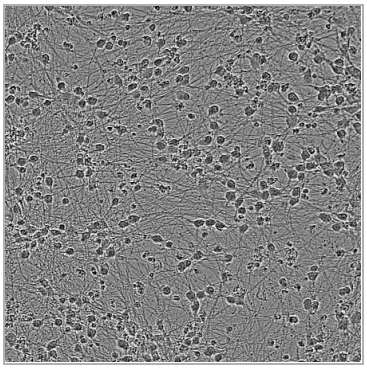

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We use this protocol and it's working

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iNDI Transcription Factor-NGN2 differentiation of human iPSC into cortical neurons Version 2

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ABSTRACT

Induced pluripotent stem cell (iPSC)-derived neurons are an important tool for studying diverse types of neurodegenerative disorders, including Alzheimer's Disease, Parkinson's disease, and related dementias. Understanding the molecular and cellular mechanisms associated with these diseases is an important step in developing new therapeutic targets. Here we describe a robust differentiation protocol in which we expressed the human neurogenin 2 (NGN2) transcription factor under a tetracycline-inducible promoter as previously described (Fernandopulle et al. 2018), with several modifications and using a PiggyBac system for delivery. This differentiation protocol yields high percentages of cortical neuron markers.

MATERIALS

Reagents

- Matrigel hESC-Qualified Matrix, LDEV-free **Corning Catalog #354277**
- DMEM/F-12, HEPES **Thermo Fisher Scientific Catalog #11330032**
- N2 supplement (100x supplement) **Gibco, ThermoFisher Catalog #17502048**
- MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Catalog #11140050**
- Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061**

- • Chroman I **MedChemExpress Catalog #HY-15392**
- DPBS no calcium no magnesium **Gibco - Thermo Fischer Catalog #14190250**
- StemPro™ Accutase™ Cell Dissociation Reagent **Gibco - Thermo Fisher Catalog #A1110501**
- Poly-L-Ornithine (PLO) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3655**
- Borate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #08059**
- BrainPhys™ Neuronal Medium **STEMCELL Technologies Inc. Catalog #05790**
- N21-MAX Media Supplement (50X) **R&D Systems Catalog #AR008**
- Recombinant human GDNF **peprotech Catalog #450-10**
- Recombinant Human/Murine/Rat BDNF **peprotech Catalog #450-02**
- Recombinant Human NT-3 **peprotech Catalog #450-03**
- Cultrex 3-D Culture Matrix Laminin I **R&D Systems Catalog #3446-005-01**
- Quality Bio Cell Culture Grade Water **Quality Biological Catalog #118-162-101CS**

- 5-Fluoro-2'-deoxyuridine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F0503**
- Uridine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #U3003**
- Bovine Serum Albumin **Jackson ImmunoResearch Laboratories, Inc. Catalog #001-000-173**
- Doxycycline hyclate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D9891**

Medium Preparation

1 **Induction Medium:**

For day 0 to day 3

	A	B	C	D
	Reagent	**Stock**	**Final concentration**	**Amount for 50mL of medium**
	DMEM/F12	---------	-------	48.5 mL
	N2 supplement	100X	1X	0.5 mL
	Non-essential amino acids (NEAA)	100X	1X	0.5 mL
	Glutamax	100X	1X	0.5 mL
	Doxycycline	2mg/mL	2µg/mL	0.05 mL
	Chroman I	50 µM	50 nM	0.05 mL

Neuronal Maturation Medium:

For day 4 and 7

	A	B	C	D
	Reagent	**Stock**	**Final concentration**	**Amount for 50mL of medium**
	DMEM/F12	---------	-------	24mL
	Brainphys	---------	---------	24mL
	N21MAX	50X	1X	1mL
	GDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
	BDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
	NT-3 (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
	Laminin	6 mg/mL	1 µg/mL	0.01 mL
	Doxycycline	2mg/mL	2µg/mL	0.05 mL
	5-Fluoro-2'-deoxyuridine	1 mM	1 µM	0.05 mL
	Uridine	1 mM	1 µM	0.05 mL

Neuronal Maturation Medium:

For day 10 to day 28

	A	B	C	D
	Reagent	**Stock**	**Final concentration**	**Amount for 50mL of medium**
	BrainPhys	----------	----------	49 mL
	N21MAX	50X	1X	1 mL
	GDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
	BDNF (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
	NT-3 (in 0.1%BSA/PBS)	10 µg/mL	10 ng/mL	0.05 mL
	Laminin	6 mg/mL	1 µg/mL	0.01 mL
	Doxycycline	2mg/mL	2µg/mL	0.05 mL
	5-Fluoro-2'-deoxyuridine	1 mM	1 µM	0.05 mL
	Uridine	1 mM	1 µM	0.05 mL

