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Document Summary: This document, SOP002 - Iterative RNA FISH Experimental Protocol, describes the process for in-situ fluorescence labeling of RNA transcripts in cells and tissues using a layered (encoding + readout) probe design. This protocol can be used for single molecule RNA FISH or for high-throughput identity barcoding (MERFISH). This protocol uses a cleavable disulfide (S-S) reporter molecule, attached to a readout oligo, to allow for iterative rounds of RNA FISH labeling and imaging of the same sample with minimal disruption to sample integrity between rounds. For sufficient signal:noise, samples are gel embedded, cleared and photobleached. Tissue processing, gel embedding, photobleaching and clearing are described in addition to the in-situ labeling method.

Quick Overview:

Part 1 – Tissue or Cell-Based Experiment Preparation

- Step 1 – Coverslip functionalization
- Step 2 - Mount, SDS pretreat and permeabilize sample
- Step 3 - Hybridize anchor probe RNA linker (use linker when gel embedding)
- Step 4 - Wash away residual linker
- Step 5 - Gel embed sample (optional - gel embed when clearing and digesting)
- Step 6 – Photobleach & clear sample
- Step 7 – Hybridize encoding probes
- Step 8 – Wash away residual encoding probes

Part 2 – Iterative RNA FISH Imaging with Automated Fluidics System

- Step 1 - Prepare solutions for fluidics
- Step 2 - Assemble fluidics system.
- Step 3 –ITERATIVE RNA FISH imaging protocol.

v4.6 revision notes

1. Updated protocol title to generalize as protocol can be used for MERFISH or smFISH, whether multiplexed or not.
2. Removed bDNA references and chemistries from the protocol.
3. Add optional tissue-dependent peroxide pretreatment to part 1, step 2.iii
4. Removed part 2b – manual iterative labeling without automated fluidics.

References:

- Hershberg, E. A., Close, J. L., Camplisson, C. K., Attar, S., Chern, R., Liu, Y., ... & Beliveau, B. J. (2020). PaintSHOP enables the interactive design of transcriptome- and genome-scale oligonucleotide FISH experiments. *bioRxiv*.
- Moffitt, J. R., Hao, J., Bambach-Mukku, D., Lu, T., Dulac, C., & Zhuang, X. (2016). High-performance multiplexed fluorescence in situ hybridization in culture and tissue with matrix imprinting and clearing. *Proceedings of the National Academy of Sciences*, 113(50), 14456-14461. <https://doi.org/10.1073/pnas.1617699113>
- Moffitt, J. R., & Zhuang, X. (2016). RNA imaging with multiplexed error-robust fluorescence in situ hybridization (MERFISH). In *Methods in enzymology* (Vol. 572, pp. 1-49). Academic Press. <https://doi.org/10.1016/bs.mie.2016.03.020>.
- Stellaris RNA FISH protocol for frozen tissues: https://biosearchassets.blob.core.windows.net/assets/bti_stellaris_protocol_frozen_tissue.pdf

Adapted from: Hershberg 2020, Moffitt, 2016 and Biosearch Technologies Stellaris RNA FISH Protocol for Frozen Tissue

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Full Protocol:

***All reagents for this protocol should be prepared sterile and RNase-free.*

***All incubation periods should be done in the dark.*

***Reagent/buffer preparation instructions follow protocol below.*

***Safety note: This protocol uses formamide which is a teratogen and can cause developmental malformation. Always work in a fume hood with formamide to avoid inhalation and avoid physical contact.*

Part 1 – Tissue or Cell-Based Experiment Preparation

Description: Part 1 of this protocol describes the steps to setup an iterative RNA FISH experiment for tissue or cell-based samples. These steps are focused on the biochemical requirement for tissue or cell preparation, probe hybridization and imaging. This protocol does not cover the requirements of the microscope for imaging. Additional detail can for the imaging setup can be found at <https://doi.org/10.1016/bs.mie.2016.03.020>.

Step 1: Coverslip Functionalization Refer to current version of SOP003 for protocol on Coverslip Functionalization. PDL-coated coverslips are preferable as tissue can be post-fixed to the coating using 4% PFA.

