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Generation of Regionally Specific Neural Progenitor Cells (NPCs) and Neurons from Human Pluripotent Stem Cells (hPSCs)

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Abstract

Neural progenitor cells (NPCs) derived from human pluripotent stem cells (hPSCs) are a multipotent cell population capable of long-term expansion and differentiation into a variety of neuronal subtypes. As such, NPCs have tremendous potential for disease modeling, drug screening, and regenerative medicine. Current methods for the generation of NPCs results in cell populations homogenous for pan-neural markers such as SOX1 and SOX2 but heterogeneous with respect to regional identity. In order to use NPCs and their neuronal derivatives to investigate mechanisms of neurological disorders and develop more physiologically relevant disease models, methods for generation of regionally specific NPCs and neurons are needed. Here, we describe a protocol in which exogenous manipulation of WNT signaling, through either activation or inhibition, during neural differentiation of hPSCs, promotes the formation of regionally homogenous NPCs and neuronal cultures. In addition, we provide methods to monitor and characterize the efficiency of hPSC differentiation to these regionally specific cell identities.

Keywords: Human pluripotent stem cells, Neural progenitor cells, Neurons, Anterior–posterior patterning, Disease modeling, Drug screening, Regenerative medicine

1 Introduction

Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and spinal muscular atrophy (SMA) currently affect approximately seven million Americans [1]. The already large burden these diseases have on families and the economy will continue to grow with the increasing proportion and size of the aged population, with costs for Alzheimer's disease and other dementias estimated to rise from $226 billion in 2015 to more than $1 trillion in 2050 [2]. Unfortunately, the etiology and pathophysiology of these devastating diseases remains incompletely understood limiting current therapeutic options to mainly palliative in nature.

Although the pathology of these diseases remains elusive, distinctive hallmarks include the degeneration of regionally specific neurons [3]. For example, AD largely affects neurons in the cortex,

hippocampus, and amygdala [4]. By comparison, PD results in the dysfunction of dopaminergic neurons in the substantia nigra of the midbrain [5] while ALS affects motor neurons in the hindbrain and spinal cord [6]. Because the cognitive and motor decline that is associated with these specific CNS diseases is due to the loss or dysfunction of specific neuronal subtypes, the ability to generate neuronal cultures of specific identity is critical in using hPSC-derived neurons to model and potentially treat neurodegenerative diseases.

Studies in model organisms, such as flies, worms, zebrafish, and mice have provided important insights into the role of a variety of signaling pathways in early neural development and patterning. From these studies, a general model for neural patterning has been proposed in which naïve ectodermal cells acquire an initial anterior neural identity through BMP antagonism and a subsequent WNT signaling gradient is responsible for inducing specific regional identities in these anterior cell types [7, 8]. Further examination of neural patterning in model organisms has led to the conclusion that a gradient of WNT signaling specifies neural cell identity along the anterior–posterior (A/P) axis. Specifically, loss-of-function studies in mice demonstrate that WNT-deficient embryos display posterior truncation (i.e., midbrain, hindbrain, and spinal cord structures fail to develop properly) with a significant expansion of the forebrain compartment [9–15]. On the other hand, gain-of-function in which the WNT pathway is ectopically activated leads to suppression of anterior fates and expansion of posterior neural markers [8, 16–20].

Here, we describe a recently published neural differentiation protocol based on the modulation of TGF-β and WNT signaling that allows for the differentiation of hPSCs into NPCs with specific A/P regional identity [21]. These regionally specified NPCs can be expanded and subsequently differentiated into cortical, midbrain dopaminergic, or spinal motor neurons. Overall, these differentiation methods will aid in the translation of these cell types for disease modeling, drug screening, and regenerative medicine therapies.

2 Materials

2.1 Equipment and Supplies

1. Biological Safety Cabinet.
2. CO_2 incubator with humidity and gas controls to maintain a stable environment of 37 °C, >95 % humidity, and 5 % CO_2.
3. Water bath set at 37 °C.
4. Benchtop cell culture centrifuge.
5. Orbital shaker (incubator safe).
6. Pipet Controller.

7. Serological pipettes (5, 10, and 25 ml).
8. 10-, 20-, 200-, and 1000-μl micropipettes.
9. 10-, 20-, 200-, and 1000-μl micropipette tips.
10. Tissue culture treated polystyrene dishes: 6-well, 12-well, and 24-well and 100 mm.
11. Ultra-low attachment 6-well multi-well plates (Bioexpress., cat. no. T-3326-1).
12. 1.5 ml microcentrifuge tubes.
13. Polystyrene conical tubes: 15- and 50-ml.
14. Hemacytometer.
15. Inverted light microscope with 4× and 10× phase objectives.
16. Polyethylene cell lifter (Corning Inc., cat.no. 3008).
17. Biospec Nano (Shimadzu Biotech) or comparable spectrophotometer.
18. C1000 Touch Thermal Cycler with 384 well reaction module (Biorad cat.no. 1851138).

