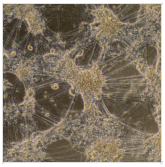

Oct 20, 2020

Generation of iPSC-derived dopaminergic neurons

Julie Jerber[1], James Haldane[1], Juliette Steer[1], Daniel Pearce[1], Minal Patel[1]

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2 *Works for me* dx.doi.org/10.17504/protocols.io.bjpgkmjw

Cellular Generation and Phenotyping

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ABSTRACT

This protocol describes a method for the production of dopaminergic neurons from human iPSCs, using a 52-day long differentiation method adapted from doi.org/10.1038/nature10648.

Steps in this protocol assume the use of a single 12 well plate for the purposes of differentiation; adjust volumes accordingly depending on labware used.

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PROTOCOL CITATION

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KEYWORDS

Dopaminergic, Differentiation, iPSC, Human, Neurons

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CREATED

Aug 13, 2020

LAST MODIFIED

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PROTOCOL INTEGER ID

40392

GUIDELINES

Unless otherwise stated, all steps should be performed under sterile conditions in a CL2 biological safety cabinet.

Below are example images showing a neuronal culture undergoing a 52-day differentiation to dopaminergic neurons.

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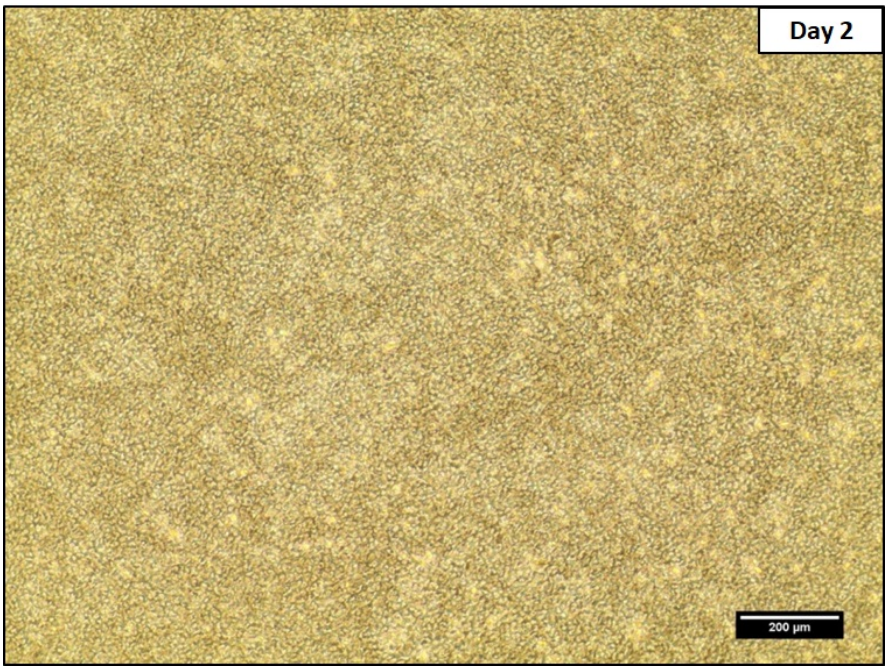

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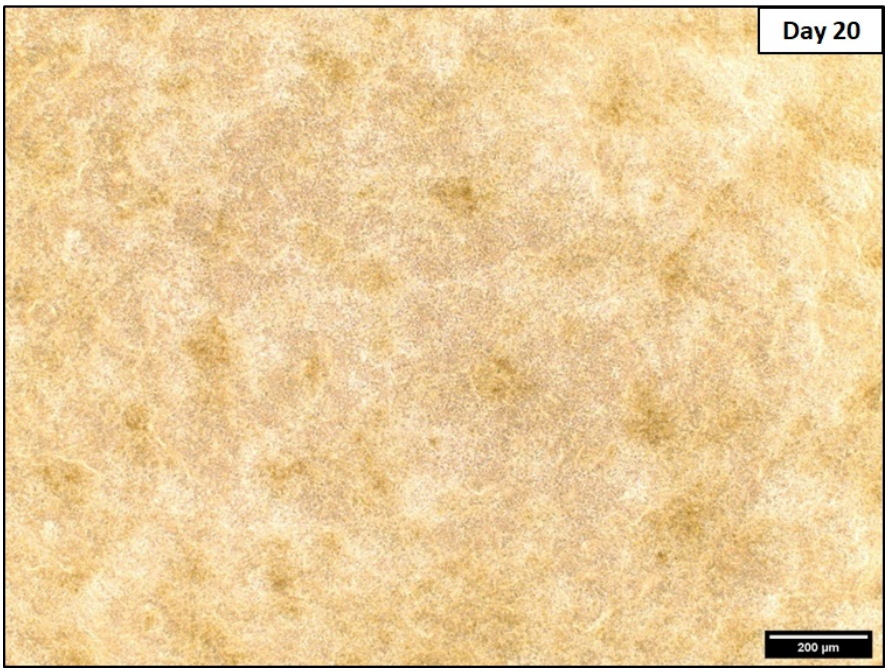

