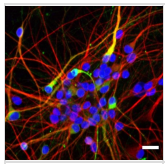

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Differentiation of iPSC into dopaminergic neurons

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on behalf of the Foundational Data Initiative for Parkinson's Disease (FOUNDIN-PD)[3]

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1 Works for me dx.doi.org/10.17504/protocols.io.bfpzjmp6

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ABSTRACT

Induced pluripotent stem cell (iPSC)-derived dopaminergic neurons is a promising tool to model Parkinson's disease and of great interest for studying disease mechanisms. Our aim is to describe the protocol for differentiation of iPSC into dopaminergic (DA) neurons currently used in our laboratory. The protocol described here is based on a previously published dual-SMAD inhibition protocol (Kriks et al. Nature 2011). Minor modifications were required for the implementation of the protocol in our automated cell culture system (Dhingra et al. JoVE 2020). The differentiation takes 65 days and produces substantial amounts of MAP2 (neuron) and TH (DA neuron) positive cells.

EXTERNAL LINK

https://www.foundinpd.org/wp/

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Dhingra A, Täger J, Bressan E, Rodriguez-Nieto S, Bedi MS, Bröer S, Sadikoglou E, Fernandes N, Castillo-Lizardo M, Rizzu P, Heutink P. Automated production of human induced pluripotent stem cell-derived cortical and dopaminergic neurons with integrated live-cell monitoring. J Vis Exp 2020 (In revision, JoVE61525).

ATTACHMENTS

Figure_1_iPSC.pdf Figure_2_DA_neuron_differentiation_protocol_schema.pdf Kriks et al. Nature 2011.pdf

MATERIALS

NAME	CATALOG #	VENDOR
BDNF (brain-derived neurotrophic factor)	450-02	peprotech
CHIR99021	4423	R&D Systems
DAPT	13197-50	Cayman Chemical Company
Db-cAMP (dibutyryl-cyclic AMP)	D0627	Sigma
Essential 8 Flex complete medium	A2858501	Gibco - Thermo Fisher
Fibronectin	356008	Corning
FGF-8b (recombinant human/murine fibro fibroblast growth factor-8b)	100-25	peprotech
GDNF (glial cell line-derived neurotrophic factor)	450-10	peprotech
Laminin	L2020	Sigma
L-ascorbic acid		Sigma
LDN193189	11802	Cayman Chemical Company
Matrigel	354277	Corning
Poly-L-Ornithine (PLO)	P3655	Sigma
Purmorphamine	1000963410	Cayman Chemical Company
SHH (recombinant human Sonic Hedgehog/Shh (C24II) N-Terminus)	1845-SH	R&D Systems

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NAME	CATALOG #	VENDOR
TGFβ3 (recombinant human transforming growth factor-beta 3)	243-B3	R&D Systems
Y-27632 dihydrochloride	1000558310	Cayman Chemical Company
Knockout DMEM/F-12	12660012	Gibco - Thermo Fisher
Knockout serum replacement (KSR)	10828028	Gibco - Thermo Fisher
GlutaMAX	35050038	Gibco - Thermo Fisher
MEAA (MEM Non-Essential Amino Acids)	11140050	Gibco - Thermo Fisher
Penicillin-streptomycin (P/S)	15140122	Gibco - Thermo Fisher
Neurobasal medium	21103049	Gibco - Thermo Fisher
B27 supplement minus vitamin A	12587010	Gibco - Thermo Fisher
N2 supplement	17502048	Gibco - Thermo Fisher
DMEM-F12 medium	31331093	Gibco - Thermo Fisher
2-Mercaptoethanol	21985023	Gibco - Thermo Fisher
Accutase cell dissociation reagent	A1110501	Gibco - Thermo Fisher
DPBS no calcium no magnesium	14190169	Gibco - Thermo Fisher
DMSO (dimethyl sulfoxide)	D2650	Sigma
HCl (hydrochloric acid)	9277	Carl Roth
HSA (human serum albumin)	A6784	Sigma
SHH (recombinant human Sonic Hedgehog/Shh (C24II) N-Terminus)	1845-SH	R&D Systems

