iPSCs

Dec 18, 2019

DIFFERENTIATION OF i^{3}NEURONS (Basic Protocol 5)

In 1 collection

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1 Works for me dx.doi.org/10.17504/protocols.io.5h6g39e

Neurodegeneration Method Development Community

ABSTRACT

This protocol describes the rapid and robust differentiation of cortical neurons from hiPSCs via induced expression of the neurogenin-2 (NGN2) transcription factor (Wang et al., 2017; Zhang et al., 2013). To begin, iPSCs with a stably integrated human or mouse neurogenin-2 transgene under a tetracycline-inducible promoter are exposed to doxycycline in neuronal induction medium (IM). Since iPSCs grow as colonies, they must be single-cell dissociated to a new plate before doxycycline treatment in order to provide the differentiating neurons adequate space to begin producing neuritic extensions.

Once the cells have been partially differentiated on Matrigel, they are re-plated onto dishes coated with poly-L-ornithine (PLO) for neuronal maturation. After 3 days of doxycycline treatment, the cells are committed to neural differentiation, although at this time they may have only minor neuritic elongations. These neurites are generally well-preserved after dissociation and replating, but the longer neuritic projections present in cells differentiated past 3 days are often damaged during the splitting process. For this reason, differentiated neurons are optimally replated on day 3. At this time, differentiating neurons can also be cryopreserved for use at a later date, enabling curation of large, standardized stocks of partially differentiated neurons for future experiments.

EXTERNAL LINK

https://doi.org/10.1002/cpcb.51

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Fernandopulle, M. S., Prestil, R., Grunseich, C., Wang, C., Gan, L., & Ward, M. E. (2018). Transcription-factor mediated differentiation of human iPSCs into neurons. Current Protocols in Cell Biology, e51. doi:https://doi.org/10.1002/cpcb.51

fernandopulle2018.pdf

MATERIALS TEXT

- iPSCs with stably integrated doxycycline-inducible NGN2 transgene (Basic Protocol 3)
- Induction Medium (IM, see Table 2)

DMEM/F-12
by Gibco, ThermoFisher
Catalog #: 11320033

N-2 Supplement (100X)
by Gibco, ThermoFisher
Catalog #: 17502001

MEM Non-Essential Amino Acids Solution (100X)
by Gibco, ThermoFisher
Catalog #: 11140050

L-Glutamine (200 mM)
by Gibco, ThermoFisher
Catalog #: 25030081

Y-27632 dihydrochloride
by Tocris
Catalog #: 1254
CAS Number: 129830-38-2

Doxycycline hyclate
by Merck Millipore Sigma
Catalog #: D9891

γ-Secretase Inhibitor XXI, Compound E - Calbiochem
by Merck Millipore
Catalog #: 565790
CAS Number: 209986-17-4

5-Bromo-2'-deoxyuridine (BrdU)
by Merck Millipore Sigma
Catalog #: B9285
CAS Number: 59-14-3

Prepare Induction Medium in sterile biosafety cabinet; medium should then be aliquotted to add additional supplements fresh; warm to 37°C before use.

Component	Amount
DMEM/F12 with HEPES	485 ml
N2 supplement, 100×	5 ml (per 500 ml)

Non-essential amino acids (NEAA), 100×	5 ml (per 500 ml)
L-glutamine, 100× (or Gluta-MAX)	5 ml (per 500 ml)
Aliquot medium and add fresh from stock:	
ROCK inhibitor Y-27632 (10 mM in PBS, 1000×)	50 µl (per 50 ml)
Doxycycline (2 mg/ml in PBS, 1000×)	50 µl (per 50 ml)
Additional components for i3LMNs:	
Compound E (2 mM in 1:1 ethanol and DMSO, 0.98 mg/ml; 10,000×, store at −20°C)	5 µl (per 50 ml)
BrdU (only for d3 replating; 40 mM in water, 12.284 mg/ml; 1000×, store at −20°C)	50 µl (per 50 ml)

Table 2: Induction Medium

- 15-cm culture dishes (Corning, cat. no. 430599)