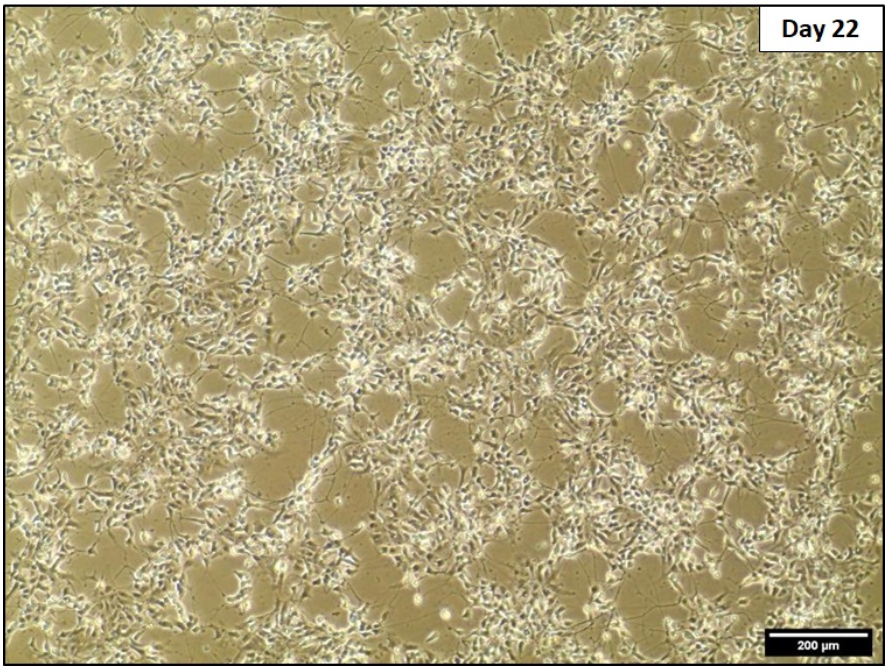

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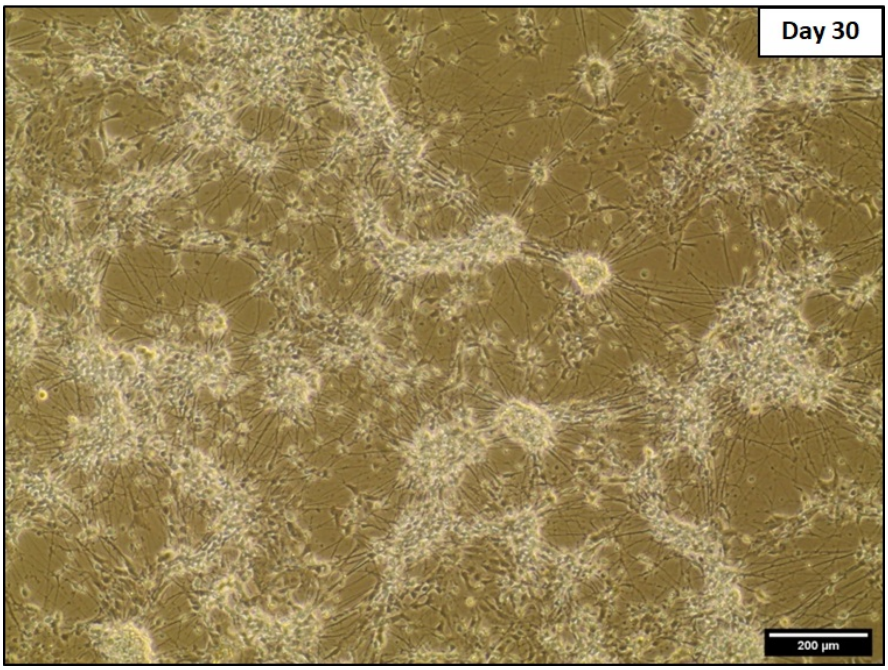

