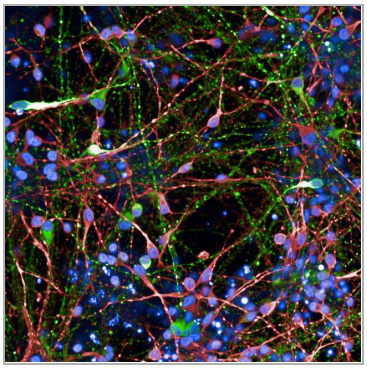

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Differentiation of human Dopamine Neurons (DaNs) from induced pluripotent stem cells (iPSCs) V.1

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ABSTRACT

This protocol employs a floor plate-based differentiation method to produce human midbrain dopaminergic neurons from induced pluripotent stem cells. Based on Kriks, S. *et al.*, 2011 with adaptations from Fedele, S. et al., 2017, this protocol incorporates expansion of floor plate progenitors which can be frozen and thawed with limited cell death. By the end of the protocol, tyrosine hydroxylase positive neurons are produced with high efficiency that fire action potentials and produce and release dopamine upon stimulus.

MATERIALS

Reagents:

- Accutase (Stem Cell Tecnologies, CAT# 07920)
- anti-anti (ThermoFisher Scientific, CAT# 15240062)
- Ascorbic acid (Sigma-Aldrich, CAS# 50-81-7, SKU# A4544-25G)
- B-27™ Supplement (50X), serum free (ThermoFisher Scientific, CAT# 17504044)
- Recombinant Human/Murine/Rat BDNF (BDNF) (PeproTech, CAT# 450-02)
- Biolaminin 521 LN (LN521) (BioLamina, CAT# LN521)

PROTOCOL integer ID: 77411

Keywords: iPSCs, differentiation, human dopamine neurons

- B-Mercaptoethanol (ThermoFisher Scientific, CAT# 21985023)
- CHIR 99021 (Bio-Techne/Tocris/R&D, CAT# 4423)
- GABA (Sigma-Aldrich, SKU# A2129)
- cCAMP (Sigma-Aldrich, CAT# D0627. CAS# 16980-89-5)
- Cytosine β-D-arabinofuranoside (AraC) (Sigma-Aldrich, CAS# 147-94-4, SKU# C1768)
- DAPT, gamma-Secretase inhibitor (Abcam, CAT# ab120633, CAS# 208255-80-5)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, CAS# 67-68-5)
- DMEM/F12 basal medium (ThermoFisher Scientific, CAT# 11320033)
- DMEM/F-12, GlutaMAX™ supplement (ThermoFisher Scientific, CAT# 10565018)
- FGF8 (Stratech, CAT# 16124-HNAE-SIB)
- GDNF (Peprotech, CAT# 450-10)
- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific, CAT# A1413202)
- KnockOut™ DMEM (KO DMEM) (ThermoFisher Scientific, CAT# 10829018)
- KnockOut™ Serum Replacement (KSR) (ThermoFisher Scientific, CAT# 10828028)
- Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma-Aldrich, CAS# 114956-81-9, SKU# L2020-1MG)
- LDN193189 hydrochloride (Sigma-Aldrich, CAS# 1062368-24-4, SKU# SML0559-5MG)
- L-glutamine (CAT# 25030-024)
- Matrigel (CAT# 354277)
- MEM Non-Essential Amino Acids Solution (100X) (NEEA) (ThermoFisher Scientific, CAT# 11140050)
- Mitomycin C (Abcam, CAT# ab120797-2mg, CAS# 50-07-7)
- mTeSR™1 (Stem Cell Technologies, CAT# 85850_C)
- N-2 Supplement (100X) (ThermoFisher Scientific, CAT# 17502048)
- Neurobasal (ThermoFisher Scientific, CAT#2113049)
- NT-3 human (Sigma-Aldrich, SKU# SRP3128-10UG)
- Penicillin-Streptomycin (10,000 U/mL) (ThermoFisher Scientific, CAT# 15140122)
- Phosphate-buffered saline, pH 7.4 (PBS) (Life Technologies, CAT# 10010056)
- Poly-D-Lysine (ThermoFisher Scientific, CAT# A3890401)
- Poly-L-ornithine (PLO) solution (Sigma-Aldrich, CAS# 27378-49-0, SKU# P4957-50ML)
- Purmophamine (Puro) (Bio-Techne, CAT# 4551/10)
- Recombinant Human Sonic Hedgehog/Shh (C24II) N-Terminus (SHH) (Bio-techne, CAT# 1845-SH-500)
- ROCK inhibitor Y-27632 (ROCKi) (CAT# 1254)
- SB 431542 (Bio-Techne | tocris, CAT# 1614, CAS# 301836-41-9)
- Sodium pyruvate (CAT# 11360070)
- Solution 18 (ChemoMetec, CAT# 910-3018)
- TGF-β3 (Peprotech, CAT# 100-36E)

