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Midbrain dopaminergic differentiation of human pluripotent stem cells

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ABSTRACT

Differentiation of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) into midbrain dopaminergic (mDA) neurons is described in this protocol. This protocol has been adapted from published protocols from three laboratories, Lorenz Studer (New York), Malin Parmar/Agnete Kirkeby (Lund), and Su-Chun Zhang (Madison). The relevant papers from these labs are PMIDs: 20362538, 22056989, 22813745, 28094017, 28858290, 22696177. This protocol instructs hESCs and hiPSCs to adopt a midbrain floor plate radial glia-like identity before maturation into mDA neurons with mixed substantia nigra pars compacta and ventral tegmental area identity. It has been applied to more than 10 hESC and hiPSC lines in our laboratory, with some cell lines producing exceptional results, and others less so. Our version the protocol is published here PMID: 30472757.

EXTERNAL LINK

<https://onlinelibrary.wiley.com/doi/full/10.1111/ejn.14286>

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Chen Y, Dolt KS, Kriek M, Baker T, Downey P, Drummond NJ, Canham MA, Natalwala A, Rosser S, Kunath T. (2019) Engineering synucleinopathy-resistant human dopaminergic neurons by CRISPR-mediated deletion of the SNCA gene. *Eur J Neurosci*. 49:510-524. doi: 10.1111/ejn.14286.

ATTACHMENTS

[ejn.14286.pdf](#) [mDA protocol Schematic .pptx](#)

MATERIALS

NAME	CATALOG #	VENDOR
Biolaminin 111 LN	LN111	Biolamina
B27 supplement without retinoic acid (50x)	17504044	Gibco, ThermoFisher
24-well cell culture plate	3524	Costar
Neurobasal Medium (1x)	21103049	Gibco, ThermoFisher
StemMACS™ iPS-Brew XF human: Basal medium and supplement	130-104-368	Miltenyi Biotec
StemMACS™ CHIR99021	130-103-926	Miltenyi Biotec
Poly-L-Ornithine	P4957	Sigma Aldrich
Dulbecco's PBS (with calcium magnesium)	14040083	Gibco, ThermoFisher
DMEM/F12	21331020	Gibco, ThermoFisher
UltraPure 0.5 M EDTA pH8	15575020	Invitrogen - Thermo Fisher
Dulbecco's PBS (without calcium magnesium)	D8537	Sigma Aldrich
N2 supplement (100x supplement)	17502048	Gibco, ThermoFisher
L-Glutamine (200mM)	25030024	Thermo Fisher Scientific
Recombinant human BDNF	450-02	peprotech
Recombinant human GDNF	450-10	peprotech
Recombinant human FGF-8b	100-25	peprotech

NAME	CATALOG #	VENDOR
Recombinant Human Sonic Hedgehog/Shh (C24II) N-Terminus	1845-SH-500	R&D Systems
L-Ascorbic acid	A4403	Sigma Aldrich
DAPT	2634	Tocris
Dibutyl cAMP	D0627	Sigma Aldrich
StemMACS™ LDN-193189	130-103-925	Miltenyi Biotec
Heparin sodium salt from porcine intestinal mucosa	H3149	Sigma Aldrich
Accutase® solution	A6964	Sigma Aldrich
Trypan Blue	T8154	Sigma Aldrich
STEM-CELLBANKER - GMP Grade	11890	amsbio
SB431542	616461-5MG	Merck Millipore
7.5% Bovine Serum Albumin solution in DPBS	A8412	Sigma Aldrich
DMSO	D2650	Sigma Aldrich
Biolaminin 521 LN	LN521	Biolamina
StemMACS™ LDN-193189 in solution	130-106-540	Miltenyi Biotec
Y-27632 dihydrochloride	1254	Tocris
200 mM L-glutamine	25030-024	Invitrogen - Thermo Fisher

BEFORE STARTING

Before starting it is helpful to create a relatively large cryopreserved bank of hESCs or hiPSCs at one particular passage (20 or more vials). Upon thawing each vial perform the mDA differentiation protocol within one or two passages of recovering the cells. It is not advisable to keep the cells 'ticking' over, and initiating mDA differentiations at different passages of the cell line, as this reduces reproducibility. This method of working is well-described by Mark Tomishima's CryoPause publication. [CryoPause_Tomishima.pdf](#)

Preparing reagents for mDA differentiation

1 Prepare 10 mM (1000x) stock of SB431542.

$C_{22}H_{16}N_4O_3 \cdot 2H_2O$. MW: 420.42 g/mol.

