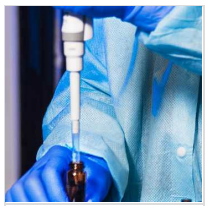

Version 3

Jun 27, 2022

Differentiation of iPSC into Microglia-Like Cells (iMGL) V.3

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Neurodegeneration Method Development Community
Tech. support email: **ndcn-help@chanzuckerberg.com**

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ABSTRACT

This protocol outlines the derivation of Hematopoietic Progenitor Cells and differentiation of iMGLs using iPSC cultures. This protocol is modified the following papers.

McQuade A, Coburn M, Tu CH, Hasselmann J, Davtyan H, Blurton-Jones M (2018). Development and validation of a simplified method to generate human microglia from pluripotent stem cells.. Molecular neurodegeneration.
https://doi.org/10.1186/s13024-018-0297-x

Abud EM, Ramirez RN, Martinez ES, Healy LM, Nguyen CHH, Newman SA, Yeromin AV, Scarfone VM, Marsh SE, Fimbres C, Caraway CA, Fote GM, Madany AM, Agrawal A, Kayed R, Gylys KH, Cahalan MD, Cummings BJ, Antel JP, Mortazavi A, Carson MJ, Poon WW, Blurton-Jones M (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases.. Neuron.
https://doi.org/10.1016/j.neuron.2017.03.042

ATTACHMENTS

Differentiation_of_iPSC_into_Microglia_Protocol_JAM_FINAL_03302020[2].docx

DOI

dx.doi.org/10.17504/protocols.io.q26g7bwqklwz/v3

PROTOCOL CITATION

Abhirami Kannan Iyer, Emma Danhash, Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M M. Karch 2022. Differentiation of iPSC into Microglia-Like Cells (iMGL). **protocols.io** https://dx.doi.org/10.17504/protocols.io.q26g7bwqklwz/v3 Version created by Jacob Marsh

WHAT'S NEW

This protocol includes more specific recommendations for cell plating density and the iMGL media formulations.

KEYWORDS

microglia, differentiation, hematopoietic progenitor cells

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OWNERSHIP HISTORY

Jun 27, 2022 Celeste M. Karch Washington University in St Louis

PROTOCOL INTEGER ID

58022

MATERIALS TEXT

Materials:

- 6-well tissue culture plate(s)

- 96-well tissue culture plate(s)
- 15 ml conical tubes
- Matrigel
- PBS
- Dispase
- Accutase
- DMEM/F12
- StemProEZPassage Disposable Stem Cell Passaging Tool
- mTesR1
- Rock Inhibitor

Medium Recipes:

iMGL Diff Base Medium (per 100 ml)

		Vendor	Cat#	vol	
phenol-free DMEM/F12 (1:1)		Thermo Fisher	11039021	92.5	mL
insulin (0.02 mg/ml)	ITS-G (100X stock)	Thermo Fisher	41400045	1	mL
holo-transferrin (0.011 mg/ml)					
sodium selenite (13.4 ug/ml)					
B27 (2% v/v) (50X stock)		Thermo Fisher	17504044	4	mL
N2 (0.5%, v/v) (100X stock)		Thermo Fisher	17502048	0.5	mL
monothioglycerol (200 uM)	11.5 M	Sigma Aldrich	M1753-100mL	1.75	uL
Glutamax (1X) (100X stock)	100X	Thermo Fisher	35050061	1	mL
non-essential amino acids (NEAA; 1X) (100X stock)	100X	Thermo Fisher	11140050	1	mL
Pen/ Strep	100X	Thermo Fisher	15140-122	1	mL
(additional insulin (5 ug/mL)) we do not add it		Sigma Aldrich	I9278-5mL	47	uL

iMGL Diff Complete Medium

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		Vendor	Cat#	dilution	
iMGL diff base medium					
IL-34 (100 ng/mL)	500 ug/mL in H2O	Peprotech	200-34	1:5000	
TGFb-1 (50 ng/mL)	100 ug/mL in 10mM Citric Acid	Peprotech	100-21	1:2000	
M-CSF (25 ng/mL)	100 ug/mL in H2O	Peprotech	300-25	1:4000	

iMGL Maturation Medium

		Vendor	Cat#	dilution	
iMGL Complete medium					
CD200 (100 ng/mL)	100 ug/mL	Novoprotein	C311-50ug	1:1000	
CX3CL1 (100 ng/mL)	100 ug/mL	Peprotech	300-31	1:1000	

