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Generation of iPSC-derived Microglia

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We use this protocol and it's working

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Disclaimer

This is a working protocol based on the work of Washer and colleagues (Washer et al., 2022), and was used to validate differentiation conditions for obtaining iPSC-derived microglia.

Abstract

This is a working protocol based on the publication of Washer et al., 2022 (Nat Sci Reports). This protocol can be used to obtain fully mature microglia to investigate neuroinflammatory mechanisms in neurodegenerative diseases. One of the advantages of this protocol is that it assessed systematically medium components, coatings, and growth factors required for iPSC differentiation to microglia, optimizing several components that were redundant for microglial identity. They use defined medium which produces both transcriptionally and functionally relevant microglia for modeling these cells' physiology in neuroinflammation and for drug discovery.

Attachments



Washer protocol over...

112KB

Image Attribution

Images proprietary of authors; schematics done with Biorender

Guidelines

For this protocol internal validation, we used H9 iPS cell lines, especially H9 WT.



Materials

1. Base Medium

	A	B	C
	MEDIUM	SUPPLIER	CAT. NO
	Stemflex	Thermo Fisher	A3349401
	X VIVO 15	Lonza	BE02-060F
	Advanced RPMI	Thermo Fisher	12633020

1. Solutions

	A	B	C
	REAGENT	SUPPLIER	CAT. NO
	Anti-adherence rising solution	STEMCELL Technologies	7010
	PBS	Thermo Fisher	10010049
	GCDR	STEMCELL Technologies	100-0485
	Accutase	Thermo Fisher	A1110501
	GlutaMAX ₁₀₀₀	Life Technologies	35050-061

1. Substrate



	A	B	C
	REAGENT	SUPPLIER	CAT. NO
	Fibronectin	Thermo Fisher	CB-40008A

1. Small molecules

	A	B	C	D
	SMALL MOLECULE	SUPPLIER	CAT. NO	QUANTITY (ug)
	BMP-4	R&D	314-BP-500	500
	VEGF	R&D	293-VE-500	500
	SCF	R&D	255-SC-01M	1000
	M-CSF	R&D	216-MC-100	100
	IL-3	R&D	203-IL-500	500
	IL-34	R&D	5265-IL-010 (ORDER BULK)	
	GM-CSF	R&D	215-GM-500	500
	TGF-B1	R&D	216-MC-100	100

Quantity as a suggestion, please evaluate how much will suit your experiments.

1. Culture Vessels



	A	B	C	D
	PLATE	# OF WELLS	SUPPLIER	CAT. NO
	AggreWell 400	24	STEMCELL Technologies	34415
	AggreWell 800	24	STEMCELL Technologies	34815
	6-well plates	6	Fisher Scientific	3516
	6-well plates	6	Fisher Scientific	140675

AggreWell400 and AggreWell800 both work, with the AggreWell800 helping avoid EBs to fuse.

1. Others

	A	B	C
	REAGENT	SUPPLIER	CAT. NO
	Y-27632	Tocris	1254

PHASE 1 - Generation of Embryoid bodies (EBs)

- 1 We maintain iPSCs in Laminin 521 (1:20 in PBS +/+) or in Cultrex (1:100 in DMEM/F12) and feed them with StemFlex supplemented every other day until they reach 70-80% confluency. After thawing, it usually takes 4 days.
- 2 Day 0: Prepare the Aggrewell plate with 0.5 mL of anti-adherence rising solution. Spin at 1300 – 3000 x g for 5-3 min to remove bubbles from the microwells. Remove the anti-adherence rising solution, and wash the wells with PBS/water 3 times before the addition of 1 mL of 2x concentrated EB medium supplemented with Rhok inhibitor (Y-27632).

2.1 **EB Media:** Add small molecules fresh.

	A	B	C	D
	Component	Stock concentration	Final concentration	Final Volume for 10 mL of EB Media (uL)
	Stem Flex supplemented	N/A	N/A	10 mL
	BMP-4	100 µg/mL	50 ng/mL	5
	VEGF	100 µg/mL	50 ng/mL	5
	SCF	100 µg/mL	20 ng/mL	2
	Y-27632 *	10 mM	20 µM	20

* Only on day 0. No need to add on the feeds at d1-6.

- 3 Dissociate iPSCs (70-80% confluency) by washing with PBS 1x and then incubate with GCDR for 30 min at 37 °C, 5% CO₂. Transfer cells to a tube containing DMEM/F12 and pellet by centrifugation at 300 x g for 5 min.
- 4 Aspirate supernatant and resuspend cells in StemFlex for counting. Cell viability needs to be >75% to ensure good iPSC quality and EB formation.



- 5 Prepare cell seeding stock: Seed 4×10^6 cells/mL (AggreWell 800) or 2×10^6 cells (AggreWell 400) per well; add 1 mL of cells to one well of the AggreWell 800 containing 1 mL of 2x concentrated EB media.
- 6 After seeding, spin plate at $100 \times g$ for 3 min to capture cells in the microwells.
- 7 Leave the EBs on the plate for 4-6 days, doing daily feeds of 50-75% media change with 1x EB media. Be careful to not dislocate the EBs and cause them to fuse. Aspirate from the meniscus to preserve the EBs on the bottom. Handle the plate gently.
- 7.1 [CHECKPOINT - OPTIONAL] On day 7, you can run a FACS as a checkpoint for hemangioblasts (KDR+/CD235a/b+) (Guttikonda et al., 2021).

