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Induced Pluripotent Stem Cells (iPSCs) Differentiation into iNeurons

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Patricia Yuste-Checa[1], F Ulrich Hartl[1]

[1]Department of Cellular Biochemistry, Max Planck Institute of Biochemistry, Martinsried, Germany

Patricia Yuste-Checa
MPI Biochemistry

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We use this protocol and it's working

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Abstract

This protocol details how to efficiently differentiate induced pluripotent stem cells (iPSCs) into iPSC-derived forebrain-type neurons using the commercial kits, STEMdiff SMADi Neural Induction Kit (08581, Stem Cell Technologies, monolayer protocol), STEMdiff Forebrain Neuron Differentiation Kit (08600, Stem Cell Technologies) and STEMdiff Forebrain Neuron Maturation Kit (08605, Stem Cell Technologies).

Materials

- Matrigel (Corning) or Geltrex (Thermo Fisher Scientific)
- ROCK inhibitor (Y27632)
- iNeurons in Neurobasal Plus medium (Thermo Fisher Scientific) supplemented with:

1. B-27 Plus Supplement 1x (Thermo Fisher Scientific)
2. [M] 0.5 millimolar (mM) GlutaMAX (Thermo Fisher Scientific)
3. 100 U/ml penicillin, 100 µL streptomycin sulfate (Thermo Fisher Scientific).

- Anti-MAP2 antibody (AB554, Merck)
- beta-3 Tubulin Monoclonal Antibody (TU-20) **Thermo Fisher Scientific Catalog #**MA1-19187

- STEMdiff™ SMADi Neural Induction Kit 1 Kit **STEMCELL Technologies Inc. Catalog #**8581
- STEMdiff™ Forebrain Neuron Differentiation Kit **STEMCELL Technologies Inc. Catalog #**08600
- STEMdiff™ Forebrain Neuron Maturation Kit **STEMCELL Technologies Inc. Catalog #**08605

iPSC Culture

1 Coat 6-well plates with Matrigel or Geltrex by dilution 1/100 in F-12 DMEM medium (for example by diluting 100 µL in 10 mL F-12 DMEM). Add 1 mL /well (6-well plate) and incubate at least 1 hour in the CO_2 incubator (37 °C).

Note

After the incubation time, you can keep the plates at 4 °C for several weeks. Make sure the liquid does not evaporate by adding more F-12 DMEM after coating was completed.

2 Thaw quickly a cryotube containing iPSCs in a water bath at 37 °C . Add the cells to 5 mL F-12 DMEM and centrifuge at 300 x g, 00:04:00 .

4m

3 Remove the supernatant, resuspend the pellet in mTeSR or mTeSR plus medium supplemented with [M] 10 micromolar (µM) ROCK inhibitor with a Pasteur plastic pipette to avoid formation of single cells, place cell suspension in the Matrigel-coated well, move the plate softly in several back-and-forth and side-to-side motions to distribute the cells evenly and incubate in the CO_2 incubator (37 °C , 5% CO_2).

Note

- iPSCs grow in colonies and are routinely passaged as small cell aggregates or clumps. Generation of single cells is stressful for the cells and can lead to genetic aberrations. Therefore, a Pasteur plastic pipette or a 1000 µL pipette (wide openings, if using 1000 µl pipette, do not pipette the cells more than 4-5 times) should be used when routinely passaging iPSCs to prevent production of single cells.
- ROCK inhibitor (Y27632) is an apoptosis inhibitor that should be added to the cells when the cells are likely to be stressed (e.g. when thawing or splitting with Accutase to generate single cells, see below).

4 Do full-medium change according to medium manufacturer's instructions (mTeSR every day, mTeSR plus every other day).

5 Once confluent, split the cells using ReLeSR (Stem Cell Technologies) or similar (PBS-EDTA) to obtain cellular aggregates and avoid making single cells for maintenance.

Generation of Neural Progenitor Cells (NPCs, Monolayer Protocol)

6 Remove the medium from the wells.

7 Add 1 mL Accutase to make single cells and incubate for 00:05:00 in the CO_2 incubator (37 °C , 5% CO_2). 5m

8 Add 1 mL F-12 DMEM supplemented with [M] 10 micromolar (µM) Y-27632 (ROCK inhibitor) (no need of ROCK inhibitor if you are quick) and collect the cells with the 1000 µL pipette.

9 Count viable cells using Trypan blue staining.

10 Centrifuge the cells at 300 x g, 00:04:00 . 4m

11 Aspirate the supernatant and resuspend the pellet with STEMdiff Neural Induction medium supplemented with SMADi and [M] 10 micromolar (µM) Y-27632 (ROCK inhibitor) to a final concentration of 1×10^6 cells/mL.

12 Add 2 mL of cell suspension per Matrigel-coated well of a 6-well plate (2×10^6 cells/well), move the plate softly in several back-and-forth and side-to-side motions to distribute the cells evenly and incubate in the CO_2 incubator (37 °C , 5% CO_2).

