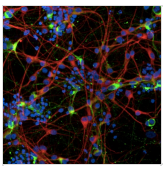

Mar 12, 2021

Optimized Derivation of Midbrain Dopaminergic Neurons from iPSCs for research application

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1 Works for me dx.doi.org/10.17504/protocols.io.bsq5ndy6

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SUBMIT TO PLOS ONE

ABSTRACT

The derivation of human induced pluripotent stem cells (iPSCs) into midbrain dopaminergic (mDA) neurons presents an exciting opportunity to access a large number of patients-specific cells in vitro, to model disease, perform target screenings, and test drug candidates. Previously published small molecule-based protocols are straightforward, implementable in automated cell culture systems (Dhingra et al. J Vis Exp. 162, 2020), and suitable for the differentiation of large sets of cell lines (Bressan et al. Protocols.io, 2020) at relatively low cost and working time. However, the derivation of mDA from a large set of human iPSC lines shows variations in differentiation efficiency between lines. In addition, the current protocols produce heterogeneous cell populations in which only a small subset represents the cells of interest. To address these issues, we optimized a previously established mDA neuron differentiation protocol (Kriks et al., Nature 480, 547–551, 2011) by: (1) adjusting the SMAD inhibition to improve and achieve more homogeneous neuron conversion between iPSC lines; (2) reinforcing the WTN signaling activation to trigger more efficient midbrain floor plate induction and conversion into mDA neurons, and (3) applying the DNA cross-linker Mitomycin-C to eliminate remaining proliferating cells. The optimized mDA neuron derivation protocol presented here resulted in more homogeneous differentiation between iPSC lines, higher yields of neurons with higher proportion of mDA neurons, and completely elimination of contaminating proliferative cells.

DOI

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PROTOCOL CITATION

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KEYWORDS

iPSC, Dopaminergic neurons, Optimization, Protocol, Differentiation, Small molecule, Mitomycin-C

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Mar 12, 2021

PROTOCOL INTEGER ID

47613

MATERIALS TEXT

1 Reagent list

2-Mercaptoethanol **Gibco - Thermo Fischer Catalog #21985023**

Accutase cell dissociation reagent **Gibco - Thermo Fischer Catalog #A1110501**

B27 supplement minus vitamin A **Gibco - Thermo Fischer Catalog #12587010**

BDNF (Brain-Derived Neurotrophic Factor) **peprotech Catalog #450-02**

CHIR99021 **R&D Systems Catalog #4423**

DAPT **Cayman Chemical Company Catalog #Cay13197**

Db-cAMP (dibutyryl-cyclic AMP) **Sigma Aldrich Catalog #D0627**

DMEM/F-12, HEPES **Gibco - Thermo Fischer Catalog #31330095**

DMSO (dimethyl sulfoxide) **Sigma Aldrich Catalog #D2650**

DPBS no calcium no magnesium **Gibco - Thermo Fischer Catalog #14190169**

Essential 8 Flex complete medium (E8) **Gibco - Thermo Fischer Catalog #A2858501**

Fibronectin (Fibro) **Corning Catalog #356008**

FGF-8b (Recombinant human/murine Fibroblast Growth Factor-8b) **peprotech Catalog #100-25**

GDNF (Glial cell line-Derived Neurotrophic Factor) **peprotech Catalog #450-10**

GlutaMAX **Gibco - Thermo Fischer Catalog #35050038**

Knockout DMEM/F-12 **Gibco - Thermo Fischer Catalog #12660012**

Knockout Serum Replacement (KSR) **Gibco - Thermo Fischer Catalog #10828028**

Laminin (Lam) **Sigma Aldrich Catalog #L2020**

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L-ascorbic acid (AA) **Sigma Aldrich Catalog #A4403**

LDN193189 **Cayman Chemical Company Catalog #Cay11802**

Matrigel hESC-Qualified Matrix, LDEV-free **Corning Catalog #354277**

Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free **Corning Catalog #356230**

MEAA (MEM Non-Essential Amino Acids) **Gibco - Thermo Fischer Catalog #11140050**

Mitomycin-C (M) **Tocris Catalog #3258**

N2 supplement **Gibco - Thermo Fischer Catalog #17502048**

Neurobasal medium **Gibco - Thermo Fischer Catalog #21103049**

Penicillin-Streptomycin **Gibco - Thermo Fischer Catalog #15140122**

Poly-L-Ornithine (PLO) **Sigma Aldrich Catalog #P3655**

Purmorphamine **Cayman Chemical Company Catalog #Cay1000963410**

HCl (Hydrochloric acid) **Carl Roth Catalog #9277**

HSA (Human Serum Albumin) **Sigma Aldrich Catalog #A6784**

SHH (recombinant human Sonic Hedgehog C24II N-Terminus) **R&D Systems Catalog #1845-SH**

Synth-a-Freeze Cryopreservation Medium **Gibco - Thermo Fischer Catalog #A1254201**

TGFβ3 (recombinant human Transforming Growth Factor-beta 3) **R&D Systems Catalog #243-B3**

Y-27632 dihydrochloride (Y) **Cayman Chemical Company Catalog #Cay10005583**

1.1 Reagent preparation and storage

1.1.1 General instructions

- Warm reagents stored at **-20 °C** to **Room temperature** before reconstitution.
- Reconstitute reagents under sterile conditions in a laminar flow hood following the instructions and dilution reagents below. Go to: Dilution of stock solutions.
- After reconstitution, aliquot stock solutions in sterile Safe-Lock tubes and store at **-20 °C** .
- Take note and control the expiration time of reagents after reconstitution.

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Note: reagents in solution might have a shorter expiration time as lyophilized reagents.

- Once thawed, reconstituted reagents can be kept for up to 5 days at 4 °C .

