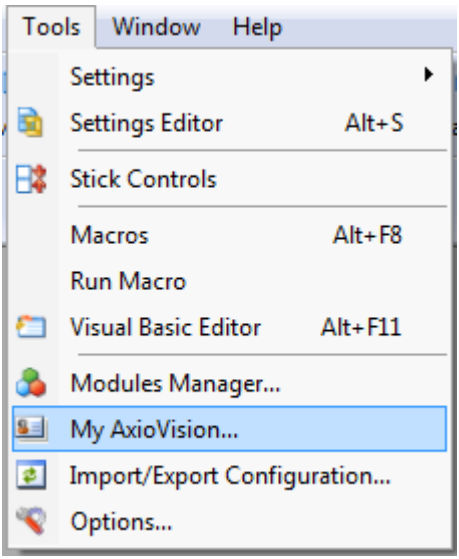
Activate the toolbar by **right-clicking** on the existing toolbars and selecting **Correlative Particle Analysis** from the shortcut menu.



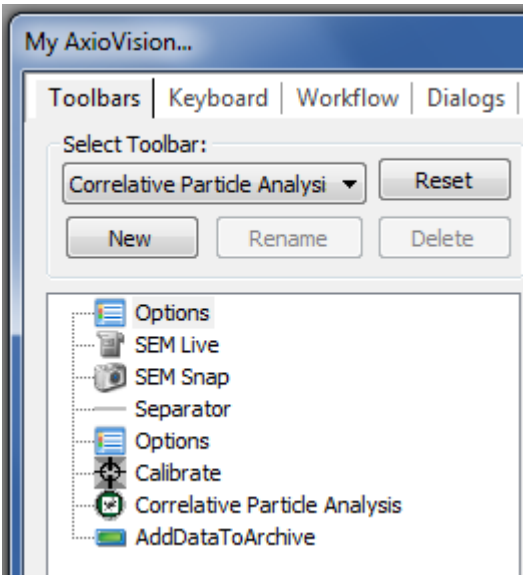
All functions can also be called up via the Application menu ⇒ Correlative Particle Analysis.



If you have installed a previous version of Correlative Particle Analysis, update the toolbar via the **Tools** menu ⇒ **My AxioVision**.



Select the **Correlative Particle Analysis** entry from the **Select Toolbar** dropdown list and click on the Reset button:



Setting the stage and camera on a light microscope

- Define the settings for the stage and camera in the same way as for the MosaiX module (see section 9.7 "MosaiX"). When the stage is moved using the stage control the objects in the live image must move in the opposite direction, i.e.:

  a) If you move the joystick forwards/move the slider upwards, the objects in the live image move down.

  b) If you move the joystick/slider to the left, the objects in the live image move to the right.

  For further information on this please refer to section 9.7 "MosaiX".

- Set the camera rotation in AxioVision in such a way that the marker in the live image on the monitor is oriented in the same way as when it is viewed through the eyepiece. To do this, insert a ZEISS holder with markers into the microscope stage and locate a marker. Set the light path to the camera.

- The correct orientation of the camera image and eyepiece image can often be achieved by rotating the camera itself together with the camera adapter on the C-mount.

  Alternatively:

  - Open the live window by clicking on the 

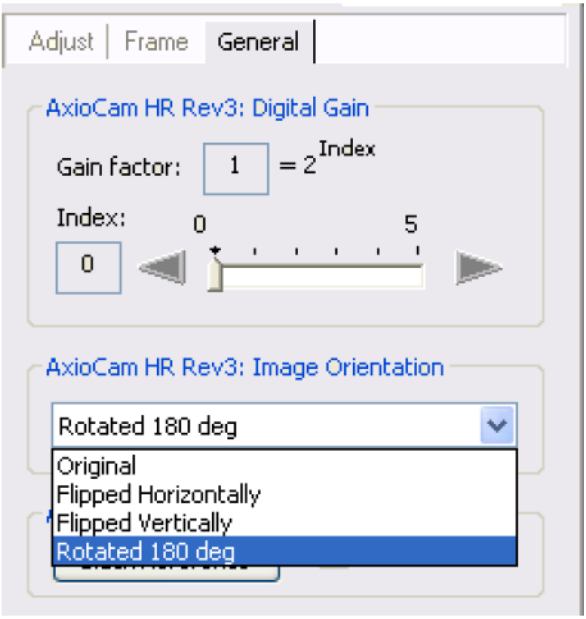
icon. Select the **Live** function from the **Acquisition** menu. Observe the marker in the live image and compare the view with the one in the eyepiece.

  - To change the image orientation, click on the **Live Properties** button. Select the **Live Properties** function from the **View** menu.



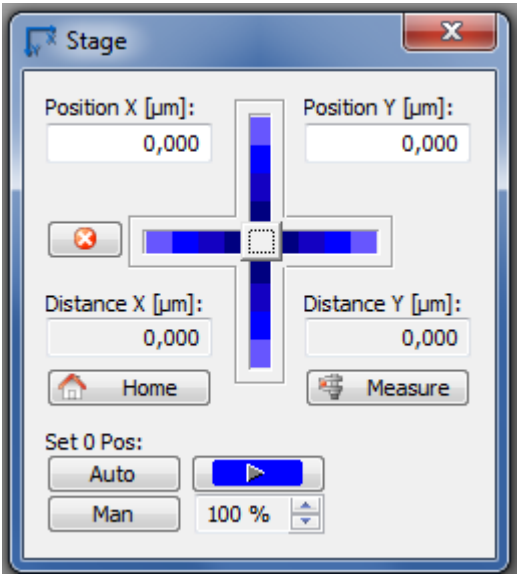
  - Select the **General** tab from the **Live Properties** dialog.

  - Here, select the appropriate **Image Orientation** from the dropdown list box so that the live image and eyepiece image correspond.



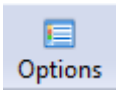
If, during calibration of the coordinate system in the normal workflow of Correlative Particle Analysis, you want to use the **To Position 1** function, always calibrate the stage beforehand with **To Position 0** Auto.

- To do this, select the microscope in the work area and go to the **XYZ** property page. Click on the **Auto** button to set the 0 Position.

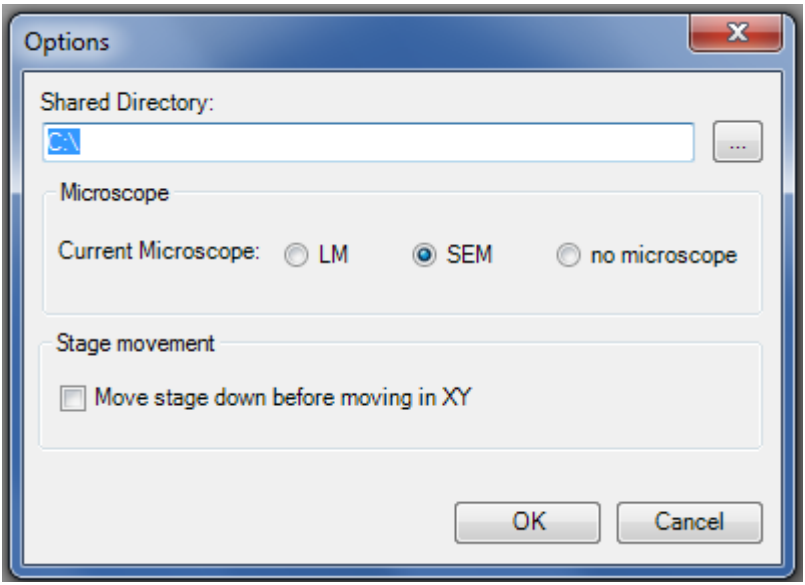


Setting CAPA options

These options are general settings for the Correlative Particle Analysis module.

Call up the options by clicking on the 

button.



- Click on the ... button to find a shared directory. With Correlative Particle Analysis you will typically be transferring data from one PC to another. The use of a network drive that both PCs can access is recommended here. Alternatively, you can transfer your data to the other PC using a USB stick, for example.

- Set the microscope type to correspond with the connected microscope: **LM** for a light microscope or **SEM** for a scanning electron microscope. If you have not connected a microscope and, for example, only want to view and process data, select **no microscope**.

- To avoid collisions, you can activate the Move stage down before moving in XY check box on microscopes with a motorized Z-drive. If this option has been checked, the stage moves downwards first in Z before each XY movement controlled by Correlative Particle Analysis. On inverse microscopes the nosepiece is lowered accordingly before the stage moves.

- On a light microscope the stage or nosepiece travels to the loading position and then, after the subsequent movement in XY, back to the working position.

- On an electron microscope the stage drops down to its lowest point and then, after the subsequent movement in XY, returns to the original position.

Please note:

- When moving to the loading position, some microscope stages can travel in the XY direction in addition to the Z direction. Make sure therefore that the stage only travels in the Z direction. You can define this setting via the TFT monitor on the light microscope:

  - Starting from the main menu, select **Configuration**, followed by **Components**.
  - Switch to the **Stage** property page.
  - Switch the **Stage in load position together with focus load** to **Off**.

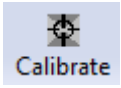
Setting calibration options

The calibration markers consist of two L-shaped markings. They have been engraved directly into the correlative holder in three positions or attached to separate marker stubs, and each consist of a large L (edge length 1 mm) and a small L (edge length 50 µm) within the large one.

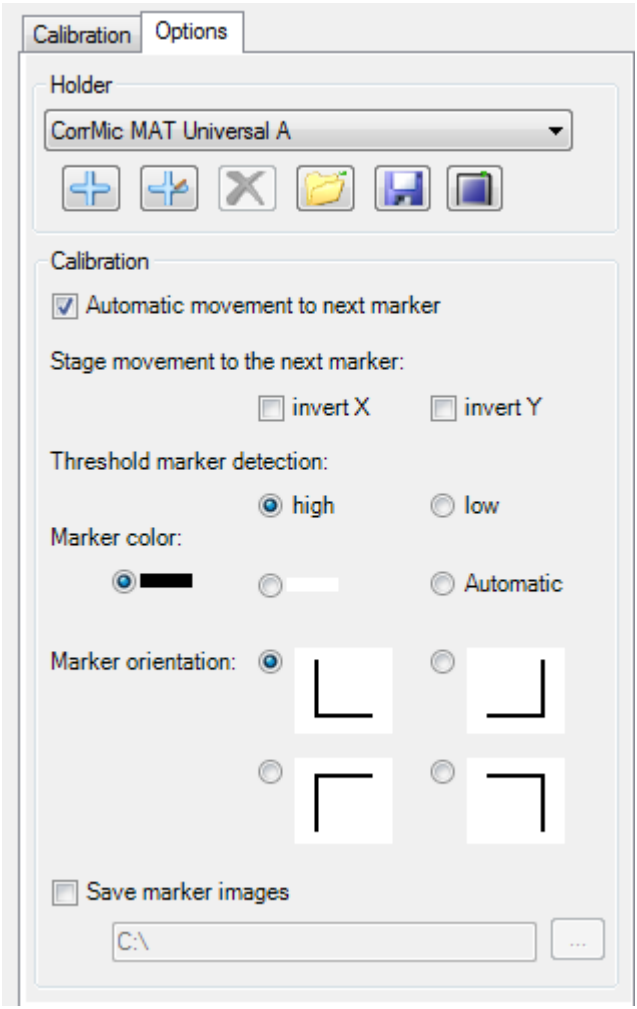


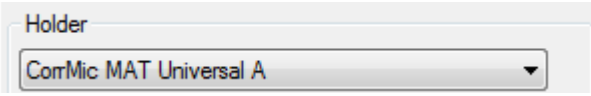
Relocalization of the particles under the electron microscope can only be guaranteed if these three markers are calibrated.

General calibration settings

Open the calibration dialog by clicking on the 

icon. Select the **Options** property page.

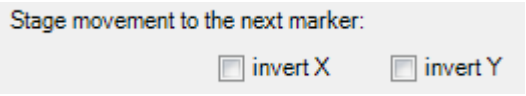




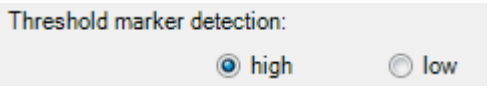
The dropdown list box shows all the holders available for selection. Make sure that the correct holder has been selected, if you wish to reuse a calibration and are working with different holders.



If this option is activated, the motorized stage automatically locates the next two marker positions once the first marker position has been correctly detected and confirmed. The route is read out from the holder file currently set.



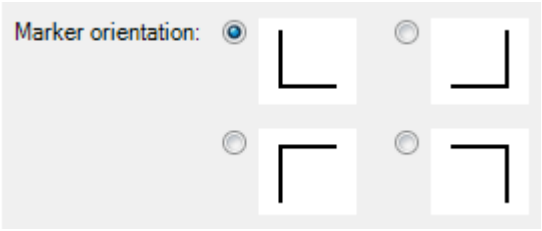
If these options are activated, the movement of the stage to the next marker is inverted in the X and/or Y direction. This setting depends on the coordinate system set for the stage.



This option affects automatic marker detection. You can set the marker detection threshold to **high** or **low**. The **high** setting is suitable for most marker structures. For damaged markers with an L structure that has a smaller aspect ratio we recommend that you specify the **low** setting.



This option affects automatic marker detection. Specify here whether the marker is black against a white background or white against a black background. The **Automatic** option uses the best result from both detections.



This option is used to specify how the marker orientation will be seen in the live image. For the light microscopy analysis, first set AxioVision in such a way that the marker orientation in the camera's live image corresponds to the orientation in the eyepiece. Then select the corresponding marker orientation in the options.



Using this option you can save marker detection control images permanently.

Buttons


Opens a dialog window in which you can create a new holder file.


Opens a dialog window in which you can edit the current holder file. Only holders you have defined yourself can be edited. The files of the Zeiss holders are write-protected.


Deletes a user-defined holder file.

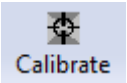

Imports a holder file.


Exports a holder file. The calibration data in the exported holder file are deleted.

 Saves the calibration data in the image currently opened or acquired.

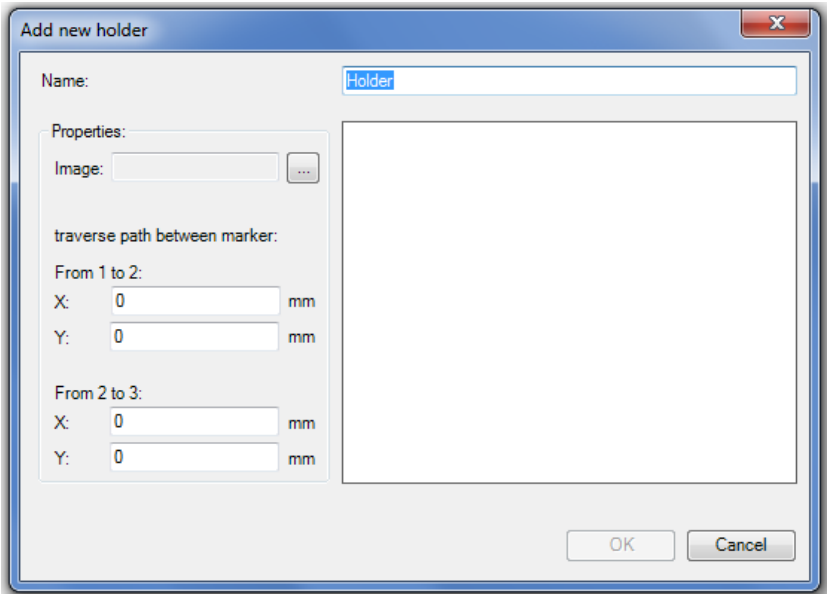
Creating new holders/importing/exporting

In the calibration dialog it is possible to define a holder yourself in addition to the existing holder files.

- Click on the 

icon to open the calibration dialog window. Switch to the **Options** property page.

- Under the **Holder** dropdown list box you will find the buttons for holder management. Click on the  button to open the dialog window for a new holder.



- Enter the name of your holder in the **Name** input field. Once you have created your holder, you can no longer edit the name.

- It is recommended that you also specify an image of your holder to obtain a better overview. Click on the  button to open an image. You can use an image in the formats .bmp, .gif, .exif, .jpg, .png or .tif.

It is always assumed that there are 3 markers on the holder. In the holder file define the distances in millimeters between the first and second markers and between the second and third markers. The values for the distances in X and Y may not both be 0.

You can determine the routes using the stage positions shown in AxioVision.

- Observe the camera's live window in the right-hand section of the calibration window.
- Activate the reticle. To do this, right-click in the live image and then click on the **Cross marker on/off** function in the shortcut menu.
- Move the microscope stage to a marker and center the stage on it using the reticle in the live image.
- Note the current stage position in the stage menu of the calibration window. Move the microscope stage to another marker, center it and read the second stage position.
- Subtract the two positions to determine the distances in the X and Y directions. To determine whether the value is positive or negative, you can use the predefined ZEISS holders as a reference.
- The maximum dimensions of the specimen holder must not exceed 78 mm if the holder is to pass through the air lock.
- In AxioVision the stage positions are displayed in µm. Divide the stage positions by 1,000 to convert them into mm. You can then enter these in the **Add new holder** dialog window.
- Click on the 

button to create the holder file and close the window.

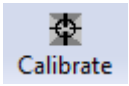
The new holder is selected in the dropdown list box as the current holder. You can also export or import your holder.

- Click on the  icon to import a holder file.

- Click on the  icon to export a holder file. Any calibration saved in a holder is deleted when the holder file is exported.

Editing holders

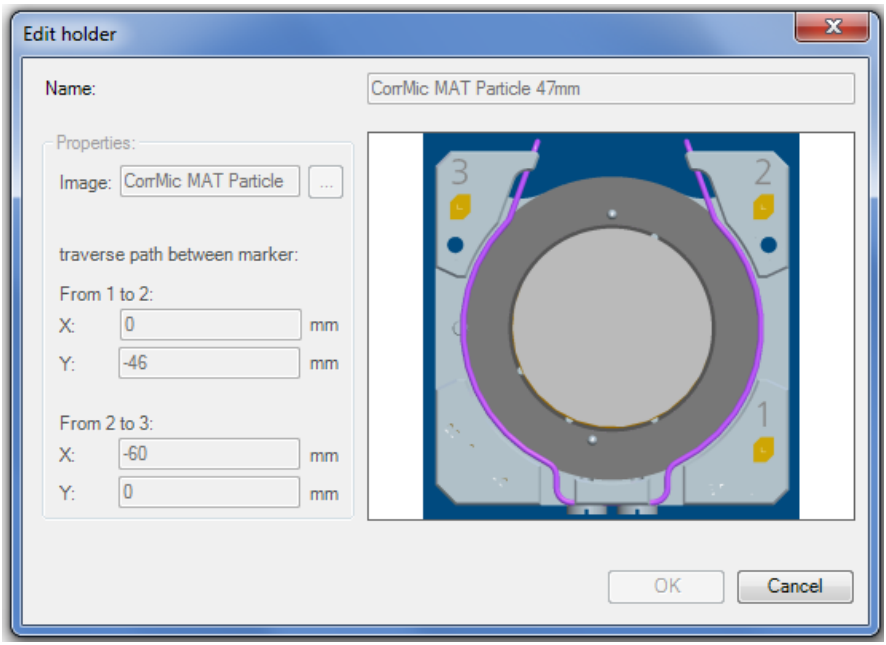
The marker distances and the image can be subsequently edited in a previously created holder file.

- Click on the 

icon to open the calibration dialog window. Switch to the **Options** property page.

- Under the **Holder** dropdown list box you will find the buttons for holder management. Click on the  icon to edit the current holder.

The predefined holders are write-protected. You can open and view the holders by clicking on the  icon. You cannot edit them, however.

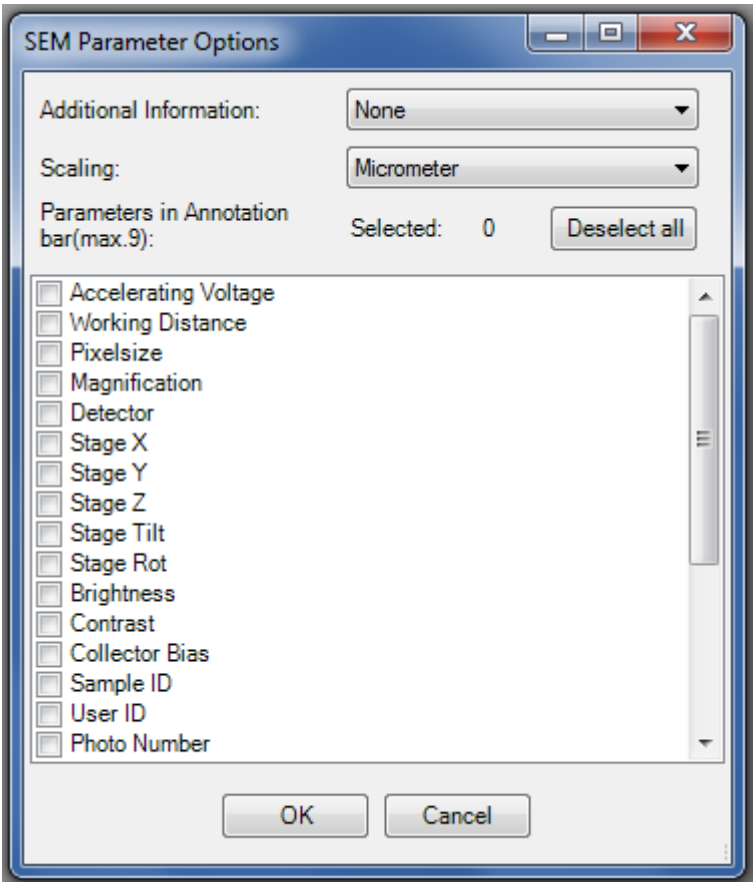


Adjusting specimen holders

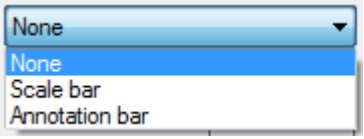
Please refer to document 432335-9110-701 "Instruction Manual Specimen holder CorrMic for Particle Analysis" for a description of how to use the correlative holder.

Setting SEM options

The **SEM Parameter Options** dialog enables you to set options for displaying SEM images. These options relate to the acquisition of SEM images using the **SEM Snap** function.

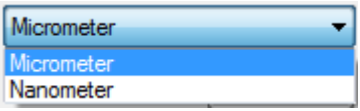


Additional information



In this dropdown list box you can set the additional display of a scale bar or a parameter bar.

Scaling



In this dropdown list box you can set the scaling in the SEM image.

