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CULTURING i^3 NEURONS (Basic Protocol 6) [↗](#)

In 1 collection

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1 *Works for me* [dx.doi.org/10.17504/protocols.io.5jpg4mn](https://doi.org/10.17504/protocols.io.5jpg4mn)

Neurodegeneration Method Development Community

ABSTRACT

Following Day 3 replating, i^3 Neurons should be cultured for at least 1 week before lysis, fixation, or other experimental endpoint, and they remain viable for at least 1 month with proper maintenance. This protocol covers medium conditions, coating of tissue culture dishes with synthetic polymers, recommended plating densities, and maintenance procedures for long-term culture and for specific experimental applications.

Cortical Neuron Culture Medium (CM) is sufficient to promote the maturation and longterm maintenance of i^3 Neurons in culture. i^3 Neurons express general markers of cortical neurons, as well as specific pre- and postsynaptic markers of glutamatergic excitatory cortical neurons. Since these cells are post-mitotic after 3 days of differentiation (see [Basic Protocol 5](#)) and prefer neuron-conditioned medium to fresh medium, maintenance conditions for these cultures are minimal. Generally, half-medium changes every 7 days with fresh, pre-warmed CM are sufficient for culturing beyond d10 (7 days after replating). Neuron attachment and growth also require a strongly adhesive substrate. Coating plates with synthetic polymers such as poly-L-ornithine (PLO) is necessary for i^3 Neuron attachment, viability, and successful outgrowth.

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

- Freshly-prepared 1× poly-L-ornithine (PLO) or polyethyleneimine (PEI) solution (see Table 3)

Poly-L-ornithine solution
by Sigma Aldrich
Catalog #: P4957

Poly-D-lysine hydrobromide
by Sigma Aldrich
Catalog #: P7405



Poly(ethyleneimine) solution

by Sigma Aldrich

Catalog #: 03880

CAS Number: 9002-98-6



Boric acid

by Sigma Aldrich

Catalog #: B6768

CAS Number: 10043-35-3



Sodium tetraborate

by Sigma Aldrich

Catalog #: 221732

CAS Number: 1330-43-4



Sodium chloride

by Sigma Aldrich

Catalog #: S7653



Sodium hydroxide solution

by Sigma Aldrich

Catalog #: S2770

CAS Number: 1310-73-2



Polymers should be resuspended to recommended concentration, filter sterilized, and diluted to 1× for use.


Borate buffer may be prepared as described here and filter sterilized, or may be purchased commercially.

Component	Amount per 50 ml
<i>Poly-L-ornithine (PLO), 10× stock</i>	
PLO	50 mg (per 50 ml)
Borate buffer (see below)	to 50 ml
<i>Poly-D-lysine (PDL), 1×</i>	
PDL	50 mg (per 50 ml)
Borate buffer (see below)	to 50 ml
<i>Polyethyleneimine (PEI), 10× stock</i>	

PEI	1 ml
Borate buffer (see below)	49 ml (per 50 ml)
<i>Borate buffer</i>	
Boric acid (100 mM)	3.09 g (per 500 ml)
Sodium tetraborate (25 mM)	4.77 g (per 500 ml)
Sodium chloride (75 mM)	2.19 g (per 500 ml)
Milli-Q water	to 500 ml
Sodium hydroxide (1 M)	As needed to adjust pH to 8.4


Table 3: Poly-L-Ornithine (PLO), Poly-D-Lysine (PDL), and Polyethyleneimine (PEI) Solutions

- Laminin




Laminin Mouse Protein, Natural
by Thermo Fisher Scientific
Catalog #: 23017015


- Freshly split or thawed 3-day differentiated i³Neurons (following completion of [Basic Protocol 5](#))
- Cortical Neuron Culture Medium (CM; see Table 4)



BrainPhys™ Neuronal Medium 500 mL
by Stemcell Technologies
Catalog #: 5790



B-27 Supplement
by Gibco - Thermo Fischer
Catalog #: 17504044



Recombinant Human/Murine/Rat BDNF
by peprotech
Catalog #: 450-02



Recombinant Human NT-3
by peprotech
Catalog #: 450-03



Laminin Mouse Protein, Natural

by Thermo Fisher Scientific

Catalog #: 23017015



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

Component	Amount per 50 ml	Final concentration
BrainPhys neuronal medium	49 ml	
B27 supplement, 50×	1 ml	1×
BDNF (10 µg/ml) in PBS containing 0.1% IgG and protease-free BSA (store at -80°C)	50 µl	10 ng/ml
NT-3 (10 µg/ml) in PBS containing 0.1% IgG and protease-free BSA (store at -80°C)	50 µl	10 ng/ml
Laminin (store at -80°C; stock concentration 1 mg/ml; thaw on ice and dispense with chilled pipets)	50 µl	1 µg/ml

Table 4: Cortical Neuron Culture Medium (CM)

- 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.

Coating dishes

- Prepare stock solutions of PLO or PEI (see [Table 3](#)).
- Add one half culture volume of the coating solution prepared in step 1 to the tissue culture dishes to be used for plating the Day 3 partially differentiated i³Neurons. Gently tilt plate to ensure full coverage.
- Incubate dishes for at least 01:00:00 at Room temperature .



For best results, incubate dishes overnight in a 37 °C incubator.

- Aspirate coating solution.
- Wash dishes with sterile water.

- 6 Repeat twice for three total washes.



Four or more total washes are recommended for **PEI-coated** plates.

- 7 Aspirate water.

- 8 Let dishes dry completely in a biosafety cabinet (typically requires 🕒 **00:30:00** to 🕒 **01:00:00**).



