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# Differentiation of Induced Pluripotent Stem Cells (iPSCs) into Microglia-like cells (iMG)

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**We use this protocol and it's working**

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## Abstract

iPSC-derived microglia are an important tool for studying the role of microglia and neuro-immune alterations during development, adulthood, and aging, both in health and disease. The use of human cells enables the identification of molecular mechanisms driven by an individual's genetic susceptibility that would not be possible with animal models. Here, we described a step-by-step protocol used in our previous manuscript (Dolan, Therrien, et al 2023). This protocol is used to induce a microglial state that arises in response to specific brain challenges and is amenable to lentiviral transduction.

## Materials

ACCUTASE™ 100 mL **STEMCELL Technologies Inc. Catalog #**7920 Equipment

- Incubator for normoxic conditions (20% $O_2$, 5% $CO_2$, 37°C)
- Humidified tri-gas incubator for hypoxic conditions (5% $O_2$, 5% $CO_2$, 37°C)
- Centrifuge
- Cell counting equipment

**iPSCs maintenance list of reagents**

| | A | B |
|---|---|---|
| | **Reagent** | **Company; Cat #** |
| | PBS 1X | Thermo Fisher; 14190250 |
| | Matrigel | VWR; 47743-722 |
| | Essential E8 medium | Thermo Fisher; A1517001 |
| | Cryostor | Stem Cell Technologies; 100-1061 |
| | Accutase | Stem Cell Technologies; 07920 |

**Microglia differentiation media composition and reagents**

| | A | B | C |
|---|---|---|---|
| | **iHPC Base Growth Medium (first 10 days)** | | |
| | **Component** | **Final concentration** | **Company; Cat #** |
| | IMDM | 50% | Thermo Fisher; 31980097 |
| | F12 | 50% | Thermo Fisher; 11765062 |

| | A | B | C |
|---|---|---|---|
| | ITSG-X | 2% v/v | Thermo Fisher; 51500-056 |
| | L-ascorbic acid 2-Phosphate | 64 µg/ml | Sigma-Aldrich; A8960-5G |
| | monothioglycerol | 400 µM | Sigma-Aldrich; M1753-100ML |
| | PVA | 10 µg/ml | Sigma-Aldrich; P8136-250G |
| | GlutaMax (1X) | 1X | Thermo Fisher; 35050079 |
| | Chemically defined lipid concentrate (1X) | 1X | Thermo Fisher; 11905031 |
| | Non-essential amino acids (NEAA) (1X) | 1X | Thermo Fisher; 11140050 |

**iHPC base media:** All reagents are combined and filtered in .22uM bottle. Base media is used within 2 weeks. L-ascorbic acid 2-Phosphate and PVA solutions are used within a week.

| | A | B | C |
|---|---|---|---|
| | **Day 0 growth factors** | | |
| | **Component** | **Final concentration** | **Company; Cat #** |
| | FGF2 | 50 ng/ml | Thermo Fisher; PHG0261 |
| | BMP4 | 50 ng/ml | Thermo Fisher; PHC9534 |
| | Activin-A | 12.5 ng/ml | Thermo Fisher; PHC9564 |
| | Rock Inhibitor (RI) | 1 µM | Selleckchem; s1049 |
| | Lithium Chloride (LiCl) | 2 mM | Sigma-Aldrich; L7026 |

**Day 0 iHPC media:** iHPC base media + growth factors listed. Growth factors are aliquoted in single use aliquots and store at -80C or -20C until expiration date following manufacturer instructions.

| | A | B | C |
|---|---|---|---|
| | **Day 2 growth factors** | | |
| | **Component** | **Final concentration** | **Company; Cat #** |
| | FGF2 | 50 ng/ml | Thermo Fisher; PHG0261 |
| | VEGF | 50 ng/ml | Peprotech; 100-20-50UG |

**Day 2 iHPC media**: iHPC base media + growth factors listed. Growth factors are aliquoted in single use aliquots and store at -80C or -20C until expiration date following manufacturer instructions.

| | A | B | C |
|---|---|---|---|
| | **Day 4 growth factors** | | |
| | **Component** | **Final concentration** | **Company; Cat #** |
| | FGF2 | 50 ng/ml | Thermo Fisher; PHG0261 |
| | VEGF | 50 ng/ml | Peprotech; 100-20-50UG |
| | TPO | 50 ng/ml | Thermo Fisher; 300-18-50UG |
| | SCF | 10 ng/ml | Thermo Fisher; PHC2115 |
| | IL-6 | 50 ng/ml | Thermo Fisher; 200-06 |
| | IL-3 | 10 ng/ml | Thermo Fisher; 200-03-10UG |

