Video Article

Rapid Detection of Neurodevelopmental Phenotypes in Human Neural Precursor Cells (NPCs)

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Abstract

Human brain development proceeds through a series of precisely orchestrated processes, with earlier stages distinguished by proliferation, migration, and neurite outgrowth; and later stages characterized by axon/dendrite outgrowth and synapse formation. In neurodevelopmental disorders, often one or more of these processes are disrupted, leading to abnormalities in brain formation and function. With the advent of human induced pluripotent stem cell (hiPSC) technology, researchers now have an abundant supply of human cells that can be differentiated into virtually any cell type, including neurons. These cells can be used to study both normal brain development and disease pathogenesis. A number of protocols using hiPSCs to model neuropsychiatric disease use terminally differentiated neurons or use 3D culture systems termed organoids. While these methods have proven invaluable in studying human disease pathogenesis, there are some drawbacks. Differentiation of hiPSCs into neurons and generation of organoids are lengthy and costly processes that can impact the number of experiments and variables that can be assessed. In addition, while post-mitotic neurons and organoids allow the study of disease-related processes, including dendrite outgrowth and synaptogenesis, they preclude the study of earlier processes like proliferation and migration. In neurodevelopmental disorders, such as autism, abundant genetic and post-mortem evidence indicates defects in early developmental processes. Neural precursor cells (NPCs), a highly proliferative cell population, may be a suitable model in which to ask questions about ontogenetic processes and disease initiation. We now extend methodologies learned from studying development in mouse and rat cortical cultures to human NPCs. The use of NPCs allows us to investigate disease-related phenotypes and define how different variables (*e.g.*, growth factors, drugs) impact developmental processes including proliferation, migration, and differentiation in only a few days. Ultimately, this toolset can be used in a reproducible and high-throughput manner to identify disease-specific mechanisms and phenotypes in neurodevelopmental disorders.

Video Link

The video component of this article can be found at https://www.jove.com/video/56628/

Introduction

The use of simpler organisms and mouse models has elucidated the mechanisms of basic brain development as well as disease pathogenesis. Despite these advances, the etiology of many neuropsychiatric disorders remains elusive because not all findings in simpler organisms are directly relevant to complex aspects of human disease. Further, the greater complexity of the human brain often makes it difficult to model human development and disorders in animals. With the evolution and progress of human induced pluripotent stem cells (hiPSCs) technology, somatic cells can be reprogrammed into stem cells and then differentiated into neuronal cells to study human disease. Advances in hiPSCs and "omic" technologies (genomics, transcriptomics, proteomics, metabolomics) promise to revolutionize the understanding of human brain development. These technologies now make possible a "precision medicine" approach to the characterization of neuropsychiatric disease on a case-by-case basis.

The current staple in the hiPSC disease-modeling field is to differentiate cells into specific neuronal subtypes in a monolayer or to use a 3D culture system called an organoid to recapitulate aspects of brain development[1,2,3]. These systems have been incredibly valuable in studying and uncovering unique aspects of human development and disease[4,5,6,7]. However, both neuronal cultures and organoids often require anywhere from weeks to months in culture before they are ready to study. The time-consuming nature of these protocols and the amount of resources needed to maintain these culture systems often limit the number of experiments that can be performed and the number of variables (like growth

factors or drugs) that can be tested. Moreover, many studies utilizing post-mitotic neurons and organoids have focused on processes such as dendrite outgrowth or synapse formation, which occur later in development. While these processes have been implicated in the pathology of developmental disorders such as autism and schizophrenia, earlier developmental events that occur before definitive neuronal differentiation are also important for disease pathogenesis[8,9,10,11,12,13]. Indeed, recent genomic studies show that the mid-fetal period, which is comprised of proliferation, process outgrowth, and migration, is particularly important in autism pathogenesis[11,14]. Thus, it is important to study neural stem and progenitor cell populations to better understand these earlier processes. Organoid systems, which are considered to better recapitulate human brain development because of their 3D nature and organized structure, do contain a progenitor pool that has been utilized to study some of these earlier events. However, the progenitor population in organoids is often sparse and more like radial glial cells than neural stem or progenitor cells[5,15]. Thus, it would be beneficial to have a high throughput method to study early stages of neurodevelopment in an actively proliferative cell population.

In the lab, we have created a protocol that uses hiPSC-derived neural precursor cells (NPCs), a mixed population of neural stem and progenitor cells that is highly proliferative, to study neurodevelopmental processes such as proliferation, cell migration, and initial process (neurite) extension. These assays were developed from techniques used in our lab for decades to successfully study neurodevelopment in rat and mouse cortical cultures[16,17,18,19,20,21,22,23]. Importantly, it was also shown that phenotypes and regulatory signals defined in the rat and mouse culture systems are highly predictive of mechanisms that are active *in vivo*, indicating the value of these techniques[16,17,18,19,24]. After initial differentiation of hiPSCs to NPCs, these methods allow us to study vital developmental processes in a matter of days. These methods have many advantages: (1) they require little sophisticated equipment and are easy to implement, (2) numerous experimental replicates can be conducted in a short period of time, allowing for rapid confirmation of the reproducibility of results, and (3) culture variables such as coating matrices, effects of growth factors, and activity of drugs can be tested quickly and cost-effectively. Furthermore, we take advantage of the well-established role of extracellular growth factors as critical regulators of diverse developmental processes. NPCs were exposed to select developmental signals that directly stimulate events like proliferation, neurite outgrowth, and cell migration, and have found they enhance the ability to identify defects that are not apparent in control conditions[19,25,26,27,28]. Likewise, the ease of assessing drugs provides a powerful avenue to adopt precision medicine techniques to test the efficacy of various therapeutic interventions. Thus, this protocol facilitates a high throughput, reproducible, and straightforward methodology to study early brain development, disease pathogenesis, and the potential beneficial effects of growth factors and drugs on neurodevelopmental phenotypes.

Protocol

1. Safety Procedures and Biosafety Cabinet Maintenance

1. **Biosafety Level-2 (BSL-2) Safety Procedures**
    1. Follow the institution's guidelines on working with BSL-2 materials. Dispose of BSL-2 materials according to the institution's practices. Indicate rooms and equipment that are used for BSL-2 materials. Wear all personal protective equipment (PPE), including lab coat and gloves.

2. **Biosafety Cabinet Maintenance**
    1. Use a biosafety cabinet certified for use of BSL-2 level materials.
    2. Turn on the UV-light in the biosafety cabinet for at least 15 min, and then spray surfaces with 10% bleach solution. Allow the bleach solution to sit for 15 min, wipe down the cabinet, and turn on the airflow before use.

3. **Radioactive Tritiated [^{3}H]-thymidine (Tritium) Safety Procedures**
    NOTE: Tritium is a Category 5 radioactive source, 'the most unlikely to be dangerous' category. Follow the institution's guidelines for working with radioactivity. Some institutions may require certifications or permits to handle tritium.
    1. Receive training from the institution on how to properly handle and dispose of tritium. Dispose of radioactive waste in proper receptacles, according to the institution's policies. Wear PPE when working with tritium.

2. Neural Induction from iPSCs

NOTE: To make NPCs, a slightly modified version of a protocol that accompanies a commercially available neural induction kit was followed. The kit consists of Neurobasal (NB) media and a 50x Neural Induction Supplement (NIS), which is used to make a 1x Neural Induction Medium (NIM). NIS is also used to make 100% Expansion Medium (see Section 3.1). A link to the protocol is found in the Materials and Equipment and References section[43].

1. Passage iPSCs when they become 70 - 80% confluent.
2. Plate 300,000 iPSCs into 1 well of a hESC-qualified extracellular matrix-mimic gel (ECM-mimic gel) coated 6-well plate containing 2 mL of feeder-free iPSC medium with 5 µM of ROCK inhibitor. See Section 3.2 for instructions on coating wells with ECM-mimic gel.
3. One day later, remove the iPSC medium + ROCK inhibitor and wash iPSCs once with 1x PBS. Add 2 mL of NIM to the iPSCs.
    NOTE: 50 mL of NIM is made by adding 1 mL of 50x NIS and 250 µL (50 units/mL, 5 µL/mL) of Penicillin/Streptomycin (P/S) to 49 mL of NB Media.
4. Change media of the neural induction every 2 days by aspirating spent medium and replacing with 2 mL of NIM until cells become confluent (around 4 - 5 days). Change media daily, once cells are confluent.
5. Passage cells after 7 days in NIM and plate into an ECM-mimic gel coated 6-well plate containing 2 mL of 100% Expansion Media and 5 µM ROCK inhibitor. The cells are considered Passage 0 (P0) NPCs at this point. See Section 3.1 below for instructions to make 100% Expansion Media.

