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Human Microglia Differentiation from hES (EB Method)

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We use this protocol and it's working

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Abstract

This protocol describes differentiation from iPSCs to microglia.

Guidelines

The differentiation protocol involves two steps.

1. iPSC differentiation into CD14 positive progenitors
2. Progenitors' differentiation into mature Microglia



Materials

Reagents required:

- ✕ Anti-Adherence Rinsing Solution **STEMCELL Technologies Inc. Catalog #07010**
- ✕ Advanced DMEM/F-12 **Thermo Fisher Catalog #12634010**
- ✕ Recombinant Human IL-34 **Thermo Fisher Scientific Catalog #200-34**
- ✕ Recombinant Human GM-CSF **Thermo Fisher Scientific Catalog #300-03**
- ✕ Recombinant Human TPO **peprotech Catalog #300-18**
- ✕ Recombinant Human Flt3-Ligand **peprotech Catalog #300-19**
- ✕ Recombinant Human IL-3 **peprotech Catalog #200-03**
- ✕ Recombinant Human M-CSF **peprotech Catalog #300-25**
- ✕ Recombinant Human SCF **peprotech Catalog #300-07**
- ✕ Recombinant Human VEGF **peprotech Catalog #100-20**
- ✕ Human BMP-4 Recombinant Protein, PeproTech® **Thermo Fisher Scientific Catalog #120-05ET-10UG**
- ✕ Corning® 96-well Clear Round Bottom Polystyrene Not Treated Microplate **Corning Catalog #3788**
- ✕ mTeSR™1 **STEMCELL Technologies Inc. Catalog #85850**
- ✕ Y 27632 dihydrochloride **Axon Medchem Catalog #1683**
- ✕ X-VIVOTM 15 Serum-free Hematopoietic Cell Medium **Lonza Catalog #04-418Q**
- ✕ GlutaMAX™ Supplement **Gibco - Thermo Fisher Scientific Catalog #35050061**
- ✕ Antibiotic/Antimycotic (100X) **Thermo Fisher Scientific Catalog #15240062**
- ✕ Gibco™ 2-Mercaptoethanol (50 mM) **Thermo Fisher Scientific Catalog #31350010**



Preparation of 96-Well Plate

1h 15m

- 1 On day -1, coat a 96-well plate (U bottom) with anti-adherence rinsing solution. 5m
- 1.1 Add  50 μL per well of the 96-well plate.
- 1.2 Allow the plate to sit at  37 $^{\circ}\text{C}$ in a tissue culture incubator for  01:00:00 . 1h
- 1.3 When ready to use the plate(s), remove the excess anti-adherence rinsing solution and wash once with PBS. 10m

Cell Collection and Preparation

10m

- 2 On day 0, collect cells from one 70–80% confluent well of the cultured plates by removing the stem cell media ( 2 mL), adding  0.5 mL of accutase, and incubating for  00:05:00 at  37 $^{\circ}\text{C}$. 5m
- 2.1 After incubation, add  3 mL of 1 $\times$ PBS to inactivate accutase enzymatic activity.
- 2.2 Gently flush the well to collect all detached cells and transfer everything to a new 15-ml Falcon tube.
- 2.3 Take an aliquot to count cell suspension in a hemocytometer while centrifuging the collected cells for  00:05:00 at  1000 rpm, Room temperature . 5m
- 3 After spinning down, take out the supernatant (~  3.5 mL) and resuspend pelleted stem cells in the required volume of mTeSR1 + BVS to get a final concentration of 10^6 cells/ml.

Media Preparation

- 4 Prepare the mTeSR1 media by taking the appropriate amount of mTeSR1 and adding mTeSR1 5 $\times$ supplements (final concentration 1 $\times$).



- 4.1 mTeSR1 media can be prepared in advance and stored at 4 °C. We recommend keeping the media no longer than 1 month at 4 °C and regularly checking for potential contamination.
- 4.2 We recommend adding BVS cytokines fresh (never store them at 4 °C for longer than 1 week).
- 5 To make mTeSR + BVS media, add 50 ng/ml of human BMP4, 50 ng/ml of human VEGF, and 20 ng/ml of human SCF to the mTeSR1 media with supplements prepared in advance.

Plating and Incubation

3m

- 6 Transfer the cell suspension in mTeSR1 + BVS + 10 µM Rock inhibitor (11 ml) into a reagent reservoir.
- 6.1 Using an 8- or 12-well multichannel pipette, plate 110 µl of stem cell suspension into each well of a 96-well treated anti-adherence rinsing solution (or U bottom ultra-low adherence plate).
- 6.2 After plating, spin down the 96-well plates at 100 r.c.f. for  00:03:00 at room temperature.
- 7 After centrifugation, check under the microscope that the stem cell suspension has clustered together into the bottom of the well.
- 7.1 Gently put the 96-well plate in the incubator at 37 °C in 5% CO₂.