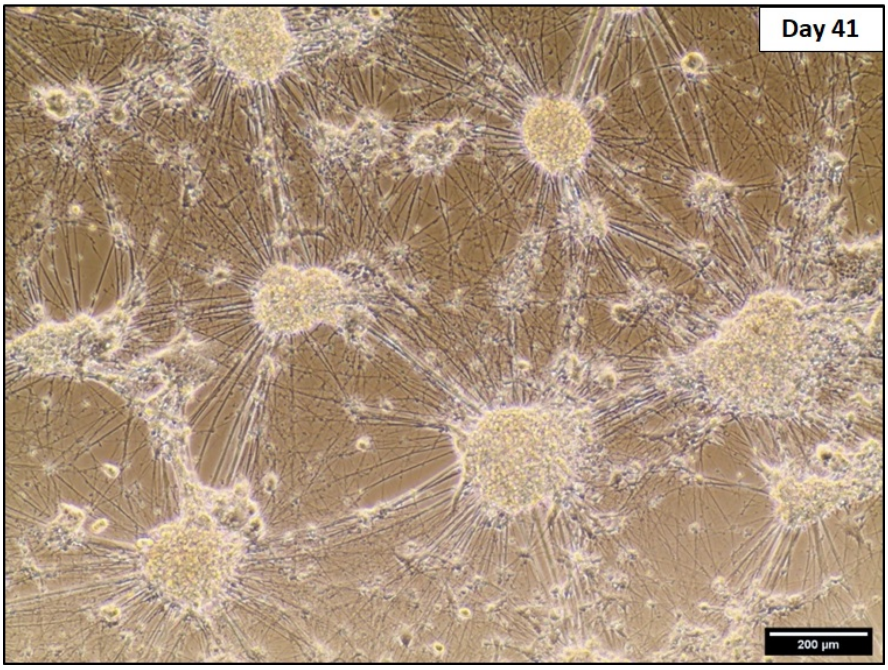

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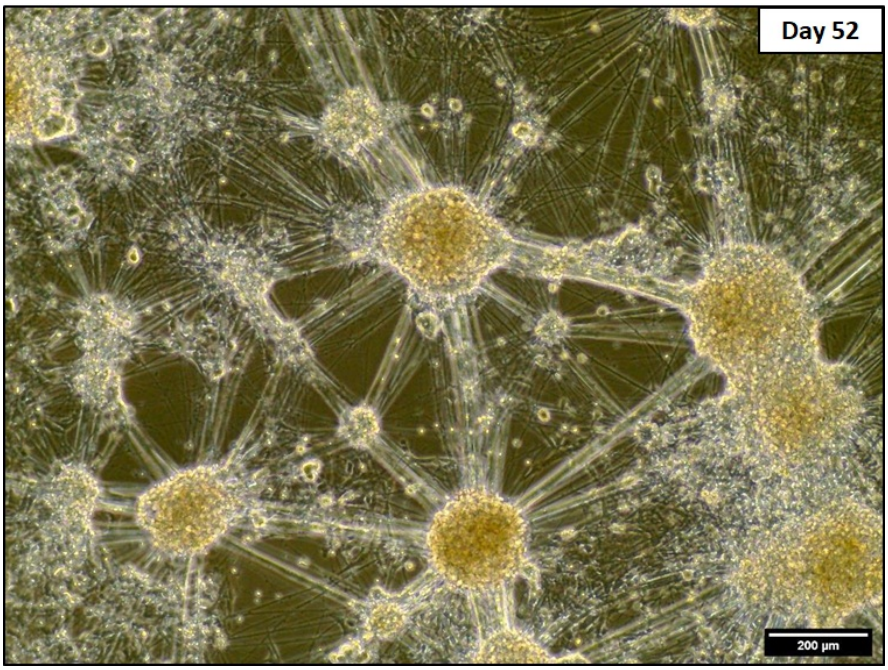

MATERIALS

NAME	CATALOG #	VENDOR
SB431542 10 mg	72234	Stemcell Technologies
Penicillin-Streptomycin (10,000 U/mL)	15140122	Thermo Fisher Scientific
Falcon™ 15mL Conical Centrifuge Tubes	14-959-53A	Fisher Scientific
DMEM/F-12, GlutaMAX™ supplement	10565018	Thermo Fisher
MEM Non-Essential Amino Acids Solution (100X)	11140050	Thermo Fisher
UltraPure™ 0.5M EDTA, pH 8.0	15575020	Thermo Fisher
DMEM/F-12, no phenol red	21041025	Thermo Fisher
Neurobasal™ Medium	21103049	Thermo Fisher
2-Mercaptoethanol (50 mM)	31350010	Thermo Fisher
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher
Essential 8™ Medium	A1517001	Thermo Fisher
KnockOut™ DMEM	10829018	Thermo Fisher Scientific
Knockout serum replacement (KSR)	10828028	Gibco - Thermo Fisher
Y-27632 dihydrochloride	Y0503	Sigma – Aldrich
DPBS	14190	Invitrogen - Thermo Fisher
Vitronectin XF™	#07180	Stemcell Technologies
DNase Vial (D2)	LK003170	Worthington Biochemical Corporation
PDS Kit Papain Vial	LK003176	Worthington Biochemical Corporation
PluriStrainer Mini 40µm	43-10040-60	pluriSelect
1.5 ml TubeOne® Microcentrifuge Tubes Natural (Sterile)	S1615-5510	StarLab
Bovine Serum Albumin	A0281	Sigma
12-well Falcon™ Polystyrene Microplates	10489482	Fisher Scientific
Recombinant Human TGF-β3	100-36E	peprotech
SAG hydrochloride Smo activator	ab145866	Abcam
Rotenone	R8875	Sigma Aldrich