6. Passage cells using the methods described in Section 3.4. Wait until cells have reached P2 and are confluent before dissociating to stain for NPC markers and plating for experiments (**Figure 1**).

3. Culture Media, Coating, and Maintenance of NPCs

1. **Media Preparation for Maintenance of NPCs (100% Expansion Media):**
    1. Make 50 mL of 100% Expansion Media by combining 24.5 mL of DMEM/F12 with 24.5 mL of NB.
    2. Add 1 mL of 50x NIS to the DMEM/F12 + NB solution. Add 250 µL of P/S solution to media.
    NOTE: Media can be refrigerated (4 °C) and used for up to 2 weeks. Smaller volumes of media can be made by adjusting the volumes of DMEM/F12+ NB+NIS using the same ratios as for the 50-mL volume.

2. **Preparation of ECM-mimic Gel Coated Culture Plates for NPC Maintenance**
    1. Aliquot ECM-mimic gel at the volume needed to make 6 mL of working solution. Calculate aliquot volume by looking at the Certificate of Analysis Sheet since ECM-mimic gel dilution factor varies from batch to batch and lot to lot.
    2. Thaw an ECM-mimic gel aliquot on ice and dissolve into 6 mL of cold DMEM/F12 media.
    3. Add 1 mL of ECM-mimic gel/DMEM/F12 solution to each well of a 6 well plate. Incubate the 6-well plate with ECM-mimic gel-solution for 30 min at 37 °C.
    4. Aspirate ECM-mimic gel solution after 30 min of incubation and replace with 100% Expansion Media or refrigerate plates (4 °C, up to 1 week) without aspirating ECM-mimic gel-solution.

3. **Maintenance of NPCs**
    1. Plate NPCs at a density of 1.5 million cells into an ECM-mimic gel-coated well containing 2 mL of 100% Expansion Media.
    2. Incubate NPCs at 37 °C in a moist environment with 5% CO_2.
    3. Add 5 µM of ROCK inhibitor to the media for NPCs at P3 or lower, or for thawed NPCs, to prevent excessive cell death. Change media after 24 h to remove ROCK inhibitor.
    4. Passage cells every 4 - 9 days, depending upon when they become confluent. Cells are considered confluent when they become a densely packed monolayer covering the entire bottom of the dish surface.
    5. Plate NPCs at a density of 1 to 1.5 million cells per one well of a 6-well plate (see Section 3.4). Remove spent media every 48 h and replace with 2 mL of 100% Expansion Media.

4. **Lift, Dissociate, and Pellet NPCs for Maintenance and/or Plating for Experimental Conditions**
    1. Aspirate medium, wash cells once with 1x PBS. Aspirate PBS and add 500 µL of 1x cell detachment solution into 1 well of confluent NPCs. Incubate for 10 min at 37 °C.
    2. Add 500 µL of room temperature (RT) PBS and wash the well using a P-1000 pipette to ensure removal of cells. Collect the cells + PBS solution into a 15-mL conical tube. Wash the plate again with 1 mL of PBS and add liquid to the tube.
    3. Spin the cells down at 300 x g for 5 min to pellet.
    4. Remove supernatant from the cell pellet and re-suspend the cells in 1-5 mL of pre-warmed DMEM/F12 media. Dilute cells to a density of 1 to 4 million cells/mL of media. Quantify cells using a hemocytometer.
    5. Plate the required number of cells, specific for either NPC maintenance (Section 3.3) or the individual assay being conducted (see subsequent sections for more details).
    6. Adjust cell suspension volume with media so that between 15 to 100 µL of cells are used for each well/dish. This small plating volume ensures that there is even cell distribution and that growth factors, drugs, or substrates in the medium are not diluted.
    7. Incubate cells at 37 °C. Change media every 48 h for NPC maintenance or see the specific details for individual assays.

5. **Media Preparation for Experimental Conditions (30% Expansion Media)**
    1. Dilute the 100% Expansion Media by 70% (termed 30% Expansion) by adding 1:1 DMEM/F12 + NB solution to make media for experimental conditions.
    2. Make 20 mL of 30% Expansion Media by adding 6 mL of 100% Expansion Media and diluting with 7 mL of NB and 7 mL of DMEM/F12 media. Add 5 µL/mL of P/S solution.
    NOTE: If 100% media already contains P/S, then add 5 µL/mL for combined- DMEM/F12+ NB= (14 mL) x (5 µL/mL) = 70 µL of P/S instead of 100µL.
    3. Add coating substrates and growth factors at desired concentrations to the 30% Expansion Media.

4. Assessing DNA Synthesis, S-Phase Entry, and Cell Numbers of NPCs

1. **Preparation for DNA Synthesis, S-phase Entry, and Cell Number Assay**
    1. Make a 1 mg/mL stock solution of poly-D-lysine (PDL) in dH_2O and filter sterilize. Dilute 1:10 in dH_2O to make a 0.1 mg/mL PDL solution and add 300 µL to each well of a 24 well plate or 1 mL to a 35-mm dish. Incubate for 20 min at RT.
    2. Wash PDL wells 3 times for 5 min with dH_2O. Aspirate dH_2O and add 300 µL/well or 1 mL/dish of laminin (5 µg/mL) diluted in 1x PBS. Cover the plates with parafilm and keep in a sterile, biosafety cabinet overnight at RT (12 to 24 h).
    3. Prepare 30% Expansion Media (see Section 3.5) after 12 to 24 h. Add vehicles, growth factors, or drugs of interest at the desired concentration in volumes that are less than 10% of the total solution to avoid diluting NIS in the 30% Expansion Medium.
    4. Wash each well of the 24-well plate twice with 1x PBS (5 min each). Aspirate 1x PBS and add 450 µL of media (without or with drugs/growth factors). Incubate the plate at 37 °C for at least 15 min before plating cells.
    5. For each experiment, set up 2 - 3 wells or dishes per condition.
    6. Plate NPCs (see Section 3.4):
    7. NPC DNA Synthesis Assay: 100,000 cells/well in a 24 well plate
    8. S-Phase Entry: 500,000 cells/35 mm dish

9. Cell Number Assay: 50,000 cells/well in a 24-well plate

2. **Neural Precursor Cell DNA Synthesis Assay**
    1. Plate 100,000 cells/well in a 24-well plate and assess in triplicate/condition.
    2. Add radioactive, tritiated [^{3}H]-thymidine to the culture medium (1.5 µCi/mL) in each well after 46 h in culture. Incubate cells at 37 °C for 2 h.
       NOTE: When using radioactive materials, receive training from your institution, follow radioactivity safety protocols, and dispose of radioactive materials in the appropriately designated waste receptacles.
    3. Remove and properly dispose of radioactive media after 2 h. Add 300 µL of pre-warmed 0.25% trypsin-EDTA (0.5 mM) to each well and incubate for 20 min at 37 °C.
    4. Turn on the cell harvester (see Materials and Equipment section) and the pump and ensure that the pump pressure is below 200 PSI. Place filter paper through the space in the cell harvester and tear off the right corner to mark paper orientation.
    5. Place collecting tubes of cell harvester into an empty "blank" tray and press prewash to moisten the filter paper. Place collecting tubes into sample wells and run (start).
    6. When the cell harvester has finished collecting samples, lift clamp and advance filter paper to repeat for all sample sets. Always prewash in an empty, "blank" plate.
    7. Dry filter paper under a light source and set up corresponding vials in a tray. Punch out paper chads into vials and add 2 mL of liquid scintillation cocktail to each vial. Cap and label vials.
    8. Incubate vials in liquid scintillation cocktail for at least 1 h before reading the counts per minute (CPMs) on a scintillation machine.

