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Differentiation of WTC11 and KOLF2.1 iPSCs to dopaminergic neurons

Forked from Optimized Derivation of Midbrain Dopaminergic Neurons from iPSCs for research application

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We use this protocol and it's working

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Abstract

This protocol describes the differentiation of iPSCs (WTC11 and KOLF2.1) to dopaminergic neurons according to **Bressan et al 2021**.

Materials

1 Reagent list

- 2-Mercaptoethanol **Gibco - Thermo Fisher Scientific** **Catalog #**21985023
- Accutase cell dissociation reagent **Gibco - Thermo Fisher Scientific** **Catalog #**A1110501
- B27 supplement minus vitamin A **Gibco - Thermo Fisher Scientific** **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) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D0627
- DMEM/F-12, HEPES **Gibco - Thermo Fisher Scientific** **Catalog #**31330095
- DMSO (dimethyl sulfoxide) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D2650
- DPBS no calcium no magnesium **Gibco - Thermo Fisher Scientific** **Catalog #**14190169
- Essential 8 Flex complete medium (E8) **Gibco - Thermo Fisher Scientific** **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 Fisher Scientific** **Catalog #**35050038
- Knockout DMEM/F-12 **Gibco - Thermo Fisher Scientific** **Catalog #**12660012
- Knockout Serum Replacement (KSR) **Gibco - Thermo Fisher Scientific** **Catalog #**10828028
- Laminin (Lam) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L2020
- L-ascorbic acid (AA) **Merck MilliporeSigma (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 Fisher Scientific** **Catalog #**11140050
- N2 supplement **Gibco - Thermo Fisher Scientific** **Catalog #**17502048
- Neurobasal medium **Gibco - Thermo Fisher Scientific** **Catalog #**21103049
- Penicillin-Streptomycin **Gibco - Thermo Fisher Scientific** **Catalog #**15140122
- Poly-L-Ornithine (PLO) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P3655
- Purmorphamine **Cayman Chemical Company** **Catalog #**Cay1000963410

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

HSA (Human Serum Albumin) **Merck MilliporeSigma (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 Fisher Scientific 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

Supplement K (BrainXell)

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.
  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

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)
- 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
- 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

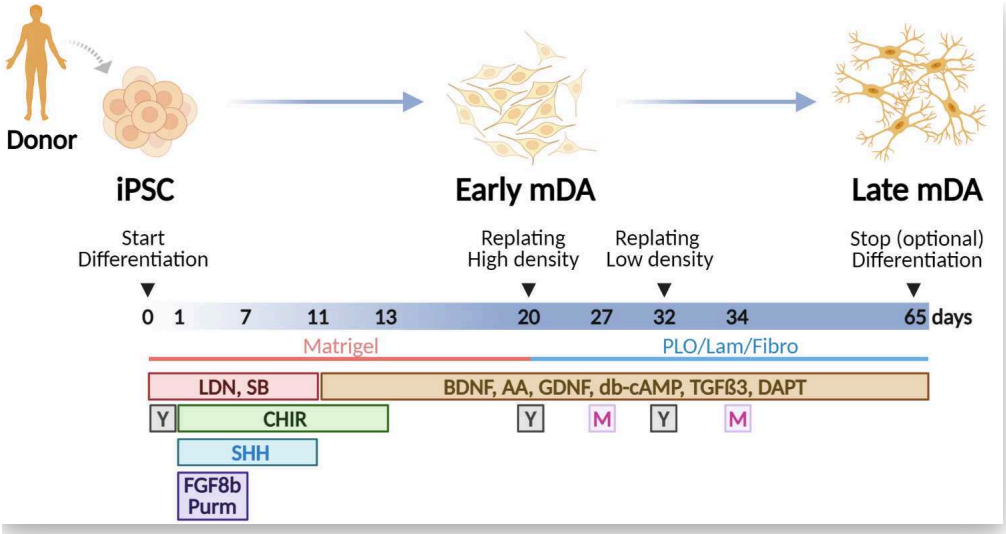
May form combustible dust concentrations in air

- L-ascorbic acid

Toxic by inhalation and ingestion. Cause skin and eye irritation

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

Before start



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

Differentiation of iPSCs into midbrain dopaminergic (mDA) neurons

1 **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

Day -2: Coating of 6-well plates with Geltrex

1d

1.1 Thaw Geltrex Overnight On ice and dilute to 100x in ice cold DMEM/F-12.

12h

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

12h

Day -1: Single cell dissociation and seeding for differentiation

1m

1.3 Discard Geltrex solution and add 2 mL per well of E8 Flex medium supplemented with [M] 10 µM Y-27632 avoiding the coating to dry out.

Day -1: Single cell dissociation and seeding for differentiation

1m

1.4 Keep the plate at 37 °C for 00:05:00 before seeding cells.

5m

Day -1: Single cell dissociation and seeding for differentiation

1m

1.5 Warm PBS, Accutase and E8 Flex medium to Room temperature .

15m

1.6 Add 1 mL per well of Accutase to iPSCs in 6-well plate.

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

1.8 Perform a quick microscope inspection of the cells. A single cell suspension should be obtained.

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

1.10 Centrifuge the cell suspension at 300 x g, 23°C, 00:05:00 . 5m

1.11 Discard the supernatant carefully and resuspend the cell pellet in 1 mL E8 Flex medium supplemented with [M] 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.

1.12 Adjust the volume with E8 Flex medium supplemented with [M] 10 µM Y-27632 to 12 mL . Mix well.

1.13 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.

Expected result

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.

