

# Microglia differentiation from hPSC (v2; optimised protocol)

Owner: Nina Lydia Kazakou | Created at: 2024-07-15

## Description

Protocol for the directed differentiation of hPSCs towards erythro-myeloid progenitors and further to microglia. Based on Washer et al, Sci Rep, 2022 with input from Bioneer A/S. Option to use "De Strooper" protocol variant (Fattorelli et al, Nature Protocols, 2021) is described.

## Materials

Reagent	Vendor	Catalog number
mTeSR1 Basal Medium and 5x supplement kit	STEMCELL Technologies	85850
X-VIVO 15	Lonza	BE04-418
Advanced DMEM/F12	Gibco	12634010
GlutaMAX	Life Technologies	35050-061
$\beta$ -mercaptoethanol	Life Technologies	31350-010
EDTA	Thermo Fisher	15575020
DPBS	Thermo Fisher	A1285601
Y-27632 dihydrochloride	Miltenyi	130-106-538
KnockOut™ Serum Replacement	Thermo Fisher	10828028
Pen/Strep	Life Technologies	17502-048
BMP4	Miltenyi	130-111-165
VEGF	Miltenyi	130-109-385
SCF	Miltenyi	130-096-692
M-CSF	Miltenyi	130-096-492
IL-3	Miltenyi	130-095-069
GM-CSF	Miltenyi	130-093-864
IL-34	Miltenyi	130-108-977
TGFb1	Peprotech	100-21C-100UG
TPO	Thermo Fisher (Peprotech)	300-18
FLT3L	Thermo Fisher (Peprotech)	300-19
Aggrewell 800 (24 wells)	STEMCELL Technologies	34811
Reversible Strainer	StemCell	27250
Anti-Adherence Rinsing Solution	STEMCELL Technologies	7010

Matrigel	Corning	354230
----------	---------	--------

**Attachments**

## Media Compositions

**EB formation (Aggrewell) medium**

Day 0-6:

Reagent	Stock Concentration	Final Concentration	Final Volume = 50ml	Dilution
mTeSR1			49.7ml	
BMP4	10 µg/ml	50 ng/ml	250 µl	1:200
VEGF	50 µg/ml	50 ng/ml	50 µl	1:1000
SCF	20 µg/ml	20 ng/ml	50 µl	1:1000
Pen/Strep	5,000 U/mL	100 U/mL	100 µl	1:500

**preMACS differentiation (Factories) medium - "Cowley" protocol, Washer et al 2022**

Day 6 onwards:

Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
X-VIVO15			98.2 ml	
M-CSF	100 µg/ml	100 ng/ml	100 µl	1:1000
IL-3	25 µg/ml	25 ng/ml	100 µl	1:1000
GlutaMAX	200 mM	2 mM	1 ml	1:100
β-mercaptoethanol	50 mM	50 µM	100 µl	1:1000
Pen/Strep	5,000 U/mL	100 U/mL	200 µl	1:500

**preMACS differentiation (Factories) medium - "De Strooper" protocol, Fattorelli et al 2021**

Day 6-12:

Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
X-VIVO15			98.2 ml	
M-CSF	100 µg/ml	50 ng/ml	50 µl	1:2000
IL-3	25 µg/ml	50 ng/ml	200 µl	1:500
SCF	20 µg/ml	50 ng/ml	250 µl	1:400
TPO	5 µg/ml	5 ng/ml	100µl	1:1000

FLT3L	50 µg/ml	50 ng/ml	100 µl	1:1000
GlutaMAX	200 mM	2 mM	1 ml	1:100
β-mercaptoethanol	50 mM	50 µM	100 µl	1:1000
Pen/Strep	5,000 U/mL	100 U/mL	200 µl	1:500

Day 12 onwards:

Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
X-VIVO15			98.2 ml	
M-CSF	100 µg/ml	50 ng/ml	50 µl	1:2000
GM-CSF	10 µg/ml	25 ng/ml	250 µl	1:400
FLT3L	50 µg/ml	50 ng/ml	100 µl	1:1000
GlutaMAX	200 mM	2 mM	1 ml	1:100
β-mercaptoethanol	50 mM	50 µM	100 µl	1:1000
Pen/Strep	5,000 U/mL	100 U/mL	200 µl	1:500