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

Derivation of Hematopoietic Progenitor Cells and Differentiation of iMGLs – Timeline

1. iPSCs Culture (2-3 Days)
2. iPSCs Aggregates Plating (1 Day) *Critical: Go/No-Go Decision*
3. iPSCs Induction into Hematopoietic Stem Cells (12 Days) *Critical: Go/No-Go Decision*
4. FACs Sorting $CD43^{+}CD34^{+}$ $CD45^{+}$ Cells (1 Day)
5. Freezing Down Sorted Hematopoietic Stem Cells (1 Day)
6. Thawing Hematopoietic Stem Cells (1 Day)
7. Differentiation of Hematopoietic Stem Cells into Induced Microglia (28 Days)

iPSCs Culture

1 Thaw and culture iPSC line per the following protocol:

iPSC Cell Culture – Maintenance and Expansion
by Celeste M. Karch,
Washington University in St Louis
PREVIEW RUN

1.1 To resuspend, thaw aliquot **On ice** .

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1.2 

Add **12.5 mL** cold DMEM/F12.

1.3 

Pipette up and down twice.

1.4 

Add **1 mL** of Matrigel per well of 6 well plate.

1.5 Store diluted Matrigel at **4 °C** .

1.6 Prior to thawing cells, coat plate with Matrigel for **01:00:00** .

> 1 vial of iPSC should be thawed into 1 well of a 6 well plate.

1.7 

Add **9 mL** DMEM/F12 to a 15 ml conical tube labeled with the iPSC line name and passage number.

1.8 Remove cells from liquid nitrogen storage.

1.9 Quickly thaw cells in **37 °C** water bath and/or in hands.

1.10 Just prior to complete thaw, remove vial from water bath.

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1.11 

Transfer the contents of the cryo-vial (~ **1 mL**) into the 15 ml conical tube.

1.12 

Spin at **750 rpm** for **00:03:00** at **Room temperature** .

1.13 Aspirate media.

1.14 

Resuspend cells in **2 mL** mTesR1 (supplemented with **5 micromolar (µM)** – **10 micromolar (µM)** Rock Inhibitor) by **pipetting two times**.

1.15 

Transfer the cell solution to one well of a 6-well plate.

1.16 

Incubate at **37 °C** **Overnight** in **6 % CO_2**.

1.17 **Replace** the **media daily** until cells are ready to split or analyze.

1.18

> Media should be changed daily. It is okay to skip a media change one time each week if double feeding is performed; however, this is largely dependent on the density of the cells and volume of media (do **not double feed** if cells are **more than 70% confluent**).

Aspirate media.

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1.19

Gently add fresh mTesR1 to cells (volume depends on cell density and well size).

- **0.5 mL** per well to 24 well plate
- **2 mL** — **4 mL** per well to 6 well plate
- **5 mL** — **10 mL** to 10 cm^2 plate

1.20

Incubate at **37 °C** in **6 % CO_2.**

1.21

When differentiating cells appear in the culture, it is important to remove all the cells promptly.
Repeated cleaning may be necessary over the course of several days to remove all the material. If differentiation is excessive and line is precious, perform subcloning.

Under microscope, remove differentiated cells with p20 or p200 tip (depending on the amount of differentiation). Transfer the cells/media to a biohazard bag.

1.22

Gently wash cells with 1x PBS.

1.23

Add fresh mTesR1.

- **0.5 mL** per well to 24 well plate
- **2 mL** — **4 mL** per well to 6 well plate
- **5 mL** — **10 mL** to 10 cm^2 plate

1.24

Incubate at **37 °C** in **6 % CO_2** until cells are **60 — 80 % confluent**. Change mTesR1 media **daily** until
cells are needed. Repeat cleaning as necessary.

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1.25

iPSCs grow on Matrigel. Plates should be coated with Matrigel at least 1 hour prior to plating and no
longer than 24 hours prior to plating cells:

- **0.5 mL** in 12 well plate
- **1 mL** in 6 well plate
- **4 mL** in 10 cm^2 plate

> It is critical to keep Matrigel on ice while coating. Prior to plating cells, ensure Matrigel has not
> evaporated from well.

1.26 Aspirate media.

1.27

Gently wash cells with 1x PBS (**2 — 3 ml/well**).