Differentiation Protocol

1h 45m

2 Day 0

The iPSCs with a stably integrated human NGN2 (plasmid Addgene #198397) using PiggyBac system under a tetracycline-inducible promoter were exposed to doxycycline as follows:

2.1 Coat a well of 6 well plate or 10cm dish to be used for differentiation with 1 mL or 4 mL respectively of Matrigel solution, tilting to ensure coverage of entire surface area. Place in 37 °C incubator for 00:30:00 to 01:00:00 . 1h 30m

Note

Overnight *incubation gives better results*

2.2 Prepare Induction Medium and place in 37 °C water or bead bath to warm during dissociation.

2.3 Observe iPSCs under a phase contrast microscope to assess confluency and presence of cells debris. Dish should be dissociated at ~70% to 80% confluency.

2.4 Aspirate culture medium and wash with PBS 1X.

2.5 Aspirate PBS and add half of culture volume of Accutase

2.6 Transfer to 37 °C incubator for 00:10:00 10m

> Note
>
> *The time can vary by cell line and density (the optimal density is 70-80% and time can go up to 20 minutes) and the goal to use accutase is singularize as single cells.*

2.7 When Incubation is ready, tilt the plate and pipet the accutase solution two to three times up and down to singularize as single cells.

2.8 Quench the Accutase adding half of the culture volume of PBS. Transfer to a new conical tube and rinse with more PBS the culture surface, combine with the cell solution in the tube.

2.9 Centrifuge 00:05:00 at 200 - 300 x g at Room temperature 5m

Note

While centrifuge, aspirate Matrigel solution from plates and add Induction Medium.

2.10 Aspirate supernatant and resuspend cell pellet with Induction Medium.

2.11 Count cells, Gently transfer 25,000 iPSCs per cm^2 (i.e. For a 6 well plate, seed ~2.5 x 10^5 cells/well).

2.12 Gently rock plate to evenly distribute cells and leave it at Room temperature for 00:10:00 to let the cells settle down then place in 37 °C incubator. 10m

3 Day 1

Check cells under the microscope, nascent neuritic extensions should begin to be evident after 24 h of doxyxycline exposure.

3.1 Prepare Induction Medium but without Chroman I and warm it.

3.2 Aspirate medium, wash once with PBS 1X and replace with warm induction medium.

4 Day 2

Check cells under the microscope, neuritic extensions should be more evident.

4.1 Repeat medium change with induction medium as on day 1.

5 Day 3

Check cells under a microscope. Neurites should be obvious by this time.

5.1

1. Repeat medium change with induction media as on day 1.

6 Day 4

Check cells under a microscope. Pre-differentiated neurons are ready to be re-plated.

6.1 Coating dishes

1h

Freshly prepared poly-L-ornithine (PLO), at final concentration at [M] 0.1 mg/mL :

- Using Sodium Borate Buffer pH 8.2, make a [M] 1 mg/mL stock PLO solution.
- To prepare working solution dilute to a [M] 0.1 mg/mL with cell culture water then filter through a 0.22µm sterile filter and it is ready to use.
- Add half of the culture volume of PLO working solution to dishes and Place in 37 °C incubator for 01:00:00 to Overnight .
- Aspirate PLO working solution from the dishes.
- Wash dishes with cell culture water three times.
- Let dry completely in a culture hood.
- Dishes are ready to use.

Note

Overnight *incubation gives better results.*

7 Plating pre-differentiated neurons day 4

Once cells are confirmed to be healthy, they should be dissociated with Accutase and either frozen or re-plated onto final dishes for neuronal maturation and experimental manipulation

7.1 Prepare fresh **Neuronal Maturation Medium** for day 4 as referred above.

7.2 After dissociating cells with Accutase as step 2.4 to 2.9 resuspend cell pellet with Neuronal Maturation Medium for day 4 and count.

7.3 Plate 1.5 x 10^6 pre-differentiated neurons onto a PLO-coated 6 well with 3-4 mL of **Neuronal Maturation Medium.**

> Note
>
> *The number of pre-differentiated neurons to be re-plated varies depending of the final assay but it can be as follows:*
>
> - *384 well plate (imaging) 7,000 to 9,000 in 100 µL medium/well.*
> - *96 well plate (imaging) 4x 10^4 in 300 µL medium/well.*
> - *12 well plate (Biochemistry) 7x 10^5 in 2 mL medium/well.*
> - *6 well plate (Biochemistry) 1.5 x 10^6 in 4 mL medium/well*
> - *10 cm dish 8 x 10^6 in 10- 12 mL medium.*

7.4 Gently rock plate to evenly distribute cells and leave it at Room temperature for 00:10:00 to let the cells settle down then place in 37 °C incubator. 10m

7.5 After day 4 do half of the medium change every 3-4 days with Neuronal Maturation Medium.