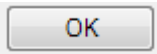
SEM Parameter

In the list of parameters you can set which SEM parameters (maximum of 9) should be displayed in the parameter bar.

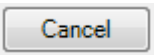
Buttons



Deselects all selected parameters.



Saves the settings and closes the dialog window.



Closes the dialog window.

SEM Live

The live window displays the current SEM image in a dialog window. Under the live image you will find functions for acquiring an SEM image. **SEM Live** currently requires the standard resolution of 1024x768.

Buttons

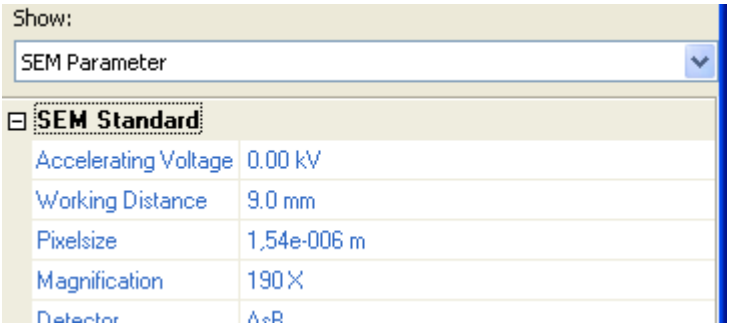


Acquires the current SEM image and opens it as a new image in AxioVision. Depending on the settings you have configured using the **SEM Parameter** dialog, additional information is displayed. The live window remains open after image acquisition. You can close it by clicking on the cross in the top right-hand corner.

Freeze Image Freezes the live image to allow perfect image acquisition. After you have clicked on the button it changes to Unfreeze Image. You can then switch back to the normal live image.

SEM Acquisition

This function allows you to acquire an image using the scanning electron microscope (SEM). A new image is generated automatically. This means that you can acquire individual images very quickly and simply. In addition, settings from the SEM are also read out as parameters and saved in the image file. In the image's property window, which can be opened via the shortcut menu if you right-click with the mouse, these can be displayed on the **Info** property page by selecting the **SEM Parameter** display.



Workflow with Correlative Particle Analysis

Correlative workflow on a light microscope

First perform the steps on the light microscope as part of a guided workflow in AxioVision using Particle Analyzer Projects/Correlative Particle Analysis as follows:

(1) Open an archive.

(2) Start the particle analysis using the **Correlative Particle Analysis** test specification.

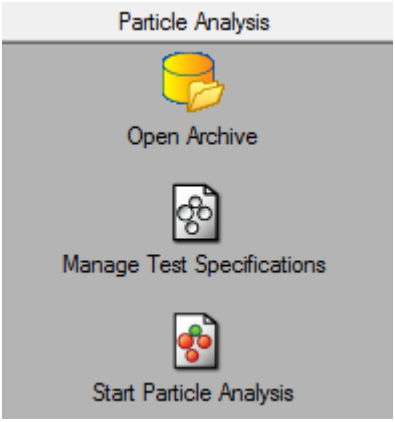
(3) Complete the input mask as far as necessary.

(4) Perform a 3-point calibration to define the reference coordinate system for the light microscopy analysis.

(5) Structure the rest of the workflow for particle analysis using Particle Analyzer Projects in the usual way. Answers to questions on particle analysis using Particle Analyzer Projects can be looked up in the online help or in the manual under 11.4.

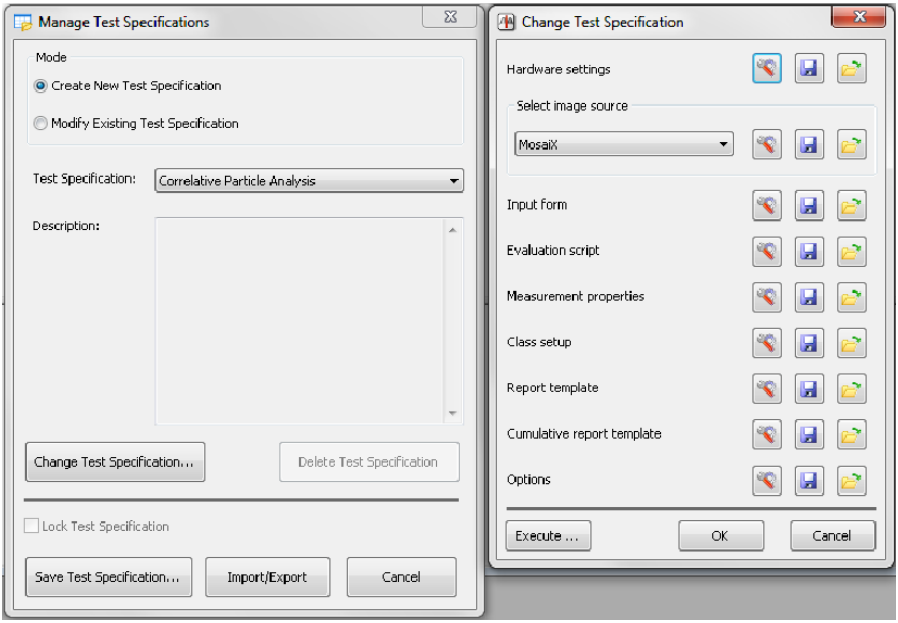
(1) Open an archive - see section 7.6.

(2) Start the particle analysis using the Correlative Particle Analysis test specification.

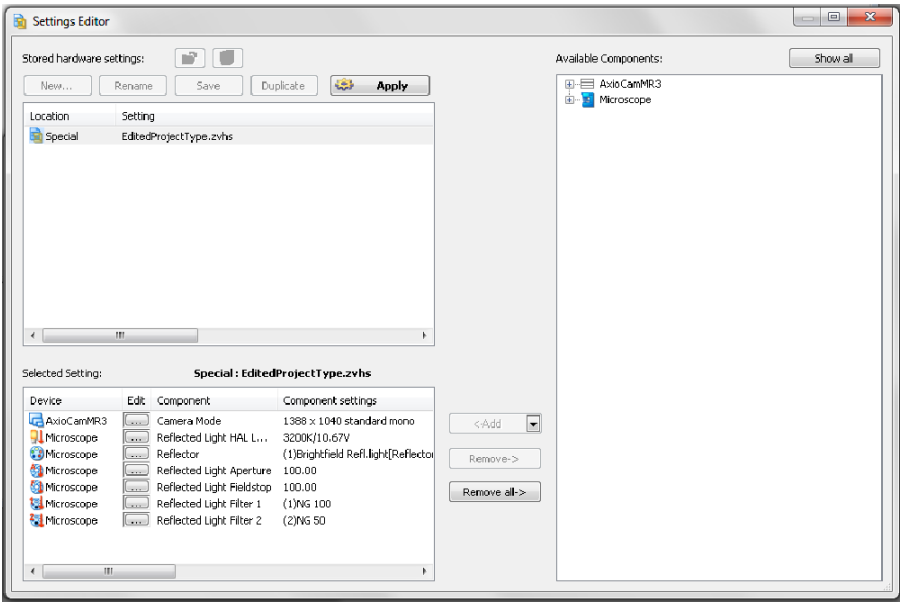


Please refer to 11.4 "Particle Analyzer Projects" for a description of how to set up the test specification correctly.

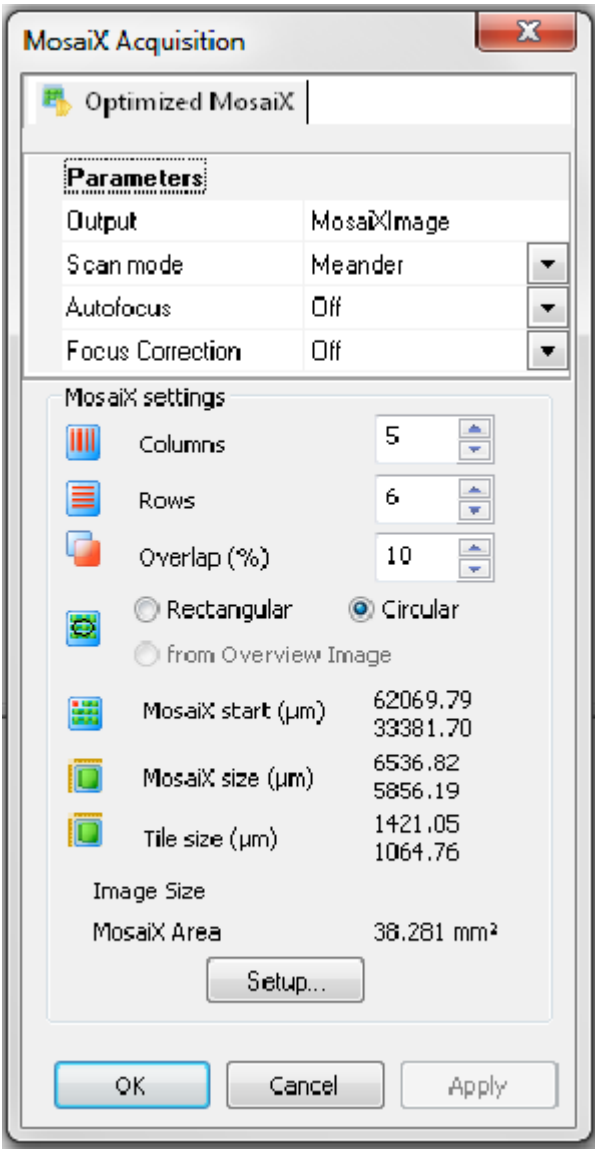
A few basic settings are shown below:



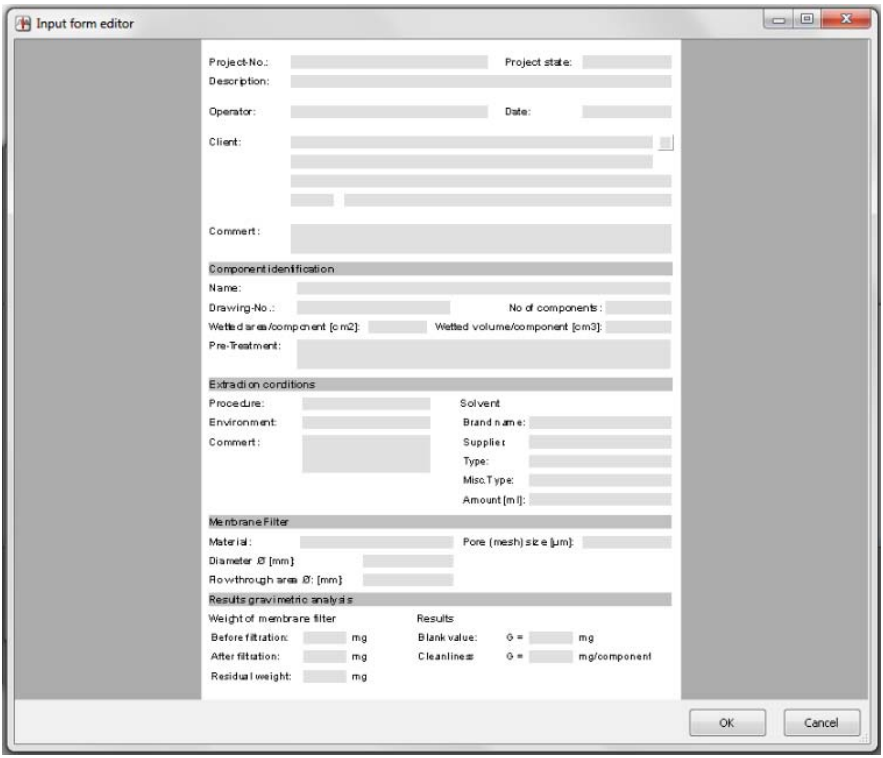
Hardware settings



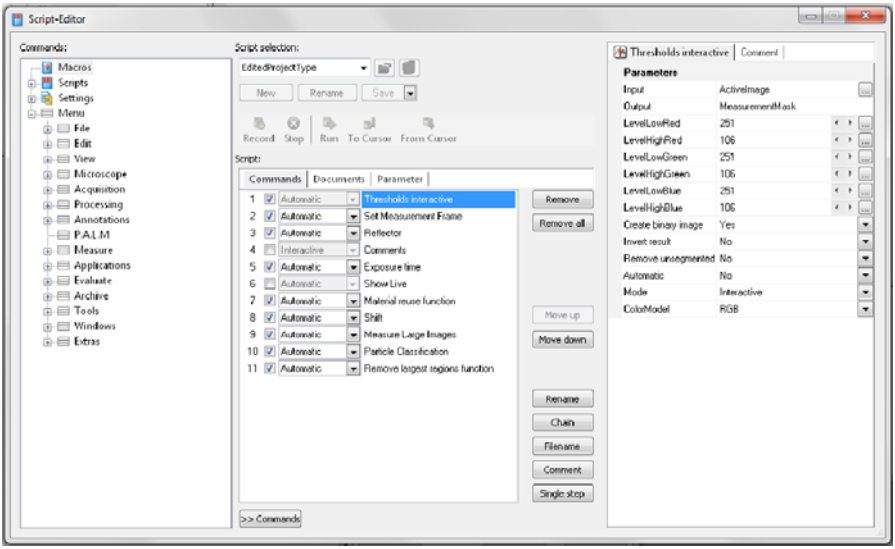
Selecting the image source

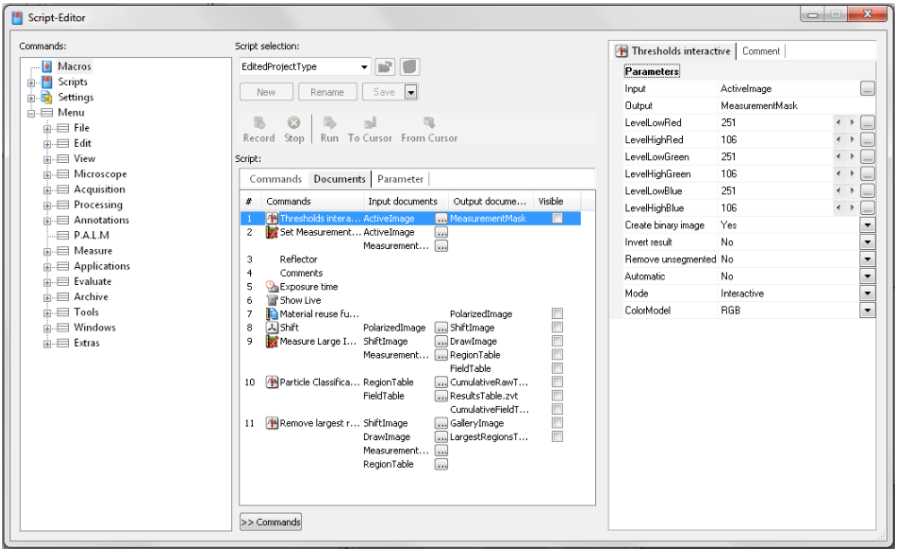


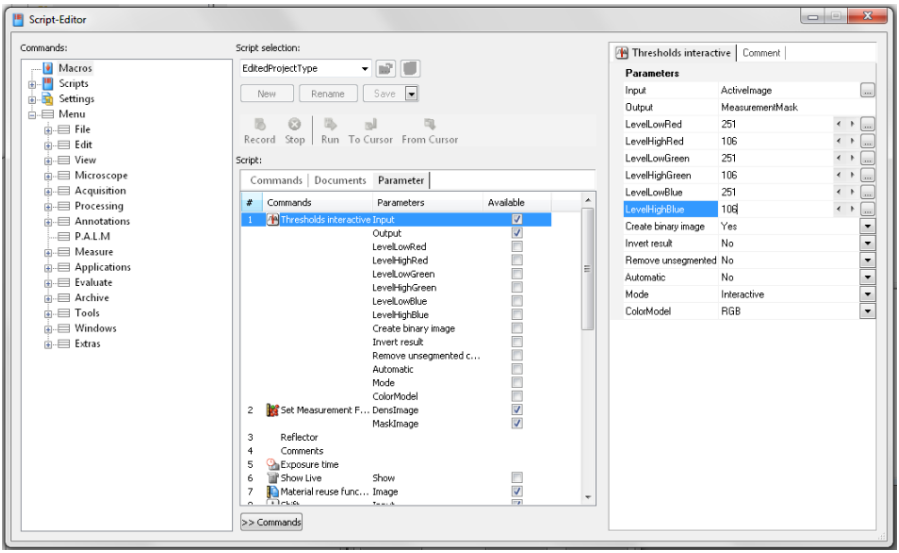
Input form


Input form editor
Project-No.:
Project state:
Description:
Operator:
Date:
Client:
Comment:
Component identification
Name:
Drawing-No.:
No of components:
Wetted area/component [cm2]:
Wetted volume/component [cm3]:
Pre-Treatment:
Extradion conditions
Procedure:
Environment:
Comment:
Solvent
Brand name:
Supplier
Type:
Misc.Type:
Amount [ml]:
Membrane Filter
Material:
Pore (mesh) size [µm]:
Diameter Ø [mm]
Flowthrough area Ø: [mm]
Results gravimetric analysis
Weight of membrane filter
Before filtration:
mg
After filtration:
mg
Residual weight:
mg
Results
Blank value:
G =
mg
Cleanliness:
G =
mg/component
OK
Cancel

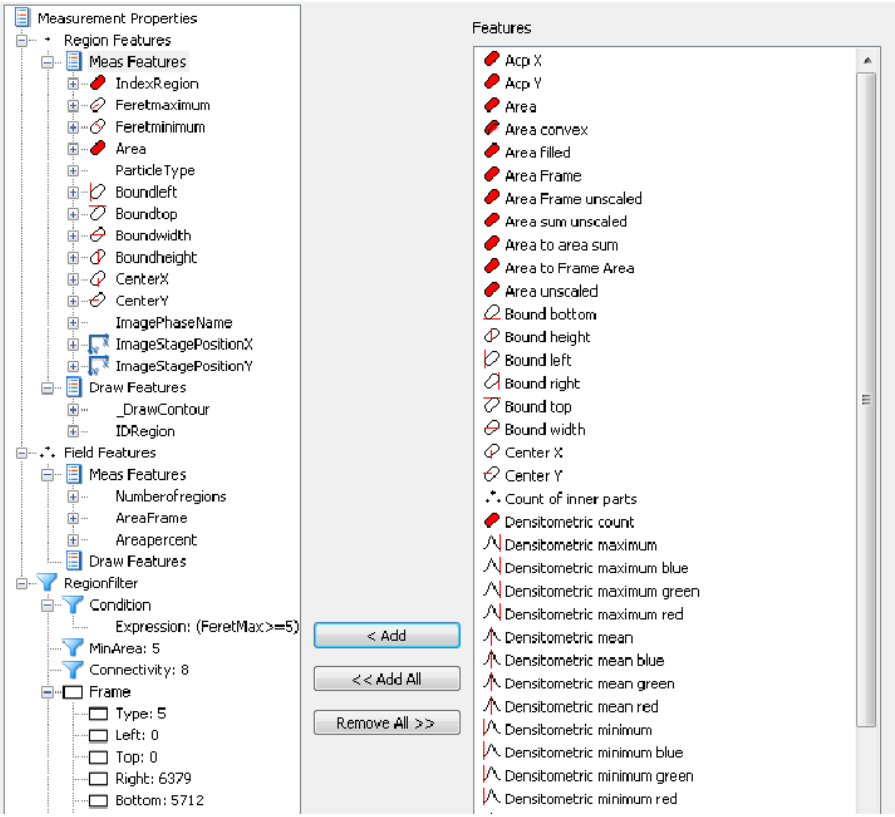
Evaluation script



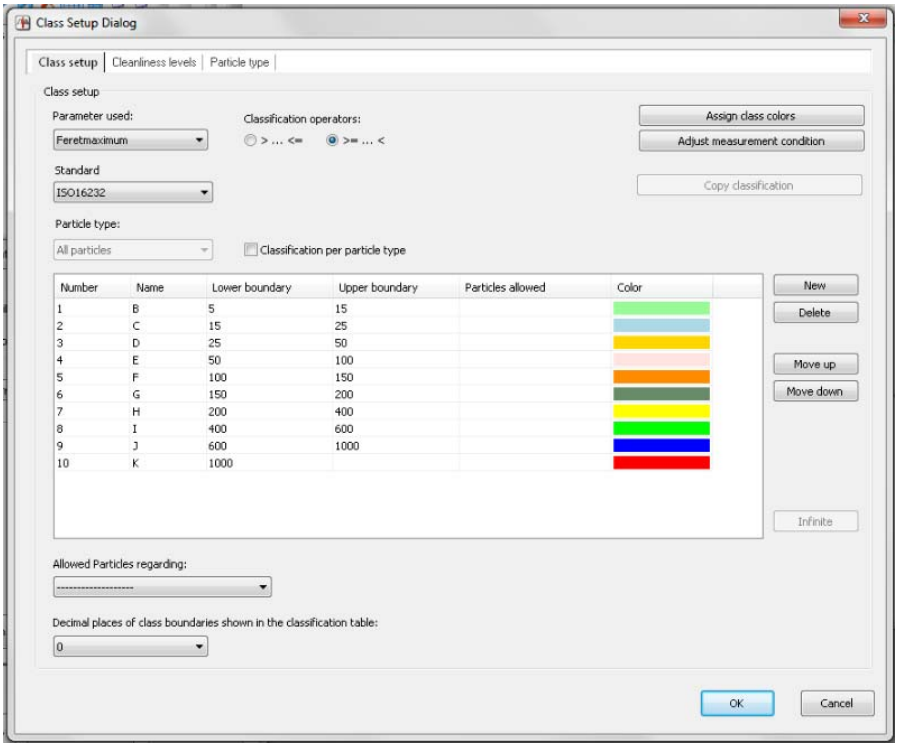


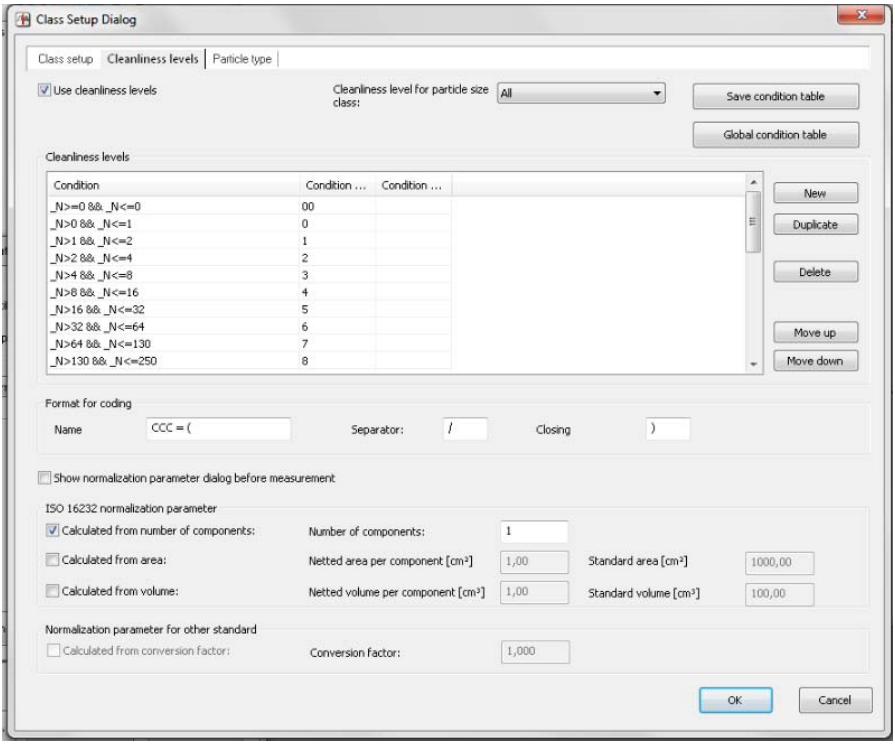

Script-Editor
Commands:
Macros
Scripts
Settings
Menu
File
Edit
View
Microscope
Acquisition
Processing
Annotations
P.A.L.M
Measure
Applications
Evaluate
Archive
Tools
Windows
Extras
Script selection:
EditedProjectType
New
Rename
Save
Record
Stop
Run
To Cursor
From Cursor
Script:
Commands
Documents
Parameter
Commands Parameters Available
1 Thresholds interactive Input
Output
LevelLowRed
LevelHighRed
LevelLowGreen
LevelHighGreen
LevelLowBlue
LevelHighBlue
Create binary image
Invert result
Remove unsegmented c...
Automatic
Mode
ColorModel
2 Set Measurement F... DensImage
MaskImage
3 Reflector
4 Comments
5 Exposure time
6 Show Live Show
7 Material reuse func... Image
>> Commands
Thresholds interactive
Comment
Parameters
Input ActiveImage
Output MeasurementMask
LevelLowRed 251
LevelHighRed 106
LevelLowGreen 251
LevelHighGreen 106
LevelLowBlue 251
LevelHighBlue 106
Create binary image Yes
Invert result No
Remove unsegmented No
Automatic No
Mode Interactive
ColorModel RGB