2.2 Stock Solutions and Reagents

1. Essential 8™ animal protein free, defined, feeder-independent medium for maintenance of undifferentiated, human ESCs and iPSCs (Thermo Fisher Scientific, Cat. No. A1517001).
2. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Life Technologies, cat.no. 11320-033).
3. Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, cat no. 11965-118).
4. 100× N-2 supplement (Life Technologies; cat.no. 17502-048).
5. 50× B-27 serum-free supplement (Life Technologies, cat.no. 17504-044).
6. Penicillin–Streptomycin (P/S) 5000 U/ml (Life Technologies; cat.no. 15070-063). Make aliquots of 5 ml and store at −20 °C.
7. GlutaMAX™ supplement (Life Technologies; cat.no. 35050-061). Make aliquots of 5 ml and store at −20 °C.
8. Phosphate-Buffered Saline (PBS), pH 7.4 (Life Technologies, cat.no. 10010023).
9. StemPro® Accutase® cell dissociation reagent (Life Technologies, cat.no. A1110501). Make aliquots of 5 ml and store at −20 °C.
10. Matrigel™, Growth Factor Reduced (BD Biosciences, cat.no. 354230). Make aliquots per lot instructions and store at −20 °C.
11. Rho-associated protein kinase inhibitor (ROCKi, Y-27632; EMD Millipore, cat.no. SCM075). Dissolve in DMSO at a

concentration of 5 mM. Make aliquots of 50 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. Protect from light. A final concentration of 5 μM will be used for the first day after passaging hPSCs and the first day of embryoid body (EB) formation.

12. Human recombinant Noggin (R&D Systems, cat.no. 6057-NG). Reconstitute in sterile, distilled water at a concentration of 200 μg/ml. Make aliquots of 25 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. A final concentration of 50 ng/ml will be used during NPC generation.
13. Dorsomorphin dihydrochloride (DM; Tocris Biosciences, cat. no. 3093). Dissolve in DMSO at a concentration of 25 mM. Make aliquots of 10 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. A final concentration of 0.5 μM will be used during NPC generation.
14. Human recombinant bFGF (Life Technologies, cat.no. PHG6014). Reconstitute in sterile, distilled water at a concentration of 30 μg/ml. Make aliquots of 50 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. A final concentration of 30 ng/ml will be used to culture and expand NPCs.
15. Human recombinant EGF (R&D Systems, cat.no. 236-EG). Reconstitute in sterile, distilled water at a concentration of 30 μg/ml. Make aliquots of 50 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. A final concentration of 30 ng/ml will be used to culture and expand NPCs.
16. Human recombinant BDNF (R&D Systems, cat.no. 248-BD-005). Reconstitute in sterile, distilled water at a concentration of 20 μg/ml. Make aliquots of 25 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. A final concentration of 20 ng/ml will be used during the 4 weeks of neuronal differentiation.
17. Human recombinant GDNF (R&D Systems, cat.no. 212-GD). Reconstitute in sterile, distilled water at a concentration of 20 μg/ml. Make aliquots of 25 μl in 1.5 ml microcentrifuge tubes and store at −20 °C. A final concentration of 20 ng/ml will be used during the 4 weeks of neuronal differentiation.
18. *N*-[(3,5-Diflurophenyl)acetyl-]-L-alanyl-2-phenyl]gylcine-1,1-dimethlethyl ester (DAPT) γ-secretase inhibitor (Tocris Biosciences; cat.no. 2634). Dissolve in DMSO at a concentration of 5 mM. Make aliquots of 50 μl and store at −20 °C. A final concentration of 1.0 μM will be used during the 4 weeks of neuronal differentiation.
19. N6, 2′-O-Dibutyryladenosine 30, 50-cyclic monophosphate sodium salt (db-cAMP; Sigma, cat.no. D0260). Dissolve in sterile, distilled water at a concentration of 25 mM. Make aliquots of 1.0 ml and store at −20 °C. A final concentration

of 0.5 mM will be used during the 4 weeks of neuronal differentiation.