13 Do a full-medium change with STEMdiff Neural Induction Medium supplemented with SMADi every day for the next 6 days.

14 On day 7 (Day in vitro (DIV) 7), remove the medium from the well, add 1 mL Accutase and incubate for 00:05:00 in the CO_2 incubator (37 °C , 5% CO_2). 5m

15 Add 1 mL F-12 DMEM supplemented with [M] 10 micromolar (µM) Y-27632 (ROCK inhibitor) (no need of ROCK inhibitor if you are quick) and collect the cells with the 1000 µL pipette.

16 Centrifuge the cells at 300 x g, 00:04:00 . 4m

17 Aspirate the supernatant and resuspend the pellet with 2 mL STEMdiff Neural Induction Medium supplemented with SMADi and [M] 10 micromolar (µM) Y-27632 (ROCK inhibitor).

18 Count viable cells using Trypan blue staining.

19 Dilute cells to a final concentration of 1×10^6 cells/mL with STEMdiff Neural Induction Medium supplemented with SMADi and 10 µM Y-27632 (ROCK inhibitor).

20 Add 2 mL of cell suspension per Matrigel-coated well of a 6-well plate (2×10^6 cells/well), move the plate softly in several back-and-forth and side-to-side motions to distribute the cells evenly and incubate in the CO_2 incubator (37 °C , 5% CO_2).

21 Do a full-medium change with STEMdiff Neural Induction Medium supplemented with SMADi every day for the next 6 days.

22 Repeat splitting on DIV 13 (steps 14-20).

23 On DIV 20, split cells as previously described (steps 14-18).

24 Centrifuge cells and freeze neural progenitor cells (NPCs) using commercial freezing media or STEMdiff Neural Induction Medium supplemented with SMADi and 10% DMSO ($2\text{-}4\times10^6$ cells/cryotube).

Note

On DIV 20 split, you can also plate the cells directly to continue the differentiation as described in the next section.

Generation of iPSC Derived-Forebrain-Type Neurons from NPCs

25 Coat Poly-L-Ornithine/Laminin (PO/L) wells. Coat wells of a 6-well plate with 500 µL poly-Ornithine solution (0.01% in PBS) during 04:00:00 or more hours at Room temperature. Aspirate solution, then add 500 µL Laminin solution (10-20 µg/mL Laminin in PBS) per well of a 6-well plate during 4 hours to Overnight (better) at Room temperature. 4h

Note

1. To coat a 12-well plate use 300 µL - 350 µL PO and L and for a 24-well plate use 200 µL - 250 µL PO and L.
2. You can keep the coated plates at 4 °C for several weeks. Make sure the liquid does not evaporate by adding more PBS after coating was completed.

26 Thaw NPCs quickly in a water bath, add cells to 5 mL of F-12 DMEM media and centrifuge at 300 x g, 00:04:00.

27 Discard supernatant, resuspend the pellet with STEMdiff Neural Induction Medium supplemented with SMADi to a final concentration of 1×10^6 cells/mL.

28 Add 2 mL of cell suspension per PO/L-coated well of a 6-well plate (2×10^6 cells/well), move the plate softly in several back-and-forth and side-to-side motions to distribute the cells evenly and incubate in the CO_2 incubator (37 °C , 5% CO_2).

29 Do full-medium change with STEMdiff Forebrain Neuron Differentiation Medium for the next 5 days.

30 On day 6 perform the final plating of the cells. Remove medium from the well, add 1 mL Accutase and incubate for 00:05:00 in the incubator (37 °C , 5% CO_2). 5m

31 Add 1 mL F-12 DMEM and collect the cells with the 1000 µL pipette.

32 Centrifuge cell at 300 x g, 00:04:00 . 4m

33 Aspirate the supernatant, resuspend the pellet with 4 mL STEMdiff Forebrain Neuron Maturation Medium and pass the cells through a strainer to make single cells.

34 Count viable cells using Trypan blue staining.

35 Dilute cells to the desired final concentration with STEMdiff Forebrain Neuron Maturation Medium, dispense them in PO/L-coated wells, move the plate softly in several back-and-forth and side-to-side motions to distribute the cells evenly and incubate in the CO_2 incubator (37 °C , 5% CO_2).

> Note
>
> Dispense 5x10^5 cells per well of a 6-well plate, 2.5x10^5 cells per well of a 12-well plate and 1x10^5 cells per well of a 24-well plate.

36 Do half-medium change every other day with STEMdiff Forebrain Neuron Maturation Medium supplemented with [M] 5 micromolar (µM) 5-Fluorouracil/Uridine (Merck) to stop growth of non-differentiated cells.

37 After 8 days in STEMdiff Forebrain Neuron Maturation Medium, maintain iNeurons in Neurobasal Plus medium supplemented with B-27 Plus Supplement 1x, 0.5 mM GlutaMAX and 100 U/ml penicillin, 100 µL streptomycin sulfate. Perform half-medium change every 2-3 days.

Note

Neuronal differentiation may be assessed by immunofluorescence using the anti-MAP2 antibody, 1/500 dilution and the anti-β-3-tubulin, 1/100 dilution or similar.