1.1.2 Protect from the light

- Db-cAMP
- L-ascorbic acid
- LDN193189
- Mitomycin-C

1.1.3 Minimize exposure to air

- L-ascorbic acid

1.1.4 Reconstitution of reagents

- BDNF: Reconstitute BDNF in 0.1% HSA/PBS to obtain a stock concentration of [M]20 µg/mL .
- CHIR99021: Reconstitute CHIR99021 in DMSO to obtain a stock concentration of [M]4 mM .
- DAPT: Reconstitute DAPT in DMSO to obtain a stock concentration of [M]10 mM .
- Db-cAMP: Reconstitute db-cAMP in deionized sterile water to obtain a stock concentration of [M]200 mM . Filter the stock solution with a 0.22 µm pore size hydrophilic PVDF membrane. Protect from the light.
- Fibronectin: Reconstitute fibronectin in deionized sterile water to obtain a stock concentration of [M]1 mg/mL .
- FGF-8b: Reconstitute FGF-8b in 0.1% HSA/PBS to obtain a stock concentration of [M]100 µg/mL .
- GDNF: Reconstitute GDNF in 0.1% HSA/PBS to obtain a stock concentration of [M]20 µg/mL .
- Laminin: No reconstitution required. Aliquot Laminin On ice . Store aliquots at -20 °C .
- L-ascorbic acid: Reconstitute l-ascorbic acid in deionized sterile water to obtain a stock concentration of [M]0.2 M . Minimize exposure to air. Protect from the light.
- LDN193189: Reconstitute LDN193189 in DMSO to obtain a stock concentration of [M]500 µM . Protect from the light.
- Matrigel: No reconstitution required. Aliquot Matrigel On ice . Store aliquots at -80 °C . Dilute Matrigel matrix with ice-cold DMEM/F-12 medium for coating plates.
- Poly-L-Ornithine: Reconstitute poly-l-ornithine in PBS to obtain a stock concentration of [M]10 mg/mL . Filter the stock solution with a 0.22 µm pore size hydrophilic PVDF membrane.
- Purmorphamine: Reconstitute purmorphamine in DMSO to obtain a stock concentration of [M]2 mM .
- SHH: Reconstitute SHH in 0.1% HSA/PBS to obtain a stock concentration of [M]100 µg/mL .
- SB431542: Reconstitute SB431542 in DMSO to obtain a stock concentration of [M]10 mM .
- TGFß3: Reconstitute TGFβ3 in 0.1% HSA/4 mM HCl/PBS to obtain a stock concentration of [M]20 µg/mL .
- Y-27632: Reconstitute Y-27632 in DMSO to obtain a stock concentration of [M]10 mM .

2 Equipment

- 37°C/CO2 incubator
- Cell counter
- Centrifuge for 15 mL conical tubes
- Laminar flow hood
- Light microscope
- Pipette boy
- Vacuum aspirator and tips
- Water Bath

3 Materials

1.5 mL Safe-Lock

Tubes **Eppendorf Catalog #5409331**

2 mL Cryogenic

vials **Nalgene Catalog #V5007 (Sigma Distributor)**

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6-well plate **greiner bio-one Catalog #657160**

15 mL conical centrifuge tube **greiner bio-one Catalog #188271**

50 mL conical centrifuge tube **greiner bio-one Catalog #227261**

Cell Carrier Ultra 96 black **Perkin Elmer Catalog #6055308**

CoolCell LX container **Corning Catalog #432002**

P1000, P200, P20, P10, P2.5 pipettes and filter tips **Eppendorf**

Sterile 50, 25, 10, 5 mL serological pipettes **greiner bio-one Catalog #768180**

SAFETY WARNINGS

Fatal if swallowed. Suspected of causing cancer. Toxic by ingestion

- Mitomycin-C

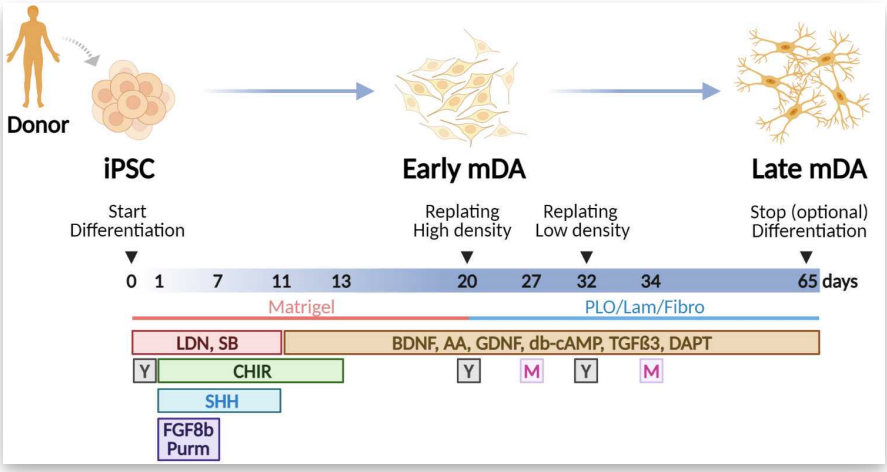
May form combustible dust concentrations in air

- L-ascorbic acid

Toxic by inhalation and ingestion. Cause skin and eye irritation

- 2-Mercaptoethanol
- CHIR99021
- DMSO
- Y-27632

BEFORE STARTING



Schematic representation of the optimized derivation of mDA neurons from iPSCs. Created with BioRender.com

Maintenance of iPSCs before start of differentiation

1 Grow iPSCs on Matrigel hESC-Qualified Matrix-coated 6-well plates in Essential 8 Flex (E8) medium for 4-5 days. Cells can be passaged once they reach 70-80% confluency (i.e., 70-80% of plate growth area is covered by a cell monolayer)

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with Gentle Dissociation Reagent (GDR) followed by seeding at a ratio of 1:6 (i.e., 1 well of a 6-well plate passaged into 6-wells).

Note: Grow thawed iPSC lines for at least five passages before the start of differentiation.

Passaging of iPSCs with Gentle Differentiation Reagent (GDR) 1h

1.1 1h

Before starting:
Coat 6-well plates with **1 mL per well** Matrigel hESC-Qualified Matrix at **37 °C** for **01:00:00** following manufacturer dilution and preparation instructions.

1.2 15m

Discard coating and add **2 mL per well** of E8 medium. Keep the plate at **37 °C** for **00:15:00** before seeding cells.

1.3 15m

Warm PBS and GDR to **Room temperature** .

1.4

Discard old culture medium and wash cells once with **1 mL per well** of PBS.

1.5

Discard PBS and add **1 mL per well** of GDR.

1.6 3m

Incubate cells at **37 °C** for **00:03:00** .

1.7

Bring the plate back to **Room temperature** and discard GDR very gently.

Critical step: Do not shake or tap the plate after incubation with GDR to avoid cell colony dislodgement and loss of cells during removal of the dissociation reagent.

1.8

Flush the wells once using a P1000 pipette filled with **1 mL** of E8 medium to dislodge cell colonies.

Critical step: do not pipette up and down cell colonies to avoid single cell dissociation.

1.9 Collect cell colonies using a P1000 pipette and seed at a ratio of 1:6 on Matrigel-coated recipient plates

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prepared in advance.

1.10

Perform a quick microscope inspection of the parent plate to check if all cell colonies were dislodged. If cell colonies are still attached to the plate, **go to step #1.8** , repeat the procedure, and seed the remaining cell colonies in the recipient plate **go to step #1.9** .

1.11 After seeding, rock the plate back-forth and side-to-side for **00:00:10** to achieve an even spread of cell colonies in the plate well. 10s

1.12 Place the plate back to **37 °C** **5 % CO2** and keep intact **Overnight** allowing cells to recover from passaging. 12h

1.13 Perform medium change after overnight recovery from passaging and, subsequently, every two days until cells reach 70-80% confluency.

Differentiation of iPSCs into midbrain dopaminergic (mDA) neurons

2

Note: This protocol is optimized for mDA neuron differentiation in **6-well plate** format.