MATERIALS TEXT

Reagent preparation and storage

The reagent preparation should be conducted under sterile conditions in a laminar flow cabinet. After acquisition, reagents stored at -20 °C should reach room temperature (RT) before reconstitution. Note that some reagents must be protected from the light and/or humidity. After reconstitution, fresh-made stock solutions should be immediately aliquoted in sterile vials and stored at -20°C. Keep thawed aliquots at 4 °C for up to one week. Note the the expiration time for each product.

BDNF: Reconstitute BDNF in 0.1% HSA/PBS to obtain a stock concentration of 20 ng/mL.

CHIR99021: Reconstitute CHIR99021 in dimethyl sulfoxide (DMSO) to obtain a stock concentration of 3 mM.

DAPT: Reconstitute DAPT in DMSO to obtain a stock concentration of 10 mM.

Db-cAMP: Reconstitute db-cAMP in deionized sterile water to obtain a stock concentration of 200 mM. Filter the stock solution with a 0.22 µm pore size hydrophilic PVDF membrane. Protect from the light and humidity.

Fibronectin: Reconstitute fibronectin in deionized sterile water to obtain a stock concentration of 1 µg/µL.

FGF-8b: Reconstitute FGF-8b in 0.1% HSA/PBS to obtain a stock concentration of 100 µg/mL.

GDNF: Reconstitute GDNF in 0.1% HSA/PBS to obtain a stock concentration of 20 ng/mL.

Laminin: No reconstitution required. For further dilutions, take into consideration the protein concentration published in the certificate of analysis of the product.

L-ascorbic acid: Reconstitute L-ascorbic acid in deionized sterile water to obtain a stock concentration of 200 mM. Minimize exposure to air. Protect from the light.

LDN193189: Reconstitute LDN193189 in DMSO to obtain a stock concentration of 100 µM. Protect from the light.

Matrigel: No reconstitution required. Aliquots should be done on ice according the dilution factor indicated in certificate of analysis of the product.

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Poly-L-Ornithine: Reconstitute poli-l-ornithine in PBS to obtain a stock concentration of 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 2 mM.

SHH: Reconstitute SHH in 0.1% HSA/PBS to obtain a stock concentration of 100 µg/mL.

SB431542: Reconstitute SB431542 in DMSO to obtain a stock concentration of 10 mM.

TGFβ3: Reconstitute TGFβ3 in 0.1% HSA/4 mM HCl/PBS to obtain a stock concentration of 20 µg/mL.

Y-27632: Reconstitute Y-27632 in DMSO to obtain a stock concentration of 10 mM.

iPSC preparation for differentiation 1w

1 Grow iPSC on matrigel-coated plates with Essential E8 Flex medium until they cover 70-80% of the well area.

2 Check if iPSC appear pluripotent and undifferentiated using a bright-field microscope. The iPSC should show a typical morphology with high nuclear-to-cytoplasm ratio, prominent nucleolus and densely packed colonies (Figure 1).

3 Dissociate iPSC into single cells with accutase (aprox. 30 min at 37 °C) and replate at 200,000 per cm^2 on matrigel-coated plates and Essential 8 Flex medium (200 µL/cm^2) supplemented with 10 µM Y-27632 (until day 0 of differentiation).

Matrigel coating: Use the same matrigel concentration applied for iPSC growth advised in the certificate of analyis of the product. Increase the coating time to 12 hours at 37 °C for differentiation. Use plates immediately after coating.

4 Once iPSC cover 100% of the well area, usually 24 to 48 after single cell replating, start the differentiation into dopaminergic neurons.