Preparing KO DMEM KSR base media:

1. Remove 90mL of KO DMEM (total 500 mL) and save for spinning tubes.
2. Add to KO DMEM:
   - 75 mL of KSR (15%)
   - 10µM B-mercaptoethanol
   - 1:100 L-glutamine
   - 1x NEAA

Preparing NNB base media:

1. Add to 500 mL Neurobasal (without Vitamin A) media:
   - 1:200 N2 (2.5 mL in 500 mL)
   - 1:100 B27 (5 mL in 500 mL)
   - 1:100 L-glutamine (5 mL in 500 mL)

Preparing NB base media (use within 3 weeks):

1. Add to 500 mL Neurobasal (without vitamin A) media:
   - 1:50 B27 (10 mL in 500 mL)
   - 1:100 L-glutamine (5 mL in 500 mL)

Preparing Final Maturation Media:

1. Add to NB base media:
   - 1:5000 BDNF
   - 1:5000 GDNF
   - 1:10 000 TGFβ3
   - 1:10 000 DAPT
   - 1:1000 Ascorbic Acid
   - 1:200 dCAMP
   - Optional: 1:100 anti-anti

Preparing MitoC media:

1. Add to NB base media:
   - 1:1000 Mitomycin C

BEFORE START INSTRUCTIONS

All growth-factor containing medias should be prepared fresh the day of the feedings. All medias throughout the entirety of the protocol should be filtered using a 0.22µm filter prior to their use to prevent risk of contamination.

Rock inhibitor should be included at any steps containing a split or replating of cells, and its use should typically be followed by a full feed containing media with no rock inhibitor.

These cells are particularly temperature sensitive, so media should always be at least at room temperature before use but preferentially briefly warmed to 37ºC

Cells should be checked daily at least until final replating day. The most success will come from gauging best practices from experience based on how cells look under a brightfield microscope at each stage.

Differentiation of iPSCs into Neuronal Progenitor Cells (NPC..

1 **Day -2: Preparing plates for replating**

Two days before intending on starting the differentiation (Day -2), add 1 mL/well in a 6-well plate of Geltrex one day prior to replating the iPSCs to begin the differentiation.

Note

Geltrex should be prepared in KO DMEM basal medium based on manufacturer's dilution instructions and should be kept cold at all times.

Cells are typically replated the day before beginning the differentiation.

2 **Day -1: Replating iPSCs for differentiation**

Replating iPSCs for differentiation is identical to described in **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**, however, includes a cell counting step.

2.1 ***Prepare for splitting***

Follow steps described in **steps 6 and 7** of **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**.

2.2 ***Prepare for cell counting***

2.2.1. Add 49 µL of Phosphate Buffered Saline (PBS) to one Eppendorf per cell line for cell

dilution.

2.2.2. Add 1 µL of Solution 18 to a separate Eppendorf for counting.

2.3 ***Replate iPSCs***

As described in **step 7** of **Protocol: Expansion and maintenance of human induced pluripotent stem cells (iPSCs)**, pausing when cell pellet is suspended in 1 mL of mTesR media (i.e. mTesR plus their accompanying Supplement and 1% Penicilline/Streptomycin) + ROCKi (1:1000) to count cells.