Medium change schema:
Day 0-20: **4 mL per well**

Day 21-65: **3 mL per well**

Day 0-15: daily media changes
Day 16-20: media changes every 2 days
Day 21-65: media changes every 2-3 days

Before starting:
Perform a quick microscope inspection of the iPSCs. Ensure that iPSC colonies appear healthy (homogeneous colonies with clear borders and absence of differentiating cells; **Figure 1**). A manual removal of spontaneous differentiating cells might be required.

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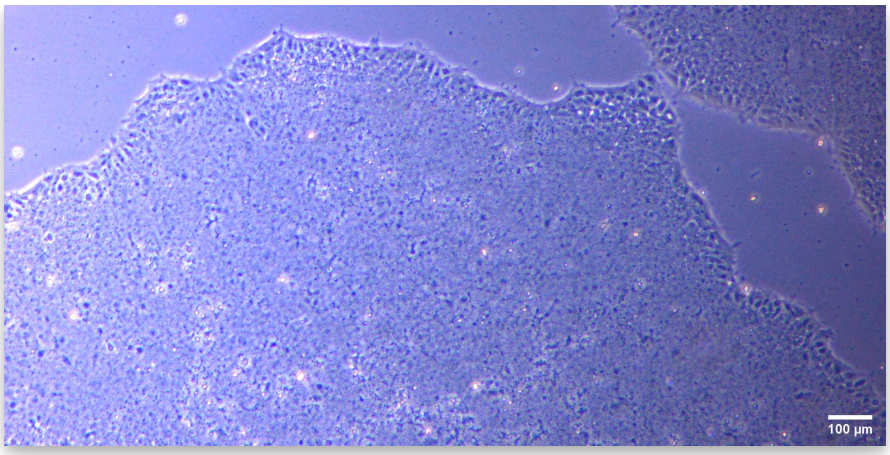

Figure 1. Representative bright-field image of iPSC colonies 4 days after passaging. Note densely packed iPSC colonies growing as cell monolayer and absence of spontaneous differentiated cells.

Day -2: Coating of 6-well plates with Growth Factor Reduced (GFR) Matrigel 1d

2.1 12h

Thaw GFR Matrigel **Overnight** **On ice** and dilute to **8.5 µg/mL** in ice cold DMEM/F-12.

2.2 12h

Coat 6-well culture plates with **1 mL per well** of GFR Matrigel solution **Overnight** at **37 °C** **5 % CO2** .

Day -1: Single cell dissociation and seeding for differentiation 1m

2.3

Discard GFR Matrigel solution and add **2 mL per well** of E8 medium supplemented with **10 µM** Y-27632 avoiding the coating to dry out.

2.4 15m

Keep the plate at **37 °C** for **00:15:00** before seeding cells.

2.5 15m

Warm PBS, Accutase and E8 medium to **Room temperature** .

2.6

Discard old culture media and wash cells once with **1 mL per well** of PBS.

2.7 Discard PBS and add **1 mL per well** of Accutase supplemented with **10 µM** Y-27632.

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2.8 30m

Incubate cells at **37 °C** for **00:30:00** .

2.9 Add **1 mL per well** of E8 medium supplemented with **10 µM** Y-27632.

2.10 Dissociate cells with a P1000 pipette by pipetting the cell suspension up and down for 3-6 times.

2.11

Perform a quick microscope inspection of the cells. If still cell colonies are visible, **go to step #2.10** and repeat the procedure. A single cell suspension should be obtained.

2.12 Transfer the cell suspension to a 15 mL conical centrifuge tube. A total volume of **12 mL** should be obtained from a full 6-well plate.

2.13 5m

Centrifuge the cell suspension at **200 x g, 23°C, 00:05:00** .

2.14 Discard the supernatant carefully and resuspend the cell pellet in **1 mL** E8 medium supplemented with **10 µM** Y-27632 by gently pipetting the cell suspension up and down for 3-6 times with a P1000 pipette to obtain a homogeneous cell suspension.

2.15 Adjust the volume with E8 medium supplemented with **10 µM** Y-27632 to **12 mL** . Mix well by 10x full inversions of the conical tube.

2.16

Perform two separate live-cell counts using a hemocytometer or an automated cell counter.
Critical step: Adjust the cell suspension volume accordingly to obtain an accurate counting. Calculate the mean achieved from the two counts and determine the concentration of live cells per milliliter.

A cell viability of 95-99% should be obtained.

Note: Working with a different brand of Accutase might impact the cell viability when following the dissociation protocol described above. If cell viability is lower than expected, reduce the incubation time with Accutase to **00:20:00** and the number of pipetting to dissociate the cell colonies to 3-4 times.

2.17 Seed 200,000 cells per cm2 in a total volume of **4 mL per well** on 6-well plates coated with GFR

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Matrigel (i.e., to start a differentiation in one well of a 6-well plate, seed 1.9 x 10^6 cells per well, considering the growth area of one well equal to 9.5 cm2).

2.18 Rock the plate back-forth and side-to-side for **00:00:10** to achieve an even spread of cells in the plate well. 10s

2.19 12h

Keep cells intact **Overnight** at **37 °C** **5 % CO2** .

Day 0: Start differentiation 1h

3 **Before starting:**

a) Prepare enough amount of Knockout Serum Replacement (KSR) medium.

For **500 mL** of KSR medium, add:

413.5 mL Knockout DMEM/F-12 medium

75 mL Knockout Serum Replacement

5 mL MEM Non-Essential Amino Acids

5 mL GlutaMAX

500 µl 2-mercaptoethanol

1 mL Penicillin-Streptomycin

Storage: KSR medium can be stored for 5 days at **4 °C** or for up to one month at **-20 °C** .

b) Reconstitute lyophilized reagents following the instructions and stock concentrations indicated in Materials (1.1.4 Reconstitution of reagents).

Use the following final concentrations:

500 nM LDN193189

10 µM SB431542

100 ng/mL SHH

2 µM Purmorphamine

100 ng/mL FGF8

4 µM CHIR99021

20 ng/mL BDNF

0.2 mM Ascorbic acid

20 ng/mL GDNF

0.5 mM db-cAMP

1 ng/mL TGFβ3

10 µM or **10 nM** DAPT

Storage: Once thawed, the stocks of small molecules and growth factors can be stored for up to 5 days at **4 °C** .

Notes: Small molecules and growth factors must be freshly added immediately before each medium change. It is strongly advised to avoid mixing different lots of reagents in the same differentiation.

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3.1

Perform a quick microscope inspection to the cells to check confluency.

Critical step: Start the differentiation with a 100% confluent cell culture, meaning that the culture area of the plate should be completely covered with a cell monolayer. Not confluent cell cultures might affect differentiation efficiency. If cell lines did not achieve 100% confluency on day 0, the number of cells seeded per cm2 on day -1 should be adjusted accordingly.

