Transcription Factor–Mediated Differentiation of Human iPSCs into Neurons

Michael S. Fernandopulle,[1,3] Ryan Prestil,[1,3] Christopher Grunseich,[1] Chao Wang,[2] Li Gan,[2] and Michael E. Ward[1]

[1]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland
[2]Gladstone Institute of Neurological Disease, Gladstone Institutes, San Francisco, California
[3]These authors contributed equally to this work.

Accurate modeling of human neuronal cell biology has been a long-standing challenge. However, methods to differentiate human induced pluripotent stem cells (iPSCs) to neurons have recently provided experimentally tractable cell models. Numerous methods that use small molecules to direct iPSCs into neuronal lineages have arisen in recent years. Unfortunately, these methods entail numerous challenges, including poor efficiency, variable cell type heterogeneity, and lengthy, expensive differentiation procedures. We recently developed a new method to generate stable transgenic lines of human iPSCs with doxycycline-inducible transcription factors at safe-harbor loci. Using a simple two-step protocol, these lines can be inducibly differentiated into either cortical (i^3Neurons) or lower motor neurons (i^3LMN) in a rapid, efficient, and scalable manner (Wang et al., 2017). In this manuscript, we describe a set of protocols to assist investigators in the culture and genetic engineering of iPSC lines to enable transcription factor–mediated differentiation of iPSCs into i^3Neurons or i^3LMNs, and we present neuronal culture conditions for various experimental applications.

Keywords: iPSC • iPSC-derived neurons • i^3Neurons • i^3LMN • transcription factor–mediated differentiation

How to cite this article:
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: 10.1002/cpcb.51

INTRODUCTION

Neurons are the primary information-processing cells in higher eukaryotes, performing essential functions in stimulus reception, signal transmission, and adaptive responses. In humans, neuronal dysfunction can cause a variety of clinical disorders, including developmental conditions such as autism, psychiatric illnesses such as schizophrenia, and degenerative diseases such as Alzheimer's dementia. Neurons are unique among cell types in their large and functionally polarized structure, post-mitotic state, and electrochemical activity. The human nervous system is unique in both its complexity and susceptibility to disease, and animal models often fail to recapitulate key characteristics of human neuronal disorders (D'Alton et al., 2014; Kitazawa, Medeiros, & Laferla, 2012). In order to understand normal human neuronal physiology as well as how cells

Current Protocols in Cell Biology e51
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpcb.51

and networks malfunction in disease, better experimental models of neuronal cell biology are needed.

Historically, the two main *in vitro* model systems for studying cellular and molecular neuroscience have been rodent primary neurons and human immortalized cell lines. Primary rodent neurons have specialized machinery unique to neurons, but, as they originate from another species, these cells may not recapitulate relevant aspects of human genetics or disease pathophysiology (Kloskowska et al., 2010; Nuber et al., 2013). Practically, primary neurons are time-consuming to isolate, can vary in quality from preparation to preparation, are difficult to scale for some applications, and are difficult to genetically engineer once isolated. Human immortalized lines such as HeLa, HEK293T, and U2OS, along with neuroblastoma lines such as SH-SY5Y, circumvent many of these challenges; they are easily cultured, relatively homogenous, scalable, and readily manipulated genetically. However, they have widespread and unstable genotypic abnormalities and lack a truly neuronal phenotype, so they are poorly suited to study neuron-specific biology such as axonal or synaptic phenomena.

The first derivation of human embryonic stem cell lines in 1998 was soon followed by techniques to manipulate developmental pathways in order to promote differentiation into cell types of interest (Bain & Gottlieb, 1998; Kawai et al., 1998; Shimazaki, Arsenijevic, Ryan, Rosenfeld, & Weiss, 1999; Thomson et al., 1998). The scalability and genetic tractability of stem cells finally permitted large populations of normal human cells to be grown *in vitro*, and for the first time human neuronal cell culture became a viable model system. The landmark development of induced pluripotent stem cell (iPSC) technology further enabled the reprogramming of patient-derived cells to establish stem cell lines capable of differentiating and recapitulating cellular disease phenotypes in culture (Takahashi & Yamanaka, 2007). The field quickly embraced these transformative tools (Chambers et al., 2009; Wernig et al., 2008), and, together, their success revolutionized the study of human neurobiology; no longer were scientists limited to choosing between cell-type and species specificity, and the use of stem cells greatly facilitated genetic engineering. Still, initial methods for differentiating iPSCs to neurons or neural precursors were far from simple; all involved complex media formulations and lengthy protocols (Hu & Zhang, 2009; Karumbayaram et al., 2009; Zeng et al., 2010). Recent approaches have simplified the process by employing primarily small molecules, and the use of overexpressed or inducibly-expressed transcription factors have simplified it further.

Most small molecule–based differentiation approaches rely on a combination of pathway inhibitors (e.g., noggin, SB431542) to drive ES or iPS cells toward neuroectodermal development. This process results in neural progenitor cells (NPCs), which must be coaxed to differentiate further with different small molecules and growth factors to finally produce the desired neuronal subpopulation. There are three major drawbacks with these strategies: small molecules are not highly efficient, individual cells transduce signals at different rates, and different iPSC clones (even from the same patient) can respond differently to the same small molecules. Together, this leads to a mixed population of neural progenitors and various neural and glial cell types, producing batch-to-batch and line-to-line variability. Particularly for long-term cultures, proliferative cells can quickly outcompete post-mitotic neurons of interest, and the presence of multiple cell types complicates downstream analysis, especially for high-throughput microscopy screens or "–omics" applications. Finally, small molecule–based methods are often laborious, expensive, and slow. From a survey of articles describing small molecule–mediated iPSC differentiation to neurons in the past year (Cao et al., 2017; Kikuchi et al., 2017; Qi et al., 2017), timelines extended from 13 to 70 days, and involved between four and six medium-formulation changes over those periods. This technical and time burden

decreases laboratory output, increases the likelihood of contamination, and creates barriers to entry for new users.

Transcription factor overexpression is a new approach to neuronal differentiation from iPSCs that circumvents many challenges associated with small molecule pathway inhibitors. Initially demonstrated by Zhang et al. (2013), overexpression of the master neuronal transcriptional regulator neurogenin-2 (NGN2) results in rapid, one-step differentiation of iPSCs to functionally mature glutamatergic cortical neurons. Similar results were independently obtained by the study of Busskamp et al. (2014), in which NGN2 was virally delivered and inducibly expressed in iPSCs, resulting in synaptically mature cells in 14 days. Both studies recorded upwards of 90% differentiation efficiency and purity, as measured by immunostaining of characteristic cortical neuron markers. With fewer medium changes and relatively rapid differentiation time, NGN2 overexpression offers an appealing alternative to small molecule differentiation strategies.

The set of protocols described here follows an improvement to the NGN2 method by Wang et al. (2017). In that study, the neurogenin-2 transgene was stably integrated into a safe-harbor locus in iPSCs under a doxycycline-inducible promoter. Clonal isolation of this stably integrated line enables near 100% efficiency and purity of differentiation to glutamatergic cortical neurons within the previously observed 14-day timeline, and simplifies differentiation to a two-step protocol. These cells, termed i^3Neurons (integrated, inducible, and isogenic), offer a substantial improvement in efficacy and ease-of-use over other existing iPSC-to-neuron differentiation strategies.

Here we provide a detailed set of protocols for the generation and use of i^3Neurons, and a related technique to generate lower motor neurons (i^3LMNs), which also includes overexpression of the transcription factors Islet-1 (ISL1) and LIM Homeobox 3 (LHX3) along with NGN2. The described techniques mostly require only basic laboratory instrumentation and reagents, and can be completed without any specific training beyond mammalian cell culture proficiency, making them appealing to a wide range of laboratories. Basic Protocols 1 to 4 provide an update on the state of the art of human iPSC culture and transgenic-line generation, with Support Protocol 1 describing a genotyping strategy for confirming stably integrated lines. Basic Protocols 5 to 8 discuss the differentiation and culture of i^3Neurons and i^3LMNs, with Support Protocols 2 to 5 providing specific instructions on immunocytochemistry, transfection, transduction, and live imaging of differentiated neurons. Support Protocols 6 to 7 provide instructions on assessing induction efficiency, as well as optional culture supplementation with astrocytes.

MAINTENANCE CULTURE OF iPSCs

BASIC PROTOCOL 1

Human iPSCs are an ideal system for studying human biology due to their rapid proliferation, genomic stability, and ability to differentiate into many somatic cell types. Historically, specialized culture practices and costly reagents have hindered widespread adoption of iPSCs by the cell biology community. In recent years, however, development of new culture techniques and improved media formulations have dramatically simplified iPSC culture and reduced costs.

The protocols described in this unit are adapted from a collection of publications that establish optimal practices for the maintenance of human iPSC cultures (Beers et al., 2012; Ludwig et al., 2006; Chen et al., 2011). While these publications provide useful guidelines for the stem cell novice, here we distill the fundamental procedures necessary for maintaining iPSCs in a pluripotent state and highlight critical steps that may need to be optimized for individual applications. In practice, iPSC lines of interest are usually

Table 1 Essential 8 Medium[a]

Component	Product no.	Amount
DMEM/F12 with HEPES	Gibco, 11330032	500 ml
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	Sigma, A8960	32 mg (per 500 ml)
Sodium selenite (dissolve 0.1 mg/ml in PBS; handle in fume hood)	Sigma, 214485	7 µg (70 µl) (per 500 ml)
Sodium bicarbonate	Sigma, S3817	271.5 mg (per 500 ml)
Sodium chloride	Sigma, S7653	As needed to adjust osmolarity to 340 mOsm
Sodium hydroxide (1 M)	Sigma, 71463	As needed to adjust pH to 7.4
Hydrochloric acid (1 M)	Sigma, H9892	As needed to adjust pH to 7.4
Aliquot and add fresh to each bottle:		
Insulin (supplied at 1000×, store at 4°C)	Sigma, I9278	500 µl (per 500 ml)
TGF-β1 (2 µg/ml in PBS; 1000×, store at −80°C)	Peprotech, 100-21	500 µl (per 500 ml)
FGF-basic (100 µg/ml in PBS; 1000×, store at −80°C)	Peprotech, 100-18B	500 µl (per 500 ml)
Holo-transferrin (10.7 mg/ml in PBS; 1000×, store at −80°C)	Sigma, T0665	500 µl (per 500 ml)

[a]E8 may be made as described here and filter-sterilized before use (can be formulated in bulk and stored at –80°C) or may be purchased commercially.

maintained in an undifferentiated state in small cultures (1 to 3 wells of a 6-well plate) to reduce reagent use before being expanded as needed for experimentation.

Essential 8 (E8) is the simplest defined medium for hiPSC culture. E8 may be prepared from its components by the consumer (Table 1; Chen et al., 2011) or purchased as a pre-formulated kit. Other commercially available media may be substituted, such as mTeSR1 or StemFlex. A volume of 12 ml of medium should be added to each 10-cm tissue culture dish or distributed evenly across each standard multiwell plate (i.e., 2 ml/well for a 6-well plate). E8 should be aspirated and replaced with fresh medium daily, although a double volume may be added at low confluency to permit an extra day of culture without medium changes. StemFlex and E8 Flex contain components that stabilize the recombinant growth factors present in the medium, permitting medium exchange every other day as a general practice. Some iPSC lines (e.g., WTC11) tolerate every-other-day medium changes of standard E8 medium without loss of pluripotency or cell death, further reducing costs of medium and consumables. Finally, mTeSR1 may promote cell survival in stressful conditions better than E8, especially for finicky iPSC lines, although supplementation with a ROCK inhibitor (RI) is also recommended in such scenarios. Use of standard E8 will be assumed throughout this basic protocol.

Additionally, this protocol uses Matrigel-coated tissue culture plates. Matrigel works well for iPSC culture and has been widely adopted for research applications. However, since Matrigel is derived from murine sarcoma cells, it is not chemically defined and exhibits batch-to-batch variability. Alternative defined coatings include recombinant laminin or vitronectin, although these substrates are typically more costly. Notably, downstream neural differentiation described in these protocols occurs in fully defined conditions (see

Basic Protocols 5 to 8), so the choice of iPSC substrate is of minimal scientific importance to all but clinical-grade applications.

Materials

Matrigel, hESC-qualified (Corning, cat. no. 354277)
DMEM/F12 medium (Gibco, cat. no. 11320033)
Human induced pluripotent stem cells (hiPSCs; e.g., WTC11, Corriell Institute, cat. no. GM25256)
E8 medium (may be user-formulated per Table 1 or purchased pre-formulated as Gibco, cat. no. A1517001; may also be substituted with E8 Flex, Gibco, cat. no. A2858501; StemFlex, Gibco, cat. no. A3349401; or mTeSR1, STEMCELL Technologies, cat. no. 85850; or similar)
70% ethanol
Rho-associated protein kinase (ROCK) inhibitor Y-27632 (e.g., Tocris Bioscience, cat. no. 1254 or Selleck Chemicals, cat. no. S1049), reconstituted to 10 mM in PBS
Phosphate-buffered saline (PBS) without calcium or magnesium (e.g., Gibco, cat. no. 10010049)
0.5 mM EDTA in PBS (diluted from Gibco, cat. no. AM9260G or Sigma, cat. no. E6758; may also be purchased as Versene, Gibco, cat. no. 15040066)
Accutase (Gibco, cat. no. A1110501 or StemCell Technologies, cat. no. 07920)
Dimethylsulfoxide (DMSO; Sigma, cat. no. 472301)
Fetal bovine serum (FBS), qualified, heat inactivated (Gibco, cat. no. 16140071)
Liquid nitrogen

CoolRack M30 (BioCision BCS-108)
P2, P20, P200, and P1000 micropipettors and tips (e.g., Gilson)
Sterile 5, 10, and 25-ml serological pipets (e.g., Corning, cat. no. 356543, 356551, and 357535, respectively)
Sterile 15- and 50-ml polypropylene conical tubes (e.g., Corning, cat. nos. 352096 and 352070, respectively)
Sterile polystyrene 10-cm tissue-culture dishes and 6-well, 12-well, and 24-well plates (e.g., Corning, cat. no. 353003, 353046, 353043, and 353047, respectively)
Laminar flow biological safety cabinet (BSC)
Vacuum aspirator and aspirating pipets (Fisher, cat. no. 1367820) *or* reusable Corning vacuum aspirator (cat. no. 4930) with disposable tips (e.g., Pure XLG pipet tips, Andwin Scientific, cat. no. 46600-020)
Phase-contrast and fluorescent microscope with 4×, 10×, 20×, and Object Marker objectives (e.g., Nikon Eclipse Ti)
Cell counting apparatus [hemacytometer or automated cell counter; also see Phelan & May (2015)]
50-ml, 250-ml, and 500-ml sterile filters, 0.2-µm pore (Millipore, cat. no. SCGP00525; Thermo, cat. no. 568-0020 and cat. no. 566-0020, respectively)
1.5-ml cryogenic tubes (Thermo, cat. no. 5000-1020)
Microcentrifuge for 1.5-ml tubes
Cryovial freezing container (e.g., CoolCell LX, BioCision, cat. no. BCS-405; or Mr. Frosty, Thermo, cat. no. 5100-0001)
Picking microscope inside sterile laminar flow enclosure (e.g., Etaluma LS620)

Matrigel coating

1. Aliquotting concentrated Matrigel:

   a. Gradually thaw a 5 ml bottle of Matrigel stock solution overnight at 0°C by burying in ice in a Styrofoam container placed within a refrigerator. Additionally,

pre-chill microcentrifuge tubes by placing in an aluminum cool rack on ice before use.

b. Before pipetting concentrated Matrigel into pre-chilled microcentrifuge tubes, chill a 1-ml pipet tip by pipetting ice-cold DMEM/F12 up and down several times, then immediately use this chilled tip to aliquot the Matrigel stock.
c. Aliquot 500 µl concentrated Matrigel into each microcentrifuge tube, and re-freeze aliquots at −80°C.

Matrigel polymerizes rapidly at room temperature when concentrated, so it is imperative to aliquot stocks with pre-chilled tips and tubes and to thaw the concentrated stock solution on ice.

2. Making Matrigel coating solution:

a. Aliquot 50 ml of cold DMEM/F12 into a conical tube.
b. Using a P1000, pipet 1 ml of cold DMEM/F12 from the conical tube into the microcentrifuge tube containing 500 µl concentrated Matrigel stock. Pipet up and down several times, and then transfer what has thawed to the conical tube containing cold DMEM/F12.
c. Repeat until the frozen concentrated Matrigel has been completely transferred to the 50-ml conical tube containing DMEM/F12. Invert several times to mix.

3. Add one half of the normal culture volume of the Matrigel coating solution to the tissue culture surface. Gently agitate plates to ensure full coverage.

For example, add 1 ml per well of a 6-well plate.

4. Transfer plates to a 37°C incubator.

Plates may be used after 1 hr, but better long-term morphology typically results from overnight coating. It is also possible to prepare plates in bulk by adding additional DMEM/F12 to a full culture volume in order to prevent wells from drying. These plates may be stored in a 37°C incubator or wrapped in Saran wrap or Parafilm and stored at 4°C. Plates should be used within 2 weeks of preparation.

