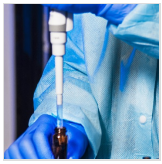

bkrvkv66

Sep 03, 2020

Differentiation of iPSC into Microglia-Like Cells (iMGL) V. (bkrvkv66)

Fabia Filipello[1], Jacob Marsh[1], Rj Martinez[1], Celeste M Karch[2]

[1]Washington University in Saint Louis - WUSTL (MO); [2]Washington University in St Louis

1 Works for me dx.doi.org/10.17504/protocols.io.bkrvkv66

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


Celeste Karch
Washington University in St Louis

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.bkrvkv66

PROTOCOL CITATION

Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch 2020. Differentiation of iPSC into Microglia-Like Cells (iMGL). **protocols.io** https://dx.doi.org/10.17504/protocols.io.bkrvkv66 Version created by Celeste Karch

KEYWORDS

microglia, differentiation, hematopoietic progenitor cells

CREATED

Sep 03, 2020

LAST MODIFIED

Sep 03, 2020

PROTOCOL INTEGER ID

41493

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

		Vendor	Cat#	dilution	
iMGL diff base medium					
IL-34 (100 ng/mL)	500 ug/mL in H2O	Peprotech	200-34	1:5000	

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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 Karch,
Washington University in St Louis
PREVIEW RUN

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

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** .

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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.

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 **[M]5 Micromolar (µM)** – **[M]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**.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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.

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.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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).

- 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.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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:
  - **10 Micromolar (µM)** concentration for new iPSC lines, lines thawed from 96 well after editing.
  - **5 Micromolar (µM)** concentration if thawing from a line without knowledge of its Rock sensitivity.
  - **1 Micromolar (µM)** concentration for all other lines (for lines still exposed to Rock Inhibitor, use **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).

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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

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)

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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

A single 6-well tissue culture plate will yield approximately 1-1.5 million HPCs

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

- Dispase
- DMEM/F12
- PBS
- mTesR1

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

Aspirate media from well.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

5.1

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

5.3 Aspirate **PBS** from well.

5.4 Add **Dispase** to cells **1 mL per well** .

5.5

Incubate at **37 °C** for between **00:07:00** and **00:08:00** .

Edges of iPSC clusters should begin to lift off the Matrigel coated plate

Cells will not completely dissociate from Matrigel coated plate. The majority of cells will remain attached after the incubation.

5.6 Aspirate the **Dispase** from the wells.

5.7

Gently wash cells 2-3 times with **2 mL of DMEM/F12** per well, aspirating after each wash.

The major goal of this step is to remove the Dispase without substantially disturbing the cells.

5.8 Add **2 mL of DMEM/F12** to each well.

5.9 Using the **StemProEZPassage Disposable Stem Cell Passaging Tool**, cut the iPSCs into aggregates that are approximately 100-200µm in diameter.

To accomplish this, the StemPro EZPassage Disposable Stem Cell Passaging Tool should be

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

To accomplish this, the StemPro EZPassage Disposable Stem Cell Passaging Tool should be rolled throughout the entirety of the well horizontally (twice) and vertically (twice). This will result in the appropriate sized aggregates for plating.

5.10 

Transfer the detached aggregates with a serological pipette into a 15 ml conical tube.

Check tissue culture plate under microscope to ensure that the majority of aggregates have been transferred from the plate.

If aggregates still remain, wash well with 2mL of DMEM/F12 and transfer to 15mL conical tube

5.11 Centrifuge 15 ml conical tubes containing cell aggregates at 750 rpm for **00:03:00** .

5.12 Aspirate supernatant from cell pellet.

5.13 Resuspend pellet gently in **2 mL of mTesR1**

Obtaining a uniform suspension of aggregates approximately 100-200µm in diameter is optimal. The StemPro EZPassage Disposable Stem Cell Passaging Tool should be used to ensure these aggregate sizes.

It is essential that pellet is resuspended by pipetting only one time, this ensures a larger degree of cell survival – as cells are incredibly delicate at this stage.

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

6.1 Pipette **40 µl pf DMEM/F12** into three individual wells of a 96-well flat bottom tissue culture plate.

6.2 Pipette **5 µl of aggregate suspension** to each well.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

6.3 Manually count the number of aggregates in each well.

Do not count aggregates smaller than 100µm

6.4 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

6.5 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.