20. Poly-L-ornithine 10 mg/ml solution (PLO; Sigma, cat.no. P4957). Make aliquots of 1.0 ml in 1.5 ml microcentrifuge tubes and store at 4 °C.
21. Laminin (LN) from Engelbreth-Holm-Swarm murine sarcoma basement membrane, 1 mg/ml solution (Sigma, cat.no. L2020). Make aliquots of 1.0 ml in 1.5 ml microcentrifuge tubes and store at −20 °C.
22. N6-[2-[[4-(2,4-Dichlorophenyl)-5-(1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]-3-nitro-2,6-pyridinediamine (CHIR 98014) glycogen synthase kinase-3 (GSK-3) inhibitor (Sigma-Aldrich, cat.no. SML1094-5MG). Dissolve in DMSO at a concentration of 10 mM. Make aliquots of 50 μl and store at −20 °C.
23. *N*-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2 d]pyrimidin-2-yl)thio]-acetamide (IWP2) WNT inhibitor (Stemgent, cat.no. 04-0034). Dissolve in DMSO at a concentration of 5 mM. Make aliquots and store at −20 °C.
24. Nucelospin RNA Isolation Kit (Macherey-Nagel, cat. no. 740955.25).
25. TaqMan® Gene Expression Mastermix (Life Technologies, cat. no. 4369016).
26. β-Mercaptoethanol (Life Technologies, cat. no. 21985-023).
27. Ethyl alcohol, pure, Molecular Biology Grade (Sigma, cat. no. E7023-500 ml).
28. Nuclease Free Water (Life Technologies, cat. no. AM9939).
29. BD Cytofix™ Fixation Buffer (BD Biosciences, cat. no. 554655).
30. BD Phosflow™ Perm Buffer III (BD Biosciences, cat. no. 558050).
31. Taqman® Gene Expression Assays (*see* Table 1).
32. Primary and Secondary Antibodies (*see* Table 2).
33. Hoechst 33342 10 mg/ml (Life Technologies, cat. no. H3570).

2.3 Medium

1. Neural Base Medium (NBM). Combine 500 ml Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Life Technologies, cat.no. 11320-033), 5 ml 50× B27 Supplement, 2.5 ml 100× N2 Supplement, 5 ml GlutaMAX, and 5 ml P/S.
2. Neural Induction Medium (NIM). In a 50 ml conical tube, combine 50 ml NBM, 12.5 μl of 200 μg/ml Noggin, and 1 μl

Table 1
Taqman® gene expression assays used for qPCR

Gene	Taman® gene expression assay
18s	Hs99999901_s1
CTIP2 (BCL11B)	Hs00256257_m1
CUX1	Hs00738851_m1
DLX2	Hs00269993_m1
EMX1	Hs00417957_m1
EN1	Hs00154977_m1
FOXG1	Hs01850784_s1
GATA2	Hs00231119_m1
GATA3	Hs00231122_m1
HOXA2	Hs00534579_m1
HOXB4	Hs00256884-m1
HOXB6	Hs00980016_m1
LMX1A	Hs00892663_m1
LMX1B	Hs00158750_m1
MNX1 (HB9)	Hs00907365_m1
NURR1 (NR4A2)	Hs00428691_m1
PITX3	Hs01013935_g1
SATB2	Hs00392652_m1
SIX3	Hs00193667_m1
TH	Hs00165941_m1

of 25 μM Dorsomorphin. The medium can be stored at 4 °C for up to 2 weeks.

3. Neural Expansion Medium (NEM). In a 50 ml conical tube, combine 50 ml NBM, 15 μl of 100 μg/μl FGF, and 15 μl of 100 μg/μl EGF. The medium can be stored at 4 °C for up to 2 weeks.
4. Neural Differentiation Medium (NDM). In a 50 ml conical tube, combine 50 ml Neural Base Medium, 50 μl of 20 ng/ml BDNF, 50 μl of 20 ng/ml GDNF, 10 μl of 5 mM DAPT, and 1.0 ml of 25 mM db-cAMP. The medium may be stored at 4 °C for up to 2 weeks.

Table 2
Primary and secondary antibodies used for immunofluorescence

Antibody	Vendor	Catalog #	Concentration used
Goat anti-SOX2	Santa Cruz	SC-17320	1:50
Goat anti-OTX2	R&D Systems	AF1979	1:200
Mouse anti-B3T	Fitzgerald	10R-T136A	1:1000
Mouse anti-MNX1	DSHB	81.5C10	1:100
Mouse anti-SOX1	BD	560749	1:10
Rabbit anti-FOXG1	Abcam	AB18259	1:100
Rabbit anti-HOXB4	Abcam	AB76093	1:10
Rabbit anti-LMX1A	Abcam	AB139726	1:100
Rabbit anti-NURR1	Millipore	AB5778	1:200
Rabbit anti-TBR1	Abcam	AB31940	1:200
Alexa 647 Donkey Anti-Goat	Life Technologies	A-21447	1:200
Alexa 647 Donkey Anti-Rabbit	Life Technologies	A-31573	1:200
Alexa 647 Donkey Anti-Mouse	Life Technologies	A-31571	1:200
Alexa 546 Donkey Anti-Goat	Life Technologies	A-11056	1:200
Alexa 546 Donkey Anti-Rabbit	Life Technologies	A-10040	1:200
Alexa 546 Donkey Anti-Mouse	Life Technologies	A-10036	1:200
Alexa 488 Donkey Anti-Goat	Life Technologies	A-11055	1:200
Alexa 488 Donkey Anti-Rabbit	Life Technologies	A-21206	1:200
Alexa 488 Donkey Anti-Mouse	Life Technologies	A-21202	1:200

3 Methods

An overview of the protocol for generation, expansion, and neuronal differentiation of regionally specific NPCs is presented in Fig. 1a. Although this protocol can be modified to generate NPCs of any A/P regional identity, we present methods to generate NPCs of (1) forebrain/cortical, (2) midbrain, and (3) hindbrain/spinal cord identity. These NPCs can be expanded and subsequently differentiated into (1) cortical, (2) midbrain GABAergic or dopaminergic, and (3) hindbrain or spinal motor neurons, respectively.