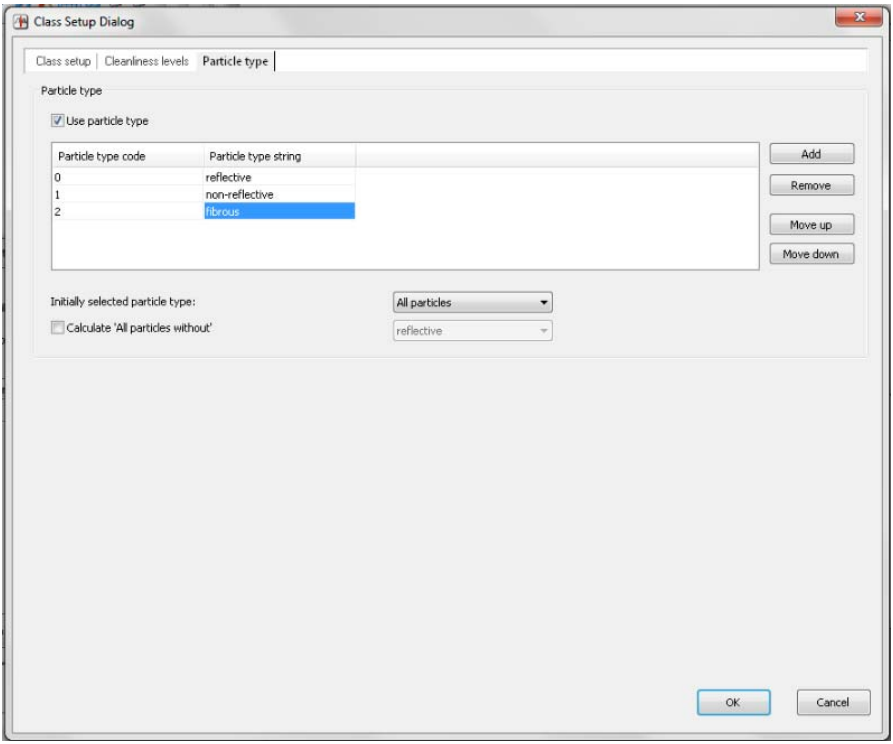
Measurement properties



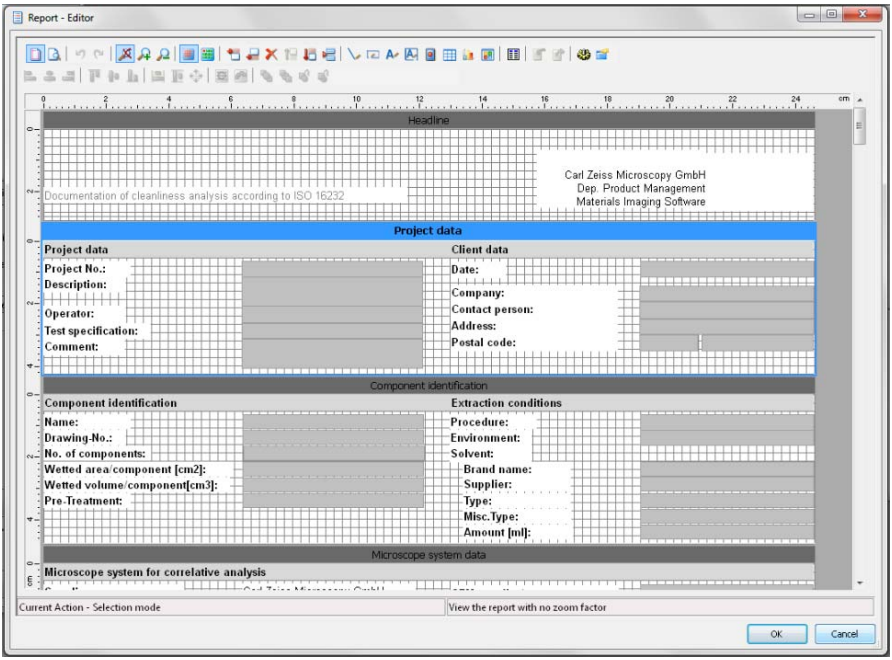
Class definition



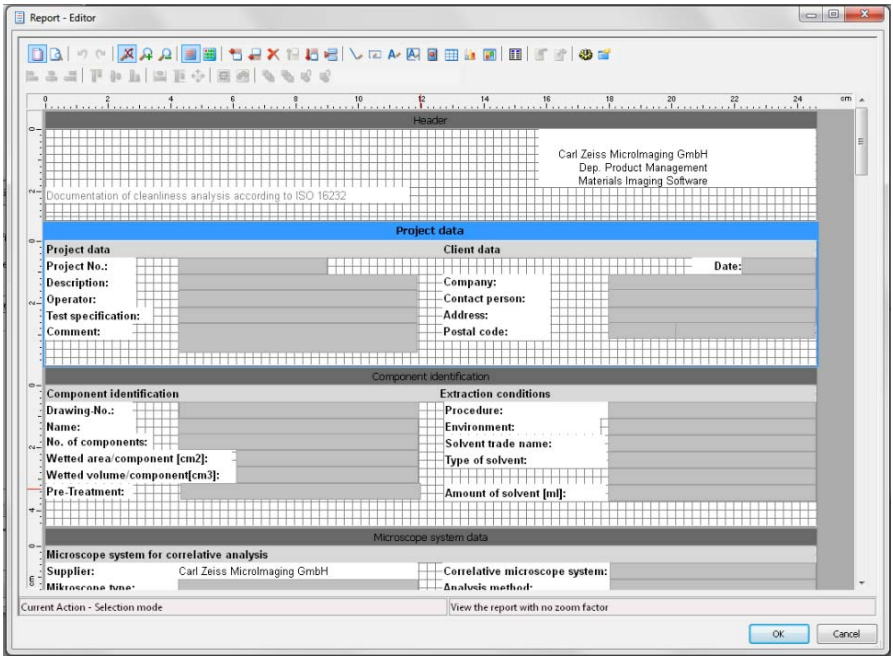

Class Setup Dialog
Class setup
Cleanliness levels
Particle type
Use cleanliness levels
Cleanliness level for particle size class:
All
Save condition table
Global condition table
Cleanliness levels
Condition
Condition ...
Condition ...
_N>=0 && _N<=0 00
_N>0 && _N<=1 0
_N>1 && _N<=2 1
_N>2 && _N<=4 2
_N>4 && _N<=8 3
_N>8 && _N<=16 4
_N>16 && _N<=32 5
_N>32 && _N<=64 6
_N>64 && _N<=130 7
_N>130 && _N<=250 8
New
Duplicate
Delete
Move up
Move down
Format for coding
Name
CCC = (
Separator:
/
Closing
)
Show normalization parameter dialog before measurement
ISO 16232 normalization parameter
Calculated from number of components:
Number of components:
1
Calculated from area:
Netted area per component [cm²]
1,00
Standard area [cm²]
1000,00
Calculated from volume:
Netted volume per component [cm³]
1,00
Standard volume [cm³]
100,00
Normalization parameter for other standard
Calculated from conversion factor:
Conversion factor:
1,000
OK
Cancel


Class Setup Dialog
Class setup
Cleanliness levels
Particle type
Particle type
Use particle type
Particle type code
Particle type string
0
reflective
1
non-reflective
2
fibrous
Add
Remove
Move up
Move down
Initially selected particle type:
All particles
Calculate 'All particles without'
reflective
OK
Cancel

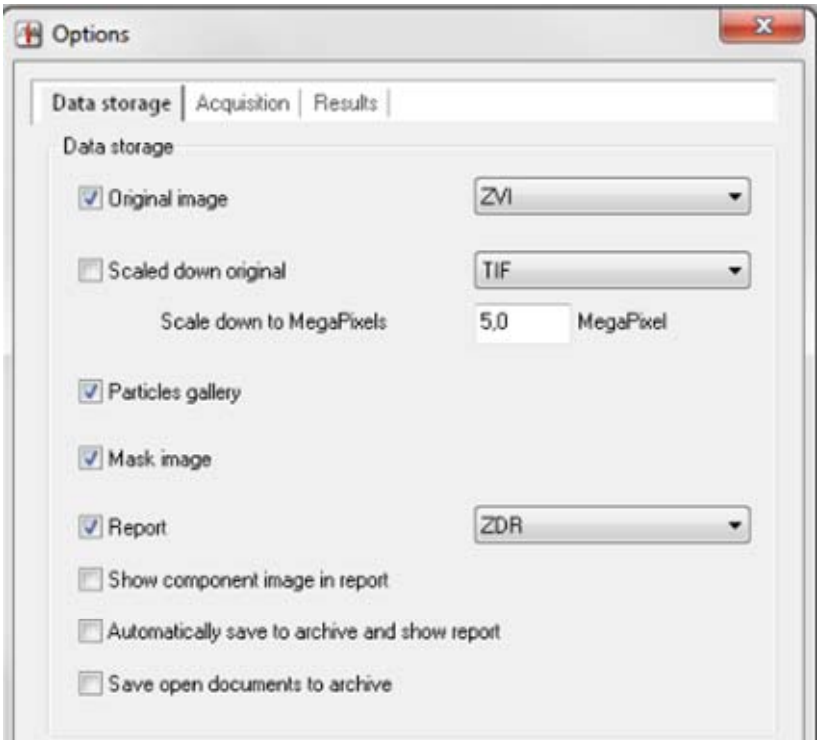
Report template

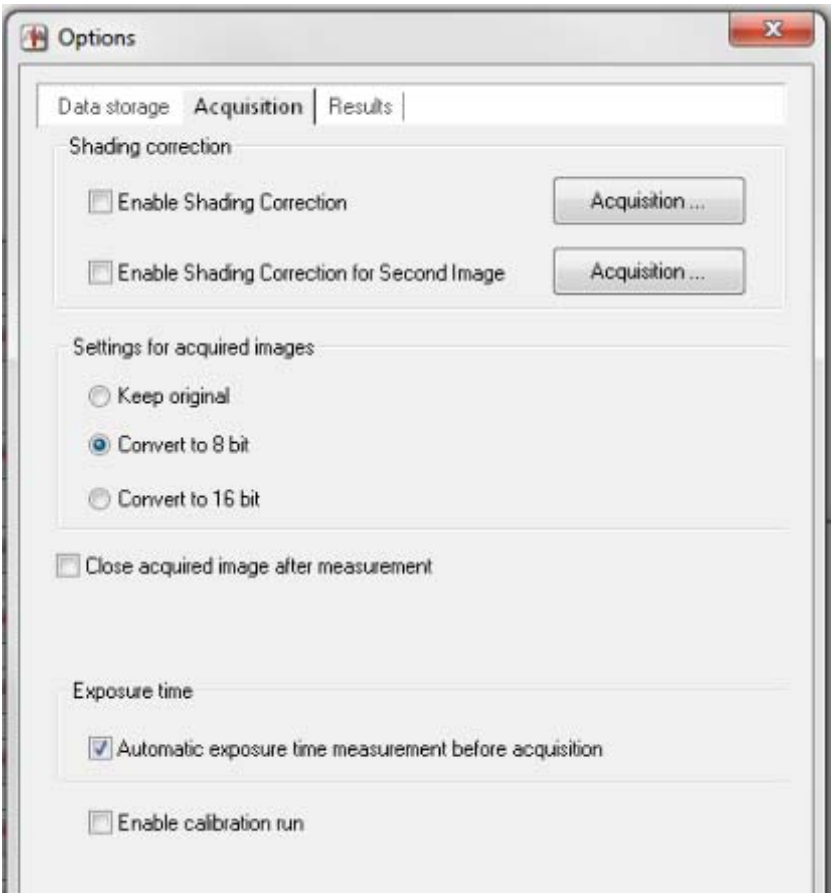


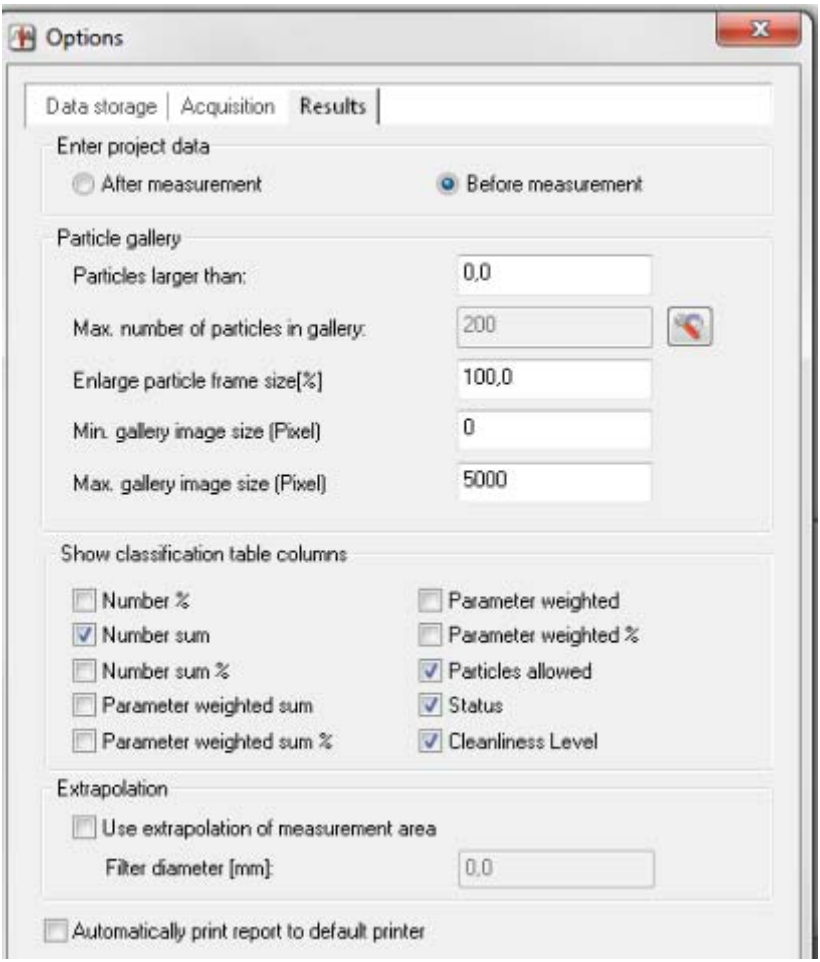
Cumulative report template



Options

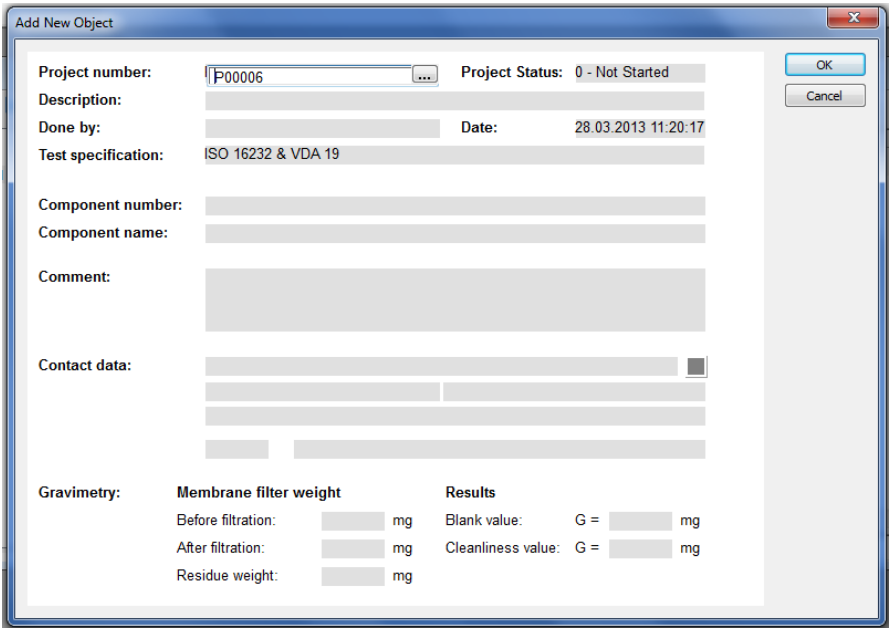

Options
Data storage
Acquisition
Results
Data storage
Original image
ZVI
Scaled down original
TIF
Scale down to MegaPixels
5,0
MegaPixel
Particles gallery
Mask image
Report
ZDR
Show component image in report
Automatically save to archive and show report
Save open documents to archive


Options
Data storage
Acquisition
Results
Shading correction
Enable Shading Correction
Acquisition ...
Enable Shading Correction for Second Image
Acquisition ...
Settings for acquired images
Keep original
Convert to 8 bit
Convert to 16 bit
Close acquired image after measurement
Exposure time
Automatic exposure time measurement before acquisition
Enable calibration run



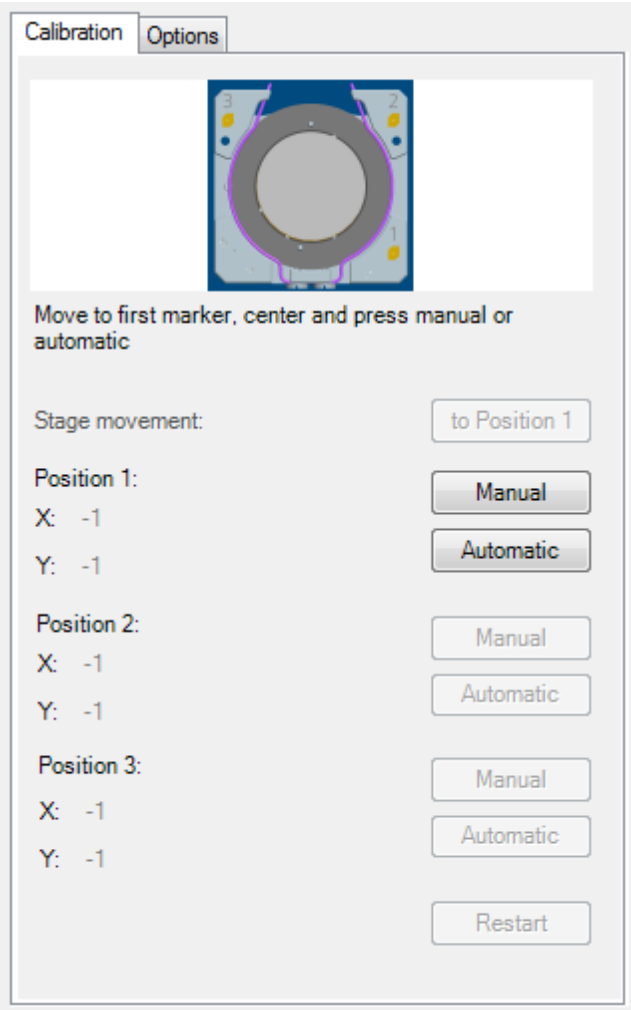
On the light microscope system, as part of the particle analysis workflow, you are first prompted to complete a report template and then automatically prompted to perform a calibration, provided that you have started the particle analysis using the **Correlative Particle Analysis** test specification supplied.

(3) Complete the input mask as far as necessary



(4) 3-point calibration

The marker positions can be set in two ways: using the **manual** or **automatic** option.



Buttons



Sets the current stage position as the marker position.



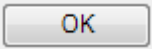
Sets the marker position with automatic marker detection. Once the marker has been detected automatically, a control image of the marker is displayed with a red reticle. From this you can see whether the marker has been detected correctly.



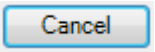
Enables the three marker positions to be recalibrated. A calibration that has already been saved remains unaffected by this until the new calibration is confirmed by means of the **OK** button.



For this function make sure that you have **automatically** calibrated the stage first. Locates the first marker position. This button is only active if you have saved a calibration in a holder file or the first position has been set during a calibration.



Saves the calibration and the settings on the **Options** property page and closes the dialog window. The button only becomes active once the calibration of the three markers has been performed in full. The calibration is saved in the holder file currently selected. A saved calibration can be adopted by clicking on the **OK** button without any need to run through the marker positions 1-3 again. This is only useful, however, if the position of the holder has not changed on the motorized stage.