1.1 To prepare 1190 µl of 10 mM SB431542:

Add **1190 µl** (2 x **595 µl**) of **DMSO** ([Sigma Aldrich, cat no. D2650](#)) directly to vial containing **5 mg** of **SB431542** ([Merck Millipore, cat no. 616461-5MG](#)) and mix by pipetting until powder is completely dissolved.

Prepare **20 µl** - **50 µl** aliquots of **10 mM SB431542** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Once thawed, keep **SB431542** aliquot at **Room temperature** in the dark for up to two weeks.

2 Prepare 10 mM (100,000x) and 0.1 mM (1000x) stocks of LDN193189.

$C_{25}H_{22}N_6$. MW: 406.48 g/mol.

2.1 To prepare 492 µl of 10 mM LDN193189:

Add **492 µl** of **DMSO** ([Sigma Aldrich, cat no. D2650](#)) directly to vial containing **2 mg** of **LDN193189** ([Miltenyi Biotec, cat no. 130-103-925](#)) to obtain a **10 Milimolar (mM)** solution.

Warm briefly (3-5min) at δ **37 °C** to dissolve powder.

Alternatively, **LDN193189** can be obtained in solution at 10 mM in DMSO ([Miltenyi Biotec, cat no. 130-106-540](#)).

Dilute **10 mM LDN193189** at 1 in 100 with **DMSO** ([Sigma Aldrich, cat no. D2650](#)) to create **[M]0.1 Milimolar (mM)** (1000x) stocks.

Prepare \square **20 μ l** - \square **50 μ l** aliquots of **0.1 mM LDN193189** in sterile 1.5-ml Eppendorfs tubes and store at δ **-80 °C** for up to one year. Once thawed, keep **LDN193189** aliquot at δ **Room temperature** in the dark for up to two weeks.

3 Prepare 600 μ g/ml (1000x) stock of Sonic Hedgehog (SHH-C24II).

3.1 To prepare 833 μ l of 600 μ g/ml SHH-C24II:

Add volume of \square **822 μ l** of **DPBS without Mg^{2+} and Ca^{2+}** ([Thermo Fisher, Cat no. 14190086](#)) directly to vial containing \square **500 μ g** of **SHH-C24II** ([R+D Systems, cat no. 1845-SH-500](#)).

Add \square **11 μ l** of **7.5% BSA** ([Sigma Aldrich, cat no. A8412-100ML](#)) directly to the vial.

Mix by pipetting up and down several times until the powder is dissolved.

Prepare \square **20 μ l** - \square **50 μ l** aliquots of **[M]600 μ g/ml SHH** in sterile 1.5-ml Eppendorfs tubes and store at δ **-80 °C** for up to one year. Once thawed, keep **SHH** aliquot at δ **4 °C** for up to two weeks.

4 Prepare 10 mM stock of CHIR99021.

4.1 To prepare 2414 μ l of 10mM CHIR99021:

Add \square **2149 μ l** of **DMSO** ([Sigma Aldrich, cat no. D2650](#)) directly to vial containing \square **10 mg** of **CHIR99021** ([Miltenyi Biotech, Cat no 130-103-926](#)) to obtain a **[M]10 Milimolar (mM)** solution. Mix by pipetting up and down several times to dissolve the powder. If precipitate is observed, warm briefly (2-5min) at δ **37 °C**.

Alternatively, **CHIR99021** can be obtained in solution at 10 mM in DMSO ([Miltenyi Biotech, Cat no. 130-106-539](#)).

Prepare \square **10 μ l** aliquots of **10 mM CHIR99021** in sterile 1.5-ml Eppendorfs tubes and store at

⌄ -80 °C for up to one year. Once thawed, keep CHIR99021 aliquot at ⌄ Room temperature in the dark for up to two weeks.

5 Prepare 100 µg/ml (1000x) stock of recombinant FGF8b

5.1 To prepare 250 µl of 100 µg/ml FGF8b:

Add 246 µl of DPBS without Mg²⁺ and Ca²⁺ ([Thermo Fisher, Cat no 14190086](#)) directly to vial containing 25 µg of FGF8b ([R+D Systems, Cat no 423-F8-025](#))

Add 3.4 µl of 7.5% BSA ([Sigma Aldrich, cat no. A8412-100ML](#)) directly to the vial.

Mix by pipetting up and down several times to dissolve the powder.

Prepare 20 µl - 50 µl aliquots of 100 µg/ml FGF8b in sterile 1.5-ml Eppendorfs tubes and store at ⌄ -80 °C for up to one year. Once thawed, keep FGF8b aliquots at ⌄ 4 °C in the dark for up to two weeks.