3. **NPC S-phase Entry Assay**
    1. See Section 3.4 for steps to lift, dissociate, pellet, and re-suspend cells.
    2. Plate 500,000 cells per dish into the 35 mm dishes prepared in Section 4.1, only 1 dish/condition for this step.
    3. Agitate dishes back and forth in all directions to ensure even distribution of cells. Incubate cells at 37 °C for 46 h.
    4. Prepare three 35 mm plates coated with PDL/Laminin per condition after 24 h. For example, if there is one 35 mm dish of 30% Expansion Media and one 35 mm dish of 30% Expansion Media + FGF (10 ng/mL) then coat 6 PDL/Laminin plates for use on the next day.
    5. Add 2 µL/mL of 5 mM EdU to cultures after 46 h. Incubate for 2 h.
    6. Dissociate and pellet the cells (see Section 3.4). Re-suspend the cell pellet in 3 mL of 30% Expansion Media or 3 mL of 30% Expansion Media + desired growth factors/drugs.
    7. Plate 1 mL per dish on pre-coated PDL/Laminin dishes. Agitate dishes back and forth in all directions to ensure even distribution of cells. Incubate at 37 °C for 2 h to allow the cells to adhere to the dish. See **Figure 2** for a simplified timeline.

4. **S-phase Entry Analysis**
    1. Fix dishes with ice-cold 4% Paraformaldehyde (PFA in 1x PBS) for 20 min. Then, wash dishes 3 times for 5 min with 1x PBS.
    2. Add 1 mL of 1x PBS with 0.05% Sodium Azide to prevent bacterial and fungal growth. Cells can be stored at 4 °C for up to 6 months if dishes (sealed with parafilm) are kept in the PBS + Sodium Azide solution, though not all immunological antigens are well-preserved after long delays in the analysis.
    3. Assay cells using a commercial EdU reaction kit (see manufacturer's protocol). Stain cells using DAPI or another nuclear marker, and image using fluorescence microscopy.
    4. Assess the proportion of EdU positive cells over total live cells (total cell number) blind in 10 systematically random fields (10x). Do not include dead cells in analysis.
    5. Utilize both phase contrast images and fluorescent images to determine positive EdU staining and to ascertain which cells are dead or alive (**Figure 3**).
    6. Count all cells that have both smooth and even cell membrane in phase contrast settings, and a large unfragmented nucleus by fluorescent DAPI nuclear stain, as these cells are live (**Figure 3A-B**).
    7. Exclude all cells that are phase bright, have a broken uneven cell membrane, and have a small, condensed nucleus as visualized by DAPI fluorescent imaging, as these cells are dead (**Figure 3A-B**). Assess live cells for expression of EdU by identifying cells that have bright fluorescent EdU stain covering the entire nucleus or speckled fluorescent staining in the nucleus (**Figure 3C**).

5. **NPC Cell Number Assay**
    1. After 2 days in culture, label and prepare 1.5 mL microcentrifuge tubes and 0.5 mL microcentrifuge tubes. In each 0.5 mL tube, add 5 µL of Trypan Blue.
    2. To each well of a 24 well plate, remove medium and add 200 µL of 1x cell detachment solution and place in incubator for 10 to 15 min.
    3. After the allotted time, once cells have lifted, add the desired amount of 1x PBS to each well.
       NOTE: Typically, on day 2, add 300 µL of 1x PBS to the well containing cells plus 200 µL of the detachment solution, for a total volume of 500 µL. With additional culture incubation time, as cells become more confluent, increase dilution volume as necessary. For example, on Day 4, add 500 µL of 1x PBS, and on Day 6, add 800 µL of 1x PBS. Total volumes will be 700 µL and 1 mL, respectively.
    4. Using a P-1000 pipette, pipette up and down in each well 4 to 5 times to remove cells. Examine the plate under a microscope to ensure that all cells have detached. Transfer cells to 1.5 mL tubes.
    5. Invert 1.5 mL tubes with diluted cells 2 to 3 times. Then take a 50 µL aliquot of cells from the middle of the tube and add to 0.5 mL tubes with Trypan Blue (See 4.5.1).
    6. Pipette cell solution up and down 2 to 3 times. Add cells to the hemocytometer and analyze immediately. Waiting for longer than 10 min may increase cell death or the presence of cells that have taken up Trypan Blue.
    7. Carefully add 10 µL of cell + Trypan Blue mixture to each side of hemocytometer to perform replicate counts. Count cells using the phase contrast microscope. Do not count dead cells or the dark blue cells that have taken up Trypan Blue.
    8. To obtain the total cell number, use the average cell number counted from 4 corners of the hemocytometer and apply the following equation:
       Mean cell number x media volume (mL) x 10,000 = Total cell number/well

9. Aspirate and repeat the procedure on remaining wells. Change the media on the cells that are not being counted, every 48 h. Repeat assay on Days 4 & 6.

5. NPC Neurite Assay

1. **Preparation of Dishes and Media for Neurite Assay**
   1. Make a 1 mg/mL stock solution of poly-d-lysine (PDL) in dH_2O and filter sterilize. Dilute 1:10 in dH_2O to make a 0.1 mg/mL PDL solution and add 1 mL to each 35-mm dish. Incubate for 20 min at RT.
   2. In the meantime, prepare 30% Expansion Media (see Section 3.5) and add 5 µg/mL (5 µL/mL) of Fibronectin solution (1 mg/mL stock) to the media.
   3. Once the media is prepared, add vehicles, growth factors, or drugs of interest at desired concentrations.
      NOTE: It is best to add vehicle, growth factors, or drugs, at volumes <10% of the total solution to avoid diluting the fibronectin and other components in the 30% expansion medium.
   4. After 20 min, wash PDL dishes 3 times for 5 min with dH_2O to remove excess PDL. Ensure dishes are dry before adding media.
   5. Place 1 mL of media (with or without drugs/growth factors) + Fibronectin solution into each PDL coated dish.
   6. Incubate dishes at 37 °C for at least 30 min before plating cells to ensure proper attachment of the Fibronectin to the PDL.
   7. For each experiment, set up 2 - 3 dishes per condition (*e.g.*, 3 vehicle-containing dishes, 3 drug-containing dishes).

2. **Plating NPCs for Neurite Assay**
   1. See Section 3.4 for steps to lift, dissociate, pellet, and resuspend cells.
   2. Plate 50,000 cells per dish into the dishes prepared in Section 5.1. Agitate dishes back and forth in all directions to ensure an even distribution of cells.
   3. Incubate cells at 37 °C for 48 h.

3. **Analysis of Neurites**
   1. After incubating cells for 48 h, aspirate medium, and fix dishes with ice cold 4% PFA for 20 min.
   2. After 20 min, wash dishes 3 times for 5 min with 1x PBS. After the final wash, add 1 mL of 1x PBS with 0.05% Sodium Azide.
   3. Analyze dishes blindly on a phase contrast microscope at 32X. Count total cells and cells with neurites in 3, 1 cm rows, chosen at random but reproducible positions. Count a minimum of 150 cells per dish to ensure adequate sampling.
      NOTE: A neurite is defined as an extension (process) from the cell body that is >2 cell body diameters in length. For cells with multiple processes, the longest process is considered for the criterion. Cells with processes that are <2 cell body diameters in length are not included (**Figure 4**).
   4. Add together the total number of cells and the total number of cells with neurites in each dish. Calculate the % of cells with neurites. Average the percentage of cells with neurites across replicate dishes. Confidence in experimental reproducibility is established when all rows within each dish are similar, and averages among dishes are highly similar, with the standard error of the mean (SEM) <10%.
   5. Alternatively, analyze neurites by conducting immunocytochemistry for markers such as beta-III-tubulin (TUJ1) or MAP2. After staining for the marker of interest, take 10 systematically random images on a fluorescent microscope at 10X. Image at least 200 cells. In this case, acquire the images not too close to the edge of the dish. Analyze the percentage of cells with TUJ1 or MAP2+ neurites (see **Figure 10** for an example).