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NAME	CATALOG #	VENDOR
Recombinant Human FGF-8a	100-25A	peprotech
LDN193189 hydrochloride	SML0559	
Human BDNF (powder)	T10583	Cambridge University
L-Ascorbic acid	A4544	Sigma Aldrich
Purmorphamine	4551	Tocris
Water (for embryo transfer sterile-filtered BioXtra suitable for mouse embryo cell culture)	W1503	Sigma Aldrich
Dibutyryl cAMP sodium salt	https://www.sigmaaldrich.com/cat	Sigma Aldrich
Citric Acid	C2404	Sigma Aldrich

MATERIALS TEXT

Equipment:

- Centrifuge (for both 15mL & 1.5mL tubes)
- Pipette boy
- Sterile 5/10/25/50mL stripettes
- P1000, P200, P20, P10 pipettes and filter tips
- Vacuum aspirator and tips
- Microbiology Safety Cabinet (MSC)
- Light Microscope
- Scale (for making up BSA)
- Method of Cell Counting (NucleoCounter®NC-200™)
- **37 °C** , 5% CO2 incubator

SAFETY WARNINGS

- Rock inhibitor (Y-27632) - **Harmful** if swallowed, in contact with skin or inhaled.
- CHIR99021 - Powder is **Toxic** by inhalation and ingestion.
- Citric acid powder - **Irritant**
- 2-Mercaptoethanol solution - **Irritant**
- Chemicals dissolved in DMSO (CHIR99021, DAPT, LDN193189, Purmorphamine, SAG, SB431542) - Increased risk of absorption through skin.

ABSTRACT

This protocol describes a method for the production of dopaminergic neurons from human iPSCs, using a 52-day long differentiation method adapted from doi.org/10.1038/nature10648.

Steps in this protocol assume the use of a single 12 well plate for the purposes of differentiation; adjust volumes accordingly depending on labware used.

BEFORE STARTING

iPSCs should be prepared prior to use of this protocol; cultured for at least 2 passages post-thaw in Essential 8 medium on vitronectin (VN-XF)-coated plates.

Supplement Preparation

1 **Supplement Preparation:**

Supplements to be added to media (on day of use) in this protocol are listed in the table below, with recommended stock concentrations for aliquots, concentrations to be used on cells and the diluent needed for each reagent. It is recommended to aliquot supplements in volumes for single use.

Aliquots should be stored at **-80 °C** .

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Supplement	Recommended Stock Concentration	Final Concentration to be used on cells	Diluent
Ascorbic Acid	200 mM	0.2 mM	Water for Cell Culture
hBDNF	100 µg/mL	20 ng/mL	0.1% BSA in Water for Cell Culture
CHIR99021	10 mM	3 µM	DMSO
DAPT	50 mM	10 µM	DMSO
dibutyryl cAMP	100 mM	0.5 mM	Water for Cell Culture
hFGF8a	100 µg/mL	100 ng/mL	0.1% BSA in Water for Cell Culture
hGDNF	100 µg/mL	20 ng/mL	0.1% BSA in Water for Cell Culture
LDN193189	1 mM	100 nM	DMSO
Purmorphamine	20 mM	1 µM	DMSO
SAG	1 mM	100 nM	DMSO
SB431542	10mM	10µM	DMSO
TGF-β3	25µg/mL	1ng/mL	10mM Citric Acid & 0.1% BSA in Water for Cell Culture (1:3)*

List of media supplements used in the protocol.
* When reconstituting, add 1 part 10mM Citric acid first, followed by 3 parts 0.1% BSA.

10% BSA stock solution (100 mg/mL):
Dissolve Bovine Serum Albumin powder in sterile Water for Cell Culture at 100 mg/mL. Filter sterilise with a 0.2 µm sterilising grade filter. Aliquot into sterile microcentrifuge tubes. Store at -20 °C. Thaw at room temperature and resuspend prior to use.

0.1% BSA Solution (1mg/mL):
Dilute a thawed aliquot of 10% BSA solution 100-fold with sterile Water for Cell Culture to create a 0.1% BSA solution; e.g. add 100µL 10% BSA to 9.9mL water.

Plate Preparation (Day -2 & 19)

2 Preparing Geltrex-coated Plate(s) (Day -2 & 19):

This section covers the preparation of the 12 well Geltrex-coated plate(s) that:

- iPSCs will be seeded onto for neural induction (Day -1)
- Early neural progenitors will be seeded onto for neuronal maturation (Day 20)

We recommend preparing the coated plates 1 day prior to the day of use.

