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Ventral midbrain dopaminergic (mDA) Neuron Differentiation Protocol

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We use this protocol and it's working

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Abstract

Generating authentic functional ventral midbrain dopaminergic neurons, those most vulnerable in Parkinson's disease, from human induced pluripotent stem cells (iPSCs) has enabled cell replacement therapy clinical trials and increasingly sophisticated in vitro disease models. This protocol features further optimizations on a recently updated developmental patterning approach (Kim et al., 2021), focusing on optimizing key stages of t including cell maintenance, induction, cryopreservation, reassembly, and maturation. This comprehensive protocol details the conditions required for the development of midbrain cell types including caudal ventral midbrain dopaminergic neurons and other resident neural cell types, including morphogens, media, substrates, timing, and enables selective enrichment of DA neurons as well as other regional cell types, and integrations with organoid and assembloid cultures. Importantly, variability between different cell line genetic backgrounds, and isogenic pairs, is reduced by by reducing the impact of non-disease-related varitation in iPSC line-intrisic phenotypic variablkes like plating and repassaging efifciency at multiple bottlenecks,and leverages strategies for further inter-line normalization.

Guidelines

1. Day -X: PSC expansion

- iPSC are cultured following Croft lab Protocols. It should have perfectly undifferentiated appearance and high viability. Almost all other substrates and media are fine as well, with some caveats (Eb, see discussion) .https://docs.google.com/document/d/1mgfOIq4uSawHrjZtxbICilmuu64zIxRIk6SAWFPGm3Y/edit

Day -2/-1 Form standardized pluripotent 3D aggregates (embryoid bodies); see **Aggrewell Plates** protocol

Seed in Stemfleex media + RI or CEPT, 2000 cells/microwell (2.4M cells per well of 400/800 aggrewell 24wp wells)

Note: smooth spherical EBs should have formed the next day.

Note: EBs can be formed 1 or 2 days before differentiation. Increase cells/microwell if using day -1. Day 2 is standard

Transfer EBs to ULA plates

- ULA 6wp, 10cm non-tc-treated dishes can be used based on user preference
- All culture plates are kept on a shaker at 80 rpm (after release for 2 days)

CHIR Dose:
CHIR99021 concentration may have to be altered to obtain the highest mDA neuron differentiation efficiency for different iPSC / ESC cell lines due to line-intrinsic differences in Wnt signaling tone and interacting signalling pathways,

Day 1 and Day 3 = CHIR99021 range of 0.7 µM to 2 µM; Standard is 1 µM
Day 4, Day 6, and Day 9 = CHIR99021 range of 5-6 µM, Standard is 6 µM

Materials

	A	B	C
	Neurobasal	Life Technologies	21103049
	N2	Life Technologies	17502-048
	B27 without RA	Life Technologies	12587-010
	SB431542	Selleck Chemicals	S1067
	LDN191389	Reprocell	27,120.00
	Puromorphamine	Selleck Chemicals	S3042
	SAG	EMD Millipore	566660-1MG
	NEAA	Life Technologies	11,140,050.00
	Albumax	Thermo Scientific	11,020,021.00
	CHIR	R&D Systems	4423/50
	BDNF	R&D Systems	248-BDB-050
	GDNF	R&D Systems	212-GD-050
	AA	Sigma-Aldrich	A4403-100MG
	TGFb3	R&D Systems	7754-BH-025
	dbCAMP	Sigma-Aldrich	D0627-1G
	GLUTAMAX	Thermo Scientific	31,980,030.00
	DAPT	Tocris	2,634.00
	Neurobrew521	Miltenyi Biotec	130-093-566
	Reconstitution buffer	R&D Systems	RB02
	DMSO	Sigma-Aldrich	D2650

Safety warnings

CHIR99021 concentrations above 7.5 µM results in dose-dependent cell death (Kim et al., 2021)

Notes: Before starting the protocol

1 PSC cultures should be completely undifferentiated

Embryoid bodies (EB) formation

2 Form EB aggregates (Day -2): iPSC are first nucleated and generated into embryoid bodies. Aggrewells, iPSC medium + CEPT (or Y27632) using the following protocol EB protocols.io

Neural Induction and patterning

3 Neural induction and patterning (Day 0-11), CHIR boost 4-10

3.1 Static or shaking suspension culture (80 RPM) in 6-well plates or 10 cm plate . in Neurobasal media.

	Components	Concentration
	Neurobasal	
	N2	10.0%
	B27 without retinoic acid (RA)	20.0%
	SB-431542	10 µM
	LDN193189	100 nM
	Purmorphamine	1 µM
	SAG	1 µM
	NEAA	10%
	Albumax	1%
	CHIR-99021	1 µM

Media Day 4-6 in Neurobasal media.

3.2 Followed by CHIR BOOST from Day 4-6

	Component	Concentration
	Neurobasal	
	N2	10%
	B27 without retinoic acid (RA)	20%
	SB-431542	10 µM
	LDN193189	100 nM
	Purmorphamine	1 µM
	SAG	1 µM
	NEAA	10%
	Albumax	1%
	CHIR99021	6 µM

Media day 4-6

3.3 Remove SB/LDN+SAG/PURO on Day 6

	Component	Concentration
	Neurobasal	
	N2	10%
	B27 without retinoic acid (RA)	20%
	CHIR99021	3 µM
	Albumax	1%

Media day 7-9

3.4 Add trophic factors from Day 10

	Component	Concentration
	Neurobasal	
	N2	10%
	B27 without retinoic acid (RA)	20%
	CHIR99021	3 µM
	BDNF	20 ng/mL
	GDNF	20 ng/mL
	Ascorbic Acid	0.2 mM
	TGFb3	1 ng/mL
	dbCAMP	0.2 mM
	Albumax	1%

Media 10-11

3.5 Remove CHIR followed by Expansion and trophic support (Day 12-33)

	Components	Concentration
	Small Molecules	Conc. (ng/mL)
	Neurobasal	
	N2	
	B27without RA	
	BDNF	20ng/ml
	GDNF	20ng/ml
	AA	0.2mM
	TGFb3	1ng/ml
	dbCAMP	0.2mM

Media Day 12-35

4 Cultures can be harvested and cryopreserved or reconstituted at (Day 12-33)

4.1 see Neural dissociation protocol .io version, See Worthington protocol.io version, See crypreservation protocol.io version,

5 Further maturation and extended Culture can be performed in the following ways
1. Reseed as Mono-, co- or tri-culture (see survival assay protocol.io versions)
2. Organoids / Assembloids (See Assembloid protocol)

Protocol references

Kim TW, Piao J, Koo SY, Kriks S, Chung SY, Betel D, Socci ND, Choi SJ, Zabierowski S, Dubose BN, Hill EJ, Mosharov EV, Irion S, Tomishima MJ, Tabar V, Studer L. Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use. Cell Stem Cell. 2021 Feb 4;28(2):343-355.e5. doi: 10.1016/j.stem.2021.01.005. PMID: 33545081; PMCID: PMC8006469