5. Aspirate Matrigel solution immediately before use and replace with culture medium and cells.

Thawing iPSCs

Often, iPSCs are stored and distributed as frozen stocks, so thawing is the first procedure performed. Routine use of antibiotics in stem cell culture medium is strongly discouraged, since these compounds can interfere with cell biochemistry and differentiation potential. Consequently, proper sterile technique is critical to prevent contamination, and cells received from other environments should be quarantined for at least two passages and tested for mycoplasma.

6. Prepare biological safety cabinet (BSC) with tube racks, DMEM/F12, P1000 tips, conical tubes, and culture medium.

DMSO is toxic to cells at room temperature, so steps 2 to 5 should be completed as quickly as possible.

7. Transfer cryovial of hiPSCs from liquid nitrogen or dry ice and thaw in 37°C water bath or bead bath

Thaw should be completed rapidly to limit exposure to DMSO. A small frozen core may remain, as pipetting and rinsing will complete thaw.

8. Sterilize cryovial by spraying with or dipping into 70% ethanol and transfer into the BSC.

9. Pipet cell solution to new 15-ml conical tube, rinse cryovial twice with 1 ml DMEM/F12, and add each rinse to the tube.

10. Centrifuge tube 5 min at 300 × *g*, room temperature.

    Speeds of 200 to 300 × g are well tolerated by iPSCs. For the purposes of this protocol, 300 × g is recommended to maximize capture of small cell numbers. For standard procedures, 200 × g is recommended.

11. Aspirate supernatant, resuspend in culture medium supplemented with 10 μM Y-27632 ROCK inhibitor, and transfer to Matrigel-coated plate.

    Maintaining high cell density maximizes survival, so it is recommended to plate each vial (typically 1 × 10^6 cells) in one well of a 6-well plate. This may be modified depending on specific cell number or viability.

12. Return plate to 37°C incubator and evenly distribute cells by gently shaking plate front-to-back and side-to-side.

    This procedure is critical any time cells are replated, and should be performed as soon as cells are transferred. Swirling or otherwise agitating culture medium before cells attach can cause higher cell densities in the middle of the well.

13. The next day, aspirate the medium and replace with fresh E8 culture medium (2 ml/well for 6-well dish). If colonies are small and/or if cell death is noted after the medium change, use of E8 with Y-27632 ROCK inhibitor may be necessary until colonies have expanded, after which inclusion of the ROCK inhibitor is not required.

Splitting

An advantage of using iPSCs as a model system is their rapid rate of proliferation; however, culture health is superior if cultures are only split to modestly low densities. Furthermore, cells will rapidly differentiate and die if allowed to grow into a monolayer. When colonies grow too large and/or begin to converge (approximately 80% confluency), they must be dissociated in order to maintain proper growth and pluripotency (Fig. 1A). Dissociating cells with EDTA is gentler and faster, and typically results in improved survival over enzymatic dissociation methods, making it ideal for general culture maintenance. EDTA acts by chelating the calcium necessary for cell attachment and transfers cells as small clumps, which promotes colony formation and growth (Fig. 1B). Alternatively, Accutase provides gentle enzymatic dissociation for iPSCs and should be used for any applications that require single-cell suspensions, such as for clonal derivation, cell counting, or flow sorting (Fig. 1C). Supplementation of culture medium with ROCK inhibitor is optional following EDTA treatment, but is required after Accutase treatment to prevent apoptosis of single cells. Additionally, cultures of stem cells can often spontaneously differentiate, particularly after overgrowth or stressful procedures (Fig. 1D). Isolated loci of differentiated cells may be removed directly by aspirating areas of the well, and this is particularly effective during EDTA splitting. For highly differentiated cultures, however, several passages are often required to regain a healthy pluripotent population. Plating at high density following EDTA splitting (1:3 ratio) can promote iPSCs to outcompete differentiated cells. Alternatively, a modified version of the EDTA split is also included below to remove spontaneously differentiated cells, which takes advantage of higher adhesion of differentiated cells compared to iPSCs.

EDTA

14. Aspirate culture medium and rinse with one-half culture volume of PBS.

    Since EDTA works by chelating the calcium ions necessary for iPSC attachment, be sure the PBS does not contain calcium.

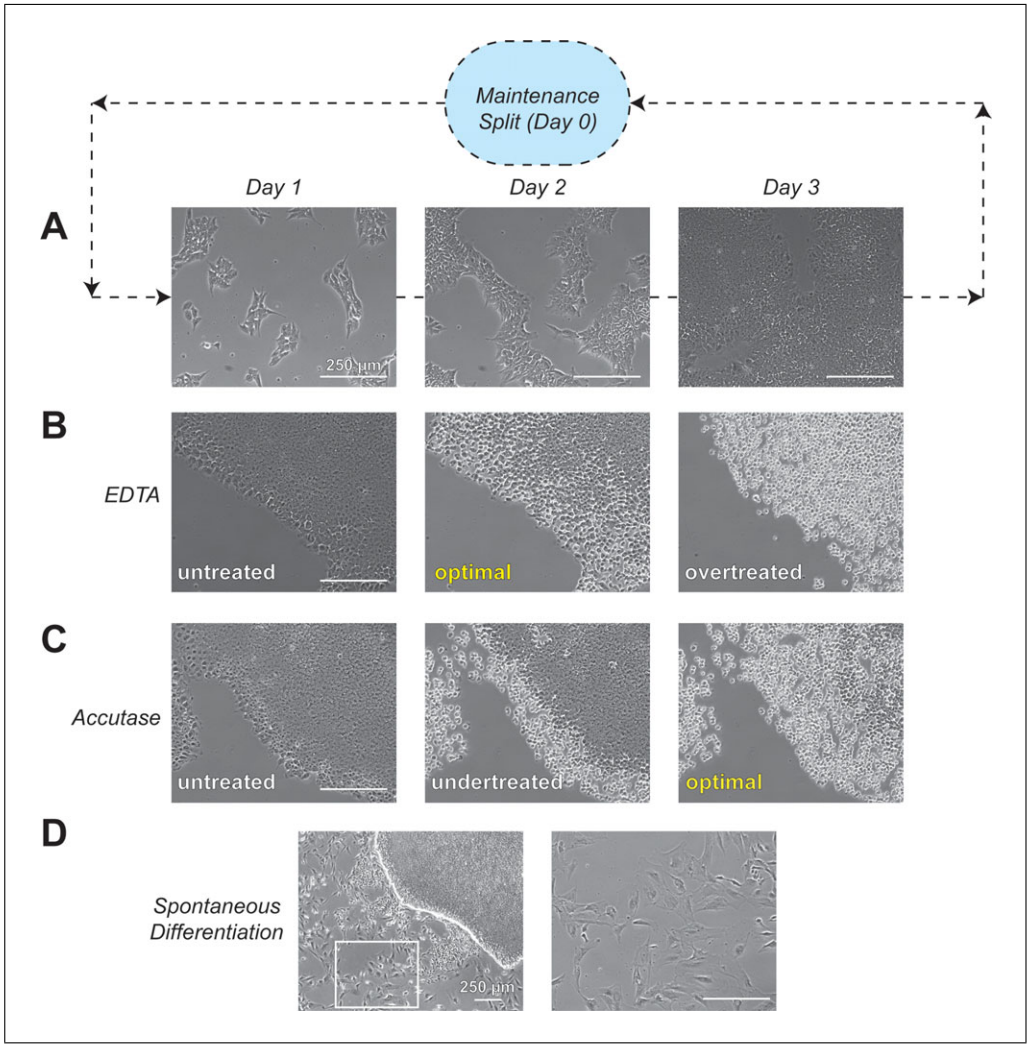

Figure 1 Schematic of routine hiPSC culture maintenance. (**A**) Cells typically grow to confluence within 3 to 4 days after plating, and must be split in order to maintain health. Splitting can be done with either EDTA or Accutase dissociation solutions. (**B**) Time course for EDTA hiPSC dissociation. When the edges of the colony just begin to singularize (evidenced by bright halos around individual cells), cells are ready for EDTA aspiration and trituration in new medium. Singularization of the entire colony ("overtreated") results in lifting and loss of cells with EDTA aspiration. (**C**) Time course for Accutase hiPSC dissociation. Cells are ready for trituration and collection only when the entire colony has singularized. (**D**) Images of spontaneous differentiation observed in hiPSC cultures. hiPSCs are typically small and form cobblestone-like colonies. Spontaneously differentiated cells (e.g., "flat cells") are typically much larger and tend to form on the outskirts of healthy colonies.

15. Aspirate PBS and add one-half culture volume of EDTA solution (0.5 mM in PBS).
16. Incubate for 5 to 10 min at room temperature (Fig. 1B).

    Exact timing varies by cell line, but 7 min is typical for hiPSCs. Cell colonies should be opaque to the naked eye, and colony edges should be just starting to detach when viewed under a microscope. Single-cell dissociation may be achieved by incubating for up to 15 min, and time may be reduced by incubating plates at 37°C. If colonies begin sloughing off, collect EDTA and cells in a conical tube, rinse with culture medium and add to the tube, centrifuge 5 min at 200 × g, aspirate supernatant, resuspend in culture medium supplemented with 10 µM ROCK inhibitor, and plate cells. Reduce EDTA incubation time in future passages.

17. While incubating, prepare recipient Matrigel-coated plates by aspirating Matrigel solution and loading with three-quarters volume of culture medium.

For example, 1.5 ml medium/well of a 6-well dish. Supplementation of 5 μM ROCK inhibitor is optional to improve survival.

18. Aspirate EDTA solution, taking care not to disturb cell colonies, which should remain attached.

    Small-scale differentiation may be removed at this step by directly aspirating areas of the well which have noticeable bumps (particularly in the middle of large colonies) or by designating areas for removal beforehand with an object marker microscope objective.

19. Dissociate cells by pipetting two to three times with culture medium equivalent to half of the denominator of the splitting ratio.

    Typical splitting ratios for 6-well plates are between 1:6 and 1:12, for which 3 and 6 ml medium should be used, respectively. Mix well, but avoid pipetting up and down more than three times in order to keep cell clumps intact. If colonies remain attached, dispense medium, gently scrape the bottom of the well with the end of the pipet, and pipet up and down twice to mix. Increase EDTA incubation time in future passages.

20. Add one-fourth of the volume of culture medium with cells to each recipient well, and discard any excess cells.

21. Return plate to 37°C incubator and evenly distribute cells by gently shaking plate front-to-back and side-to-side.

EDTA-mediated removal of spontaneously differentiating cells

22. Prepare cells as for an EDTA split (see above)

23. Incubate cells at room temperature and view by phase-contrast microscopy every 2 min.

24. When colony edges begin to detach (typically 5 to 10 min), gently tap the plate three to five times against your hand until most iPSC colonies are in suspension.

25. Gently pipet once with the plate tilted to collect cells at the bottom of the well and transfer the solution to a 15-ml conical tube.

    Some iPSCs and most differentiated cells should remain attached, but avoid further washing steps. Differentiated cells usually require longer EDTA incubation and higher shear forces to dissociate than iPSCs.

26. Add 3 ml DMEM/F12 directly to the tube to inactivate the EDTA.

27. Centrifuge 5 min at 300 × *g*, room temperature.

28. Aspirate supernatant, resuspend in culture medium, and plate to a new Matrigel-coated dish.

    Plating at high density and with ROCK inhibitor promotes survival and proliferation of iPSCs in order to outcompete differentiated cells. Repeat this method of splitting until cell culture is nearly pure; consistently high levels of spontaneous differentiation suggest inherent problems with the cell line and/or culture conditions.

Accutase

29. Aspirate culture medium and rinse with one-half culture volume of PBS. Aspirate PBS and add up to one-half culture volume of Accutase.

    To save reagent, only enough Accutase is required to cover the culture surface (i.e., 0.5 ml/well of a 6-well dish).

30. Transfer to 37°C incubator for 5 min, or until most cells have detached (Fig. 1C).

If colonies remain attached, gently tap the plate against your hand three to five times or extend incubation to at most 15 min in total.

31. While incubating, prepare recipient Matrigel-coated plates by aspirating Matrigel solution and loading with culture medium supplemented with 10 μM ROCK inhibitor.

32. Tilt the plate, pipet the Accutase solution twice down the culture surface to break apart clumps, and transfer to conical tube.

33. Rinse culture surface with culture medium and combine with cell solution in the tube.

    DMEM/F12 or PBS may also be used to wash and are recommended if cells tend to clump in culture medium. However, addition of at least 5% culture medium aids in subsequent pelleting and attachment if PBS is used.

34. Centrifuge 5 min at 300 × *g*, room temperature.

35. Aspirate supernatant.

    Residual Accutase can interfere with cell attachment and downstream applications such as transfection, so remove as much as possible without disturbing the cell pellet. For example, remove a majority of the supernatant by vacuum aspiration, then finish with a P1000.

36. Resuspend in culture medium.

    If desired, count cells using a hemacytometer or automated cell counter (Phelan & May, 2015) and calculate plating volume.

37. Add cells to recipient plate.

38. Return recipient plate to 37°C incubator and evenly distribute cells by gently shaking plate front-to-back and side-to-side.

Freezing

Culturing over many passages may result in mutations and genetic drift, and stressful events can select for abnormal genotypes such as oncogene mutations or chromosomal deletions and rearrangements (Merkle et al., 2017). To circumvent these adverse outcomes and to provide backup in case of contamination, several vials of cells should be cryopreserved immediately after cell line isolation. Additionally, freezing cell clones during validation reduces reagent use, and expanding cultures and freezing a large, pooled batch of cells on the same date with the same passage number provides useful downstream reproducibility.

39. Prepare cells as for an EDTA split (see above). During EDTA incubation, label cryovials and formulate cryopreservation medium by combining culture medium with 10% DMSO.

    Optionally, the addition of 20% fetal bovine serum or knockout serum replacement to cryopreservation medium may improve viability. 90% fetal bovine serum/10% DMSO solutions (i.e., no culture medium) have also been used successfully. Note that if doxycycline-inducible promoters are integrated into the iPSC lines, we recommend the use of validated, tetracycline-free FBS for such purposes (e.g., Tet System Approved FBS from Clontech). Cryopreservation medium may also be purchased commercially (e.g., CryoStor CS10, STEMCELL Technologies cat. no. 07930)

40. Aspirate EDTA and dissociate cells with cryopreservation medium.

    It is optional to dissociate with culture medium and count cells (Phelan & May, 2015) in order to ensure that standard cell numbers are frozen across samples, typically

1×10^6 cells/vial. This is followed by centrifugation, aspiration of culture medium, and resuspension in an appropriate volume of cryopreservation medium. For routine applications, however, 1 to 2 ml cryopreservation medium per well of a 6-well plate at approximately 80% confluency provides adequate cell density for a healthy thaw. Steps 3 to 4 should be completed as quickly as possible to reduce DMSO exposure.

41. Transfer 1 ml cryopreservation cell suspension to each 1.5-ml cryovial and freeze in a CoolCell freezing container or Mr. Frosty isopropanol caddy at −80°C for two hours to overnight.

    Gradually reducing temperature by 1°C/minute improves viability.

42. Transfer cryovials to liquid nitrogen for long-term storage.

    Cryovials should be placed on dry ice for transit from the freezer to the liquid nitrogen tank to prevent thawing.

Manual manipulation

Manual changes in the composition of an iPSC population may be accomplished by either isolating a desired colony into a separate culture (manual passage, or pick-to-keep) or by scraping away undesired cells for aspiration (pick-to-remove). In practice, manual passaging is an important part of the clonal isolation protocol below, and picking-to-remove is most commonly used to remove isolated areas of spontaneously differentiated cells from maintenance cultures or to provide more room for the desired cells to grow prior to picking-to-keep. Proper sterile technique is essential during both of these procedures, as plates may be uncovered in the biosafety cabinet for extended periods of time.

Pick-to-keep

43a. Prepare a recipient 24-well plate by aspirating Matrigel solution and adding 250 µl/well of culture medium supplemented with 10 µM Y-27632 ROCK inhibitor. Place the cell culture dish on a picking microscope in a sterile enclosure.

    A microscope (e.g., Lumascope) connected to a screen or tablet computer may be sterilized and kept inside a BSC. Alternatively, a PCR enclosure with a stereoscope that can be sterilized by ethanol and/or UV treatment provides sufficient protection.

44a. Remove the cover of the culture dish, center the colony desired to be picked, flush the P1000 tip with medium to avoid cell retention, and align the end of a P1000 pipet tip just above the colony.

    Colonies are often pre-selected with a marking objective, which leaves a 1.8 mm ring on the bottom of the culture dish. Colonies approximately the size of the inside of the ring are of ideal size for picking, and a wide-diameter tip is desirable to reduce shear forces on the cells. P200 or smaller tips should not be used. Set the pipet to 250 µl. Balance the tip on your opposite index finger to improve stability and control.

45a. Lower the pipet tip until it makes contact with the culture surface.

    If necessary, raise the plunger of the pipettor slightly to remove the air bubble for better viewing.

46a. Gently and slowly scrape the bottom of the well with the tip, slowly raise the plunger to detach cells in strips, and collect in the pipet tip.