2.4 ***Count cells (automatically using cell counter)***

2.4.1. Dilute cells by adding 1 µL of cell suspension to 49 µL of previously prepared PBS in an Eppendorf.

2.4.2. Mix thoroughly.

2.4.3. Take 19 µL of diluted cell mixture and add to Eppendorf containing 1 µL of Solution 18.

2.4.4. Mix thoroughly and count cells.

2.4.5. Calculate and plate cells based on the following optimal density for Day -1 plating: 1.425 million cells / well of 6-well plate (150 000/ cm^2).

Note

Cells may also be replated 2 days before starting the protocol, for which the following optimal density should be used for calculations:

Day -2 plating: 1.19 million cells/ well of a 6 well plate (125 000/$cm^{2)}$

2.4.6. Transfer cells to a 6-well plate after aspirating the Matrigel, and top up with mTesR media to have 2 mL media total.

Differentiation of iPSCs into Dopamine Neurons (DaNs)

3 Before starting, check the confluency. The iPSCs should be at least >80% confluent to start, otherwise feed the cells and wait another day. If in doubt, more confluent is better.

Thaw growth factors at room temperature and make every media fresh daily and filter immediately before use.

Days 0 through 20 of the protocol contain no antibiotics and therefore, it is extra pertinent to use the most aseptic technique.

If at any point Day 1 through Day 10 the cells peel, they can be replated by collecting the media and, if any cells remain adhered, using Accutase as described in **step 4.3.**

3.1 *Day prior to preparing differentiation medias*

3.1.1. Thaw supplements (KSR, B27 and N2) (ideally in fridge overnight).

3.1.2. Prepare KO DMEM KSR base media and NNB base media (see **Materials**).

Note

Full media changes are 4 mL / well of a 6 well plate.
Half media changes are 2mL / well of a 6 well plate.

3.2 *Day 0: Full media change*

1. Add to KO DMEM KSR base media:
1:10 000 LDN
1:1000 SB

3.3 *Day 1 : Full media change*

1. Add to KO DMEM KSR base media:
1:10 000 LDN
1:1000 SB
1:1000 SHH
1:5000 FGF8a
1:5000 Puro

3.4 *Day 2: Half media change*

1. Add to KO DMEM KSR base media:
1:10 000 LDN
1:1000 SB
1:1000 SHH
1:5000 FGF8a
1:5000 Puro

3.5 *Day 3: Full media change*

1. Add to KO DMEM KSR base media:
1:10 000 LDN
1:1000 SB
1:1000 SHH
1:5000 FGF8a
1:5000 Puro

3.6 *Day 4: Half media change*

1. Add to KO DMEM KSR base media:
1:10 000 LDN
1:1000 SB
1:1000 SHH
1:5000 FGF8a

1:5000 Puro
1:3333 CHIR

3.7 ***Day 5 : Full media change***

75% KO DMEM KSR base media
25% NNB base media
1:10 000 LDN
1:1000 SHH
1:5000 FGF8a
1:5000 Puro
1:3333 CHIR

3.8 ***Day 6: Half media change***

75% KO DMEM KSR base media
25% NNB base media
1:10 000 LDN
1:1000 SHH
1:5000 FGF8a
1:5000 Puro
1:3333 CHIR

3.9 ***Day 7: Full media change***

50% KO DMEM KSR base media
50% NNB base media
1:10 000 LDN
1:3333 CHIR

3.10 ***Day 8: Half media change***

50% KO DMEM KSR base media
50% NNB base media
1:10 000 LDN
1:3333 CHIR

3.11 ***Day 9: Full media change***

25% KO DMEM KSR base media
75% NNB base media
1:10 000 LDN
1:3333 CHIR

3.12 ***Day 10: Full media change (D10 media)***

25% KO DMEM KSR base media
75% NNB base media
1:10 000 LDN
1:3333 CHIR

Expansion of NPCs

4 **This section is optional.** If not desired, continue on to **Section: Patterning NPCs into dopaminergic neurons Day 11 to 22+**.

Expansion allows for the freezing and thawing of progenitors midway through the protocol, as well as evidence suggests the final neurons may be more mature (Fedele. S. *et al.*, 2017). It consists of maintaining progenitors cells on *Day 10 media* (D10; **step 3.12**) for 1 to 21 days with splits throughout. Therefore, time points are labelled ($D10^{+exp}$), for instance, cells in *Day 10 media* for 4 days are labelled $D10^{+4exp}$.