6. NPC Neurosphere Migration Assay

1. **Neurosphere Formation**
   1. Add 1 mL of 100% Expansion Media into a 35-mm dish with no coating substrate. Incubate dishes for at least 15 min at 37 °C before plating NPCs. Prepare 2 - 3 dishes to ensure that there will be enough neurospheres for the cell migration assay.
      NOTE: The absence of a coating substrate ensures that NPCs remain suspended in the media, which is essential for neurosphere formation. Coating dishes will prevent neurosphere formation.
   2. See Section 3.4 on steps to lift, dissociate, and pellet cells. Resuspend cell pellet in 2-5 mL of pre-warmed 100% Expansion Media. Plate 1 million NPCs into each 35-mm dish prepared in section 6.1.1.
   3. Incubate NPCs at 37 °C for 48 to 96 h to allow NPCs to aggregate and form neurospheres. Assess sphere size using a live ruler on a phase-contrast microscope. Wait for most spheres to reach an approximate diameter of 100 µm (± 20 µm) (**Figure 5**). Smaller spheres will completely disperse and break apart during the migration assay.

2. **Preparation of Plates for Neurosphere Migration Assay**
   1. Dissolve ECM-mimic gel aliquots (see Section 3.2) into 6 mL of 30% Expansion Media. Once ECM-mimic gel/30% Expansion Media solution is prepared, add vehicles, growth factors, or drugs of interests at desired concentrations.
      NOTE: Vehicle, drug, and growth factor concentrations need to be increased to account for the addition of 200 µL of neurospheres in Section 6.3.
   2. Plate 1 mL of the ECM-mimic gel /30% Expansion Media solution (± vehicles, growth factors, or drugs) into one well of a 6-well plate. Make 2 - 3 wells per experimental condition. Alternatively, 35-mm dishes may be used. Incubate plates for at least 30 min at 37 °C.
      NOTE: DO NOT aspirate the ECM-mimic gel/30% Expansion Media solution for this assay. Plating spheres onto an aspirated ECM-Mimic gel will lead to rapid and excess migration.

3. **Plating Neurospheres**
   1. Collect neurospheres formed in Section 6.1 and place them into a conical tube. Wash the 35 mm dishes with 1 mL of 1x PBS to ensure all neurospheres are collected. Spin down the collected neurospheres at 100 x g for 5 min.

2. Re-suspend the neurospheres in 1 - 3 mL of pre-warmed 30% Expansion Media. If 1 dish of neurospheres is collected, 1 mL of media is used to resuspend spheres. If 2 dishes are collected, 2 mL of media are added to resuspend spheres, *etc.* Pipette gently and use only a P-1000 to ensure spheres are not broken.
3. Plate 200 µL of the resuspended neurospheres into the ECM-mimic gel/30% Expansion solution made in Section 6.2. Rock plates in all directions to evenly distribute neurospheres. Incubate the plates for 48 h at 37 °C.
4. Remove ECM-mimic gel/30% Expansion Media solution and fix cells in 4% PFA, wash, and keep cells in 1x PBS + 0.05% Sodium Azide.

4. **Analysis of Neurospheres**
   1. Acquire images of entire neurospheres using phase contrast settings at 10X. Ensure spheres are not touching each other. Measure average migration using the ImageJ software.
   2. Trace the outer contour of the neurosphere using the freehand line tool. The freehand line can be accessed by right-clicking on the "straight" line icon. Manually trace using a mouse.
   3. Use the measure function to calculate the area of the trace. Ensure that "area" is selected as a read-out in the "Set Measurements" window found under the Analyze tab. See **Figure 6** for trace of outer contour in blue.
   4. Trace the inner cell mass of the sphere and measure area. See **Figure 6** for trace of inner contour in red. Quantify average migration by subtracting the inner cell mass from the total neurosphere area.
   5. Measure neurospheres that exhibit a densely packed inner cell mass with cells migrating out as a contiguous carpet (**Figure 6**).
   6. Do not include cells outside of the carpet or detached from the neurosphere for measurement. See **Figure 6** for examples of cells (circled in white) that are excluded from the outer carpet measurement. Analyze a minimum of 20 neurospheres for each condition.

Representative Results

One goal of these studies is to define the proliferative activity of the NPCs, that is, an increase in cell numbers. This is achieved by assessing DNA synthesis of the total cell population, a high-throughput approach that measures the incorporation of radioactive tracer tritiated thymidine into cell extracts, and reflects all cells engaged in S-phase, whether they are synthesizing for 5 minutes or the entire two hours. Additionally, these assays allow the determination of the proportion of cells that enter S-phase and total cell numbers, a more labor-intensive assay of single cells. Cells synthesize DNA in S-phase, a step that precedes mitosis and cell division, which must occur in order to increase cell numbers. Since these processes take some time, changes in DNA synthesis assessed at 48 hours may not be associated with changes in cell numbers at this time point. Nonetheless, it was found that changes in DNA synthesis at 48 hours reliably predict increases of cell numbers at days 4 and 6.

In assessing DNA synthesis, cells are plated at a density of 100,000 cells (~50% confluent) in a 24-well plate and are allowed to grow for 48 hours before making measurements. Using this density ensures that the cells resemble their monolayer environment, but also do not grow so quickly in a 48 h period that the media becomes too acidic. Media that is too acidic can significantly affect cell metabolism and thus, alter proliferation results. If specific cell lines are highly proliferative, the researcher should consider altering cell density, media volume, or media exchange frequency to prevent highly acidic conditions. If conditions are changed, it is critical to be consistent when comparing different cell lines because cell-to-cell contact dependent changes certainly affect growth rates. The straightforward design of these assays allows us to test different growth factors. As seen in **Figure 7**, the addition of fibroblast growth factor (FGF, 10 ng/mL) for 48 hours increases DNA synthesis by ~40%. Furthermore, the DNA synthesis assay is reproducible as all clones and individuals show an increase in DNA synthesis after FGF stimulation. The potential for variability of baseline and FGF stimulated DNA synthesis among different unaffected individuals, as well as the potential for clonal variability in the same individual is demonstrated in **Table 1**. Due to this variability, it is important to test multiple iPSC clones per individual, as well as assess a minimum of 3 to 5 NPC lines derived from each different iPSC clone. A minimum of 3 experiments for each NPC line was performed.

The DNA synthesis assay allows us to rapidly assess numerous experimental groups in a high-throughput fashion and the measure reflects the sum total of DNA synthesis regardless of S-phase duration (5 minutes to 2 hours). To define the proportion of cells engaged in S-phase, the S-phase entry assay was employed. For this assay, cells are grown at the same density as previously mentioned to allow monolayer-like dynamics to occur, but then they are dissociated after 2 days and allowed to briefly adhere to plates to conduct single-cell analysis. Counting cells in a monolayer can be difficult due to high cell-to-cell contact and regional variability in the plate. This paradigm allows us to model the cells as a monolayer and then analyze them as single cells. It also acts as a methodologically independent confirmation of data obtained in the DNA synthesis assay. As seen in **Figure 8**, 48 hours of FGF (10 ng/mL) stimulation increases the proportion of cells entering S-phase by ~25%.

In assessing cell numbers, a lower cell density is used than in the aforementioned assays, with 50,000 cells being plated per well of a 24 well plate. Again, this density was chosen to ensure that faster cell lines do not grow so quickly over the 6-day period that the pH of the medium becomes too acidic and turns yellow. In **Figure 9**, while cell numbers may not be significantly different between control and FGF (10 ng/mL) groups at 2 days, the changes in DNA synthesis at 48 h (**Figure 7**) are predictive of changes in cell numbers at 4 and 6 days.

While NPCs are typically cultured at high density, the neurite assay is conducted at a density of 50,000 cells plated into a 35-mm dish in order to assess single cells. Even at this low density, the NPC cultures express cytoskeletal proteins and transcription factors characteristic of NPCs such as NESTIN, SOX2, and PAX6 (**Figure 10 A-D**). This indicates that a low-density culturing does not significantly alter cell fate in this time frame. Moreover, similar low-density conditions have been used in the rat and mouse culture systems to detect phenotypes that were ultimately reproducible *in vivo*[16,17,18,19,20,21,22,23]. After 48 hours of incubation, a small proportion of NPCs begin to extend neurites as seen in **Figure 11A** and **B,** and quantified in the graph in **Figure 11C**. The proportion of cells that extend neurites, the length of neurites, and the number of neurites/cell can be measured to assess developmental parameters. In order to accurately assess the percentage of cells with neurite outgrowth, it is important that cells are plated as single cells or clusters of <5 cells and not as large aggregates. As seen in **Figure 10 E-F**, cells bearing neurites (white arrow) also express immature neuronal marker beta-III tubulin (TUJ1). As mentioned in the methods, fluorescent images can be acquired of TUJ1 stained NPCs and then these images can be counted for the proportion of TUJ1+ neurites to ensure neuronal origin of processes. In our lab, analyses by either method have yielded statistically similar results.