Corning® 150 mm TC-treated Culture Dish
by Corning
Catalog #: 430599

- Additional reagents and equipment for general iPSC culture (Basic Protocol 1) and counting cells (Phelan & May, 2015)

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

BEFORE STARTING

NOTE: The following steps will assume the experimenter has a 70 % to 80 % confluent 15-cm dish of iPSCs with a stably integrated inducible NGN2 transgene. It is critical that the health of iPSCs be optimal prior to differentiation; poorly maintained, spontaneously differentiated, or overly confluent iPSC cultures tend to differentiate poorly or variably.
Apart from observation under the microscope, counting, and centrifugation, all steps should be carried out in a sterile biological safety cabinet.

Day 0

1 Coat a new 15-cm tissue culture dish to be used for differentiation with **7 ml** to **9 ml** of Matrigel solution, tilting to ensure coverage of entire surface area. Place in **37 °C** incubator for **00:30:00** to **01:00:00** prior to beginning dissociation and re-plating.

Matrigel can be re-used once or twice after 30-min incubation periods (aspirate with serological pipet and store solution at **4 °C**). If desired, Matrigel can also be kept on coated plates for 1 week in the incubator. If pursuing this strategy, use 10% to 20% more Matrigel solution than for a 30-min incubation and check plate regularly to ensure complete coverage of the surface area (evaporation and/or non-level incubator shelves can cause parts of the plate to dry). Matrigel incubated at 37°C for more than 1 to 2 total hours should not be re-used.

1.1 Coat a new 15-cm tissue culture dish to be used for differentiation with **7 ml** to **9 ml** of Matrigel solution, tilting to ensure coverage of entire surface area.

1.2 Place in **37 °C** incubator for **00:30:00** to **01:00:00** prior to beginning dissociation and re-plating.

2 Prepare **20 ml** IM with **2 µg/ml** doxycycline and **10 Micromolar (µM)** Y-27632 ROCK inhibitor (see Table 2). Place in **37 °C** water or bead bath to warm during the dissociation protocol.

Doxycycline is light-sensitive, so keep cool (**4 °C**) and dark when not in use.

3 Observe iPSCs under a phase contrast microscope to assess confluency and presence of cell debris. Dish should be dissociated at around **70% to 80% confluency** (i.e., $2.5–3 \times 10^7$ cells).

4 Wash plate with PBS. To wash, aspirate medium with an aspirating pipet connected to a vacuum apparatus. Pipet **10 ml** PBS onto plate by tilting dish and slowly dispensing PBS onto the sidewall of the dish. After dispensing PBS, tilt dish in all directions to spread solution around the plate surface. Aspirate PBS and repeat once more. Observe cells under the microscope to assess clearance of debris. Continue washing until debris are absent (typically one or two washings are needed).

Cell debris can increase inaccuracy of downstream cell counting and obstruct pellet formation and resuspension after centrifugation. Debris should be minimal in healthy iPSC cultures.

4.1 Wash plate with PBS. To wash, aspirate medium with an aspirating pipet connected to a vacuum apparatus.

4.2 Pipet **10 ml** PBS onto plate by tilting dish and slowly dispensing PBS onto the sidewall of the dish.

4.3 After dispensing PBS, tilt dish in all directions to spread solution around the plate surface.

4.4 Aspirate PBS and repeat once more.

4.5 Observe cells under the microscope to assess clearance of debris.

4.6 Continue washing until debris are absent (typically one or two washings are needed).

5 Split cells with **7 ml** Accutase (see Basic Protocol 1) and collect dissociated cells in the Accutase and an additional **7 ml** PBS in 15-ml conical tube.

6 Count cells, transfer 2 to 2.5 × 10^7 iPSCs per 15-cm dish to be differentiated into a 15- or 50-ml conical tube, and centrifuge **00:05:00** at **200 x g** , **Room temperature** .

Aspirate the supernatant and resuspend cells in **10 ml** to **12 ml** of IM with **2 µg/ml** doxycycline and **10 Micromolar (µM)** Y-27632 ROCK inhibitor.

