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Human iPSC culture and directed differentiation to Midbrain Dopaminergic neurons.

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ABSTRACT

Generation of hiPSC derived midbrain dopaminergic neurons.

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iPSC culture and maintenance 1d

1
- Human induced pluripotent stem cells (hiPSCs) were maintained in feeder-free monolayers on Geltrex (ThermoFisherScientific) and fed daily with Essential 8 medium (Life Technologies).
- Grow cells on 6-well tissue culture plates.
- When confluent, hiPSCs were passaged using 0.5 µM EDTA (Life Technologies). All cells were maintained at 37°C and 5% carbon dioxide.

Midbrain Dopaminergic Neuron protocol

2 **Media for neuronal induction is Neurobasal and DMEM-F12 supplemented as follows:**

- **Neurobasal (B27):**

Neurobasal phenol red free (500ml): thermofisher cat number: 12348017
Add the following supplements into 1x 500ml bottle:
B27 Supplement (10ml): thermofisher cat number: 17504044
Glutamax (5ml): thermofisher gibco cat number: 35050-038
Pen/Strep (2.5ml): thermofisher gibco cat number: 15140122 10,000U/ml

- **DMEM-F12 (N2):**

DMEM-F12 (500ml): thermofisher cat number: 10565018
Add the following supplements into 1x 500ml bottle:
N2 Supplement (5ml) : thermofisher cat number: 17502048
MEM Non-essential amino acids (5ml): thermofisher gibco cat number: 11140-050
Pen/Strep (2.5ml): thermofisher gibco cat number: 15140122 10,000U/ml
Beta-mercaptoethanol (910ul): thermofisher gibco cat number: 21985-023
Insulin solution human (250ul): sigma cat number: I9278

Media we use is a 1:1 mix of the supplemented Neurobasal (B27) and supplemented DMEM-F12 (N2), termed "N2B27".

2.1 Before starting an induction, make the media above. Then make **"Media A"** which consists of:
- 50ml N2B27
- 2uM Dorsomorphin (Tocris 3093/10)
- 5uM SB 431542 (Tocris 1614/10)
- 1uM CHIR99021 (Miltenyi Biotec 130-104-172)

2.2 On Day 0, check iPSCs to see if they are ~100% confluent. Aspirate iPSC media and wash cells one with 2ml PBS (ThermoFisher Gibco) per well. Then add 5ml of **"Media A"** to each 6-well plate to initiate differentiation.

2.3 On Day 1, aspirate media and replace with fresh 5ml of **"Media A".**

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2.4 On Day 2, prepare **"Media B"** which consists of:

- 50ml N2B27
- 2uM Dorsomorphin (Tocris 3093/10)
- 5uM SB 431542 (Tocris 1614/10)
- 1uM CHIR99021 (Miltenyi Biotec 130-104-172)
- 1uM Purmorphamine (Merck Millipore SML0868)

Aspirate media and replace with 5ml of "Media B".

2.5 On Day 3, aspirate media and replace with fresh 5ml of **"Media B".**

2.6 On Day 4, split the cells in a 1:2 dilution following the **"Dispase protocol"** below, and plate the split cells into new Geltrex pre-coated 6-well plates in 2ml per well of **"Media B" + 10µM Y-27632 dihydrochloride** (Tocris).

2.7 On Day 5 till and including day 7 aspirate the media and replace with fresh 5ml of **"Media B".** Daily media change.

2.8 On Day 8, prepare **"Media C"** which consists of:

- 50ml N2B27
- 2uM Dorsomorphin (Tocris 3093/10)
- 1uM Purmorphamine (Merck Millipore SML0868)

Aspirate media and replace with 5ml of **"Media C"**.

2.9 On Day 9, aspirate media and replace with fresh 5ml of **"Media C".**

2.10 On Day 10, split the cells in a 1:2 dilution following the **"Dispase protocol"** below, and plate the split cells into new Geltrex pre-coated 6-well plates in 2ml per well of **"Media C" + 10µM Y-27632 dihydrochloride** (Tocris).