**Day 4-10 iHPC media:** iHPC base media + growth factors listed. Growth factors are aliquoted in single use aliquots and store at -80C or -20C until expiration date following manufacturer instructions.

| | A | B | C |
|---|---|---|---|
| | **iMG differentiation basal medium** | | |

| | A | B | C |
|---|---|---|---|
| | **Component** | **Final concentration** | **Company; Cat #** |
| | DMEM | 50% | Thermo Fisher; 10569044 |
| | F12 | 50% | Thermo Fisher; 11765062 |
| | ITS-G | 2x v/v | Thermo Fisher; 41400045 |
| | B-27 | 2x v/v | Thermo Fisher; 17504044 |
| | N2 | 0.5x | Thermo Fisher; 17502048 |
| | Monothioglycerol | 200 µM | Sigma-Aldrich; M1753-100ML |
| | GlutaMax (1X) | 1X | Thermo Fisher; 35050079 |
| | Non-essential amino acids (NEAA) (1X) | 1X | Thermo Fisher; 11140050 |
| | Insulin | 5 µg/ml | Sigma-Aldrich; I2643 |

**Mg base media:** All reagents are combined and filtered in .22uM bottle. Base media is used within 2 weeks. B27 and N2 are frozen in single used aliquots.

| | A | B | C |
|---|---|---|---|
| | **Day 10 growth factors** | | |
| | **Component** | **Final concentration** | **Company; Cat #** |
| | M-CSF | 25 ng/ml | Thermo Fisher; 300-25-100 |
| | IL-34 | 10 ng/ml | Thermo Fisher; 200-34-250UG |
| | TGFβ | 50 ng/ml | Thermo Fisher; 100-21-100UG |

**Day 10 Mg media: Mg** base media + growth factors listed. Growth factors are aliquoted in single use aliquots and store at -80C or -20C until expiration date following manufacturer instructions.

| | A | B | C |
|---|---|---|---|
| | **Day 30 growth factors** | | |
| | **Component** | **Final concentration** | **Company; Cat #** |
| | CD200 | 100 ng/ml | Thermo Fisher; 103872-010 |
| | CX3CL1 | 100 ng/ml | Thermo Fisher; 300-31 |
| | M-CSF | 25 ng/ml | Thermo Fisher; 300-25-100 |
| | IL-34 | 10 ng/ml | Thermo Fisher; 200-34-250UG |
| | TGFβ | 50 ng/ml | Thermo Fisher; 100-21-100UG |

**Day 10 Mg media: Mg** base media + growth factors listed. Growth factors are aliquoted in single use aliquots and store at -80C or -20C until expiration date following manufacturer instructions.

Materials for microglia cell growth

| | A | B |
|---|---|---|
| | Product name | Company; Cat # |
| | 6-well Ultra-Low Attachment plate | Corning Life Sciences; 3471 |
| | Cell lifter | Life Sciences; 76036-004 |

## Protocol materials

- Essential 8 **Life Technologies** **Catalog #**A1517001
- TeSR™-E8™ Kit for hESC/hiPSC Maintenance 1 Kit **STEMCELL Technologies Inc.** **Catalog #**5990
- Corning® Matrigel® Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL **Corning** **Catalog #**356237
- CryoStor CS10-100ml **STEMCELL Technologies Inc.** **Catalog #**07930
- ACCUTASE™ 100 mL **STEMCELL Technologies Inc.** **Catalog #**7920
- DPBS, no calcium, no magnesium **Thermo Fisher Scientific** **Catalog #**14190144
- Y-27632 (ROCK inhibitor) (Selleck Chemicals Catalog # S1049) **Selleckchem** **Catalog #**S1049
- Costar® 6-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates **Corning** **Catalog #**3471
- Fisherbrand™ Cell Lifters **Thermo Fisher Scientific** **Catalog #**08-100-240

## iPSC maintenance

1 Maintenance of stem cells

Successful differentiation is achieved with high quality stem cells. It is recommended to split iPSCs at least 2 times after thawing before starting differentiation and check frequently at their morphology to avoid spontaneous differentiations that may interfere with mesodermal induction. Make sure iPSCs are growing in

Essential 8 **Life Technologies Catalog #**A1517001 or

TeSR™-E8™ Kit for hESC/hiPSC Maintenance 1 Kit **STEMCELL Technologies Inc. Catalog #**5990

otherwise change accordingly.