    Keep a shallow angle with the plate and avoid pressing down on the plate. Raise the plunger more quickly to provide more force if cells remain stuck or are close to the well wall.

47a. Deposit the picked cells (in 250 µl medium) into the destination well.

    If picking has been slow, cells may be stuck to the inside of the tip, so check the well under the microscope before disposing of the tip to ensure that cells are present. Try to

avoid pipetting multiple times in the well in order to keep cells clumped. If the clone was not completely picked, the same tip may be reused to acquire more cells; otherwise, change pipet tips between each clone.

Pick-to-remove

Depending on the scale of cells to be removed, picking may be accomplished with a pipet tip (without a pipettor) or with a more specialized cell scraper, either purchased commercially or made from a borosilicate Pasteur pipet (Kent, 2009).

43b. Place the cell culture dish on a picking microscope in a sterile biological safety cabinet.

44b. Remove the cover of the culture dish, center the cells to be removed, and align the end of the picking implement just above the cells.

Hold the pipet tip near the end or balance the picking implement on your opposite index finger to improve stability and control. Be careful not to hold your hand above the well or to otherwise compromise sterility.

45b. Slowly lower the implement until it makes contact with the culture surface.

46b. Gently scrape until cells are in suspension.

Be careful not to scratch the culture surface, as this can disrupt the Matrigel coating. For pipet tips, hold at an angle of approximately 45˚ from the plate and align the lower edge with the cells to be removed.

47b. Between removals, swirl medium to collect picked cells in the center of the well.

48b. Aspirate medium and rinse with PBS.

49b. Check for full removal, and scrape away remaining cells if necessary.

50b. Swirl PBS, aspirate, and replace with fresh culture medium.

BASIC PROTOCOL 2

LIPID-MEDIATED TRANSFECTION OF iPSCs

This protocol describes the lipid-mediated transfection of iPSCs maintained in E8 medium on Matrigel. While several lipid-based transfection reagents are commercially available, Lipofectamine Stem is used here because it is specifically optimized for delivery of DNA plasmids into hiPSCs. If preferred, similar results may also be achieved by electroporation or nucleofection, and other lipid reagents are available for *in vitro*–transcribed RNA or *in vitro*–translated ribonucleoproteins (RNPs). Furthermore, while this protocol provides the steps for a general transfection, specific details are provided below regarding insertion of the transgene cassettes relevant for neural differentiation (See Basic Protocols 5 and 7).

Passaging with Accutase immediately before transfection improves efficiency by generating a single-cell suspension that increases exposure to the Lipofectamine reagent; however, if Accutase passaging for certain iPSC lines results in low viability, transfection may also be performed on EDTA-passaged cells or on adherent cells at low confluency (20% to 30%). Transfection efficiency may be monitored by including a fluorescent protein reporter under a promoter that is active in human stem cells (e.g., CAG, PGK, EF-1α containing introns; not CMV) and viewing the cells 1 day after transfection. This reporter does not need to be integrated, as transient expression should persist for 3 to 4 days after transfection. Finally, increased cell death is typical for 1 to 2 days after transfection, and can result in the accumulation of debris, so the culture medium should be changed daily, and cells may also be washed with PBS after aspiration of spent medium to further reduce debris carryover. The transfected iPSCs should be passaged for expansion, enrichment,

and/or clonal selection (Basic Protocol 3 or 4) after the cells have reached approximately 80% confluency, which commonly occurs 2 to 4 days after transfection.

Materials

Lipofectamine Stem (Invitrogen, cat. no. STEM00001) or other lipid-based transfection reagent
Opti-MEM I Reduced Serum Medium (Gibco, cat. no. 31985062)
DNA plasmid(s) (e.g., CRISPR-Cas9 and guide RNA, TALENs, and/or DNA insert with appropriate homology arms; DNA obtained from an endotoxin free maxi-prep kit)

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

1. Grow a sufficient number of iPSCs for transfection and prepare cells as for an Accutase split (see Basic Protocol 1).

   One or two wells of a 6-well dish at 80% confluency should provide more than enough cells for one transfection.

2. Count the cells (Phelan & May, 2015), transfer 1.5×10^6 cells to a 15-ml conical tube, and centrifuge 5 min at $300 \times g$, room temperature.

3. Aspirate the supernatant and resuspend in 2 ml of E8 medium supplemented with 10 µM Y-27632 ROCK inhibitor.

   If iPSCs are normally maintained in a Flex medium, it is best to transition to regular E8 on the day of transfection to improve efficiency.

4. Pipet the medium and cells into 1 well of a 6-well dish pre-coated with Matrigel and return plate to the incubator. Gently shake the plate front-to-back and side-to-side.

5. Allow the cells to adhere in the incubator for 1 to 2 hr before adding the transfection solution.

6. For each transfection, add 100 µl of Opti-MEM and 3 µg of total DNA to one 1.5-ml microcentrifuge tube and vortex for 2 to 3 sec. In a second tube, combine 100 µl of Opti-MEM and 10 µl of Lipofectamine Stem reagent, and vortex for 2 to 3 sec.

   For TALEN-mediated insertion to the AAVS1 or CLYBL locus, such as for the hNGN2 (Addgene, cat. no. 105840) and hNIL (Addgene. cat. no. 105841) differentiation cassettes, use a 2:1:1 ratio of 1.5 µg donor construct with 0.75 µg of each of the site-specific TALENs. For AAVS1: 0.75 µg of pTALdNC-AAVS1_T2 (Addgene, cat. no. 80496) and 0.75 µg of pTALdNC-AAVS1_T1 (Addgene, cat. no. 80495) per transfection. For CLYBL: 0.75 µg of pZT-C13-R1 (Addgene, cat. no. 62197) and 0.75 µg of pZT-C13-L1 (Addgene, cat. no. 62196) per transfection.

7. Combine the contents of the two tubes and vortex again for 2 to 3 sec. Incubate this mixture for 10 min at room temperature.

8. Retrieve the cells plated in steps 3 to 4, and, using a P200 pipet tip and repeat pipettor, add 200 µl of the complete transfection solution from step 6 dropwise, evenly across the surface of the well. Return the cells to the incubator overnight.

9. 24 hr after transfection, aspirate transfection medium and replace with fresh E8. If applicable, evaluate transfection efficiency by fluorescence microscopy.

   All cells transfected with the hNGN2 (Addgene, cat. no. 105840 or 110492) and hNIL constructs (Addgene, cat. no. 105841 or 105842) will transiently express mCherry for 3 to 4 days, while only those cells with transgene insertion will maintain stable expression

of mCherry for longer periods of time. See Basic Protocols 3 and 4 for options for enrichment and clonal isolation.

10. Change medium daily with normal culture medium, and wash with PBS if necessary to remove debris. Once the cells have reached 80% confluency, they may be passaged to a new dish for expansion or used for enrichment or clonal selection.

BASIC PROTOCOL 3

SELECTION AND ENRICHMENT OF TRANSGENIC CELL POPULATIONS

While lipid-mediated transient transfection and expression of transgenes in hiPSCs is often quite high (>50%), even an optimal transfection with the best editing tools can result in very low genomic integration efficiency (<1%). For this reason, establishing pure cell populations is typically the major bottleneck of any transgenic workflow. Commonly, frontline selection and enrichment are used to facilitate later single-cell cloning, which remains necessary to ensure that transgene insertion did not result in mosaicism or off-target mutagenesis (See Basic Protocol 4).

In order to remove non-edited cells, positive selection markers such as genes coding for fluorescent proteins or antibiotic resistance are often included in the insert construct under constitutive promoters, and this protocol describes general methods for the former strategy. However, constant expression of a fluorophore interferes with immunocytochemistry, and antibiotic selection can incorrectly select for cells with multiple aberrant transgene insertions. To counter these shortcomings, we developed a platform for enrichment from heterogeneous cell populations through cell-surface affinity for magnetic streptavidin beads mediated by expression of a streptavidin binding peptide (SBP) fused to the truncated extracellular and transmembrane domains of low-affinity nerve growth factor receptor (LNGFR). The SBP tag is commonly used to facilitate co-immunoprecipitation with streptavidin-coated beads, and, with fusion to the LNGFR, it is efficiently localized to the extracellular surface where it can bind the beads more readily.

This construct was developed based on one initially designed for purification of transgenic T cells in suspension (Matheson, Peden, & Lehner, 2014), and it is included in Addgene cat. no. 105842 for direct use with hNIL or excision and insertion into other constructs. Here, we extend the original protocol in order to optimize enrichment following safe-harbor locus insertion in iPSCs, and we present evidence to support its use as either a positive or negative selection marker in a variety of transgenic applications. However, we have found that the SBP-LNGFR construct should be the only gene expressed under a dedicated, highly active promoter, as it appears to interfere with 2A-mediated ribosome skipping. For example, a different construct, with the reverse tetracycline transactivator (rtTA) linked to SBP-LNGFR by a T2A sequence, led to disrupted differentiation due to reduced rtTA activity (see Support Protocol 6).

When structured correctly, however, SBP-LNGFR permits highly selective enrichment that does not require specialized equipment as in fluorescence-activated cell sorting (FACS) and does not require extensive tuning as in antibiotic selection (Fig. 2A). Notably, the efficiency of enrichment increases exponentially with a lower frequency of positive cells in the population (Fig. 2E). In addition, the protocol is easily modified to permit negative selection, e.g., for removing a floxed transgene with Cre recombinase or a gene knockout reporter (Fig. 2F). Furthermore, cells expressing SBP-LNGFR may be transiently labeled at the cell surface by incubating with any fluorophore conjugated to streptavidin (Fig. 2B and C). After washing to remove unbound fluorophore, these cells may be imaged, marked for clonal picking, or FACS-purified at an equivalent efficacy to cells constitutively expressing a fluorophore marker of insertion. Fluorescence then

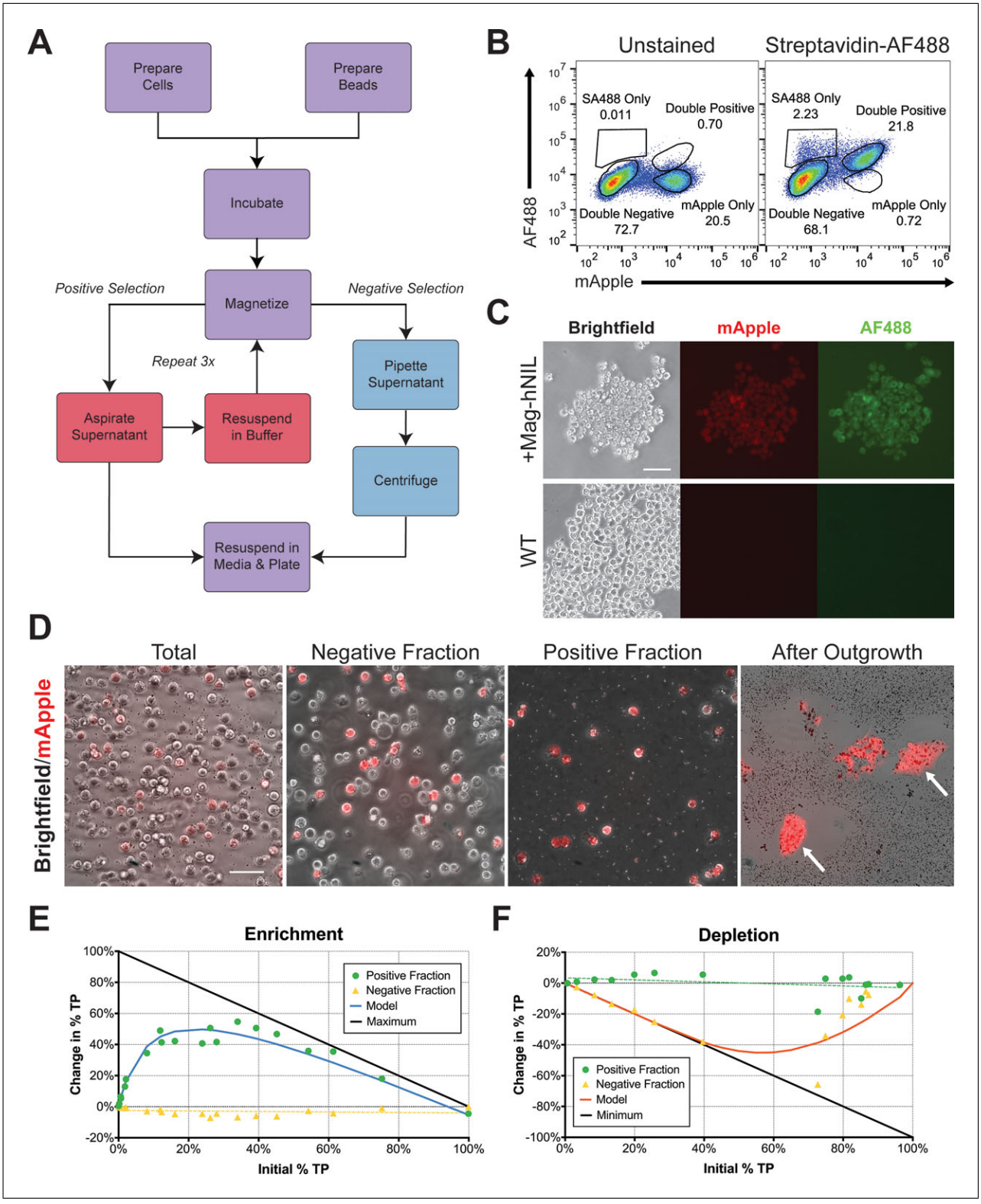

Figure 2 *(legend appears on next page)*

returns to undetectable levels within one passage and does not interfere with downstream immunocytochemistry applications.

Precise, label-free editing of endogenous loci may be enriched by FACS sorting for transient expression of the components necessary for editing 1 day after transfection [i.e., using a combined gRNA, Cas9, and GFP expression plasmid with an integration plasmid expressing RFP outside of the insertion sequence or by using functionalized RNPs such as S1mplex with Qdots or fluorescent streptavidin (Carlson-Stevermer et al., 2017)]. This can considerably increase the prevalence of correctly edited cells, but the combined stress of transfection and sorting can decrease cell survival. To compensate, it is recommended to transfect and sort (2.4×10^6 cells) in triplicate and to plate cells at high density following the sort. In addition, extensive downstream cloning and genotyping is often required due to the propensity for non-homologous end joining leading to mutagenesis at the target locus rather than homology-directed repair-mediated integration of the desired insert.

This protocol will continue with the assumption of integration of a transgene cassette into a safe-harbor locus as described in Basic Protocol 2. Label-free gene knockouts caused by NHEJ-mediated indel formation are typically prevalent (>15%), so frontline enrichment is optional for these applications. Following enrichment, small stocks of heterogeneous cell populations should be frozen, and the cells should be taken directly to Basic Protocol 4 for clonal isolation.

Materials

Heterogeneous post-transfection iPSC population (Basic Protocol 2)
1 M EDTA stock
10% (w/v) bovine serum albumin (BSA; Miltenyi, cat. no. 130091376) stock
autoMACS Rinsing Solution (optional; Miltenyi, cat. no. 130091222)
Magnetic streptavidin beads (Dynabeads MyOne Streptavidin C1; Thermo, cat. no. 65001)
Biotin (optional; Sigma, cat. no. B4639)

Fluorescence-activated cell sorter (FACS)
Vacuum source
DynaMag-2 magnetic tube rack (Thermo, cat. no. 12321D)

Figure 2 *(image appears on previous page)* Enrichment of transgenic iPSCs by magnetic streptavidin bead affinity. **(A)** Workflow of positive and negative selection protocols. See protocol for considerations on key steps, such as cell number, bead volume, and incubation time. (**B**) A mixed population of unedited cells and cells transfected with the Mag-hNIL construct and of unedited cells were incubated with streptavidin conjugated to AlexaFluor 488 and washed with PBS once. Staining is mostly specific for edited cells, and mApple is strongly co-expressed with SBP-LNGFR. (**C**) Pure populations of cells with integrated Mag-hNIL or of unedited cells were incubated with streptavidin conjugated to AlexaFluor 488 and washed with PBS once before fluorescent imaging to confirm co-expression and specificity of streptavidin binding. (**D**) A mixed population of cells was dissociated by EDTA, and the total, negative, and positive fractions were imaged and quantified. The total fraction contains 21% mApple-expressing cells, the negative fraction contains 17%, and the positive fraction contains 97%. The positive fraction was then plated, and multiple pure clones were observed following outgrowth. (**E**) Pure populations of true positive (TP) or true negative (TN) cells were mixed at various ratios, and the enrichment protocol was performed as suggested. Total, negative, and positive fractions were collected from each sample and quantified for %TP by flow cytometry for mApple expression. Data shown is the difference between the total fraction and positive or negative fraction %TP, and maximum represents a pure positive fraction. Enrichment is highly predictable as a function of the initial %TP, with optimal gains in initial populations of 10% to 50%TP, often resulting in positive fractions >90%TP. It is also especially effective for rare populations in proportion to their initial %TP, resulting in up to 10-fold increases. (**F**) The experiment from E was performed using the depletion protocol as suggested, and minimum represents a pure negative fraction. A similar predictable relationship was observed between initial %TP and reduction in the %TP of the negative fraction was observed, although low levels of cell recovery in high initial %TP samples resulted in increased variability.