Step 2: Mount, SDS Pretreat and Permeabilize Sample

- a. *Using 4% PFA-fixed and OCT-embedded tissue.*
 - i. Slice tissue and blot tissue slice to a sample coverslip. Leave tissue slice on coverslip for 5 min in cryostat to re-freeze the sample to the coverslip surface.
 - ii. Post-fix the sample to the coverslip using RT PFA for 10min, quench PFA with 100mM Ammonium chloride for 5-20 min then rinse with 1x PBS 3x for 5 min at RT.
 - iii. Pretreat tissue
 1. (*optional – tissue dependent*) Immerse tissue in 5-6 drops of 3% peroxide solution at RT for 10 min then rinse sample with then rinse out well with 1x PBS 3x at RT.
 2. Incubate in 4% SDS Clearing Solution (SDS-CS) for 5 minutes at RT then rinse out well with 1x PBS 3x for 5 min at RT.
 - iv. To permeabilize the tissue, immerse the slip mounted tissue in 70% (vol/vol) ethanol overnight at 4°C (recommended) in a Pyrex 60mm petri dish.
***For faster results, sample can be incubated in 70% EtOH for 1 hour at RT.*
 - v. Following permeabilization, aspirate off the 70% ethanol and move to step c.
 - vi. Rehydrate sample with ethanol series of 50%, 30% and 10% EtoH in nuclease free water.

- b. *Using 4% PFA-fixed cells grown on coverslip (optionally, use 8-chamber well or similar)*
 - i. Pretreat cells for 2min at RT in 2% SDS clearing solution (50% diluted 4% clearing solution in 1x PBS).
 - ii. Rinse the sample 3x for 5 min in 1x PBS solution.
 - iii. To permeabilize the cells, immerse the slip mounted sample in 70% (vol/vol) ethanol overnight at 4°C (recommended) in a Pyrex 60mm petri dish (Fisher 08-747A). (For faster results, sample can be incubated in EtOH for 1 hour at RT).
 - iv. Aspirate rinse from the sample and let dry.
Alternative permeabilization method for cells:
 1. Pipette 100µL permeabilization buffer (PBS-t) and incubate at RT for 10 min with gentle rocking.
 2. Rinse with permeabilization buffer rinse (PBS-tw) and rinse again with 1x PBS.
 3. Let the sample dry and move on to step c.

- c. Using a hydrophobic pen, draw a barrier around your sample and let the barrier dry before hybridizations. You may want to add a very small volume of PBS to a tissue sample during this process to prevent tissue sample desiccation.

Step 3: Hybridize anchor probe RNA linker (use linker when gel embedding).

1. Wash & equilibrate sample by immersing slip-mounted sample in 52°C pre-heated 100µL-250µL Wash Buffer A for 5-30 min.
2. Assemble a humidified chamber per figure 1.
3. Remove Wash Buffer A from the sample.
4. Dispense 125µL 47°C prewarmed Encoding Hybridization Buffer containing 1µM** linker to your sample, add an overlay coverslip and assemble the sample in the hybridization chamber per figure 1.
***Adjust the concentration of the linker depending on the sample size and thickness.*
5. Incubate at 37-41°C** in a humidified chamber for 18-24 hours, up to 36 hours.

Step 4: Wash Away Residual Linker

1. Remove the hybridization buffer and carefully remove the excess buffer surrounding the sample.
2. Wash the sample in pre-heated 47°C Wash Buffer A for 30 min, two times.
3. Wash two times in a 47°C pre-heated Encoding Wash Buffer (SSC-tw) for 5 mins, two times.
4. Rinse two times with RT 1x PBS.

Step 5: Gel Embed Sample (optional – gel embed when clearing and digesting)

1. Incubate sample for 2 min for cells or up to 3hr for 100 µm-thick tissue sections with de-gassed PA monomer solution.
2. Incubate sample for 2 min with PA Gel Solution and remove.
3. Cast a thin PA film by adding ~50-100µL gel solution (*including fluorescent beads*) to the sample and then invert a smaller (25mm-diameter) Gel Slick (Lonza, 50640) coated coverslip overlay onto the gel solution, being careful to avoid air bubbles. Adjust the gel solution volume and make sure your gel film is thin by aspirating any extra gel solution away. You can also carefully overlay a Kim wipe to blot extra gel solution from the sample.
4. Allow casting for 1.5 hour at RT.
5. After casting, carefully remove the overlay coverslip from your sample. If the coverslip is stuck, you can loosen the coverslip by immersing SDS-CS at 37°C.