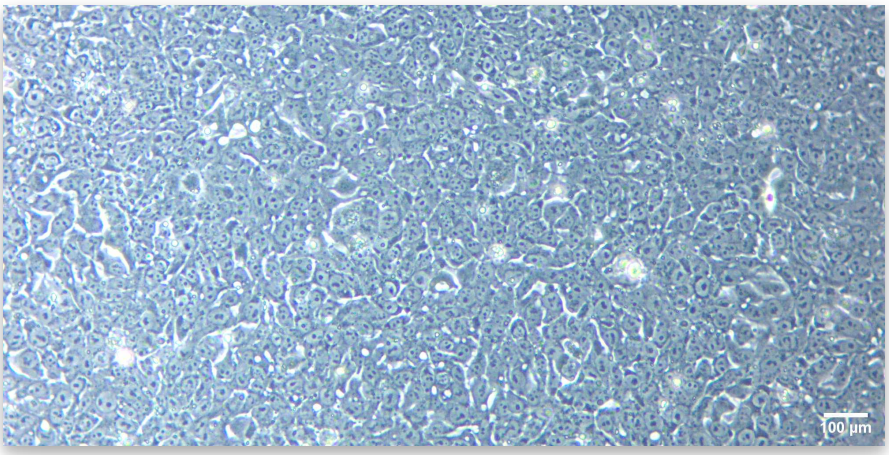

Figure 2. Representative bright-field image of iPSC on day 0 of differentiation. Note cells covering 100% of the plate culture area.

3.2 15m

Prepare differentiation medium:

Warm KSR medium at **37 °C** .

Add:

500 nM LDN193189

10 µM SB431542

Mix well by 20x full inversions of the conical tube or flask.

Critical step: Do not add small molecules to cold medium to avoid inadequate dissolution.

3.3

Perform full media change:

Discard old culture medium and add **4 mL per well** of differentiation medium very carefully avoiding touching the bottom of the well.

Critical step: To prevent cells from drying out during full media changes, change the medium of one 6-well plate at each time. Add differentiation medium very gently (dropwise) to avoid perturbation of the cell layer. Cell detachments might affect the differentiation efficiency. In case of cell detachment, a confluency above 95% is desired to continue the differentiation.

Media changes during differentiation

4 From day 1, change 75% of the differentiation medium daily until day 15, and then, every 2 days until day 20.

Note: to perform 75% medium change of a working volume of **4 mL per well** , discard **3 mL** of old medium and

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add **4 mL per well** of fresh prepared differentiation medium.

Day 1 and 2 15m

15m

4.1 Warm KSR medium at **37 °C** .

Add:

[M] **500 nM** LDN193189

[M] **10 µM** SB431542

[M] **100 ng/mL** SHH

[M] **2 µM** Purmorphamine

[M] **100 ng/mL** FGF-8b

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **4 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 3 and 4 15m

15m

4.2 Warm KSR medium at **37 °C** .

Add:

[M] **500 nM** LDN193189

[M] **10 µM** SB431542

[M] **100 ng/mL** SHH

[M] **2 µM** Purmorphamine

[M] **100 ng/mL** FGF-8b

[M] **4 µM** CHIR99021

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **4 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 5 and 6 1h

4.3 **Before starting:**

Prepare enough amount of N2 medium.

For **500 mL** of N2 medium, add:

479 mL Neurobasal medium

10 mL B27 supplement without vitamin A

5 mL N2 supplement

5 mL GlutaMAX

1 mL Penicillin-Streptomycin

Storage: N2 medium can be stored for 5 days at **4 °C** or for up to one month at **-20 °C** .

Warm KSR and N2 medium at **37 °C** .

Mix:

75 % KSR medium

25 % N2 medium

Add:

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[M] **500 nM** LDN193189

[M] **10 µM** SB431542

[M] **100 ng/mL** SHH

[M] **2 µM** Purmorphamine

[M] **100 ng/mL** FGF-8b

[M] **4 µM** CHIR99021

Mix well by 20x full inversions of the conic tube or flask.

Perform medium change: **4 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 7 and 8 15m

4.4 15m

Warm KSR and N2 medium at **37 °C** .

Mix:

50 % KSR medium

50 % N2 medium

Add:

[M] **500 nM** LDN193189

[M] **10 µM** SB431542

[M] **100 ng/mL** SHH

[M] **4 µM** CHIR99021

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **4 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 9 and 10 15m

4.5 15m

Warm KSR and N2 medium at **37 °C** .

Mix:

25 % KSR medium

75 % N2 medium

Add:

[M] **500 nM** LDN193189

[M] **10 µM** SB431542

[M] **100 ng/mL** SHH

[M] **4 µM** CHIR99021

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **4 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 11 and 12 1h

4.6 **Before starting:**

Prepare enough amount of NB/B27 medium.

For **500 mL** NB/B27 medium, add:

484 mL Neurobasal medium

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10 mL B27 supplement without vitamin A

5 mL GlutaMAX

1 mL Penicillin-Streptomycin

Storage: NB/B27 medium can be stored for 5 days at 4 °C or for up to one month at -20 °C .

Warm NB/B27 medium at 37 °C .

Add:

[M]4 µM CHIR99021

[M]20 ng/mL BDNF

[M]0.2 mM Ascorbic acid

[M]20 ng/mL GDNF

[M]0.5 mM db-cAMP

[M]1 ng/mL TGFβ3

[M]10 µM DAPT

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: 4 mL per well .

Place cells back at 37 °C 5 % CO2 .

Day 13 -15, 17 and 19 15m

15m

4.7 Warm NB/B27 medium at 37 °C .

Add:

[M]20 ng/mL BDNF

[M]0.2 mM Ascorbic acid

[M]20 ng/mL GDNF

[M]0.5 mM db-cAMP

[M]1 ng/mL TGFβ3

[M]10 µM DAPT

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: 4 mL per well .

Place cells back at 37 °C 5 % CO2 .

Day 20: Replating of mDA neuron precursors at high cell density 2d

2d

5

Note: At day 20 of differentiation, mDA neuron precursors can be replated as describe below or cryopreserved go to Step 12 .

Before starting:

Coating of 6-well plates step 1

Coat 6-well culture plates with 1 mL per well [M]0.1 mg/mL Poly-L-Ornithine (PLO) in PBS. Incubate plates Overnight at 37 °C . Wash plates three times with PBS. Discard PBS and proceed to coating step 2.

Coating of 6-well plates step 2

Coat 6-well culture plates with 1 mL per well [M]10 µg/mL Laminin plus [M]2 µg/mL Fibronectin, both diluted in

Citation: Elisangela Bressan, Ashutosh Dhingra, Stella Donato, Peter Heutink (03/12/2021). Optimized Derivation of Midbrain Dopaminergic Neurons from iPSCs for research application. https://dx.doi.org/10.17504/protocols.io.bsq5ndy6

PBS. Incubate plates **Overnight** at **37 °C** . Do not store coated plates. Proceed with preparation of plates for seeding cells.

Preparation of 6-well plates for seeding cells

Warm NB/B27 medium at **37 °C** .

Make NB/B27 complete medium by adding:

20 ng/mL BDNF

0.2 mM Ascorbic acid

20 ng/mL GDNF

0.5 mM db-cAMP

1 ng/mL TGFβ3

10 nM DAPT

10 µM Y-27632

Discard coating reagents and add **2 mL per well** of NB/B27 complete medium.