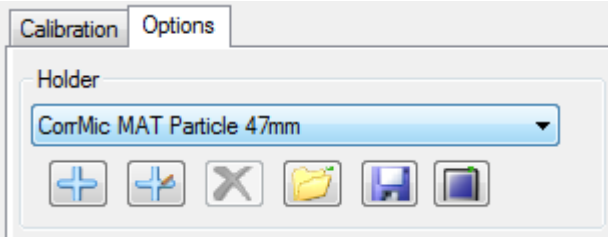


Closes the **Calibration** dialog window and continues the correlative workflow.

3 steps to calibration

We recommend that you initialize the stage before starting calibration. After the light microscope has been restarted, the initialization is performed automatically as soon as the Particle Analyzer workflow is started.

- **(1) Select the holder** under **Options** and ensure the settings have been configured correctly for detecting the L markers.



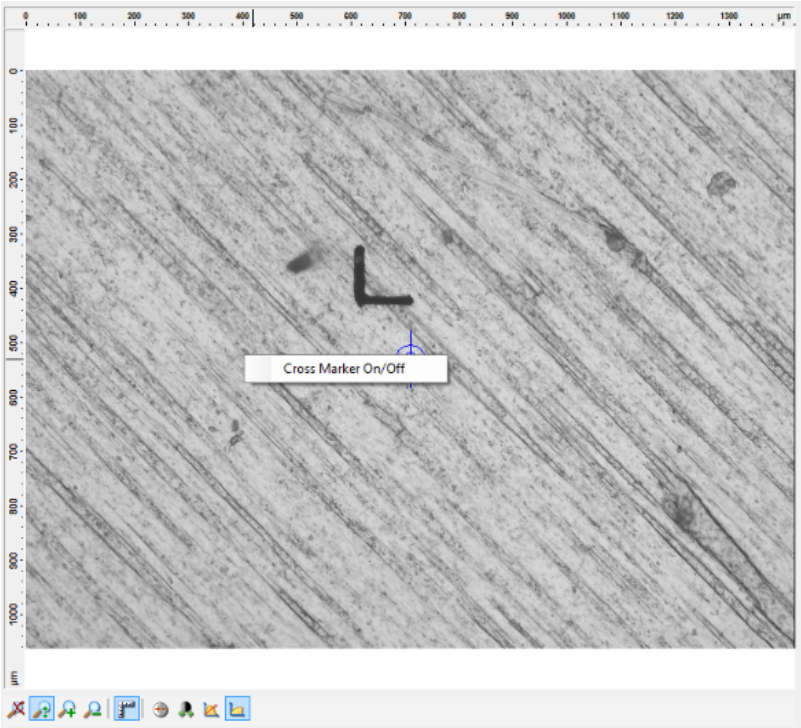
- **(2) Return to Calibration and move the stage manually to the first marker**. Set the microscope to ensure that the small marker can be seen clearly and in full.

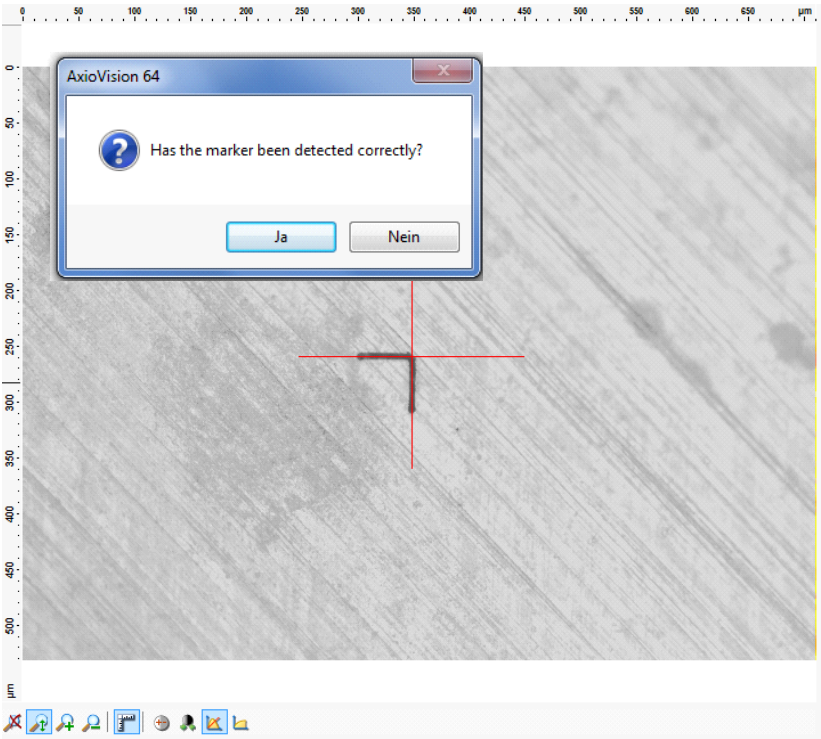
  We recommend that you initially use a weakly magnifying objective for the rough positioning of the large L and only then switch to the objective you want to use for calibration and subsequent acquisition, depending on the particle size to be detected. Bring the objective into focus. This can either be done manually or, if your system is equipped accordingly, using autofocus.

  Next you can decide whether you want to use the automatic detection function for the marker or whether you prefer the manual procedure. Experience has shown that our automatic marker detection works very reliably. We therefore recommend that preference is given to this method. The manual version is only advisable if the automatic function fails or if markers are used that are not L-shaped.

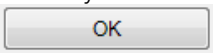
➢ **Automatic marker detection:**

Click on the Automatic **button** to adopt the current stage position as marker position 1. The position of the corner of the small marker is used for the marker position.

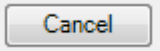




AxioVision now displays a control image of the acquired marker together with a red reticle. The image above shows an example of correct marker detection. If you select **Yes** in the **Has the marker been detected correctly?** window, the system locates the next marker position (marker position 2). Repeat this process until all three marker positions have been correctly detected. Close the **LM Calibration** window by clicking on

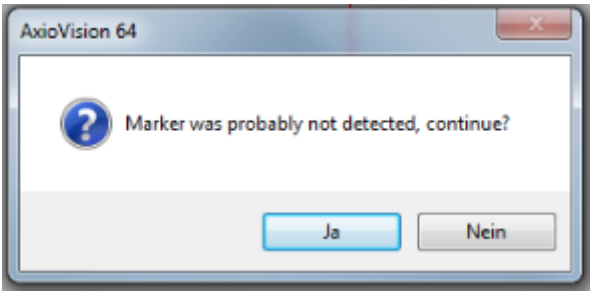


.



exits the dialog and the Correlative Particle Analysis workflow continues, i.e. the calibration that has previously been saved is used.

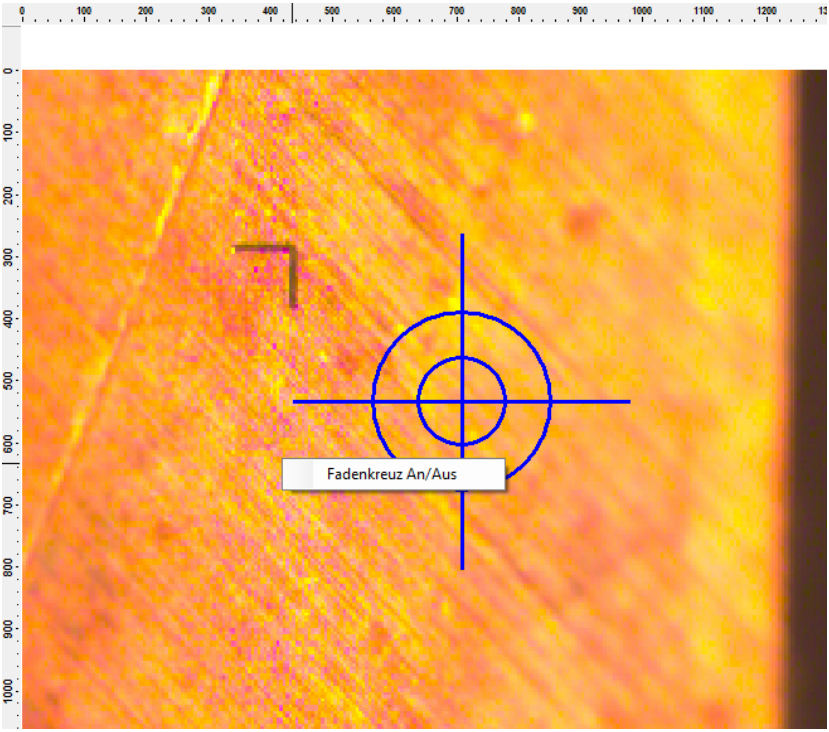
If the marker has been poorly detected or has not been detected at all, a dialog window opens:



If you click on **Yes**, the marker coordinates are adopted. Clicking on **No** gives you the opportunity to perform a manual calibration.

- **(3) Manual marker calibration**

  If you set the markers manually, no automated marker detection is performed. Instead you are able to use dirty or self-defined markers, which have to be centered manually in the image and can then be used for calibration. If automatic marker detection fails or you are using other markers which are not L-shaped, please use manual marker detection. A reticle can be displayed by right-clicking with the mouse in the live image:



Position the reticle precisely over the marker by moving the stage in the X/Y direction.

Clicking on the Manual button adopts the marker position into the system. The rest of the procedure is the same as for the automatic calibration process.

Please note:
Due to possible center deviations between individual objectives we recommend, whenever possible, that you perform calibration and image acquisition using the same objective! If you have **parcentricity correction**, you can switch the objective between calibration and measurement without any problem.

Fine calibration

Fine calibration is used to improve the relocation of particles in special cases, e.g. in the event of a systematic deviation in particle positions in the X and/or Y direction. The fine calibration dialog is accessible as soon as particles have been selected for the EDS analysis (particle selection).

In the left-hand area of the window the gallery images from the light microscopy analysis that correspond to the results data table are displayed. In the right-hand area of the window a live electron microscope image is generated.



Steps

(1) Select a particle that you want to use for fine calibration.

(2) Define a calibration position (see red reticle in the graphic) at a prominent location on the particle in the LM view by clicking with the mouse (left mouse button).

(3) Move the motorized stage of the electron microscope in such a way that the selected calibration position for the particle is at the center of the blue reticle in the graphic and confirm by clicking on **OK**.

(4) Start the automatic EDS analysis.

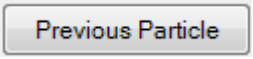
Settings

Once the calibration position has been set in the LM gallery image (red reticle in the graphic), the motorized stage of the electron microscope automatically moves to this position, which should be at the center of the frame. If this is not the case, this indicates a shift between the particle positions on the light and electron microscope. The shift is compensated for by moving the motorized stage on the electron microscope in such a way that the two particle positions are aligned. **This offset correction is applied to all selected particles during the automatic EDS analysis**.

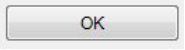
Buttons



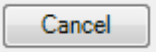
Displays the next LM particle gallery image listed in the results data table. Used to select a particle for fine calibration.



Displays the previous LM particle gallery image listed in the results data table. Used to select a particle for fine calibration.



Moves the motorized stage of the electron microscope to the selected calibration position. A dialog opens that prompts you to move the stage manually to align the particle position. Confirm this dialog by clicking on **OK** to conclude fine calibration.



Cancels the **Fine calibration** dialog and shows the **Correlative Particle Analysis** dialog again in which the particles can be selected.

After confirming the calibration by clicking on the **OK** button you can continue the particle analysis.

(5) Structure the rest of the workflow for particle analysis using Particle Analyzer Projects

The instructions for the Particle Analyzer Projects module can be found in section 11.4.

Correlative workflow on an electron microscope

Remove the correlative holder with the filter sample from the motorized stage of the light microscope and attach it to the motorized stage of the electron microscope.

Then perform the steps on the electron microscope as part of a guided workflow using Correlative Particle Analysis and Smart PI as follows:

(1) Import the particle results data from the light microscopy analysis using Correlative Particle Analysis.

(2) Perform a 3-point calibration to define the reference coordinate system for the electron microscopy analysis.

(3) Select the desired **reflective** particles for the EDS analysis from the particle results data table.

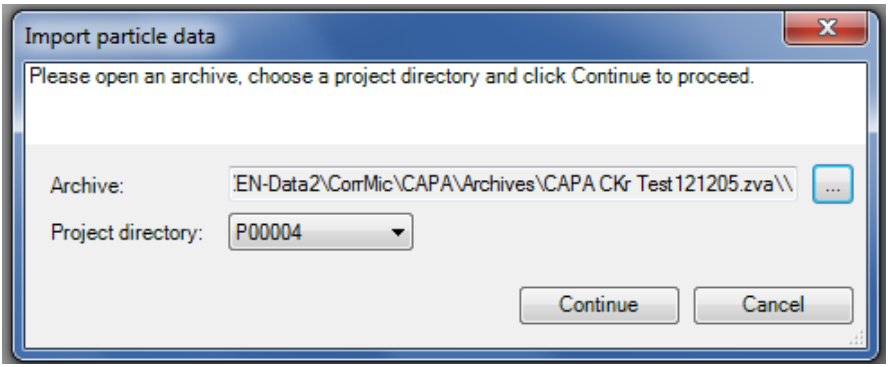
(4) Start the automatic EDS analysis of the selected reflective particles. Answers to further questions on the SEM/EDS analysis can be looked up in the SmartPI online help or in the SmartPI manual.

Depending on the selected presetting, you will either directly obtain a report containing the combined results from the light and electron microscopy analyses or an interactive view that allows you to examine the analysis results and gives you the option of performing a new EDS analysis. It is also possible to generate a combined results report from the interactive view.

(1) Import the particle results data

If you click on the 

button in the toolbar, the **Import particle data** dialog opens.



Steps

(1) Confirm the Particle Analyzer archive displayed or select an alternative archive containing the data to be correlated from the light microscopy analysis.

(2) Confirm the project directory displayed or select an alternative project directory from the list. Continue the correlative workflow by clicking on **Next**.

Settings

Archive: Displays the archive currently selected.

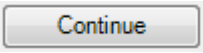
... Opens the **Archiv Öffnen** dialog; allows alternative archives to be selected.



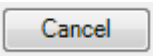
Displays a list of the project directories available in the selected archive.

As part of the correlative workflow, the **Import particle data** dialog automatically shows the last Particle Analyzer archive used with the most recently edited project. If desired, you can also select an alternative archive in this dialog.

Buttons



Continues the correlative workflow and opens the **Calibration** dialog.



Cancels the correlative workflow and closes the **Import Particle data** dialog.

(2) Calibration on an electron microscope

The calibration to define the reference coordinate system is the most important step in the correlative workflow. This process must be performed once before each new analysis is started. On the electron microscope the calibration dialog starts automatically after the dialog to import the particle data.

Steps

(1) Locate the first marker position manually so that this appears centrally in the frame and confirm this position by clicking on the **manual** or **automatic** button.

(2) Confirm the positions for the second and then for the third marker in the same way.

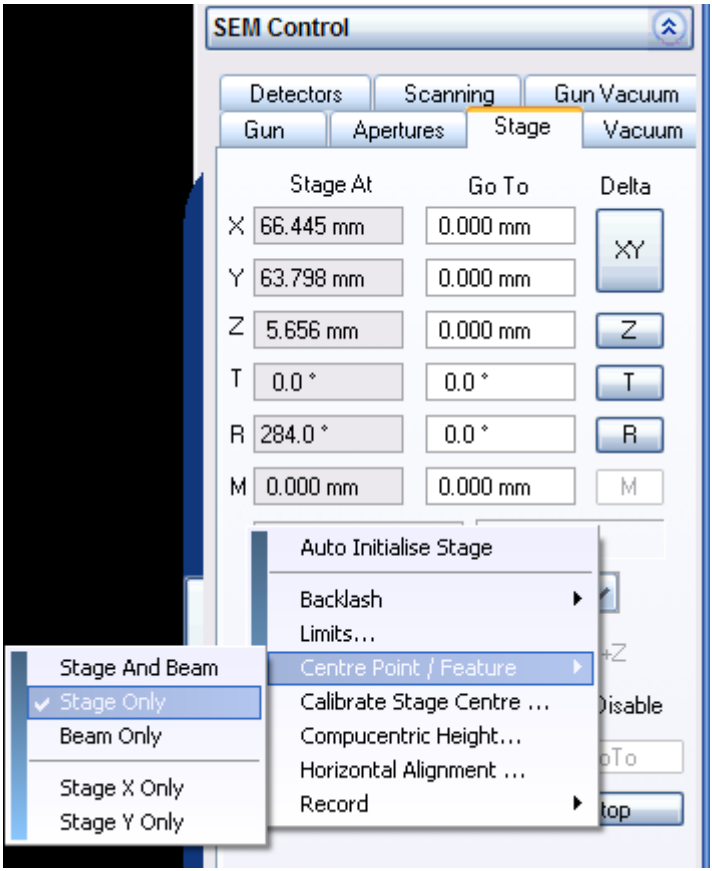
(3) Continue the correlative workflow by clicking on the **OK** button.

On the **Calibrate** property page you will find a detailed description of the individual steps and on the **Options** property page you will find the setting options.

Additional information

On an electron microscope there are a few points to bear in mind for Correlative Particle Analysis:

- The stage rotation is not taken into account.
- If you are using a ZEISS holder, perform a one-off mechanical rotation of the stage to ensure that the L marker is straight in the image.
- With CAPA the beam shift must be switched off.
- If you are using **Center Point Feature**, deactivate the use of the beam shift. Call up the shortcut menu by right-clicking on the **Stage** property page. Select **Center Point / Feature** ⇒ **Stage Only**.

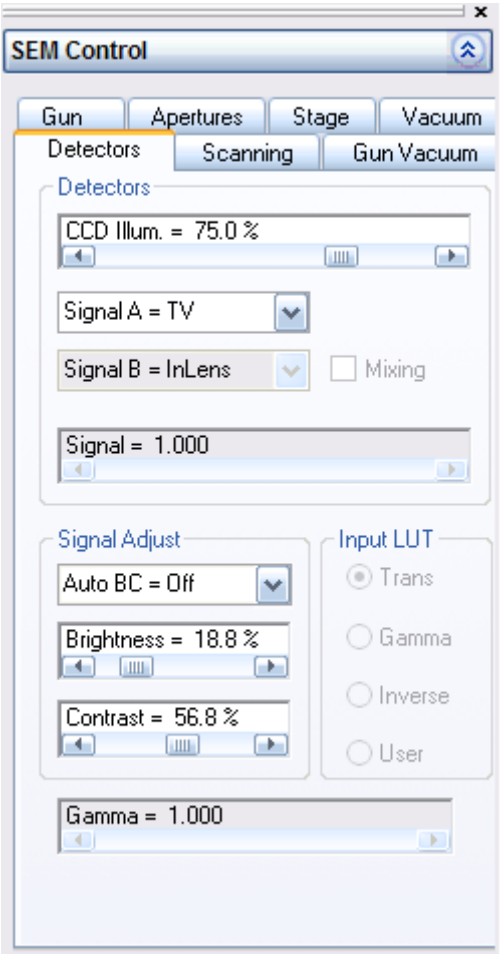


- Do not use scan rotation.

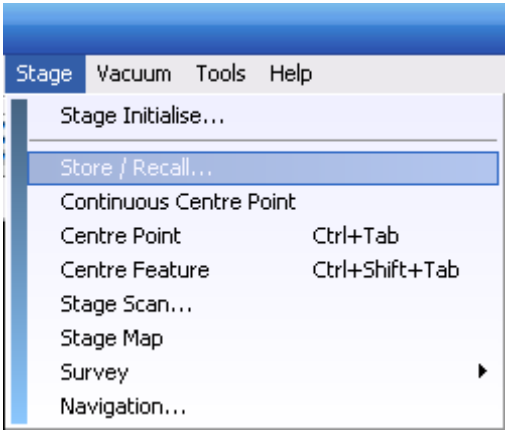
- The stage on an SEM may move left/right to the next marker rather than forwards/backwards. In such cases rotate the stage by 90°.

- Each time before you click on the **Automatic** button, set the SEM parameters so that the small L marker can clearly be seen in the image. Focus and contrast/brightness are most important. You can either adjust the focus manually or by means of the autofocus function, using the **Ctrl+F** key combination.

- The **Contrast/Brightness** setting can be adjusted manually or you can set **Auto BC** correction on the **Detectors** property page in the **Signal Adjust** field.



- If you have located the position of the first marker and the stage is correctly oriented, it is best to save this position in SmartSEM in the **Stage Points List** via the **Stage** menu ⇒ **Store / Recall...**.

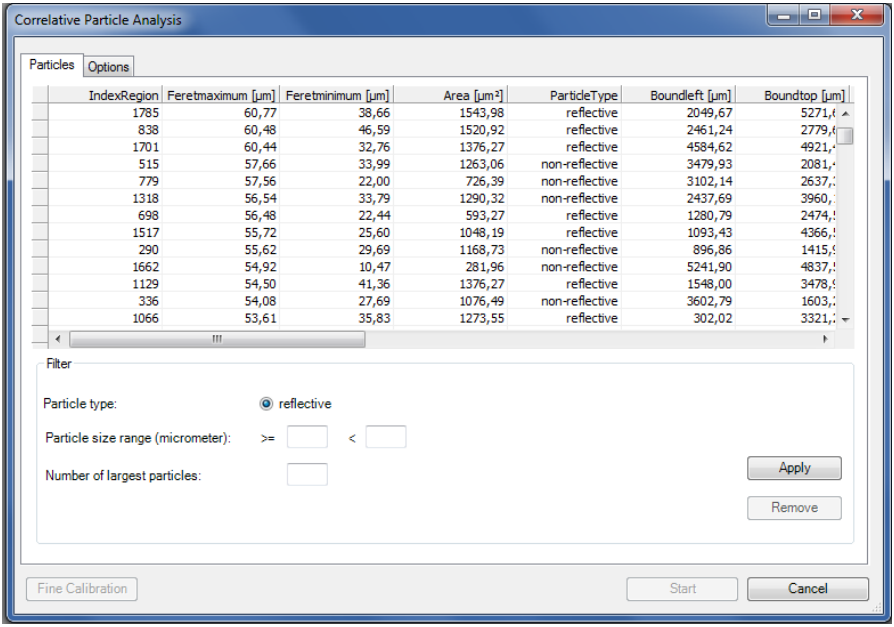


- The **Stage Points List** dialog window opens. Click on the **Add** button to save the current stage position. You can locate a saved position by double-clicking on an existing entry in the list.


Stage Points List
Stage List = Stage
Save/Load
On Goto
Add
Del
Edit
Undo Stage Goto
Stage stop
Goto
First
Next
Prev
Selected
No Image Available

(3) Particle selection

In the **Correlative Particle Analysis** dialog you can select specific **reflective** particles for automatic EDS analysis.



The **Particle Size range** and **Number of largest particle filter** functions are available for this purpose on the **Particle data** property page.