1.28

Add Accutase (Gibco A11105-01) directly to the cells and incubate at **37 °C** for
00:03:00 — **00:04:00** .

- 6 well plate, add **0.75 mL** — **1 mL** per well
- 24 well plate, add **0.5 mL**
- 10 cm^2 dish, add **3 mL**

1.29 Tap dish to aid in dislocation of cells.

1.30

Add DMEM/F12 directly to cells and scrape gently to remove all cells (use p1000 for 24 well plate, and cell scraper for 6 well plate and 10cm^2 dish).

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- 6 well plate, add **2 mL** — **4 mL** per well
- 24 well plate, add **1 mL**
- 10 cm^2 dish, add **9 mL**

1.31 Collect cells in conical tube (15 ml/50 ml depending on volume).

1.32 

If necessary, add **2 mL** — **5 mL** DMEM/F12 to dish to remove all cells from the dish and add to conical tube.

1.33 

Centrifuge cells at **750 rpm** for **00:03:00** at **Room temperature** .

1.34 

Carefully aspirate supernatant.

> To avoid aspirating cell pellet, it is OK to leave a small amount of media (**0.5 mL** — **1 mL**).

1.35 

Resuspend cell pellet with mTesR1 (Rock Inhibitor addition varies, see below).

- **2 mL** mTesR1 per well of a 6 well plate
- Our goal is to maintain iPSC lines without using Rock Inhibitor; however, this must be done through careful weaning off Rock Inhibitor
- All cells should be thawed in Rock Inhibitor:

- [M]**10 micromolar (µM)** concentration for new iPSC lines, lines thawed from 96 well after editing.

- [M]**5 micromolar (µM)** concentration if thawing from a line without knowledge of its Rock sensitivity.

- [M]**1 micromolar (µM)** concentration for all other lines (for lines still exposed to Rock Inhibitor, use

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[M] **1 micromolar (µM)** . Otherwise, do not use Rock Inhibitor.)

1.36

Pipet cells **2 times** only to preserve clumps.

1.37

Transfer cell suspension to appropriate plate (pre-coated with Matrigel for at least **01:00:00**).

- For maintenance, dilute cells 1:3 in mTesR1
- For expansion, plate all cells

1.38

Incubate at **37 °C** in **6 % CO_2** until cells are **60 — 80% confluent**. Change mTesR1 media **daily** until cells are needed.

1.39 Aspirate media.

1.40

Gently wash cells with 1x PBS (Use **2 mL** — **3 mL** per well in 6 well plate).

1.41

Add Accutase (Gibbco A11105-01) directly to the cells and incubate at **37 °C** for **00:03:00** — **00:04:00** .

- 6 well plate, add **0.75 mL** — **1 mL** per well
- 10cm^2 dish, add **3 mL**

1.42 Tap dish to aid in dislocation of cells.

1.43

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Add DMEM/F12 directly to cells.

- 6 well plate, add **2 mL** — **4 mL** per well
- 10cm^2 dish, add **9 mL**
- If cells remain attached, use a cell scraper to gently dislodge cells (apply gentle pressure and use 1 — 2 passes to remove cells)

1.44 Collect cells in conical tube (15 ml/50 ml depending on volume).

1.45 

Add **2 mL** — **5 mL** DMEM/F12 to dish to remove all cells from the dish and add to conical tube.

1.46 

Centrifuge cells at **750 rpm** for **00:03:00** at **Room temperature** .

1.47 

Carefully aspirate supernatant.

> To avoid aspirating cell pellet, it is OK to leave a small amount of media (**0.5 mL** — **1 mL**).

1.48 

Resuspend cell pellet with mTesR1 (No Rock Inhibitor).

- Use volume appropriate for freezing
- Assume **1 mL** per cryovial total and add ½ total volume of mTesR1
- Pipet cells 1 — 2 times only to preserve cell clumps

> **Example**: to freeze 10 tubes, you will need **10 mL** total and will add **5 mL** mTesR1 to cell pellet (and **5 mL** of 2x Freezing Media below)

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1.49 

Add an equal volume of cold 2x Freezing Media (20 % DMSO, FBS). Pipet cells 1 time only to preserve cell clumps.

1.50 

Transfer cell suspension to pre-labeled cryovials (**1 mL** per cryovial).