### Microglia Maturation Medium

Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
ADMEM/F12			98ml	
Glutamax	200mM	2mM	1ml	1:100
M-CSF	100ug/ml	25ng/ml	25ul	1:4000
GM-CSF	10ug/ul	10ng/ml	100ul	1:1000
IL-34	100ug/ul	100ng/ml	100ul	1:1000
TGFb1	50ug/ml	50ng/ml	100ul	1:1000
Pen/Strep	5,000 U/mL	100 U/mL	200ul	1:500

## Embryo Bodies (EB) generation

**Preparation of Aggrewell-800 plates**

1	<p>Pre-treat wells with Anti-Adherence Rinsing Solution. For 24-well plates, add 500ul per well</p> <p>NOTE: Do not expose AggreWell plates to organic solvents, including ethanol or isopropanol</p>	00:00:00
2	<p>Centrifuge plate at 1300 x g for 3 minutes in a swinging bucket rotor fitted with plate holders</p> <p>NOTE: Plates must be well-balanced. Prepare a balanced plate using a standard plate filled with water to match the weight and position of the Aggrewell plate</p>	00:00:00
3	<p>Observe the plate under a microscope to ensure bubbles have been removed from microwells. If bubbles remain trapped in any microwells, centrifuge at 1300 x g for an additional 3 minutes</p>	00:00:00
4	<p>Aspirate Anti-Adherence Rinsing Solution from the wells</p>	00:00:00
5	<p>Rinse each well with 500ul of DMEM/F12 medium</p> <ul style="list-style-type: none"> <li>• Centrifuge at 1300 x g for an additional 3 minutes</li> </ul> <p>Remove media and add 1ml of fresh DMEM/F12 medium (second wash).</p>	00:00:00
6	<p>Remove DMEM/F12 medium and add 1ml of EB medium + 10<math>\mu</math>M Y-27632 ROCK inhibitor to each well</p>	00:00:00

**hPSC dissociation: DIV 0**

1	<p>Cultures should be 70–80% confluent:</p> <ul style="list-style-type: none"> <li>• homogeneous-appearing colonies with clear borders</li> <li>• absence of obvious differentiating zones</li> </ul>	00:00:00												
2	Prepare appropriate volume of EB medium + 10 $\mu$ M Y-27632 ROCK inhibitor	00:00:00												
3	Aspirate medium from hPSC cultures	00:00:00												
4	Wash cells with appropriate PBS volume	00:00:00												
5	<p>Add the appropriate volume of 50<math>\mu</math>M EDTA in PBS</p> <table border="1"> <thead> <tr> <th>Plate</th> <th>Seeding area (Sarstedt)</th> <th>EDTA 50<math>\mu</math>M (75 <math>\mu</math>L/cm<sup>2</sup>)</th> </tr> </thead> <tbody> <tr> <td>6-well</td> <td>9.07 cm<sup>2</sup></td> <td>1.9ml</td> </tr> <tr> <td>12-well</td> <td>3.65 cm<sup>2</sup></td> <td>700<math>\mu</math>l</td> </tr> <tr> <td>24-well</td> <td>1.82 cm<sup>2</sup></td> <td>300<math>\mu</math>l</td> </tr> </tbody> </table>	Plate	Seeding area (Sarstedt)	EDTA 50 $\mu$ M (75 $\mu$ L/cm <sup>2</sup> )	6-well	9.07 cm <sup>2</sup>	1.9ml	12-well	3.65 cm <sup>2</sup>	700 $\mu$ l	24-well	1.82 cm <sup>2</sup>	300 $\mu$ l	00:00:00
Plate	Seeding area (Sarstedt)	EDTA 50 $\mu$ M (75 $\mu$ L/cm <sup>2</sup> )												
6-well	9.07 cm <sup>2</sup>	1.9ml												
12-well	3.65 cm <sup>2</sup>	700 $\mu$ l												
24-well	1.82 cm <sup>2</sup>	300 $\mu$ l												
6	<p>Incubate at 37°C for 7 min</p> <p>NOTE: If very confluent leave cells in the incubator for up to 10min</p>	00:00:00												