Before you start the automatic EDS analysis, you should ensure that the desired SmartPI presettings, such as the selection of the material database, application type and application recipe, have been configured on the **Options** property page. You can also select your preferred results view on the **Options** property page.

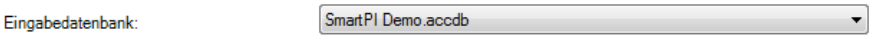
(3.1) Presettings for Correlative Particle Analysis

The settings configured on the **Options** property page determine the workflow for the automatic SEM/EDS analysis on the electron microscope.

The input databases, application types and application recipes available for selection are read out from the SmartPI software. Such SmartPI configuration files are essential for performing the correlative analysis on the electron microscope using Correlative Particle Analysis. A quick guide to configuring the required settings and defining the parameters can be found in the SmartPI manual (from version 08.08.12) under "*Appendix 1 Application Recipe – quick guide*".

(3.2) Optionen property page: Selection of SEM/EDS parameters

Settings



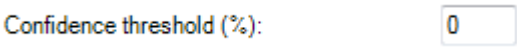
Shows the material classification databases made available by SmartPI.



Shows a list of the application types made available by SmartPI. This configuration file contains information and settings for all the application recipes assigned to it.



Shows a list of the application recipes made available by SmartPI that are accessible via the selected application type. An application recipe stores basic settings relating to the holder setup, device calibration (SEM & EDS), and brightness and contrast settings for image processing and analysis.



Defines a lower limit for the confidence threshold. The confidence value is a percentage measure of the similarity between the particle found on the light microscope and on the SEM. All particles below the set threshold value are not analyzed further and are also not displayed in the results table. Generally speaking, the greater this number is, the better the correspondence between the particle found on the light microscope and on the SEM.



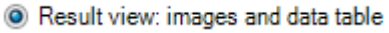
Determines the actions that the electron microscope performs after the analysis: electron beam off, EHT off, activate standby mode, enter high vacuum mode (HV).

(3.3) Options property page: Selection of results documentation

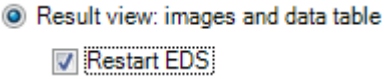
Settings



Generates a combined LM/EM results report after completion of the EDS analysis.

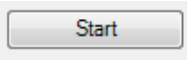


Generates a results view with correlative LM/EM images and spectra as well as an interactive data table with LM/EM measurement parameters, element composition percentages and material classification.

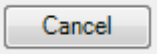


Generates a results view with correlative LM/EM images and spectra as well as an interactive data table with LM/EM measurement parameters, element composition percentages and material classification. Also offers the option of performing another EDS analysis with an alternative application recipe.

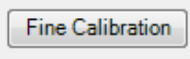
Buttons



Starts the automatic EDS analysis procedure. An EDS analysis of the previously selected particles is performed.



Cancels the workflow and closes the **Correlative Particle Analysis** dialog.



Opens the **Fine Calibration** dialog. Fine calibration is used to improve the precision with which particles are relocated in special cases and is described in the *"Fine calibration"* section under 3.1 (4).

Now return to particle selection.

Steps

(1) Select the particles you intend to use for an EDS analysis with the help of a filter function.

(2) Apply the selected filter.

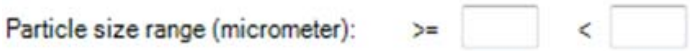
(3) Start the automatic EDS analysis of the previously selected particles.

(3.4) Particle data property page

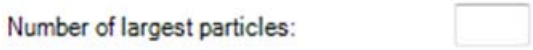
Settings



Presetting of the particle type. The correlative workflow provides for the EDS analysis of reflective particles. If you want to expand the analysis to include non-reflective particles, you also need the AxioVision Shuttle&Find module.



Filters the particle data table on the basis of the particle size range.



Filters the particle data table on the basis of the N largest (FeretMax) reflective particles.

Buttons



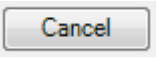
Filters the particle data table by reflective particles on the basis of the selected **Particle size range** or the preset **Number of largest particles**. After the filter has been applied the particle data table shows only the previously selected particles.



Removes the filter applied to the particle data table. After the filter has been removed the unfiltered particle data table containing all particles is displayed once again.



Starts the automatic EDS analysis procedure. An EDS analysis of the previously selected particles is performed.



Cancels the workflow and closes the **Correlative Particle Analysis** dialog.

Documentation of results

Report and results view

For the documentation of your results you can choose between three options, which are activated in the **Correlative Particle Analysis** dialog on the **Options** property page ⇒ **Result view** selection option.

(1) Report view

(2) Result view: images and data tables

(3) Result view: images and data tables with option to perform another EDS analysis

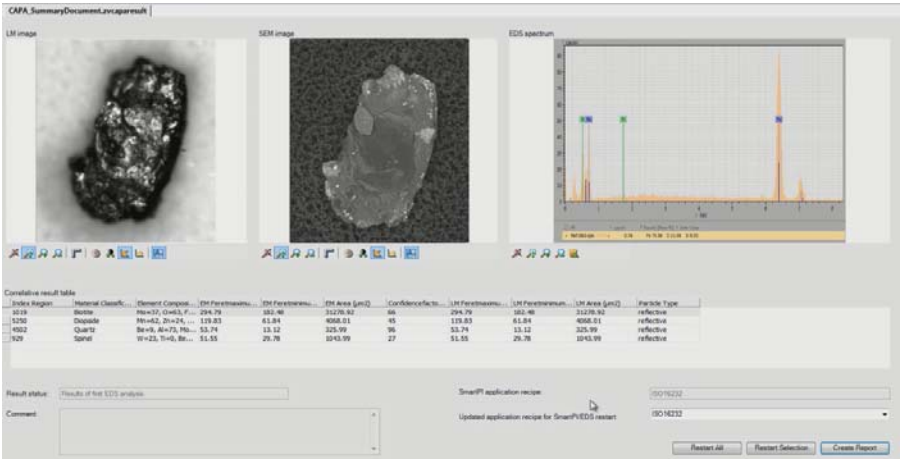
If you select the **Report view** option, immediately after the EDS analysis a combined results report is generated containing the results from the light and electron microscopy analyses. This concludes the correlative analysis.

If you select the **Result view: Images and data table**, an interactive results document is generated that shows the results from the light and electron microscopy analyses and also offers the option of relocating the particles analyzed by means of SEM/EDS in a targeted way on the electron microscope. You can view these particles in the electron microscope's live image mode. At the touch of a button you can then generate a combined results report as described and in this way conclude the correlative analysis.

If you select the **Result view: Images and data table with Option to restart EDS analysis**, in addition to the functions described it is possible to perform a second EDS analysis of individual or all selected particles on the basis of a modified application recipe. Once this EDS analysis has been completed, a second results document is generated. Each additional EDS analysis overwrites the results of the second EDS analysis, which means that a maximum of two results documents are generated irrespective of the number of repeated EDS analyses ?? the document for the first and the document for the repeated EDS analysis. To conclude the correlative analysis, you can generate a combined results report as described.

Steps when viewing results

The following results table appears following the completion of the automatic EDS analysis:



(1) Select a particle that you want to view on the electron microscope by left-clicking once on the row in question in the correlative results table.

(2) Repeat step 1 until you have examined all the particles that are of interest to you.

(3) Generate a combined results report by clicking on the **Create Report** button.

Settings

In the results view the following are shown for each selected particle (1) the gallery image from the light microscopy analysis, (2) the corresponding gallery image from the electron microscopy analysis, (3) the corresponding element spectrum with the element distribution percentages and (4) the correlative results table containing the combined data from the light and electron microscopy analyses. The correlative results table has been designed interactively and offers you the option of relocating particles on the electron

microscope and of selecting particles in a targeted way for another EDS analysis.

 Displays the image in its original size.

 Adjusts the zoom factor to the window size.

 Increases the zoom factor (F7)

 Decreases the zoom factor (F8)

 Shows or hides the ruler.

 Best Fit: Optimum display of the highest and lowest gray or color value present, excluding defined min. and max. values.

 Min/Max: Optimum display of the highest and lowest gray or color value present.

 Maximum range: Linear display of the maximum possible value range.

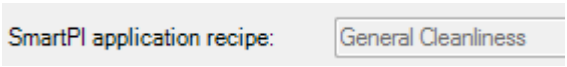
 Gamma 0.45: Display curve presetting for best color reproduction.

 Shows or hides annotations.

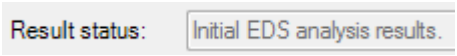
 Displays the EDS spectrum in its original size as an AxioVision document.



Allows comments to be linked to a particle result.



Shows the SmartPI application recipe used for the EDS analysis.



Shows whether the result relates to the first or a repeated EDS analysis.

Buttons



Generates a results report containing combined results from the light and electron microscopy analyses.

Steps when viewing results with performance of another EDS analysis

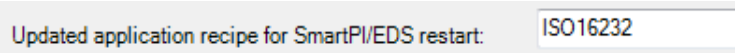
(1) Use a key combination to select individual particles for the new EDS analysis. Hold down the **Ctrl** key and left-click on the rows in question in the correlative results table.

(2) Select an adjusted application recipe for the new EDS analysis.

(3) Start the new EDS analysis.

Alternatively, in step 1 you can also restart the EDS analysis of all particles shown in the correlative results table.

Settings



Selects an alternative SmartPI application recipe for the new EDS analysis.

Buttons



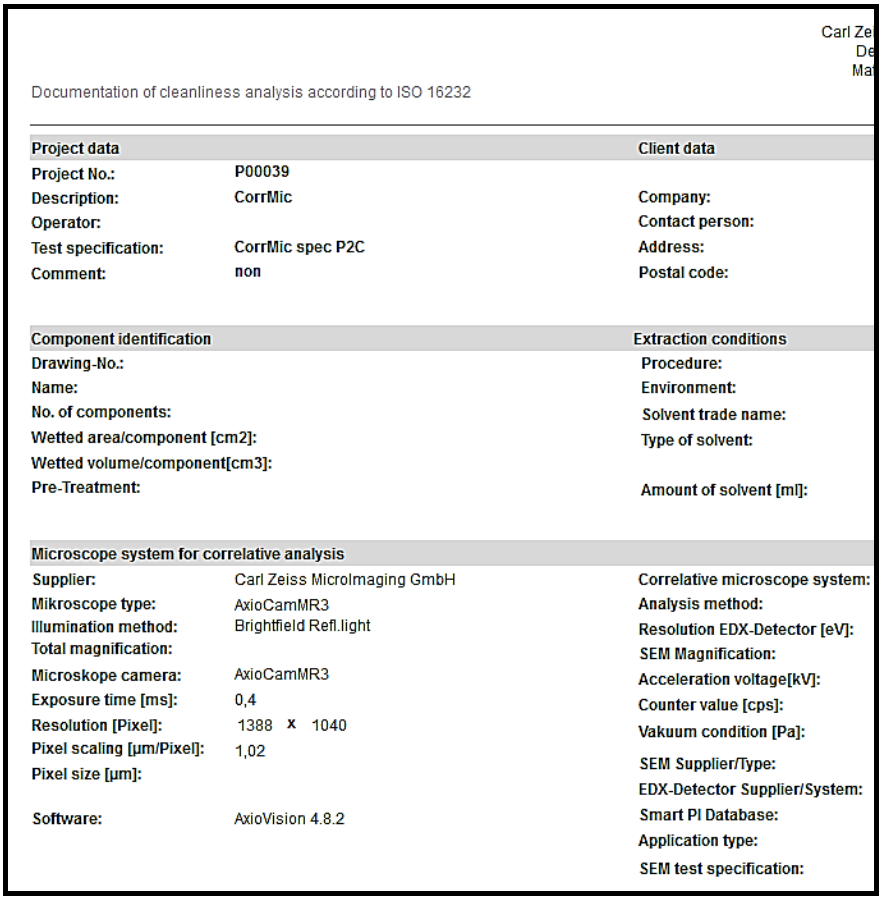
Starts the EDS analysis of all particles shown in the correlative results table.



Starts the EDS analysis of a selection of the particles shown in the correlative results

Report

Overview page



Carl Ze
De
Ma

Documentation of cleanliness analysis according to ISO 16232

Project data		Client data	
Project No.:	P00039		
Description:	CorrMic	Company:	
Operator:		Contact person:	
Test specification:	CorrMic spec P2C	Address:	
Comment:	non	Postal code:	

Component identification		Extraction conditions	
Drawing-No.:		Procedure:	
Name:		Environment:	
No. of components:		Solvent trade name:	
Wetted area/component [cm2]:		Type of solvent:	
Wetted volume/component[cm3]:			
Pre-Treatment:		Amount of solvent [ml]:	

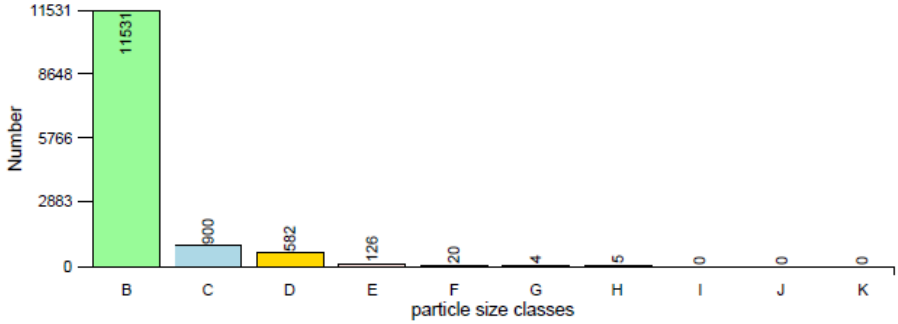
Microscope system for correlative analysis			
Supplier:	Carl Zeiss MicroImaging GmbH	Correlative microscope system:	
Mikroscope type:	AxioCamMR3	Analysis method:	
Illumination method:	Brightfield Refl.light	Resolution EDX-Detector [eV]:	
Total magnification:		SEM Magnification:	
Microskope camera:	AxioCamMR3	Acceleration voltage[kV]:	
Exposure time [ms]:	0,4	Counter value [cps]:	
Resolution [Pixel]:	1388 x 1040	Vakuum condition [Pa]:	
Pixel scaling [µm/Pixel]:	1,02	SEM Supplier/Type:	
Pixel size [µm]:		EDX-Detector Supplier/System:	
Software:	AxioVision 4.8.2	Smart PI Database:	
		Application type:	
		SEM test specification:	

The first page of the report shows all the **project data**, e.g. the project number, operator and specification used, as well as the **parameters used**, e.g. the extraction method, type and amount of rinsing fluid, and also **information on the microscopes**, e.g. the microscope type, microscope magnifications, camera, exposure time, resolution on the LM and EM, etc. The LM parameters can be found on the left and the EM parameters on the right.

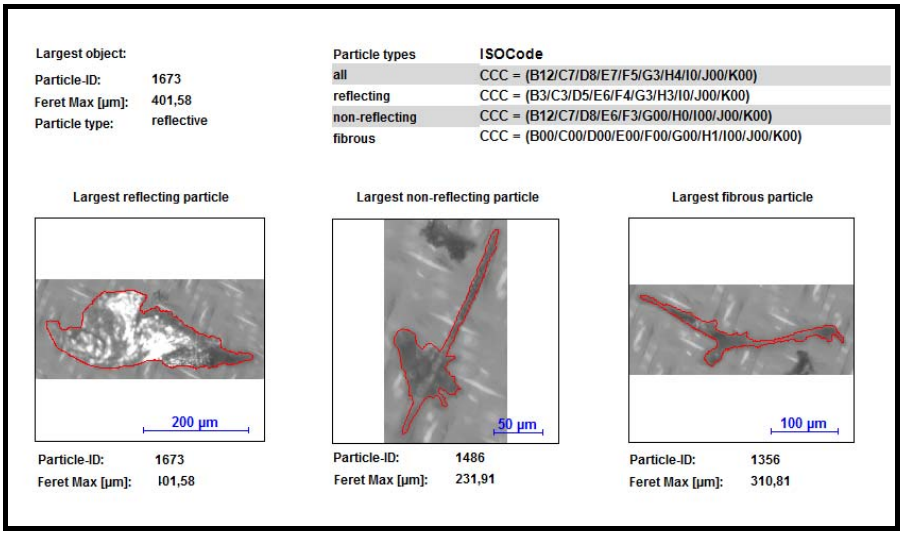
LM report section

Classification results all particles incl. fibres

Class	Lower boundary	Operators	Upper boundary	Number	Number sum	Parameter weighted
B	5	>= ... <	15	2296	2296	16119,50
C	15	>= ... <	25	115	2411	2301,31
D	25	>= ... <	50	186	2597	6546,39
E	50	>= ... <	100	106	2703	7108,70
F	100	>= ... <	150	21	2724	2594,58
G	150	>= ... <	200	5	2729	882,47
H	200	>= ... <	400	9	2738	2413,85
I	400	>= ... <	600	1	2739	401,58
J	600	>= ... <	1000	0	2739	0,00
K	1000	>= ... <		0	2739	0,00



Page two presents an overview of the particle classification, including reflecting, non-reflecting and fibrous particles and the associated histogram.



On page three you will find a gallery of the largest reflecting, non-reflecting and fibrous particles as well as their "Cleanliness Level" Code.

In the tables that follow, the size distributions for the reflecting, non-reflecting and fibrous particle types are displayed again. The "Cleanliness Level" is also indicated in addition to the size classification.

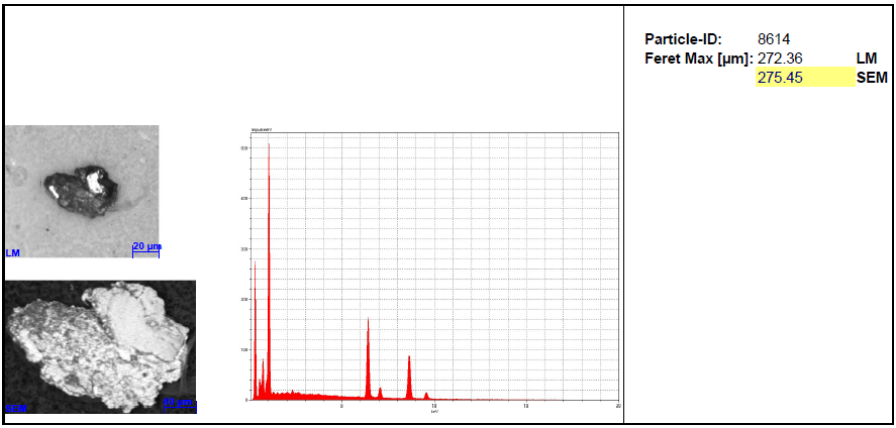
EM report section

Results SEM / EDS analysis

Element composition of selected particles - part 1

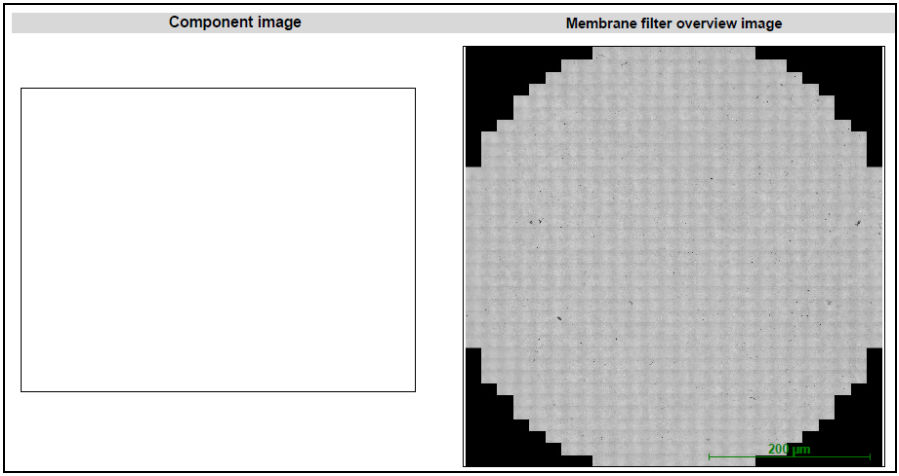
Index Region	IA Classification	Material Classification	Element Composition (%)	Particle Classification	Feretmaximum (µm)	Feretminimum (µm)	Area (µm2)	Diameter (µm)	CenterX	CenterY	Confidencefactor (%)
8861	400 - 600	Zn-haltig	Al=9, S=5, Zn=85	Zn-haltig	529.13	211.93	0.06	281.84	0.00	0.00	54
15311	200 - 400	Zn-haltig	Fe=5, Zn=95	Zn-haltig	368.18	149.80	0.03	202.84	0.00	0.00	54
627	200 - 400	Zn-haltig	Zn=100	Zn-haltig	275.97	126.39	0.02	171.70	0.00	0.00	52
8614	200 - 400	Zn-haltig	Fe=42, Zn=58	Zn-haltig	275.45	181.28	0.03	202.39	0.00	0.00	54
226	5 - 15	Unclassified	Ca=64, S=31, Na=4	Unclassified	8.86	6.45	0.00	5.91	0.00	0.00	24
16793	150 - 200	Al-haltig	S=4, Al=96	Al-haltig	173.87	54.15	0.01	81.46	0.00	0.00	53
17549	150 - 200	Stahl unlegiert	Fe=100	Stahl unlegiert	163.94	101.17	0.01	101.45	0.00	0.00	52
892	150 - 200	Al-haltig	Al=90, Si=10	Al-haltig	151.90	81.35	0.01	95.30	0.00	0.00	54
13253	100 - 150	Al-haltig	Si=13, Al=82, Cu=5	Al-haltig	146.54	112.52	0.01	106.84	0.00	0.00	53
14103	100 - 150	Stahl unlegiert	Fe=100	Stahl unlegiert	141.82	82.22	0.01	96.12	0.00	0.00	55
16549	100 - 150	Stahl unlegiert	Fe=100	Stahl unlegiert	133.43	80.99	0.01	99.85	0.00	0.00	54

This table displays the element compositions, size, area and the confidence factor, which indicates the degree of correspondence between the LM particle and the particle measured on the EM.



In addition, the report presents a comparison in an LM and EM view of all the particles measured using EDS and also displays the spectrum. To check the measurement it is possible to refer to the **Feret Max** value, which should not

be too far from the LM value. If it is, the segmentation must be adjusted in the SmartPI software.



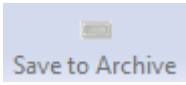
On the last page of the report you can insert an image of the component by selecting one under **Manage Test Specification** ⇒ **Options** ⇒ **Show component in report**.

For further information on setting the test specification please refer to sections 3.1 "General" and 11.4. "Particle Analyzer Projects" of this manual.

