Microglia differentiation from human pluripotent stem cells - Kirkeby Lab

Reagents required

Reagents	Supplier	Cat no
BMP4	Miltneyi	130-111-165
SCF	Miltenyi	130-096-692
VEGF	Miltneyi	130-109-385
X-VIVO 15	Lonza	BE04-418
M-CSF	Miltneyi	130-096-492
IL-3	Miltneyi	130-095-069
Advanced DMEM/F12	Life Technologies	12634-010
N2 supplement	Life Technologies	17502-048
GlutaMAX	Life Technologies	35050-061
2-mercaptoethanol	Life Technologies	31350-010
Pen/Strep	Life Technologies	17502-048
IL-34	Miltneyi	130-108-977
GM-CSF	Miltneyi	130-093-864
Aggrewell 800 well (24 wells)	STEMCELL Technologies	34811
Anti-Adherence Rinsing Solution	STEMCELL Technologies	7010
6 well ULA plates	Corning®	CLS3471
Nunc™ EasYFlask™ Cell Culture Flasks	Thermo Fisher	156499
Strainers, 40 mm	Corning	352340
RPMI medium	Thermo Fisher	11875093
IMDM medium	Thermo Fisher	12440053
B-27 supplement minus vitamin A	Thermo Fisher	12587010
Accutase	Thermo Fisher	A1110501
D-PBS –Ca2+/–Mg2+ CTS	Thermo Fisher	A1285601
Y-27632 dihydrochloride	Miltenyi	130-106-538
KnockOut™ Serum Replacement	Thermo Fisher	10828028

Preparation of aggrewell 800 plates

- Warm basal medium (DMEM-F12 with KOSR) and complete medium (Desired medium to plate cells).
- Open AggreWell plates in a biosafety cabinet. NOTE: Do not expose AggreWell plates to organic solvents, including ethanol or isopropanol.
- Pre-treat wells with Anti-Adherence Rinsing Solution as described below. Add Anti-Adherence Rinsing Solution to each well to be used, as follows: 24-well plate - 500 µL, 6-well plate - 2 mL.
- Centrifuge plate at 1300 x *g* for 5 minutes in a swinging bucket rotor fitted with plate holders. NOTE: Plates must be well balanced. Prepare a balance plate using a standard plate filled with water to match the weight and position of the AggreWell plate.
- Observe the plate under a microscope to ensure that bubbles have been removed from microwells. If bubbles remain trapped in any microwells, centrifuge at 1300 x *g* for an additional 5 minutes. Aspirate Anti-Adherence Rinsing Solution from the wells.
- Rinse each well with warm basal medium, as follows: • 24-well plate: 2 mL. Aspirate medium from the well.

- Add warm complete medium to each well to be used, as follows: • 24-well plate: 1 mL

Generation of EBs

- Prepare a single-cell suspension in the desired medium
- To achieve EBs size of 10,000 cells per EB, plate 3.0 x 10^6 in each well and add complete medium to each well to achieve a final volume as follows: • 24-well plate: 2 mL/well
- Prepare a centrifuge balance plate using a standard plate filled with water to match the weight and position of the AggreWell plate.
- Pipette cells up and down gently several times to ensure even distribution of cells throughout the well. Be careful not to introduce bubbles into the microwells.
- Immediately centrifuge the AggreWell plate at 100 x *g* for 3 minutes to capture cells in the microwells, using the balance plate prepared.
- Observe the plate under a microscope to verify that cells are evenly distributed among the microwells.
- Incubate the plate at 37°C with 5% CO^2 and 95% humidity for 24 hours. Observe the cells under a microscope.

Changing medium in aggrewell plate

- Warm complete medium.
- Perform a 50 - 75% medium change as follows: 24-well plate: Slowly remove 1 - 1.5 mL of medium from each well.
- Replace with 1 - 1.5 mL of the fresh complete medium by slowly pipetting down the wall of the well. Slowly dispensing the medium helps to prevent displacement of EBs/spheroids from the microwells.

Harvesting EBs from aggrewell plates

- Warm basal medium and complete medium.
- Using a serological pipette (Alternative option: This step can be achieved by using P1000 cut tips):
    - Remove approximately half of the culture medium from the well.
    - Dispense the medium firmly back onto the surface of the plate to dislodge the EBs/spheroids from the microwells. Do not triturate.
- Select the appropriate strainer and conical tube for separation of EBs/spheroids from single cells: 24-well plate, For harvesting from a single well, use 37 µm Reversible Strainer, Small and a 15 mL conical tube.
- Place strainer on top of the tube with the arrow pointed upward. Add 1ml of PBS to wet the surface of the strainer.
- Gently aspirate the dislodged EBs/spheroids. Pass the EB/spheroid suspension through the strainer. NOTE: The aggregates will remain on the filter; any unincorporated single cells will flow through.
- Using a serological pipette, dispense 1 mL (24-well plate) or 3 mL (6-well plate) of the warm basal medium across the entire surface of the well to dislodge any remaining EBs/spheroids. Collect wash and pass over the strainer. Repeat this wash step 3 times. wash can be repeated until you do not see more EBs under the microscope.
- Invert the strainer, and place over a new conical tube of the same size. Collect the EBs/spheroids by washing with 2 - 5 mL of complete medium per well harvested.