Keep the plate at **37 °C** for **00:15:00** before seeding cells.

5.1 Warm PBS, Accutase and NB/B27 medium to **Room temperature** .

5.2

Discard old culture media and wash cells once with **1 mL per well** of PBS.

5.3 Discard PBS and add **1 mL per well** of Accutase supplemented with **10 µM** Y-27632.

5.4 45m

Incubate cells at **37 °C** for **00:45:00** .

5.5 After incubation, block Accutase with **1 mL per well** NB/B27 medium supplemented with **10 µM** Y-27632.

5.6 Dissociate cells with a P1,000 pipette by pipetting the cell suspension up and down for 3-6 times.

5.7

Perform a quick microscope inspection of the cells. If a significant number of cell clumps are visible, **go to step #5.6** and repeat the procedure to dissociate remaining cell clumps. A single cell suspension should be obtained.

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5.8 Transfer the cell suspension to a conical tube. A total volume of **12 mL** should be obtained from a full 6-well plate.

5.9 5m

Centrifuge the cell suspension at **200 x g, 23°C, 00:05:00** .

5.10 Discard the supernatant carefully and add **1 mL** NB/B27 complete medium. Resuspend the cell pellet very gently with a P1,000 pipette by pipetting the cell suspension up and down for 3-6 times.

5.11 Complete the volume to **12 mL** with NB/B27 complete medium and mix well by 10ix full inversions of the conical tube.

5.12

Perform two separate live-cell counts using a hemocytometer or an automated cell counter.
Critical step: Adjust the cell suspension volume accordingly to obtain an accurate counting. Calculate the mean achieved from the two counts and determine the concentration of live cells per milliliter.

A cell viability of 90-99% should be obtained. If lower, reduce the number of pipetting to dissociate the cell clumps to 3-4 times.

5.13 Seed 300,000 cells per cm2 in a total volume of **3 mL per well** on 6-well plates coated with PLO/Laminin/Fibronectin (i.e., to make one well of a 6-well plate, seed 2.85 x 10^6 cells per well, considering the growth area of one well equal to 9.5 cm2).

5.14 Rock the plate back-forth and side-to-side for **00:00:10** to achieve an even spread of cells in the plate well. 10s

5.15 12h

Keep cells intact **Overnight** at **37 °C** **5 % CO2** .

Day 21, 23 and 25: Medium change 15m

6 From day 21, change 75% of the differentiation medium every 2-3 days. 15m

Warm NB/B27 medium at **37 °C** .

Add:

20 ng/mL BDNF

0.2 mM Ascorbic acid

20 ng/mL GDNF

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[M]**0.5 mM** db-cAMP

[M]**1 ng/mL** TGFβ3

[M]**10 nM** DAPT

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **3 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

6.1 Images of mDA neurons differentiated until day 26.

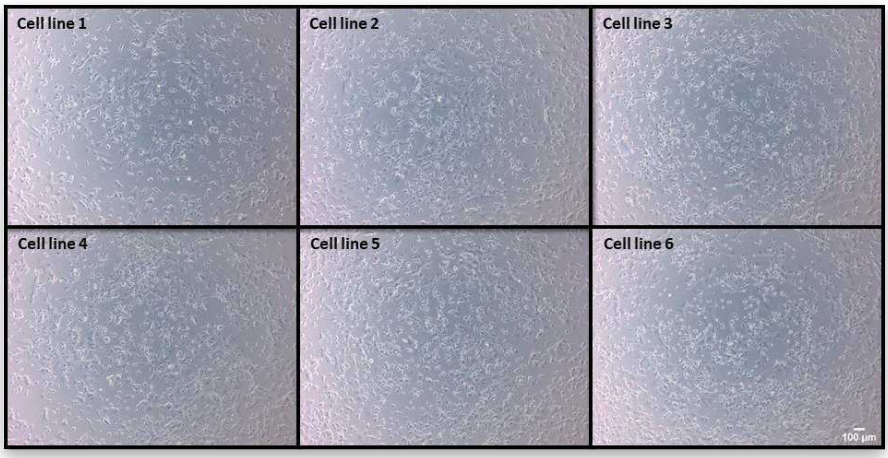

Figure 3. Representative bright-field images of mDA neurons on day 26 of differentiation. Note homogeneous differentiation between iPSC lines.

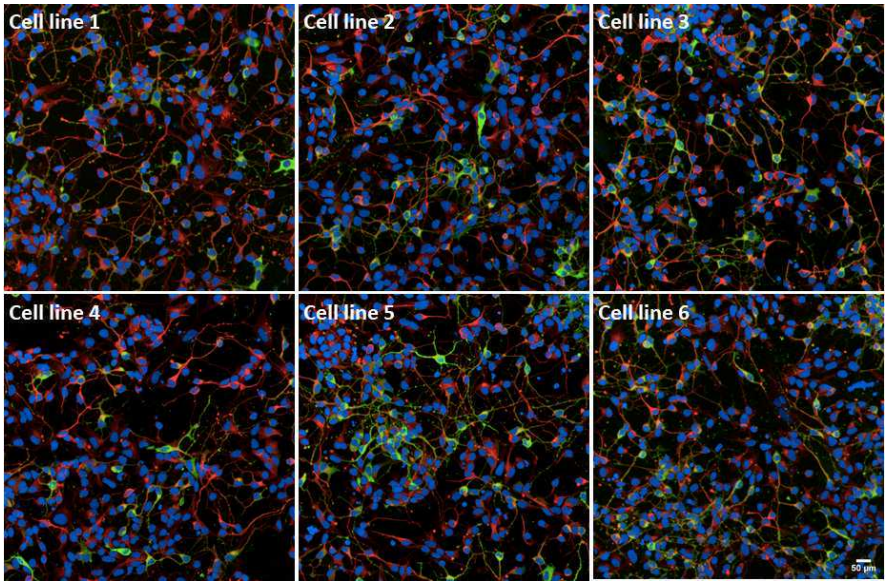

Figure 4. Representative fluorescent images of mDA neurons on day 26 of differentiation. Green: TH, Tyrosine Hydroxylase (mDA neuron); Red: TUJ1, Neuron-specific class III beta-tubulin (neuron); Blue: DAPI (nuclei).

Day 27: Treatment with Mitomycin-C 15m

15m

7 Before starting:

Warm NB/B27 medium at **37 °C** .

Make NB/B27 complete medium by adding:

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[M]**20 ng/mL** BDNF

[M]**0.2 mM** Ascorbic acid

[M]**20 ng/mL** GDNF

[M]**0.5 mM** db-cAMP

[M]**1 ng/mL** TGFβ3

[M]**10 nM** DAPT

Prepare enough amount of NB/B27 complete medium supplemented with [M]**1 µg/mL** Mitomycin-C. Mix well by 20x full inversions of the conical tube or flask.

7.1 Discard old culture medium and add **1.5 mL per well** of NB/B27 complete medium supplemented with Mitomycin-C.

7.2 1h

Incubate cells for **01:00:00** at **37 °C** .