Undifferentiated hPSCs are grown in feeder-free conditions on Matrigel™-coated plates. Undifferentiated hPSCs are directed to the neural lineage through the stepwise formation of embryoid bodies (EBs; Fig. 1b left panel) and neuroepithelial-like rosettes

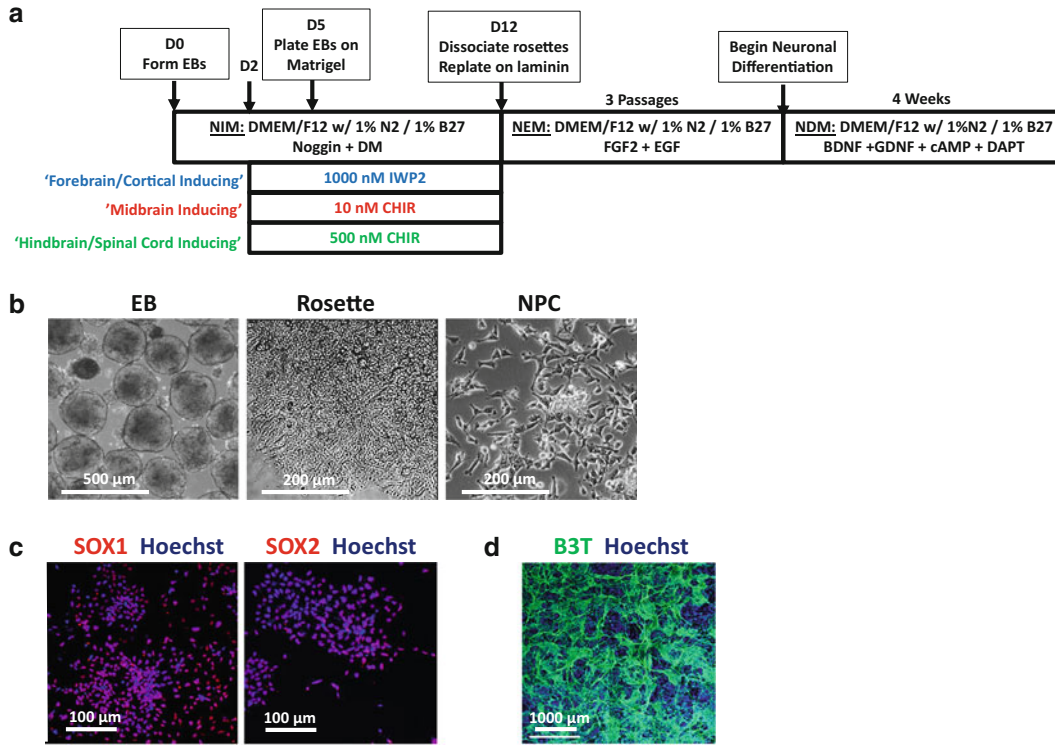

Fig. 1 Generation of regionally specific NPCs and neurons through exogenous manipulation of WNT signaling. (**a**) Outline of protocol to generation regionally specific NPCs and neurons from hPSCs. (**b**) Phase contrast images of EBs (*left panel*), neural rosettes (*center panel*), and NPCs (*right panel*). (**c**) Immunofluorescent analysis of NPC cultures for pan-neural markers SOX1 and SOX2. (**d**) Immunofluorescent analysis of neuronal cultures for pan-neuronal marker B3T. Figure and legend adapted with permission from [21]

(Fig. 1b center panel) in the presence of the TGF-β/BMP antagonists Noggin and Dorsomorphin. Patterning of these cells to specific A/P identities is achieved by fine tuning the level of WNT signaling activity through treatment of differentiating cultures with IWP2 (a small molecule that acts on PORCN to block WNT processing and secretion; [22] or increasing concentration of CHIR 98104 (CHIR; a potent inhibitor of GSK-3β and activator of the WNT pathway). Neural rosettes are enzymatically dissociated and replated on laminin (LN)-coated plates to form NPCs (Fig. 1b right panel). NPCs derived from this can be maintained as proliferative, multipotent SOX1+SOX2+ (Fig. 1c) cells in the presence of FGF2 and EGF on LN-coated plates. Subsequent differentiation of NPCs to B3T+ neurons (Fig. 1d) is achieved through the withdrawal of FGF2 and EGF and addition of BDNF, GDNF, db-cAMP, and DAPT. NPC and neuronal cell populations can be characterized by qPCR and immunofluorescence.

