Seeding iPSC-derived neurons onto 384-well plates

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Abstract

iPSC-derived neurons are an excellent in vitro model of neurological diseases and it is hoped they will be successfully used in drug screening applications. For drug screening it is imperative to use cells in multi-well plates to increase throughput of assays; it is also useful for disease mechanism research as it reduces the number of cells and amount of reagents needed for experiments. Here we describe a method of seeding iPSC-derived neurons into 384-well plates towards the end of the differentiation procedure. This method covers coating the plates with substrates to aid attachment, dissociation of the cells into a single cell suspension, and seeding onto 384-well plates to give an even distribution of neurons. The cells can then be used for imaging or whole-well assays.

Keywords

Induced pluripotent stem cells, neuronal cells, 384-well plates, microplates, cell culture, drug discovery.

1. Introduction

iPSC-derived neurons have attracted attention as an exciting new model of neurological diseases. Since these cells are of human origin and can be generated using cells from a patient with a particular disease they are more relevant than animal cells or immortalized cell lines. Further to enabling new discoveries of mechanisms of neurological diseases it is hoped that iPSC-derived neurons may be the key to improving the productivity crisis in drug discovery. For drug discovery applications cells need to be seeded into microplates to enable the screening of large numbers or compounds in a short space of time. The use of microplates is also important to researchers as they reduce the amount of expensive reagents needed to perform experiments. This is particularly important when using iPSC-derived neurons as the differentiation of these cells takes a long time and uses expensive cytokines, therefore efficient use of this precious resource is important. Some recent studies have used iPSC-derived neurons in 384-well plates [1-3] and multiple protocols exist which include seeding of iPSC-derived neurons [4-7] however there are no detailed protocols for seeding iPSC-derived neurons onto 384-well plates.

To coat the plates this protocol uses poly-l-ornithine, a synthetic positively charged amino acid chain widely used to enhance cell attachment to plasticware and glassware alongside laminin and fibronectin, which are extracellular matrix proteins that are regularly used to maintain attachment of neuronal cell cultures. Here we use a two-step coating method whereby a tissue culture-treated plastic microplate is first coated for 24 hours with poly-l-ornithine. This is then removed and the plate is coated for a further 24 hours

with a combination of laminin and fibronectin. This solution is then removed immediately before iPSC-derived neurons are seeded onto the coated plate. This coating method ensures that cells are evenly distributed throughout the well and do not become detached during subsequent media changes or downstream applications. Furthermore the cell suspension is passed through a 40um nylon strainer to remove any cell aggregates that would negatively effect the even distribution of cells. Once seeded the cells can undergo multiple wash steps involved in downstream applications such as immunofluoresence staining without detaching from the plate (Figure 1). Furthermore this method has been tested using a liquid dispenser to seed the cells with equivalent results to manual pipetting (Figure 1 b).

2. Materials

1. Coating solutions
    a. Poly-L-Ornithine, molecular weight 30,000-70,000 0.01% sterile-filtered solution, stock concentration 100ug/ml, store at 4°C, working concentration 15ug/ml, diluted in PBS on day of use
    b. Laminin and fibronectin, stock concentration of each 1mg/ml, store at -20°C, combine and dilute in PBS to give working concentration of 5ug/ml or each on day of use
2. Cell dissociation
    a. Accutase solution, sterile-filtered, used as supplied.
    b. Cell lifter
    c. Falcon 40µm Cell Strainer

3. Neuronal medium (see Note 1): Neurobasal medium, 50% DMEM / F12 medium, 1 x B27 supplement, 1 x N2 supplement, 400uM L-glutamine, 2-mercaptoethanol, BDFN (20ng/ml), GDNF (20ng/ml).