Cell strainer, 40-µm pore diameter (Corning, cat. no. 352340)
Rotator Genie benchtop spinner (Scientific Industries, SI-2100)

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

Cell preparation

1. Culture cells after transfection and expand to at least one full 6-well plate or one 10 cm dish by EDTA split (see Basic Protocol 1).

   Post-transfection recovery and expansion should take about 1 week; this enables the degradation/dilution of transient plasmids and permits expansion of edited cells to ensure enough cells are present for effective selection. Genomic DNA may be collected from unpurified cells to test for integration efficiency, and extra heterogeneous cells should be frozen throughout the purification process to provide a low-passage backup should contamination occur or should validation fail at any point.

For fluorescent proteins (FACS)

2a. When cells reach 70% to 80% confluency (day 3 to 4 after plating), dissociate with Accutase (see Basic Protocol 1).

3a. Transfer cells and Accutase solution to conical tube, rinse with DMEM/F12, and spin 5 min at 200 × *g*, room temperature.

4a. Aspirate supernatant and resuspend in E8 supplemented with 10 µM Y-27632 ROCK inhibitor.

   Using medium at this step helps to improve cell viability; however, if FACS is not performed rapidly, cells can clump and reduce purification efficacy. Cold PBS with 3% BSA can be substituted to reduce clumping.

5a. Transfer cell solution to FACS tube and place on ice.

6a. Perform FACS using appropriate fluorophore gating and nozzle diameter (see Critical Parameters and Troubleshooting for more detail).

   Sort directly onto a Matrigel-coated cell culture plate loaded with E8 supplemented with 10 µM Y-27632 ROCK inhibitor (even if PBS is used in step 4 instead of E8) since typically small volumes are deposited. Alternatively, positive cells can be deposited into a separate tube, centrifuged, and replated. Some iPSC lines tolerate single cell/well sorting into 96- or 384-well dishes containing E8 supplemented with 10 µM Y-27632 ROCK inhibitor, which can further expedite clonal isolation and expansion. Survival of a polyclonal population can then be maximized by depositing any additional positive cells into the remaining well.

7a. Expand selected cells for clonal isolation.

   Colonies of positive cells may be marked and picked to keep, and/or negative cells may be marked and removed.

Magnetic streptavidin bead affinity purification (see Fig. 2)

Prepare bead incubation buffer

1b. Prepare bead incubation buffer by supplementing PBS with 2 mM EDTA and 0.5% BSA.

   For example, 47.4 ml PBS, 100 µl of 1M EDTA stock, and 2.5 ml of 10% BSA stock. Buffer may be formulated by the consumer or purchased (e.g., autoMACS buffer).

2b. Vacuum filter.

Components may already be sterile, but applying vacuum helps to degas the solution and reduce bubbling during subsequent use.

3b. Store at 4°C.

Bead preparation

4b. Resuspend the bead stock by vortexing or inversion.

Several streptavidin-coated magnetic beads are commercially available, but in our experience Dynabeads MyOne Streptavidin C1 has provided the best cell binding and enrichment efficiency.

5b. Pipet the total amount of bead solution to be used into a sterile microcentrifuge tube.

10 µl beads per sample is recommended for positive selection, and 100 µl beads per sample is recommended for negative selection. In general, efficacy is maximized by using fewer beads for positive selection and more beads for negative selection; however, this should be balanced with reduced recovery and survival for small volumes and increased reagent cost for large volumes.

6b. Add 1 ml bead incubation buffer to beads and invert several times to mix.

7b. Place the tube on a magnetic rack, invert two to three times, and let rest for at least 1 min.

8b. Aspirate and discard supernatant.

9b. Remove tube from magnetic rack.

10b. Repeat steps 6b to 9b.

For bead volumes above 100 µl, repeat at least twice.

11b. Resuspend beads in buffer equal to the initial volume of beads.

Cell preparation and binding

12b. Prepare cells as for an EDTA split (see Basic Protocol 1, steps 14 and 15) and incubate at 37°C for 10 to 15 min, until colonies begin to detach without physical dissociation.

Do not use Accutase or other enzymatic dissociation reagents, as extracellular SBP-LNGFR is easily digested.

13b. Prepare beads during incubation (see above).

14b. Tilt the plate and pipet the EDTA solution two to three times down the well to dissociate and collect cells. Transfer to 15-ml conical tube.

Cells may be optionally filtered through a cell strainer to remove remaining clumps, but proper incubation and trituration should result in full homogenization.

15b. Wash with PBS to collect remaining cells, and add to the conical tube.

16b. Count cells (Phelan & May, 2015) and calculate total number of cells.

Initially, 5×10^6 cells per sample is recommended for both positive and negative selection. Efficacy may be maximized by using higher cell numbers for positive selection and by using fewer cells for negative selection; however, at least 1×10^6 cells should be used to improve recovery.

17b. Centrifuge 5 min at 200 × *g*, room temperature, and discard supernatant.

18b. Resuspend with 1 ml buffer per sample and transfer each sample to a separate sterile 1.5-ml microcentrifuge tube.

19b. Add washed beads (see above for volume considerations and preparation instructions).

20b. Incubate at room temperature for 30 min on a benchtop spinner at approximately 10 rpm.

Efficacy may be maximized by reducing incubation time to 10 min for positive selection and by increasing time to 45 min for negative selection. Shorter times may not permit adequate binding, and longer times may lead to reduced survival due to osmotic stress.

21b. Place on magnet, invert two to three times, and let sit for at least 1 min.

Beads should be completely removed from suspension and stuck against the side of the microcentrifuge tube. Buffer should be clear or white but not golden or brown.

Positive selection

22b. Aspirate negative cell fraction.

Suction may be used to increase throughput, but use narrow pipet tips to avoid interfering with the beads.

23b. Add 1 ml buffer, remove from magnetic rack, and invert several times to fully resuspend beads.

24b. Repeat steps 22b and 23b at least three times.

The optimal number of washes depends on the initial number of cells used, with three washes ideal for 5×10^6 cells. In general, repeat washes until the removed negative fraction is clear. Performing four or more washes may improve enrichment if 1×10^7 or more cells were used, but it also reduces recovery.

25b. Remove tube from magnetic rack, and either perform an optional additional incubation to release cells from beads (steps 26b to 29b) or resuspend in E8 and plate directly (skip to step 30b).

Directly plating cells with beads has not been shown to interfere with cell viability, and cells can be grown and passaged normally. However, removal of beads may be desired for downstream analysis applications or to hasten cell growth.

26b. Resuspend remaining beads and cells with E8 supplemented with 10 μM Y-27632 ROCK inhibitor and 2 mM biotin.

Biotin is able to outcompete SBP for streptavidin binding sites on the beads, resulting in cell release.

27b. Incubate at room temperature for 30 min on a benchtop spinner at approximately 10 rpm.

28b. Place on magnet for 1 min, invert two to three times, and let sit for 1 additional min.

29b. Remove supernatant with P1000.

30b. Pipet cells and medium to a recipient culture well.

Biotin is normally present in culture medium at low levels, so supplementation should not affect the cells. If the high levels are of concern, the cells may be centrifuged and plated in fresh E8 supplemented with 10 μM Y-27632 ROCK inhibitor.

31b. Return recipient plate to 37°C incubator and evenly distribute cells by gently shaking plate front-to-back and side-to-side.

32b. The following day, wash twice with PBS, then add fresh E8 supplemented with 10 μM Y-27632 ROCK inhibitor. Repeat this step daily until colonies are well established, then return to normal E8 culture conditions.

Negative selection

33b. Pipet supernatant (negative fraction) into a 15-ml conical tube.

Pipet from the top of the tube and leave 50 to 100 μl at the bottom of the tube to reduce carryover of beads. Normally, fewer than 0.1% of beads are carried over into the first negative fraction. No further washes are necessary, and the positive fraction may be discarded.

34b. Add 1 ml E8 to the tube to improve cell pelleting.

35b. Centrifuge tube 5 min at 300 × *g*, room temperature.

36b. Aspirate buffer and resuspend in E8 supplemented with 10 μM Y-27632 ROCK inhibitor.

37b. Pipet cells and medium to a recipient culture well.

38b. Return recipient plate to 37°C incubator and evenly distribute cells by gently shaking plate front-to-back and side-to-side.

BASIC PROTOCOL 4

ISOLATION AND VALIDATION OF CLONAL TRANSGENIC LINES

While transgenic populations may be enriched to near-purity using the techniques described above, the derivation of a clonal iPSC line descended from a single parent cell is necessary for proper genotyping and for many downstream applications, and it is thus standard practice following any genetic edit (Fig. 3). One common method for isolating such clones, as described below, is by serial dilutions on two 6-well plates, followed by manual picking. As with all single-cell dissociations of iPSCs, the use of Y-27632 ROCK inhibitor is required until colonies are properly established; wells with fewer cells may require treatment for 2 to 3 days. 6-well plates are used because their wells have a larger surface area than other multiwell dishes, facilitating downstream picking. For highly enriched populations, serial dilutions may be reduced to one 6-well plate loaded with 1×10^5 cells in the first well, as there is a higher probability of identifying a purely positive colony.

Following isolation, this protocol further describes basic genotyping in parallel with the gradual expansion of clonal lines, utilizing QuickExtract to prepare genomic DNA and PCR to test for the presence of a transgene of interest. Primer sequences and other specifics are included in more detail for the particular differentiation cassettes in Support Protocol 1. In general, basic genotyping by PCR should be performed as quickly as possible to screen out negative clones, while potentially positive clones should be confirmed by more stringent methods such as Sanger sequencing and western blotting as appropriate. To save culture reagents, negative clones may be immediately discarded, while all others should be expanded and frozen pending confirmation.

Materials

Genomic DNA extraction kit (e.g., QuickExtract, Epicentre, cat. no. QE09050 or DNeasy kit, Qiagen, cat. no. 69504)
Matrigel-coated 6- and 12-well plates (Basic Protocol 1, steps 1 to 4)
Phase-contrast and fluorescent microscope
Cell Dotter marking objective (SEO Enterprises, cat. no. MBW10020)

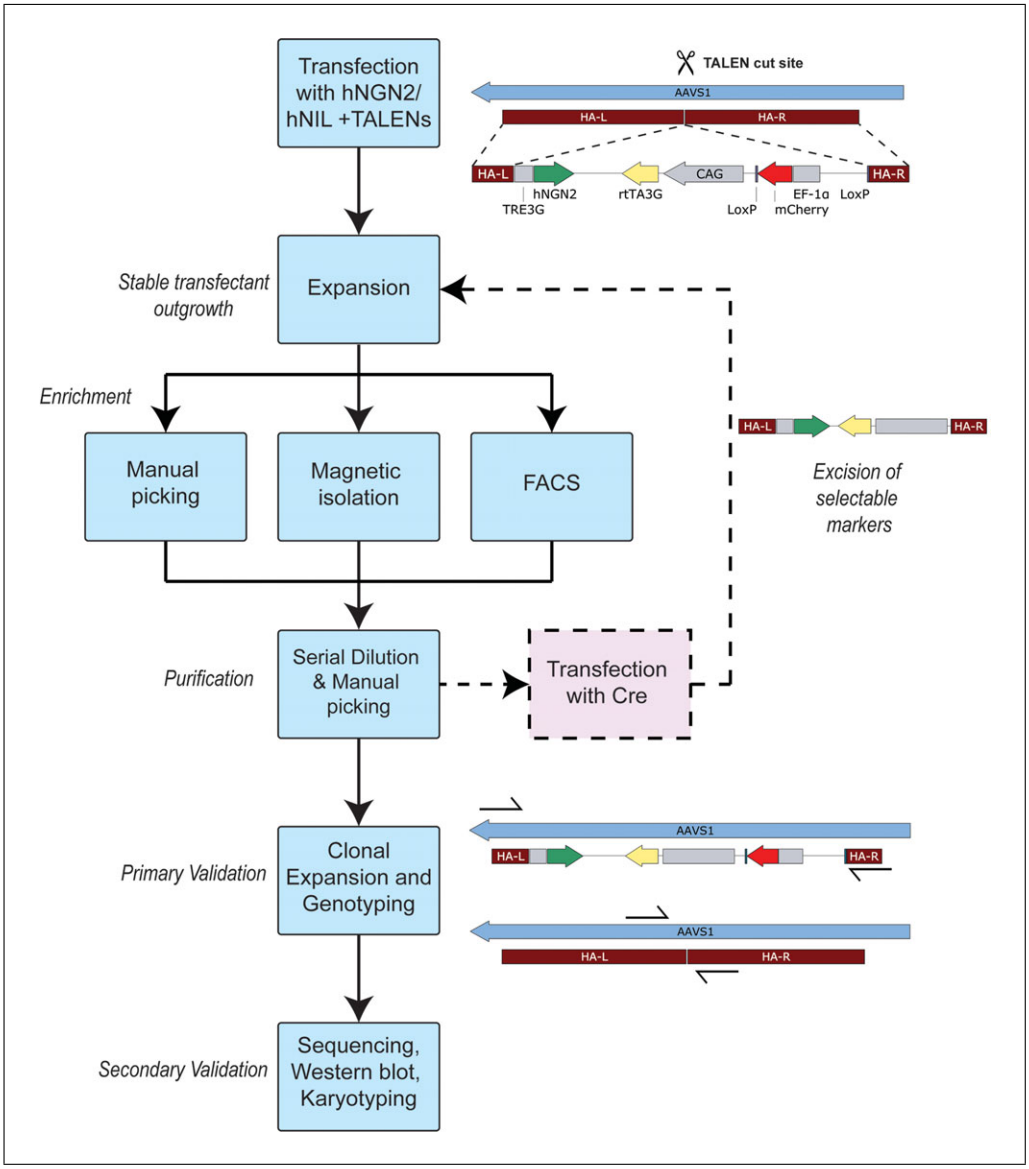

Figure 3 Overview of the general workflow for transgenic hiPSC line development, from transfection to cell line purification and validation methods. Generation of a new line requires about 1 month, though high transfection efficiency and FACS or magnetic enrichment methods can accelerate this timeline significantly.

Additional reagents and equipment for cell culture techniques (Basic Protocol 1) and counting cells (Phelan & May, 2015), PCR (Kramer & Coen, 2001), and agarose gel electrophoresis (Voytas, 2001)

Serial dilution

1. Prepare one well of 80% confluent post-transfected cells from a 6-well plate as for an Accutase split (see Basic Protocol 1, step 27).

   Clonal isolation may be performed immediately after transfection; however, permitting outgrowth for one or two passages can improve subsequent growth rate and viability, and performing frontline enrichment (Basic Protocol 3) can dramatically improve the percentage of positive clones.

2. Aspirate the Matrigel solution from two 6-well plates and add 2 ml of E8 supplemented with 10 µM Y-27632 ROCK inhibitor to all but one well; leave the final well empty.

3. After centrifugation of the Accutase-dissociated cells, aspirate the supernatant and resuspend in 2 ml of E8 supplemented with 10 μM Y-27632 ROCK inhibitor.
4. With a 5-ml pipet, add the full 2 ml of cells and medium to the first well (A1) and pipet up and down twice to mix.
5. With the same pipet, transfer 2 ml of this mixture into the second well and mix by pipetting up and down twice.
6. Continue pipetting and mixing for each well, performing a one-half dilution, and dispense the final 2 ml into the empty well.
7. Return the plates to the incubator and gently shake the plates front-to-back and side-to-side.
8. Track colony formation daily with a phase-contrast microscope.

   Typically, the first plate will rapidly become confluent and may be EDTA split and combined for other uses, such as freezing a heterogeneous population. Meanwhile, the second plate should be kept for picking (see below).

9. Once colonies are properly established, aspirate medium and replace with fresh culture medium.

   Medium should only be changed after a majority of cell colonies contain at least 8 cells. As the rate of cell division is typically faster in wells with a higher cell density, any wells seeded with at least 50,000 cells should have their medium changed after 1 day, while wells seeded with fewer than 5,000 cells will likely require 3 days.

Marking and picking clones

10. Using the plates seeded above, identify the wells at which colonies are well spaced and of a standard size and shape.

    Look for compact, round colonies to indicate single-cell origin, adequate separation from other colonies to permit further outgrowth, and fluorescence (if applicable).

11. Mark colonies on day 3 to 4 with a Cell Dotter.

    For fluorescent markers, allow colonies to grow to at least 500 μm in diameter in order to be easier to discern. Ten to twenty clones should be sufficient; however, marking and picking more clones can help ensure proper genotyping and avoid the need to re-pick. For label-free editing, frontline enrichment is highly recommended (Basic Protocol 3), and it is often necessary to screen 50 to 100 clones. The Cell Dotter attaches directly to the objective turret, enabling rapid marking of the underside of the plate with 1.8-mm rings. Center the colony of interest in the field of view using a 10× objective, then switch to the Cell Dotter and gently extend the marker to make contact with the plate.