3.1 Maintenance of hPSCs in Feeder Free Conditions

1. hPSCs are maintained in feeder-free conditions on Matrigel™ coated 100 mm plates with E8 culture medium.
2. Prior to passaging hPSCs prepare a working solution of Matrigel™ by thawing one aliquot of Matrigel™ on ice (*see* **Note 1**).
3. Make a 1:25 dilution of Matrigel™ in cold DMEM in a 50 ml conical. The working solution of Matrigel™ should be maintained on ice and can be stored at 4 °C for up to 2 weeks.
4. Coat a 100 mm dish with 5 ml of Matrigel™ working solution per dish in a tissue culture hood and transfer to 37 °C incubator for 20 min (*see* **Note 2**).
5. Warm E8, DMEM, and Accutase® solution in 37 °C water bath.
6. Aspirate medium from 100 mm plate of 75 % confluent hPSCs and add 5 ml of Accutase®.
7. Incubate the plate in a CO_2 incubator for 5 min. After 5 min check cell dissociation under microscope to determine if additional incubation is required for cell detachment.
8. Add 5 ml of DMEM medium to the plate.
9. Using a 10 ml serological pipette, gently wash off the remaining cells and transfer the cell suspension to a 15 ml conical tube
10. Centrifuge the conical tube at 200 × *g* for 5 min.
11. Aspirate the supernatant and resuspend the cells in 5 ml E8 + 5 μl ROCKi. Gently pipette up and down 3–4 times with a 1000 μl pipette to break up any visible cell clumps.
12. Take 10 μl of the cell suspension to perform a cell count using a hemacytometer (*see* **Note 3**).
13. Using the cell count from the hemacytometer calculate the volume of cell suspension needed for 1.2×10^6 cells.
14. In a separate 15 ml conical add 9 ml E8, 9 μl ROCKi and the volume of cell suspension equivalent to 1.2×10^6 cells (*see* **Note 4**).
15. Aspirate Matrigel™ solution from previously coated 100 mm plate (*see* **Note 5**).
16. Add the cell suspension to the Matrigel™ coated plate.
17. Place the plate in the CO_2 incubator. Gently move the plate in several quick horizontal and vertical motions to disperse cells evenly across the cell culture surface.
18. Change the medium daily by aspirating the old medium and adding 10 ml fresh E8 (*see* **Note 6**). After 2–3 days, bright colonies should appear. hPSCs need to be passaged when the colonies begin to merge and the culture reaches approximately 75–80 % confluency, which typically occurs 3–4 days after passaging (*see* **Note 7**).

3.2 Formation and Growth of EBs

1. Warm Neural Induction Medium (NIM), DMEM, and Accutase® in a 37 °C water bath.
2. Aspirate E8 medium from healthy, 75–80 % confluent hPSC grown in a 100 mm plate.
3. Add 5 ml of Accutase® solution and incubate in the CO_2 incubator at 37 °C for 5 min. Observe under the microscope to determine if additional incubation is required for cell detachment.
4. Add 5 ml of warmed DMEM to the plate.
5. Using a 10 ml serological pipette gently wash off the remaining attached cells until the plate is clear.
6. Gently triturate the cell suspension until all noticeable cell clumps are broken up.
7. Transfer the cell suspension to a 15 ml conical tube.
8. Take 10 μl of the cell suspension to perform a cell count using the hemocytometer.
9. Centrifuge the conical tube at 200 × *g* for 5 min.
10. Aspirate the supernatant and resuspend the cells in NIM such that the final concentration of cells is 5.0×10^5 cells/ml (*see* **Note 8**).
11. Add 4 ml of cell suspension per well to an ultra-low attachment 6-well plate. A total of 2×10^6 cells will be present in each well.
12. Add 4 μl of 5 mM ROCKi per well of EBs.
13. Place the plate on an orbital shaker inside a CO_2 incubator. Set the shaker speed at 95 RPM.
14. After 24 h, examine the cells under the microscope. Small clusters of cells should be visible (Fig. 1b left panel). Do not change the medium.
15. After 48 h carefully aspirate approximately ½ (2 ml) of the medium from each well using a 5 ml serological pipette (*see* **Note 9**).
16. Add 2 ml of NIM to each well. Add the appropriate amount of IWP2 or CHIR so that the *final concentration* is as follows: (*see* **Notes 10** and **11**).
    (a) Forebrain/Cortical Induction: 1000 nM IWP2.
    (b) Midbrain Induction: 10 nM CHIR.
    (c) Hindbrain/Spinal Cord Induction: 500 nM CHIR.
17. Place the plate back on the orbital shaker inside a CO_2 incubator.
18. Continue to remove ½ (2 ml) of medium and replace with 2 ml fresh NIM each day. Add the appropriate amount of IWP2 or CHIR so that the final concentrations are the same as stated in **Step 16.**