3m

Media Change and EB Growth

15m

- 8 Every day from days 1 to 3, change the media of the 96-well plates where EBs are growing by following these steps:
- 8.1 Prepare 10 ml of fresh mTeSR1 + BVS and pour into a plastic bucket adequate for multichannel pipettes.
- 8.2 To change the media, first remove  50 µL using an 8- or 12-well multichannel pipette.

15m



8.3 Add  50 μ L of fresh mTeSR1 + BVS.

8.4 Repeat this procedure for a total of 75% medium change. For the second media change, rotate the plate 180° to ensure complete removal of dead cells on both sides of the EBs.

EB Collection and Differentiation

20m

9 On day 4, collect the EBs one by one using a P1000 pipette and P1000 tips with large orifices.

20m

9.1 Place the P1000 tip on the center and bottom of the well.

9.2 Carefully aspirate the media and the EBs and immediately transfer everything to a 50-ml Falcon tube.

9.3 Repeat this procedure in all the wells of the 96-well plate.

9.4 Check under the microscope that all the EBs have been collected.

10 Let the EBs in the 50-ml Falcon tube sink by gravity for a couple of minutes.

10.1 Once they have sunk, carefully remove the medium (~8–9 ml of mTeSR1 + BVS) using a 10-ml serological tip without taking out the EBs.

10.2 To avoid EB loss, some medium (<2 ml) can be left in the 50-ml Falcon tube.

11 Using a 25-ml serological pipette, resuspend the EBs in 20 ml of fresh differentiation medium + SMIFT and plate 4 ml per well containing ~20 EBs per well in a six-well plate (no coating is applied to the plate from this step onward).

Differentiation Medium Preparation

12 Prepare the differentiation media by taking the appropriate amount of X-VIVO 15 media and adding 2 mM GlutaMAX, 100 U/ml of Antibiotics–Antimycotic, and 0.055 mM 2-



mercaptoethanol.

- 12.1 Differentiation media can be prepared in advance and stored at 4 °C.
- 12.2 We recommend keeping the media no longer than 1 month at 4 °C and regularly checking for potential contamination.
- 12.3 We recommend adding SMIFT cytokines fresh (never store them at 4 °C for longer than 1 week).
- 13 To make differentiation media + SMIFT, add 50 ng/ml of human SCF, 50 ng/ml of human M-CSF, 50 ng/ml of human IL3, 50 ng/ml of human FLT3, and 5 ng/ml of human TPO to the differentiation media prepared in advance.

Media Change and Progenitor Collection

- 14 On day 8, use a 1 ml pipette to take up the medium of the six-well plate containing EBs very carefully and transfer everything to a 50-ml Falcon tube.
 - 14.1 Some of the bodies will be attached to the bottom of the six-well plate; some will not.
 - 14.2 Attached EBs can be left in the wells of the six-well plate.
 - 14.3 If floating EBs are picked up, put them back in the well.
- 15 Add  4 mL of fresh differentiation medium + SMIFT per well.
- 16 On day 11, perform a media change by repeating the same procedure as in Steps 14–15.
 - 16.1 In this case, remove medium (~4 ml of differentiation medium + SMIFT) from each well and add  4 mL of fresh differentiation medium + FMG.
 - 16.2 Over the next 7 days, progenitors will accumulate in the media, which is not changed until day 18.



Differentiation Medium + FMG Preparation

17 We recommend adding FMG cytokines fresh (never store them at 4 °C for longer than 1 week).

17.1 To make differentiation media + FMG, add 50 ng/ml of human FLT3, 50 ng/ml of human M-CSF, and 25 ng/ml of human GM-CSF to the differentiation media prepared in advance.

Harvesting Microglial Progenitors

5m

18 On day 18, check that microglial progenitors are visible in the supernatant and are ready to be harvested.

18.1 If there are only few or no progenitors, repeat step 16 and leave the culture until day 25.

19 Collect the progenitors along with the media leaving behind the EBs in the well into a fresh 15 ml falcon tube and fresh differentiation medium + FMG for collection at day 32.

19.1 Spin down the collected progenitors at 300 r.c.f. for 5 min.

5m

20 Efficiency can be assessed by two markers (CD14 and CX3CR1) expression by Flow Cytometry analysis.

20.1 CX3CR1 expression used to be slower than CD14 (but it will go up if you culture them more), and we used to see over 90% of cells are CD14 positive at Day 18 and Day 25.

21 Microglial progenitors also can be used for mono-culture with advanced DMEM/F12 supplemented with N2, Glutamax, Penicillin-Streptomycin, 2-Mercaptoethanol with the addition of IL-34 (100 ng/μl) and M-CSF (25 ng/μl), and at least culture them up to two weeks for maturation.

21.1 Change fresh media every 2-3 days.



Protocol references

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