7.3 Discard medium containing Mitomycin-C and add **3 mL per well** of NB/B27 complete medium.

7.4 Place cells back at **37 °C** **5 % CO2** .

Day 29 and 31: Medium change 15m

8 15m

Warm NB/B27 medium at **37 °C** .

Add:

[M]**20 ng/mL** BDNF

[M]**0.2 mM** Ascorbic acid

[M]**20 ng/mL** GDNF

[M]**0.5 mM** db-cAMP

[M]**1 ng/mL** TGFβ3

[M]**10 nM** DAPT

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **3 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 32: Replating of mDA neurons at low cell density (optional) 2d

9 2d

A second replating of the mDA neurons at low cell density in 96-well plate format can be performed for imaging assays requiring single cell analysis.

Before starting:

Coating of 96-well plates step 1

Citation: Elisangela Bressan, Ashutosh Dhingra, Stella Donato, Peter Heutink (03/12/2021). Optimized Derivation of Midbrain Dopaminergic Neurons from iPSCs for research application. https://dx.doi.org/10.17504/protocols.io.bsq5ndy6

Coat 96-well culture plates with **100 µL per well** **0.1 mg/mL** Poly-L-Ornithine (PLO) in PBS. Incubate plates **Overnight** at **37 °C** . Wash plates three times with PBS. Discard PBS and proceed to coating step 2.

Coating of 96-well plates step 2

Coat 96-well culture plates with **100 µL per well** **10 µg/mL** Laminin plus **2 µg/mL** Fibronectin, both diluted in PBS. Incubate plates **Overnight** at **37 °C** . Do not store coated plates. Proceed with preparation of plates for seeding cells.

Preparation of 96-well plates for seeding cells

Warm NB/B27 medium at **37 °C** .

Make NB/B27 complete medium by adding:

20 ng/mL BDNF

0.2 mM Ascorbic acid

20 ng/mL GDNF

0.5 mM db-cAMP

1 ng/mL TGFβ3

10 nM DAPT

10 µM Y-27632

Cool down NB/B27 complete medium to **Room temperature** .

Add:

10 µg/mL Laminin

2 µg/mL Fibronectin

Discard coating reagents and add **100 µL per well** of NB/B27 complete medium supplemented with Laminin and Fibronectin.

Keep the plate at **37 °C** for **00:15:00** before seeding cells.

9.1 Dissociate cells following the steps described for replating mDA neurons on day 20 **go to step #5** .

9.2 Seed 100,000 cells per cm2 in a total volume of **200 µl per well** in 96-well plates (i.e., to make one well of a 96-well plate, seed 32,000 cells per well, considering the growth area of one well equal to 0.32 cm2).

9.3 Rock the plate back-forth and side-to-side for **00:00:10** to achieve an even spread of cells in the plate well. 10s

9.4 Keep cells intact for **48:00:00** at **37 °C** **5 % CO2** . 2d

Day 34: Treatment with Mitomycin-C 15m

10 **Before starting:** 15m

Warm NB/B27 medium at **37 °C** .

Make NB/B27 complete medium by adding:

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[M] **20 ng/mL** BDNF

[M] **0.2 mM** Ascorbic acid

[M] **20 ng/mL** GDNF

[M] **0.5 mM** db-cAMP

[M] **1 ng/mL** TGFβ3

[M] **10 nM** DAPT

Prepare enough amount of NB/B27 complete medium supplemented with [M] **1 µg/mL** Mitomycin-C. Mix well by 20x full inversions of the conical tube or flask.

10.1 Discard old culture medium and add **100 µl per well** of NB/B27 complete medium supplemented with Mitomycin-C.

10.2 1h

Incubate cells for **01:00:00** at **37 °C** .

10.3 Discard medium containing Mitomycin-C and add **200 µL per well** of NB/B27 complete medium.

10.4 Place cells back at **37 °C** **5 % CO2** .

Every 2-3 days: Change medium for terminal differentiation of mDA neurons 15m

11 Warm NB/B27 medium at **37 °C** . 15m

Add:

[M] **20 ng/mL** BDNF

[M] **0.2 mM** Ascorbic acid

[M] **20 ng/mL** GDNF

[M] **0.5 mM** db-cAMP

[M] **1 ng/mL** TGFβ3

[M] **10 nM** DAPT

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **3 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Every 10 days: Supplement differentiation medium with Laminin and Fibronectin 15m

11.1 Warm NB/B27 medium at **37 °C** . 15m

Make NB/B27 complete medium by adding:

[M] **20 ng/mL** BDNF

[M] **0.2 mM** Ascorbic acid

[M] **20 ng/mL** GDNF

[M] **0.5 mM** db-cAMP

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[M] **1 ng/mL** TGFβ3

[M] **10 nM** DAPT

Cool down NB/B27 complete medium to **Room temperature** .

Add:

[M] **10 µg/mL** Laminin

[M] **2 µg/mL** Fibronectin

Mix well by 20x full inversions of the conical tube or flask.

11.2 Discard old culture medium. Perform medium change as following:

3 mL per well in 6-well plates

200 µl per well in 96-well plates

11.3 Place cells back at **3 °C** **5 % CO2** .

11.4 **Images of mDA neurons differentiated until day 65.**

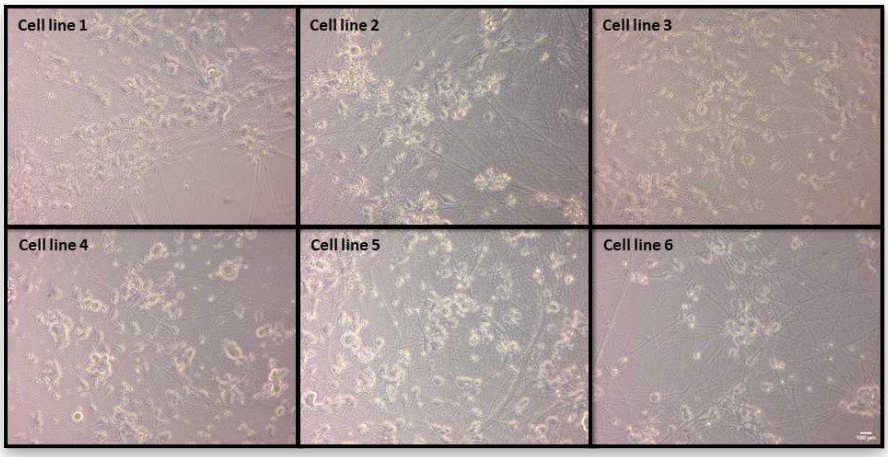

Figure 5. Representative bright-field images of mDA neurons on day 65 of differentiation. Note high number of neurons.