6 Prepare 1 mg/ml (1000x) stock of Heparin Sodium Salt from porcine intestinal mucosa

6.1 To prepare 55 ml of 1 mg/ml Heparin:

Add 55 ml of DPBS without Mg²⁺ and Ca²⁺ ([Thermo Fisher, Cat no 14190086](#)) directly to vial containing 55 mg of Heparin ([Sigma Aldrich, Cat no H3149](#)).

Mix by pipetting up and down several times to dissolve the powder.

Filter the reconstituted Heparin solution using an appropriately sized syringe for the volume and 0.33µM filter

Prepare 500 µl aliquots of 1 mg/ml Heparin in sterile 1.5-ml Eppendorfs tubes and store at ⌄ 4 °C for up to two years.

7 Prepare 20 µg/ml (1000x) stock of recombinant Bone-Derived Growth Factor (BDNF).

7.1 To prepare 5 ml of 20 µg/ml BDNF:

Add 4930 µl of DPBS without Mg²⁺ and Ca²⁺ ([Thermo Fisher, Cat no 14190086](#)) directly to vial containing 100 µg of BDNF ([PeproTech, Cat no 450-02](#)).

Add 70 µl of 7.5% BSA ([Sigma Aldrich, cat no. A8412-100ML](#)) directly to the vial.

Mix by pipetting up and down several times to dissolve the powder.

Prepare **20 µl** - **50 µl** aliquots of **20 µg/ml BDNF** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Once thawed, keep **BDNF** aliquots at **4 °C** for up to two weeks.

8 Prepare 20 µg/ml (2000x) stock of recombinant Glial Derived Growth Factor (GDNF).

8.1 To prepare 5 ml of 20 µg/ml GDNF:

Add **4930 µl** of **DPBS without Mg²⁺ and Ca²⁺** ([Thermo Fisher, Cat no 14190086](#)) directly to vial containing **100 µg** of **GDNF** ([PeproTech, Cat no 450-10](#)).

Add **70 µl** of **7.5% BSA** ([Sigma Aldrich, cat no. A8412-100ML](#)) directly to the vial.

Mix by pipetting up and down several times to dissolve the powder.

Prepare **20 µl** - **50 µl** aliquots of **20 µg/ml GDNF** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Once thawed, keep **GDNF** aliquots at **4 °C** for up to two weeks.

9 Prepare 0.2 M (1000x) stock of Ascorbic Acid by Sigma Aldrich. C₆H₈O₆, MW: 176.12 g/mol.

9.1 To prepare 2841 µl 0.2 M Ascorbic Acid:

Add **2841 µl** of **sterile water** directly to vial containing **100 mg** of **Ascorbic Acid** ([Sigma Aldrich, Cat no A4403](#)).

Mix by pipetting up and down several times to dissolve the powder.

Filter the reconstituted **Ascorbic Acid** solution using an appropriately sized syringe for the volume and a 0.33µM filter.

Prepare **20 µl** - **50 µl** aliquots of **0.2 Molarity (M) Ascorbic Acid** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Once thawed, keep **Ascorbic Acid** aliquots at **4 °C** in the dark for up to two weeks.

10 Prepare 0.5 M (1000x) stock of dcAMP (N⁶,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt) by Sigma Aldrich. C₁₈H₂₃N₅O₈PNa, MW: 491.3 g/mol.

10.1 To prepare 4070 µl 0.5 M dcAMP:

Add **4070 µl** of **sterile water** directly to vial containing **1000 mg** of **dcAMP** ([Sigma Aldrich, Cat no D0627](#)).

Mix by pipetting up and down several times to dissolve the powder.

Filter the reconstituted **dcAMP** solution using an appropriately sized syringe for the volume and a 0.33µM filter.

Prepare **20 µl** - **50 µl** aliquots of [**0.5 Molarity (M)**] **dcAMP** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Once thawed, keep **dcAMP** aliquots at **4 °C** in the dark for up to two weeks.

11 Prepare 10 mM (10,000x) stock of DAPT

$C_{23}H_{26}F_2N_2O_4$, MW: 432.46 g/mol.

11.1 To prepare 2312 µl 10 mM DAPT:

Add **2312 µl** of **DMSO** ([Sigma Aldrich, cat no. D2650](#)) directly to vial containing **10 mg** of **DAPT** ([Tocris, Cat no 2634](#)).

Mix by pipetting up and down several times to dissolve the powder. If precipitate is observed, warm the solution to **37 °C** for 2-5 mins.

Prepare **20 µl** aliquots of [**10 Milimolar (mM)**] **DAPT** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Once thawed, keep **DAPT** aliquots at **4 °C** in the dark for up to 1 month.