4.1 ***Day 10: Preparation on day prior to first expansion split***

Geltrex 6-well plates as described in **step 1.**

4.2 ***Day 10^{+1}: Preparation on the day of the first expansion split.***

4.2.1. Pre-warm spinning falcons containing 9mL of KO DMEM base media.
4.2.2. Prepare D10 media (**step 3.12**) + ROCKi (1:1000).
4.2.3. Allow an aliquot of desired volume (1mL per well of a 6-well plate) to reach room temperature.

Note

If cells have recently peeled and been replated, or are not very confluent, it is fine to maintain cells in D10 media with daily media changes and wait until cells reach confluency before splitting.

4.3 ***Day 10^{+1}: Splitting Day 10 NPCs***

Some lines may survive 1:2, but it is advisable to split cells 2:3, as there will be quite some cell death and the cells need to be fairly confluent to maintain viability and cell identity. If split too harshly, they may differentiate into other cell types.

4.3.1. Aspirate media and wash each well with 1mL of PBS.
4.3.2. Immediately aspirate PBS and add 1mL of Accutase.
4.3.3. Incubate at 37^{0}C for 5 minutes.
4.3.4. Gently collect cells using a 1000 µL pipette and place in pre-warmed spinning falcon.
4.3.5. Spin cells for 5 minutes at 350g.
4.3.6. While cells are spinning, aspirate Geltrex and replace with 3mL of pre-warmed D10 media (**step 3.12**)+ ROCKi (1:1000).
4.3.7. Aspirate media from pelleted cells, re-suspend pellet in 1mL of D10 media (**step 3.12**) + ROCKi and add dropwise to well of 6-well plate.
4.3.8. Gently swirl to distribute cells evenly around dish.

4.4 *Day 10^{+2}: Maintaining NPCs in Day 10 Expansion Media*

4.2.1. Day following expansion split, full feed cells with D10 media (**step 3.12**).

4.2.2. Subsequently feed neurons daily with D10 media (also named as D10 expansion media), alternating between half and full feeds.

4.2.3. Passage cells when confluent, generally success is found with a 1:2 split on Day 10^{+8}, Day 10^{+15} and Day 10^{+20}.

Note

Cells may be frozen down during the expansion stage of the protocol, but it is recommended to do so at the later stages for improved success rates.

4.5 *Freezing Day 10^{+exp} NPCs*

4.5.1. Passage cells as described in **step 4.3.**

4.5.2. Resuspend pellet in freezing media containing 90% D10 media (**step 3.12**) and 10% DMSO.

4.5.3. Store in liquid nitrogen.

Note

Wells of cells can be frozen and thawed at a ratio of 1 well: 1 well, particularly at the later stages of the expansion. More caution should be taken if freezing at the earlier stages of the expansion step as they can be more vulnerable (freeze/thawing at a ratio of 1.3:1 for example).

To plan accordingly for experiments, 1 well of 6 well plate will give roughly 5-10 million cells/ well, but can vary greatly for each line and differentiation.

The last split of expansion is typically seen as the last opportunity to freeze cells as freezing neurons during the patterning stage or later is not recommended due to low viability.

Patterning NPCs into Dopaminergic Neurons (DaNs)

5 Day 11: Full media change

1. Add to NB base media:
1:3333 CHIR
1:5000 BDNF
1:5000 GDNF
1:10 000 TGFβ3
1:10 000 DAPT

1:1000 Ascorbic Acid
1:200 dCAMP

6 **Day 12: Half media change**

1. Add to NB base media:
1:3333 CHIR
1:5000 BDNF
1:5000 GDNF
1:10 000 TGFβ3
1:10 000 DAPT
1:1000 Ascorbic Acid
1:200 dCAMP

Note

While plenty of cell death is present throughout this protocol, it might be especially apparent the first few days of the patterning stage.

7 **Day 13 - Day 19: Daily media changes**

Alternate half and full feeds with Final Maturation Media (see **Materials**).