Comparison of data between light and electron microscope

Save to Archiv

You can also use the light microscope for the particle analysis of additional filter samples while a correlative analysis is in progress on the electron microscope.

The presence of new data from the electron microscopy analysis is indicated by the 

button becoming enabled.

Updates the archive with data from the electron microscopy analysis. The data are saved in the project that formed the database for the selection of particles from the light microscopy analysis.

Overlaying

To overlay the information acquired from the light and electron microscope, you need the overlay function of the **Shuttle&Find** module.

Depending on how much memory the system has, it may not be possible for images larger than 200 MB to be loaded in the overlay window.

EDS mapping of a particle

If you want to perform EDS mapping to analyze the distribution of the elements on the particle as a whole, please refer to the manual for the detector you are using.

Notes on operation

- Make sure that the holder and, in particular, the markers do not get dirty. Work in an environment that is clean and as free of dust as possible. Make sure that air is not blown vertically onto the holder and consequently onto the markers. This could cause dust particles to accumulate, which would result in the accuracy of the marker detection being reduced. If the marker is a little dirty, it can be helpful for detection purposes to defocus it slightly.

- Make sure that you protect the filter sample from external contamination. Always keep the filter horizontal when transferring it from the light to the electron microscope to prevent the particles moving on the filter sample.

- Once you have calibrated, the most recent calibration that you have set always remains in the holder file. This means that you do not need to perform calibration again, provided that the position of the holder does not change relative to the stage. Generally speaking, however, accuracy is reduced if a calibration is re-used.

- Make sure that the correct holder is always set under the calibration options. As the calibration is saved in the holder file, the calibration from the currently selected holder is always used. The holder files are not user-specific. Your calibration data will be lost if another user performs his or her own calibration using the same holder file.

- If the stage moves in the wrong direction during calibration, check the settings for XY inversion on the **Options** property page.

- For marker detection it is sufficient to have the L marker in the frame. It does not have to be positioned precisely at the center.

- In the case of stitched MosaiX images accuracy is reduced.

- You can increase the accuracy by performing a fine calibration.

On the light microscope

- Initialize the stages before you use Correlative Particle Analysis.

- To achieve a good calibration, the objectives must have been geometrically calibrated correctly. We urgently recommend that you scale the objectives manually with the help of a stage micrometer and do not use the **Automatic Scaling** function on the **Measure** menu!

- The **To Position 1** function on light microscopes requires stage calibration. Always make sure that you have selected the correct holder in the calibration options. The calibration is saved in the file of the current holder. When a holder is exported the calibration in the file of the current holder is deleted.

On the electron microscope

- Use an electron microscope with VP (variable pressure) mode to prevent excessive charging of the filter.

- The SE2 detector is well suited to marker detection.

- Switch off the beam shift and scan rotation.
- It is recommended that on the electron microscope the position of the first marker is saved in the SmartSEM software. During calibration you will then be able to locate the first position again quickly.
- The **Stage only** setting should be activated under **Center Point** / Feature.
- Rotate the stage mechanically before the calibration so that the markers are as straight as possible in the SEM image. Do not otherwise rotate the stage.
- Use the **stage backlash** to increase the stage's accuracy in locating positions.

General information on accuracy

Influencing accuracy

There are various factors that can influence the accuracy of CAPA:

- The accuracy with which the microscope stages locate positions
- The centering and scaling of the objectives on the light microscope
- The accuracy of the calibrations
- The transfer to other microscopes, if the sample moves in the holder or the position of a particle changes during the transfer.
- CAPA has a location accuracy of 10 µm. You can increase the accuracy by performing a fine calibration or using a more accurate stage, for example. Below you will find tips on how to achieve maximum accuracy.

How can maximum accuracy be achieved?

- Make sure that CAPA is set up correctly (please read the "Setting up Correlative Particle Analysis" section in this chapter)
- If possible, the objective should not be changed on the LM after calibration.
- The scaling set for the objectives should be as accurate as possible
- Calibration should be as accurate as possible. Check the automatic marker detection by relocating the positions and, if necessary, recalibrating or setting the positions manually.



- In the drawing the position on the small marker at which the positions are set has been indicated.
- Make sure that you have calibrated the stage on the LM using



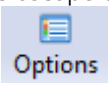
.

- When you switch to another microscope, do not move the filter sample in the holder and ensure that it can move as little as possible.

Troubleshooting

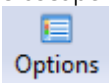
Calibration

➢ The window always says **SEM**- or **LM Calibration**, even though the microscope is of the other type:

- Set the correct microscope type in the options. You can call these up by clicking on the 

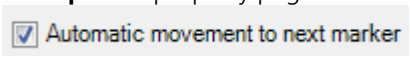
button.

➢ I cannot set a marker position

- On a light microscope make sure that the connection to the stage is in order.
- Have you set the correct microscope type in the options? You can call these up by clicking on the 

button.
- Switch on the light microscope before starting AxioVision.
- Have you set the inversion settings in the options?

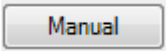
➢ The stage does not move to the next marker

- Indicate in the options that you want the stage to move. Switch to the **Options** property page and activate the 

check box.
- Check the settings for stage inversion. On an electron microscope the stage does not move on if the settings are incorrect and the value would extend beyond the travel limits of the stage. For orientation with X and Y you can view the holder files on the **Options** property page by clicking on the  icon.
- On a light microscope check the connection to the motorized stage.
- If you have clicked on **automatic**, it is possible that in some cases the marker has been poorly detected or has not been detected at all. A dialog window appears in which you must either confirm or cancel the continued movement of the stage.
- Check your selected holder. Have you selected the correct holder file? Or, in a user-defined holder: are the marker distances correct?

- The **To Position 1** function is not working
  - Has a Position 1 been set? If there is not one available, it cannot be located.
  - Has the stage been calibrated on the light microscope? If not, click on 

to calibrate it automatically.
  - Had the stage on the light microscope been calibrated when you set the first marker position?

- Automatic marker detection during calibration takes a long time
  - Perform the centering manually and use the function for setting the marker position manually by clicking on the 

button.

- The marker is not detected automatically
  - Adjust the marker orientation on the **Options** property page.
  - Are the scalings of the objectives and therefore also the pixel size correct on the light microscope?
  - Have you set the color of the marker correctly? If in doubt, switch to **automatic**.
  - Are you also using the small marker for marker detection?
  - Is the small marker located completely within the image and clearly visible? Adjust the contrast and brightness.
  - Try changing the threshold value in the options from **high** to **low**. **High** is the default value.
  - Can reflections or charges be seen? These can influence detection. Try to eliminate them.
  - Is the marker dirty and the L difficult to see? Try performing detection with the marker slightly defocused or clean the markers.

- If none of the above measures help, use the function for setting the marker position manually. In this case the current stage position/the center of the image in the live image is used. Make sure that you center the corner of the small L marker in the image beforehand.

➢ I have lost my calibration

- Have you already performed calibration? Calibration is only completed once all 3 positions have been set and you have clicked on **OK**.
- Have you selected the holder with which you performed calibration?
- If you imported the holder, no calibration is used. The calibration is deleted when a file is exported.
- Did you click on **OK** after calibration to save the calibration?
- Have you reinstalled Shuttle&Find? The holder files and therefore the calibration are deleted if the software is uninstalled.

➢ I have lost my holder

- Have you deleted the holder? It is also possible that another user has deleted the holder. The holder files can be used by all users.
- Have you created the holder? Click on **OK** in the **Add new holder** dialog window to save a new holder.

➢ The connection to the SEM does not appear to be working

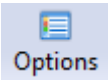
- Close AxioVision and start the SmartSEM interface. Log on and then restart AxioVision.
- Have you closed SmartSEM? Follow the procedure in the point above. To control the SEM, you need to start the SmartSEM interface.
- Have you started SmartSEM but are not logged on? Log on to SmartSEM.
- Do you have sufficient user rights in your SmartSEM account?

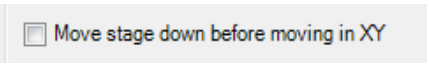
- The **Correlative Particle Analysis** button is not active.
  - Have you activated the **Archive** module and the Particle Analyzer Projects module? (**Tools** menu ⇒ **Module Manager**). Press **Ctrl+9** to open the **Event Log** dialog.

Relocation

- Relocation is not accurate
  - Has the current holder been calibrated correctly and did you complete calibration by clicking on **OK**?
  - Has the correct holder been selected in the calibration options?
  - Have you selected the correct holder on the current microscope?
  - Is the inversion of the axes on the LM/EM set correctly? Is the MosaiX displayed correctly?
  - Are the EM image and LM image incorrectly oriented/mirror-inverted in relation to each other? You can adjust the mirror inversion of the image under **Calibrate** ⇒ **Options**.
  - In the case of stitched images or images for which the automatic stitching function was used the accuracy with which positions are located is reduced. Such images can still be used, however.
  - Although it is possible to use previous calibrations, this reduces the accuracy with which positions are located.
  - Was it possible for the sample to move in the holder when it was transferred to another microscope? This reduces the accuracy.
  - Did you keep the filter holder horizontal when transferring it to the next microscope? If not, it is possible that particles on the filter moved. Repeat the complete workflow from LM to EM.
  - You can improve the accuracy by performing a fine calibration. Please read the "Fine calibration" section in this chapter.

➢ The stage moves down before each movement

- The setting has been configured in the options that the stage should move down before each XY movement. Open the options by clicking on 

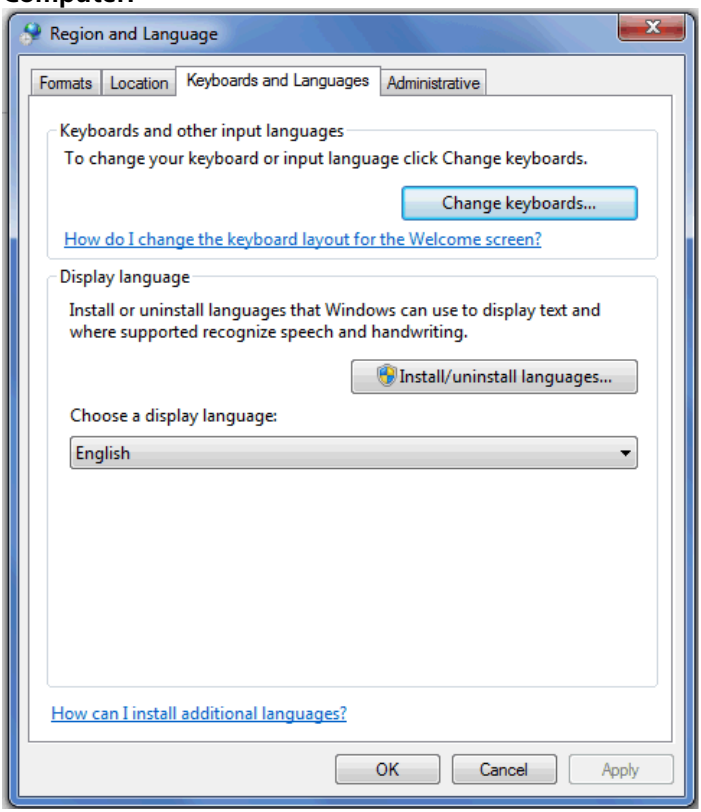
and deactivate the 

check box.

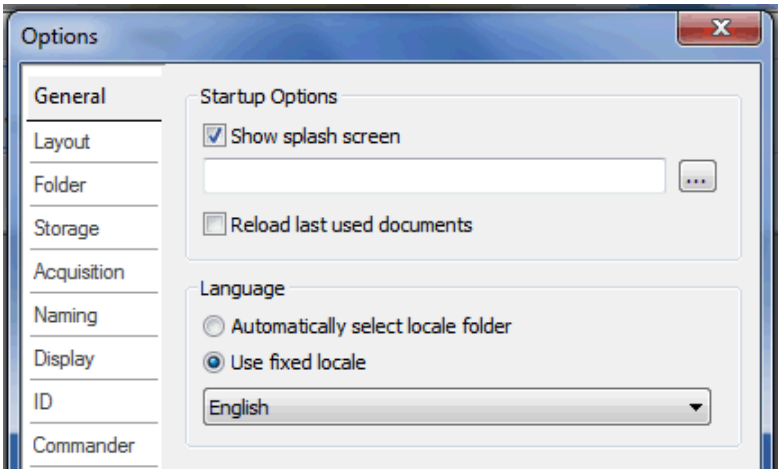
➢ There are unrealistic values in the report

- Please make sure to have the same language on your computer and in AxioVision.

Computer:



AxioVision:



Correlative Particle Analysis checklist

Make sure that the device is fully functional and that the necessary settings have been made. The Correlative Particle Analysis module (like other AxioVision modules) is influenced by incorrect settings, incorrect calibrations and parts that are fully or partially defective. It is not an error of the module if objectives have not been correctly centered, for example, and this leads to a reduction in accuracy.

LM = light microscope, EM = electron microscope

Point	Device	X
Have all objectives been centered?	LM	
Is a motorized scanning stage available and fully functional?	LM	
Are all hardware components available, such as the holder frame and/or adapter plate?	LM	
Has CAPA been set up?		
Is the CAPA toolbar active and are all icons visible?	LM, EM	
Have the settings for stage inversion been activated in MTB2011 and for the MosaiX module?	LM	
Is the view through the eyepiece the same as that in the live image window (e.g. L shape of the marker)? If not, adjust	LM	

the camera rotation.		
Has the current microscope been selected as the microscope type in the **Options**?	LM, EM	
Have you created a shared directory in the **Options**?	LM, EM	
Has **Automatic movement to next marker** been checked in the **Options** of the **Calibrate** dialog window?	LM, EM	
Does the stage move in the correct X direction during calibration? If not, set the X inversion in the calibration options.	LM, EM	
Does the stage move in the correct Y direction during calibration? If not, set the Y inversion in the calibration options.	LM, EM	
Does the marker color in the calibration options correspond to that of the marker in the microscope image?	LM, EM	
Is the marker orientation in the calibration options the same as that of the marker in the live image window?	LM, EM	
Have the scalings been defined for all objectives and are the defined scalings correct?	LM	
Are the scalings for the objectives in micrometers?	LM	
Has the position of the first marker been saved in SmartSEM in the **Stage points list**?	EM	
Have you initialized the stage?	EM	
Has the beam shift been switched off?	EM	
Has the scan rotation been switched off?	EM	
Has the **Stage only** setting been activated under Center Point/Feature?	EM	
Is the marker straight in the image? If not, rotate the stage mechanically.	EM	
If desired, activate the stage backlash to achieve better accuracy in locating positions!	EM	
Have the SmartPI settings been completed? (please refer to the user manual)	EM	

12 Configuration Modules

12.1 Commander

Introduction

AxioVision's operating concept is based on the principle that in most cases, rather than being faced with completely new tasks all the time, the user has to carry out a certain number of recurring routine activities. Even the basic system takes this into account, by offering the possibility of running through routine activities step by step using workflows, without having to check the last functions used – and their menu positions, each time.

With the Commander module, this concept has been taken further, and enhanced in a consistent way. Essentially, the Commander offers the following possibilities:

- Recording steps to be executed in sequence.
- Adding to, editing and refining the sequence recorded ("script").
- Batch processing: automatically applying a script to several images.

The Commander has numerous areas of application. As well as being a means of "simply" automating the process of retrieving functions, a script can also be regarded as an independent command in AxioVision, and can even be used in workflows (for example). If used in combination with the VBA module, you can even include VBA macros that you have created yourself in a script as commands.

Note:

- On the Product DVD you will find a selection of script examples.

Special points relating to the creation of scripts

Almost all of the functions available in AxioVision's main menu can be included in a script.

Once the process of recording the script has started, a button (which is otherwise hidden) will become available. The button can be used to adopt the command into the active script. Functions are not, therefore, "simply recorded", as numerous commands that you do not require would then be included too, and you would have to delete them. Often it is difficult to keep track of this once you are inside a script.

Functions that do not have a function dialog (e.g. annotations and interactive measurement functions) actually have to be executed before they can be included in a script. Further information can be found in the **Script Editor's** online help.

Batch processing - application of a script to several images

The automation of a number of commands is usually only one of the aspects involved in the creation of scripts. The next step is applying them to the desired number of images. With this in mind, the Commander module has a separate function for batch processing. This allows you to specify the number of images you want to process using a script, and where these images come from:

- The first possibility involves acquiring the images directly via the camera, and executing the script for these images. The script is started after an image has been acquired. Once it has been executed, you can acquire the next image, and so on, until you want to end the processing.
- The second possibility involves acquiring images first, and saving them on the hard disk before starting processing using a script. The function for automatically saving images after acquisition is useful here (**Tools** menu ⇒ **Options** ⇒ **Naming** and **Acquisition**).
- Another option involves processing all of the images that are currently open in AxioVision.

Limitations of scripts

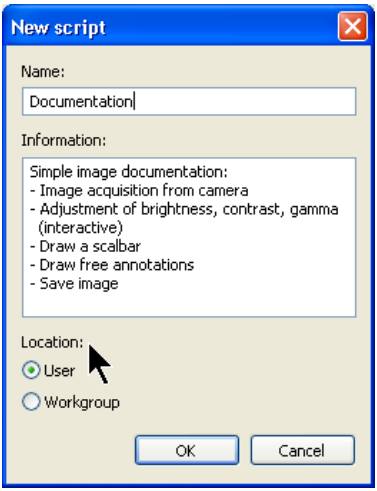
The aim of the Commander is to enable the user to execute the commands available in AxioVision automatically. It is therefore an aid to automation. The Commander should not be confused with a programming language, as the typical elements of programming (generation of function dialogs, variables, loops etc.) are not available. The VBA module should be used in such cases.

Use of scripts by several users of a system

Microsoft Windows XP and Microsoft Windows Vista, which are designed to allow several users to work on a system, are fully supported by AxioVision. More specifically, this means that each user can create his/her own AxioVision environment without other users of the system being able to access the configurations and files that he/she generates (see also section 8.3 "User and Data Administration").

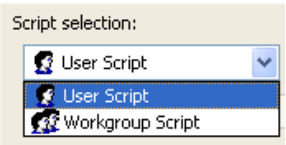
AxioVision therefore saves scripts by default in the folder *My Documents\Carl Zeiss\Data\Script.*

Frequently, however, you will want to make a script that has been created available to all users of a system. When you are saving a script, you therefore have the option of selecting whether you want to save the current script for yourself only, or for all users.



The scripts are now saved in the folder *Shared Documents\Carl Zeiss\Data\Script*.

Icons in the list of scripts in the Script Editor or on the toolbar indicate whether a script has been saved for the current user only, or whether it may be accessed by all users:



Installation of scripts on other systems

Scripts can easily be transferred to other systems. The Commander module must not be present on the target system. The installed scripts can be executed at any time via the menu, or via the Commander toolbar.

Commander scripts form part of an individual AxioVision configuration. They are therefore also saved when you save a configuration using the **Import/Export Configuration** function on the **Tools** menu. To avoid problems, we recommend that you use this function to save and transfer scripts to other systems. Further information on the use of this function can be found in section 8.7 "Exporting and Importing User Configurations".

Note:

- If functions that are part of a module are used within a script, you must ensure that the relevant module is also available on the target system, and has been activated in the **Module Manager** (**Tools** menu ⇒ **Modules Manager**).

Recording a script

In this exercise you will learn how to record and save a script using the script editor. A simple documentation task will be used as an example: acquire image, enhance contrast, draw in scale bar, save image.

Specifically, the following steps are covered:

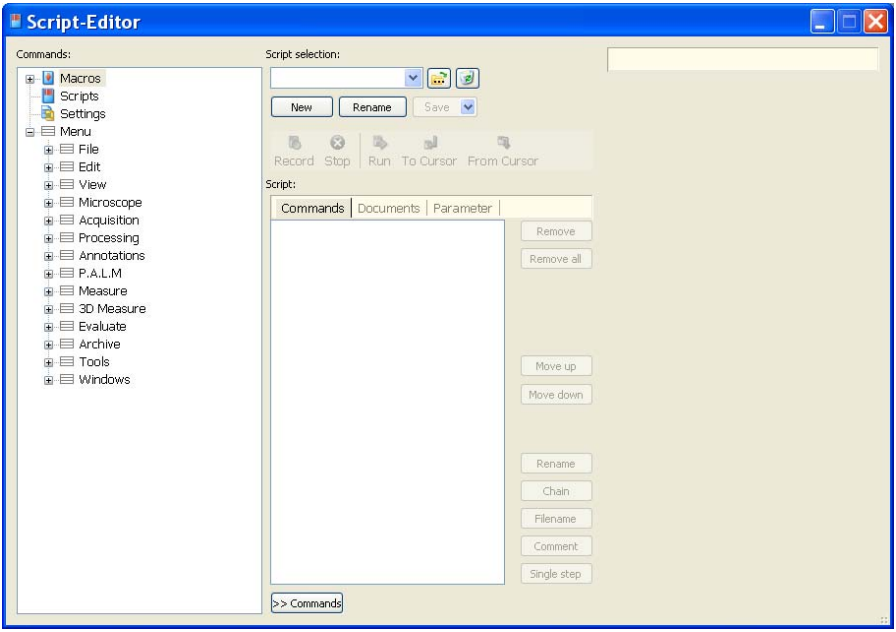
- creating new scripts, entering a description
- starting the recording of a script
- behavior of the script editor during recording
- adding commands to a script
- saving a script

Notes:

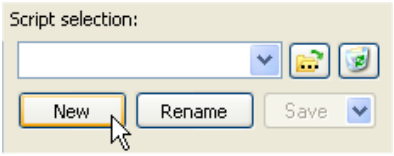
- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

- Before you start to record a script, you can determine the behavior of the editor during recording with the function **Commander – Options**.

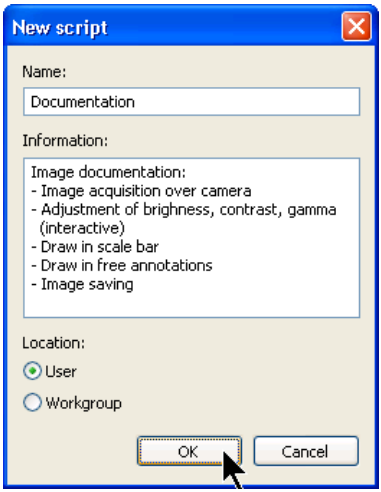
Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu. The script editor is the "control center" for creating and revising scripts.



- To record a new script, click on **New** under **Script selection**.



- Enter a name in the **New script** dialog that describes the script as clearly as possible. You can also describe the content of the script in more detail in the **Information** field.
- Via **Location** you can specify whether the script should be available only to you or also to all other users of the system (the **Workgroup**).
- Click on **OK**, to create the new script.