1.14 Seed 800,000 cells per well in a total volume of 4 mL per well on 6-well plates coated with Geltrex.

1.15 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

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

Day 0: Start differentiation 1h

2 **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:

[M] 500 nM LDN193189

[M] 10 µM SB431542

[M] 100 ng/mL SHH

[M] 2 µM Purmorphamine

[M] 100 ng/mL FGF8

[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 or [M] 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.

2.1 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.

2.2 **Prepare differentiation medium:** 15m

Warm KSR medium at 37 °C .

Add:

500 nM LDN193189

10 µM SB431542

Mix well.

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

2.3 **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

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

Day 1 and 2

15m

3.1 Warm KSR medium at 37 °C. 15m

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.
Perform medium change: 4 mL per well.
Place cells back at 37 °C 5 % CO2.

Day 3 and 4

15m

3.2 Warm KSR medium at 37 °C. 15m

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.
Perform medium change: 4 mL per well.
Place cells back at 37 °C 5 % CO2.

Day 5 and 6

1h

3.3 **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:
[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.
Perform medium change: 4 mL per well .
Place cells back at 37 °C 5 % CO2 .

Day 7 and 8

15m

3.4 Warm KSR and N2 medium at 37 °C . 15m
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.

Perform medium change: 4 mL per well .

Place cells back at 37 °C 5 % CO2 .

Day 9 and 10

15m

3.5 Warm KSR and N2 medium at 37 °C . 15m

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.

Perform medium change: 4 mL per well .

Place cells back at 37 °C 5 % CO2 .

Day 11 and 12

1h

3.6 **Before starting:**

Prepare enough amount of NB/B27 medium.

For 500 mL NB/B27 medium, add:

484 mL Neurobasal medium

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.

Perform medium change: 4 mL per well .

Place cells back at 37 °C 5 % CO2 .

Day 13 -15, 17 and 19

15m

3.7 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 µM DAPT

Mix well.

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

4 **Note:** At day 20 of differentiation, mDA neuron precursors can be replated as describe below or cryopreserved.

2d

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 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:

[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

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.

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

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

4.3 Discard PBS and add 1 mL per well of Accutase.

4.4 Incubate cells at 37 °C for 00:15:00 . 15m

4.5 After incubation, block Accutase with 1 mL per well NB/B27 medium supplemented with [M] 10 µM Y-27632.

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

4.7 Perform a quick microscope inspection of the cells. A single cell suspension should be obtained.

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

4.9 Centrifuge the cell suspension at 300 x g, 23°C, 00:05:00 . 5m

4.10 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.

4.11 Complete the volume to 12 mL with NB/B27 complete medium and mix well.

4.12 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.

Expected result

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.

4.13 Seed desired density (300,000 to 500, 000 cells) on MatTek dishes or 6-well plate coated with PLO/Laminin.

4.14 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

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

Day 23: Treatment with antimitotic inhibitor (Supplement K) 15m

5 **Before starting:** 15m

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

Prepare enough amount of NB/B27 complete medium supplemented with [M] 1 Parts per Million (PPM) Stock: 1000x Supplement K (BrainXell). Mix well.

5.1 Discard old culture medium and add 2 mL per well of NB/B27 complete medium supplemented with Supplement K.

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

Every 5 days: Change medium for terminal differentiation of mDA neurons 15m

6 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: 2 mL per well .

Place cells back at 37 °C 5 % CO2 .

Every 10 days: Supplement differentiation medium with Laminin

15m

6.1 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

[M] 1 ng/mL TGFβ3

[M] 10 nM DAPT

Cool down NB/B27 complete medium to Room temperature .

Add:

[M] 10 µg/mL Laminin

Mix well.

6.2 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

6.3 Place cells back at 3 °C 5 % CO2 .

Cryopreservation of mDA neurons on day 20

5m

7 **Before starting:**

Thaw Synth-a-Freeze cryopreservation medium from -20C.

7.1 Perform cells dissociation and previously described go to step #1.5 .

7.2 After determining the number of live cells, centrifuge the cell suspension at 400 x g, 23°C, 00:05:00 . 5m

7.3 Resuspend the cell pellet very gently in Synth-a-Freeze medium to 3-5×10^6 cells/mL.

7.4 Distribute 500 µL of the cell suspension in cryogenic vials.

7.5 Transfer cryogenic vials in a container at -80 °C Overnight .

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

Thawing cryopreserved mDA neurons 12h

8 **One day before thawing:** 12h

Coat 6-well culture plates with Geltrex diluted in ice cold DMEM/F-12 medium Overnight at 37 °C .

Day of thawing 15m

8.1 **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 .

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 .

8.2 Thaw cryopreserved mDA neurons by placing the cryogenic vial containing cells in a 37 °C water bath for approximately 00:01:00 or until no ice is visible but the liquid is still cold. 1m

8.3 Fill a 5-mL serological pipette with 4 mL wash medium and collect the thawed cell suspension very carefully.

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

8.5 Centrifuge the cell suspension at 400 x g, 23°C, 00:05:00 . 5m

8.6 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.

8.7 Seed cells in a total volume of 2 mL per well on 6-well plates coated with Geltrex.

Treat cells with Supplement K following the same procedure described on day 23 of differentiation go to step #5 .

8.8 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

Day 7 after thawing: Replate mDA neurons 1m

8.9 Replate mDA neurons 7 days after thawing on MatTek or 6-well plates at appropriate cell densities following the procedure described for replating mDA neurons on day 20 go to step #4.15 .

8.10 For long-term culture of mDA neurons, change differentiation medium every 5 days as described previously go to step #6.1 .