Ensure that cryovials are labeled with the following:

- Cell Type
- Line Name
- Passage #
- Date
- Your Name

1.51 Freeze vials at **-80 °C** in foam racks for **48:00:00** — **72:00:00** .

1.52 Transfer vials to **liquid nitrogen** for long-term storage.

iPSCs Aggregate Plating

2 Once iPSCs are 70-80% confluent in 2-3 wells of a 6-well tissue culture plate, passage and plate the iPSCs as aggregates

> Aggregates should be approximately 100-200µm in diameter

3 Coat a 6-well tissue culture plate with **Matrigel** for a least **01:00:00** prior to passaging cells

4 Prepare desired volume of mTesR1 and 5-10µM ROCK Inhibitor (ROCKi = 1:2000 or 1:1000). After 1 hour of Matrigel coating, aspirate and replace with 2mL per well mTesR1 + Desired

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Concentration of ROCK Inhibitor. Pre-warm plates with media at 37°C and 6% CO_2 until aggregates are ready to be plated.

5 Set the following media out to warm to **Room temperature** :

- ReLeSR
- DMEM/F12
- PBS
- mTesR1

6 After plate has been coated for **01:00:00** and media has warmed to **Room temperature** , proceed to passage aggregates as described below:

6.1 Aspirate media from well.

6.2 Wash cells with **2 mL of PBS** per well

6.3 Aspirate **PBS** from well.

6.4 Add ReLeSR to cells **1 mL per well** .

6.5 2m 30s

Incubate at **Room temperature** for between **00:01:00** and **00:01:30** .

6.6 Aspirate the ReLeSR from the wells using a p1000 micropipette and allow cells 12m to continue sitting without any reagent in the wells for **00:05:00** to

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00:07:00 .

At approximately 4.5 minutes, iPSC appear to be lifted from the base of the wells

6.7

Add 1mL per well of mTesR1 + 5-10µM by gently allowing to trickle down the wall of the well. Tap the plate to release lifted cells to form aggregates of various sizes. Following this, use a p1000 to transfer 1mL of aggregates from each well to a 15mL conical tube without pipetting up and down

The amount of iPSC aggregates released after ReLeSR treatment from one well of a 6-well plate is sufficient to perform aggregate plating in 1, 6-well plate or more depending on aggregate count. You may also notice some unattached aggregates after tapping, you can repeat this process and harvest another round, if required.

7 Perform triplicate aggregate counts to determine the average number of cell aggregates.

7.1 Pipette **100 µL of mTesR1** into three individual wells of a 96-well flat bottom tissue culture plate.

7.2 Pipette **5 µL of aggregate suspension** to each well.

7.3 Manually count the number of aggregates in each well.

A uniform suspension of aggregates (50-200µm size) is optimal. Do not count aggregates smaller than 100µm

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7.4 Calculate the average number of aggregates per well.

Add the number of aggregates per well and then divide by 3 to find the average number of aggregates per well

7.5 Next calculate the *Concentration of Aggregates* or *Aggregates/uL.*

Take the average number of aggregates per well and divide by 5 (the dilution factor) to obtain the number of aggregates per microliter.

8 Determine the number of aggregates to plate in a 12-well or 6-well tissue culture plate.

For a 12 well tissue culture plate it is recommended to plate 40 - 60 aggregates/well (10 aggregates/cm^2) to achieve 16 colonies/well (4 - 10 colonies/cm^2) adhered to the culture ware after 24 hours of incubation; however, multiple plating densities may need to be tested.

For a 6 well tissue culture plate it is recommended to plate 40-60 aggregates/well to 6-well plates with mTESR1 + ROCKi media as prepared in Step 4. Multiple plating densities may need to be tested for each donor-derived iPSC line Prior to plating tap the conical tube once or twice to dislodge the pelleted aggregates (earlier wells may have bigger aggregates than later ones).

9 Place the plate in a 37°C, 6% CO_2 incubator. Move the plate in several quick, short, back-and-forth, and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

Usually the aggregates attach in about 6 hours, we have also performed aggregate plating early in the morning and after confirmation of attached aggregates, switched to

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Medium A on the same day in the evening

iPSCs Induction into Hematopoietic Stem Cells

10 ⚠

After 24 hours, confirm that 20- 38 colonies/well (6-well plate) or 16 - 40 colonies/well (p12 well-plate) are adhered to the plate. Ensure to count all colonies, including tiny colonies with only a few cells.