Citation: Elisangela Bressan, Ashutosh Dhingra, Stella Donato, Peter Heutink (03/12/2021). Optimized Derivation of Midbrain Dopaminergic Neurons from iPSCs for research application. https://dx.doi.org/10.17504/protocols.io.bsq5ndy6

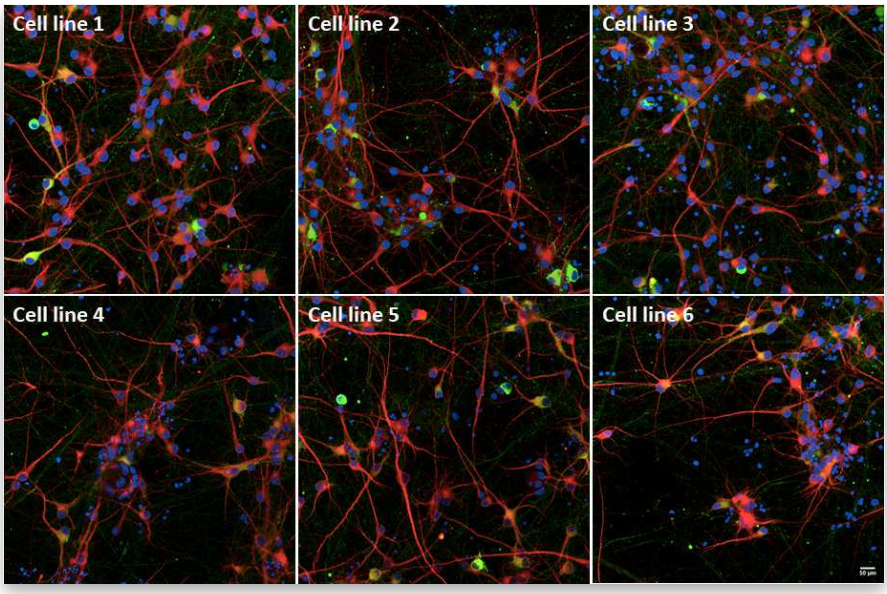

Figure 6. Representative fluorescent images of mDA neurons on day 65 of differentiation. Green: TH, Tyrosine Hydroxylase (mDA neuron); Red: TUJ1, Neuron-specific class III beta-tubulin (neuron); Blue: DAPI (nuclei).

Cryopreservation of mDA neurons on day 20 5m

12 **Before starting:**

Supplement Synth-a-Freeze cryopreservation medium with [M]**10 µM** Y-27632.

Place the cryopreservation medium and a CoolCell LX container on ice.

12.1 Perform cells dissociation and previously described **go to step #5** .

12.2 5m

After determining the number of live cells, centrifuge the cell suspension at **200 x g, 23°C, 00:05:00** .

12.3 Resuspend the cell pellet very gently in ice cold Synth-a-Freeze medium to 3-5x10^6 cells/mL.

12.4 Distribute **1 mL** of the cell suspension in cryogenic vials.

12.5 Transfer cryogenic vials to a pre-cooled CoolCell LX container.

12.6 Keep the CoolCell LX container at **-80 °C** **Overnight** .

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12.7 Transfer cells to the vapor phase of a liquid nitrogen storage facility.

Thawing cryopreserved mDA neurons 12h

13 12h

One day before thawing:

Coat 6-well culture plates with [M]**8.5 µg/mL** GFR Matrigel diluted in ice cold DMEM/F-12 medium **Overnight** at **37 °C** .

Day of thawing 15m

13.1 **Before starting**

Prepare:

Wash medium

Warm NB/B27 medium at **37 °C** .

Add: [M]**10 µM** Y-27632.

Recovery medium

Warm NB/B27 medium at **37 °C** .

Make NB/B27 complete medium by adding:

[M]**20 ng/mL** BDNF

[M]**0.2 mM** Ascorbic acid

[M]**20 ng/mL** GDNF

[M]**0.5 mM** db-cAMP

[M]**1 ng/mL** TGFβ3

[M]**10 nM** DAPT

[M]**10 µM** Y-27632

Cool down NB/B27 complete medium to **Room temperature** .

Add [M]**8.5 µg/mL** GFR Matrigel and mix well by 20x full inversions of the conical tube or flask.

6-well plates

Discard coating reagent and add **2 mL per well** of recovery medium.

Keep plates at **37 °C** for **00:15:00** before seeding cells.

Conical centrifuge tubes

Label 15-mL conical tubes and fill with **5 mL** wash medium. Keep at **Room temperature** .

13.2 Thaw cryopreserved mDA neurons by placing the cryogenic vial containing cells in a **37 °C** water bath for approximately **00:02:00** or until no ice is visible but the liquid is still cold. 2m

13.3 Fill a 5-mL serological pipette with **4 mL** wash medium and collect the thawed cell suspension very

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carefully.

13.4 Transfer the cell suspension dropwise to the 15-mL conical tube containing **5 mL** of wash medium.

13.5 Wash the cryogenic vial with **1 mL** wash medium to collect residual cells and transfer to the 15-mL conical tube.

13.6  5m

Centrifuge the cell suspension at **200 x g, 23°C, 00:05:00** .

13.7 Discard the supernatant carefully and resuspend the cell pellet with **1 mL** recovery medium by gently pipetting up and down 3-6 times to obtain a homogeneous cell suspension.

13.8 Complete the volume with recovery medium to **12 mL** and mix well by 10x full inversions of the tube.

13.9 Perform two separate live-cell counts using a hemocytometer or an automated cell counter.
Critical step: Adjust the cell suspension volume accordingly to obtain an accurate counting. Calculate the mean achieved from the two counts and determine the concentration of live cells per milliliter.

A cell viability of 85-98% should be obtained. A lower cell viability than expected might require more gentle manipulation of the cells during thawing. Reduce the number of pipetting and thaw less cells lines at once to speed the process and seed cells quickly.

13.10 Seed 400,000 cells per cm2 in a total volume of **3 mL per well** on 6-well plates coated with GFR Matrigel (i.e., to make one well of a 6-well plate, seed 3.8 x 10^6 cells per well, considering the growth area of one well equal to 9.5 cm2).

13.11 Rock the plate back-forth and side-to-side for **00:00:10** to achieve an even spread of cells in the plate well. 10s

13.12 Keep cells intact **Overnight** at **37 °C** **5 % CO2** . 12h

Day 1, 3 and 5 after thawing: medium change 15m

13.13 Warm NB/B27 medium at **37 °C** . 15m

Add:

[M] **20 ng/mL** BDNF

[M] **0.2 mM** Ascorbic acid

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[M]**20 ng/mL** GDNF

[M]**0.5 mM** db-cAMP

[M]**1 ng/mL** TGFβ3

[M]**10 nM** DAPT

Mix well by 20x full inversions of the conical tube or flask.

Perform medium change: **3 mL per well** .

Place cells back at **37 °C** **5 % CO2** .

Day 7 after thawing: Replate mDA neurons 1m

13.14 Replate mDA neurons 7 days after thawing following the procedure described for replating mDA neurons on day 32 **go to step #9** .

13.15 Seed cells at desired cell density and plate format.
Examples:
High cell density in 6-well plates: seed 300,000 cells per cm2.
Low cell density in 96-well plates: seed 100,000 cells per cm2.