Step 6: Photobleach and Clear Sample

1. Depending on the tissue type (e.g. lung tissue), you may need to modify the clearing process. For lung tissue, start with step 6.2.2.1 - 6.2.2.3. For brain tissues, skip to step 6.3.
2. (*optional for lung tissue*) Enzymatic digestion
 - 2.1. Incubate sample in 3mL PBS with 10% collagenase/elastase at 20,000U/mL for 3 hours at 37°C.
 - 2.2. Wash sample with a quick rinse of RT 1x PBS followed by two 5min washes of 1x PBS at RT.
 - 2.3. Wash the sample on the coverslip twice with 1mL SDS Clearing Solution (SDS-CS) for 5 min each wash at 37°C.
3. (optional) Photo bleach the sample overnight in 5x SSC storage buffer with 3mg/mL pvsd and protected using a UV filter and aluminum foil.
4. Incubate with 3-5mL prewarmed 37°C SDS Clearing Solution with 1% Proteinase K in a humidified chamber for 1-24h at 37°C, depending on the sample.
5. Wash the sample by immersing it in Wash Buffer B 3 to 4 times for 5 min each.
6. Rinse sample in 2x SSC, 3x.

Step 7: Hybridize Encoding probes.

1. Wash & equilibrate sample by immersing slip-mounted sample in 52°C pre-heated 100µL-250µL Wash Buffer A for 5-30 min.
2. Assemble a humidified chamber per figure 1.
3. Remove Wash Buffer A from the sample.
4. Dispense 125µL 47°C prewarmed Encoding Hybridization Buffer containing 5-200**µM encoding probes (depending on the number of unique encoding probes in the probe set and sample size) and add an overlay coverslip and assemble the sample in the hybridization chamber per figure 1.
***Adjust the concentration of encoding probe depending on the sample size and thickness.*
5. Incubate at 43°C** in a humidified chamber for 18-24 hours, up to 72 hours.
***Note: hybridization temperature may need to be adjusted based on Tm of probes as designed. PaintSHOP probes hybridized at 43°C; Moffitt/Zhuang probes hybridized at 37°C.*

Step 8: Wash Away Residual Encoding Probes.

1. Remove the hybridization buffer and carefully remove the excess buffer surrounding the sample.
2. Wash the sample in pre-heated 47°C Wash Buffer A for 30 min, two times.
3. Wash two times in a 47°C pre-heated Encoding Wash Buffer (SSC-tw) for 5 mins, two times.
4. Rinse two times with RT 1x PBS.

Part 2 – ITERATIVE RNA FISH Imaging with Automated Fluidics System

Description: Imaging for ITERATIVE RNA FISH involves multiple rounds of fluid exchange to hybridize, image, cleave and rinse samples. Automated fluid exchange and imaging approach is recommended.

Step 1: Prepare Solutions for fluidics.

- i. Readout Hybridization Buffer
- ii. Readout Wash Buffer
- iii. Imaging Buffer (store under mineral oil)
- iv. TCEP Cleavage Buffer
- v. 2x SSC Wash Buffer (Wash B)
- vi. DAPI Staining Solution

Suggested buffer volumes for use with pump-driven fluidics setup:

- Imaging buffer - 2.5mL per round.
- TCEP Cleavage buffer - 4.5mL per round.
- Wash D - 4.5mL per round.

Step 2: Assemble Fluidics System

1. Pre-rinse the fluidics setup following SOP002a before use.
2. Make sure that all tubing is properly connected. MULTIPLEXED ITERATIVE FISH probes and sample preparation time are costly so leaks need to be avoided.
3. Ensure the system is fully assembled, plugged in and turned on.
4. Double-check correctness of the details for the pump program for the MULTIPLEXED ITERATIVE FISH Fluidics for the current project.
5. Load the sample to the flow cell and connect.
6. Carefully load all solutions to the proper reservoirs.
7. Prime the solutions using the solution pre-treatment program to reduce bubbles in lines and bring solutions to the distribution manifold.

Step 3: ITERATIVE RNA FISH Imaging Protocol. Once the fluidics system is setup, solutions are prepped and loaded and the sample is in place in the chamber, an automated program should run the following cycle.

