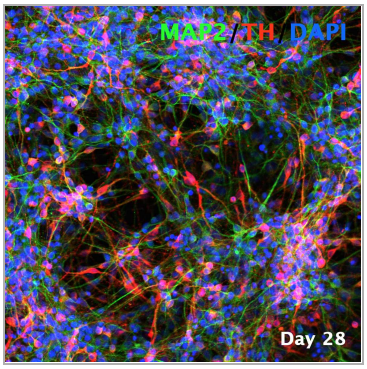

JAN 25, 2023

OPEN ACCESS

External link:
https://doi.org/10.1016/j.stemcr.2022.10.016

Protocol Citation: Kaneyasu Nishimura, Emilia Síf Ásgrimsdottir, Shanzheng Yang, Ernest Arenas 2023. A human development-based protocol for the differentiation of human ESCs into midbrain dopaminergic neurons.. **protocols.io**
https://protocols.io/view/a-human-development-based-protocol-for-the-differe-cmftu3nn

MANUSCRIPT CITATION:
Nishimura K, Yang S, Lee KW, Ásgrímsdóttir ES, Nikouei K, Paslawski W, Gnodde S, Lyu G, Hu L, Saltó C, Svenningsson P, Hjerling-Leffler J, Linnarsson S, Arenas E. Single-cell transcriptomics reveals correct developmental dynamics and high-quality midbrain cell types by improved hESC differentiation. Stem Cell Reports. 2022 Nov 8:S2213-6711(22)00512-4. doi: 10.1016/j.stemcr.2022.10.016

A human development-based protocol for the differentiation of human ESCs into midbrain dopaminergic neurons.

Kaneyasu Nishimura[1], Emilia Síf Ásgrimsdottir[1], Shanzheng Yang[1], Ernest Arenas[1]

[1]Karolinska Institutet, Department of Medical Biochemistry and Biophysics, Laboratory of Molecular Neurobiology, Stockholm 171 77, Sweden

Ernest Arenas

ABSTRACT

Protocol for the differentiation of human embryonic stem cells into midbrain dopaminergic neurons. This protocol recapitulates the activation of developmental pathways known to be important for mDA neuron development and mimics key sequence of events taking place in human midbrain development.

The protocol involves activation of not only Shh and WNT/b-catenin signaling, but also the sequential activation of additional pathways that have not been previously implemented in hESC differentiation, including: activation of non-canonical WNT signaling by WNT5A, of integrin/YAP signaling by Laminin 511, and of the nuclear receptors NR1H3 and NR1H2 by GW3965.

In an accompanying publication (Nishimura et al., 2022; doi: 10.1016/j.stemcr.2022.10.016) single cell RNA-sequencing is used to confirm:
(i) that the cells generated *in vitro* follow critical developmental steps as endogenous human midbrain DA neurons *in vivo*,
(ii) that contaminating/inappropriate cells (from outside the ventral midbrain) are not present in the cultures, and
(iii) that the quality of the cells *in vitro* is comparable to that of endogenous human ventral midbrain developmental standards *in vivo*.
Additional experiments show that the neurons are functional *in vitro*, as assessed by electrohysiological properties and dopamine release. We conclude that this differentiation protocol models human midbrain DA neuron development *in vitro.* We suggest a possible application of this protocol to generate either DA progenitors for cell replacement in Parkinson's disease (PD) or DA neurons for i*n vitro* modeling of PD and drug testing in human PD-iPS cells.

IMAGE ATTRIBUTION

Image taken by Emilia Síf Ásgrimsdottir showing MAP2 (green), TH (red) and DAPI (bue) in a culture differentiated for 28 days.

MATERIALS

Protocol status: Working
We use this protocol and it's working

Created: Jan 08, 2023

Last Modified: Jan 25, 2023

PROTOCOL integer ID: 74963

Keywords: Human, midbrain, dopamine, neuron, embryonic stem cell, progenitor, differentiation, maturation, function, single cell, RNA-sequencing, development, Parkinson's disease, cell replacement, disease modeling, cell type

List of reagents

	A	B	C
	Reagent	**Source**	**Cat. no**
	Nutristem hPSC XF	Saveen-Werner	05-100-1A
	TeSR™-E6	STEMCELL Technologies	05946
	Neurobasal™ Medium	Thermo Scientific	21103049
	Y27632	Tocris	1254
	LDN193189	Stemgent	04-0074-base
	SB431542	Tocris	1614/10
	Purmorphamine	Tocris	4551
	CHIR99021	Sigma Aldrich	SML1046
	WNT5A	R&D	645-WN
	FGF8B	Peprotech	100-25-100
	BDNF	R&D	248-BDB-010
	Ascorbic Acid	Sigma-Aldrich	A4403
	DAPT	Tocris	2634
	GW3965	Sigma-Aldrich	G6295
	GDNF	R&D	212-GD-010/CF
	dbcAMP	Sigma-Aldrich	D0627-1G
	TGFb3	R&D	243-B3-010/CF
	PD0325901	Sigma-Aldrich	PZ0162
	SU5402	Sigma-Aldrich	SML0443
	Laminin 511 Laminin 521	Biolamina	LN511-0502 LN521-05
	B27	Thermo Scientific	17504044
	L-Glutamine	Thermo Scientific	25030081

	A	B	C
	NEAA	Thermo Scientific	11140035
	2-Mercaptoethanol	Thermo Scientific	31350010
	TrypLE Select	Thermo Scientific	12604021
	Defined Trypsin Inhibitor	Thermo Scientific	R007100
	DNase I	Sigma-Aldrich	11284932001

SAFETY WARNINGS

PLease check each of the reagents before usage.