A
Separate 100 uL of EB suspension. Spin down at $300 \times g$ for 5 min, aspirate media and start dissociation with Accutase for 30 min. It may need mechanical dissociation as well to break the EBs. Verify if dissociation was successful before next step. ALWAYS have a non-stained control for cell autofluorescence.
Add FACS Buffer to tubes containing dissociated EBs and Accutase. BSA fraction V may help stop Accutase reaction. Spin down at $300 \times g$ for 5 min and aspirate media.
Add FACS Buffer containing the KDR-PE (1:20) and CD235a (1:20). Incubate on ice or 4°C for 30 min.
Prepare Sytox solution (1 to 1000 in FACS buffer). Make enough solution so all the samples can be resuspended in 300 uL
Spin down at $300 \times g$ for 5 min and add 300 uL of FACS Buffer.
Read on Attune. At least 10-15% of double-positive is great to proceed with the differentiation. 20% and above is exceptional and will have a high yield.

PHASE 2 - Microglia precursor generation (monolayer)

- 8 Day 5: Thaw enough aliquots of fibronectin in 4 °C overnight.



- 9 Day 6: Coat the culture vessels you will need with Fibronectin 10 $\mu\text{g}/\text{mL}$ in PBS +/- (containing calcium and magnesium). Let it coat in 4 °C overnight, or you can do it fresh on d7 (coat for 3 h at room temperature).
- 10 Day 7: Pipette up and down to collect all the EBs from the AggreWell. Collect them in a 15ml/50mL conical.
- 11 Spin at 120 - 300 x g for 5 min or just let the EBs sediment in the tube.
- 12 Prepare "FACTORY" / Precursor Medium (see below)

12.1 **FACTORY / Precursor Medium:** Add small molecules fresh.

A	B	C	D
Component	Stock concentration	Final concentration	Final Volume for 10 mL of EB Media (uL)
X VIVO 15	N/A	N/A	10 mL
GlutaMAX	100X	1X	100
M-CSF	100 $\mu\text{g}/\text{mL}$	100 ng/mL	10
IL-3	100 $\mu\text{g}/\text{mL}$	25 ng/mL	2.5

- 13 Aspirate the old supernatant and resuspend the EBs in Precursor Medium.
- 14 Seed approximately 100-200 uL of EB suspension in one well of a 6-well plate coated with fibronectin (for pooling 5 wells from a AggreWell400; reference value), or alternatively use a T-75 flask.
- 15 Distribute the EBs fairly in the well and leave the plate at 37 °C. Check the attachment on the next day.



OBS: It can take 48 - 72 h before they recover and form a reasonable monolayer - leave the plate alone for this period, then change half the medium if necessary. Otherwise, half media changes can be done once a week using enough volume (minimum of 3 mL per well in a 6-well plate).

PHASE 3 - Maturation to microglia

16 Day 14-35: When changing the Precursor Media, collect precursors (floating cells), and filter through a 40 μ m strainer. Spin down at 300 x *g* for 5 min.

17 Resuspend in Maturation media 1. COUNT CELLS.

Maturation Media 1:

Component	Stock concentration	Final concentration	Final Volume for 10 mL of EB Media (μ L)
Advanced RPMI	N/A	N/A	10 mL
GlutaMAX	100X	1X	100
IL-34	100 μ g/mL	100 ng/mL	10
M-CSF	100 μ g/mL	25 ng/mL	2.5
GM-CSF	100 μ g/mL	10 ng/mL	1
TGF-Beta1	50 μ g/mL	50 ng/mL	10

17.1 [OPTIONAL] Take an aliquot to run Attune on CD43/CD45. Cells are expected to be >90% double-positive. Alternatively, use CD11c-PE or CD11c-647 as a dendritic cell marker to observe the precursors under the microscope.

18 FOR MATURATION: Plate in a ULA or TC-treated / fibronectin-coated vessel at 50,000 cells/cm² with enough Maturation Media volume.



OBS: If your plating density is higher, you will have to feed the cells more often, as the IL-34 will be limited, and you will start seeing cell death. Plating at 50,000 cells/cm² allows you to feed twice a week with enough media volume. Half media changes are encouraged.

- 19 Mature for 12-14 days with Maturation medium 1.
After, maintain cells in Maturation medium 2 until experiments.

Maintenance: Change media every 3 days, being consistent in the time of the feeds (e.g., around 2pm every time).

19.1 Maturation

Medium 2 (Maintenance medium):

Component	Stock concentration	Final concentration	Final Volume for 10 mL of EB Media (uL)
Advanced RPMI	N/A	N/A	10 mL
GlutaMAX	100X	1X	100
IL-34	100 µg/mL	100 ng/mL	10
M-CSF	100 µg/mL	25 ng/mL	2.5

- 20 FOR CRYOPRESERVATION - Resuspend the counted cells in 500 µL of Maturation medium (base media) per 0.5M - 2.0 M cells. Add 500 µL of 2xFM per cryovial. The next day, remove cryovials from -80 and store them in a liquid nitrogen tank.



Protocol references

Washer SJ, Perez-Alcantara M, Chen Y, Steer J, James WS, Trynka G, Bassett AR, Cowley SA. Single-cell transcriptomics defines an improved, validated monoculture protocol for differentiation of human iPSC to microglia. *Sci Rep*. 2022 Nov 14;12(1):19454. doi: 10.1038/s41598-022-23477-2. PMID: 36376339; PMCID: PMC9663826.

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