7	<p>Add 1 mL of wash medium to the well and pipette off the colonies from all sides of the well using a P1000 pipette</p> <ul style="list-style-type: none"> <li>• The cells should easily be removed by washing the well with a medium</li> <li>• Prepare a single-cell suspension</li> </ul>	00:00:00
8	Transfer the colonies to the 15 mL tube containing 10ml Wash Buffer	00:00:00
9	<p>Count cells with Nucleocounter:</p> <ul style="list-style-type: none"> <li>• Determine the concentration of cells/ml</li> <li>• Determine the total volume of cell suspension to initiate differentiation</li> </ul>	00:00:00
10	Transfer the appropriate cell suspension volume to a new tube	00:00:00
11	Centrifuge cells at 400g for 5min at RT	00:00:00

**EB generation: DIV 0**

1	To achieve EB size of 15,000 cells per EB, plate $4 \times 10^6$ cells in each well	00:00:00
2	Aspirate medium and resuspend cells in 1ml of EB medium per well	00:00:00
3	Add 1ml of cell suspension to each 24-well already containing 1ml of EB medium	00:00:00

4	Prepare a centrifuge balance plate using a standard plate filled with water to match the weight and position of the AggreWell plate	00:00:00
5	Pipette cells up and down gently several times to ensure even distribution of cells throughout the well NOTE: Be careful not to introduce bubbles into the microwells	00:00:00
6	Centrifuge the AggreWell plate at 100 x g for 3 minutes to capture cells in the microwells	00:00:00
7	Incubate the plate at 37°C with 5% CO <sub>2</sub> and 95% humidity	00:00:00

**EB medium change: DIV 1 - DIV 6**

1	Perform 75% medium change (EB medium) every day	00:00:00
2	Slowly remove 1.5ml of medium from each well	00:00:00
3	Replace with 1.5m of the fresh EB medium by slowly pipetting down the wall of the well; Slowly dispensing the medium prevents displacement of EBs/spheroids from the microwells	00:00:00

## preMACS - "Cowley" version

**Coat Flasks with Matrigel**

1	Remove a pre-diluted aliquot of matrigel (5ml of 5% matrigel) from the freezer and in the fridge overnight	00:00:00
2	Add 5ml of DMEM/F12 (w/ Glutamax) to each aliquot <ul style="list-style-type: none"> <li>• Matrigel should always be handled on ice</li> </ul>	00:00:00
3	Add 10ml of the matrigel in the flask	00:00:00
4	Incubate the flask at 37°C with 5% CO <sub>2</sub> and 95% humidity for at least 1h	00:00:00
5	Remove coating before seeding the EBs	00:00:00

**Transfer EBs into matrigel coated T75 flask**

1	Remove EBs carefully from Aggrewells using a 5ml stripette	00:00:00
2	Transfer EBs to a 40µm invertible cell strainer; Use a 50ml Falcon tube as liquid waste beneath the strainer <ul style="list-style-type: none"> <li>• This step ensures that only EBs are transferred into the flask</li> <li>• Single &amp; dead cells are captured on the strainer membrane</li> </ul>	00:00:00

3	Flash the remaining EBs from the Aggrewell using 2ml PBS and transfer to the strainer	00:00:00
4	Repeat step 3	00:00:00
5	Wash dead cells away from the EB in the strainer using 4 mL of PBS	00:00:00
6	Invert the strainer over a new 50ml Falcon tube and wash off EBs off using 4 mL of preMACS differentiation medium	00:00:00
7	Divide EBs evenly into two matrigel-coated T75 flasks, each containing 18ml of preMACS differentiation medium (total V = 20ml)	00:00:00
8	Incubate the plate at 37°C with 5% CO <sub>2</sub> and 95% humidity	00:00:00

### preMACS Factories media change: ~ till DIV35

1	Factories are fed weekly by <b>adding</b> 10ml of preMACS differentiation media until macrophage/microglia precursors (preMACS) cells start to appear in the supernatant; This process usually takes ~5 weeks NOTE: At this point, preMACS can be used for microglia maturation experiments	00:00:00
---	--	----------

