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Human ESC/iPSC Microglia Directed Differentiation

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Hematopoietic Matrigel preparation

1. Thaw Matrigel (Corning, Cat #354234, 10 mL) by submerging the vial in ice in a 4°C refrigerator, overnight (ON).
2. Next day, get a new ice bucket.
3. Add 30 mL cold IMDM (Thermo, Cat #12440053) to a 50mL conical tube on ice.
4. Prepare and label 15mL conical tubes (i.e., Hematopoietic Matrigel, date, name). Then, place them on ice.
5. Take thawed Matrigel from the fridge and place it on ice.
6. Swirl Matrigel bottle to mix well.
7. Add 10 mL Matrigel to the 30 mL IMDM in the 50 mL conical tube.
8. Wash off bottom of glass containing concentrated Matrigel using the diluted form from the 50mL conical tube.
9. Make 5 mL aliquots.
10. Store at -20°C until use.

Once aliquot is thawed, keep it at 4°C, it can be stored for a long time.

Hematopoietic Matrigel plate coating process

1. Thaw the Hematopoietic Matrigel aliquot ON in the fridge (4°C).
2. Place the 6-well plates in the freezer (-20°C) at least 1h before using. This step is crucial to ensure coating success.
3. After that time, place the plates on ice to prepare the coating.
4. Add half the volume of Matrigel to the plate filling the first line of wells. Cover well by moving the plate over the ice. Transfer that volume to the next line of wells. When all wells are covered, recover the remaining Matrigel and save it in the fridge. This Matrigel can be used for the next experiment.
5. Place the coated plates in the fridge after sealing with parafilm for 20 min (no ice is necessary).
6. Transfer the plates to the incubator (37°C) and keep in it for another 20 min.
7. The plate is ready to use.

Hematopoietic Differentiation (Heinze *et al.*, Stem Cell Reports, 2022)

Day -2

1. Coat a 6-well plate with Hematopoietic Matrigel prior to plating cells.
2. Use hiPSC at 80% of confluence growing in a 6 well-plate.
3. Aspirate the medium, wash with PBS, add 1 mL ReLeSR per well and take it out within 1min.
4. Incubate the cells 5 min at 37°C in the incubator.
5. Add 1 mL mTeSR per well and passage the cells at low confluence (~30–50 × 10^3 cell aggregates/well) into a Matrigel coated 6-well plate. The cell aggregates should be ≥ 50 µm in diameter.

Day -1

6. Check the above confluence indicated and feed the cells with mTeSR. If the confluence is too high or low, discard the plate and start again because the differentiation will be compromised.

Day 0

7. Start the differentiation. Remove the mTeSR completely, add the differentiation media for *Day 0-1*, and transfer the plate to the **hypoxia incubator** (5% O2).

Day 2

8. Remove the medium and add the differentiation media for *Day 2-3*. Place the plate in the **hypoxia incubator**.

Day 4

9. Repeat procedure of step 8 but using the differentiation media for *Day 4-5*.

Day 6

10. Floating cells can often be seen in culture starting day 6, and they will increase in number for the remainder of the protocol. To feed the cells, collect, and transfer them into a conical tube, centrifuge at 300 *x g* for 5 min, aspirate the supernatant and resuspend the pellet in 2mL of differentiation media for *Day 6-11*. Plate the cells in the same 6-well plate and place it in the **hypoxia incubator**.

Day 8

11. Repeat step 10 but transfer the plate in the **normoxia incubator**.

Day 10

12. Repeat step 11.

Day 12

13. Harvest hematopoietic progenitors. Collect and transfer them into a conical tube, perform two washes with DMEM/F-12 (don´t remove the adherent cells), and centrifuge at 300 *x g* for 5 min. Aspirate the supernatant and resuspend each pellet by pooling them together in 1 mL of DMEM/F-12. Count the cells using Trypan Blue and a hemocytometer and separate them to induce the microglia differentiation and perform their characterization.
NOTE: from a full 6-well plate is possible to collect 2 – 5 x 10^6 progenitors cells depending on the line.

Characterization by flow cytometry:
Criteria: > 90% CD43+, > 20% CD34+/CD45+
CD34-Bv421 (1 µl)
CD45-APC (3 µl)
CD43-PE (3 µl)

Differentiation Medium (volumes for a 6-well plate)

	Reagents	Stock Conc.		Final Conc.		Volume	
D0-1	StemPro34					11.42	mL
	StemPro34 suppl					312	uL
	L-glut	100	x	1	x	120	uL
	Primocin	500	x	1	x	24	uL
	aMTG	11.5	M	0.45	mM	0.47	uL
	Transferrin	30	mg/mL	200	ug/mL	80.00	uL
	AA	50	mg/mL	88.06	ug/mL	21.13	uL
	BMP4	10	ug/mL	5	ng/mL	6.00	uL
	VEGF	100	ug/mL	50	ng/mL	6.00	uL
	CHIR99021	3	mM	2	uM	8.00	uL