## Generation of HPC

47m

2 Day -1: Generation of embryoid bodies

2.1 Detach cells

15m

- Aspirate the culture media and wash with 1X PBS.
- Dissociate iPSCs by incubating with

  ACCUTASE™ 100 mL **STEMCELL Technologies Inc. Catalog #**7920

  00:10:00 (time may vary depending on different cell lines) at 37 °C , 5% $CO_2$. Lightly tap the side of the plate to dislodge the cells.
- Collect the entire plate volume and transfer the cell suspension to a 15 ml conical tube.
- Wash the plate again with

  DPBS, no calcium, no magnesium **Thermo Fisher Scientific Catalog #**14190144

  to collect cells left behind and transfer to the same 15 ml tube.
- ·Centrifuge the cells at 300 x g, 00:05:00 . Aspirate supernatant to waste and resuspend the pellet to triturate to create a single cell suspension using

  Essential 8 **Life Technologies Catalog #**A1517001 with [M] 10 micromolar (µM)

  Y-27632 (ROCK inhibitor) (Selleck Chemicals Catalog # S1049) **Selleckchem Catalog #**S1049

2.2 Count the cells and adjust cell density to 5 × $10^5$ per well in a Costar® 6-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates **Corning Catalog #**3471 in Essential 8 **Life Technologies Catalog #**A1517001 + Y-27632 (ROCK inhibitor) (Selleck Chemicals Catalog # S1049) **Selleckchem Catalog #**S1049 (2 ml in a 6-well plate).

- Count cells using Trypan blue. Viability should be at least 90%.
- Plate evenly on growth surface and begin normoxic culture conditions (20% $O_2$, 5% $CO_2$, 37°C).

Note

Because of the different proliferation rates among cell lines, it is recommended to plate different concentrations (2 x $10^5$ and 8 x $10^5$) and start differentiation with the one with 50-100 embryoid bodies (EBs).

3 Day 0: Change media 5m

- Carefully collect the entire plate medium with EBs in suspension by using a 5 ml pipette to not dislodge them, and transfer to a 15 ml tube.
- Centrifuge EBs at 100 x g, 00:05:00 Aspirate supernatant to waste and resuspend EBs in iHPC Base Growth medium supplemented with 2 mL Day 0 growth factors in a new low-attachment plate well.
- Plate evenly on growth surface and begin hypoxic culture conditions by incubating cells in a humidified tri-gas incubator with 5% $O_2$, 5% $CO_2$, 37 °C .

4 Day 2: Making HPC media and change media 5m

- Carefully collect the entire plate medium with EBs in suspension by using a 5 ml pipette to not dislodge them, and transfer to a 15 ml tube.
- Centrifuge EBs at 100 x g, 00:05:00 . Aspirate supernatant to waste and scroll the tune on the grill of the hood to dislodge the pellet. Resuspend EBs in 2 mL iHPC Base Growth medium supplemented with Day 2 growth factors in a new low-attachment plate well.

- Plate evenly on the growth surface and incubate under hypoxic culture conditions (5% $O_2$, 5% $CO_2$, 37 °C ).

5 Day 4: Change media 5m

- Carefully collect the entire plate medium with EBs in suspension by using a 5 ml pipette to not dislodge them, and transfer to a 15 ml tube..
- Centrifuge EBs at 100 x g, 00:05:00 . Aspirate supernatant to waste and scroll the tube on the grill of the hood to dislodge the pellet. Resuspend EBs in 2 mL iHPC Base Growth medium supplemented with Day 4 growth factors in a new low-attachment plate well.
- Plate evenly on growth surface and incubate under normoxic culture conditions (20% $O_2$, 5% $CO_2$, 37 °C ).

6 Day 6: Feed cells

Add 1 mL of iHPC Base Growth medium supplemented with Day 4 growth factors.

7 Day 8: Feed cells

Add 1 mL of iHPC Base Growth medium supplemented with Day 4 growth factors.

8 Day 10: Collect HPC 7m

- Carefully collect the entire plate medium with EBs in suspension and transfer to a 15 ml tube.
- Let the EBs precipitate by gravity (around 00:02:00 ).
- Collect the supernatant containing hematopoietic progenitor cells (hpc) in single cells in a tube and filter through a 40 µm screen (to avoid any residual EBs).
- Centrifuge the single cell suspension at 300 x g, 00:05:00 Aspirate supernatant to waste and resuspend the pellet in Mg differentiation basal medium supplemented with Day 10 growth factors.
- Count the cells using Trypan blue