Prepare 10% Geltrex stock aliquots:

1. Thaw a vial of Geltrex at **4 °C** overnight.
2. Dilute 1:10 with ice-cold DMEM:F12 w/o phenol red.
3. Aliquot into sterile tubes in single use volumes. Ensure the temperature does not exceed **15 °C** while working with Geltrex.

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4. Store aliquots at **-80 °C**

Prior to coating plates, thaw 10% Geltrex aliquots at **4 °C** overnight or on the day required.

3 Prepare 1% Geltrex by adding **1.25 mL** of 10% Geltrex to **11.25 mL** of ice-cold **DMEM:F12 w/o phenol red,** keeping the Geltrex on ice during the process.

Note: if lumps are observed in the Geltrex aliquot then it should not be used (to avoid premature gelling).

4 Add **1 mL** of cold 1% Geltrex to each well of the 12 well plate.

5 Incubate the plate at **37 °C** for at least **02:00:00** , but not more than 24 hours.

Equilibrate plates to room temperature for 1 hour before use.

Seeding iPSCs for neural induction (Day -1)

6 **Seeding iPSCs for Neural Induction (Day -1):**

This section covers the seeding of iPSCs onto a prepared 12 well Geltrex-coated plate for neural induction.

Note: Our lab carried out Day -1 steps on a Friday and any days mentioned in the protocol assume this to be the case.

Cells are seeded at a density of 200,000 cells/cm^2 or 760,000 cells per well in a 3.8cm^2 12 well. The total number of cells required to seed a 12 well plate is at least **$9.2x10^6$ cells**.

Select an adequate number of high quality iPSC wells/ plates/ flasks at a suitable density for harvesting. As a general guide a 60-80% confluent well on a 6 well plate (9.6cm^2) should yield 2-4 million cells.

7 Prepare **E8-ROCKi** medium by supplementing **E8** with **ROCK inhibitor (Y-27632)** to a final concentration of 10µM.

8 Aspirate the media from the well(s) of the iPSC culture and add **2 mL** of DPBS (-/-) to wash.

9 Aspirate the DPBS (-/-) and add **1 mL** of warm Accutase to each well.

10 Incubate cells for **00:10:00** at **37 °C**

11 Inspect the cultures after incubation to confirm cell detachment.

If cells are still partially adhered, incubate for a further 3 minutes.

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12 Add **2 mL** of **E8-ROCKi** to each well and repeatedly wash the media over the well(s) (*with a P1000 pipette*) to detach the cells and dissociate them to a single-cell suspension.

Confirm a majority single cell suspension has been obtained under the microscope before proceeding to the next step.

If there are a lot of cell aggregates still present then triturate the cell suspensions further with a pipette. Alternatively, centrifuge the cell suspension at 250xg for 3 minutes and resuspend the cell pellet with 1mL E8-ROCKi using a P1000 pipette.

13 Collect the cell suspension from each dissociated well and transfer into 15mL centrifuge tube(s) capped with a 40µm mini cell strainer.

14 Centrifuge cells at **250 x g, Room temperature , 00:03:00** .

15 *Note: These next steps describe a 1/10 dilution to prepare a 500uL suspension for cell counting with a Nucleocounter. Adjust as necessary for your chosen method of counting.*

Prepare a 1.5mL tube for cell counting. Add **450 µl** **E8-ROCKi** to the tube.

16 Remove supernatant and gently re-suspend the cell pellet(s) in **1 mL** of **E8-ROCKi** medium with a P1000 pipette. Pipette up and down several times to ensure a single cell suspension is created.

If there are mutiple tubes then combine the cell suspensions together in one tube.

Top up to **5 mL** with **E8-ROCKi** and mix well.

17 Immediately transfer **50 µl** of the re-suspended cell suspension into the 1.5mL tube prepared in step 15 and perform a viable cell count.

18 Prepare a cell suspension of **9.9x10^6 viable iPS cells** in **13 mL** **E8-ROCKi.** This is enough to seed 12 wells at a seeding density of 760,000 cells/well (or 2x10^5 cells/cm^2).

If seeding a different number of wells always prepare a slightly higher volume than required to account for volume loss during processing.

19 Prepare the Geltrex-coated 12 well plate for seeding by aspirating excess Geltrex and adding **1 mL** of **E8-ROCKi** into each coated well to be used. Do not let the coated wells dry out.

20 Thoroughly mix and resuspend the cell suspension prepared in step 18 by pipetting up and down with a stripette and then transfer **1 mL** to each well of the 12 well plate.

21 Transfer the plate to a tissue culture incubator set at **37 °C** and 5% CO_2. Agitate plate gently on the incubator

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shelf to ensure even distribution of cells across wells and then leave undisturbed overnight.

22 Neural Induction (Day 0):

Inspect the plate(s) and verify that the cells are well-distributed and ~90-100% confluent.