2.11 On Day 11 till and including day 13 aspirate the media and replace with fresh 5ml of **"Media C".** Daily media change.

2.12 On Day 14, split the cells in a 2:3 dilution following the **"Dispase protocol"** below, and plate the split cells into new Geltrex pre-coated 6-well plates in 2ml per well of just **N2B27 + 10µM Y-27632 dihydrochloride** (Tocris). At this

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point, NPCs can also be cryopreserved

2.13 On day 15, aspirate the media and replace with 5ml of **N2B27.** The cells are now midbrain NPCs, and media can be changed every 2 days until final plating.

2.14 On day 19 prior to final plating, coat plates, ibidi chambers, 96-well plates, coverslips with Geltrex for final plating of NPCs for terminal differentiation into neurons. At this point, NPCs can also be cryopreserved

- Using Accutase (ThermoFisher Gibco), get cells into a single cell suspension and count the cells.
- Make up N2B27 + 10µM Y-27632 dihydrochloride to plate cells in.
- Prepare cell stocks to obtain a final plating density of 500,000 cells per ml.

Plate the cells into final chambers:

- 100,000 cells per well of a µ-Slide 8 Well (Ibidi chamber).
- 50,000 cells per well of a 96-well plate.
- 500,000 cells of a 12-well plate.

2.15 On day 20, prepare Terminal Differentiation media:

- 50ml N2B27
- 10µM Y-27632 dihydrochloride
- 0.1µM Compound E (Enzo Life Sciences).

Aspirate media and replace with **"Terminal Differentiation media".**

Cells need to be changed with Terminal Differentiation media twice weekly until desired differentiation time point (3-5 weeks).

Dispase Protocol

3 On the days indicated above, cells need to be enzymatically dissociated using Dispase. Before beginning coat 6-well plates with Geltrex. On day 4, and 10 cells are split 1:2 (1 well of a 6-well plate is split into 2 wells).
On day 14, cells are split 2:3 (2 wells of a 6-well plate are split into 3 wells).

- Prepare dispase: Dissolve dispase powder in sterile PBS (ThermoFisher Gibco) to obtain a concentration of 1mg/ml. Make sure dispase is completely dissolved. Can increase speed of dissolving by shaking the falcon tube.
- Filter the dissolved dispase in a sterile environment through a 0.22um filter.
- Add a 1:10 dilution to each well of the 6-well plate containing the cells (500ul of dispase to each well containing 5ml of media).
- Leave in the incubator for ~20 minutes checking the plate every 10 minutes.
- Whilst cells are in the dispase, add 10ml of sterile PBS to a 15ml falcon, 1 falcon for each iPSC line.
- Add DNase I (Roche) to each falcon containing PBS to achieve a final concentration of 0.1mg/ml DNase I (stock is made up to be 10mg/ml). Add 100ul per falcon containing 10ml of PBS.

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- Once cells have lifted (they should lift as a continuous sheet), add the cells to its respective 15ml falcon containing PBS and DNase I. Invert the falcon 2-3 times to break up, and wash the cells.
- Allow the cells to settle to the bottom of the falcon with gravity flow.
- Once they have settled, aspirate the PBS and 10ml of fresh sterile PBS to each falcon. Invert the falcon again 2-3 times to break up cells into smaller clumps.
- Allow the cells to settle to the bottom of the falcon with gravity flow.
- In the meantime prepare the required media with the extra addition of **10µM Y-27632 dihydrochloride.**
- When cells have all settled to the bottom, aspirate the PBS and add the required amount of media to each falcon to achieve a final volume of 2ml per well of a 6-well plate (for eg. splitting 1 well into 2, the total will be 4ml in the falcon. 2ml will then be added to each well).
- Pipette up and down using a 5ml stripette to break up clumps of cells.
- Add the cell suspension to the 6-well plate achieving the desired cell split. Move the cells in several quick side-to-side and back-and-forth motions to disperse the cells uniformly across the well. Place them in the incubator.

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