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Dopaminergic neuron differentiation V.1

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We use this protocol and it's working

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Abstract

This protocol has been used to differentiate dopaminergic neurons from hPSC adapted to feeder free culture systems.

Protocol overview

- A. Plate preparation
- B. Media recipes
- C. Dopaminergic neuron differentiation

Attachments



Dopaminergic neuron ...

64KB

Materials

	Item	Vendor	Catalog number
	DMEM/F12	Gibco	11320033
	mTESR-plus Medium	StemCell Tech	100-0276
	Neurobasal medium	Gibco	21103049
	N2 supplement	Gibco	17502048
	B27 supplement without vitamin A	Gibco	12587-010
	L-Glutamine	Sigma	G8450
	Penicillin-Streptomycin	Gibco	15140122
	Y-27632 Dihydrochloride Rock inhibitor	ToCris	CD0141
	DPBS (No calcium, No magnesium)	Cytiva	SH30028
	Accutase	Innovative Cell Tech	AT104
	Matrigel	Corning	354230
	Laminin	R&D Systems	3400-010-02
	Poly-L-Ornithine	Sigma	P3655
	LDN	Stemgent	04-0074
	SHH C25II	R&D Systems	1845-SH-100
	SB431542	SelleckChem	S1067
	CHIR99021	ToCris	4423
	GNF	PeProtech	450-10
	BDNF	PeProtech	450-02
	Dibutyryl-cAMP (Bucladesine)	SelleckChem	S7858
	Sodium L-Ascorbate	Sigma	A40-34
	TGFβ3	R&D Systems	8420-B3-005
	DAPT	Tocris	2634



Plate preparation

1 Plate preparation:

Note

This protocol has been used to differentiate dopaminergic neurons from hPSC adapted to feeder free culture systems. Our version of the protocol has been adapted from [Kim et al. 2021; Cell Stem Cell 28, 343–355 e5, February 4, 2021](#) and [Piao et al. 2021; Cell Stem Cell 28, 217–229 e7, February 4, 2021](#).

Note

We have used two alternative protocol variants with minor modifications which will be outlined at the respective steps as **SP** (Soldner Protocol) and **HP** (Hockemeyer Protocol).

Note

It is important to start the differentiation from pristine, undifferentiated feeder free cultures. For more details consult: <https://doi.org/10.17504/protocols.io.b4mcqu2w>

- 1.1 **Matrigel/Geltrex coating:** Prepare matrigel/geltrex (1:30) in cold DMEM/F12 or DPBS in a 15 ml tube as described by the manufacturer. Coat each well with 1-1.5 ml (6 well plate size) or 0.5 ml (12w plate size) of solution and incubate at 37 °C incubator for at least 00:30:00 to 1 hour. 30m
- 1.2 **Laminin coating:** Prepare laminin (2 µg/ml) in cold DPBS and coat each well of a 6-well plate with 1.5ml laminin solution (2 µg/ml) and incubate Overnight at 37 °C . 1h
- 1.3 **Poly-L-Ornithine (PLO) + Fibronectin (F) + Laminin (L) coating:** Prepare PLO (15 µg/ml) in sterile water and coat each well with 0.5-1 ml making sure it covers the entire surface. Incubate at 37 °C for 06:00:00 to overnight. The day after, wash each well with sterile water 4 times and add a new solution with fibronectin (1 µg/ml) and laminin (2 µg/ml) (in water) and incubate Overnight at 37 °C . Do not let the wells dry. 12h
- 1.4 **Poly-L-Ornithine (PLO) + Laminin (L) coating:** Prepare PLO (15 µg/ml) in sterile water and coat each well of a 12-well plate with 0.5-1 ml making sure it covers the entire 12h



surface. Incubate at 37 °C for 06:00:00 to overnight. The day after, wash each well with sterile water 2 times and add a new solution with laminin (2 µg/ml) (in cold DPBS) and incubate Overnight at 37 °C . Do not let the wells dry.

Media Preparation

2 Media recipes:

- 2.1 **Media A:** Neurobasal media + N2 supplement (1% vol/vol) + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + SHH (200 ng/ml) + CHIR99021 (0.7 µM) + LDN (250 nM) + SB431542 (10 µM).
- 2.2 **Media B:** Neurobasal media + N2 supplement (1% vol/vol) + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + SHH (200 ng/ml) + CHIR99021 (7.5 µM) + LDN (250 nM) + SB431542 (10 µM).
- 2.3 **Media C:** Neurobasal media + N2 supplement (1% vol/vol) + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + CHIR99021 (7.5 µM).
- 2.4 **Media D:** Neurobasal media + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + BDNF (20 ng/ml) + GDNF (20 ng/ml) + Ascorbic acid (200 µM) + Dibutyryl-cAMP (0.5 mM) + TGFβ3 (1ng/ml) + CHIR99021 (3 µM).
- 2.5 **Precursor splitting media:** Neurobasal media + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + BDNF (20 ng/ml) + GDNF (20 ng/ml) + Ascorbic acid (200 µM) + Dibutyryl-cAMP (0.5 mM) + TGFβ3 (1ng/ml).
- 2.6 **Maturation media:** Neurobasal media + B27 supplement without vitamin A (2% vol/vol) + L-Glutamine (2 mM) + Penicillin-Streptomycin (100U/ml) + BDNF (20 ng/ml) + GDNF (20 ng/ml) + Ascorbic acid (200 µM) + Dibutyryl-cAMP (0.5 mM) + TGFβ3 (1ng/ml) + DAPT (10 µM).