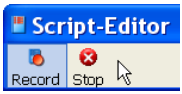
- An empty script window is now displayed.



➢ Now start recording the script by clicking on the **Record** button.



➢ If you have activated the **Minimize Script Editor while recording** option in the function **Commander - Options**, the script editor is hidden and only a window with the buttons **Record** and **Stop** is displayed. This means that there is more space on your screen for working in AxioVision.



➢ You can now work entirely as normal in AxioVision: First open the live image via the **Acquisition** menu ⇒ **Live** function.



➢ Select a scaling that is suitable for the objective you are using from the **List of available scalings** in the area at the bottom of the live window.

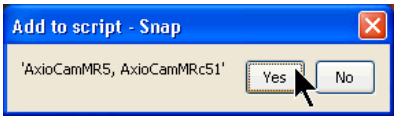
➢ Close the **Live** window.

Note:

- The live window does not have to be adopted into the script. It is automatically displayed if you execute the **Snap** function interactively.

➢ Now select the **Snap** function from the **Acquisition** menu.

- The image is acquired and the **Add to script** dialog is then displayed. Using this dialog, you can decide whether or not each command should be included.

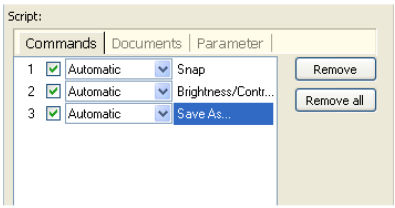


- Click on **Yes** to adopt the command into the script. This closes the **Add to script** dialog window.

- Now execute the following functions in addition to the **Live** function, and adopt them into the script as described above:

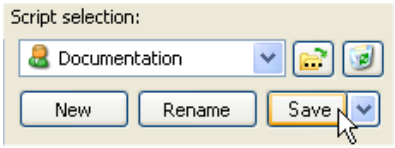
  - **Processing** menu, **Adjust** group, **Brightness/Contrast/Gamma** function.

  - **File** menu, **Save As** function.



- Then save the script by clicking on **Save** under **Script selection**.

- Once the current version of the script has been saved, the **Save** button is displayed in gray.





- Now stop recording the script.



Inserting commands into an existing script

Often, after recording a script, you will discover that the sequence does not meet your requirements. Perhaps commands are missing, or the commands that are included do not function as expected.

This exercise describes how to expand a previously recorded script in the appropriate way:

- executing a script step by step
- inserting new commands
- copying and moving commands

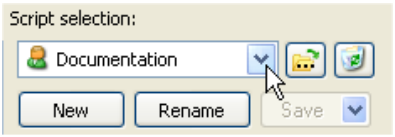
Notes:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

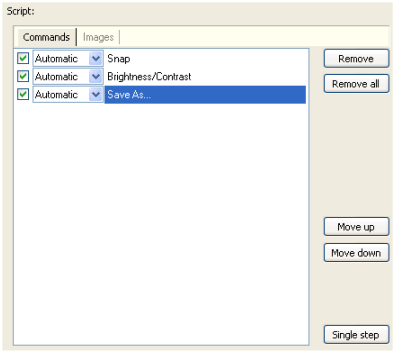
- The "Documentation" script used in the following example is the result of the previous exercise "Recording a scrip". It is assumed that you have carried out the exercise as described and that the script is therefore available.

➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.

➢ In the list under **Script selection**, select the script that you want to change.

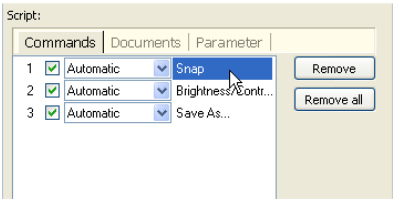


- The selected script is immediately displayed in the script editor.



- Below, you will incorporate the possibility of drawing in a scale bar and adding free annotations after the **Brightness/Contrast/Gamma** command.

- To insert further commands, you should first execute the script up to the line after which the new command is to be inserted.



- Highlight the first line **Snap** and then click on **Single step**. The image that is currently set is acquired.

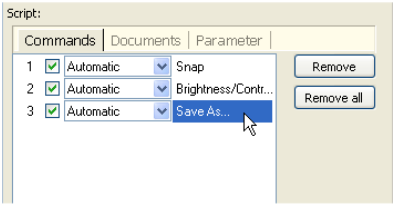


Note:

- If the image is unsatisfactory, adjust it first via the live image.

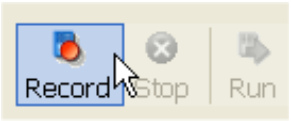
- Click again on **Single step**. The **Brightness/Contrast/Gamma** function is executed.

- After the execution of **Brightness/Contrast/Gamma** the last line is highlighted, as the cursor moves on automatically. When you insert functions, however, they are inserted ***after*** the position that is highlighted at that moment.

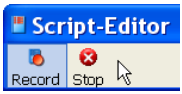


- You should therefore activate the **Brightness/Contrast/Gamma** function by clicking on it.

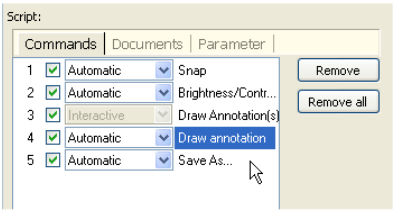
- Commands are inserted simply as a result of the desired functions being recorded.



- If you have activated the **Minimize Script Editor while recording** option in the function **Commander - Options**, the script editor is hidden and only a window with the buttons **Record** and **Stop** is displayed. This means that there is more space on your screen for working in AxioVision.



- Now execute the following functions one after the other and adopt them into the script:
  - **Annotations** menu, **Scale bar** function
  - Draw the scale bar in the image.
  - **Annotations** menu, **Draw Annotations** function.

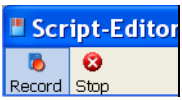


The new functions are now displayed after the **Brightness/Contrast/ Gamma** command.

The script now contains all the commands required. However, to ensure smooth operation there are two settings that need to be done:

1. The live window is not closed in every case, but only if the **Close after Snap** function in the live window's shortcut menu has been activated.

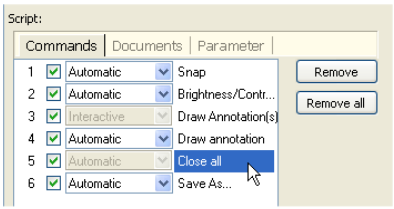
2. Documents may still be open. At the start you should therefore ensure that all documents have been closed.

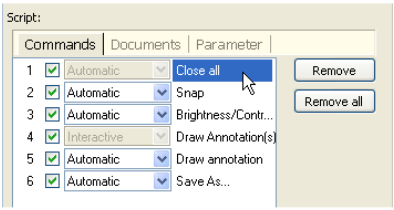
3. Start the recording again 

and insert the **Close all** command from the **Windows** menu.

- The **Close all** command is now displayed in the last but one line. It should, however, be executed at the start of the script.

- To move it to the first position in the list, highlight the line and click on the **Move up** button until it moves to the first line in the script.



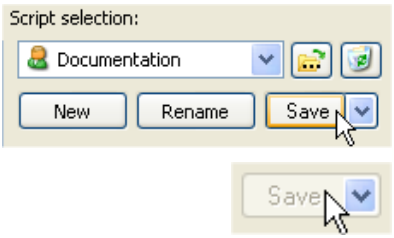


Note:

- You can also duplicate individual lines by using **Ctrl C** (copy) and **Ctrl V** (paste) keys. The line is then included twice in the script.

- Then save the script by clicking on Save under Script selection.

- Once the current version of the script has been saved, the Save button is displayed in gray.



Changing the way individual commands are executed, selecting images to be displayed

This example shows you how to influence the way in which individual commands behave during the execution of the script. In addition, you will be shown how to specify which images are displayed/not displayed during and/or after execution:

- displaying function dialogs during execution so that you can adapt parameters (interactive execution of commands).
- switching individual commands on and off so that commands are not executed (useful for testing a script).
- specifying which images should be displayed during execution, to prevent too many images from "blocking" the screen.

Notes:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

- The "Documentation" script used in the following example is the result of the previous exercises "Recording a script" and "Inserting commands into an existing script". It is assumed that you have carried out the exercises as described and that the script is therefore available.

In the following example the "Documentation" script is modified to meet the requirements described below.

The **Close all** function should simply close all images, including those that have not been saved, without asking whether the images should be saved.

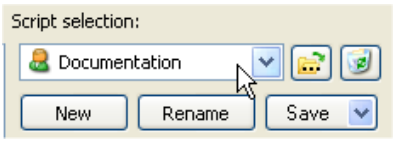
The execution of the script should be interrupted at the following commands so that settings and parameter values can be entered in dialogs:

- **Record**, so that you can position the specimen correctly and focus.
- **Brightness/Contrast/Gamma**, so that you can adapt the settings to the image that is currently being acquired.
- **Save As**, so that you can change the folder, file name and image format.

Only the resulting image should be displayed in AxioVision, not the acquired image.

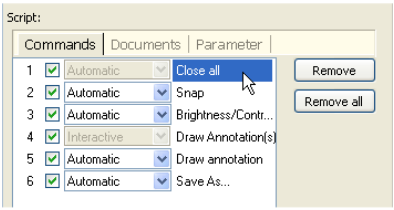
➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.

➢ In the list under **Script selection**, select the script that you want to change.



➢ The selected script is immediately displayed in the script editor.

➢ Click on the **Close all** function.

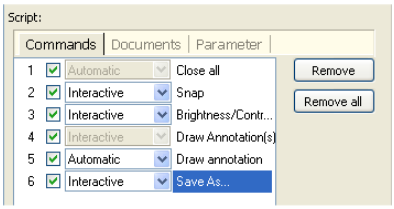


➢ Under **Parameters** on the right-hand property page, switch the setting for **Force close** from **No** to **Yes**.



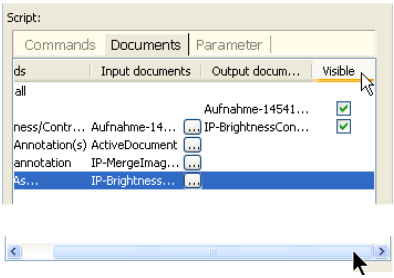
The **Close all** function will now close all open documents without asking whether unsaved documents should be saved.

- Click on the **Snap** function and switch the **Automatic** parameter to **Interactive**.



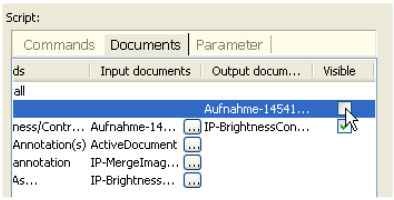
- Carry out the same action for the **Brightness/Contrast/Gamma** and **Save As** functions.

- To specify which images are displayed during execution of the script and which are not, switch to the **Documents** property page.



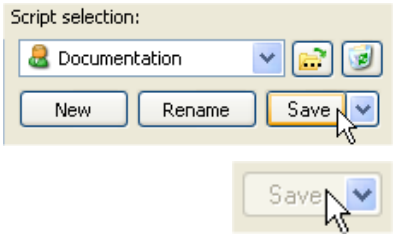
- If the **Visible** column is not displayed, move the slider at the bottom edge completely to the right.

- Now deactivate the **Visible** option for the **Snap** command.



- Only the resulting image is then displayed during automatic execution (see next exercise).

- Then save the script by clicking on Save under Script selection.



- Once the current version of the script has been saved, the Save button is displayed in gray.

Executing a script automatically

This example shows you how to execute a script and how to influence the way in which individual commands are executed:

- displaying the **Commander** toolbar.
- executing a script automatically.
- displaying script information.

Notes:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

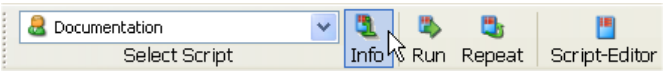
➢ The "Documentation" script used in the following example is the result of the previous exercises. It is assumed that you have carried out the exercises as described and that the script is therefore available.

The simplest way to execute a script is via the **Commander** toolbar. If the toolbar is not displayed, select the **Toolbars** group from the **View** menu and there select the **Commander** entry.

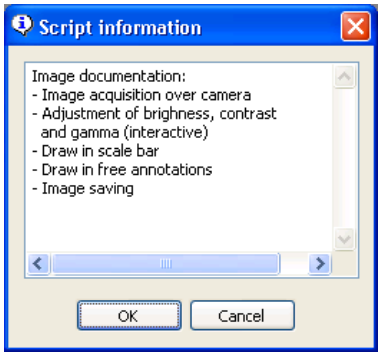
➢ Then select the script that you wish to execute under **Select Script**.



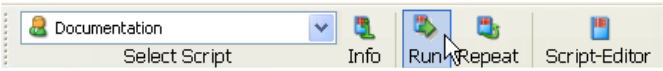
➢ If you are not sure whether it is the right script, click on **Info** after making your selection.



➢ The **Script Information** window is then displayed. This contains a more detailed description of the script (if this was entered by its creator).



➢ Click on **Run** to start the execution of the script (the **Script batch** option is explained in the following exercise).



Preparing a script for batch processing

Processing several images using a single script is the real area of application of the Commander, or rather of the scripts that are created with it. This exercise shows you the conditions that a script has to meet in order to be executed via the **Script batch** function.

- Preparing a script for batch processing
- Executing the function for batch processing
- Selecting the image source

Notes:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and Operating Concept".

➢ The "Documentation" script used in the following example is the result of the previous exercises. It is assumed that you have carried out the exercises as described and that the script is therefore available.

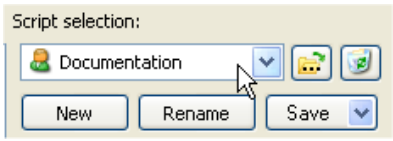
➢ If you want to execute a script via the **Script batch** function, it must meet the following conditions:

1. It must not include a function that opens an image file or acquires an image via the camera.

2. The first function within the script that requires an image as input must use the image that is currently loaded. Since, however, at the time the script is created it is not known where this image will come from and what name it will have, a general place-marker must be used (what is known as the "active image").

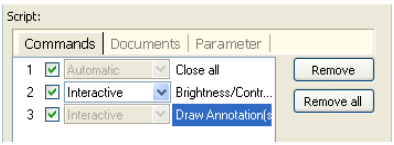
➢ Open the script editor. To do this, select the **Script Editor** function from the **Commander** menu.

➢ Select the script that you want to be changed in the **Script selection** drop-down list box.



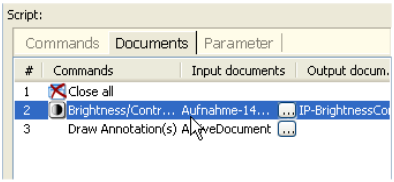
The selected script is immediately displayed in the Script Editor. In the **Script** field, select the **Snap** and **Save As** commands, one after the other, and delete them using the **Remove** button.

- The commands that are executed automatically by the **Script batch** function have now been removed.

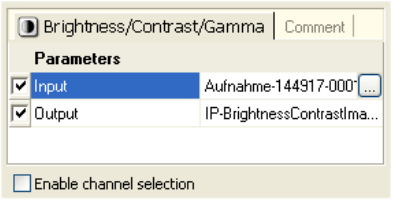


You must now ensure that the first image-processing command (**Brightness/Contrast/Gamma**) actually uses the loaded or acquired image as the input image.

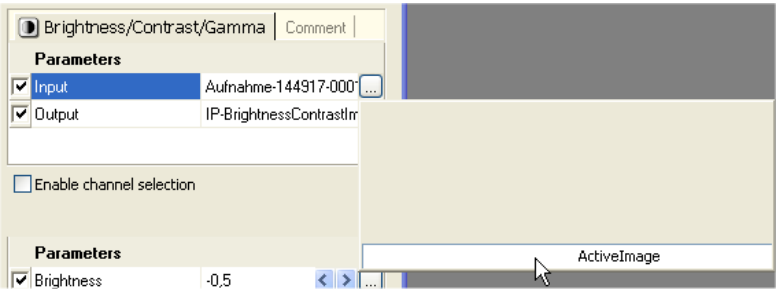
- On the **Documents** tab sheet select the **Brightness/Contrast/Gamma** command.



- On the right side of the script editor page the parameters will be shown.
- To change the input image, select the ... button under **Input documents** to open a gallery for selecting an image.



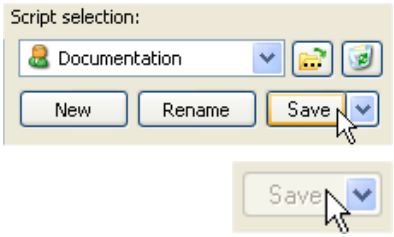
- If you have not previously loaded or acquired an image, the gallery will be empty, as shown in the illustration on the right.



- Here, select the **ActiveImage** command. The "active" image is then always used as the input image for the **Brightness/Contrast/Gamma** function – irrespective of the source from which it originates (camera, file) and its name.

- Then save the script by clicking on Save under Script selection.

- Once the current version of the script has been saved, the Save button is displayed in gray.



- Close the **Script Editor**.

Notes:

- Before you save the script, think about whether you will only want to use it with the **Script batch** function in the future. If not, you should use a different name.

- In this case, click on the arrow to the right of **Save**, under **Script selection**, and select **Save As**. This function allows you to save the script under a new name.

Repeating scripts

The **Repeat** function allows you to apply a script to any number of images. The images may already be available as files in a folder or may already have been loaded in AxioVision. You may, of course, also acquire them via the camera. In addition, you can specify, for each resulting image within the script, whether or not you want the image in question to be saved. The following steps are carried out in this exercise:

- Executing the function for repetition
- Selecting the image source

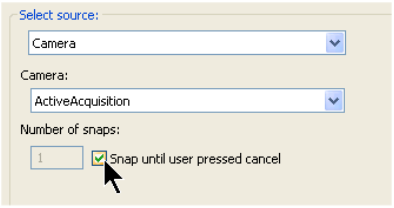
Notes:

- The following example describes the selection of the commands to be used via the AxioVision main menu. You can, of course, also call up the appropriate commands via toolbars, workflows and your own customized dialogs. Please also read chapter 2 "Overview and operating concept".

- Before you perform this exercise, you should ensure that you have worked through the previous example "Preparing a script for batch processing".

➢ To execute the script for several images, select the **Repeat script** function from the **Commander** menu.

➢ First, select where the images should originate from in the **Select source** drop-down list box. In most cases you will presumably acquire them via the camera.

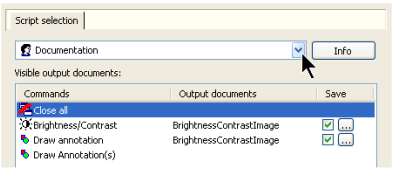


➢ If you work with several cameras, select the **ActiveAcquisition** setting in the **Camera** drop-down list box. The camera activated in the **Acquisition** menu will then be used each time.

➢ Finally, specify how many images you wish to acquire. If the number is not known, activate the **Snap until user pressed cancel** option.

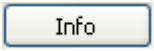
➢ Now select, under **Script selection**, the script that you wish to use.

➢



➢ Now select, under **Script selection**, the script that you wish to use.

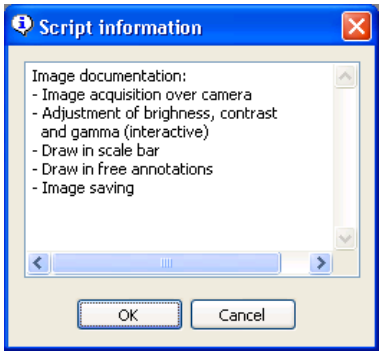


- If you are not sure whether it is the right script, click on 

after making your selection.

- The **Script information** window is then displayed. This contains a more detailed description of the script (if this was entered by its creator).

- Start the execution of the script by clicking on **Start**.



The commands are now run through as if you were selecting them interactively via the menu. At any time you can use the **Cancel** button to finish the script.

12.2 VBA

General

The VBA module enables you to simplify routine tasks with the help of macros, and to program your own applications.

AxioVision contains the most recent version, VBA 6. In this version the language features of VBA are on a par with Visual Basic.

If you have never done any programming before, we advise you to build up your knowledge in advance. As Visual Basic/**V**isual **B**asic for **A**pplications is a very widely used standard, you will easily find a wide range of training programs and literature to meet the needs of people with almost any level of prior knowledge.

Section 8.5 "Adapting the User Interface" describes how workflows, toolbars and shortcut keys can be used to adapt AxioVision to your personal work situations, and to make your work a great deal easier and more convenient. With the additional possibilities offered by the VBA module, you can let your imagination run free, as there are no longer any restrictions when it comes to configuring AxioVision to meet your individual requirements.

First steps

To make it easier to enter the world of programming in AxioVision, the following resources are available to you:

- VBA online help (Microsoft Visual Basic Help, AxioVision Visual Basic Help)
- AxioVision VBA website for support, downloads and forum: www.zeiss.de/vba
- VBA user course for AxioVision (for detailed information please visit the website www.zeiss.de/vba)
- AxioVision online help

The VBA online help is supplied with the VBA module. An up-to-date version can be downloaded from the VBA website.

The VBA website and forum are a platform where customers, specialists and developers can discuss VBA for AxioVision. Here you will find information about programming in AxioVision, e.g. in the form of tutorials and tips & tricks. You can exchange information with other forum members and obtain support relating to specific questions and problems.

The VBA user course for AxioVision offers you entry into the "VBA for AxioVision world". It will provide you with detailed basic information, such as an introduction to the AxioVision object model and how it is programmed.

Lastly, the AxioVision online help is another useful source of information on programming, offering you a precise description of the parameters of image processing functions, for example.

The VBA online help

In addition to a description of the general VBA functions (Microsoft Visual Basic Help), you will find an online help in the VBA Editor via the **Help** menu together with the documentation on the AxioVision objects (AxioVision Visual Basic Help).

Welcome to Standard Visual Basic documentation

The documentation on Visual Basic is extremely diverse. Each individual component has been created to help you to familiarize yourself with particular aspects of the product. The documentation supplied with Visual Basic covers the components described below.

Help with the Visual Basic user interface

Here you will find information on interface elements of the Visual Basic Editor, such as commands, dialog boxes, windows and toolbars.

Visual Basic concepts

The *context*-sensitive *help* contains information that will help you build up your knowledge of programming in Visual Basic.

Help with Visual Basic procedures

In this area of the help you will find useful information on general procedures, such as the use of the object catalogue or the specification of Visual Basic environment options.