Add 5% IM to dissociated cells in PBS before centrifugation to improve pelleting.

6.1 Count cells.

6.2 Transfer 2 to 2.5 × 10^7 iPSCs per 15-cm dish to be differentiated into a 15- or 50-ml conical tube.

6.3 Centrifuge **00:05:00** at **200 x g** , **Room temperature** .

6.4 Aspirate the supernatant and resuspend cells in **10 ml** to **12 ml** of IM with **2 µg/ml** doxycycline and **10 Micromolar (µM)** Y-27632 ROCK inhibitor.

7 Aspirate Matrigel solution from coated 15-cm dish.

Alternatively, if the plate was freshly coated, the Matrigel solution may be transferred to a new dish (see step 1).

8 Gently pipet the cell suspension onto the newly aspirated 15-cm dish. Rinse the 15-ml conical tube with an additional **8 ml** IM with **2 µg/ml** doxycycline and **10 Micromolar (µM)** Y-27632 ROCK inhibitor and add to the dish for a total volume of **18 ml** to **20 ml** . Gently tilt dish in all directions to evenly distribute cells throughout surface area.

Higher cell densities at the time of plating can interfere with robust neuronal differentiation. If more cells are needed for downstream experiments, it is best to increase the number of dishes used rather than the number of cells plated per dish. In general, iPSCs divide one to two times after doxycycline administration and are then post-mitotic. If fewer cells are needed, scale the number of iPSCs plated and volume of medium used according to the surface area of the dish (e.g., 0.8 – 1.0 × 10^7 cells per 10-cm dish or 1.5 × 10^6 cells/well of a 6-well plate). If multiple iPSC lines are being simultaneously differentiated, plate the same number of cells for each line to minimize density-dependent differentiation variability.

8.1 Gently pipet the cell suspension onto the newly aspirated 15-cm dish.

8.2 Rinse the 15-ml conical tube with an additional **8 ml** IM with **2 µg/ml** doxycycline and **10 Micromolar (µM)** Y-27632 ROCK inhibitor and add to the dish for a total volume of **18 ml** to **20 ml** .

8.3 Gently tilt dish in all directions to evenly distribute cells throughout surface area.

9 Observe cells under a microscope to ensure even distribution and high proportion of live cells (identified by light halo around each cell) versus dead cells and debris (darker and smaller than live cells).

10 Place in **37 °C** incubator and gently slide dish side-to-side and front-to-back to evenly distribute cells.

Day 1

11 Check cells under a microscope.

Nascent neuritic extensions should begin to be evident after about 24 hr of doxycycline exposure.

12 Make **20 ml** of IM (+ **2 µg/ml** doxycycline, but without ROCK inhibitor; see Table 2) and warm in **37 °C** water/bead bath for approximately **00:20:00** , or until warm to the touch.

13 Aspirate old medium, wash one to two times with PBS, and replace with **18 ml** of warm IM+ **2 µg/ml** doxycycline.

If significant cell debris are noted, wash additional times with PBS prior to adding fresh medium.

13.1 Aspirate old medium.

13.2 Wash with PBS. (1/2)

13.3 Wash with PBS. (2/2)

13.4 Replace with **18 ml** of warm IM+ **2 µg/ml** doxycycline.

If significant cell debris are noted, wash additional times with PBS prior to adding fresh medium.

Day 2

14 Check cells under a microscope. Neuritic extensions should be more evident.

15 Repeat medium exchange with IM+doxycycline as on day one.

Day 3

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

If neurites are not present or cells appear misshapen or otherwise unhealthy, cells should be discarded and the protocol re-attempted with a new batch of d0 iPSCs. Make up new IM medium with fresh doxycycline and ROCK inhibitor.

17 Once cells are confirmed to be healthy, cells should be dissociated with Accutase and either frozen or re-plated onto final dishes for neuronal maturation and experimental manipulation. Dissociation should follow steps 3 to 8 from Day 0 of this protocol; freezing should be done as described in Basic Protocol 1, and plating for neuronal maturation will be detailed in Basic Protocol 6.