7 Determine the number of aggregates to plate in a 6-well tissue culture plate.

It is recommended plating 130-160 aggregates per well (optimal for control iPSC lines). This density typically results in 20-38 colonies per well after 24 hours of incubation.

The number of aggregates plated can be adjusted depending on how particular iPSC lines behave.

Plating more aggregates at this stage does not result in more HPCs obtained at the end of the protocol.

7.1 Calculating the plating volume of the cell aggregate mixture is accomplished by dividing the number of aggregates to plate by the concentration of the cell aggregates. For example:

1. Plate 130 aggregates per well
2. Concentration of Cell Aggregates =3 Aggregates/µL
3. 130 Aggregates per Well / 3 Aggregates per µL= Plate ~43µL of Aggregate Mixture per Well

8 After calculating the number of aggregates to plate per well, aspirate **Matrigel** from previously coated 6-well plate(s) (refer to **Step 2**), and add **2 mL of mTesR1** containing **2.5µM Rock Inhibitor** to each well.

8.1 Gently mix the cell aggregate mixture by inverting 15mL conical tube.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

Do not vortex or pipette mixture.

8.2 Add the previously calculated volume of aggregate suspension to each well in the 6-well plate.

8.3

Place the plate containing **mTesR1** and aggregate suspension in incubator at **37 °C** for **24:00:00** .

Prior to placing plate in incubator, perform a “t” shaped motion with the plate to ensure that aggregates are distributed evenly throughout the wells.

8.4 After 24 hours, carefully aspirate **mTesR1** containing **2.5µM Rock Inhibitor** from each well.

8.5 Gently wash cells with **2 mL of PBS** per well.

8.6 Aspirate **PBS**.

8.7 Add **2 µl of pre-warmed fresh mTesR1** to each well.

8.8 Visualize plate under microscope and manually count the number of adhered aggregates per well.

Aim for at least 20 adhered aggregates per well of a 6-well tissue culture plate

Do not proceed further if < 20 aggregates or > 40 aggregates have adhered to 6-well tissue culture plate as differentiation will be compromised

iPSCs Induction into Hematopoietic Stem Cells

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

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

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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

10 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

11 Change media on the cell aggregates using the following schedule.

11.1 **Day 0** - Aspirate medium from wells and add **2 mL of Medium A** per well.

Day 0 starts 24 hours after aggregate plating

11.2 **Day 2** - Gently remove **1 mL of Medium A** from each well and gently replace with **1 mL of fresh Medium A** per well.

It is best to use a serological pipette or a 1mL micropipette to perform the media removal.

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

11.4 **Day 5** - Gently remove **1 mL of Medium B** from each well and gently replace with **1 mL of fresh Medium B** per well.

11.5 **Day 7** - Gently remove **1 mL of Medium B** from each well and gently replace with **1 mL of fresh Medium B** per well.

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.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

11.6 **Day 10** - Gently remove **1 mL of Medium B** from each well and gently replace with **1 mL of fresh Medium B** per well.

As the number of floating cells in the culture increases, it is imperative that media changes are done slowly and gently to avoid removing a large number of the floating cells.

12 Harvesting Cells for FACS Sorting:

12.1 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

12.2 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.

12.3 Transfer floating cells and media to appropriately sized conical tube.

12.4 Wash well with **1 mL of DMEM/F12** and transfer to same collection tube, this will ensure the majority of floating cells have been collected.

Be sure that collected cells are kept on ice to avoid cell death.

12.5 Centrifuge the collection tube at 300 x g for **00:05:00** at **4 °C** .

12.6 Aspirate supernatant.

12.7 Re-suspend pellet in **5 mL of sterile FACS Buffer (PBS and 2% FBS)** .

12.8 Filter the suspension through a 40µm filter into collection tubes.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