Dopaminergic neuron differentiation

1h 35m

- 3 Day 0: Dissociate hPSCs using accutase (00:10:00 - 37 °C). Quench dissociation by diluting the accutase solution with mTeSR-plus media + 10 µM Y-27632 and collect the cells into a 15ml conical tube. Spin the cells down at 105 rcf for 00:05:00 . Remove the supernatant, resuspend the cells in mTeSR-plus media + 10 µM Y-27632 and count them.

15m



- 3.1 **SP:** Plate the cells at 400-600k/cm² in media A + 10 µM Y-27632 onto geltrex coated plates (adjust cell number to the plate size being used).
- 3.2 **HP:** Plate the cells at 400-600k/well in a 6-well plate in mTeSR-plus + 10 µM Y-27632 onto matrigel coated plates.
- 4 Day 1-3: Change the media - media A (change daily or every other day as necessary).
- 5 Day 4 and day 6: Change the media- media B (change daily or every other day as necessary).
- 6 Day 7 and Day 9: Change the media - media C (change daily or every other day as necessary).
- 7 Day 10: Change the media - media D.
- 8 Day 11: Passage #1. Dissociate cells using accutase (approximately  00:10:00 -  37 °C but longer incubations may be necessary to properly detach the cells). Quench dissociation by diluting the accutase solution with 1 ml/well media D + 10 µM Y-27632. Collect cells in 15ml conical tubes and spin them down at  300 rcf -  00:05:00 . Remove the supernatant, resuspend the cell in media D + 10 µM Y-27632.
- 8.1 **SP:** Count the cells and plate them at 800k/cm² in media D + 10 µM Y-27632 onto PLO+F+L coated plates.
- 8.2 **HP:** Split 1:2 in precursor splitting media + 10 µM Y-27632 onto laminin-coated plates.
- 9 Day 12-15: Change the media - maturation media (change daily or every other day as necessary).
- 10 Day 16: Passage #2. Dissociate cells using accutase (approximately  00:10:00 -  37 °C but longer incubations may be necessary to properly dissociate collect the cells). Quench dissociation by diluting accutase with 1 ml/well precursor splitting media + 10 µM Y-27632. Collect the cells in 15 ml conical tubes and spin them down at

15m

15m



300 rcf - 00:05:00 . Remove the supernatant, resuspend the cell in precursor splitting media + 10 μ M Y-27632 and count them.

- 10.1 **SP:** Plate the cells at 800k/cm² in precursor splitting media + 10 μ M Y-27632 onto PLO+F+L coated plates.
- 10.2 **HP:** Plate the cells at >1 million cells per well of 12-well plate in maturation media + 10 μ M Y-27632 onto PLO+L coated plates.
- 11 Day 17-24: Change the media - maturation media (change daily or every other day as necessary).
- 12 Day 25: Passage #3.
- 12.1 **SP:** Dissociate cells using papain solution (00:30:00 - 37 °C). Inactivate papain solution using ovomucoid trypsin inhibitor (10mg/ml). Collect your cells in 15 ml conical tubes and spin them down at 300 rcf - 00:05:00 . Remove the supernatant, resuspend the cells in maturation media + 10 μ M Y-27632 and count them. Plate the cells at 200-300k/cm² in maturation media + 10 μ M Y-27632 onto PLO+L+F coated plates. 35m
- 12.2 **HP:** Dissociate cells using accutase (approximately 00:10:00 - 37 °C is the usual standard but longer incubations may be necessary to properly detach the cells). Quench accutase by adding maturation media + 10 μ M Y-27632. Collect your cells in 15 ml conical tubes and spin them down at 300 rcf - 00:05:00 . Remove the supernatant, resuspend the cells in maturation media + 10 μ M Y-27632 and count them. Plate the cells at >1 million cells per well of 12-well plate in maturation media + 10 μ M Y-27632 onto PLO+L coated plates. 15m
- 13 Day >26: Change media to maturation media (perform media changes every 2-3 days). Cells can be maintained in this media for several months or until experiment.