The simple design and the rapid nature of the neurite assay also allow us to test the effects of developmentally relevant growth factors, cytokines, and peptides. For example, **Figure 11** shows that the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP, 3 nM) increases neurite outgrowth in NPCs. PACAP is an important developmental factor that has wide expression in the CNS and has been shown to be important in brain development. Rodent studies in our lab and other labs have found that PACAP has widespread stage-dependent developmental effects such as regulating neurite outgrowth, migration, and proliferation in both the hindbrain and forebrain[16,22,29,30,31]. Recent studies by Ataman *et al.* (2016) using cultured human fetal cortical cells indicate that neuronal activity induces a 9-fold increase in PACAP gene expression, indicating the peptides' importance in human neuronal development[32]. Indeed, **Table 2** shows the percentage of neurites in control and PACAP (3 nM) conditions between numerous lines derived from unaffected individuals. As seen in **Table 2**, there is some variability in the percentage of neurites expressed in cell lines derived from different clones from the same individual and from NPCs derived from different individuals. However, these unaffected individuals have an increase in neurite outgrowth in response to PACAP, indicating the reproducibility of the assays.

Like neurite outgrowth, cell migration is an important developmental process essential for the proper connection, organization, and wiring of the brain. Neurospheres allow us to study NPC migration in a typical high-density condition that maintains cell-cell contact amongst NPCs (**Figure 12**). Developmentally relevant factors can also be tested on neurospheres to assess their effects on migration. For example, **Figure 12** shows that PACAP (10 nM) increases migration of NPCs.

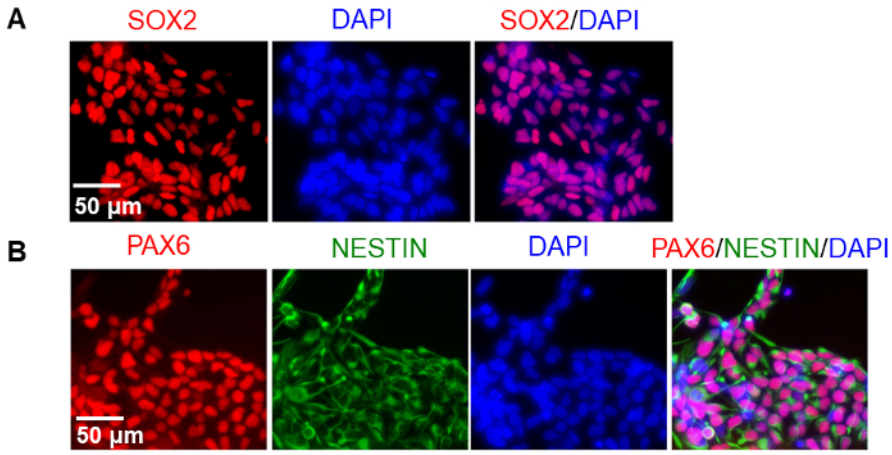

Figure 1: NPCs at passage 3. NPCs at P3 express multiple stage-specific markers including **(A)** pluripotent transcription factor, SOX2 **(B)** transcription factor specific for forebrain NPCs, PAX6, and NPC cytoskeletal protein NESTIN. Please click here to view a larger version of this figure.

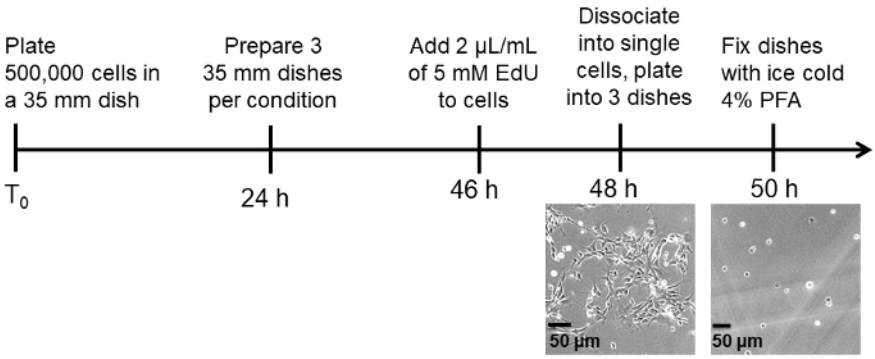

Figure 2: Schematic of S-phase entry assay. Timeline of S-phase Entry Assay. Please click here to view a larger version of this figure.

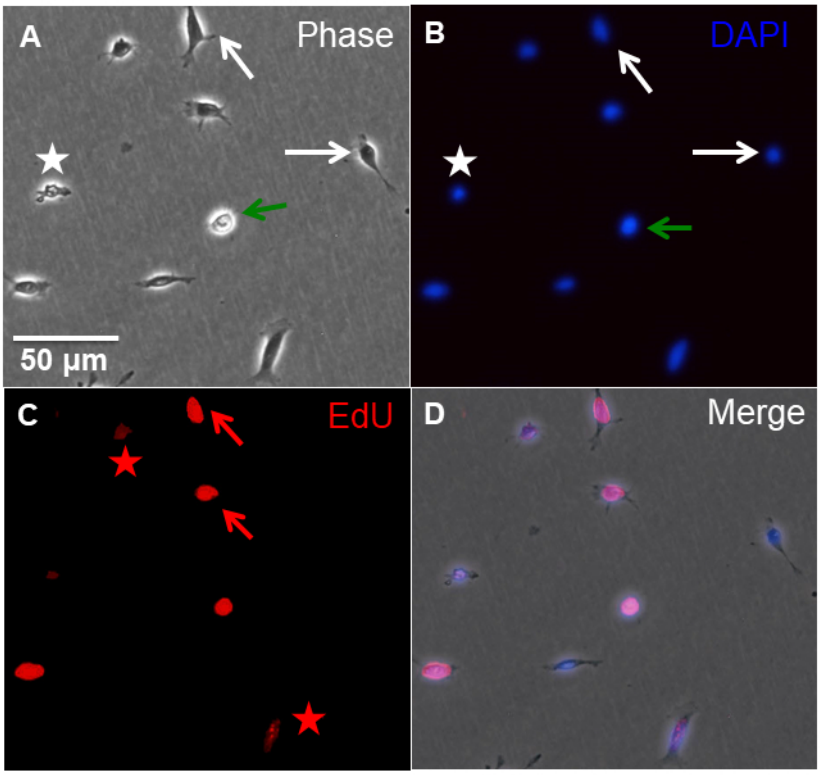

Figure 3: Quantifying S-phase entry. (A) Phase image showing phase-dark live cells (white arrows), a phase-bright dead cell (white star) and a phase-bright live cell (green arrow). **(B)** Fluorescent DAPI stain showing condensed nucleus in a dead cell (white star) and large nuclei in a live cell (white and green arrows). **(C)** Fluorescent EdU image showing bright EdU positive nuclei (red arrow) and speckled EdU positive nuclei (red star). **(D)** Phase and fluorescent merge of images 3A-3C.

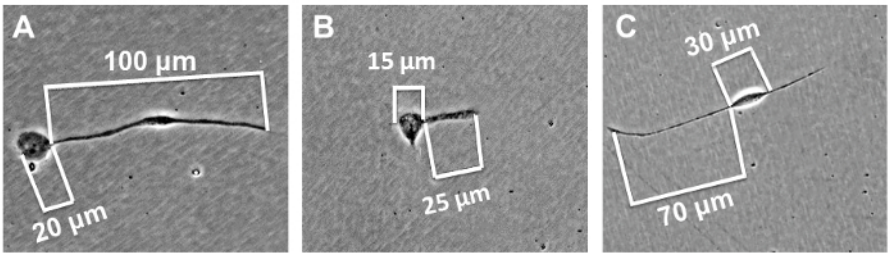

Figure 4: Identifying neurites. Phase-contrast images of NPCs. **(A)** A cell with a process >2 cell bodies in length, thereby meeting the criterion for a neurite. **(B)** A cell with a process <2 cell bodies in length, and therefore not considered neurite-bearing. **(C)** Represents a cell with 2 processes- the longer process is assessed for the neurite criterion. Please click here to view a larger version of this figure.