12 Prepare 10 mM (1000x) stock of Y-27632 dihydrochloride

$C_{14}H_{21}N_3O \cdot 2HCl$, MW: 320.26 g/mol.

12.1 To prepare 15.61 ml 10 mM Y-27632:

Add **15.61 ml** of **DPBS without Mg^{2+} and Ca^{2+}** ([Thermo Fisher, Cat no 14190086](#)) directly to vial containing **50 mg** of **Y-27632 dihydrochloride** ([Tocris, Cat no 1254](#)).



Mix by pipetting up and down several times to dissolve the powder.

Prepare aliquots of [**10 Milimolar (mM)**] **Y-27632 dihydrochloride** in sterile 1.5-ml Eppendorfs tubes and store at **-80 °C** for up to one year. Prepare smaller **50 µl** aliquots for working use and larger **500 µl** aliquots which can be aliquoted out at a later date.


Once thawed, keep **Y-27632** aliquots at **4 °C** in the dark for up to 1 month.



13 Prepare aliquots of N2

N2 stocks should be stored at **-20 °C**. To avoid freeze thawing it is helpful to create working aliquots.

- 13.1 Prepare  **0.5 ml** aliquots of **100x N2** ([Thermo Fisher, Cat 17502048](#)) in sterile 1.5-ml Eppendorfs tubes and store at  **-20 °C** for up to one year.

14 Prepare aliquots of B27 (without RA)

B27 stocks should be stored at  **-20 °C**. To avoid freeze thawing it is helpful to create working aliquots.


- 14.1 Prepare  **1 ml** aliquots of **50x B27 (without RA)** ([Thermo Fisher, Cat no 17504044](#)) in sterile 1.5-ml Eppendorfs tubes and store at  **-20 °C** for up to one year.

Preparation for mDA D0

- 15 On mDA day 0 cells hPSCs are lifted as clumps using EDTA and seeded onto plates that have been pre-coated with Laminin-111.

Plates need to be coated with Laminin 111 at least 12 hours prior to the beginning the differentiation.

15.1 Pre-Coating plates with Laminin 111:


Laminin-111 stocks (0.1mg/ml) ([Biolamina, Cat no LN111](#)) should be thawed at RT and then diluted 1:20 in **DPBS (with Ca²⁺ and Mg²⁺)** ([ThermoFischer, cat no. 14040083](#)) to create a final working stock solution of  **5 µg/ml Laminin-111**.

The diluted laminin should be mixed thoroughly and added to the wells at a volume recommended for the well size (see below table).

Plate type	Approx Area of Well (cm ²)	Volume of 5 µg/ml L521 (ul) required for optimal coating
24 well plate	2	300
48 well plate	1	150

Move the plate gently around to ensure the well is evenly covered

Wrap the plate in parafilm to reduce evaporation

Incubate the plate at  **4 °C** overnight for at least 12 hours (overnight incubation is recommended).

Coated plates can be stored at  **4 °C** for 3 weeks.

16 Preparation of 0.5mM EDTA from ThermoFisher.

To lift hPSCs as clumps for seeding, a stock of **0.5mM EDTA** is prepared from **50mM Ultra Pure EDTA** ([ThermoFisher, Cat no 15575020](#)).

16.1 To prepare 50ml of 0.5mM EDTA:

Take an **50 µl** aliquot of **50 Milimolar (mM) Ultra Pure EDTA** ([ThermoFisher, Cat no 15575020](#)), and add to a 50ml falcon tube.

Add **50 ml** of **DPBS (without Ca²⁺ and Mg²⁺)** ([ThermoFischer, cat no. 14040083](#)) directly to the 50ml falcon tube and mix to create a **0.5 Milimolar (mM) EDTA** stock

Filter the diluted **EDTA** through a 0.33µM filter into a fresh 50ml falcon tube.

Label and store **0.5 mM EDTA** stock at **Room temperature**.

0.5mM EDTA can be kept at **Room temperature** for 6 months.

17 Preparation of Neural Induction Medium (NIM):

50% DMEM/F12 + 50% Neurobasal + 1x N2 + 1x B27 + 2mM L-Glutamine.

Between mDA D0 and mDA D4 cells are cultured within NIM.

The components which form the base media NIM (N2 supplement, B27 supplement, DMEM/F12 and Neurobasal media) are in themselves only stable for 2 weeks due to the presence of light sensitive components. Thus, fresh base media should be made up at the start of each differentiation and kept in the dark at **-4 °C** for up to 2 weeks.