BEFORE START INSTRUCTIONS

Prepare the culture media

E6 media

TesR-E6	500 mL
L-Glutamine	5 mL (x100)
NEAA	5 mL (x100)
50 mM 2-ME	1 mL (x500)

Neurobasal media

Neurobasal	500 mL
L-Glutamine	5 mL (x100)
B27	10 mL (x50)

Coating of the well of choice

	A	B	C	D	E	F
		96-well	**48-well**	**24-well**	**12-well**	**6-well**
	Surface area	(0.33cm2)	(0.75cm2)	(1.82cm2)	(3.8cm2)	(9.6cm2)
	Laminin (521 for hESCs or 511 for DA differentiation)	3.2 uL	10 uL	20 uL	40 uL	100 uL
	DPBS(+/+)	50 uL	150 uL	300 uL	600 uL	1500 uL

Maintenance of hESCs cells

1 Prior to the start of differentiation, hESCs are cultured in NutriStem® XF media on LN521 (1 ug/cm^2) coated plates. Once the cells reach approximately 70-90% confluency, cells are passaged at a seeding density of 20.000 cells/cm^2 with ROCK inhibitor (10 uM Y-27632), only during the first 24h.

2 Prior to the passage of hESCs, prepare the LN521 coated plate by diluting LN521 (1 ug/cm^2) in a sufficient volume of DPBS (+/+) to completely cover the well. Incubate the plate for a minimum of 2h at 37°C or overnight at 4°C. Before seeding the cells, wash the coated plates two times with DPBS (+/+). Laminin coated plates can be wrapped in Parafilm and stored for up to two weeks at 4°C

To passage the hESCs, remove the media and wash the cells once with DPBS(-/-). Add pre-warmed TrypLE (100 uL/cm^2) to the cells and incubate at 37°C for 4 minutes. After 4 minutes, disassociate the cells by gently pipetting up and down using a 1 mL pipette to yield a single cell solution. Inactivate the TrypLE by either adding pre-warmed defined trypsin inhibitor in a 1:1 ratio to the TrypLE or by diluting the TrypLE in a 1:5 ratio with NutriStem® XF medium with ROCK inhibitor (10 uM Y-27632).

Remove a 10 uL aliquot for counting and count cells using either a hemocytometer or an automated cell counter. Determine the required volume of cell suspension needed to achieve a plating density of 20.000 cells/cm^2 and then spin down the cells at 1200 rpm for 5 minutes at RT. After spinning, aspirate the medium and re-suspend the cells in NutriStem® XF with ROCK inhibitor (10 uM Y-27632) and plate the cells on pre-warmed LN521 coated plates.

Midbrain DA differentiation of hESCs

3 To start the differentiation protocol, passage the hESCs onto a LN511 coated (1 ug/cm^2) plate according to the protocol outlined above . Cells are plated at a seeding density of 500.000 cells/cm^2 and the media volume is approximately 1mL/cm^2. In the first 11 days of the protocol it is normal and expected that the media becomes orange-yellow overnight but if it becomes completely yellow it may be good to increase the media volume slightly.

The example protocol below is given for a differentiation done in a 12 well plate.

4 **Day -2**
Cells are plated in NutriStem® XF + 10 uM Y-27632

5 **Day -1**
Add fresh NutriStem® XF media.

Cells should be very dense, covering the bottom of the well completely and the media should have turned *orange* overnight.

6 **Day 0**

Cultivate cells in:	Volume (stock)
Essential 6™ media	4 mL
0.5 mM LDN193189	1.6 uL (x2500)
10 mM SB431542	4 uL (x1000)
10 mM Y27632	2 uL (x2000)

7 **Days 1-2**

Cultivate cells in:	Volume (stock)

Essential 6™ media	4 mL
0.5 mM LDN193189	1.6 uL (x2500)
10 mM SB431542	4 uL (x1000)
2 mM Purmorphamine	4 uL (x1000)

As the cells are plated very densely at the start of the protocol, it is common to observe some shrinkage at the outer ring of the well. If this happens, it is very important to remove and add the media gently, using a 1 mL pipette. If the shrinkage is severe at the time of changing media, it usually helps to only remove the media partially, leaving enough old media to just barely cover the cells.