13.16 Treat cells with Mitomicyn-C **48:00:00** after replating following the same procedure described on day 34 of differentiation **go to step #10** .

13.17 For long-term culture of mDA neurons, change differentiation medium every 2-3 days as described previously **go to step #11** .

13.18 **Images of thawed mDA neurons differentiated until day 65.**

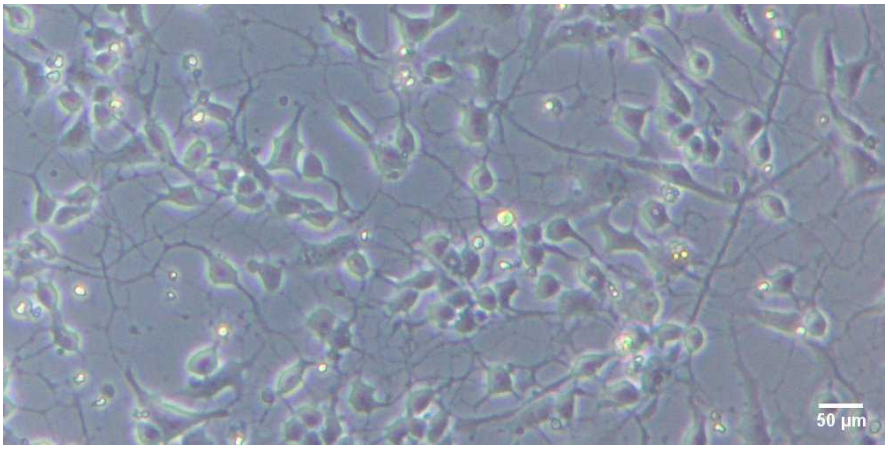

Figure 7. Representative bright-field image of day-33 mDA neurons thawed on day 20 and replated on day 32 of differentiation. Cell line 5.

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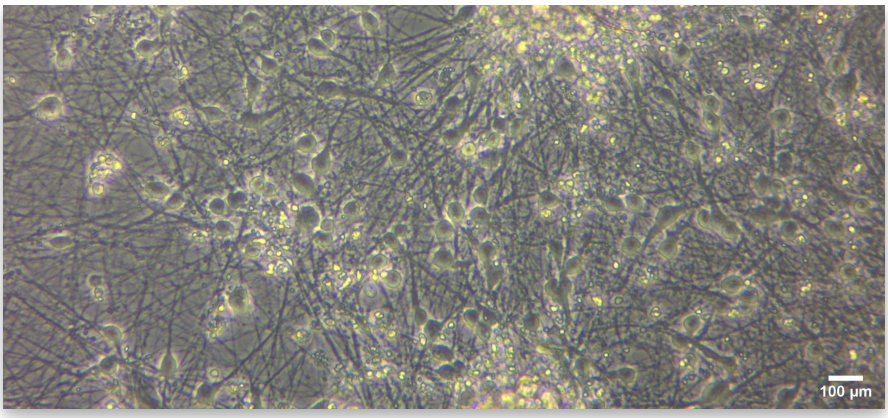

Figure 8. Representative bright-field image of day-65 mDA neurons thawed on day 20 and replated on day 32 of differentiation. Cell line 5. Note high number of neurons.

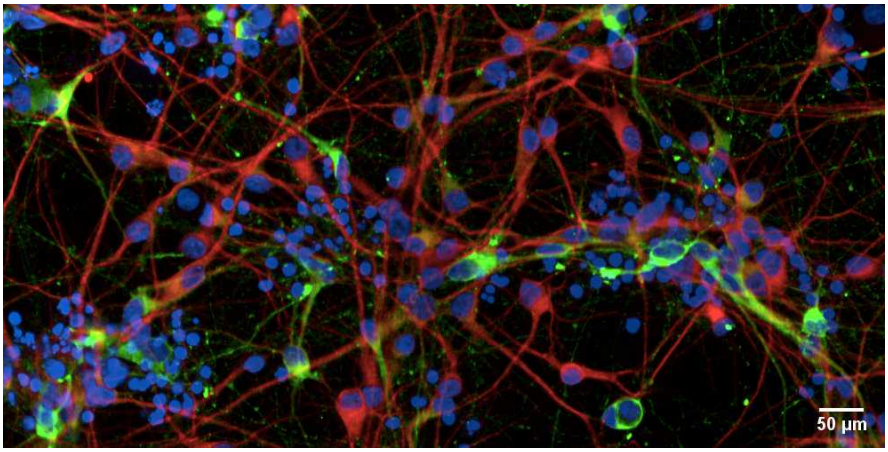

Figure 9. Representative fluorescent image of day-65 mDA neurons thawed on day 20 and replated on day 32 of differentiation. Cell line 5. Green: TH, Tyrosine Hydroxylase (mDA neuron); Red: TUJ1, Neuron-specific class III beta-tubulin (neuron); Blue: DAPI (nuclei).

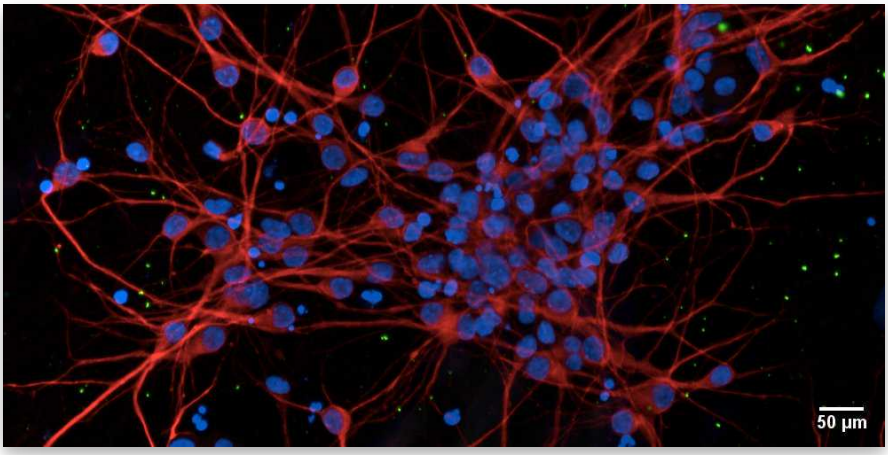

Figure 10. Representative fluorescent image of day-65 mDA neurons thawed on day 20 and replated on day 32 of differentiation. Cell line 5. Green: MKI67, Proliferation marker protein Ki-67 (proliferating cells); Red: MAP2, Microtubule-associated protein 2 (neuron); Blue: DAPI (nuclei). Note high numbers of neurons and absence of nuclei (DAPI+ cells, blue) co-stained with the proliferation marker MKI67 (green).

Citation: Elisangela Bressan, Ashutosh Dhingra, Stella Donato, Peter Heutink (03/12/2021). Optimized Derivation of Midbrain Dopaminergic Neurons from iPSCs for research application. https://dx.doi.org/10.17504/protocols.io.bsq5ndy6