2	<p>Factories can be maintained successfully for at least 4mo:</p> <ul style="list-style-type: none"> <li>• Factories are fed weekly by <b>adding</b> 10ml of preMACS differentiation media <ul style="list-style-type: none"> <li>◦ This is okay for most cell lines, including RC17, but it needs to be adjusted to twice a week for other lines, like KOLF2.1; Keep an eye on the media color and adjust as necessary</li> </ul> </li> <li>• When the flask becomes too full, transfer the supernatant containing the preMACS in the suspension into a 50ml Falcon tube, centrifuge for 5min at 400g, remove supernatant, resuspend preMACS into 10ml of preMACS differentiation media, and return the freshly resuspended preMACS into the relevant flask</li> </ul>	00:00:00
---	---	----------

## preMACS - "De Strooper" version

Follow the steps described for the "Cowley" protocol - the only difference is the preMAC media composition (see table above)

## Monolayer microglia differentiation

From ~DIV35 onwards, free-floating preMACS in the Factories supernatant can be collected and matured into microglia w/ Microglia Maturation Medium (MMM).

preMACS can be seeded on plastic plates or glass coverslips.

Microglia can be used between preMACS seeding day +7d of MMM until preMACS seeding day +2mo of MMM.

preMACs for maturation into microglia are seeded at 100,000 cells/cm<sup>2</sup>

### Seeding monolayer microglia

1	Aspirate all medium from a preMACs Factory containing flask	00:00:00
2	Transfer into a 50ml Falcon through a 40um strainer	00:00:00

3	Count cells using a nuclecounter to determine the concentration of cells/ml	00:00:00																		
4	<p>Determine the total volume of cell suspension needed and transfer the appropriate volume into a new Falcon tube</p> <p>NOTE: Do not leave the preMACs in the tube for too long, as cells tend to stick to everything, including the tube walls</p>	00:00:00																		
5	Centrifuge cells at 400g for 5min at RT	00:00:00																		
6	<p>Aspirate medium and resuspend cells in MMM (according to the table below)</p> <table border="1" data-bbox="196 892 1308 1488"> <thead> <tr> <th data-bbox="196 892 566 991">Plate</th> <th data-bbox="566 892 937 991">Seeding area</th> <th data-bbox="937 892 1308 991">MMM (V)</th> </tr> </thead> <tbody> <tr> <td data-bbox="196 991 566 1092">6-well</td> <td data-bbox="566 991 937 1092">9.07 cm<sup>2</sup></td> <td data-bbox="937 991 1308 1092">2.5ml</td> </tr> <tr> <td data-bbox="196 1092 566 1192">12-well</td> <td data-bbox="566 1092 937 1192">3.65 cm<sup>2</sup></td> <td data-bbox="937 1092 1308 1192">1ml</td> </tr> <tr> <td data-bbox="196 1192 566 1293">24-well</td> <td data-bbox="566 1192 937 1293">0.64 cm<sup>2</sup></td> <td data-bbox="937 1192 1308 1293">500ul</td> </tr> <tr> <td data-bbox="196 1293 566 1394">48-well</td> <td data-bbox="566 1293 937 1394">1.82 cm<sup>2</sup></td> <td data-bbox="937 1293 1308 1394">300ul</td> </tr> <tr> <td data-bbox="196 1394 566 1488">96-well</td> <td data-bbox="566 1394 937 1488">0.29 cm<sup>2</sup></td> <td data-bbox="937 1394 1308 1488">100ul</td> </tr> </tbody> </table>	Plate	Seeding area	MMM (V)	6-well	9.07 cm <sup>2</sup>	2.5ml	12-well	3.65 cm <sup>2</sup>	1ml	24-well	0.64 cm <sup>2</sup>	500ul	48-well	1.82 cm <sup>2</sup>	300ul	96-well	0.29 cm <sup>2</sup>	100ul	00:00:00
Plate	Seeding area	MMM (V)																		
6-well	9.07 cm <sup>2</sup>	2.5ml																		
12-well	3.65 cm <sup>2</sup>	1ml																		
24-well	0.64 cm <sup>2</sup>	500ul																		
48-well	1.82 cm <sup>2</sup>	300ul																		
96-well	0.29 cm <sup>2</sup>	100ul																		
7	Incubate the plate at 37°C with 5% CO <sub>2</sub> and 95% humidity	00:00:00																		

**Monoayer microglia media change**

1	Perform 50% medium change (MMM) every other day	00:00:00
2	Microglia monolayers can be maintained successfully for up to 2mo	00:00:00