3.3 EB Plating and Formation of Neural Rosettes

1. After 5 days of EB growth, plate EBs on Matrigel™ coated plates to induce formation of neural rosettes (Fig. 1b center panel).
2. Prior to plating EBs prepare a working solution of Matrigel™ by thawing one aliquot of Matrigel™ on ice.
3. Make a 1:25 dilution of Matrigel™ in cold DMEM in a 50 ml conical (*see* **Note 1**).
4. Coat each well of a 6-well tissue culture plate with 1.5 ml of Matrigel™ working solution per well. Incubate in a CO_2 incubator at 37 °C for 20 min (*see* **Note 2**).
5. Warm NIM in a 37 °C water bath.
6. Aspirate Matrigel™ solution from each well (*see* **Note 5**).
7. Add 4 ml of NIM. Add the appropriate amount of IWP2 or CHIR so that the *final concentration* is as follows:
   (a) Forebrain/Cortical Induction: 1000 nM IWP2.
   (b) Midbrain Induction: 10 nM CHIR.
   (c) Hindbrain/Spinal Cord Induction: 500 nM CHIR.
8. Using a 1000 μl pipette carefully aspirate 75 % of the medium from wells with EBs. Carefully transfer EBs to Matrigel™ coated wells. One well of EBs should be split equally to two Matrigel™ coated wells.
9. Quickly place the plate in the CO_2 incubator at 37 °C. Gently move the plate in several quick horizontal and vertical movements to evenly disperse the EBs (*see* **Note 12**).
10. After 24 h, carefully examine the cells under the microscope. EBs should have settled and adhered to the plate. Do not change the medium (*see* **Note 13**).
11. After 48 h, carefully remove ½ (2 ml) of the medium from each well using a 1000 μl pipette and replace 2 ml fresh NIM with patterning factors:
    (a) Forebrain/Cortical Induction: 1000 nM IWP2.
    (b) Midbrain Induction: 10 nM CHIR.
    (c) Hindbrain/Spinal Cord Induction: 500 nM CHIR.
12. Place plate back inside a CO_2 incubator. Changes of half of the medium should be made daily. After 3 days, the EBs should spread out on the Matrigel™ substrate and neural rosette structures should be visible (Fig. 1b center panel).

3.4 Generation, and Expansion of NPCs

1. After 7 days of culture, rosettes should be dissociated and replated onto poly-L-ornithine/Laminin (PLO/LN) coated plates.

2. To make PLO/LN coated plates make a 400 ng/ml working solution of poly-L-ornithine (PLO) by combining 2 ml 0.01 % PLO with 48 ml DPBS.
3. Coat a 100 mm dish with 10 ml of the PLO working solution at 37 °C for 4 h.
4. After 4 h of incubation, aspirate the PLO working solution and wash the PLO-coated 100 mm plates three times with 10 ml of PBS.
5. Thaw an aliquot of laminin (LN) on ice. Prepare a 4 μg/ml working solution of LN by adding 400 μl of 0.5 mg/ml LN to 50 ml of sterile PBS in a 50 ml conical tube.
6. Coat the PLO-coated 100 mm plate with 10 ml of LN working solution and incubate at 37 °C for 4 h (*see* **Note 14**).
7. Aspirate the LN working solution and rinse the plates one time with 10 ml of PBS.
8. Warm DMEM, Neural Expansion Medium (NEM) and Accutase® solution in a 37 °C water bath.
9. Gently aspirate NIM from the day 7 rosette cultures.
10. Add 1 ml of Accutase® to each well and incubate in the CO_2 incubator at 37 °C for 10 min. After 10 min, gently tap the sides of the plate against a solid surface to ensure complete cell dissociation (*see* **Note 15**).
11. Using a 10 ml serological pipette, gently wash off the remaining attached cells until the plate is clear.
12. Gently pipette the cell suspension until visible cell clumps are broken up.
13. Transfer the cell suspension to a 15 ml conical with 10 ml warm DMEM and centrifuge the tube at 200 × *g* for 5 min.
14. Resuspend the cells in the appropriate amount of NEM medium with ROCKi at 5 μM so that the final cell concentration is approximately 1–2 × 10^6 cells/ml (*see* **Note 3**).
15. Take 10 μl of the cell suspension to perform a cell count using the hemocytometer.
16. In a separate 15 ml conical tube add 9 μl ROCKi, 9 ml of fresh NEM, and cell suspension to equal 1.1 × 10^6 cells.
17. Aspirate PBS from PLO/LN coated plates and add the approximately 10 ml cell suspension onto a PLO/LN coated plate.
18. Place the plate in the CO_2 incubator. Gently move the plate in several quick horizontal and vertical motions to disperse the cells evenly across the cell culture surface.
19. Change the medium every other day by aspirating the old medium and adding 10 ml of fresh NEM.