12. Pick-to-remove other cells in close proximity to marked colonies (See Basic Protocol 1).

    Any cells within or immediately surrounding the ring formed by the marking objective should be removed.

13. Check cells daily for growth and pick-to-keep around days 5 to 7.

    Pick when colonies are about as large as the interior of the marking ring.

14. Before picking, rinse wells once with PBS and add at least 5 ml medium to each well to allow picking of multiple clones in succession.
15. Aspirate the Matrigel solution from a 24-well plate and load each well with 250 μl of E8 supplemented with 10 μM Y-27632 ROCK inhibitor.

16. Pick-to-keep each colony into an individual well of the 24-well plate (see Basic Protocol 1).

17. Grow cells in 24-well plates for 5 to 7 days until colonies are large (at least 2 mm in diameter).

    Be wary of spontaneous differentiation, especially at the center of colonies which tend to grow upwards and turn brown. It is possible to pick away these areas for better splitting, or continue to expand and perform later EDTA-mediated removal (see Basic Protocol 1), but clones with excessive spontaneous differentiation may harbor genetic abnormalities and should simply be discarded.

Expansion and genotyping

18. Prepare clones in the 24-well plate as for an EDTA split (using 250 µl of 0.5 mM EDTA in PBS).

19. During incubation, for each clone, prepare a microcentrifuge tube labeled with the clone number for genomic DNA collection and load a new well for growth loaded with one half volume E8 supplemented with 10 µM Y-27632 ROCK inhibitor.

    Ideally, use one well of a 12-well plate to maximize surface area and promote outgrowth.

    If cells are precious, e.g., if only a few clones were successfully derived, use two wells of a 24-well plate to immediately have a backup well. This well may also be used for later genomic DNA collection if desired.

    If colonies are small, use one well of a 24-well plate to improve survival. Genomic DNA collection may also be postponed until this well is split.

20. Aspirate EDTA and gently dissociate with a P1000 pipet tip by pipetting 1 ml of medium two to three times.

21. Deposit half of the medium in the microcentrifuge tube.

22. Deposit the other half of the medium in the new well.

23. Return recipient plates to the incubator and gently shake the plates front-to-back and side-to-side.

24. Spin down the microcentrifuge tube 5 min at 500 × *g*, room temperature.

    Arrange all the tubes in the same orientation so the cell pellet forms in the same location for each tube.

25. Aspirate as much of the medium as possible.

    The cell pellet may not be readily visible, so use a filterless pipet tip on the end of an aspirating pipet if suction is used, or use a P1000 for better control.

26. Extract genomic DNA from the cell pellet as per manufacturer's instructions.

    QuickExtract (Epicentre) is recommended due to its scalability and ease-of-use. 30 µl is usually sufficient for small cell pellets (<50,000 cells), and any samples which are overly viscous after thermocycling may be diluted with DNase-free water.

27. Test the extracted genomic DNA by PCR (Kramer & Coen, 2001).

    For inserts at a safe-harbor locus, testing for integration at the correct locus can be accomplished by using one primer beyond the homology arm and one primer unique to the insert cassette. Insertion zygosity can also be determined by using primers on either side of the insertion locus, as insertion will prevent amplification of that allele. Use 1 µl of the QuickExtract solution in a 10-µl PCR preparation; additional cleanup is typically not necessary for amplification (See Supporting Protocol 1).

28. Run each PCR sample on an agarose gel (Voytas, 2001) in order to confirm amplification.

    Clones that fail to amplify the test fragment but properly amplify a positive control fragment should be immediately discarded to save culture reagents.

    The genomic DNA of clones with inconclusive genotyping results (i.e., variable band intensities, multiple bands, etc.) should be checked for quality and either re-amplified with different PCR conditions or re-extracted.

    Clones with positive initial genotyping should be PCR purified and Sanger sequenced to confirm integration. In particular, further selection may be desired for scarless integration and/or retaining an unedited wild-type allele.

29. Expand clones with positive PCR results via EDTA splitting 1:4 into larger wells at 50% to 60% confluency. Polyclonal populations may be discarded or combined and subcloned if desired.

    Split the entire population at low ratio to enhance growth rate and outcompete differentiation. For example, split one well of a 24-well plate to two wells of a 12-well plate, one of which can be split into two wells of a 6-well plate.

30. For each clone in two wells of a 6-well plate at 80% confluency, prepare both wells as for an EDTA split (see Basic Protocol 1).

31. During incubation, prepare a recipient plate with E8 medium supplemented with 10 μM Y-27632 ROCK inhibitor, prepare a 15-ml conical tube with 1 ml medium containing the components for 3 ml cryopreservation medium, and label three cryovials with the clone number.

    For example, combine 300 μl DMSO and 700 μl E8 medium.

32. Detach the cells from one well with 3 ml medium and transfer 0.5 ml to two wells to accomplish a 1:6 split for maintenance. Use the remaining 2 ml medium and cells to detach the second well and add to the 15-ml conical tube to constitute 1× cryopreservation medium. Add 1 ml to each of the three cryovials and freeze.

    If cells are not fully dissociated from the plate, rinse the wells again with the full cryopreservation medium to maximize cell density.

    Maintenance cultures should be further split 1:12; if sequencing results are not readily available, reduce reagent use by freezing all of the cells and not retaining a maintenance culture. All clones should be kept frozen until a few sequenced clones are confirmed, and any clones which sequence incorrectly may be discarded.

33. Clones confirmed by sequencing should be further expanded to a full 6-well plate and frozen into six to eight vials, in addition to keeping the clone in culture for other characterization assays.

    While only one clone is necessary to move forward, isolating at least three sequence-confirmed clones is ideal in case off-target effects or other genetic abnormalities are found later.

    Common options for further validation include: (1) western blot to confirm transgene expression; (2) karyotype to ensure no chromosomal abnormalities have arisen; (3) sequencing of potential CRISPR off-target loci; and (4) exome or whole-genome sequencing of pre- and post-edited cells to search for genetic drift or CRISPR off-target effects.

DIFFERENTIATION OF i^{3}NEURONS

BASIC PROTOCOL 5

This protocol describes the rapid and robust differentiation of cortical neurons from hiPSCs via induced expression of the neurogenin-2 (NGN2) transcription factor (Wang

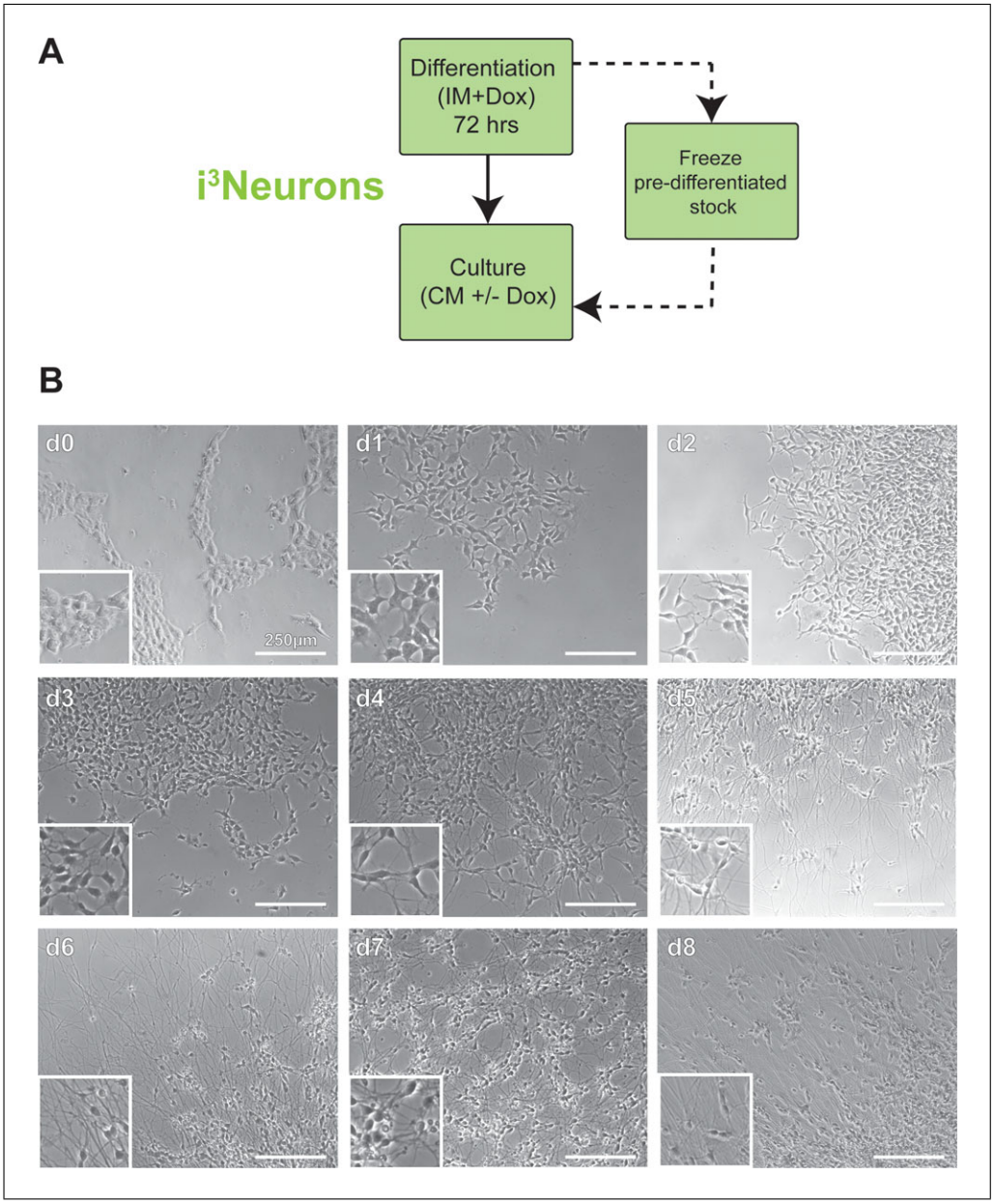

Figure 4 Outline of i^{3}Neuron differentiation. (**A**) General workflow for i^{3}Neuron differentiation and culturing. Freezing large batches of pre-differentiated (d3) i^{3}Neurons is the preferred method, enabling reproducibility of multiple downstream experiments from a single round of differentiation. (**B**) Bright-field images of i^{3}Neurons throughout the differentiation process. Cells change morphology within 24 hr, and neurites can be identified within 48 hr. Note the drastic difference in neuritic elongation between d3 and d4. i^{3}Neurons at d3 can be safely dissociated and frozen; florid neuritic elongation by d4 hinders singularization and makes these neurons susceptible to damage from the splitting process.

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) (Fig. 4A). 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

Table 2 Induction Medium[a]

Component	Product no.	Amount
DMEM/F12 with HEPES	Gibco, 11330032	485 ml
N2 supplement, 100×	Gibco, 17502048	5 ml (per 500 ml)
Non-essential amino acids (NEAA), 100×	Gibco, 11140050	5 ml (per 500 ml)
L-glutamine, 100× (or Gluta-MAX)	Gibco, 25030081	5 ml (per 500 ml)
Aliquot medium and add fresh from stock:		
ROCK inhibitor Y-27632 (10 mM in PBS, 1000×)	Tocris, 1254	50 µl (per 50 ml)
Doxycycline (2 mg/ml in PBS, 1000×)	Sigma, D9891	50 µl (per 50 ml)
Additional components for i^{3}LMNs:		
Compound E (2 mM in 1:1 ethanol and DMSO, 0.98 mg/ml; 10,000×, store at −20°C)	Calbiochem, 565790	5 µl (per 50 ml)
BrdU (only for d3 replating; 40 mM in water, 12.284 mg/ml; 1000×, store at −20°C)	Sigma, B9285	50 µl (per 50 ml)

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

this time they may have only minor neuritic elongations (Fig. 4B, d3). 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.

Materials

iPSCs with stably integrated doxycycline-inducible NGN2 transgene (Basic Protocol 3)
Induction Medium (IM, see Table 2)
15-cm culture dishes (Corning, cat. no. 430599)

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

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 to 9 ml of Matrigel solution, tilting to ensure coverage of entire surface area. Place in 37°C incubator for 30 min to 1 hr 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.

2. Prepare 20 ml IM with 2 μg/ml doxycycline and 10 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.

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 $\times$ 10^7 iPSCs per 15-cm dish to be differentiated into a 15- or 50-ml conical tube, and centrifuge 5 min at 200 $\times$ g, room temperature. Aspirate the supernatant and resuspend cells in 10 to 12 ml of IM with 2 μg/ml doxycycline and 10 μM Y-27632 ROCK inhibitor.

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

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 μM Y-27632 ROCK inhibitor and add to the dish for a total volume of 18 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 $\times$ 10^7 cells per 10-cm dish or 1.5 $\times$ 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.

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 (Fig. 4B, d1).

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 20 min, 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.

Day 2

14. Check cells under a microscope. Neuritic extensions should be more evident (Fig. 4B, d2).

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 (Fig. 4B, d3).

    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.

BASIC PROTOCOL 6

CULTURING i^{3}NEURONS

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 long-term 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 (Fig. 5A and B). 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.

Materials

Freshly-prepared 1× poly-L-ornithine (PLO) *or* polyethyleneimine (PEI) solution (see Table 3)
Laminin (Gibco, cat. no. 23017015)
Freshly split or thawed 3-day differentiated i^{3}Neurons (following completion of Basic Protocol 5)

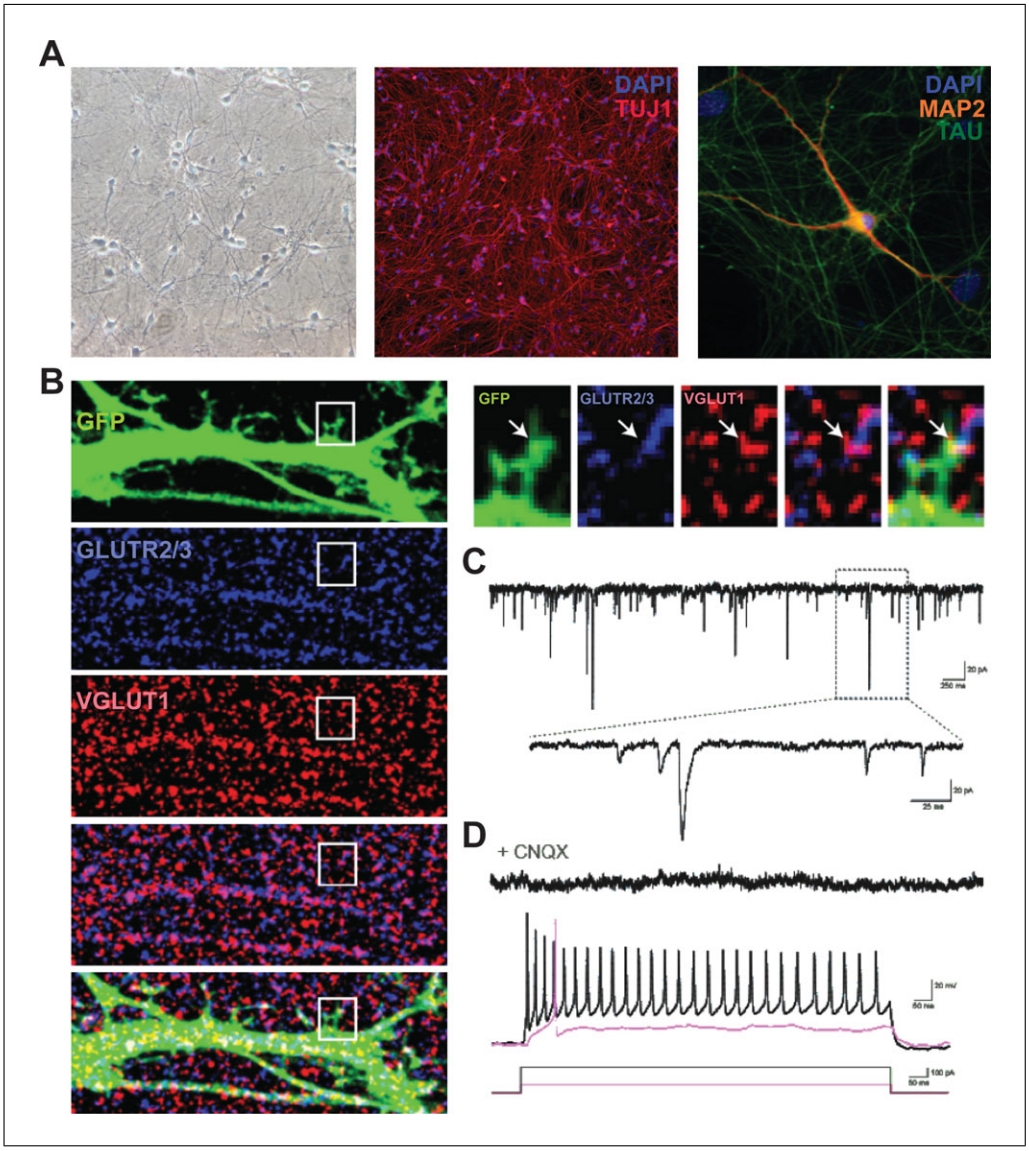

Figure 5 i^{3}Neuron validation. (**A**) Phase-contrast and immunofluorescence images of d14 differentiated i^{3}Neurons. Phase-contrast image demonstrates neuronal morphology, and IF images demonstrate staining for characteristic cortical neuron markers (Tuj1, Map2, Tau). (**B**) Dendritic spines in mature i^{3}Neurons express pre- and postsynaptic markers of excitatory neurons. (**C**) Mature i^{3}Neurons co-cultured with astrocytes exhibit spontaneous excitatory currents that can be inhibited by CNQX, a glutamate receptor antagonist. (**D**) i^{3}Neurons can fire spontaneous trains of action potentials.