17.1 To prepare 50ml of NIM:

Add **24 ml** of **DMEM/F12** ([Thermo Fisher, Cat no 21331020](#)) + **24 ml** of **Neurobasal™ Medium** ([Thermo Fisher, Cat no 21103049](#)) + **500 µl** of **100x N2** ([Thermo Fisher, Cat no 17502048](#)) + **1 ml** of **50x B27** ([Thermo Fisher, Cat no 17504044](#)) + **500 µl** of **200mM L-Glutamine** ([Thermo Fisher, Cat no 25030032](#)).

mDA Day 0

18 mDA Day 0 plating

On mDA Day 0 cells are lifted as clumps using 0.5mM EDTA and then seeded onto Laminin-111 coated plates. Before beginning; self-renewing cultures of hPSCs must be free of spontaneous differentiation and confluency should be between 70% and 90% before use.

Prior to mDA D0 seeding, human ESC/iPSC cultures are grown on Laminin-521 (5µg/ml) matrix coated 6-well plates with daily feeding using 2 ml StemMACS iPS-BrewXF (Miltenyi Biotec). Each well of a 6-well plate should be pre-coated with 1200 µl of Laminin-521 overnight at 4°C before use to ensure even hPSC adherence and efficient growth. One well of a 6-well plate should provide enough cells to seed between 6 and 10 wells of a 24-well plate for mDA differentiation.

18.1 Preparation of wash media for lifted hPSCs:

Per each well of a 6 well plate of hPSCs prepare 4ml of wash media.

Wash media: 50% iPS-Brew XF medium (self renewal media for hPSCs)

+ 50% DMEM/F12 + 10 μ M Y27632.

For example when lifting one well of hPSCs from a 6 well plate prepare:

▢ 2 ml iPS-Brew XF medium ([Miltenyibiotec, Cat no 130-104-368](#)) + ▢ 2 ml DMEM/F12 ([Thermo Fisher, Cat no 21331020](#)) + ▢ 2 μ l 10mM Y27632.

18.2

Prepare an adequate volume of plating medium:

Neural Induction Media (NIM) + 10 μ M Y27632 + 10 μ M SB431542, 100nM LDN, 600 ng/ml Shh and between 0.7 and 1.2 μ M CHIR99021*.

To start differentiation in 24-well plates, you will need 400 μ l per well.

**The concentration of CHIR99021 recommended can vary between cell lines. A change in 0.1 μ M can have a suprisingly large effect on the puritiy of cells obtained. Therefore it is recommended when first starting to try out and compare a range of CHIR99021 concentrations.*

18.3 Lifting hPSCs with EDTA

Aspirate the **iPS-Brew XF medium** from the hPSCs (in 6-well plate).

Wash cells once with ▢ 1 ml of [M]0.5 Milimolar (mM) EDTA (prepared step 16.1).

Add ▢ 1 ml of [M]0.5 Milimolar (mM) EDTA to the cells and incubate at 37 °C for approximately 5-7 minutes or when most of the cells appear 'refractile' by light microscopy. **The incubation time should depend on the confluency of the cells.*

After the incubation period; remove the EDTA and use ▢ 1 ml of the Wash medium (*prepared in step 18.2*) to lift the cells gently from the well. Aim to lift cells in a few pipettes as possible to ensure optimal survival.

Transfer the lifted cells to the tube with remaining Wash medium.

Gently invert the tube a few times to ensure the cells are thoroughly mixed for cell counting.

18.4 Counting the cells:

By using EDTA the cells are lifted as clumps. For more accurate counting of the cells take an aliquot to be broken into single cells.

To count the cells, take 2 x 10 μ l aliquots of cells into new 0.5ml Eppendorfs. Mix each with ▢ 10 μ l of Trypan Blue ([Sigma Aldrich, Cat no T8154](#)).

Count the cells using a haemocytometer*. Calculate the amount of cells within the wash media.

18.5 Plating cells:

On mDA Day 0 we recommend using seeding into a 24 well plate.

From the cell count, estimate the total volume of cells you will need to initiate differentiation at a

density of 40,000 cells/cm².*

For example, for 4 wells of a 24-well plate, you will need 4 (number of wells) x 2 (area of each well) x 40,000 (required density) = 320,000 cells.

**Note the optimal plating density can vary depending on the cell line used. For example RC17 a plating density of 40,000 cells/cm² is recommended but for AST18 a plating density of 60,000 cells/cm² is recommended.*

Transfer 110% of the required volume (an extra 10% is transferred to account for any cell death) from the remaining cell suspension within the wash media (that has not been triturated) to a new tube for centrifugation 300 g for 3 minutes.