Final replating of Dopamine Neurons (DaNs)

8 Replate the dopamine neurons into final desired dish on Day 20±1 of the protocol (this has been tested to be equally successful on Day 19 and 21). **Replating neurons at later stages of their maturation is not recommended and will decrease viability.**

8.1 ***One to two days prior to final replating: Dish coating***

Choice of coating will affect cell distribution and likelihood of peeling. Geltrex and PLO/Biolaminin are both suitable for replating the neurons. PLO/Biolaminin is thought to reduce peeling and provide a more distributed layer of neurons advantageous for imaging.

If using Geltrex:

1 day prior to Day 20 replating:

- Coat dishes with Geltrex prepared in neurobasal media

If using PLO/Biolaminin:

2 days prior to Day 20 replating:

- Coat plates with undiluted PLO

1 day prior to Day 20 replating:

- Wash 3x with PBS without letting plates dry.
- Dilute Biolaminin 1:10 in cold, sterile PBS.
- Aspirate and add Biolaminin at same volume as PLO.

8.2 *Replating of Day 20 Dopamine Neurons*

Note

Before starting:

- Prepare and pre-warm spinning falcons containing 9mL of neurobasal media.
- Prepare, filter and pre-warm Final Maturation Media (see **Materials**) with ROCKi (1:1000) and optional anti-anti (1:100).
- Warm Accutase to room temperature.

On Day 20 and onwards, cells may be kept in anti-anti if desired without significant effects to their morphology or activity, but notably, extensive testing has not been taken to see its effects on maturing dopamine neurons

If replating many lines, media may be aliquoted and kept in the fridge throughout the day and pre-warmed immediately before use.

8.2.1. Wash cells 1x with 1mL PBS and replace with 1 mL Accutase, incubate at 37^{o}C for 5 minutes.

8.2.2. Gently collect cells into a pre-warmed spinning falcon.

8.2.3. Spin at 350g for 5 minutes.

8.2.4. Resuspend pellet in 1 mL of pre-warmed Final Maturation Media (see **Materials**) and count cells using Solution 18.

8.2.5. Replate at final desired concentrations (**Table 1**).

	A	B	C	D
	Plate Type	**Surface Area (cm2)**	**Useful for**	**Ideal density**
	12 well	3.8	Protein & RNA collections	1 x106/ well
	24 well	1.9	Protein & RNA collections	5 x 105/ well
	24 well + 10 mm coverslip	0.78	Electrophysiology	1 x 105/ well
	96 well half area	0.15	Imaging	3-5 x 104/ well

Table 1: Recommended densities for various types of experiments.

Remove non-neuronal proliferating cell types (Mitomycin C...

9 It is normal to produce a small number of non-neuronal cells throughout this protocol, but ideally these should be at a minimum. We try to reduce the amount remaining in our culture following the final replating of post-mitotic dopamine neurons (previous section) by treating the cells with Mitomycin C on Day 22.

9.1 ***Day 22: Prepare medias***

9.1.1. Prepare MitoC media (see **Materials**), Neurobasal (wash media) and Final Maturation Media (see **Materials**).

9.1.2. Pre-warm and filter all three medias.

9.2 ***Day 22: Mitomycin C treatment***

9.2.1. Aspirate Final Maturation Media from DaNs.

9.2.2. Add pre-warmed and filtered MitoC media from **step 9.1.1.**

9.2.3. Incubate at 37^{0}C for 1 hour.

9.2.4. Aspirate Mitomycin C and wash 1x with pre-warmed Neurobasal media.

9.2.5. Aspirate Neurobasal media and replace with Final Maturation Media.

Maintaining and maturing Dopaminergic Neurons (DaNs) in...

10 Dopaminergic neurons can be kept in culture for multiple months before obvious demise. The length of survival will depend on feeding schedule, density and various other factors.

10.1 ***Day 24 - Day 25: Full media change***

First feed immediately following Mitomycin C treatment should be a full feed with Final Maturation Media (see **Materials**).

10.2 ***Day 25^{+}: Biweekly Half media changes***

10.2.1. From Day 25 onwards, half feed neurons with Final Maturation Media (see **Materials**) two to three times a week.

10.2.2. Monitor cells regularly using a brightfield microscope for cell health and contamination.

Note

The optimal time point at which to perform experiments varies depending on type of experiment. Measuring synaptic function is recommended post-Day 70, while organelle biology can be assessed as early as Day 35.