Cortical Neuron Culture Medium (CM; see Table 4)

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

Coating dishes

1. Prepare stock solutions of PLO or PEI (see Table 3).
2. 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 i^3 Neurons. Gently tilt plate to ensure full coverage.
3. Incubate dishes for at least 1 hr at room temperature.

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

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

Component	Product no.	Amount per 50 ml
Poly-L-ornithine (PLO), 10× stock		
PLO	Sigma, P3655	50 mg (per 50 ml)
Borate buffer (see below)		to 50 ml
Poly-D-lysine (PDL), 1×		
PDL	Sigma, P7405	5 mg (per 50 ml)
Borate buffer (see below)		to 50 ml
Polyethyleneimine (PEI), 10× stock		
PEI	Sigma, 03880	1 ml
Borate buffer (see below)		49 ml (per 50 ml)
Borate buffer[b]		
Boric acid (100 mM)	Sigma, B6768	3.09 g (per 500 ml)
Sodium tetraborate (25 mM)	Sigma, 221732	4.77 g (per 500 ml)
Sodium chloride (75 mM)	Sigma, S7653	2.19 g (per 500 ml)
Milli-Q water		to 500 ml
Sodium hydroxide (1 M)	Sigma, 71463	As needed to adjust pH to 8.4

[a]Polymers should be resuspended to recommended concentration, filter sterilized, and diluted to 1× for use.
[b]Borate buffer may be prepared as described here and filter sterilized, or may be purchased commercially.

Table 4 Cortical Neuron Culture Medium (CM)

Component	Product no.	Amount per 50 ml	Final concentration
BrainPhys neuronal medium	STEMCELL Technologies, 05790	49 ml	
B27 supplement, 50×	Gibco, 17504044	1 ml	1×
BDNF (10 μg/ml) in PBS containing 0.1% IgG and protease-free BSA (store at −80°C)	PeproTech, 450-02	50 μl	10 ng/ml
NT-3 (10 μg/ml) in PBS containing 0.1% IgG and protease-free BSA (store at −80°C)	PeproTech, 450-03	50 μl	10 ng/ml
Laminin (store at −80°C; stock concentration 1 mg/ml; thaw on ice and dispense with chilled pipets)	Gibco, 23017015	50 μl	1 μg/ml

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

4. Aspirate coating solution, then wash dishes with sterile water. Repeat twice for three total washes.

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

5. Aspirate water and let dishes dry completely in a biosafety cabinet (typically requires 30 min to 1 hr).

   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.

6. 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 long-term 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 2 hr at 37°C before aspirating and plating neurons directly. This is optional for PLO and highly recommended for PEI.

Plating cells

7. Prepare CM in a sterile biosafety cabinet and place in 37°C water/bead bath for approximately 20 min or until warm to the touch.

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 i^3Neuron 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).

8. 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 × 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 to 3 ml one day after plating).
   d. 10-cm dish (biochemistry): 1–1.2 × 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 × 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

9. 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.

10. 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.

11. *Optional:* Supplementation with astrocytes or astrocyte-conditioned medium have been shown to improve the overall health and electrophysiological activity of i^3Neurons in long-term cultures (Fig. 5C and D). 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.

BASIC PROTOCOL 7

DIFFERENTIATION OF i^3LMNs

This protocol describes the rapid and robust differentiation of hiPSCs into lower motor neurons (i^3LMNs) via induced expression of the transcription factors NGN2, ISL1, and LHX3 (hNIL) (Mazzoni et al., 2013; Shi et al., 2017). In particular, a donor construct containing these factors under the tetracycline response element (TRE3G) (Gossen & Bujard, 1992), a CAG promoter driving constitutive expression of the reverse tetracycline transactivator (rtTA3G), and an EF-1α promoter driving constitutive expression of selection genes (mCherry for Addgene, cat. no. 105841 and SBP-LNGFR and mApple for Addgene, cat. no. 105842) was stably integrated into the safe-harbor *CLYBL* locus via TALENs (Cerbini et al., 2015). Motor neuron differentiation efficiency can vary between iPSC lines, and homozygous insertion into both CLYBL alleles may result in improved efficiency. Reduced differentiation efficiency of mCherry positive clones can occur with off-target integration of the donor construct. Additionally, the mCherry reporter is flanked by loxP sites, permitting excision by transient transfection of Cre recombinase if desired.

Materials

iPSCs with stably integrated doxycycline-inducible hNIL transgenes (Basic Protocols 2 to 4)
Induction Medium (see Table 2)
Compound E (Gamma-Secretase Inhibitor XXI, Calbiochem, cat. no. 565790); 500 μg reconstituted in 255 μl ethanol and 255 μl DMSO (2 mM stock) (store light-protected at −20°C up to 6 months)

Inverted microscope
Cell strainer, 40-μm pore diameter (Corning, cat. no. 352340)

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

NOTE: 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. This protocol assumes differentiation of one 10-cm dish, which should be seeded with 1.5×10^6 cells. Each dish in turn provides 6 to 8×10^6 cells at Day 3. Medium volumes and cell numbers may be scaled by surface area (i.e., seed 2×10^4 iPSCs/cm^2, or 1.8×10^5 iPSCs/well of a 6-well plate).

Day 0

1. Prepare a sufficient amount of hNIL-iPSC for differentiation.

   Approximately two wells of a 6-well dish at 80% confluency are sufficient to begin the differentiation of a 10-cm dish, and cell numbers may be scaled by well surface area. Typically, hNIL lines proliferate for longer than hNGN2 lines, and this protocol calls for plating in E8 before switching to IM on d1, so hNIL cells are seeded at lower density on d0.

2. Prepare cells as for EDTA split (see Basic Protocol 1), and incubate at room temperature for 10 to 15 min (until colonies begin to detach).

3. Without aspirating the EDTA, tilt the plate and pipet 3 ml DMEM/F12 into each well with a 10-ml pipet and remove the cells from the bottom of the dish. Gently triturate the cells by pipetting the cells up and down two to three times in each well.

4. Count the cells (Phelan & May, 2015) and transfer 1.5×10^6 iPSCs per 10 cm dish to be differentiated to a 15-ml conical tube. Centrifuge 5 min at $300 \times g$, room temperature. Aspirate the supernatant and plate the resuspended cells in 12 ml E8 medium supplemented with 10 μM ROCK inhibitor on a 10-cm Matrigel-coated dish.

Day 1

5. Gently rock and swirl the plate by hand to resuspend and collect debris, then aspirate off the medium and wash once with PBS.

6. Add 12 ml of IM freshly supplemented with 10 μM Y-27632 ROCK inhibitor, 2 μg/ml doxycycline, and 1:10,000 Compound E from stock.

   Doxycycline will induce the expression of the hNIL construct and promote the differentiation into motor neurons. Morphological changes should be evident after 24 hr of doxycycline treatment. The induction efficiency can be tested by comparing parallel wells treated with and without doxycycline, or by performing a separate dox-GFP experiment as described in Support Protocol 6.

Day 2

7. Observe differentiating cells under an inverted microscope. Cells should be beginning to spread out and form processes.

8. If long-term culture is desired immediately, coat plates overnight with PLO, PDL, or PEI (see Basic Protocol 8).

DAY 3

9. Treat the differentiating cells with Accutase (see Basic Protocol 1). Use 3 ml Accutase to digest each 10-cm dish.

Table 5 Motor Neuron Culture Medium (MM)

Component	Product no.	Amount per 50 ml	Final concentration
Neurobasal medium	Gibco, 21103049	47.5 ml	
B27 supplement, 50×	Gibco, 17504044	1 ml	1×
N2 supplement, 100×	Gibco, 17502048	500 µl	1×
Non-essential amino acids (NEAA), 100×	Gibco, 11140050	500 µl	1×
L-glutamine, 100× (or Gluta-MAX)	Gibco, 25030081	500 µl	1×
(Optional) CultureOne supplement, 100×	Gibco, A3320201	500 µl	1×
Laminin (store at −80°C; stock concentration 1 mg/ml)	Gibco, 23017015	50 µl	1 µg/ml

[a]Prepare in sterile biosafety cabinet; medium should then be aliquotted to add additional supplements fresh; warm to 37°C before use.
[b]While not required, addition of BDNF and NT-3 as described for CM (Table 4) improves long-term cell health.
[c]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).

10. Tilt the plate and add 6 ml PBS to the Accutase and cell solution using a 10-ml pipet. Gently triturate four to five times to break the cells apart.

    For best results, pass the cells and solution through a 40-µm cell strainer to ensure full dissociation. Add 1 ml IM after straining to improve pelleting.

11. Centrifuge the cells 5 min at 300 × *g*, room temperature.

12. Aspirate the supernatant and resuspend in 5 ml IM.

13. Count the cells (Phelan & May, 2015).

14. Cells may be re-plated immediately (see Basic Protocol 8) or frozen for future use (see Basic Protocol 1).

    Cells to be frozen should be diluted to a standard cell density (i.e., 1 × 10^6 cells/ml) in IM containing a final concentration of 10% DMSO.

BASIC PROTOCOL 8

CULTURING i^{3}LMNS

Induction and differentiation of i^{3}LMNs is nearly identical to the first 3 days of differentiation for i^{3}Neurons (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 (Fig. 6A).

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 (Fig. 6B and C), 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 (Fig. 6D). 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

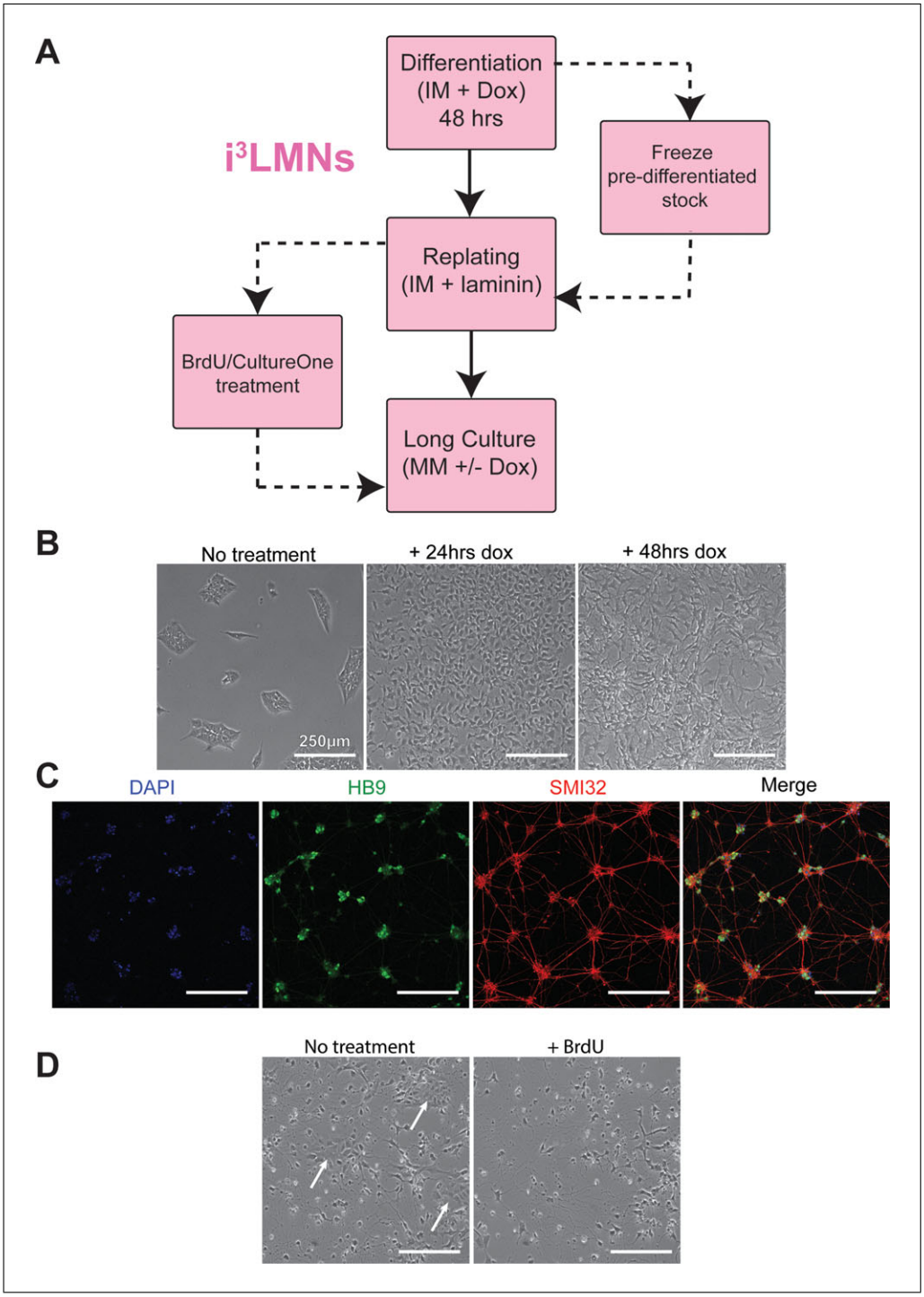

Figure 6 i^{3}LMN validation. (**A**) General workflow for i^{3}LMN differentiation and culturing. Note key differences versus i^{3}Neurons, including shorter doxycycline differentiation period, different media for initial replating and long-term culture, and the option for BrdU treatment to eliminate mitotically active cells. (**B**) Time course for i^{3}LMN differentiation. Note rapid dispersal and morphological change after 24 hr, as well as formation of nascent neuritic extensions by 48 hr. (**C**) i^{3}LMNs stain for characteristic lower motor neuron markers, including Hb9 and Smi32. (**D**) Effect of BrdU on i^{3}LMN culture purity. Small colonies of undifferentiated iPSCs or other mitotically active differentiated species can quickly take over a culture of i^{3}LMNs. Treatment with BrdU rapidly extinguishes these cell populations with little toxicity to i^{3}LMNs.

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. Laminin-coated 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.

Materials

PLO, PEI, or PDL solution (see Table 3)
Laminin (Gibco, cat. no. 3017015)
Freshly split or thawed 3-day differentiated i^{3}LMNs (following completion of Basic Protocol 5)
Motor Neuron Culture Medium (MM; see Table 5)

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

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. Gently tilt the plate to ensure full coverage.
3. Incubate dishes for at least 1 hr at room temperature.

   For best results, incubate dishes overnight in a 37°C incubator
4. Aspirate coating solution, then wash dishes with sterile water. Repeat twice for three total washes.

   An additional two washes is recommended for PEI coating.
5. Aspirate water and let dishes dry completely in a biosafety cabinet (typically requires 30 min to 1 hr).

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

   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 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 1 hr, then plate neurons directly by adding cells in an addition one half culture volume of IM.

Plating cells

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

   Additionally supplementing with 40 μM BrdU for 24 hr helps to prevent outgrowth of mitotically active cells without affecting neuronal health. Alternatively, CultureOne supplement may be added to the medium from d4 onwards.

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

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

   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.

10. 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.

    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.

11. After day 7, perform half-medium changes every 4 to 7 days with complete MM+laminin for long-term maintenance.

    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).

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

SUPPORT PROTOCOL 1

GENOTYPING OF iPSCS WITH GENE INSERTIONS

This protocol will use genomic DNA isolated from the purified iPSC clones with CLYBL or AAVS1 gene insertion to determine if integration of the transgene has occurred correctly, in a heterozygous or homozygous fashion, and if the floxed selection genes are present. Primer sequences, amplicon sizes, PCR mix composition, and thermal conditions are included below.

Materials

Primers (see recipe in Reagents and Solutions)
PCR reagents (e.g., Platinum SuperFi PCR Master Mix, Invitrogen, cat. no. 12358250)
Genomic DNA

Additional reagents and equipment for PCR (Kramer & Coen, 2001) and agarose gel electrophoresis (Voytas, 2001)

1. Set up PCR reaction (also see Kramer & Coen, 2001). For each 10 µl reaction, use the primer pairs recommended in Reagents and Solutions below for the given safe-harbor site and donor construct chosen:

   1 µl 10 µM Primer 1
   1 µl 10 µM Primer 2
   1 µl purified genomic DNA from iPSC clone
   2 µl H_2O
   5 µl 2× PCR Master Mix.

2. Run PCR reactions:

1	95°C	3 min
2	95°C	30 sec
3	64°C for CLYBL primers, 58°C for AAVS1 primers	30 sec
4	72°C	1 min
5	Repeat (to step 2) 34 times	
6	72°C	5 min
7	Hold at 12°C	Indefinitely

3. Run PCR products on 1% agarose gel (Voytas, 2001) with interpretation given above for each primer pair. If performing the PCR on samples following Cre excision, increasing the amount of time during the extension step may be necessary to detect the larger un-excised template if a heterogeneous culture is obtained.