8 **Days 3-4**

Cultivate cells in:	Volume (stock)
Essential 6™ media	4 mL
0.5 mM LDN193189	1.6 uL (x2500)
10 mM SB431542	4 uL (x1000)
2 mM Purmorphamine	4 uL (x1000)
1mM CHIR99021	6 uL (x667)

9 **Days 5-6**

Cultivate cells in:	Volume (stock)
Essential 6™ media	3 mL
Neurobasal™ media	1 mL
0.5 mM LDN193189	1.6 uL (x2500)
10 mM SB431542	4 uL (x1000)
2 mM Purmorphamine	4 uL (x1000)
1mM CHIR99021	6 uL (x667)

10 **Days 7-8**

Cultivate cells in:	Volume (stock)
Essential 6™ media	2 mL
Neurobasal™ media	2 mL
0.5 mM LDN193189	1.6 uL (x2500)
2 mM Purmorphamine	4 uL (x1000)
1mM CHIR99021	6 uL (x667)
100 ug/ml WNT5A	4 ul (x1000)

11 **Days 9-10**

Cultivate cells in:	Volume (stock)
Essential 6™ media	1 mL
Neurobasal™ media	3 mL
0.5 mM LDN193189	1.6 uL (x2500)
2 mM Purmorphamine	4 uL (x1000)
1mM CHIR99021	6 uL (x667)

100 ug/ml WNT5A	4 ul (x1000)
100 ug/mL FGF8B	4 ul (x1000)

Coat a plate with LN511 (1 ug/cm^2) ahead of re-plating on D11 at 4°C overnight.

12 **Day 11 (Replating cells at 500.000 cells/cm^2)**

Pre-warm the LN511 coated plated for one hour at 37°C. Wash the coated wells twice with DPBS (+/+) before plating the cells.

Wash cells once with DPBS(-/-) and add 500 uL of TrypLE Select with DNase I (100 ug/mL). Incubate the cells at 37°C for *10-15* min and then dissociate the cells with a 1 mL pipette to yield a single cell solution.

When the cells are dissociated, inactivate the TrypLE by adding either pre-warmed defined trypsin inhibitor with DNAseI (100 ug/mL) in a 1:1 ratio to the TrypLE or by diluting the TrypLE in a 1:5 ratio with Neurobasal medium with ROCK inhibitor (10 uM Y-27632).

Remove a 10 uL aliquot for counting and count cells using either a hemocytometer or an automated cell counter. Determine the required volume of cell suspension needed to achieve a plating density of 500.000 cells/cm^2 and then spin down the cells at 1200 rpm for 5 minutes at RT. After spinning, aspirate the medium and re-suspend the cells in the following media.

Neurobasal™ media	4 mL
2 mM Purmorphamine	4 uL (x1000)
10 mM CHIR99021	3 uL (x1333)
100 ug/mL FGF8B	4 ul (x1000)
10 mM Y27632	4 uL (x1000)

13 **Days 12-15**

Cultivate cells in:	Volume (stock)
Neurobasal™ media	4 mL
2 mM Purmorphamine	4 uL (x1000)
10 mM CHIR99021	3 uL (x1333)
100 ug/mL FGF8B	4 ul (x1000)

Coat a plate with LN511 (1 ug/cm^2) ahead of re-plating on D16 at 4°C overnight.

14 **Day 16 (Replating cells at 500.000 cells/cm2)**

On D16, cells are re-plated following the same protocol as detailed on D11 and then re-suspended in:

Neurobasal™ media	4 mL
20 ug/mL BDNF	4 uL (x1000)
200 mM AA	4 uL (x1000)
10 mM DAPT	4 uL (x1000)

10 mM GW3965	4 uL (x1000)
10 mM Y27632	4 uL (x1000)

15 **Days 17-21**

Cultivate cells in:	Volume (stock)
Neurobasal™ media	4 mL
20 ug/mL BDNF	4 uL (x1000)
200 mM AA	4 uL (x1000)
10 mM DAPT	4 uL (x1000)
10 mM GW3965	4 uL (x1000)

16 Optional re-plating step: cells can be re-plated on D22 following the same procedure as detailed on D11. This re-plating step is not necessary but it can be beneficial to replenish the LN511 and standardize the cell number if the cells are cultivated beyond D28 of the protocol.

Days 22-27

Cultivate cells in:	Volume (stock)
Neurobasal™ media	4 mL
20 ug/mL BDNF	4 uL (x1000)
20 ug/mL GDNF	2 uL (x2000)
500 mM dbcAMP	4 uL (x1000)
200 mM AA	4 uL (x1000)
2 ug/mL TGFb3	2 uL (x2000)
1 mM PD0325901	4 uL (x1000)
5 mM SU5402	4 uL (x1000)
10 mM DAPT	4 uL (x1000)

17 **Days 28- 75: Final maturation (Only half media changes from this point onwards *to reduce the risk of cell detachment*)**

Cultivate cells in:	Volume (stock)
Neurobasal™ media	4 mL
20 ug/mL BDNF	4 uL (x1000)
20 ug/mL GDNF	2 uL (x2000)
500 mM dbcAMP	4 uL (x1000)
200 mM AA	4 uL (x1000)
2 ug/mL TGFb3	2 uL (x2000)
10 mM DAPT	4 uL (x1000)