20. Once the cells reach confluency they should be Accutase®. passaged at a density of $2.0 \times 10^4/cm^2$ onto new PLO/LN coated plates which typically occurs every 5–7 days. After 2–3 passages, NPCs should have a morphology as displayed in Fig. 1b right panel.

3.5 Characterization of NPCs

NPCs can be characterized using qPCR (Fig. 2a) and immunofluorescent staining (Fig. 2b). NPCs of all A/P regional identities should express high levels of the pan neural markers SOX1, SOX2, and NESTIN (Fig. 1c). NPCs of forebrain cortical identity should express high levels of FOXG1, DLX2, SIX3, and OTX2 (blue cluster in Fig. 2a). Midbrain-specified NPCs should express high levels of LMX1A and EN1 (red cluster in Fig. 2a) while hindbrain/spinal cord-specified NPCs should express high levels of HOXA2 and HOXB4 (green cluster in Fig. 2a).

3.5.1 Characterization of NPCs by qPCR

1. After three passages, NPCs can be characterized by qPCR analysis for pan neural markers and regionalized markers.
2. Warm DMEM and Accutase® solution in a 37 °C water bath.
3. Aspirate NEM from passage 3 NPC cultures.
4. Add 5 ml of Accutase® solution to each 100 mm plate and incubate in the CO_2 incubator for 5 min. After 5 min, gently tap the sides of the plate against a solid surface to ensure complete cell dissociation.
5. Observe under the microscope to determine if additional incubation is needed.
6. Using a 10 ml serological pipette, gently wash off the remaining attached cells with 5 ml of warmed DMEM until the plate is clear (*see* **Note 15**).

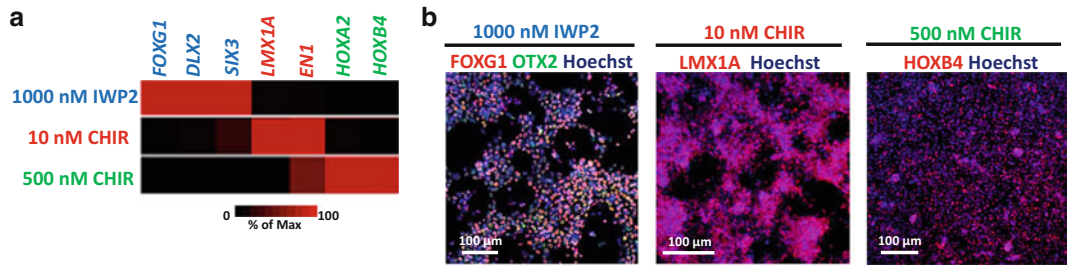

Fig. 2 Characterization of regionally specific NPCs. (**a**) Gene expression analysis of NPCs generated from hPSCs in the presence of 1000 nM IWP2, 10 nM CHIR, and 500 nM CHIR. The data are displayed in a heatmap where black corresponds to minimum expression levels and red corresponds to maximum levels. For each gene analyzed, the expression levels were normalized to the sample with the highest expression level. Primers used for gene expression analysis are listed in Table 1. (**b**) Immunofluorescent analysis of NPC cultures generated from hPSCs in the presence of 1000 nM IWP2, 10 nM CHIR, and 500 nM CHIR. Antibodies used for immunofluorescent analysis are listed in Table 2. Figure and legend adapted with permission from [21]

7. Gently pipette the cell solution into a 15 ml conical and spin down at 200 × *g* for 5 min.
8. Aspirate the supernatant and freeze the cell pellet at −80 °C or proceed directly with RNA isolation protocol with the Machery Nagel kit as described below.
9. Add 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol to each sample tube. Vortex vigorously.
10. Filtrate the lysate by placing the NucleoSpin® filter (purple ring) in a collection tube and applying the mixture to the filter. Centrifuge for 1 min at 11,000 × *g*.
11. Discard the NucleoSpin® filter and add 350 µl ethanol (70 %) to the lysate. Mix by pipetting up and down five times.
12. For each sample take one NucleoSpin® RNA column (light blue ring) placed in a Collection Tube. Pipette the sample lysate up and down 2–3 times and load the lysate to the column. Centrifuge for 30 s at 11,000 × *g*. Following centrifugation place the column in a new collection tube (2 ml).
13. Add 350 µl Membrane Desalting Buffer (MDB) and centrifuge at 11,000 × *g* for 1 min to dry the membrane.
14. Prepare DNAse reaction mixture in a sterile 1.7 ml eppendorf tube. For each isolation add 10 µl reconstituted rDNase (*see* **Notes 16** and **17**) to 90 µl Reaction Buffer for rDNase. Mix by gently flicking the tube. Apply 95 µl DNase reaction mixture onto the center of the silica membrane and incubate at room temperature for 15 min.
15. Add 200 µl Buffer RAW2 to the column and centrifuge for 30 s at 11,000 × *g*. Following centrifugation place the column in a new collection tube (2ml).
16. Add 600 µl Buffer RA3 to the column and centrifuge for 30 s at 11,000 × *g*. Aspirate flow through and place column back into the collection tube (*see* **Note 18**).
17. Add 250 µl Buffer RA3 to the column and centrifuge for 2 min at 11,000 × *g*.
18. Place the column in a nuclease free collection tube (1.5 ml) and elute the RNA in 20 µl RNase-free H_2O at 11,000 × *g* for 1 min.
19. Following RNA isolation check RNA quality and concentration using a Biospec Nano. Prior to analysis, blank the Biospec Nano by placing 1 µl of nuclease free H_2O on the pedestal and selecting "Blank."
20. Following blanking, pipette 1 µl of RNA on pedestal and click "Measure." Record values for concentration, 260/280, and 260/230 (*see* **Note 19**).