1. SSC Buffer
 - a. Prime SSC (0.5mL/min)
 - b. Flow 3mL SSC buffer at 0.5mL/min
2. Readout hybridization buffer (including readout probes)
 - a. Prime RHB (0.5mL/min)
 - b. Flow 2mL over sample at 0.4mL/min
 - c. Pause flow for 15-120 mins, depending on sample size (10 μm = 30 min, 30 μm = 60 min, 100 μm = 120 min).
2. Readout Wash Buffer
 - a. Prime RWB (0.5mL/min)
 - b. Flow 2.5mL over sample (0.2mL/min)
3. SSC Buffer
 - a. Prime SSC Buffer (0.5mL/min)
 - b. Flow 2.5mL over sample (0.2mL/min)
4. Imaging Buffer
 - a. Flow 2mL IB over sample (0.2mL/min)
 - b. Flow and additional 1mL IB over the sample (0.5mL/min).

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- c. Pause flow for 15 ins to equilibrate the sample
5. Imaging. Pause fluidics and proceed with imaging.
6. TCEP Cleavage Buffer
 - a. Prime CB (0.5mL/min)
 - b. Flow 1.3mL CB over sample (0.13mL/min)
 - c. Pause flow for 10 min.
7. SSC Buffer
 - a. Prime SSC (0.5mL/min)
 - b. Flow 3mL SSC buffer at 0.5mL/min
8. Repeat steps 1-7 for each readout round.

When all readout rounds are complete proceed with steps 9-12.

9. DAPI Stain (optional) – Wash 2mL DAPI in 2xSSC (Wash B) for 60 min.
 - a. Use 50µg/mL for thick (100µm) samples.
 - b. Use 1-10µg/mL for 10µm samples.
10. SSC Buffer
 - a. Prime SSC Buffer (0.5mL/min)
 - b. Flow 2.5mL over sample (0.2mL/min)
11. Imaging Buffer
 - a. Flow 2mL IB over sample (0.2mL/min)
 - b. Flow and additional 1mL IB over the sample (0.5mL/min).
 - c. Pause flow for 15 ins to equilibrate the sample
12. Imaging. Pause fluidics and proceed with imaging.

Solution Preparation:**Wash Buffer A (40% Formamide Wash Buffer)**

- 2x SSC Buffer (Fisher, AM9763)
- 1% (vol/vol) Tween 20 (Sigma, P9416-100ML)
- 40% (vol/vol) Formamide (Fisher, AM9342)
- Nuclease-free water

Wash Buffer A Master Mix, 45mL (for 75mL final volume with FA):

- 36.75mL nuclease-free water
- 7.5mL 20x SSC Buffer (Fisher, AM9763)
- 750µL Tween 20 (Sigma, P9416-100ML)
- Add 40% formamide (FA) to prepare on demand (Fisher, AM9342)

Wash Buffer B

- 2x SSC buffer (Fisher, AM9763) prepared in nuclease-free water.

Saber Encoding Hybridization Buffer

- Nuclease-free water
- 2x SSC Buffer (Fisher, AM9763)
- 40% (vol/vol) Formamide (Fisher, AM9342)
- 0.1% (wt/vol) Yeast tRNA (Life Technologies, 15401011)
- 1% (vol/vol) Murine RNase Inhibitor (New England Labs, M0314L)
- 1% (vol/vol) Tween 20 (Sigma, P9416-100ML)
- 10% (wt/vol) Dextran sulfate (Sigma, D8906-100g)
- Add encoding probes at 5-200µM final concentration, depending on the size of the pool.
- Prepare on demand.

Saber Encoding Hybridization Buffer Master Mix, 4.8mL (for 8.0mL prep with formamide added)

- Nuclease-free water
- 800µL 20x SSC Buffer (Fisher, AM9763)
- 320µL Yeast tRNA solution (Life Technologies, 15401011, reconstituted to 25mg/mL)
- 80µL Murine RNase Inhibitor (New England Labs, M0314L)
- 80µL Tween 20 (Sigma, P9416-100ML)
- 0.8g Dextran sulfate (Sigma, D8906-100g)
- Aliquot mix and store at -20°C.
- To prepare on demand, add 40% (vol/vol) Formamide (Fisher, AM9342) to master mix at time of use.
- Add encoding probes at 5-200µM final concentration, depending on the size of the pool.

Encoding Buffer Rinse (SSC-tw)

- Nuclease-free water
- 2x SSC (Fisher, AM9763)
- 0.1% (vol/vol) tween 20 (Sigma, P9416-100ML)
- Store at RT

PA Solution

- Nuclease-free water
- 4% (vol/vol) 19:1 acrylamide/bis-acrylamide (Bio-Rad Laboratories, 1610144)
- 60mM Tris-HCl pH8 (Fisher, AM9856)
- 0.3M NaCl (Fisher, AM9759)
- De-gas solution before use.
- Prepare on demand.

