**iPSC differentiation into Microglia**

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Thais protocol is slight modification from the original protocol published.

This protocol is adapted from the below article.

McQuade A, Coburn M, Tu CH, Hasselmann J, Davtyan H, Blurton-Jones M. Development and validation of a simplified method to generate human microglia from pluripotent stem cells. *Mol Neurodegener.* 2018;13(1):67.

**Reagents required**

1. STEMdiff™ Hematopoietic Kit (# 5310)
2. Matrigel (Corning, #356230)
3. DMEMF12 (Gibco, #11330-032)
4. NEAA (Gibco, #11140050)
5. Glutamax (Gibco, )
6. N2 (Gibco, #17502-048)
7. B27 (Gibco, #17504-044)
8. MCSF (peprotech #300-25)
9. TGFbeta (peprotech #100-21)
10. IL34 (Peprotech #200-34)
11. CX3CL1 (Peprotech #300-31)
12. CD200 (Novo protein #C311)
13. Monothioglycerol (sigma #M6145)
14. ITSN (insulin-transferrin-selenite) (Gibco # 41400045)
15. Insulin (Sigma, #I2643)

**Base media:**

DMEM/ F12

2X insulin-transferrin-selenite

2X B27, 0.5X N2

1X glutamax

1X non-essential amino acids

400μM monothioglycerol

5 μg/mL insulin

**3 cytokine media:**

Base media

25 ng/ml mCSF

100 ng/ml IL34

50 ng/ml TGFbeta

**5 Cytokine media:**

Base media

25 ng/ml mCSF

100 ng/ml IL34

50 ng/ml TGFbeta

100 ng/ml CX3CL1

100 ng/ml CD200

**Steps:**

The differentiation protocol involves two steps.

1. iPSC differentiation into CD34 positive hematopoietic progenitors

2. Hematopoietic progenitors’ differentiation into mature Microglia

Note: This differentiation protocol requires healthy iPSCs approximately 50-60% confluency with nice tight colonies.

**Day-2:**

Coat 3 wells of a 6 well pates with Matrigel. (1 hour coating also works)

**Day-1:**

1. Bring iPSC maintenance to plate cell culture hood, remove the media and rinse once with PBS.
2. Then add 1 ml of 0.5uM EDTA to the well and leave in the incubator for about 5 minutes. By this time, you will see colonies lifting from the plates. If not leave plate in the incubator for few more minutes.

Note: Do not tap or pipet to lift off the cells from the plate. This protocol requires a clumps off iPSC colonies for good yield of hematopoietic progenitors.

1. Once the 50 to 60% of colonies come off the plate, bring plate into the hood and neutralize the reaction by adding 2 ml of E8+Ri media.
2. Now gently swirl the plate to mix EDTA solution containing cells and E8+Ri media. (Never pipet, this mechanical force will disrupt the clumps)
3. After swirling the plate pipet 1 ml of the cells with 5 ml pipet into 15 ml falcon tube.
4. Spin down the cells at 1 RCF for 3 minutes (This slow spin is required for avoiding single cells smaller clumps).
5. After this spin, take aspirate the supernatant and gently tap the cell pellet.
6. Then add 3 ml of E8+Ri media and tap gently once again to mix the clumps.
7. For counting the clump number take 5ul of this mix into a 96 well plate, add 100ul of E8 media. Gently tap the plate and count the colonies under the microscope. Repeat this in 3 wells of 96 well plate and average the clump number.
8. Now bring the Matrigel coated plate, aspirate the Matrigel and add 1.5ML of E8+Ri media. (3 well were coated on day0)

Now plate 10 clumps in 1st well, 20 clumps in 2nd well and 30 clumps in 3rd well of the 6 well plate. (Since counting of clumps is arbitrary, it is never perfect. This exercise is essential till you get very good experience in plating the clumps)

Shake the plate up and down and to sides, return the plate to the incubator.

**Day 0**

1. Count the colonies in in each well, ideal colony number is between 10-30. It doesn’t have to be perfect number. Wells with as low as 5 colonies and max up to 30 can also be used. Anything above 40 should be avoided.

Note1: Each clump should have 20-40 cells. If you have smaller colonies change media to E8 only and let the colonies grow for 1-2 days.

Note2: Above 30 clumps number differentiation may not work because mesodermal cells require space to migrate and differentiate into hematopoietic progenitors.

1. Choose on well with desired number of colonies and you can discontinue maintaining remaining wells.
2. After achieving desired colony number prepare Media A (2ml base media + 10ul supplement A)
3. Aspirate E8+Ri media and add 2ml Media A and leave plate in incubator for 2 days.

**Day 2:**

1. Add 1 ml A (1ml base media + 5ul supplement A)

**Day 3:**

1. Prepare Media B (2ml base media + 10ul supplement B)
2. Aspirate media A and add 2 ml Media B

**Day5, 7,9,10** supplement the cell with 1 ml of Media B.

**Day12: Collection**

1. By day 12 you will see lot of floating hematopoietic progenitor cells.
2. Collect hematopoietic progenitor cells by gently swirling the plate with a 5 ml pipet into 15 ml falcon tube.
3. Spin down the cells 3 minutes 3RCF.
4. Remove the supernatant and resuspend the cell pellet in Macrophage differentiation media. (RPMI+ 20% FBS+100ng/ML M-CSF)
5. Count the cell by using hemocytometer and plate 100,000 in one well of 6 well plate.

**Steps for Microglia differentiation from hematopoietic progenitors:**

**Day12:**

Plate 100,000 hematopoietic progenitor on Matrigel coated 6 well plate in 3 cytokine media.

**On days 14,16,18,20 and 22 supplement with 1 ml of 3 cytokine media.**

**Day 24:**

On Day 24 collect 6 ml of cells + media into 15 ml falcon by leaving 1 ml conditioned media in the plate.

Spin down at 3 RCF for 3 minutes and remove the supernatant.

Resuspend the pellet in 2 ml of fresh 3 cytokine media and plate back into the same well containing conditioned media.

**Day 26,28,30,32,34,36 supplement with 1 ml of 3 cytokine media.**

**Day37:**

On Day 37 collect 6 ml of cells + media into 15 ml falcon by leaving 1ml conditioned media in the plate.

Spin down at 3 RCF for 3 minutes and remove the supernatant.

Resuspend the pellet in 2 ml of fresh 5 cytokine media and plate back into the same well containing conditioned media.

**Day39:**

supplement cells with 1 ml of 5 cytokine media

**Day41:**

Collect cells for experiment.