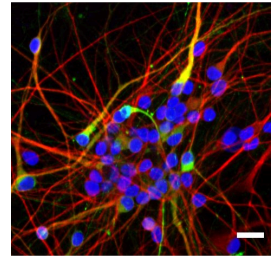


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Differentiation of iPSC into dopaminergic neurons

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We use this protocol and it's working

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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.

Attachments



[Figure_1_iPSC.pdf](#)

1.4MB



[Figure_2_DA_neuron_d..](#)

527KB



[Kriks et al. Nature ...](#)

2.3MB



Materials

MATERIALS

- ✕ BDNF (brain-derived neurotrophic factor) **peprotech Catalog #450-02**
- ✕ CHIR99021 **R&D Systems Catalog #4423**
- ✕ DAPT **Cayman Chemical Company Catalog #13197-50**
- ✕ Db-cAMP (dibutyl-cyclic AMP) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0627**
- ✕ Essential 8 Flex complete medium **Gibco - Thermo Fisher Scientific Catalog #A2858501**
- ✕ Fibronectin **Corning Catalog #356008**
- ✕ FGF-8b (recombinant human/murine fibro fibroblast growth factor-8b) **peprotech Catalog #100-25**
- ✕ GDNF (glial cell line-derived neurotrophic factor) **peprotech Catalog #450-10**
- ✕ Laminin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L2020**
- ✕ L-ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich)**
- ✕ LDN193189 **Cayman Chemical Company Catalog #11802**
- ✕ Matrigel **Corning Catalog #354277**
- ✕ Poly-L-Ornithine (PLO) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3655**
- ✕ Purmorphamine **Cayman Chemical Company Catalog #1000963410**
- ✕ SHH (recombinant human Sonic Hedgehog/Shh (C24II) N-Terminus) **R&D Systems Catalog #1845-SH**
- ✕ TGFβ3 (recombinant human transforming growth factor-beta 3) **R&D Systems Catalog #243-B3**
- ✕ Y-27632 dihydrochloride **Cayman Chemical Company Catalog #1000558310**
- ✕ Knockout DMEM/F-12 **Gibco - Thermo Fisher Scientific Catalog #12660012**
- ✕ Knockout serum replacement (KSR) **Gibco - Thermo Fisher Scientific Catalog #10828028**
- ✕ GlutaMAX **Gibco - Thermo Fisher Scientific Catalog #35050038**
- ✕ MEAA (MEM Non-Essential Amino Acids) **Gibco - Thermo Fisher Scientific Catalog #11140050**
- ✕ Penicillin-streptomycin (P/S) **Gibco - Thermo Fisher Scientific Catalog #15140122**
- ✕ Neurobasal medium **Gibco - Thermo Fisher Scientific Catalog #21103049**
- ✕ B27 supplement minus vitamin A **Gibco - Thermo Fisher Scientific Catalog #12587010**
- ✕ N2 supplement **Gibco - Thermo Fisher Scientific Catalog #17502048**
- ✕ DMEM-F12 medium **Gibco - Thermo Fisher Scientific Catalog #31331093**
- ✕ 2-Mercaptoethanol **Gibco - Thermo Fisher Scientific Catalog #21985023**
- ✕ Accutase cell dissociation reagent **Gibco - Thermo Fisher Scientific Catalog #A1110501**
- ✕ DPBS no calcium no magnesium **Gibco - Thermo Fisher Scientific Catalog #14190169**

⊗ DMSO (dimethyl sulfoxide) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D2650**

⊗ HCl (hydrochloric acid) **Carl Roth Catalog #9277**

⊗ HSA (human serum albumin) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A6784**

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

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 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.

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.

Troubleshooting

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 cm² on matrigel-coated plates and Essential 8 Flex medium (200 µL/cm²) 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 analysis 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/cm²) 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/cm²) as described below:

- 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 cm² 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/cm²).

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 differentiation.



NOTE 3: An additional replating at lower cell densities (100,000 per cm²) can be performed on day 32 of differentiation for single-cell imaging.

- 9 On day 26, perform media change (200 µL/cm²) 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.