Dopaminergic neuron differentiation 9w 2d

5 A schematic representation of the differentiation protocol is shown in Figure 2.

Start day 0 of differentiation by changing the culture medium to differentiation medium (KSR medium; 200 µL/cm^2) supplemented with small molecules as described below:

- Day 0 - 1: 100 nM LDN193189, 10 µM SB431542
- Day 1 - 3: 100 nM LDN193189, 10 µM SB431542, 1 mM SHH, 2 µM Purmorphamine, 100 ng/mL FGF-8b
- Day 3 - 5: 100 nM LDN193189, 10 µM SB431542, 1 mM SHH, 2 µM Purmorphamine, 100 ng/mL FGF-8b, 3 µM CHIR99021
- Day 5 - 7: 100 nM LDN193189, 1 mM SHH, 2 µM Purmorphamine, 100 ng/mL FGF-8b, 3 µM CHIR99021
- Day 7 - 9: 100 nM LDN193189, 1 mM SHH, 3 µM CHIR99021
- Day 9 - 11: 100 nM LDN193189, 1 mM SHH, 3 µM CHIR99021

KSR (knockout serum replacement) medium composition and storage: Mix 409.5 mL of knockout DMEM/F-12 medium with 75 mL knockout serum replacement (15%), 5 mL GlutaMAX (2 mM), 5 mL MEM Non-Essential Amino Acids (1%), 0.5 mL 2-mercaptoethanol (55 µM) and 5 mL Penicillin-Streptomycin (1%). Store media without small molecules and growth factors at 4 °C for up to one week or at -20 °C for up to four weeks.

6 From day 5, combine KSR with N2 medium and perform media changes (200 µL/cm^2) as described below:

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- Day 0 - 5: 100 % KSR
- Day 5 - 7: 75% KSR/25% N2
- Day 7 - 9: 50% KSR/50% N2
- Day 9 - 11: 25% KSR/75% N2

N2 medium composition and storage: Mix 475 mL Neurobasal medium with 5 mL N2 supplement (1%), 10 mL B27 supplement (2%), 5 mL GlutaMAX (2 mM) and 5 mL Penicillin-Streptomycin (1%). Store media without small molecules and growth factors at 4 °C for up to one week or at -20 °C for up to four weeks.

7 From day 11, replace KSR/N2 by NB/B27 medium supplemented with the following small molecules and growth factors:

- 3 µM CHIR99021 (until day 13)
- 0.2 mM Ascorbic acid
- 20 ng/mL BDNF
- 10 µM DAPT
- 1 mM db-cAMP
- 20 ng/mL GDNF
- 1 ng/mL TGFβ3

NB/B27 medium composition and storage: Mix 485 mL Neurobasal (NB) medium with 10 mL B27 supplement (2%) and 5 mL Penicillin-Streptomycin (1%). Store media without small molecules and growth factors at 4 °C for up to one week or at -20 °C for up to four weeks.

8 On day 25, dissociate dopaminergic precursors into single cells with accutase (aprox. 40 min at 37 °C) and replate at 400,000 per cm^2 on plates pre-coated with poly-l-ornithine (0.1 mg/mL), laminin (10 µg/mL) and fibronectin (2 µg/mL). Cultivate cells in differentiation medium (200 µL/cm^2).

Differentiation medium: NB/B27 medium supplemented with 0.2 mM Ascorbic acid, 20 ng/mL BDNF, 10 µM DAPT, 1 mM db-cAMP, 20 ng/mL GDNF , 1 ng/mL TGFβ3 and 10 µM Y-27632 (until day 26).

NOTE 1: It is highly recommended to avoid the storage of differentiation medium containing small molecules and growth factors.
NOTE 2: Dopaminergic precursors can be frozen on day 25 of differentiaion.
NOTE 3: An additional replating at lower cell densities (100,000 per cm^2) can be performed on day 32 of differentiation for single-cell imaging.

9 On day 26, perform media change (200 µL/cm^2) to remove dead cells and debris. Add freshly prepared differentiation medium supplemented with small molecules and growth factors, as described in step 8 (without Y-27632).

10 From day 29, perform media changes every 3-4 days as described in step 9 .

11 On day 65 of differentiation, process differentiated cells for assays as desired.

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