To facilitate counting, aspirate medium, wash with PBS and replace with fresh mTeSR™1.

CRITICAL: Do not proceed if cultures have < 16 colonies or > 40 colonies per well, as differentiation will be compromised

11 Prepare **Medium A** per the following recipe:

1. Add Supplement A to Hematopoietic Basal Medium at a concentration of 1:200

Medium A can be prepped and stored for a maximum of three days

12 Prepare **Medium B** per the following recipe:

1. Add Supplement B to Hematopoietic Basal Medium at a concentration of 1:200

Medium B can be prepped and stored for a maximum of three days

Medium A and Medium B can be prepared by adding Supplement A or B into STEMdiff Hematopoietic Basal Medium and stored frozen as 50mL aliquots in -20C until use

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13 Change media on the cell aggregates using the following schedule for a 6 well tissue culture plate.

13.1 **Day 0** - Aspirate medium from wells and add **2 mL of Medium A** per well and incubate at 37°C, 6% CO_2.

Day 0 starts 24 hours after aggregate plating

13.2 **Day 2** - Gently add **1 mL of Medium A** to each well and incubate at 37°C, 6% CO_2

13.3 **Day 3** - Aspirate **Medium A** from wells and gently add **2 mL of Medium B** per well.

13.4 **Day 5** - Gently add **1 mL of Medium B** to each well and incubate at 37°C, 6% CO_2

13.5 **Day 7** - Gently add **1 mL of Medium B** to each well and incubate at 37°C, 6% CO_2

At this point, floating cells can often be seen in culture and they will continue to increase in number for the remainder of the protocol.

13.6 **Day 10** - Gently add **1 mL of Medium B** to each well and incubate at 37°C, 6% CO_2

If desired, cells may be harvested now as described for Day 12. The cell

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yield and proportion of CD34+CD45+ cells will be much lower at Day 10 than at Day 12.

Between Days 7 and 10 add 1mL of Medium B to each well if media changes color to orange-yellowish

14 Harvesting Cells for FACS Sorting:

14.1 Pre-coat a 6 or 12 well tissue culture plate with Matrigel **01:00:00** prior to[1h] harvesting cells for FACS Sorting

Harvest both the floating cells (>90% of these are CD43+ HPCs) and the adherent cells (10-70% are CD43+ HPCs). Recommend for every step to be done in sterile conditions.

Keep HPCs cold on ice or at **4 °C** throughout the process of harvesting, staining for flow-sorting, collection after sorting and until ready to freeze them down if not proceeding with microglia differentiation immediately

14.2 Floating and adherent cells should be harvested for FACS sorting on the twelfth day of culture for presence of the following cellular markers:
1) CD43
2) CD34
3) CD45

14.3 Begin harvesting floating cells using a serological pipette or 1mL micropipette, vigorously pipette media and cells up and down approximately 2-3 times in the well to break up floating cell aggregates.

Transfer floating cells and media to appropriately sized conical tube.

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14.4

14.5 Wash well with **1 mL of DMEM/F12** , triturate, and transfer to same collection tube, this will ensure the majority of floating cells have been collected. Repeat at least one more time.

14.6 Centrifuge the collection tube at 300 x g for **00:05:00** at **Room temperature** .

14.7 Aspirate supernatant.

14.8 Re-suspend pellet in **300 µL of sterile FACS Buffer (PBS and 2% FBS)** and keep on ice.

If smaller cell pellet, resuspend in lower volume. If larger cell pellet, resuspend in larger volume

14.9 Filter the suspension through a 40µm filter before antibody staining or transfer cells into Filter FACS Tubes (Falcon 352235)

14.10 Begin harvesting Adherent Cells by first washing the well with **1 mL D-PBS** and discarding the wash

14.11 Add **1 mL of Accutase** to each well.

20m

14.12 Incubate at **37 °C** for **00:20:00** .

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14.13 Triturate vigorously with a 1mL pipette tip to dislodge the adherent cells and create a single-cell suspension. Do not scrape residual colonies from the tissue culture plate surface, as these clumps will not further dissociate.