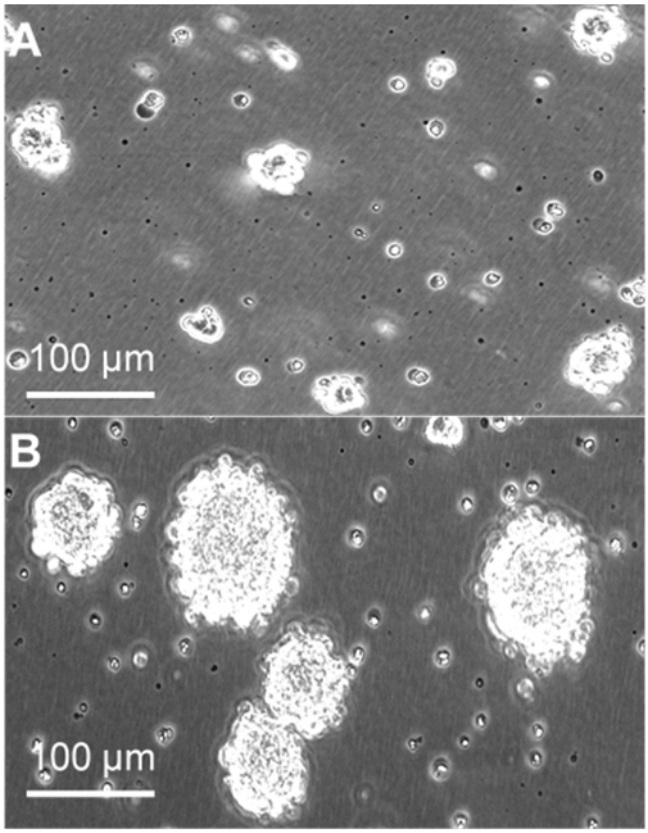

Figure 5: Selecting neurospheres for migration assay. (A) Phase contrast image of a representative field of neurospheres at 24 h. Spheres are all less than 100 µm and thus, are not collected for the migration assay. **(B)** Phase contrast image of a representative field of neurospheres at 72 h. All spheres are within the 100 µm ± 20 µm range, indicating they are ready to be collected for the migration assay.

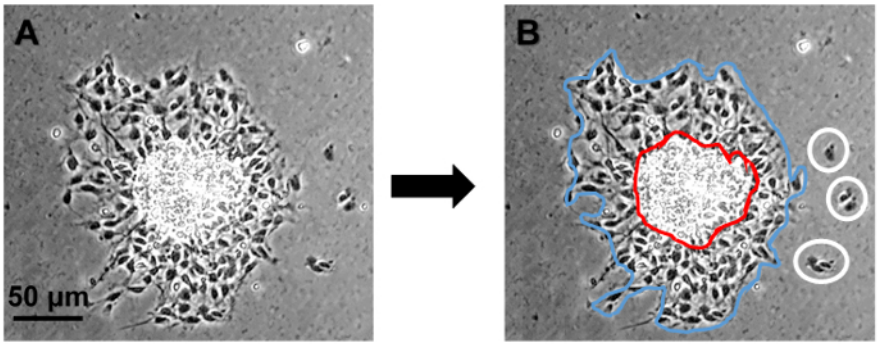

Figure 6: Quantifying Migration. (A) Phase contrast image of a representative neurosphere. **(B)** Blue outline displays the trace to measure total neurosphere area. Red shows the contour used to measure the area of the inner cell mass. Migration is defined as total neurosphere area-inner cell mass area. Note, the white circles show cells that are not in a contiguous carpet, as these cells are excluded from the migration contours.
Please click here to view a larger version of this figure.

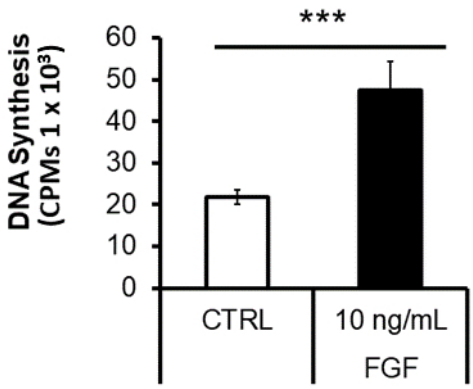

Figure 7: Assessing DNA Synthesis. Representative results of control versus FGF-treated NPCs. FGF (10 ng/mL) increases DNA synthesis at 48 h ($p \leq 1 \times 10^{-3}$). (n = 2 - 4 wells/group/experiment; 3 experiments). Error bars represent SEM.

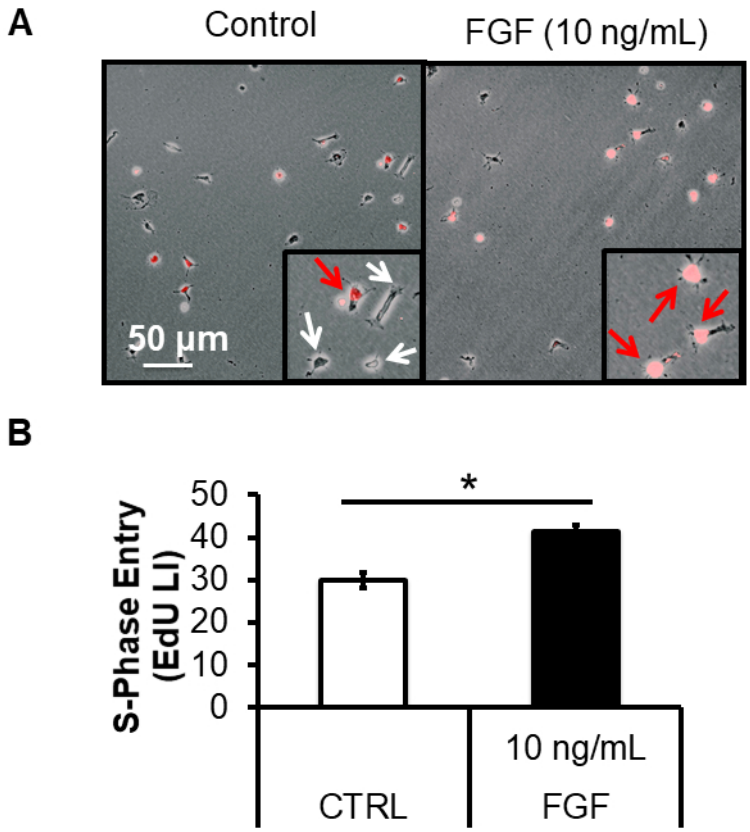

Figure 8: Proportion of Cells Entering S-Phase. (A) Phase contrast images of NPC cells incubated in control versus FGF (10 ng/mL) treated media for 48 h. Insets represent higher magnification images of cells stained for fluorescent EdU marker in control and 10 ng/mL FGF media. Red arrows indicate cells that are EdU positive and white arrows indicate cells that are EdU negative. **(B)** Graph of representative results of control versus FGF (10 ng/mL) treated NPCs. FGF increases S-phase entry at 48 h ($p \leq 1 \times 10^{-3}$). (n = 2 - 4 dishes/group/experiment; 3 experiments). Error bars represent SEM.