SUPPORT PROTOCOL 2

IMMUNOCYTOCHEMISTRY OF i^{3}NEURONS

Staining i^{3}Neurons for immunofluorescence (IF) studies is difficult due to the delicate and interconnected nature of the neuronal processes. These processes are easily disrupted in the extensive series of washes in an IF study, and initial dissociation of even a few processes often results in entire sheets of neurons lifting off the culture surface. The

best ways to minimize these events are by reducing the number of total washes and by carrying out washes slowly on a tilted dish. Additionally, if possible, IF studies should only be done in neurons 10 days old or younger. After this point, neurons tend to be very delicate and even extremely gentle washing typically causes substantial cell washout.

Materials

Fresh 16% paraformaldehyde (PFA) solution (Electron Microscopy Sciences, cat. no. 15710)
Phosphate-buffered saline (PBS; e.g., Gibco, cat. no. 10010049))
10% (w/v) saponin solution (Acros Organics, cat. no. 419231000)
Donkey serum (Sigma, cat. no. D9663)
96-well plate with neurons to be fixed and stained (see protocols above)
Antibodies (primary and secondary)
Sodium azide (Sigma, cat. no. S8032)
Hoechst/DAPI dye (20 mg/ml; 10,000×)

Sterile filters (SteriFlip, Millipore)
Liquid reagent reservoirs (Thermo, cat. no. 8096-11)
P200 8-channel pipet (for processing 96-well dishes)
Plate rocker
Imaging system

NOTE: The following protocol assumes that the experimenter is using a full 96-well dish with the recommended cell counts and medium volumes as indicated in Basic Protocols 6 and 8. Staining on other surfaces (i.e., 8-well chamber slides) may be performed by scaling volumes appropriately.

1. Make 15 ml of 8% PFA solution (7.5 ml PBS, 7.5 ml 16% PFA).
2. Make 50 ml of antibody blocking solution (3% donkey serum and 0.1% saponin in PBS). Filter sterilize.

   Detergents other than saponin (i.e., Triton X-100 or Tween-20 at 0.25%) may also be used. Concentrations should be optimized by the user.

3. Pour the 8% PFA solution into a liquid reagent reservoir.
4. Retrieve 96-well plate (45,000 to 50,000 cells/100 µl medium in each well) and slowly add 100 µl 8% PFA solution to each well with 8-channel pipet. Do not pipet up and down to mix.

   4% PFA is a typical fixative for ICC applications. This concentration is used here, but an aspiration step is eliminated by adding an equal volume of 8% PFA directly to the culture medium on the cells. Pipetting up and down eliminates this advantage by promoting cell dissociation from the culture surface.

5. Incubate at room temperature for 10 min.

   Longer fixation times and/or cold incubations may be used as per requirements for particular antibodies.

6. Tilt dish to one side and lower 8-channel micropipet tips so that they contact the wall of each well. Slowly aspirate the PFA, leaving a small amount (if necessary) at the wall–culture surface interface.

   To prevent drying, aspirate only one column of wells at a time. Dispense PFA into a waste liquid reagent reservoir.

7. With plate tilted, slowly dispense 200 µl PBS onto the same wall in each well, taking care to direct the micropipet tip toward the wall and NOT the culture surface. Liquid should flow smoothly onto culture surface.

   PBS without detergent tends to adhere to the wall and then suddenly rush onto the culture surface all at once, promoting cell dissociation. To ensure more gradual flow, gently rub the micropipet tip against the wall in a side-to-side motion while dispensing. This action disrupts the surface tension of the dispensed PBS droplet, providing a mechanical substitute for detergent.

8. Repeat steps 6 and 7 for each column of wells.
9. Once fixative solution has been replaced with PBS, gently rock for 5 min.
10. Repeat steps 6 to 9 two times, each with 100 µl washes of PBS.
11. Aspirate PBS (using plate tilt method) and add 100 µl of blocking solution to each well. Gently rock at room temperature for 30 min.
12. During blocking, make up primary antibody solution(s) in blocking buffer or 3% BSA solution.
13. Aspirate blocking solution and add primary antibody solution to plate (following procedure in steps 6 and 7). Gently rock at room temperature for 1 hr or at 4°C overnight.
14. Aspirate primary antibody solution.

    Primary antibody may be saved and re-used for up to 1 month. Sodium azide (0.02% final concentration) should be added to any saved antibody solutions to prevent microbial growth

15. Add 150 to 200 µl of blocking solution to wells (following procedure in steps 6 and 7) and gently rock for 5 min.
16. Repeat steps 14 and 15 two times for a total of three washes.
17. Aspirate blocking solution and add secondary antibody solution (following procedure in steps 6 and 7). Gently rock at room temperature for 1 hr.
18. Wash wells with PBS three times (following procedure in steps 6 and 7). The second-to-last wash can contain Hoechst/DAPI dye if nuclear visualization is desired.
19. Image cells in PBS

    Replace with mounting medium if desired

20. Store plate at 4°C.

SUPPORT PROTOCOL 3

TRANSFECTION OF i^{3}NEURONS

Transient protein expression can easily be studied in i^{3}Neurons using lipid-based transfection. This protocol is identical to that in iPSCs (see Basic Protocol 2). i^{3}Neurons are modestly transfectable, with 5% to 10% of cells showing fluorescent protein expression after 24 hr. We have found that refreshing neuronal medium 1 to 2 hr after transfection both allows successful DNA entry into cells and largely prevents cytotoxicity resulting from the transfection reagent. Unlike iPSCs, i3Neurons show increased protein expression/accumulation over time, with greater fluorescence 48 to 72 hr after transfection than at 24 hr. Transient transfections also show more durable expression in i3Neurons than iPSCs, likely because episomes are not diluted by cell division. i^{3}Neurons can be transfected in suspension (i.e., re-plating after day 3 of differentiation) or as an adherent culture, although better results are observed in adherent cultures. They are also

amenable to serial transfections (i.e., re-transfecting with the same construct 24 hr apart) if higher-percentage transfections are desired.

TRANSDUCTION OF i^{3}NEURONS

SUPPORT PROTOCOL 4

Lentiviral infection of neurons can be used for a variety of applications in microscopy and biochemistry, provided that the transgene is driven by promoters not silenced in iPSCs or neurons (e.g., CAG, PGK In EF-1α, not CMV, see Critical Parameters and Troubleshooting). The precise parameters for optimal viral production and supernatant collection/concentration will depend on the type of virus used, viral packaging mechanism, viral packaging cell line, and specific transgene introduced. Highly efficient results have been observed with lentivirus produced by Lenti-X HEK cells, with transgenes carried in the pLEX backbone.

Materials

Poly-L-ornithine (PLO) coating solution (see Table 3)
Lenti-X HEK cells (Clontech, cat. no. 632180)
Opti-MEM (ThermoFisher, cat. no. 31985062)
DMEM/F12 medium (Gibco, cat. no. 11320033) containing 10% (v/v) FBS, heat inactivated (Gibco, cat. no. 16140071)
psPAX (Addgene, cat. no. 12260), pMD302 (Addgene, cat. no. 12259), pAdvantage (Promega cat. no. E1711) viral packaging plasmids
pLEX or equivalent lentiviral vector with desired transgene
Lipofectamine 3000 with P3000 reagent (ThermoFisher, cat. no. L3000015)
500× ViralBoost reagent (ALSTEM, cat. no. VB100)
Inverted microscope
i^{3}Neurons (See Basic Protocols 5 and 6)
Cortical Neuron Culture Medium (CM, see Table 4)

Day 0

1. Coat a 6-well dish with PLO (see Basic Protocol 6).
2. Plate 2.5 × 10^6 Lenti-X HEK cells in 6-well dish well in 1.5 ml DMEM/F12 containing 10% FBS (HEK medium).

Day 1

3. Check cells for confluency under an inverted microscope. Cells should be >95% confluent for maximal viral production efficiency.

   If necessary, wait a day to transfect so that the cells are nearly confluent.

4. Warm two tubes with 150 µl Opti-MEM each to room temperature.
5. Add 1.6 µg psPAX, 0.6 µg pMD302, 0.2 µg pAdvantage, 2.4 µg pLEX viral vector, and 10 µl P3000 reagent to tube A, gently flick to mix.
6. Add 1.87 µl Lipofectamine 3000 to tube B, flick to mix.
7. Incubate tubes at room temperature for 5 min.
8. Add the contents of tube A into tube B with a P200, then immediately flick the tube several times to mix.
9. Incubate tube at room temperature for 20 to 40 min.
10. Add contents of tube dropwise to to the medium of Lenti-X cells with a P1000.
11. Agitate plate to evenly distribute transfection solution.

Day 2

12. Replace medium on Lenti-X HEK cells with 3 ml of warm, fresh HEK medium supplemented with 6 µl of 500× ViralBoost reagent.

13. Check cells with a fluorescent microscope to ensure expression of any fluorescent proteins in the viral vector.

Day 4

14. Check cells under inverted microscope for multinucleated morphology (indicating viral production) and fluorescent protein production.

15. Collect medium from well, filter to purify virus from floating HEK cells, and either use immediately or freeze aliquots at −80°C.

    Instead of filtering, medium may be centrifuged at >10,000× g for 10 min at 4°C to remove cells/debris, followed by transferring the supernatant to a new microcentrifuge tube.

16. *Optional:* Replace medium on HEK cells with 1 to 2 ml fresh HEK medium, to be collected the following day. This process can be repeated until day Day 6 to collect more virus. Be aware that viral titer decreases with each collection. Lentiviral preps can also be concentrated using various commercially available reagents (e.g., Clontech, cat. no. 631231).

For each new viral preparation introduced to iPSCs, i^3Neurons, or i^3LMNs,, titer should be assessed with a serial dilution of viral supernatant relative to the culture medium (E8, CM, or MM, respectively). This dilution typically begins with a 1:1 mixture of culture medium and viral supernatant, and reduces by 50% in each subsequent well. Viral supernatant–containing medium should be removed from cells approximately 24 hr after addition, and replaced with fresh culture medium. With lentivirus, maximal transgene expression typically occurs 3 to 5 days after infection. Depending on the particular transgene, vector, and viral packaging system, initial expression can often be assayed 2 days after infection.

SUPPORT PROTOCOL 5

LIVE IMAGING OF i^3NEURONS

Live imaging permits visualization of molecular and organellar dynamics within the neuron. While a standard confocal microscope is sufficient for short imaging experiments, extended imaging applications (>1 hr) are best served by a 37°C live imaging chamber outfitted onto the microscope. CM should also be changed to Hibernate A Low Fluorescence Medium (BrainBits LLC, cat. no. SKU#HAPR) for extended imaging. Hibernate A permits long-term maintenance of neuronal cultures in ambient carbon dioxide levels (0.04% vs. 5% for standard cell culture incubators) and provides a better imaging environment by reducing autofluorescence from phenol red-containing medium. Finally, medium (either CM for short imaging or Hibernate A for extended imaging) should be supplemented with SOS neuronal supplement (Cell Guidance Systems, M09-50) instead of B27. SOS supplement does not contain phototoxic components present in B27 and other neuronal supplements. Imaging is best done on glass-bottom slides, such as Ibidi µ-slides (Ibidi, cat. no. 80827).

SUPPORT PROTOCOL 6

ASSESSING RTTA ACTIVITY

Materials

Cell line of interest and appropriate culture medium
pBI-MCS-EGFP plasmid (Addgene, cat. no. 16542)
Doxycycline (2 mg/ml in PBS; 1000×; Sigma, cat. no. D9891)
Fluorescent microscope

Additional reagents and equipment for general iPSC culture (Basic Protocol 1) and transfection (Basic Protocol 2)

1. Prepare four wells of the cell line of interest by EDTA split (Basic Protocol 1).

   For at least the first time performing this protocol, include positive and negative control cells in order to compare with cells of interest.

2. 1 to 2 days after plating, follow Basic Protocol 2 (steps 6 to 9) for two of the wells in order to transfect with the pBI-MCS-EGFP plasmid.

3. One day later, aspirate medium and replace with fresh E8. In one transfected well and one un-transfected well, supplement medium with 2 µg/ml doxycycline.

   If cells are more than 30% confluent when transfected, doxycycline may be added at the time of transfection in order to permit imaging the next day.

4. 1 day later, aspirate medium, rinse twice with PBS, and image with a fluorescent microscope.

   eGFP typically has a half-life of 24 hr, so fluorescence should persist for at least 2 to 3 days after doxycycline treatment.

5. Since transfection is heterogeneous, only a subset of cells should express GFP. In addition, levels of expression may vary by the number of plasmid copies delivered to each cell. However, in cells with active tetracycline transactivator, the average intensity of GFP will be notably higher in the well exposed to doxycycline than in the well which was not. Some leaky expression is expected due to the sequence of the tetracycline operator in this plasmid, so un-transfected cells thus serve as a further negative control.

ASTROCYTE PRODUCTION

SUPPORT PROTOCOL 7

Materials

P0 or P1 mouse pups
DMEM, high glucose (Gibco, cat. no. 11965092) containing 10% (v/v) FBS (Gibco, cat. no. 16140071)
Trypsin (Gibco, cat. no. 23500054)

Shaker
75-cm^2 (T75) culture flasks (Thermo, cat. no. 156499)
Transwells (e.g., for 12-well plates; Corning, cat. no. 3401)

Additional reagents and equipment for cell culture (see Basic Protocol 1) and counting cells (Phelan & May, 2015)

1. Use 3 P0 or 1 P1 rat pup per uncoated T75 flask. Meninges should be completely removed from brains, and astrocytes isolated per standard mechanical and/or enzymatic dissociation protocols under sterile conditions. Expand astrocytes for 1 week or until confluent in DMEM containing 10% FBS by volume (astrocyte medium).

2. Shake at 300 rpm at 37°C to remove microglia, any surviving neurons, and other contaminating cell types.

3. When the flask nears confluency, wash with PBS and incubate with trypsin for 5 min.

4. Centrifuge 5 min at 200 × *g*, room temperature, aspirate supernatant, and resuspend in DMEM containing 10% FBS.

5. Seed cells from each T75 into three new T75 flasks and grow until nearly confluent.

   Astrocytes may be passaged in culture up to two times. Passaging more than two times reduces neutrophic and synaptogenic support qualities.

6. Alternatively, astrocytes can be frozen in liquid nitrogen (see Basic Protocol 1).

7. To add astrocytes to neural cultures, repeat steps 3 and 4 or thaw from frozen stock, and seed cells onto a transwell.

   Over-adding astrocytes is better than under-adding; if insufficient number of astrocytes are plated, neurons will likely be less healthy. The induction can largely be rescued by adding additional astrocytes the following day if it appears that the neurons are not responding well.

REAGENTS AND SOLUTIONS

Primers

CLYBL insertion of hNIL:

C1	5′	TGACTAAACACTGTGCCCCA	3′
C2	5′	AGGCAGGATGAATTGGTGGA	3′
C3	5′	CAGACAAGTCAGTAGGGCCA	3′
C4	5′	AGAAGACTTCCTCTGCCCTC	3′

Run PCR products on 1% agarose gel (Voytas, 2001). The C1/C2 primer pair will yield a 790-bp band if the cells have at least one wild-type allele (i.e., no insertion or heterozygous insertion). With homozygous insertion, no band will be produced. The C3/C4 pair will yield a band of 1080 bp with hNIL insertion to the CLYBL site; this band will be lost with successful Cre excision.

CLYBL insertion of mag-hNIL:

C1	5′	TGACTAAACACTGTGCCCCA	3′
C2	5′	AGGCAGGATGAATTGGTGGA	3′
C3	5′	CAGACAAGTCAGTAGGGCCA	3′
CM4	5′	AGGCCTTCCATCTGTTGCT	3′
CM5	5′	TGCCAAGTGGGCAGTTTAC	3′

Run PCR products on 1% agarose gel (Voytas, 2001). The C1/C2 primer pair will yield a 790-bp band if the cells have at least one wild-type allele (i.e., no insertion or heterozygous insertion). With homozygous insertion, no band will be produced. The C3/CM4 pair will yield a band of 1410 bp with mag-hNIL insertion. Upon successful Cre excision, the C1/CM5 primer pair will amplify a product of 816 bp, with an amplicon at 4139 bp if editing does not occur.

AAVS1 insertion of NGN2:
A1 5′ GGAATCTGCCTAACAGGAGGT 3′
A2 5′ CGGTTAATGTGGCTCTGGTT 3′
A3 5′ CCCCCAGAATAGAATGACACC 3′

Run PCR products on 1% agarose gel (Voytas, 2001). The A1/A2 primer pair will yield a 163-bp band if the cells have no or heterozygous insertion of hNGN2 into AAVS1. With homozygous insertion, no band will be produced. The A2/A3 pair will yield a band of 1100 bp with hNGN2 insertion to the AAVS1 site that will be reduced to 229 bp with successful Cre excision at the loxP sites.