14.14 Transfer the single-cell suspension to a collection tube containing **2 mL of DMEM/F12**

14.15 Wash the well with an additional 1mL of DMEM/F12. Add wash to the collection tube. Repeat.

14.16 Centrifuge the collection tube at 300 x g for **00:05:00** at **Room temperature** .

14.17 Aspirate supernatant.

14.18 Re-suspend pellet in **300 µL of sterile FACS Buffer (PBS and 2% FBS)** and keep on ice.

14.19 Filter the suspension through a 40µm filter before antibody staining or transfer cells into Filter FACS Tubes (Falcon 352235)

FACS Sorting CD43^{+} CD34^{+} CD45^{+} Cells

15 To stain cells for FACS sorting, add the following antibodies to the filtered cell suspension (cells and FACS Buffer) in the noted concentrations:
- CD34-FITC (1:200)
- CD43-APC (1:200)
- CD45 – Alexa Fluor700 (1:200)
- CD41-PE (1:200) (*optional*)

16 

Incubate cells and antibodies **On ice** in the dark for **00:20:00** .

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17

After incubation, add **2 mL of FACS Buffer** to each tube and centrifuge at 300 x g for **00:05:00** .

18 Aspirate supernatant.

19 Re-suspend pellet in **500 µL of FACS Buffer** .

20 Sort the $CD34^+$ and $CD43^+$cell population using a **Becton Dickinson FACSAria II** and collect the selected population in sterile tubes.

Sorting has to be performed in sterile conditions.

In order to obtain high quality HPCs, it is suggested to sort only the $CD34^+$, $CD45^+$ and $CD43^+$ triple positive cell population, discarding the single or double negative cells.

If you wish to continue with the iMGL protocol from freshly sorted cells, skip the Freezing Down Sorted Hematopoietic Stem Cells steps. Re-suspend cell pellet in iMGL Diff Complete Medium at a concentration of ~200,000/300,000 cells per well of a 6 well tissue culture plate

Freezing Down Sorted Hematopoietic Stem Cells

10m

21

Centrifuge positively sorted cells at 300 x g for **00:10:00** at **4 °C** .

22 Aspirate supernatant.

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23 Re-suspend cells at a concentration of 1 million cells per **1 mL of Cryostor CS10** .

24 Aliquot **1 mL of cell and freezing medium suspension** per cryovial.

25 1d

Place cells in **-80 °C** for approximately **24:00:00** .

26 After 24 hours, cells must be transferred to liquid nitrogen for long-term storage.

Deriving iMGLs - Thawing Hematopoietic Stem Cells

27 Using previously sorted cryopreserved cells (**Freezing Down Sorted Hematopoietic Stem Cells Section**), place frozen vial of cells in **37 °C water bath** for quick thaw.

> Thaw should take less than one minute, remove cells from water bath prior to complete thaw.

28 Transfer contents of cryovial to a conical tube containing **8 mL of DMEM/F12 containing 5% FBS** .

29 Centrifuge concial tube at 300 x g for **00:05:00** .

30 Aspirate supernatant.

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31 Re-suspend cell pellet in iMGL Diff Complete Medium at a concentration of ~500,000 cells per well of a 6 well tissue culture plate

31.1 iMGL Differentiation Basal Medium (per 500 mL)

A	B	C	D	E	F
Component	**Stock Concentration**	**Final Concentration**	**Vendor**	**Catalog #**	**Volume**
Phenol-free DMEM/F12 (1:1)			Thermofisher	11039021	462.4 mL
Insulin (0.02 mg/mL), Holo-transferrin (0.011 mg/mL), Sodium selenite (13.4 ug/mL) (ITS-G Solution)	100X	2X	Thermofisher	41400045	10 mL
B27	50X	2% V/V	Thermofisher	17504044	10 mL
N2	100X	0.5% V/V	Thermofisher	17502048	2.5 mL
Monothioglycerol	11.5M	400 uM	Sigma Aldrich	M1753-100ML	17.4 uL
Non-Essential Amino Acids (NEAA)	100X	1X	Thermofisher	11140050	5 mL
Glutamax	100X	1X	Thermofisher	35050061	5 mL
Pen/Strep	100X	1X	Thermofisher	15140-122	5 mL
Recombinant Human Insulin	20 mg/mL	5 ug/mL	Sigma Aldrich	I2643	125 uL

31.2 iMGL Diff Complete Medium Recipe:

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A	B	C	D	E
		Vendor	Cetalog #	Dilution
iMGL Diff Base Medium				
IL-34 (100 ng/ml)	500 ug/mL in H20	Peprotech	200-34	1:5000
TGFb-1 (50 ng/ml)	100 ug/mL in 10mM Citric Acid	Peprotech	100-21	1:2000
M-CSF (25 ng/ml)	100 ug/mL in H20	Peprotech	300-25	1:4000

iMGL differentiation basal medium is made at the start of each round of differentiation and more if required is made subsequently