To accelerate the drying process, stand the dishes on their sides and lean them against the back of the BSC. Lids may also be left askew to allow better airflow. In particular, PEI requires complete drying to prevent toxicity.

- 9 Coated and dried dishes should be used immediately or wrapped in aluminum foil and stored at 🌡 **4 °C** for up to 1 week.



Faster neurite outgrowth, increased neuronal survival, and reduced clumping for longterm culture may be achieved by a secondary coating with laminin prior to plating.

Thaw concentrated laminin stock solution (approximately **1 mg/ml**) slowly on ice and use chilled pipets to dispense in order to reduce polymerization. Dilute laminin stock to **10 µg/ml** in cold PBS, and add one half culture volume of this solution to the polymer-coated, washed, and dried dishes. Incubate for at least 🕒 **02:00:00** at 🌡 **37 °C** before aspirating and plating neurons directly. This is **optional for PLO** and **highly recommended for PEI**.

Plating cells

- 10 Prepare CM in a sterile biosafety cabinet.



Doxycycline (final concentration of **2 µg/ml**) can be added to this medium at the discretion of the experimenter. Neurogenin-2 requires only 24 hr of expression to induce irreversible differentiation to neurons, so the 3-day differentiation period in IM medium should be sufficient; however, prolonged induction of NGN2 has not been shown to have significant effects on neuronal health or maturation. If other doxycycline-inducible transgenes are present in the cell line, it may be desirable to restrict expression of these cassettes to prevent toxicity and/or accommodate future induction time points.

We have found that several basal media (e.g., DMEM/F12, Neurobasal A, and Brainphys), when properly supplemented (+B27/BDNF/NT3/laminin), work well for i³Neuron culture. We favor BrainPhys medium, since it facilitates synapse formation and spontaneous electrical activity, but alternative basal medium/supplement combinations may be superior for specific applications or individual iPSC lines, and other neuronal medium formulations are also worth considering (e.g., B27 Plus, Invitrogen).

- 11 Place CM in 🌡 **37 °C** water/bead bath for approximately 🕒 **00:20:00** or until warm to the touch.

12 From a thawed cryovial (see [Basic Protocol 1](#)) or freshly dissociated 3-day differentiated cells, resuspend in the appropriate amount of medium. Typical cell counts and medium volumes are as follows:

- a. 96-well plate (imaging): $1-5 \times 10^4$ cells in 100 μ l medium/well.
- b. 8-well chamber slide (imaging): $0.3-1.5 \times 10^5$ cells in 250 μ l medium/well.
- c. 6-well plate (biochemistry): $1.5-2 \times 10^6$ cells in 1.5 ml medium/well (supplement to 2 to 3 ml one day after plating).
- d. 10-cm dish (biochemistry): $1-1.2 \times 10^7$ cells in 8 ml medium (supplement to 10 to 12 ml one day after plating).
- e. 15-cm dish (biochemistry): $3-3.5 \times 10^7$ cells in 18 ml medium (supplement to 20 to 22 ml one day after plating).



Biochemistry applications typically require a high concentration of cells for a given surface area. Thus, these experiments require a greater volume of medium than would be required for an imaging experiment. However, after splitting, cells typically adhere better to a new plate with a lesser volume of medium compared to a greater volume. Thus, neurons plated on 6-well, 10-cm, or 15-cm dishes for biochemistry should be plated with 1.5 ml, 8 ml, and 18 ml of medium, respectively. These volumes should then be supplemented to the final volumes above on the day after plating.

Culture maintenance

13 Check cells daily under a phase-contrast microscope, paying particular attention to cell debris and morphological changes.



High levels of cell debris and/or cell clumping often indicate a problem with either the dish coating or culture medium. If this is seen, remove half volume of culture medium and replace with full volume of medium (50 % additional medium). If additional fresh medium does not result in less debris the next day, there has likely been insufficient coating or the coating medium was expired.

14 Biweekly half-medium changes (i.e., every 3 to 4 days) are effective for sustaining dense cultures (e.g., biochemistry applications). Weekly half-medium changes are sufficient to sustain long-term cultures at moderate densities (e.g., microscopy applications). Use the appropriate serological pipet or micropipet to slowly aspirate a measured volume from each well, and very gently replace with fresh medium.



Neurons tend to easily dissociate from the dish, so any aspiration or dispensing of medium directly onto cells is not recommended. Take care to aspirate and dissociate by tilting the dish so that medium accumulates on one side. Then, aspirate/dispense with the pipet directed toward the wall of the dish (i.e., away from the cells at the bottom). Should a full medium aspiration be necessary (i.e., for a PBS wash), initially add the medium dropwise in the middle of the well with the plate tilted until a small pool forms in the corner, at which point add the rest of the medium dropwise down the well wall onto this pool. This reduces shear forces on the edges which can cause whole wells to detach in a sheet.

Optional: Supplementation with astrocytes or astrocyte-conditioned medium have been shown to improve the overall health and electrophysiological activity of i³Neurons in long-term cultures. To maintain culture purity for biochemistry, astrocytes can be supplemented using various commercially available Transwell dish inserts. Alternatively, half-medium changes as described above can be replaced with half-medium changes of astrocyte-conditioned medium (i.e., medium extracted from independent astrocyte cultures and sterile filtered to ensure no cell or debris carryover). Primary astrocytes expanded in DMEM + 10 % fetal bovine serum (FBS) from post-natal (P0-3) mice or rats are sufficient for these purposes, provided that they are low-passage (< p3 post-harvest). Astrocytes can also be frozen in aliquots after expansion and thawed immediately prior to co-culture with partially differentiated neurons at d3. If directly co-culturing astrocytes, we recommend a ratio of 2 neurons:1 astrocyte. See [Support Protocol 7](#) for detailed information.



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