output volume:	12 mL

D2-3	Reagents	Stock Conc.	Final Conc.	Volume

	StemPro34					11.43	mL
	StemPro34 suppl					312	uL
	L-glut	100	x	1	x	120	uL
	Primocin	500	x	1	x	24	uL
	aMTG	11.5	M	0.45	mM	0.47	uL
	Transferrin	30	mg/mL	200	ug/mL	80.00	uL
	AA	50	mg/mL	88.06	ug/mL	21.13	uL
	BMP4	10	ug/mL	5	ng/mL	6.00	uL
	VEGF	100	ug/mL	50	ng/mL	6.00	uL
	bFGF	100	ug/mL	20	ng/mL	2.40	uL

output volume:	12 mL

	Reagents	Stock Conc.		Final Conc.		Volume	
D4-5	StemPro34					11.44	mL
	StemPro34 suppl					312	uL
	L-glut	100	x	1	x	120	uL
	Primocin	500	x	1	x	24	uL
	aMTG	11.5	M	0.45	mM	0.47	uL
	Transferrin	30	mg/mL	200	ug/mL	80.00	uL
	AA	50	mg/mL	88.06	ug/mL	21.13	uL
	VEGF	100	ug/mL	15	ng/mL	1.80	uL
	bFGF	100	ug/mL	5	ng/mL	0.60	uL

output volume:	12 mL

	Reagents	Stock Conc.		Final Conc.		Volume	
D6-11	StemPro34					38.05	mL
	StemPro34 suppl					1040	uL
	L-glut	100	x	1	x	400	uL
	Primocin	500	x	1	x	80	uL
	aMTG	11.5	M	0.45	mM	1.57	uL
	Transferrin	30	mg/mL	150	ug/mL	200.00	uL
	AA	50	mg/mL	50	ug/mL	40.00	uL
	BMP4	10	ug/mL	10	ng/mL	40.00	uL
	VEGF	100	ug/mL	5	ng/mL	2.00	uL
	bFGF	100	ug/mL	5	ng/mL	2.00	uL
	SCF	100	ug/mL	100	ng/mL	40.00	uL
	FLT3L	100	ug/mL	10	ng/mL	4.00	uL
	TPO	100	ug/mL	30	ng/mL	12.00	uL
	IL3	100	ug/mL	30	ng/mL	12.00	uL
	IL6	100	ug/mL	10	ng/mL	4.00	uL
	IL11	20	ug/mL	5	ng/mL	10.00	uL
	IFG1	50	ug/mL	25	ng/mL	20.00	uL

	SHH	20	ug/mL	20	ng/mL	40.00 uL

output volume:	40 mL

Microglia Differentiation

STEMdiff™ Microglia Differentiation and Microglia Maturation Kits (Cat #100-0019, #100-0020)

Day 12

1. Centrifuge the progenitor cells again at 300 *x g* for 5min. Resuspend the pellet in Microglia Differentiation Medium to obtain a final concentration of 2 x 10^5 cells/mL.
2. Add 1 mL to each well of a Matrigel-coated 6-well plate (same Matrigel for culturing hiPSC, Cat #354277), containing 1 mL Microglia Differentiation Medium. Incubate at 37°C and 5% CO2.

Day 14-22

3. Feed the cells every other day by topping up the well with half of the start volume of Microglia Differentiation Medium (1 mL). Do not remove existing medium.

Day 24

4. Transfer the entire cell suspension to a 15 mL conical tube (a conical tube for each well). If some cells remained in the plate perform a wash with DMEM/F-12. Centrifuge at 300 *x g* for 5 minutes.
5. Remove supernatant until there is ~1 mL remaining on top of the cell pellet. Using a pipettor, gently mix to resuspend.
6. Add the cell suspension to one well of a new Matrigel-coated 6-well plate containing 1 mL fresh Microglia Differentiation Medium. Place the plate in the incubator at 37°C and 5% CO2.

Day 26-34

7. Feed the cells every other day by topping up the well with half of the start volume of Microglia Differentiation Medium (1 mL). Do not remove existing medium.

Day 36

8. Transfer the entire cell suspension to a 15 mL conical tube (a conical tube for each well). If some cells remained in the plate perform a wash with DMEM/F-12. Centrifuge at 300 *x g* for 5 minutes.
9. Remove supernatant until there is ~1 mL remaining on top of the cell pellet. Using a pipettor, gently mix to resuspend.
10. Count cells using Trypan Blue and a hemocytometer. Add 1 x 10^6 cells to one well of a new coated 6-well plate containing 1 mL fresh Microglia Maturation Medium. If the total volume in the well is < 2 mL, top up to 2 mL with Maturation Medium. Incubate at 37°C and 5% CO2.
    NOTE: 1 x 10^5 cells/cm2 is the optimal density for replating. Continuing the protocol with < 5 x 10^5 cells (5.5 x 10^4 cells/cm2) may result in a decrease in the % CD11b-positive cells at the end of maturation.

Day 38 and 40

11. Feed the cells every other day by topping up the well with half of the start volume of Microglia Maturation Medium (1 mL). Do not remove existing medium.

Day 42

12. Harvest Microglia Cells following instructions in step 8.
    NOTE: starting the microglia differentiation with a full 6-well plate is possible to collect 8 – 10 x 10^6 cells depending on the line.

    Characterization by flow cytometry:
    Criteria: >80% CD45+/CD11b+, >50% TREM2+
    CD45-PE (3 µl)
    CD11b-Bv421 (1 µl)
    TREM2-APC (2 µl)