3. Methods

1. **Coating plates with poly-l-orninthine, laminin and fibronectin**
    a. Pipette 15ug/ml poly-l-ornithine in PBS into a tissue culture treated 384-well plate, 30ul per well. Place plates into incubator at 37°C and incubate for 24 hours.
    b. Aspirate poly-l-ornithine, wash wells with sterile H_2O and leave to air dry completely
    c. Add 5ug/ml laminin and fibronectin solution to each well, incubate and 4 degrees for 24 hours (see note 2).
2. **Dissociation of iPSC-derived neurons**
    a. Use neurons that have been differentiated in a 6-well plate (see note 3) for approximately 45 days from start of neuronal induction using preferred differentiation protocol (see note 4).
    b. Completely aspirate media from well, add 1ml PBS, then aspirate and add 1 ml accutase then incubate for 10 minutes at 37°C
    c. Add 1ml neuronal medium to each well, use a cell lifter to gently scrape along the surface of the place to detach the cells while tilting the place to collect the cells at the bottom edge of the well (see note 5)

d. Collect the cell suspension into a tube and centrifuge at 200 x g for 4 minutes.
e. Remove supernatant and add 2-3 ml fresh N2B27 to the centrifuged cells
f. Pipette gently up and down using a p1000 pipette to break up the cell pellet, do this 5-10 times until you can see an even distribution of cells in the solution. Avoid introducing air bubbles into the cell suspension.
g. Pass the cell suspension through a 40µm nylon cell strainer to remove any remaining cell aggregates

3. Seeding dissociated neurons

a. Count cells using preferred counting method
b. Dilute cells in the appropriate amount of neuronal medium to make a cell solution of 400,000 cells per ml. Add Y-27632 Rho kinase inhibitor to cell solution (10uM final concentration) to improve cell viability.
c. Transfer cell solution to a reagent reservoir
d. Remove laminin and fibronectin solution from coated plate
e. Add 30ul of cell solution to each well of the coated plate using a 12-channel pipette (see note 6). Each well will contain approximately 12,000 cells (see note 7).

4. Observation of plated neurons:

a. The following day change media by aspirating the media fully and adding 30µl of fresh neuronal medium to each well
b. Change media every 3-4 days (twice a week)

c. Inspect cells regularly by light microscopy, cellular processes should start to appear after 2-3 days
d. Check for cell density and presence of aggregates, cells should be evenly spaced and not too dense
e. For optimal images cells should be imaged or fixed at 7 to 10 days after plating (see note 8)

4. Notes

Note 1: The neuronal medium used here is based on the method used in Chambers et al., 2009 Nat. Biotechnol. Use whichever neuronal media the cells are growing in at time of seeding.

Note 2: plates coated with laminin and fibronectin can be stored at 4°C for up to 1 week

Note 3: Neurons can be differentiated in 6-, 12- or 24-well plates, for best results keep neurons at high density and passage at least every 2 weeks before final seeding in 384-well plates.

Note 4: Seeding can be performed on neurons aged between 35 and 65 days. The day on which it is best to plate the cells may differ depending on the differentiation protocol, if there is too much proliferation after plating try plating at a later date, if there is a lot of death after plating try plating at an earlier date.

Note 5: This can also be achieved without a cell scraper by gently pipette media onto surface of well to dissociate cells, pipetting around the well approximately 3 times to dissociate all of cells.

Note 6: It is also possible to use a liquid dispenser to transfer the cell solution to the 384-well plate

Note 7: It is important to dispense cells to the bottom of the well to ensure no air bubbles are trapped between the bottom of the well and the cell suspension, position pipette tip at the bottom corner of the well and dispense cells slowly.

Note 8: Cells can be left longer if need be, depending on confluency, the presence of proliferative cells and the assay to be used. Cells may become too confluent or may aggregate if left longer than 10 days, to reduce aggregation of cells 5ug/ml laminin can be added to neuronal media at approximately 7 days after plating.

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Figures

Figure 1. iPSC-derived neurons seeded in a 384 well plate

iPSC-derived neurons were seeded according to this protocol then fixed with 4% PFA and stained for neuronal specific class III beta-tubulin (green), neuronal specific microtubule associated protein 2 (red) and with Hoechst-33342 (blue) to highlight nuclei. Cells were either seeded manually (a & b) or seeded using a liquid dispenser (c & d). Lower panels (b & d) each show 25 images taken from one well stitched together. Scale bar represents 20um