Visual Basic language directory

In the *language directory* you will find information on the Visual Basic programming language, i.e. all of its methods, features, statements, functions, operators and objects.

Visual Basic add-in model

If you want to adapt the Visual Basic Editor, in the language directory you will find information on the object model, which will enable you to expand the environment.

Reference for Microsoft Forms

Here you will find information on *Userform* objects and control elements, and on programming with *Userform* objects and control elements in Visual Basic.

Welcome to AxioVision Visual Basic documentation

You will find the AxioVision-specific online help on the **Help** menu ⇒ **AxioVision Visual Basic Help**.

It has been devised to allow you to search quickly for code examples and offers you information as well as an overview of the main AxioVision classes. The AxioVision application and its modules consist of almost 60 class libraries (DLLs) containing several hundred classes. Only seven DLLs are elementary and relevant for programming. These form the basis of the AxioVision VBA online help.

Managing and running macros

There are two ways to run macros that you have created in the Visual Basic Editor:

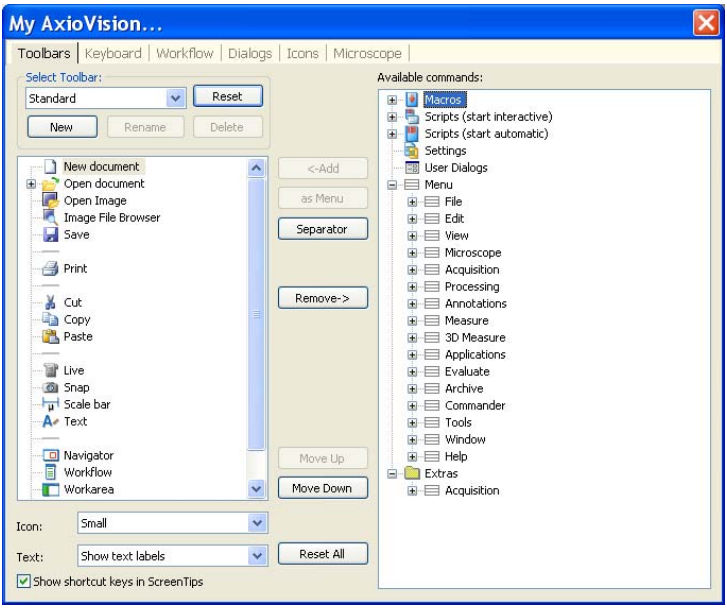
- The first is via macro management. From the **Tools** menu select the **Macros** function. Besides the possibility of running macros, here you will also find functions for deleting macros, starting the Visual Basic Editor etc.

- The second possibility is via the function for running macros. From the **Tools** menu select the **Run Macro** function. A list of the macros you have created is then displayed. Select the desired macro and click on **Start** to run it.

Running macros via workflows, toolbars and shortcut keys

A much more convenient option, however, is to integrate each macro into the user interface.

Macros are treated in the same way as any other function in AxioVision, and can therefore be used via workflows, toolbars and shortcut keys, as required. In the **Tools** menu under the **My AxioVision** function you will always find a **Macros** branch on the corresponding property pages. The list of the macros you have created is also available.



Note:

- Section 8.5 "Adapting the User Interface" describes how workflows, toolbars and shortcut keys can be used to adapt AxioVision to your personal work situations, and to make your work a great deal easier and more convenient.

- To ensure that your macro appears in the list, the procedure behind it must in principle be declared as global, i.e. as "Public", e.g. Public Sub MyMacro(); in addition, it must not have any parameters.

- If a newly created procedure is not present in the **Macros** list, you should update the list using the **Refresh** function on the AxioVision **View** menu.

12.3 Kiosk Mode

General

Kiosk Mode is available as a free, separate module in AxioVision and allows users to operate AxioVision in a mode with restricted user rights.

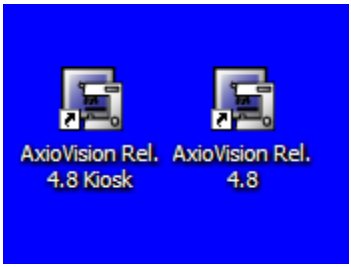
The graphical user interface can be preconfigured by one user to ensure that another user is unable to change this user interface either intentionally or unintentionally. Usually only a few elements of the graphical user interface are available to the user in Kiosk Mode, e.g. only the toolbars and workflows of one or several applications, such as Particle Analyzer or Grains.

The functionality can be compared with the **My AxioVision** concept (**Extras** menu). There, however, the user has the option of changing AxioVision's user interface again at any time.

Different Kiosk Mode user profiles

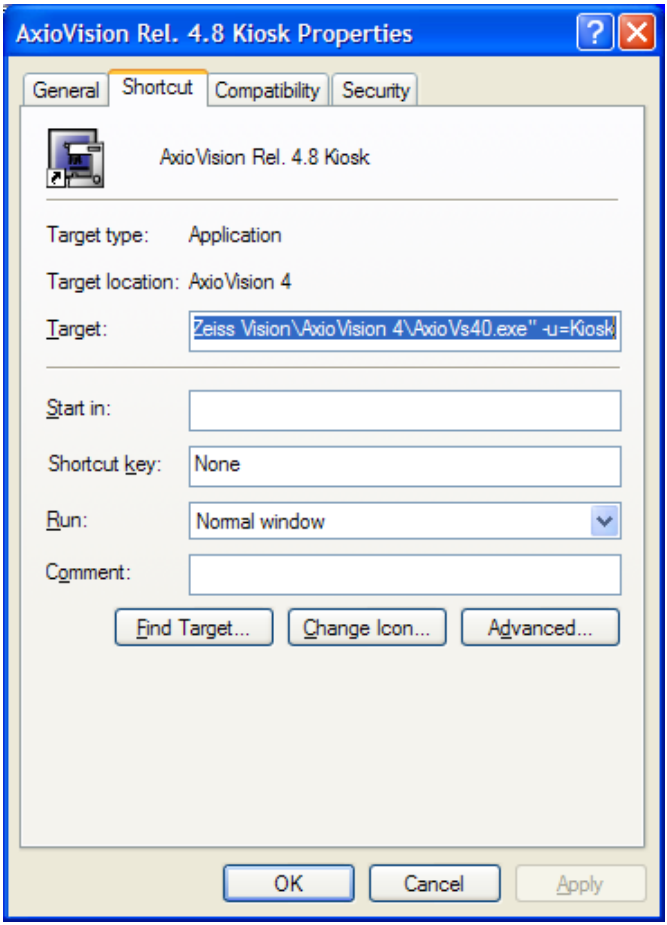
If the PC is used by several users, Kiosk Mode can be set differently for various user accounts on the operating system.

Alternatively, as described in section 8.8 of the user manual, several AxioVision icons can be created with various AxioVision profiles on the desktop of a user account on the operating system. In the example below a second **AxioVision Rel. 4.8 Kiosk** icon has been created. This icon uses the "Kiosk" profile internally and Kiosk Mode has only been activated for this profile.

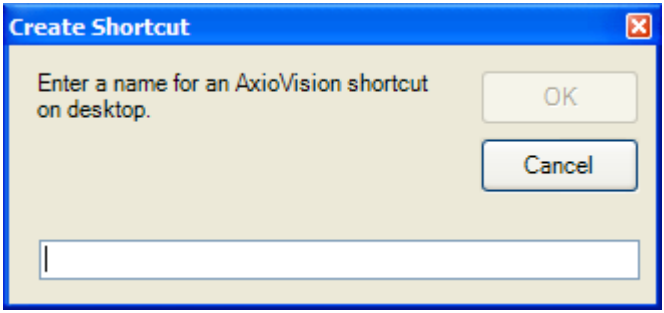


Copy the original AxioVision icon and rename this as "AxioVision Rel 4.8 Kiosk". To do this, right-click on the icon and open the **Properties** function. In the **AxioVision Rel. 4.8 Kiosk Properties dialog window**, enter the following in the **Target** field:

"C:\Program Files\Carl Zeiss Vision\AxioVision 4\AxioVs40.exe" -u=Kiosk"



Alternatively, use the function **Create Shortcut** function (**Application** menu ⇒ **Kiosk Mode**), which creates automatically the new icon after entering a name. This name is used for the icon as well as for the AxioVision profile.

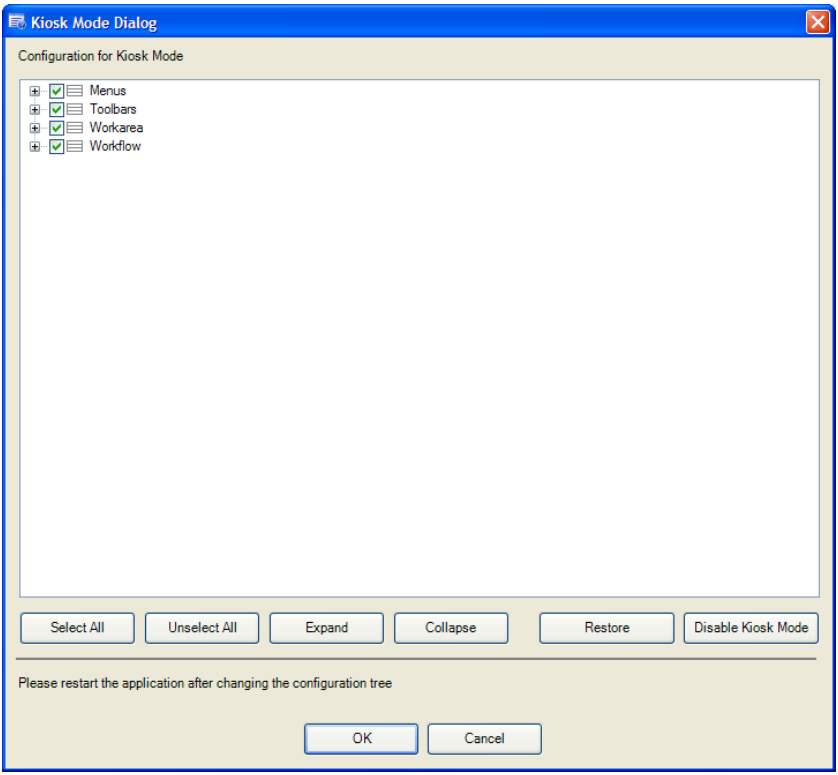


Setting up Kiosk Mode

Start AxioVision and open the **Kiosk Mode Dialog**.

To do this, activate the **Start Kiosk Mode** function (**Application** menu ⇒ **Kiosk Mode**).

Alternatively, use the key combination **Ctrl+K**. This key combination can also be used if AxioVision has already been started in Kiosk Mode in order to change or switch off Kiosk Mode.

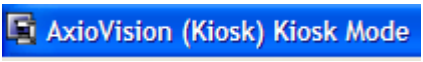


You can adapt the parts of the menu, toolbars, work areas and workflows that are visible for Kiosk Mode.

Only activate those elements of the user interface that you want to make available to your Kiosk Mode user. For a materials user only activate the necessary toolbars and workflows and deactivate all other elements, as well as the **Manage Test Specifications** button in the workflow.

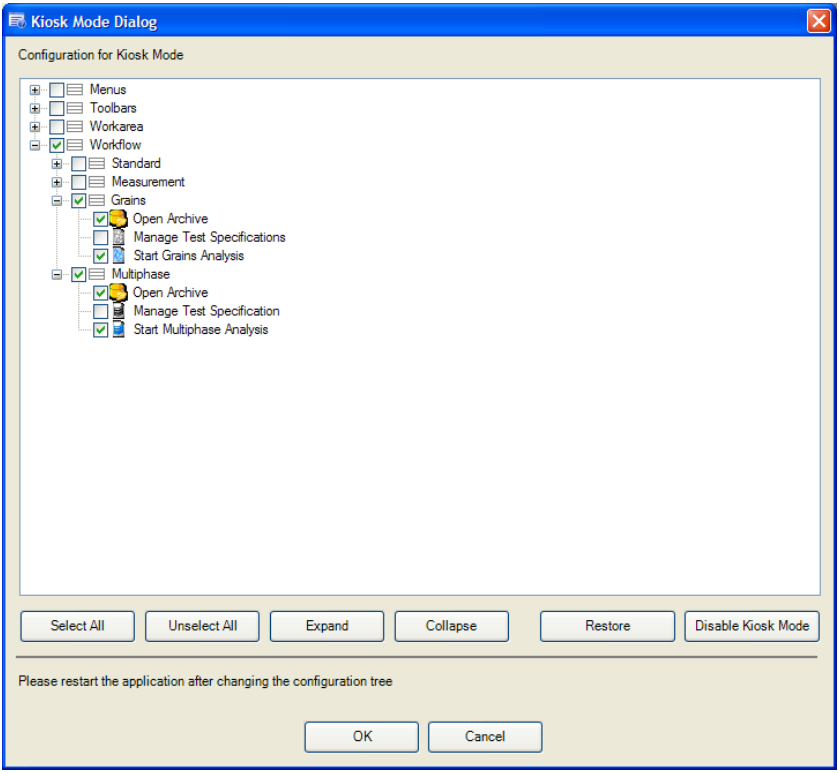
OK saves the Kiosk Mode settings for the AxioVision profile with which AxioVision has been started.

Exit AxioVision. When AxioVision is restarted, the software will appear in Kiosk Mode. This is displayed in the title bar of the software.

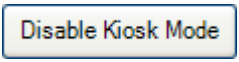


The settings are saved in the *"C:\Documents and Settings\"USER"\Application Data\Carl Zeiss\AxioVs40\Profiles\Default"* directory or in the current AxioVision profile in the *"KioskModeConfiguration.xml"* file. Deleting the file deactivates Kiosk Mode.

The following example shows a Kiosk Mode configuration for a materials user.



Switching off Kiosk Mode

The 

button in the Kiosk Mode Dialog deletes all settings.

Exit AxioVision. When it is restarted, AxioVision will appear in standard mode.

Index

A
Advanced Automatic Measurement module 11-19
allocation of scalings 6-1
animation, perform 10-51
annotations 1-2, 5-3
  draw 5-19
  format 5-19
  standard settings 5-21
ApoTome
  Bildaufnahme 9-24
  calibration of phase position 9-9
  grid allocation 9-34
  hardware configuration 9-5
  multidimensional acquisition 9-30
  objectives 9-34
ApoTome module 9-1
Archive Modules
  Asset-Archive 1-18
autofocus calibration 9-44
Autofocus module 9-43
autofocus use 9-45
Automatic Measurement module 11-5
AxioVision 4
  configuration 2-1
  overview 2-1
AxioVision concept 1-1
AxioVision ZVI format 7-1

B
backup copies 1-20
batch processing 12-2
Bedienoberfläche anpassen 10-31

C
calibrating autofocus 9-44
calibration of phase position 9-9
Calotte Grinding Measurement 241
color space transformation 10-3
Commander module 12-1
Comperative Diagrams 11-178
conditions for image acquisition 3-2
configuration 1-3, 8-1
Configuration modules
  Commander 12-1
  VBA 12-24
Configuration Modules
  Commander 1-18
  VBA 1-19
Correlative Particle Analysis-CAPA 352
creating toolbars 8-17

D
data entries 7-9
DeepView
  using z-stack image 9-77
default experiments 9-138
dialogs 2-5
display characteristic curve 3-11
display the Z-stack images in three dimensions 10-32
documentation 1-2, 7-1

E
experiment 9-137
export measurement data 6-7
Extended Focus
  acquire image 9-75
Extended Focus module 9-75

F
file format 10-51
first step for image acquisition 3-2
form for data entries 7-9
Fourier transformation 10-3
fringe projection imaging principle 9-2

G
general settings 8-27
generate scalings 8-13
Graphite 11-197
gray morphology 10-2
grid allocation 9-34

H
hardware configuration, ApoTome 9-5
hardware setting, generate 4-6
How do I create a multichannel experiment? 9-52
How do I create a time lapse series? 9-66
How do I create a Z-stack? 9-63
How do I create an experiment? 9-49
How do I manage channel settings 9-58

I
image acquisition 1-1
  first steps 3-2
Image Acquisition modules 9-1
Image Acquisition Modules
  Autofocus 1-3
  Digital High Speed Recorder 1-5
  Dual Camera 1-5
  Extended Focus 1-3
  Fast Acquisition 1-4
  Fast MosaiX 1-4
  HDR Imaging 1-5
  Mark&Find 1-4
  MosaiX 1-4
  Multichannel Fluorescence 1-4
  Panorama 1-4
  Time Lapse 1-5
  Z-Stack 1-5
image analysis 1-2, 6-1
Image Analysis Modules 11-1
  3D Measurement 1-14
  Advanced Automatic Measurement 1-8
  ASSAYbuilder 1-17
  Automatic Measurement 1-8
  AutoMeasure Plus 1-8
  Calotte Grinding Measurement 1-9
  Colocalization 1-14
  Comparative Diagrams 1-12, 1-16
  Grains 1-10
  Graphite 1-12
  Interactive Measurement 1-8
  Multiphase 1-11
  NMI 1-10
  Online Measurement 1-14
  Particle Analyzer 1-9
  Physiology 1-15
  QuantiFISH 1-13
  Ratio 1-13
  SFM 1-16
  TIC Measurement 1-9
  TMA 1-13
  Topography 1-14
  Tracking 1-13
image display, enhance 5-19
image enhancement 10-1
image file browser window 7-5
image processing 1-2, 5-1
Image Processing modules 10-1
Image Processing Modules
  2D Deconvolution 1-7
  3D Deconvolution 1-7
  ApoTome 1-6
  Imaging Plus 1-7
  Inside4D 1-7
  Widefield Multichannel Unmixing 1-8
image scaling 6-2
images
  image file browser 7-4
  manage 7-4
Imaging Plus
  color space transformation 10-3

Fourier transformation 10-3
gray morphology 10-2
image arithmetics 10-2
working with image processing functions 10-4
Imaging Plus module 10-1
incubation 1-1
Inside4D 10-32
azimuth 10-49, 10-50, 10-51
brightness 10-49, 10-50, 10-51
elongation 10-49, 10-50, 10-51
OpenGL mode 10-58, 10-63
settings 10-40
shadow mode 10-38, 10-41, 10-42
surface mode 10-38
transparency mode 10-38
viewing modi 10-38
windows control elements 10-34
installation of scripts 12-5
Interactive Measurement module 6-4
Interactive Measurement Module 11-1

K
key combinations 8-20

L
Layer Thickness Measurement 219
limitations of scripts 12-3
live image speed 3-10

M
macros 12-26
managing macros 12-26
Mark&Find
advanced image acquisition 9-68
Mark&Find Experiment 9-69
Mark&Find module 9-97
Measure Workflow 6-4
measurement conditions 6-1
measurement data, save 6-2
Measurement Programs
Interactive measurement programs 11-1
Measurement Wizard 6-6
Microhardness Measurement module 248
microscope configuration 4-2
microscope control 1-1, 4-1
microscopes
configuration 4-2
Microsoft Windows and AxioVision 1-19
Modules
Advanced Automatic Measurement 11-19
ApoTome 9-1
Archive 1-18
Autofocus 9-43
Automatic Measurement 11-5
Calotte Grinding Measurement 241
Comperative Diagrams 11-178
Configuration 1-18
Correlative Particle Analysis-CAPA 352
Extended Focus 9-75
Graphite 11-197
Image Acquisition 1-3, 9-1
Image Analysis 1-8, 11-1
Image Processing 1-6, 10-1
Imaging Plus 10-1
Interactive Measurement 6-4, 11-1
Layer Thickness Measurement 219
Mark&Find 9-97
Microhardness Measurement 248
MosaiX 9-110
Multichannel Fluorescence 9-52
Multidimensional Acquisition 9-48
NMI-Non Metallic Inclusions 11-92
Panorama 9-98
Particle Analyzer Projects 11-60
SFM-Scanning Fluorescence Microscope 270
Shuttle&Find 299

TIC Measurement 257
Time Lapse Imaging 9-66
TMA 9-132
Widefield Multichannel Unmixing 10-84
Z-Stack 9-62
MosaiX module 9-110
Multichannel Fluorescence module 9-52
Multidimensional Acquisition
assign names 9-166
experiment 9-137
save modified experiments 9-138
Multidimensional Acquisition module 9-48
multidimensional acquisition with ApoTome 9-30

N
NMI–Non Metallic Inclusions 11-92

O
online help 1-19
optimum color reproduction 3-11
own dialogs 8-22

P
Panorama module 9-98
Particle Analyzer Projects 11-60
Particle Anazyer Projects
accessories 11-62
analysis script 11-83
archive view 11-69
AxioVision settings 11-70, 11-97
calibration standard 11-63
cleaning calibration standard 11-64
cleaning sample holder 11-64
operating concept 11-65
performing measurement 11-90
preparing the system 11-70
report template 11-87, 11-112, 11-144, 11-167, 11-189, 11-208
special settings 11-74
system variants 11-60
test specifications 11-77
Partikelanalyse
Allgemeine Einstellungen 11-72, 11-98
Auswerteskript 11-109
Messung durchführen 11-114, 11-145, 11-167, 11-208
Projektvorlagen erstellen 11-102
Spezielle Einstellungen 11-99
perform animation 10-51
perform measurements 6-1
Properties window 2-7

R
running macros 12-26

S
saving measurement data 6-2
scalings 8-12
script installation 12-5
script limitation 12-3
scripts by several users 12-3
settings 8-27
Settings Editor 4-5
SFM-Scanning Fluorescence Microscope 270
Shadow mode 10-41, 10-42
shortcut menus 2-6
Shuttle&Find 299
Shuttle&Find, Application examples 330
Shuttle&Find, Checklist 348
Shuttle&Find, Creating new holders 325
Shuttle&Find, Fine calibration 328
Shuttle&Find, General Information on Accuracy 340
Shuttle&Find, Notes on operation 338
Shuttle&Find, Overlaying images 323

Shuttle&Find, Procedure for using it 307
Shuttle&Find, Setting up 299
Shuttle&Find, The SEM image 329
Shuttle&Find, Tips to ensure accuracy 339
Shuttle&Find, Troubleshooting 342
Skalierungen erstellen 11-35
speed modes 3-10
step by step to the first image 3-4

T
TIC Measurement module 257
Time Lapse Imaging module 9-66
TMA module 9-132
toolbars 2-5, 8-17

U
user interface 8-17
user specific configuration 8-3
user's guide 1-19

V
VBA module 12-24

W
why is the resulting image an optical section? 9-5
Widefield Multichannel Unmixing module 10-84
workarea 2-2
workflow 2-3
workflows 8-25
working with default experiments 9-138
working with image processing functions 5-4, 10-4

Z
Z-Stack module 9-62
ZVI Dateiformat 7-1