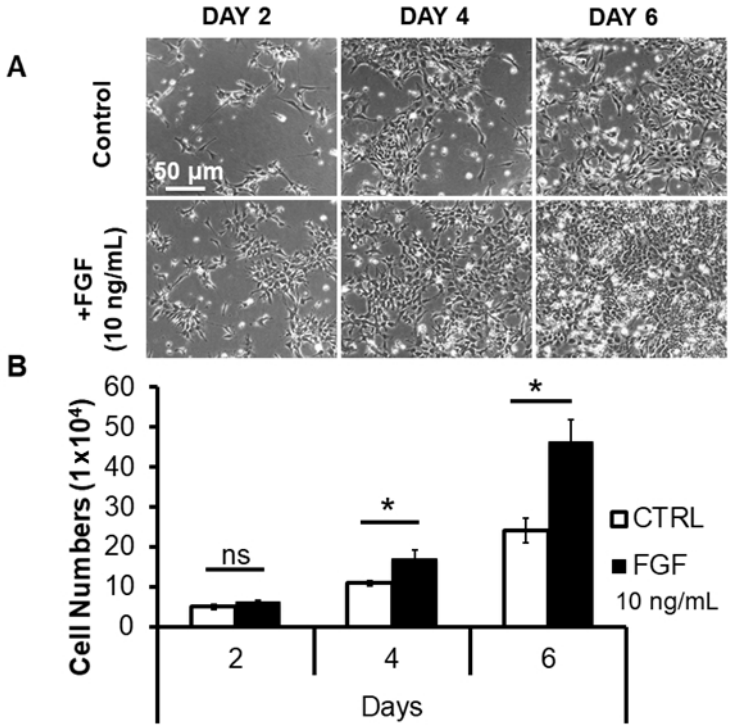

Figure 9: Enumerating cells at Days 2, 4, and 6. (A) Phase contrast images of cells in control and FGF (10 ng/mL) treated media at Days 2, 4, and 6. **(B)** Graph of representative results; note that FGF (10 ng/mL) does not always increase cell numbers at 2 days, but at 4 and 6 days increases are apparent ($p \leq 0.05$). (n = 2 - 4 wells/group/experiment; 3 experiments). Error bars represent SEM.

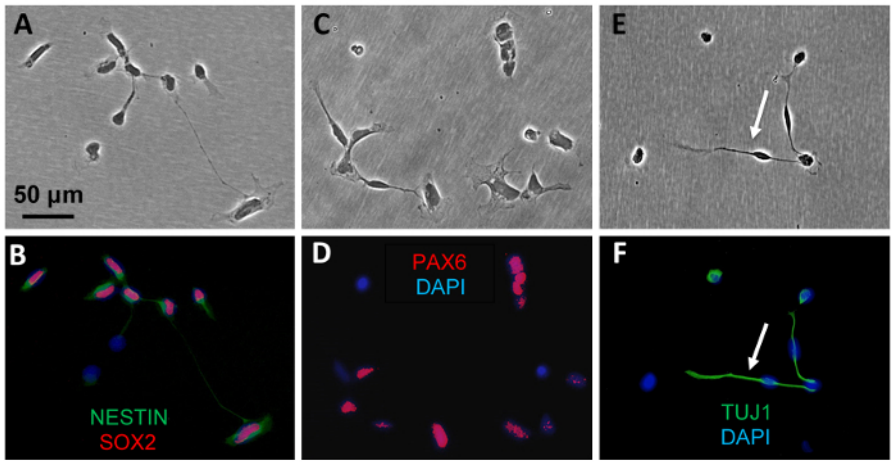

Figure 10: Characterization of NPCs in low density conditions. (A, C, E) Phase contrast images of NPCs in low-density conditions. **(B)** NPCs express stem/progenitor cell markers NESTIN (green), SOX2 (red), and nuclear marker DAPI (Blue). **(D)** PAX6 (Red), DAPI (Blue). **(F)** At low-density, cells extending neurites (white arrow) also express immature neuron marker TUJ1 (green). Please click here to view a larger version of this figure.

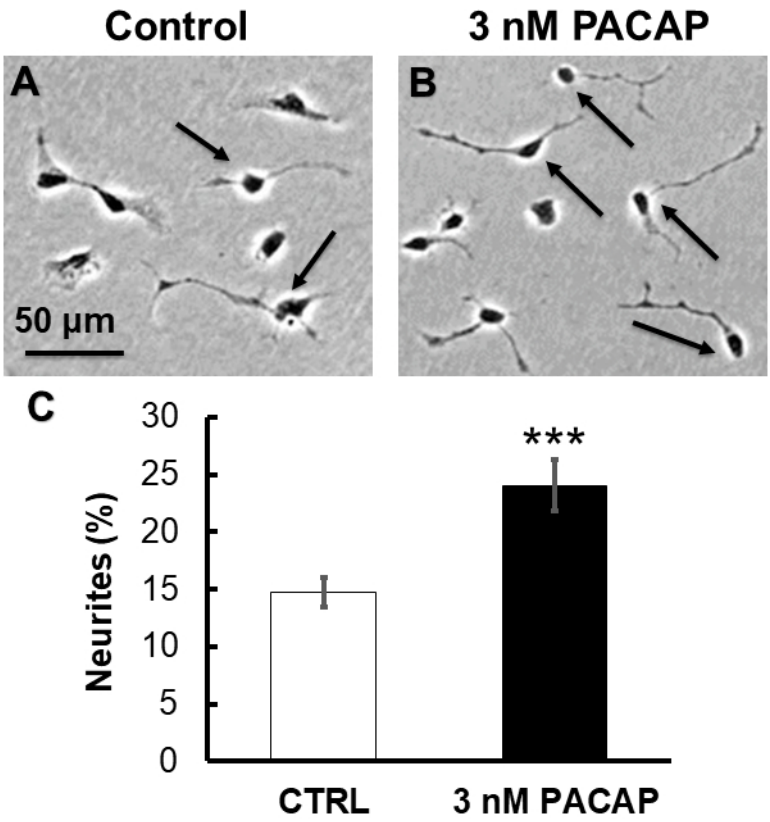

Figure 11: Neurite outgrowth. (A) Phase contrast image of NPCs. The black arrows point to the cells with neurites. **(B)** Addition of neuropeptide PACAP (3 nM) increases the percent of cells with neurites ($p \leq 1 \times 10^{-2}$). **(C)** Quantification of neurite outgrowth in control and 3 nM PACAP containing media. (n = 2 - 4 dishes/group/experiment; 3 experiments). Error bars represent SEM.

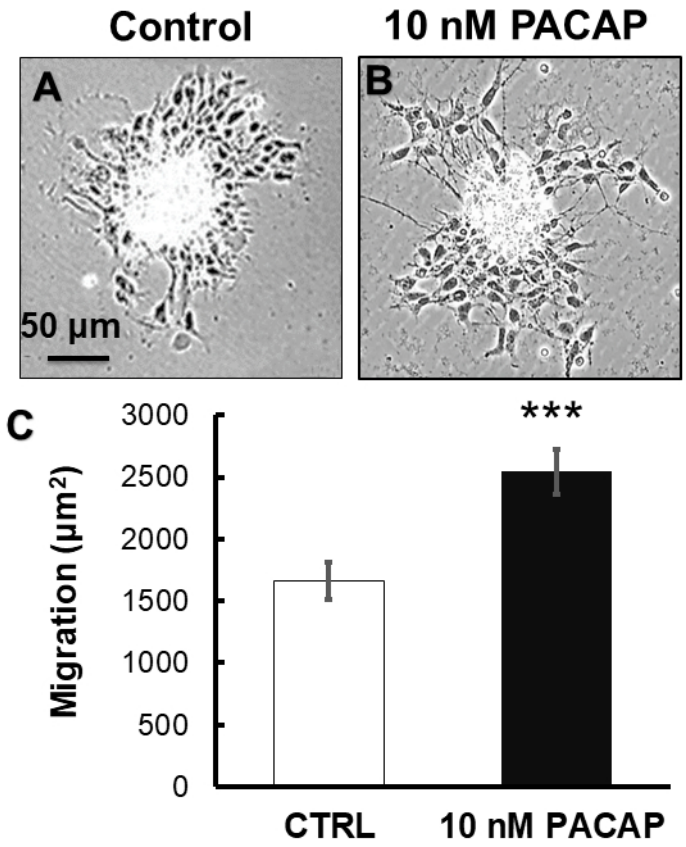

Figure 12: Neurosphere Migration. (A) Phase contrast images of neurospheres. **(B)** Addition of PACAP (10 nM) increases neurosphere cell migration ($p \leq 10^{-2}$) **(C)** Quantification of cell migration. (n = 20 spheres/group/experiment, 3 experiments). Error bars represent SEM.

		Media	
Patient	**Clone #**	**CTRL**	**FGF**
			(10 ng/mL)
Patient 1	1	21,853	47,538
	2	20,336	38,070
Patient 2	1	7,664	14,060
	2	16,573	30,087

Table 1: DNA Synthesis. A summary of the DNA synthesis values (in CPMs) of NPCs derived from two iPSC clones per two unaffected individuals.