PA Gel

- PA Solution including polymerizing agents:
 - 0.03% (wt/vol) ammonium persulfate (Sigma A3678)
 - 0.15% (vol/vol) TEMED
 - **Add desired concentration of fluorescent beads for image registration

- ***Prepare on demand. Polymerizing agents will act rapidly. Make gel in small quantities (1mL) and right before use.*
- *Optional: Add fluorescent beads for image registration purposes, as needed.*
- Prepare on demand.

Storage Buffer (SSC-SB)

- Wash Buffer B
- 0.1% (vol/vol) murine RNase Inhibitor (New England Labs, M0314L)
- Store in aliquots at -20°C.

5x SSC Storage Buffer (5xSSC-SB)

- 5x SSC buffer prepared in nuclease free water
- 3mg/mL Poly(vinylsulfonic acid, sodium salt) solution (Sigma, 278424)
- Prepare on demand

Readout Hybridization Buffer (RHB)

- 2x SSC buffer (Fisher, AM9763)
- 10% (vol/vol) ethylene carbonate (Sigma, E26258)
- 0.1% (vol/vol) Murine RNase Inhibitor (New England Labs, M0314L)
- Nuclease-free water
- Add readout probes at 3nM final concentration.
- Prepare on demand.

Readout Wash Buffer (RWB)

- 2x SSC Buffer (Fisher, AM9763)
- 10% (vol/vol) ethylene carbonate (Sigma, E26258)
- 0.1% triton X-100
- Store at RT.

Trolox Quinone Imaging Buffer

- 10% >500µM Trolox Quinone (see preparation instruction below)
- 0.2% 2.5M PCA - 3,4-Dihydroxybenzoic acid (386mg/mL in methanol) (Sigma, 37580)
- 0.5% Trolox (100mg/mL in methanol) (Sigma, 238813)
- 0.5% 1M NaOH (Sigma, S5881)
- 0.3% PVSA (Sigma, 278424)
- Prepared in 2x SSC buffer (Invitrogen, AM9763)

Trolox Quinone Preparation Instruction

- 100mM Trolox dissolved in methanol (250mg Trolox in 10mL methanol).
- Add 0.4mL 100mM Trolox to 20mL 2x SSC buffer, 8x, and vortex to mix well.
- Bake for 30min at 254nm in UVP-CX2000 or similar.
 - Remove aliquot and measure absorbance at 255nm.
 - Repeat baking step until absorbance is 1.63 or greater for ~500µM Trolox Quinone.
- Combine aliquots and measure the final combined absorbance.
- Prepare 4mL aliquots and store at -20°C.

Cleavage Buffer

- 2x SSC Buffer (Fisher, AM9763)
- 50mM Tris(2-carboxyethyl) phosphine [TCEP] (Sigma, 646547)
- Prepare on demand.

DAPI Staining Solution

- 50µg/mL DAPI stain (Fisher, D1306) in Wash Buffer B for thick (40µm) tissue.
- 1-10µg/mL DAPI stain (Fisher, D1306) in Wash Buffer B for thin (10µm) tissue.
- Prepare on demand.

Permeabilization Buffer (PBS-t)

- Nuclease-free water
- 1x PBS Buffer (Invitrogen, AM9625)
- 0.5% (v/v) Triton X-100 (Sigma, T8787-100mL)
- Store at RT

Permeabilization Buffer Wash (PBS-tw)

- Nuclease-free water
- 1x PBS Buffer (Invitrogen, AM9625),
- 0.1% (v/v) Tween 20 (Sigma, P9416-100ML)
- Store at RT

SDS Clearing Solution (SDS-CS)

- 1x PBS
- 4% SDS (Sigma, 75746)
- Nuclease Free Water
- Store at RT

PFA Quenching solution (NH4CL)

- 100mM Ammonium Chloride in nuclease free water.

