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CULTURING i^{3}LMNS (Basic Protocol 8)

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iPSCs

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We use this protocol and it's working

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Abstract

Induction and differentiation of i^{3}LMNs is nearly identical to the first 3 days of differentiation for i3Neurons (see **Basic Protocol 5**), including identical induction medium. Following replating, however, differences arise including the use of Motor Neuron Culture Medium (MM) for long-term culture (Table 5), additional reagents to reduce proliferative cells if necessary, and variable options for coating polymers.

MM is sufficient to promote the maturation and long-term culture of i^{3}LMNs. While a majority of these cells at Day 3 are committed to differentiation to post-mitotic neurons, a small subset may remain proliferative and can quickly overtake the culture. To compensate, a 1-day pulse of bromodeoxyuridine (BrdU) is recommended at the time of replating of Day 3 i^{3}LMNs and has proven effective at impairing mitosis without causing neural toxicity. Following BrdU treatment, medium should be completely exchanged the following day. CultureOne is also effective at reducing levels of proliferative cells over time, and it may be included in MM medium with usually minimal effects on neural cell health. In general, one fourth to one half of the medium should be aspirated and replaced with fresh medium every 3 to 4 days.

Neuron attachment and growth also requires a strongly adhesive substrate. Coating plates with synthetic polymers such as poly-L-ornithine (PLO), polyethyleneimine (PEI), or poly-D-lysine (PDL) is sufficient for cell attachment, and providing an optional additional coating of purified laminin improves i^{3}LMN viability and neurite outgrowth. Laminincoated wells also support proliferative cells better than polymer without laminin, so BrdU is necessary in these conditions.While these substrates are stiffer than those under biological conditions, they reduce cell migration and clumping, facilitating imaging of individual cells. In our experience, PLO has produced the best neuronal morphology, but it is also the most sensitive to cell detachment resulting from medium exchanges. Detachment is a particular concern at high cell density and after extended time in culture, as the interconnected network of neural processes can cause entire wells to detach from the edges. PEI and PDL typically promote stronger adhesion, but PEI is toxic to cells if coating is not performed properly, and rapid degradation and batch-to-batch variability complicate the use of PDL. Cells are especially susceptible to detachment during the many washes required for immunocytochemistry, so these steps should be performed with extreme care. This protocol will assume use of PLO, although coating with PEI or PDL may be performed using an identical protocol except where noted.

Attachments

PDF

fernandopulle2018.pd...

1.7MB

Materials

- PLO, PEI, or PDL solution (see **Table 3**)
- Laminin (Gibco, cat. no. 3017015)

Laminin Mouse Protein, Natural **Thermo Fisher Scientific Catalog #**23017015

- Freshly split or thawed 3-day differentiated i^{3}LMNs (following completion of **Basic Protocol 5**)
- Motor Neuron Culture Medium (MM; see Table 5)

Neurobasal™ Medium **Thermo Fisher Scientific Catalog #**21103049

B-27 Supplement **Gibco - Thermo Fisher Scientific Catalog #**17504044

N-2 Supplement (100X) **Thermo Fisher Scientific Catalog #**17502001

MEM Non-Essential Amino Acids Solution (100X) **Gibco - Thermo Fisher Scientific Catalog #**11140050

L-Glutamine (200 mM) **Gibco - Thermo Fisher Scientific Catalog #**25030081

CultureOne™ Supplement (100X) **Thermo Fisher Scientific Catalog #**A3320201

Laminin Mouse Protein, Natural **Thermo Fisher Scientific Catalog #**23017015

Note

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

While not required, addition of BDNF and NT-3 as described for CM (**Table 4**) improves long-term cell health.

Alternative basal media may be substituted and optimized by cell line (e.g., Neurobasal electro; Thermo, **cat. no. A1413701**, which includes B27 electro; or BrainPhys, STEMCELL Technologies, **cat. no. 05790**).

	Component	Amount per 50 ml	Final Concentration
	Neurobasal medium	47.5 ml	

	B27 supplement, 50×	1 ml	1×
	N2 supplement, 100×	500 µl	1×
	Non-essential amino acids (NEAA), 100×	500 µl	1×
	L-glutamine, 100× (or GlutaMAX)	500 µl	1×
	(Optional) CultureOne supplement, 100×	500 µl	1×
	Laminin (store at −80° C; stock concentration 1 mg/ml)	50 µl	1µg/ml

Table 5: Motor Neuron Culture Medium (MM)

- 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

1 Prepare stock solutions of PLO, PEI, or PDL (see **Table 3**).

2 Add one-half culture volume of 1× coating solution from step 1 to the tissue culture dishes to be used for plating Day 3 partially differentiated i^{3}LMNs.

3 Gently tilt the plate to ensure full coverage.

4 Incubate dishes for at least 01:00:00 at Room temperature.

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

5 Aspirate coating solution.

6 Wash dishes with sterile water.

7 Repeat twice for three total washes.

> Note
>
> An additional two washes is recommended for PEI coating.

8 Aspirate water and let dishes dry completely in a biosafety cabinet (typically requires 00:30:00 to 01:00:00).

> Note
>
> To accelerate the drying process, stand the dishes on their sides and lean them against the back of the biosafety cabinet. 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.

Note

Optional: Faster neurite outgrowth and increased neuronal survival during replating may be achieved by additionally coating plates with laminin prior to plating. Dilute laminin to [M] 15 µg/ml in IM, and add one half culture volume of this solution to the polymer-coated, washed, and dried wells. Incubate at 37 °C for 01:00:00, then plate neurons directly by adding cells in an addition one half culture volume of IM.

Plating cells

10 If laminin coating was not performed, prepare wells of pre-coated plates with warm IM supplemented with [M] 10 micromolar (µM) ROCK inhibitor, [M] 2 µg/ml doxycycline, 1:10,000 Compound E from stock, and [M] 1 µg/ml laminin.

Note

Additionally supplementing with [M] 40 micromolar (µM) BrdU for 24:00:00 helps to prevent outgrowth of mitotically active cells without affecting neuronal health. Alternatively, CultureOne supplement may be added to the medium from d4 onwards.

11 From frozen stock or freshly dissociated 3-day differentiated cells, resuspend (see **Basic Protocol 1**, thawing iPSCs) in the appropriate amount of medium. Typical cell counts and medium volumes are as follows:

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

Note

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

12 The next day (d4), aspirate medium and replace with pre-warmed MM supplemented with [M] 1 µg/ml laminin.

Note

For full medium changes, avoid drying wells by only aspirating one dish or a few wells at a time. Initially, add the medium very slowly dropwise in the middle of the well with the plate tilted until a small pool forms in the corner, at which point add media dropwise down the well wall onto this pool. This helps to avoid shear forces on the edges which can cause whole wells to detach in a sheet. If BrdU was used on day 3, wash with PBS before adding medium.

13 For the first 4 days (d4 to d7), check cells daily under a phase-contrast microscope, paying particular attention to cell debris and morphological changes. Medium changes should be done every 2 to 3 days by replacing one-half of the medium with fresh MM+laminin.

Note

High levels of cell debris and/or cell clumping often indicate a problem with either the dish coating or culture medium. If 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 After day 7, perform half-medium changes every 4 to 7 days with complete MM+laminin for long-term maintenance.

Note

Biweekly half-medium changes can be effective in 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 dissociate from the dish easily, 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).

15 *Optional:* Supplementation with astrocytes or astrocyte-conditioned medium have been shown to improve the overall health and electrophysiological activity of i^3LMNs in long-term cultures, as described for i^3Neurons (see **Basic Protocol 6** and **Support Protocol 7**).