Aspirate the wash media and resuspend the colonies gently in the plating medium (*prepared in step 18.2*). It is recommended to plate cells into the wells of a 24 well plate in a volume of 400 µl per well. E.g if 4 wells of a 24-well plate are being seeding for example then cells should be resuspended in 4 x 400 µl.

Aspirate the Laminin-111 from the coated plates and seed the cell suspension into the well. Shake the plate with to ensure an even distribution of cells.

mDA Day 2

19 mDA Day 2 media change.

Fresh medium should be prepared from base media NIM and growth factors.

19.1 Prepare an adequate volume of fresh day 2 medium:

Neural Induction Media (NIM) + 10 µM SB431542, 100nM LDN, 600 ng/ml SHH and between 0.6 and 1.0 µM CHIR99021.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.


For each well of a 24-well plate  400 µl of fresh media is required.

mDA Day 4

20 mDA Day 4 media change

Between mDA Day 4 and mDA Day 11 cells are no longer cultured within NIM as a base media but instead are cultured within neural proliferation media NPM.

**You should observe the cells becoming more confluent day by day. We expect cells to be close to 100% confluent between mDA D3 and mDA D7. How quickly cells become confluent varies between cell lines.*

The components which form the base media NPM (N2 supplement, B27 supplement, DMEM/F12 and Neurobasal media) are in themselves only stable for 2 weeks due to the presence of light sensitive components. Thus, fresh base media should be made up at the start of each differentiation and kept in the dark at  4 °C for up to 2 weeks.

20.1 Preparation of NPM

50% NIM + 25% Neurobasal + 25% DMEM/F12 + 2mM L-Glutamine.

To prepare NPM from NIM:

For example, to make **50 ml NPM** = **25 ml NIM** + **12.25 ml DMEM/F12** ([Thermo Fisher, Cat no 21331020](#)) + **12.25 ml Neurobasal™ Medium** ([Thermo Fisher, Cat no 21103049](#)) + **250 µl 200mM L-Glutamine** ([Thermo Fisher, Cat no 25030032](#)).

Alternatively to make NPM de novo:

50% Neurobasal, 50% DMEM/F12 + 0.5x N2 + 0.5x B27 + 2mM L-Glutamine.

For example, to make **50 ml NPM** = **24.4 ml** of **DMEM/F12** ([Thermo Fisher, Cat no 21331020](#)) + **24.4 ml** of **Neurobasal™ Medium** ([Thermo Fisher, Cat no 21103049](#)) + **250 µl** of **100x N2** ([Thermo Fisher, Cat no 17502048](#)) + **500 µl** of **50x B27** ([Thermo Fisher, Cat no 17504044](#)) + **500 µl** of **200mM L-Glutamine** ([Thermo Fisher, Cat no 25030032](#)).

20.2 Prepare an adequate volume of fresh day 4 medium:

NPM + 10 µM SB431542, 100nM LDN, 600 ng/ml Shh and between 0.6 and 1.0 µM CHIR99021.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate **600 µl** of fresh media is required.

mDA Day 7

21 mDA Day 7 media change.

Cells should be confluent by mDA Day 7 at the latest.

21.1 Prepare an adequate volume of fresh day 7 medium:

NPM + 10 µM SB431542, 100 nM LDN, 600 ng/ml Shh and between 0.6 and 1.0 µM CHIR99021.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate **600 µl** of fresh media is required.

mDA Day 9

22 mDA Day 9 media change

22.1 Prepare an adequate volume of fresh medium:

NPM + 100 ng/ml FGF8b + 1 µM Heparin solution.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

For each well of a 24-well plate, **600 µl** of fresh media is required.

mDA Day 11: preparation

23 mDA Day 11

On mDA day 11 cells can either be replated or frozen for use later on. From mDA D11 onwards cells are cultured within new base media; Neural differentiation medium (NDM). mDA cells should be replated onto new plates freshly coated Laminin-111.

23.1 Pre-coating plates with Laminin-111

Plates should be coated with L111 following the same protocol as step ?

24 Preparation of Neural Differentiation Medium (NDM):

The components which form the base media NDM (B27 supplement, and Neurobasal media) are in themselves only stable for 2 weeks due to the presence of light sensitive components. Thus, fresh base media should be made up at the start of each differentiation and kept in the dark at **4 °C** for up to 2 weeks.

24.1 Preparation of NDM:

Neurobasal + 1x B27 + 2mM L-Glutamine.