From this point, carry out a daily media change as described in the '**Media Change**' section until Day 20 when the culture is passaged.

Media Change (Day 0-52)

23 Media Change (Day 0-52):

Confirm how much media you will need for the next few days before making up base media (or for the day in the case of supplemented media).

Base media should be warmed to between **Room temperature** and **37 °C** before adding supplements. Do not put complete supplemented media in the water bath.

Perform media change very gently, especially later on into the differentiation process, as cells can detach very easily. *We recommend tilting plates and dispensing media down the side of each well to reduce force applied to the cell layer.*

Refer to the 'Media Composition' section for details on each media type and which media to use for Day 0-12.

Day	Media Change	Media Type	Media feed volume per well
0-12	Daily	Differs (See 'Media Composition')	2 mL
13-19	Daily	Maturation Medium	2 mL
20	Passage	Maturation Medium	-
21-22	Daily	Maturation Medium	1 mL
23-33	Every other day	Maturation Medium	2 mL
34-52	Every 2 (*or 3) days	Maturation Medium	2mL (*or 3mL)

Media change instructions across the differentiation process.
* Our lab fed cells with 3mL of media on Fridays from Day 34 onward to avoid media change during weekends.

Media Composition

24 Base Media:

Listed below are the reagent quantities needed for composition of the three types of base media used throughout this protocol. All of these base media require further supplements prior to media change; supplements needed are listed in the **Neural Induction Media** and **Maturation Medium** steps.

Note: Use-within dates are only for the base media, not for media with added supplements. Supplemented media (i.e. Neural Induction and Maturation media) should only be used on the day of preparation.

KOSR (use within 1 week)	100mL	1mL	[Final]
Knockout DMEM	81.98 mL	819.8 µL	-

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Knockout Serum Replacement (KSR)	15 mL	150 µL	15%
Glutamax (100X)	1 mL	10 µL	1X
MEM Non Essential Amino Acids (NEAA) (100X)	1 mL	10 µL	1mM
2-mercaptoethanol (50mM)	20 µL	0.2 µL	0.01mM
Penicillin-Streptomycin (10,000 U/mL)	*1 mL*	*10 µL*	*100 U/mL*

A base media used for days 0-10 of the differentiation process. Use of Penicillin-Streptomycin is optional.

NNB (Use within 3 days)	100mL	1mL	[Final]
Neurobasal medium	96.5 mL	965 µL	-
N2 (100X)	0.5 mL	5 µL	0.5X
B27 (50X)	1 mL	10 µL	0.5X
Glutamax (100X)	1 mL	10 µL	1X
Penicillin-Streptomycin (10,000 U/mL)	*1 mL*	*10 µL*	*100 U/mL*

A base media used for days 5-10 of the differentiation process. Use of Penicillin-Streptomycin is optional.

NB/B27 (Use within 3 days)	100mL	1mL	[Final]
Neurobasal medium	96 mL	0.96 mL	-
B27 (50X)	2 mL	20 µL	1X
Glutamax (100X)	1 mL	10 µL	1X
Penicillin-Streptomycin (10,000 U/mL)	*1 mL*	*10 µL*	*100 U/mL*

A base media used for days 11-52 of the differentiation process. Use of Penicillin-Streptomycin is optional.

25 Neural Induction Media:

This is the media that will be used for the neural induction stage (Day 0-12). Each complete medium must be used only on the day of preparation, though the unsupplemented base media (varies) can be prepared earlier and used within the associated timespan listed in the 'Base Media' tables.

During neural induction, the media is gradually altered from 100% KOSR to 25% KOSR/75% NNB.

Days listed in the table assume that iPSC seeding (Day -1) takes place on a Friday.

Day 0	**Neural Induction Medium-1**	**28 mL**	**1mL**	**[Final]**
(Sat)	KOSR	28mL	1 mL	100%
	LDN193189 (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	SB431542 (stock 10 mM)	28 µL	1 µL	10 µM
Day 1-2	**Neural Induction Medium-2**	**28 mL**	**1 mL**	
(Sun/Mon)	KOSR	28mL	1 mL	100%
	LDN193189 (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	SB431542 (stock 1 0mM)	28 µL	1 µL	10 µM
	SAG (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	Purmorphamine (stock 20 mM)	2.8 µL	0.1 µL	2 µM
	FGF8a (stock 100 µg/mL)	28 µL	1 µL	100ng/mL
Day 3-4	**Neural Induction Medium-3**	**28 mL**	**1 mL**	**[Final]**
(Tue/Wed)	KOSR	28mL	1 mL	100%
	LDN193189 (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	SB431542 (stock 10 mM)	28 µL	1 µL	10 µM