		Media	
Patient	**Clone #**	**CTRL**	**PACAP**
			(3 nM)
Patient 1	1	13.60%	18.10%
	2	16.50%	21.10%
Patient 2	1	8.90%	14.10%
	2	14.20%	21.10%

Table 2: Percentage of Cells with Neurites. A summary of the percentage of cells bearing neurites in NPCs derived from two iPSC clones per two unaffected individuals.

Discussion

The protocols presented here illustrate quick and simple methods to study fundamental neurodevelopmental processes and test growth factors and drugs using hiPSC-derived neural precursor cells. hiPSC technology has revolutionized the study of the pathogenesis of neurodevelopmental diseases by providing us with unprecedented access to live human neuronal cells from affected individuals. Indeed, there

have been numerous hiPSC studies of neurodevelopmental disorders including Rett Syndrome, Timothy Syndrome, Fragile-X syndrome, and schizophrenia, which have unearthed disease-specific aberrations in dendrites, synapses, and neuronal function[4,33,34,35,36]. Most of these studies have primarily focused on terminally differentiated, post-mitotic neurons which, though considered relatively functionally immature, excludes the study of earlier neurodevelopmental processes such as proliferation and migration. These latter processes have been heavily implicated in the pathogenesis of neurodevelopmental disorders and warrant further study[8,9,10,11,12,35,37,38]. The use of NPCs allows us to study these important earlier events while also providing the opportunity to investigate more mature processes like the ability of cells to extend immature axons/dendrites (neurites). Further, some of these assays can also be extended to study other parameters, such as neurite length, number, and branching or the furthest distance traveled by a cell.

Some newer studies have used organoid model systems to study earlier developmental events in a 3-D "mini-brain system"[1,39,40]. Yet, even in these organoid systems, the proliferative precursor cell population is limited and early maturation and migration are difficult to study[15,39]. In addition to limiting the study of earlier developmental phenomena, the use of terminally differentiated neurons or organoids is often time-consuming, costly, and limits the number of variables that can be assessed in the system. This is because making neurons and organoids may require viral induction protocols, special incubators and equipment, multiple weeks of time, and large quantities of media. In contrast, with the exception of the DNA synthesis assay (which is addressed later below), this protocol can be readily applied to the study of neurodevelopmental disorders and does not require extensive training, costly tools, and resources, or software. The ease and relatively low cost of adding drugs and growth factors in these assays, make this protocol a useful high-throughput technology to test various potential treatments for neurodevelopmental and neuropsychiatric diseases. Moreover, since growth factors act *via* defined cellular signaling pathways, they can also be used as tools to test for potential signaling defects in developing systems. Finally, since NPCs are a proliferative self-renewing population, large quantities of cells can be produced and cryopreserved allowing experiments to be conducted efficiently without having to make NPCs from iPSCs every time.

To successfully employ these assays, it is important to note the following critical steps. This protocol places NPCs in differing conditions for maintenance and expansion versus experimentation. Specifically, while the NPCs are induced, grown, and passaged in medium containing Neural Induction Supplement (NIS), our experimental conditions reduce the supplement by 70%, which places cells in a limiting environment, allowing us the opportunity to add back and test the effects of important growth factors. Secondly, it is essential to keep track of the passage of the NPCs. In our studies, we have generally restricted passage number of the cell lines from P3 - P8. In passages earlier than P3, some lines do not robustly express characteristic markers. At higher passages, while some cell lines have very consistent growth rates or responses to growth factors, other cell lines may have dramatic changes in cell growth or response. Though not routinely reported, we, and many others have experienced this dramatic change in proliferative rates at higher passages. The reason for this is uncertain, but this change may reflect a limited self-renewal capability of NPCs. Defining why and how proliferation rates change over extended passages may provide insights into development and disease pathogenesis, but further research will need to be done. Finally, the commercial neural induction protocol that we are using can sometimes yield poor quality neural stem cells, particularly if the starting iPSCs are not of high quality (*i.e.*, have differentiating cells on the borders of cell colonies, karyotype abnormalities). In some cases, cell morphology is distorted and expression of markers is not present. Do not use these cultures. In other cases, NPCs grow with flatter "contaminant" cells, which can be removed using a differential cell detachment solution treatment to ensure virtually pure NPC populations before use in experiments. Having high-quality NPCs is critical for proper results: see the Materials and Equipment section for a link to a Neural Induction Protocol where images of high and low-quality NPCs can be found.

For each of the assays presented, it is important to note the following critical steps, potential errors, and troubleshooting tips. For the cell number, DNA synthesis, and neurite assays, it is important to plate cells as dissociated single cells and not as clumps, as this can skew DNA synthesis measures, cell counts, and neurite behavior. To ensure clumps of cells are not plated, sample a small volume of cells with a P1000, plate on a slide, and check if cell clumps are present. If clumps are noted, pipette cells up and down to manually break apart clumps before plating. For the DNA synthesis and the cell number assay, cells are lifted with enzymes for counting and analysis. It is critical to visually confirm cells have completely lifted from the culture vessel to get accurate counts and if required, longer enzyme incubation periods can be used. In the case of low cell counts or low CPMs for the DNA synthesis assay, cells can be plated at higher initial densities, radioactive tritiated [^{3}H]-thymidine can be added for double the time (4 hours instead of 2), or tritiated [^{3}H]-thymidine concentration can be doubled. For the neurite assay, initial plating density can be doubled without risking increased cell-to-cell contact. Pay attention to the distribution of cells across a dish and count 1 cm rows such that each part of the dish is sampled. If the neurite percentage is too low at 48 h, the assay can be extended up to 6 days or the coating substrate type or concentration can be changed to promote greater percentage of neurites. However, it is important to note that the coating times and methods we have presented in the protocol were selected for optimal neurite outgrowth and cell health after testing numerous different substrates, substrate concentrations, and coating times. For the neurosphere assay, use of smaller pipettors (P20, P200) can lead to the shearing and breakage of larger spheres. Thus, it is imperative that pipetting is done gently and with a P1000 or a serological pipette. For pelleting the neurospheres, lower speeds (100 x g instead of 300 x g) are also recommended to prevent neurosphere dissociation. During sphere plating, ensure that spheres are appropriately spaced apart as sphere-to-sphere contact can influence migration. In cases where migration is too fast or too slow, incubation time can be decreased or increased respectively. Coating substrates can also be altered to change migration rates.

While the techniques used are rapid, simple and applicable to the study of neurodevelopmental disorders, there are certain limitations. For one, many of the analyses presented (cell number assay, neurite assay, migration assay) require investigators to make subjective decisions (*e.g.*, Is this a neurite? Is this cell dead?) potentially leading to investigator bias and lower reproducibility. However, conducting analyses blind and setting strict standards for each decision made within an assay, as illustrated in the methods, can ameliorate these biases. Similarly, these assays require manual measurements and counts, which can be time-consuming and labor intensive. However, in labs that have the equipment and technical resources, these assays can be sped up with the use of automated cell counters and programs that can conduct automated measurements[41,42]. In the case of the DNA synthesis assay, these methods are specific to our cell harvester and scintillation machine (see Materials and Equipment); however, there are other available models and methods that can be used to gain the same information, such as the Omnifilter-95 cell harvester. For some institutions, the use of radioactive sources may not be feasible. In this case, an alternative method using a fluorescent thymidine analog, such as EdU, analyzed on a fluorescent microplate reader, will allow for acquisition of the same information on bulk analysis of DNA synthesis[44].

Our low-density culture system separates NPCs into individual cells or small clumps, a condition that differs from the densely packed nature of NPCs in the developing neural tube. Yet, the NPCs are healthy and express appropriate markers (**Figure 1, Figure 10**). Moreover, our prior

studies of mouse and rat cortical cultures showing parallel findings in *in vitro* cultures, and *in vivo* models indicate the utility and value of using this approach[16,17,18,19,24]. Additionally, this system provides a powerful approach to understand maturation of cells and study cell sub-populations. For example, immunohistochemistry can be conducted on the neurite assay to determine what specific neuronal cell type is extending a neurite. Ultimately, despite some limitations, this unique protocol provides straightforward, powerful, and rapid methods to study neurodevelopmental disorders.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

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