**QC preMACS by FACS**

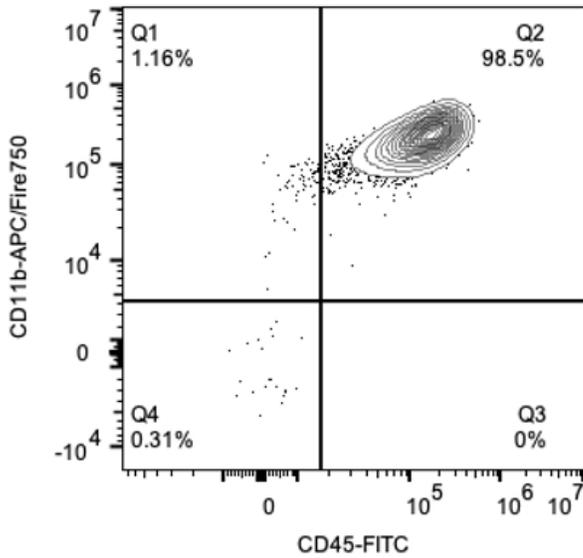
From around DIV30 onwards, preMACs can be analysed by flow cytometry for the co-expression of CD45 and CD11b using the protocol and antibodies linked below. The time of QC depends on the cell line, e.g. RC17 are typically not ready before DIV40.

**Protocol:** [Flow cytometry - surface markers \(live cells\)](#)

**Antibodies** (click on the link for full details):

Antibody	Fluorophore	Dilution (Cytex Aurora)
<a href="#">CD45</a>	FITC	1:100
<a href="#">CD11b</a>	APC/Fire750	1:500

**Example**



## QC microglia by RT-qPCR

QC panel for RT-qPCR:

#	Primer name	Version	Sequence Fw	Sequence Rv
436	P2RY1 2	v2	TGCCAAACTGGGAACA GGACCA	TGGTGGTCTTC TGGTAGCGAT C
437	TMEM1 19	v1	AGTCCTGTACGCCAAG GAAC	GCAGCAACAG AAGGATGAGG
438	TREM2	v2	ATGATGCGGGTCTCTA CCAGTG	GCATCCTCGA AGCTCTCAGA CT
419	CSF1R		TCCAAAACACGGGGAC CTATC	CGGGCAGGGT CTTTGACATA
439	CX3CR 1	v2	CACAAAGGAGCAGGC ATGGAAG	CAGGTTCTCTG TAGACACAAG GC
420	IRF8		TGGCTGATCGAGCAGA TTGACAGT	AAGGGATCCG GAACATGCTCT TCT
421	PU.1		GAGCCCCCACTGGA GGT	TGGTACAGGC GGATCTTCTTC T

417	CD68		CGAGCATCATTCTTTCA CCAGCT	ATGAGAGGCA GCAAGATGGA CC
440	AIF1/ IBA1	v1	CCCTCCAAACTGGAAG GCTTCA	CTTTAGCTCTA GGTGAGTCTT GG
418	ITGAM/ CD11b		GGAACGCCATTGTCTG CTTTCG	ATGCTGAGGT CATCCTGGCA GA
233	CD44		GGACACCATGGACAA GTTTTGG	CACGTGGAAT ACACCTGCAA AG
412	MERTK		CAGGAAGATGGGACC TCTCTGA	GGCTGAAGTC TTTCATGCACG C
441	CD45		CTTCAGTGGTCCCATT GTGGTG	CCACTTTGTTC TCGGCTTCCA G
442	CCR2		TGG CTG TGT TTG CTT CTG TC	TCT CAC TGC CCT ATG CCT CT
45	GAPDH		TTGAGGTCAATGAAGG GGTC	GAAGGTGAAG GTCGGAGTCA
174	ACTB_v 2		ATGTGGCCGAGGACTT TGATTG	ATGGCAAGGG ACTTCCTGTAA C

## QC microglia by ICC

After 7 days in MMM, monolayer microglia should be positive for the following markers:

#	Primary ab	Species	Concentration	Secondary ab	Concentration
111	CD45	Ms	1:300	dk aMS 488	1:500
82	PU.1	Rb	1:300	dk aRb Cy3	1:500
80	IBA1	Gt	1:500	dk aGt 647	1:500

### Example