COMMENTARY

Background Information

The use of transcription factors (TFs) to specify iPSC differentiation follows naturally from the use of Yamanaka factors (Oct4/Sox2/Klf4/c-Myc) to generate iPSCs from differentiated cells. Whereas small molecules elicit inconsistent effects based on cell-to-cell variability in uptake and metabolism, stably integrated transcription factors enable uniformly efficient differentiation. These strategies are gaining momentum in the iPSC field, with recent reports detailing TF-based strategies for iPSC-derived motor neurons, oligodendrocytes, and even pancreatic beta cells (Goto et al., 2017; Major, Powers, & Tabar, 2017; Zhu, Liu, Zhou, & Ikeda, 2017). Ultimately, TF-based differentiation offers higher efficiency, higher purity, and a shorter timeline for producing the desired cell model when compared to small molecule differentiation methods. For laboratories with expertise in iPSC culture, TF-based methods have the potential to accelerate productivity, while for laboratories new to iPSC technologies, TF-based methods offer a low barrier to entry. The i^3Neuron and i^3LMN strategies described in this article express these TFs in an integrated and inducible fashion, offering the user maximal facility and control. For instance, multi-transgenic iPSC lines can be constructed piecemeal over time, with no concerns over loss of hNGN2 or hNIL expression. These lines can then be differentiated on a small or large scale, matured and assayed experimentally, or cryopreserved in a convenient pre-differentiated stock.

The main limitation with our strategy is the unavailability of the Tet-On promoter system for precise control of other transgenes, such as toxic genes. The 3-day neuronal differentiation period with doxycycline will also induce any other genes controlled by the Tet-On promoter, and overexpression of toxic genes (e.g., TDP-43 overexpression for ALS modeling) at this critical time might compromise the quality of the resulting neurons. Methods that we have used to counter this challenge include implementing a shorter differentiation period (terminal differentiation is induced within 24 hr of continuous doxycycline exposure; Busskamp et al., 2014), inserting Kozak sequences with short upstream open reading frames (uORFs) to attenuate expression from the Tet-On promoter, and employing orthogonal inducible systems.

Critical Parameters and Troubleshooting

DNA quality for transfections

Efficient transfections are critical for facilitating downstream enrichment and clonal isolation when constructing a new iPSC line. Low efficiency transfections will require several weeks of enrichment, whereas an exceptionally high efficiency transfection could result in clonal isolation after the initial serial dilution or FACS enrichment of transfected cells. We have observed that the best indicators of DNA quality (apart from proper 260/280 and 260/230 spectrophotometric ratios) are concentration and preparative scale. Concentrating DNA, either through a new prep or ethanol precipitation, almost always improves efficiency. DNA for transfection should ideally be at a concentration higher than 1 μg/μl, and should almost certainly be concentrated if below 300 ng/μl. Miniprep DNA tends to produce less efficient transfections than either midi- or maxiprep DNA at the same concentration, likely due to higher bacterial endotoxin levels in smaller preps. Since endotoxins cannot be removed from existing preps, DNA to be used for transfection should ideally be prepared in a maxiprep.

Coating and freshness of medium for neurons

Plate coating for neurons (PLO for hNGN2 neurons and PLO/PEI with laminin for hNIL neurons) is the single most important parameter determining long and short-term neuronal health. Coating solutions should always be freshly prepared, and any stocks should be used within 1 week. Coated and washed dishes may also be stored covered at 4°C for only 1 week. Though we have not observed a striking cutoff point for coating viability, we have observed a steady decline in neuronal health when older coating solutions have been used. Likewise, neuronal media (IM, CM, or MM) should be prepared fresh with supplements in small scale batches (i.e., 50 to 100 ml), and supplemented media should be used within 1 week.

FACS conditions for iPSC sorting

FACS conditions must be carefully selected to ensure iPSC viability. Microfluidic flow sorting machines (e.g., Sony SH800S, NanoCellect WOLF Cell Sorter) employ low pressures for sorting, and are therefore ideal

to preserve iPSC viability. If only conventional sorting machines are available, the nozzle should be adjusted to 100 μm (versus the 30-μm standard nozzle) in order to lower the sorting pressure. For bulk sorting, iPSCs should be resuspended for sorting in E8 + ROCK inhibitor, and they should be sorted into Matrigel-coated dishes with E8+ ROCK inhibitor. Improved survival and outgrowth from single cells may be accomplished by resuspending in StemFlex medium (Gibco, cat. no. A3349401) with ROCK inhibitor and plating on wells that have been coated with rhLaminin-521 (Gibco, cat. no. A29249). If routine passaging was done in E8 and E8 + ROCK inhibitor, these cells should be pre-equilibrated to StemFlex for at least 2 passages.

Immunocytochemistry

Neurons can be stained in situ, but special care must be taken to prevent dissociation of individual cells or sheets of neurons from the dish. It is therefore critical to reduce the total number of washes (e.g., using 2× fixative solution added directly to the neuronal medium) and carry out washes slowly (see Support Protocol 2).

Lentiviral vectors for i^{3}Neuron and i^{3}LMN transduction

We have found that iPSCs and iPSC-derived neurons (i^{3}Neurons and i^{3}LMNs) silence certain viral elements in transgenic constructs. In particular, CMV promoters and IRES elements consistently fail to express. We suggest replacing CMV promoters with CAG, PGK, or EF-1α promoters, and replacing IRES elements with T2A linkers for polycistronic gene expression.

Enhancing stable cell line generation

Establishing stable lines of iPSCs using the safe-harbor integration strategy requires induction of double-stranded breaks at defined genomic loci (e.g., AAVS1 or CLYBL). One of the major roadblocks in the clonal enrichment and isolation process is the inefficiency of stable transgene integration (<1%). In addition to the low frequency of homologous recombination events between the donor plasmid and the genomic integration locus, a high frequency of p53-mediated death has been observed in cells that successfully undergo TALEN- or CRISPR/Cas9-mediated double-stranded breaks (Ihry et al., 2017). To counter the latter problem, we co-transfect a non-integrating dominant-negative p53 construct (Addgene #41856) along with donor DNA and TALENs when establishing a new line. We have seen major improvements in donor construct integration through this strategy, as measured by increased numbers of fluorescent cells both 24 hr after transfection and 1 week after FACS.

Anticipated Results

The construction of i^{3}Neuron and i^{3}LMN iPSC lines enables versatility in studying various aspects of neuronal cell biology through additional gene knockouts, knockdowns, or overexpression systems. The integrated, inducible, and isogenic transgene system allows the production of a more pure and mature population of neurons in less time than neurons differentiated by growth factors and small molecules alone. These cells are furthermore easily amenable to multiple experimental applications, including microscopy, biochemistry, -omics, and electrophysiology (with addition of glia).

Time Considerations

The estimated time to generate a stable hNGN2 or hNIL-expressing iPSC line is approximately 1 month (transfection, manual or FACS fluorophore enrichment, clonal isolation, genotyping, karyotyping). The doxycycline differentiation period is 3 days, and culture to mature neurons takes an additional 11 days (mature d14 neurons). These neurons can also be cultured longer, as we have observed good viability and morphology through 30 days after initial doxycycline induction.

Acknowledgements

This protocol is based on work that was partially carried out at the Gladstone Institutes (San Francisco) and implemented at the NIH. The work was financially supported by an NIH K08 award (M.E.W.), as well as by the NINDS intramural research program. We thank Kurt Fischbeck (NINDS/NIH) and Daniel Felkner (WiCell) for their helpful comments and suggestions.

Literature Cited

Bain, G., & Gottlieb, D. I. (1998). Neural cells derived by in vitro differentiation of P19 and embryonic stem cells. *Perspectives on Developmental Neurobiology*, *5*, 175–178.

Beers, J., Gulbranson, D. R., George, N., Siniscalchi, L. I., Jones, J., Thomson, J. A., & Chen, G. (2012). Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nature Protocols*, *7*, 2029–2040. doi: 10.1038/nprot.2012.130.

Busskamp, V., Lewis, N. E., Guye, P., Ng, A. H., Shipman, S. L., Byrne, S. M., ... Church, G. M. (2014). Rapid neurogenesis through transcriptional activation in human stem cells. *Molecular Systems Biology*, *10*, 760–760. doi: 10.15252/msb.20145508.

Cao, S.-Y., Hu, Y., Chen, C., Yuan, F., Xu, M., Li, Q., ... Liu, Y. (2017). Enhanced derivation of human pluripotent stem cell-derived cortical glutamatergic neurons by a small molecule. *Scientific Reports*, *7*, 3282. doi: 10.1038/s41598-017-03519-w.

Carlson-Stevermer, J., Abdeen, A., Kohlenberg, L., Goedland, M., Molugu, K., Lou, M., & Saha, K. (2017). Assembly of CRISPR ribonucleoproteins with biotinylated oligonucleotides via an RNA aptamer for precise gene editing. *Nature Communications*, *8*, 1711. doi: 10.1038/s41467-017-01875-9.

Cerbini, T., Funahashi, R., Luo, Y., Liu, C., Park, K., Rao, M., ... Zou, J. (2015). Transcription activator-like effector nuclease (TALEN)-mediated CLYBL targeting enables enhanced transgene expression and one-step generation of dual reporter human induced pluripotent stem cell (iPSC) and neural stem cell (NSC) lines. *PloS One*, *10*, e0116032. doi: 10.1371/journal.pone.0116032.

Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., & Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nature Biotechnology*, *27*, 275–280. doi: 10.1038/nbt.1529.

Chen, G., Gulbranson, D. R., Hou, Z., Bolin, J. M., Ruotti, V., Probasco, M. D., ... Thomson, J. A. (2011). Chemically defined conditions for human iPSC derivation and culture. *Nature Methods*, *8*, 424–429. doi: 10.1038/nmeth.1593.

D'Alton, S., Altshuler, M., Cannon, A., Dickson, D. W., Petrucelli, L., & Lewis, J. (2014). Divergent phenotypes in mutant TDP-43 transgenic mice highlight potential confounds in TDP-43 transgenic modeling. *PloS One*, *9*, e86513. doi: 10.1371/journal.pone.0086513.

Gossen, M., & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the U.S.A.*, *89*, 5547–5551. doi: 10.1073/pnas.89.12.5547.

Goto, K., Imamura, K., Komatsu, K., Mitani, K., Aiba, K., Nakatsuji, N., ... Inoue, H. (2017). Simple derivation of spinal motor neurons from ESCs/iPSCs using sendai virus vectors. *Molecular Therapy—Methods & Clinical Development*, *4*, 115–125. doi: 10.1016/j.omtm.2016.12.007.

Hu, B.-Y., & Zhang, S.-C. (2009). Differentiation of spinal motor neurons from pluripotent human stem cells. *Nature Protocols*, *4*, 1295–1304. doi: 10.1038/nprot.2009.127.

Ihry, R. J., Worringer, K. A., Salick, M. R., Frias, E., Ho, D., Theriault, K., Kommineni, S., ... Kaykas, A. (2017). P53 toxicity is a hurdle to CRISPR/CAS9 screening and engineering in human pluripotent stem cells. *bioRxiv*, *168443*. doi: https://doi.org/10.1101/168443.

Karumbayaram, S., Novitch, B. G., Patterson, M., Umbach, J. A., Richter, L., Lindgren, A., ... Lowry, W. E. (2009). Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. *Stem Cells*, *27*, 806–811. doi: 10.1002/stem.31.

Kawai, H., Sango, K., Mullin, K. A., & Proia, R. L. (1998). Embryonic stem cells with a disrupted GD3 synthase gene undergo neuronal differentiation in the absence of b-series gangliosides. *Journal of Biological Chemistry*, *273*, 19634–19638. doi: 10.1074/jbc.273.31.19634.

Kent, L. (2009). Culture and maintenance of human embryonic stem cells. *Journal of Visualized Experiments*, *pii*, 1427. doi: 10.3791/1427.

Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., ... Takahashi, J. (2017). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature*, *548*, 592–596. doi: 10.1038/nature23664.

Kitazawa, M., Medeiros, R., & Laferla, F. M. (2012). Transgenic mouse models of Alzheimer disease: Developing a better model as a tool for therapeutic interventions. *Current Pharmaceutical Design*, *18*, 1131–1147. doi: 10.2174/138161212799315786.

Kloskowska, E., Pham, T. M., Nilsson, T., Zhu, S., Oberg, J., Codita, A., ... Benedikz, E. (2010). Cognitive impairment in the Tg6590 transgenic rat model of Alzheimer's disease. *Journal of Cellular and Molecular Medicine*, *14*, 1816–1823. doi: 10.1111/j.1582-4934.2009.00809.x.

Kramer, M. F., & Coen, D. M. (2001). Enzymatic amplification of DNA by PCR: Standard procedures and optimization. *Current Protocols in Cell Biology*, *10*, A.3C.1–A.3C.14. https://doi.org/10.1002/0471143030.cba03fs10.

Ludwig, T. E., Bergendahl, V., Levenstein, M. E., Yu, J., Probasco, M. D., & Thomson, J. A. (2006). Feeder-independent culture of human embryonic stem cells. *Nature Methods*, *3*, 637–646. doi: 10.1038/nmeth902.

Major, T., Powers, A., & Tabar, V. (2017). Derivation of telencephalic oligodendrocyte progenitors from human pluripotent stem cells. *Current Protocols in Stem Cell Biology*, *39*, 1H.10.1–1H.10.23. doi: 10.1002/cpsc.17.

Matheson, N. J., Peden, A. A., & Lehner, P. J. (2014). Antibody-free magnetic cell sorting of genetically modified primary human CD4+ T cells by one-step streptavidin affinity purification. *PloS One*, *9*, e111437. doi: 10.1371/journal.pone.0111437.

Mazzoni, E. O., Mahony, S., Closser, M., Morrison, C. A., Nedelec, S., Williams, D. J., ... Wichterle, H. (2013). Synergistic binding of transcription factors to cell-specific enhancers programs motor neuron identity. *Nature Publishing Group*, *16*, 1219–1227. doi: 10.1038/nn.3467.

Merkle, F. T., Ghosh, S., Kamitaki, N., Mitchell, J., Avior, Y., Mello, C., ... Eggan, K. (2017). Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. *Nature*, *545*, 229–233. doi: 10.1038/nature22312.

Nuber, S., Harmuth, F., Kohl, Z., Adame, A., Trejo, M., Schönig, K., ... Riess, O. (2013). A progressive dopaminergic phenotype associated with neurotoxic conversion of α-synuclein in BAC-transgenic rats. *Brain*, *136*, 412–432. doi: 10.1093/brain/aws358.

Phelan, K., & May, K. M. (2015). Basic techniques in mammalian cell tissue culture. *Current Protocols in Cell Biology*, *00*, 1.1.1–1.1.22. https://doi.org/10.1002/0471143030.cb0101s66.

Qi, Y., Zhang, X. J., Renier, N., Wu, Z., Atkin, T., Sun, Z., ... Studer, L. (2017). Combined small-molecule inhibition accelerates the derivation of functional cortical neurons from human pluripotent stem cells. *Nature Biotechnology*, *35*, 154–163. doi: 10.1038/nbt.3777.

Shi, Y., Lin, S., Staats, K. A., Li, Y., Chang, W., Hung, S., ... Ichida, J. K. (2017). Haploinsufficiency Leads to Neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. *Nature Medicine*, *24*(3), 313-325. doi: 10.1038/nm.4490.

Shimazaki, T., Arsenijevic, Y., Ryan, A. K., Rosenfeld, M. G., & Weiss, S. (1999). A role for the POU-III transcription factor Brn-4 in the regulation of striatal neuron precursor differentiation. *EMBO Journal*, *18*, 444–456. doi: 10.1093/emboj/18.2.444.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., ... Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, *131*(5), 861–872. doi: https://doi.org/10.1016/j.cell.2007.11.019.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*, *282*(5391), 1145–1147. doi: https://doi.org/10.1126/science.282.5391.1145.

Voytas, D. (2001). Agarose gel electrophoresis. *Current Protocols in Molecular Biology*, *51*, 2.5A.1–2.5A.9. doi: 10.1002/0471142727.mb0205as51.

Wang, C., Ward, M. E., Chen, R., Liu, K., Tracy, T. E., Chen, X., ... Gan, L. (2017). Scalable production of iPSC-derived human neurons to identify tau-lowering compounds by high-content screening. *Stem Cell Reports*, *9*, 1221–1233. doi: 10.1016/j.stemcr.2017.08.019.

Wernig, M., Zhao, J. P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., ... Jaenisch, R. (2008). Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 5856–5861. doi: 10.1073/pnas.0801677105.

Zeng, H., Guo, M., Martins-Taylor, K., Wang, X., Zhang, Z., Park, J. W., ... Xu, R. H. (2010). Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells. *PloS One*, *5*, e11853. doi: 10.1371/journal.pone.0011853.

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., ... Südhof, T. C. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron*, *78*, 785–798. doi: 10.1016/j.neuron.2013.05.029.

Zhu, Y., Liu, Q., Zhou, Z., & Ikeda, Y. (2017). PDX1, Neurogenin-3, and MAFA: Critical transcription regulators for beta cell development and regeneration. *Stem Cell Research & Therapy*, *8*, 240. doi: 10.1186/s13287-017-0694-z.