For example, to make **50 ml NDM** = **48.5 ml** of **Neurobasal™ Medium** ([Thermo Fisher, Cat no 21103049](#)) + **1 ml** of **50x B27** ([Thermo Fisher, Cat no 17504044](#)) + **500 µl** of **200mM L-glutamine** ([Thermo Fisher, Cat no 25030032](#)).

mDA D11 replating

25 mDA D11 replating:

On day 11 cells can be (i) lifted and replated for continued differentiation, or (ii) cryopreserved

To replate; mDA D11 cells are lifted as single cells using Accutase and replated into a new L111-coated plate at a density of 800,000cells/cm².

25.1 Preparation of wash medium:

NPM + 10 µM Y27632

For each well of a 24 well plate prepare **1.2 ml** wash media. ** This volume is 5x the volume of Accutase added per well.*

25.2 Preparation of plating medium:

NDM + 10 µM Y-27632 + 20 ng/ml BDNF + 10 ng/ml GDNF 100 ng/ml FGF8b + 0.2 mM Ascorbic Acid + 1 µg/ml heparin solution.

Prepare plating medium according to the number of the wells to be re-plated, remember to account for extra dead volume.

It is recommended to re-plate cells into either a 24 or 48 well format.

Volume for day 11 plating:

µl/well	area (cm ²)	Volume (µl)
24 well plate	2	600
48 well plate	1	300

25.3 Lifting cells as single cells with Accutase

Wash the cells once briefly Accutase (300 µl/well for 24-well plate) ([Sigma Aldrich, Cat no A6964](#))

Add 300µl/well of Accutase ([Sigma Aldrich, Cat no A6964](#)) and incubate **37 °C** for 10min.

Following the incubation period, to dissociate into single cells pipette up and down. *

**Place a P1000 tip in a P200 tip, set the P1000 to 600. Tilt the plate towards you, place the pipette tip in the bottom corner of the well. Pipetting the cells up and down against this corner will aid in breaking up the cells. Pipetting ~15 times is usually sufficient to break into single cells. Try to avoid the production of bubbles where possible.*

Transfer the lifted cells to the wash media.

Repeat the process for each well.

25.4 Counting cells

To count the cells, take 2 x 10 µl aliquots of cells into new 0.5ml Eppendorfs. Mix each with **10 µl** of Trypan Blue ([Sigma Aldrich, Cat no T8154](#)).

Count the cells using a haemocytometer*. Calculate the amount of cells within the wash media.

**Alternatively, can use automatic cell counter to count the cells. E.g TC20™ Automated Cell Counter Biorad ([BioRad, Cat no 1450102](#)).*

25.5 Plating cells:

From the cell count, estimate the total volume of cells needed for re-plating. For mDA day 11 replate at a density of 800,000 cells/cm².

For example: cells needed = (number of wells) x 2cm² (area of a well in a 24-well plate) x 800,000 cells/cm² (required density). The volume to spin down = cells needed / cell count.

Transfer the required volume from the remaining cell suspension within the wash media (that has not been triturated) to a new tube for centrifugation 300 g for 3 minutes.

Aspirate the wash media and resuspend the colonies gently in the mDA Day 11 plating medium (*prepared in step 24.2*). E.g if 4 wells of a 24-well plate are being seeding for example then cells should be resuspended in 4 x 600 µl.

Aspirate the Laminin-111 from the coated plates and seed the cell suspension into the wells. Shake the plate to ensure even distribution of cells.

25.6 Alternative to replating: freezing mDA Day 11 cells

Instead of replating cells can be cryopreserved at this stage.

See cryopreservation protocol described in:

<https://www.biorxiv.org/content/10.1101/2020.02.11.944272v1>.

mDA Day 14

26 mDA Day 14 media change.

26.1 Prepare an adequate volume of fresh medium:

NDM + 20 ng/ml BDNF + 10 ng/ml GDNF + 100 ng/ml FGF8b + 0.2 mM Ascorbic Acid + 1 µg/ml heparin solution.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

Volume needed per well:

Plate Type	area (cm ²)	Volume (µl/ well)
24 well plate	2	600
48 well plate	1	300

Preparation for mDA Day 16

27 mDA Day 16 replating preparation

On mDA day 16 cells can either be replated or cryopreserved for use later on. mDA cells should be replated onto new plates freshly coated Laminin-111.

27.1 Pre-coating plates with Laminin-111

Plates should be coated with L111 following the same protocol as step 15.

mDA Day 16

28 mDA Day 16 replating

On day 16 cells can be (i) lifted and replated for continued differentiation, (ii) cryopreserved, or (iii) transplanted into an animal model.

As with mDA Day 11 replating, mDA Day 16 cells are lifted as single cells using Accutase and replated into a new L111-coated plate at a density of 800,000 cells/cm².