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	SAG (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	Purmorphamine (stock 20 mM)	2.8 µL	0.1 µL	2 µM
	FGF8a (stock 100 µg/mL)	28 µL	1 µL	100ng/mL
	CHIR99021 (stock 10 mM)	8.4 µL	0.3 uL	3 µM
Day 5-6	**Neural Induction Medium-4**	**28 mL**	**1 mL**	**[Final]**
(Thu/Fri)	KOSR	21 mL	0.75 mL	75%
	NNB	7 mL	0.25 mL	25%
	LDN193189 (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	SAG (stock 1mM)	2.8 µL	0.1 µL	100 nM
	Purmorphamine (stock 20mM)	2.8 µL	0.1 µL	2 µM
	FGF8a (stock 100 µg/mL)	28 µL	1 µL	100ng/mL
	CHIR99021 (stock 10 mM)	8.4 µL	0.3 uL	3 µM
Day 7-8	**Neural Induction Medium-5**	**28 mL**	**1 mL**	**[Final]**
(Sat/Sun)	KOSR	14 mL	0.5 mL	50%
	NNB	14 mL	0.5 mL	50%
	LDN193189 (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	CHIR99021 (stock 10 mM)	8.4 µL	0.3 uL	3 µM
Day 9-10	**Neural Induction Medium-6**	**28 mL**	**1 mL**	**[Final]**
(Mon/Tue)	KOSR	7 mL	0.25 mL	25%
	NNB	21 mL	0.75 mL	75%
	LDN193189 (stock 1 mM)	2.8 µL	0.1 µL	100 nM
	CHIR99021 (stock 10 mM)	8.4 µL	0.3 uL	3 µM
Day 11-12	**Neural Induction Medium-7**	**28 mL**	**1 mL**	**[Final]**
(Wed/Thu)	Maturation Medium	28 mL	1 mL	100%
	CHIR99021 (stock 10 mM)	8.4 µL	0.3 µL	3 µM

Various base media supplemented for days 0-12 of the differentiation process. Note that volumes are based on recommended stock concentrations (column 'B'). Column 'C' gives recommended volumes to prepare, assuming use of a single 12 well plate for this protocol (2mL per well plus excess).

26 Maturation Medium:

This is the medium that will be used for the majority of this protocol (Day 13 onward). The complete supplemented medium must be used only on the day of preparation, though the base medium (NB/B27) can be prepared earlier and used within 3 days.

Maturation Medium (Use within 1 day)	28mL	*1mL*	[Final]
NB/B27			
Neurobasal medium	26.88 mL	960 µL	-
B27 (50X)	560 µL	20 µL	1X
Glutamax (100X)	280 µL	10 µL	1X
Penicillin-Streptomycin (10,000 U/mL)	280 µL	10 µL	100 U/mL
Supplements			
BDNF (stock 100 µg/mL)	5.6 µL	0.2 µL	20 ng/mL
DAPT (stock 50 mM)	5.6 µL	0.2 µL	10 uM
GDNF (stock 100 µg/mL)	5.6 µL	0.2 µL	20 ng/mL
TGFB3 (stock 25 µg/mL)	1.12 µL	0.04 µL	1 ng/mL
Dibutyryl cAMP (stock 100mM)	140 µL	5 µL	0.5 mM
Ascorbic acid (stock 200mM)	28 µL	1 µL	0.2 mM

The NB/B27 base media supplemented for days 13-52 of the differentiation process. Note that volumes are based on recommended stock concentrations (column 'A'). Use of Penicillin-Streptomycin is optional. Column 'B' gives recommended volume to prepare for most days, assuming use of a single 12 well plate for this protocol (2mL per well plus excess).

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Passage (Day 20)

27 **Passaging of Neuro-epithelial sheet (Day 20):**

This section covers the passaging of the neuro-epithelial sheet on Day 20. Cells are lifted and re-seeded for neuronal maturation at **3.5x10^5 cells/cm^2** on a 12 well Geltrex-coated plate (***prepare prior*** to these steps as described in the '**Plate Preparation**' section). This is the only passage during the differentiation process.

Note: These steps assume seeding back onto one 12 well plate, however we found that we consistently had more than enough cells to seed two 12 well plates after this passage; adjust volumes as needed.

28 Prepare 30mL of **Wash Buffer 1** and 12mL **Wash Buffer 2** for every 12 well plate to be dissociated.

28.1 Supplement **42 mL** DMEM:F12 + GlutaMAX with ROCK inhibitor (Y-27632) to a final concentration of **10 Micromolar (µM)** .

28.2 Split this into two tubes of **30 mL** (for **Wash Buffer 1**) and **12 mL** (**Wash Buffer 2**).

Wash Buffer 1 *will be further supplemented with DNase1 in step 34.*

1 vial DNase (D2) should be added for every 15mL of ***Wash Buffer 1*** *prepared. However,* ***DNase should only be added immediately prior to use of the buffer*** *when the cells are nearing the end of their dissociation. Keep the DNase at 4 °C until use.*

29 Prepare **Dissociation Buffer** by supplementing **10 mL** Accutase (warmed) with 2 vials Papain (PDS Kit).

Reconstitute the lyophilised papain with the Accutase, replace the lid on the vial and invert several times ensuring all powder in the vial and on the lid is dissolved. Transfer solution back into the Dissociation Buffer tube.