Differentiation protocol

1) Maintain the pluripotent hPSCs/IPSCs on Lam-521 (0.5 µg/cm^2) in iPS-Brew medium. Cells can be passaged every 7 days with 0.5 mM EDTA, followed by seeding at a density of 2,500 cells per cm^2 with ROCK inhibitor (10 µM Y-27632) included in the medium for the first 24 h after plating.
2) Day 1: $3x10^6$ iPSCs or ES cells were seeded into an Aggrewell 800 well (STEMCELLTechnologies) to form EBs with ROCK inhibitor (10 µM Y-27632), in iPS-Brew medium and fed daily medium with BMP4 (50 ng ml^{-1}), VEGF (50 ng ml^{-1}), SCF (20 ng ml^{-1}).
3) Day 2- 4: Change medium gently in the Aggrewell 800 well plate without agitating the EBs on the plate.

4) Day 4: Four-day EBs were then differentiated in either 6-well plates (15 EBs/well), T75 (75 EBs), or T175 flasks (150 EBs) with X-VIVO15 Supplemented with 100 ng/mL M-CSF, 25 ng/mL IL-3, 2 mM GlutaMAX, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.055 mM b-mercaptoethanol with fresh medium added weekly. (Cells can be maintained in this medium for up to 6months).
5) pMacpre (Stem cell-derived macrophage/microglia progenitors) emerging into the supernatant after approximately 1 month were collected weekly and differentiation cultures were replenished with fresh medium. Harvested cells were strained (40 mm, Corning) and used: either directly as pMacpre; or plated onto tissue-culture treated plastic or glass coverslips at 100,000 per cm^2 and differentiated for 7 days or more. Note: At this point, pMacpre can be harvested and frozen down for further use.
6) Cells in suspension were collected and either (a) Cultured in RPMI with 10% FBS, l-glutamine, and pen/strep with IL-34 (100 ng ml^{-1}) and M-CSF (10 ng ml^{-1}) for 7–11 d until cells were adherent and elongated to transition to primitive macrophages, or (b) Co-cultured with DA neurons in Neurobasal medium containing B-27 supplement (1:500), l-glutamine (1:1000) and BDNF (20 ng ml^{-1}), ascorbic acid (0.2 mM) GDNF (20 ng ml^{-1}), cAMP (500 µM), and IL-34 (100 ng ml^{-1}) and M-CSF (20 ng ml^{-1}) for 7-10 days for direct transition to microglia.
7) For serum-free differentiation, cells in suspension were harvested and cultured in 75% IMDM, 25% RPMI medium containing B-27 supplement (1:500), l-glutamine (1:1000) and IL-34 (100 ng ml^{-1}) and M-CSF (20 ng ml^{-1}) for 11–14 days.

Quality control for pMacpre and microglia

FAC-sorting for $CD11b^+$ and $CD45^+$ on both pMacpre and Microglia.

Antibodies	Supplier	Cat no
CD11b	Biolegend	301351
CD45	BD Pharmingen	560976

ICC for IBA-1, PU.1, TMEM119 on differentiated Microglia.

Antibodies	Supplier	Cat no
IBA-1	AbCam	ab5076
PU.1	Cell Signaling	2258S
CD11b	AbCam	ab8878
CD45	BioLegend	304002
TMEM119	Sigma	HPA051870

Reference

A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response.
Walther Haenseler, Stephen N. Sansom, Julian Buchrieser, Sarah E. Newey, Craig S. Moore, Francesca J. Nicholls, Satyan Chintawar, Christian Schnell, Jack P. Antel, Nicholas D. Allen, M. Zameel Cader, Richard Wade-Martins, William S. James, Sally A. Cowley.

Fully defined human pluripotent stem cell-derived microglia and tri-culture system model C3 production in Alzheimer's disease
Sudha R. Guttikonda, Lisa Sikkema, Jason Tchieu, Nathalie Saurat, Ryan M. Walsh, Oliver Harschnitz, Gabriele Ciceri, Marjolein Sneeboer, Linas Mazutis, Manu Setty, Paul Zumbo, Doron Betel, Lot D. de Witte, Dana Pe'er and Lorenz Studer.