Aliquots of cytokines are stored at -80°C and added fresh to an aliquot of required volume of basal medium on teh day of media addition to iMGLs. Remaining volume of aliquots are stored at 4°C and used up first before thawing new aliquots for further days of media addition

iMGL differentiation complete medium + 1x cytokines refers to required volume of basal medium + 1:5000 hIL-34 + 1:2000 hTGF-β1 + 1:4000 hM-CSF while complete medium + 2x cytokines refers to required volume of basal medium + 1:2500 hIL-34 + 1:1000 hTGF-β1 + 1:2000 hM-CSF

For splitting steps, N refers to existing no. of wells and N' refers to newer wells pre-coated with Matrigel at least for 1 h or O/N

During splitting, all collected supernatants also will contain floating iMGLs while some are left behind attached to the wells

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IL-34, hTGF-β1 and M-CSF are cytokines that promote iMGL survival and homeostasis and are referred to as maintenance cytokines while CD200 and CX3CL1 cytokines that induce iMGL maturation and are accordingly referred to as maturation cytokines

Differentiation of Hematopoietic Stem Cells into iMGLs 6m

32 **Day 2** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

33 **Day 4** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

34 **Day 6** - Split and add **1 mL of IMGL Diff Complete Medium** + 2X Cytokines per well of a 6-well tissue culture plate.

34.1 Collect all but 1mL of media from each well and transfer to a 50mL conical[6m] tube. Spin this supernatant media at 300 xg for **00:06:00** at **Room temperature**

34.2 While above centrifugation is ongoing, split 3 existing wells (N) of iMGLs into 1 new well (N')

There should be ~1 mL of cells in each well after harvesting supernatant in prior step. To split the cells, ~300 µL from each of the 3 existing wells is transferred to one new Matrigel-coated well for each line. iMGLs tend to be more confluent around the center of each well, therefore while taking up ~300 µL for splitting into new well, ensure to collect cells from the center of each well and pipette up/down one time around the center and then transfer the ~300 µL to the new well.

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34.3 After spin step, aspirate supernatant after freezing an aliquot (~500 µL) for future ELISA experiments and resuspend cells in (N+N') mL of iMGL differentiation complete medium + 2X cytokines and evenly distribute 1ml/well to N+N' wells (this means you continue the culture in existing wells in addition to expansion into new wells with every splitting performed)

35 **Day 8** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

36 **Day 10** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

Use extreme caution as plate is nearly full with media.

37 **Day 12** - Split and add **1 mL of IMGL Diff Complete Medium** + 2X Cytokines per well of a 6-well tissue culture plate (Refer to Step 34)

38 **Day 14** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

39 **Day 16** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

40 **Day 18** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

41 **Day 20** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

42 **Day 22** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

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43 **Day 24** - Add **1 mL of IMGL Diff Complete Medium** + 1X Cytokines per well of a 6-well tissue culture plate.

Use extreme caution as plate is nearly full with media.

At any point during the culture from Days 12 to 36, if cells look stressed either due to thawing, centrifugation or other reasons, add iMGL Differentiation Complete Medium + 2X Cytokines

44 Split and add **1 mL of IMGL Diff Maturation Medium** + 2X Cytokines per well of a 6-well tissue culture plate (Refer to Step 34)

iMGL Maturation Media Recipe:

A	B	C	D	E
		Vendor	**Catalog #**	**Dilution**
iMGL Complete Medium				
CD200 (100 ng/mL)	100 ug/mL	Novoprotein	C311-50ug	1:1000
CX3CL1 (100 ng/mL)	100 ug/mL	Peprotech	300-31	1:1000

Every 2 days, supplement cells with 1mL per well of **iMGL Maturation Media** (after day 37 feeding, maturation media + 1x maintenance and maturation cytokines unless if cells look stressed, in which case feed maturation media + 2x maintenance cytokines + 1x maturation cytokines)

45 **Day 28** - Cells should have reached maturity by this step and are ready for experimental use.

Continue feeding cells with **iMGL Maturation Media**. Mature Microglia-Like Cells can

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be used for approximately 2-3 weeks.

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