0.5mL Dissociation Buffer is required per well to be harvested. 5 wells of a 12 well plate (2.5mL Dissociation Buffer) will provide enough cells to seed onto one 12 well plate; adjust volumes as needed.

30 Prepare **Seeding Medium** by supplementing **30 mL** of **Maturation Medium** with ROCK inhibitor (Y-27632) to a final concentration of 10µM.

31 Aspirate the media from the well(s) of the neuronal culture and gently add **1 mL** of DPBS (-/-) without disturbing the cell layer.

32 Aspirate the DPBS (-/-) and add **0.5 mL** of **Dissociation Buffer** to each well.

33 Transfer cells to a **37 °C** 5% CO_2 tissue culture incubator. Incubate for **00:20:00** .

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34 Complete the preparation of **Wash Buffer 1** within the last 5 minutes of incubation of the cells.

- Retrieve the **DNase (D2)** vial(s) from **4 °C** storage.
- Reconstitute the vial(s) by adding **250 µl** **Wash Buffer 1** to each vial to make a **2 mg/ml** solution. Make sure to reconstitute all the powder (check the lid). Avoid vigorous mixing - do not vortex.
- Transfer the entire contents of the reconstituted vial(s) back into the Wash Buffer 1 tube. **Use 250µL (1 vial) for every 15 mL buffer.**

35 Following incubation inspect the cells under a microscope.

The cells should be detaching from the plate and the cell layer should have a darkened appearance. Cells will have a rounded appearance as they dissociate and individual cells should be visible at the edges or in gaps in the cell layer.

36 ***Optional:*** Test for ease of dissociation by gently pipetting ~ **100 µl** of the Dissociation Buffer against the cells with a P1000 pipette. If cells do not dissociate easily, extend digestion by **00:03:00** and repeat this step, but do not exceed 30 mins.

37 Add **2 mL** of **Wash Buffer 1** per well.

38 ⚠

Repeatedly wash the buffer over the well(s) with a P1000 pipette to detach the cells and dissociate them to a single-cell suspension.

Sufficient dissociation is critical at this step. It is recommended to check the cell suspension under a microscope and if necessary pipette the cell suspension up and down further until the majority is single cells. Try to avoid over-pipetting the cells as this could affect viability.

39 Transfer the cell suspension into a 15mL centrifuge tube capped with a 40µm cell strainer.

40 ***Optional:*** If there are residual cells in the plate, wash well(s) with **0.5 mL** of **Wash Buffer 1** and transfer this suspension into the tube from the previous step.

41 Centrifuge cells at **150 x g, Room temperature , 00:03:00**

42 Aspirate supernatant and gently re-suspend the cell pellet in **12 mL** (~1mL per well harvested) of **Wash Buffer 2.**

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43 Centrifuge cells at **150 x g, Room temperature , 00:03:00** .

44 *Note: These next steps describe a 1/10 dilution to prepare a 500uL suspension for cell counting with a Nucleocounter. Adjust as necessary for your chosen method of counting.*

Prepare a 1.5mL tube for cell counting. Add **450 µl** **Wash Buffer 2** to the tube.

45 Aspirate supernatant and gently re-suspend the cell pellet in **5 mL** of **Seeding Medium**.

46 Immediately transfer **50 µl** of the re-suspended cell suspension into the 1.5mL tube prepared in step 44 and perform a viable cell count.

47 Prepare a cell suspension of **1.729x10^7 viable cells** in **13 mL** **Seeding Medium.** This is enough to seed 12 wells at a density of 1.33x10^6 cells per well.

If seeding a different number of wells always prepare a slightly higher volume than required to account for volume loss during processing.

48 Aspirate excess Geltrex from the prepared 12 well plate and immediately add **1 mL** of **Seeding Medium** into each coated well to be used. Do not let the coated wells dry out.

49 Thoroughly mix and resuspend the cell suspension prepared in step 47 by pipetting up and down with a stripette. Transfer **1 mL** of the cell suspension to each well of the prepared Geltrex 12 well plate.

50 Transfer the plate to a tissue culture incubator set at **37 °C** and 5% CO_2. Agitate the plate gently on the incubator shelf to ensure even distribution of cells across wells and then leave undisturbed overnight.

51 On Day 21 inspect the plate(s) and verify that the cells are attached and well distributed.

Continue to maintain the culture with media changes as described in the '**Media Change**' section up to Day 52 (or beyond, according to your requirements)

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