28.1 Preparation of wash media:

Wash media is prepared from NDM + 10 µM Y-27632.

For each well of a 24-well plate prepare **1.2 ml** wash media.

For each well of a 48-well plate prepare **0.6 ml** wash media.

**This volume is 5x the volume of Accutase added per well.*

28.2

Preparation of plating media:

NDM + 10 μ M Y27632 + 20 ng/ml BDNF + 10 ng/ml GDNF + 0.2 mM Ascorbic Acid + 0.5 mM dcAMP + 1 μ M DAPT.

Prepare plating medium according to the number of the wells to be re-plated, remember to account for extra dead volume.

It is recommended to re-plate cells into either a 24 or 48-well format.

Volume for day 16 plating:

Plate Type	area (cm ²)	Volume (μ l/ well)
24 well plate	2	600
48 well plate	1	300

28.3 Lifting cells as single cells with Accutase

Aspirate off the NDM from each well.

Wash the cells once briefly Accutase (300 μ l/well for 24-well plate, 150 μ l/well for 48-well plate) ([Sigma Aldrich, Cat no A6964](#)) to remove excess medium.

Add Accutase (300 μ l/well for 24-well plate, 150 μ l/well for 48-well plate) ([Sigma Aldrich, Cat no A6964](#)) and incubate at 37 °C for 10 mins.

Following the incubation period, to dissociate into single cells pipette up and down. ***

****Place a P1000 tip in a P200 tip, set the P1000 to 600. Tilt the plate towards you, place the pipette tip in the bottom corner of the well pipetting the cells up and down against this corner will aid in breaking up the cells. Pipetting ~15 times is usually sufficient. Try to avoid the production of bubbles.*

Transfer the lifted cells to the wash media.

Repeat the process for each well.

28.4 Counting cells

To count the cells, take 2 x 10 μ l aliquots of cells into new 0.5ml Eppendorfs. Mix each with 10 μ l of Trypan Blue ([Sigma Aldrich, Cat no T8154](#)).

Count the cells using a haemocytometer*. Calculate the amount of cells within the wash media.

**Alternatively, can use automatic cell counter to count the cells. E.g TC20™ Automated Cell Counter Biorad ([BioRad, Cat no 1450102](#)).*

28.5 Plating cells:

From the cell count, estimate the total volume of cells needed for re-plating. For mDA day 16 replate at a density of 800,000 cells/cm².

For example: cells needed = (number of wells) x 2 cm² (area of a well in a 24-well plate) x 800,000 cells/cm² (required density). The volume to spin down = cells needed / cell count.

Transfer the required volume from the remaining cell suspension within the wash media (that has not been triturated) to a new tube for centrifugation 300 g for 3 minutes.

Aspirate the wash media and resuspend the colonies gently in the mDA Day 16 plating medium (*prepared in step 27.2*).
E.g if 4 wells of a 24-well plate are being seeding for example then cells should be resuspended in 4 x 600 µl.

Aspirate the Laminin-111 from the coated plates and seed the cell suspension into the wells with generous shaking to ensure even distribution.

28.6 Alternative to replating: freezing mDA Day 16 cells

Instead of replating, cells can be frozen at this stage.

See freezing protocol described in: <https://www.biorxiv.org/content/10.1101/2020.02.11.944272v1>.

mDA Day 18

29 mDA Day 18 media change

29.1 Prepare an adequate volume of fresh day 18 medium:

NDM + 20 ng/ml BDNF + 10 ng/ml GDNF + 0.2 mM Ascorbic Acid + 0.5 mM dcAMP + 1 µM DAPT.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

Volume for day 18 media change:

Plate Type	area (cm ²)	Volume (µl/ well)
24 well plate	2	600
48 well plate	1	300

mDA Day 18 onwards

30 Media changes mDA day 18-day 45:

From mDA Day 18 onwards cells should be fed every 2-3 days until day 45. At day 45 the mDA cells are expressing high levels of mature mDA markers e.g TH.

30.1 Prepare an adequate volume of fresh medium:

NDM + 20 ng/ml BDNF + 10 ng/ml GDNF + 0.2 mM ascorbic acid + 0.5 mM dcAMP + 1 µM DAPT.

Prepare medium according to the number of the wells to be fed, remember to account for extra dead volume.

Volume for media change:

Plate Type	area (cm ²)	Volume (µl/ well)
24 well plate	2	600
48 well plate	1	300

mDA neurons can be kept in culture for over 1 year feeding once or twice a week. DAPT should be removed from the feeding medium after day 45.