Keywords:

In-situ hybridization, FISH, fluorescence, RNA, iterative FISH, formamide, amplified probes, bDNA, branched DNA, fluidics, acrydite, linker probe, anchor probe, encoding probes, tissue, cells, thick tissue, clearing, digestion, polyacrylamide gel

Hybridization Chamber Rendering

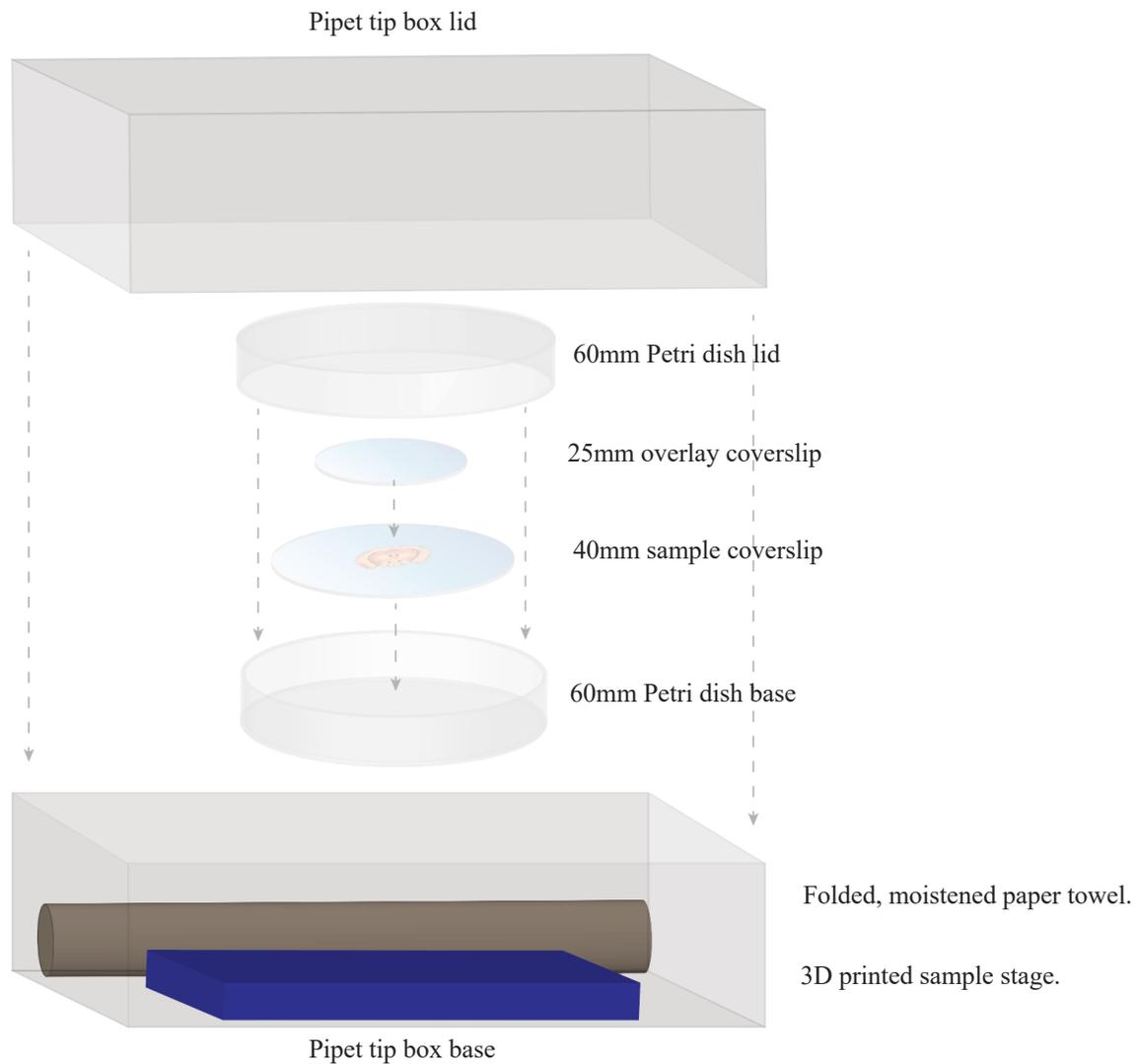


Figure 1. Hybridization chamber rendering. For hybridization incubations, it is critical to prevent evaporation. For hybridizations, pipet the hybridization buffer on the sample then overlay with a cleaned and RNase-pretreated 25mm coverslip (or parafilm). Place the sample coverslip in a 60mm petri dish (Fisher 08-747A), replace the petri dish lid then Parafilm the edges of the dish to prevent evaporation. Wet a rolled paper towel and place in a clean, empty pipet tip box along with a sample stage which keeps the sample away from any standing water.