12.9 Centrifuge the collection tube at 300 x g for **00:05:00** at **4 °C** .

12.10 Aspirate supernatant.

12.11 Re-suspend pellet in **5 mL of sterile FACS Buffer (PBS and 2% FBS)** .

12.12 Begin harvesting Adherent Cells by first adding **1 mL of Accutase** to each well.

12.13 Incubate at **37 °C** for **00:15:00** .

12.14 Add **2 mL of DMEM/F12** to each well.

12.15 Triturate vigorously to achieve a single cell suspension.

12.16 Transfer cell suspension to appropriately sized collection tube.

12.17 Centrifuge the collection tube at 300 x g for **00:05:00** at **4 °C** .

12.18 Aspirate supernatant.

12.19 Re-suspend pellet in **5 mL of sterile FACS Buffer (PBS and 2% FBS)** .

12.20 Filter the suspension through a 40µm filter into collection tubes.

12.21 Centrifuge the collection tube at 300 x g for **00:05:00** at **4 °C** .

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

12.22 Re-suspend pellet in **5 mL of sterile FACS Buffer (PBS and 2% FBS)** .

FACS Sorting CD43$^+$ CD34$^+$ CD45$^+$ Cells

13 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) (*optional*)
- CD41-PE (1:200) (*optional*)

14 

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

15 

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

16 Aspirate supernatant.

17 Re-suspend pellet in **500 µl of FACS Buffer** .

18 Sort the CD34$^+$ and CD43$^+$cell population using a **Becton Dickinson FACSAria II**.

Sorting has to be performed in sterile conditions.

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

Freezing Down Sorted Hematopoietic Stem Cells

19 

Centrifuge positively sorted cells at 300 x g for **00:05:00** at **4 °C** .

20 Aspirate supernatant.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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

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

23

Place cells in **-80 °C** for approximately **48:00:00** .

24 After 48 hours, cells can be transferred to liquid nitrogen for long-term storage.

Deriving iMGLs - Thawing Hematopoietic Stem Cells

25 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.

26 Transfer contents of cryovial to a conical tube containing **8 mL of DMEM/F12 supplemented with 2% FBS** .

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

28 Aspirate supernatant.

29 Re-suspend cell pellet in iMGL Diff Complete Medium at a concentration of ~500,000 cells per **2 mL** .

29.1 iMGL Diff Complete Medium Recipe:

		Vendor	Cetalog #	Dilution
iMGL Diff Base Medium				
IL-34 (100 ng/ml)		Peprotech	200-34	1:5000
TGFb-1 (50 ng/ml)		Peprotech	100-21	1:2000
M-CSF (25 ng/ml)		Peprotech	300-25	1:4000

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

iMGL Diff Base Medium can be kept in storage at 4°C for approximately one month. It is important to make fresh *iMGL Diff Complete Medium* (addition of fresh cytokines to the base medium) every time you need to feed the culture

Differentiation of Hematopoietic Stem Cells into iMGLs

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

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

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

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

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

Use extreme caution as plate is nearly full with media.

35 **Day 12** - Collect all but **1 mL of media** from wells and centrifuge at 300 x g for **00:05:00** at **Room temperature** .

35.1 Aspirate the media and add **1 mL of iMGL Diff Complete Medium** per well to re-suspend cell pellet.

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

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

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

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

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66

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

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

Use extreme caution as plate is nearly full with media.

42 **Day 25** - Collect all but **1 mL of media** from wells and centrifuge at 300 x g for **00:05:00** at **Room temperature** .

42.1 Aspirate the media and add **1 mL of iMGL Maturation Medium** per well to re-suspend cell pellet.

iMGL Maturation Media Recipe:

		Vendor	Catalog #	Dilution
iMGL Complete Medium				
CD200 (100 ng/ml)	100 ug/ml	Novoprotein	C311-50 ug	1:1000
Cx3CL1 (100 ng/ml)	100 ug/ml	Peprotech	300-31	1:1000

iMGL Diff Base Medium can be kept in storage at 4°C for approximately one month. It is important to make fresh *iMGL Diff Complete Medium* (addition of fresh cytokines to the base medium) every time you need to feed the culture

43 **Day 28** - Cells should have reached maturity by this step.

Continue feeding cells with **iMGL Maturation Media**. Mature Microglia-Like Cells can be used for approximately 2-3 weeks.

Mature iMGLs can be also be detached with **Accutase** (**00:05:00** at **37 °C**) and replated on plastic or glass culture ware that has been pre-coated with **Matrigel**.

Citation: Fabia Filipello, Jacob Marsh, Rj Martinez, Celeste M Karch (09/03/2020). Differentiation of iPSC into Microglia-Like Cells (iMGL). https://dx.doi.org/10.17504/protocols.io.bkrvkv66