Note

**Quality control:** To assess that differentiation is successful, on Day 10, it is recommended to run a quality control to check viability, and the expression of CD45 and CD43 markers via flow cytometry. Viaiblity should be > 70%, CD43 positive cells > 95% and CD45 positive cells > 50%

Note

Cells can be frozen at this time in CryoStor CS10-100ml **STEMCELL Technologies Inc.** **Catalog #**07930 . We recommend freezing at least 300 000 cells/aliquot so they can be thawed in 1 well of a 6-well plate

8.1 Cell replating to continue differentiation

- Resuspend HPC in Mg differentiation basal media with Day 10 growth factors
- Adjust cell density to 3 × $10^5$ per well in a Corning® Matrigel® Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL **Corning** **Catalog #**356237 coated 6-well plate.
- Incubate under normoxic culture conditions (20% $O_2$, 5% $CO_2$, 37 °C ).

9 Every 2 days: Feed cells

Add 1 ml of Mg differentiation basal medium supplemented with Day 10 growth factors

10 Day 22: Collect, count and replate cells

10.1 Collect and count cells 10m

- Collect the entire plate medium and transfer to a 15 ml tube (do not aspirate, as many cells might still be in suspension).
- Collect early iMGL by adding DPBS, no calcium, no magnesium **Thermo Fisher Scientific** **Catalog #**14190144 to the well and let sit for 00:05:00 at room temperature.

- Gently detach cells with a
  Fisherbrand™ Cell Lifters **Thermo Fisher Scientific Catalog #**08-100-240 .
  Wash the well with
  DPBS, no calcium, no magnesium **Thermo Fisher Scientific Catalog #**14190144
  and transfer to a new 15 ml tube.
- Centrifuge both tubes (one containing plate medium and one PBS) at
  300 x g, 00:05:00
- From the tube with PBS, aspirate supernatant to waste. From the tube with conditioned Mg medium, collect the supernatant, and transfer to a new tube, leaving 1 ml to cover the pellet. Resuspend the pellet with the remaining 1 ml and transfer this cell suspension into the PBS tube.
- Combine and gently triturate the two pellets together.
- Count using Trypan blue

10.2 Replate cells

- Adjust cell density to 3 × $10^5$ per well in a
  Corning® Matrigel® Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL **Corning Catalog #**356237
  coated 6-well plate.
- Plate cells in 50% old conditioned medium + 50% fresh Mg differentiation basal medium supplemented
  with Day 10 growth factors.

> Note
>
> For high-proliferating cell lines, it might be necessary to split cells at Day 20 instead.

## Microglia generation and maturation

5m

11 Starts from either freshly collected iHPC or from frozen iHPC vials. If cells were frozen, thaw rapidly at 37 °C water bath, resuspend cells in warm DMEM-F12, centrifuge at 300 x g, 00:05:00 and count to plate at the appropriate density.

5m

12 Every 2 days: Feed cells
Add 1 ml of Mg differentiation basal medium supplemented with Day 10 growth factors

13 Day 30: Collect, count and replate cells

Note

Cells at Day 30 must be plated in the final experimental vessel.

13.1 Collect and count cells

- Collect the entire plate medium and transfer to a 15 ml tube (do not aspirate, as many cells might still be in suspension).
- Collect early iMGL by adding
  DPBS, no calcium, no magnesium **Thermo Fisher Scientific Catalog #**14190144
  to the well and let sit for 00:05:00 at room temperature.
- Gently detach cells with a
  Fisherbrand™ Cell Lifters **Thermo Fisher Scientific Catalog #**08-100-240 Wash the well with
  DPBS, no calcium, no magnesium **Thermo Fisher Scientific Catalog #**14190144
  and transfer to a new 15 ml tube.
- Centrifuge both tubes (one containing plate medium and one PBS) at
  300 x g, 00:05:00
- From the tube with PBS, aspirate supernatant to waste. From the tube with conditioned Mg medium, collect the supernatant, and transfer to a new tube, leaving 1 ml to cover the pellet. Resuspend the pellet with the remaining 1 ml and transfer this cell suspension into the PBS tube.
- Combine and gently triturate the two pellets together.
- Count using Trypan blue.

13.2 Replate cells

- Adjust cell density to 3 × $10^5$ per well in a
  Corning® Matrigel® Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL **Corning Catalog #**356237
  coated 6-well plate.
- Plate cells in 50% old conditioned medium + 50% fresh Mg differentiation basal medium supplemented
  with Day 30 growth factors.

14 Every 2 days: Feed cells

Add 1 ml of Mg differentiation basal medium supplemented with Day 30 growth factors.

15 Day 40: Collect cells for desired experiments.