21. Using the previously measured concentration of RNA, calculate the volume of RNA equivalent to 1000 ng.
22. In 0.2 μl PCR tubes prepare the following volumes for each sample: 4 μl iScript RT Supermix, 1000 ng of RNA (volume previously calculated), and nuclease free water to 20 μl volume.
23. Place the 0.2 μl PCR tubes in a thermal cycler and run for 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C.
24. Following reverse transcription, cDNA may be stored at −80 °C or used directly to perform RT-qPCR.
25. Prepare the 384 well qPCR plate as follows.
26. In order to prepare a master mix for each sample of cDNA to be analyzed. First calculate the number of wells required for each sample (x) by multiplying the number of genes to be analyzed by 3 (for 3 technical replicates/sample of cDNA) (*see* **Note 20**). Add 3 to this number to account for residual volume losses ($y = x + 3$).
27. Take the number calculated in **step 27** (y) and multiply it by the following values to determine the volumes to add to a 1.7 ml eppendorf tube in order to prepare cDNA sample master mixes (*see* **Note 21**).
    (a) TaqMan® Gene Expression Mastermix: 5(y) μl.
    (b) cDNA: 0.1(y) μl.
    (c) Nuclease free H_2O: 4.4(y) μl.
28. Add 9.5 μl of sample master mix to each well (*see* **Note 23**).
29. Add 0.5 μl of primer for a gene to each well. Primers used for qPCR analysis are listed in Table 1. Include an endogenous reference gene such as 18S (*see* **Note 23**).
30. Once the plate is fully loaded, seal with optical cover being careful not to touch the surface of the optical cover (*see* **Note 24**).
31. Spin down the 384 well plate at 200 × *g* for 1 min.
32. Load the 384 well plate into the thermal cycler and run for 10 min at 95 °C, then 40 cycles (15 s at 95 °C, 1 min at 60 °C).
33. Following completion, use to Ct values to calculate relative fold changes in gene expression using the $2^{-\Delta\Delta CT}$ method [23].

3.5.2 Characterization of NPCs by IF Staining

1. After three passages, NPCs can be characterized by immunofluorescent (IF) staining.
2. Warm DMEM, NEM, and Accutase® solution in a 37 °C water bath.
3. Aspirate NEM from passage 3 NPC cultures.

4. Add 5 ml of Accutase® solution to each 100 mm plate and incubate in the CO_2 incubator for 5 min. After 5 min, gently tap the sides of the plate against a solid surface to ensure complete cell dissociation (*see* **Note 15**).
5. Observe under the microscope to determine if additional incubation and tapping is required.
6. Add 5 ml warmed DMEM medium to the plate.
7. Using a 10 ml serological pipette, gently wash off the remaining attached cells until the plate is clear.
8. Gently pipette the cell solution up and down until all visible cell clumps are broken up.
9. Transfer the cell suspension to a 15 ml conical tube.
10. Centrifuge the tube at 200 × *g* for 5 min.
11. Resuspend the cells in the appropriate amount of NEM medium so that the final cell concentration is about 1.0–2.0 × 10^6 cells/ml (*see* **Note 3**).
12. Take 10 μl of the cell suspension to perform a cell count using the hemacytometer.
13. Dilute cell suspension in NEM such that the final cell concentration is 1.0 × 10^5 cells/ml.
14. Into three wells of a PLO/LN coated 24-well plate add 1.0 ml of cell suspension.
15. Place the plate back inside a CO_2 incubator.
16. After 24 h, aspirate the NEM medium from each well.
17. Wash each well twice with 1 ml of sterile PBS.
18. Fix the NPCs by adding 1 ml of Fixation Buffer to each well. Incubate the cells in Fixation Buffer for 10 min at room temperature.
19. Aspirate the Fixation Buffer and wash each well twice with 1 ml of sterile PBS.
20. Incubate the primary antibodies overnight at 4 °C. See Table 2 for list of antibodies. Antibodies should be diluted in PBS to concentrations listed in Table 2.
21. Aspirate the primary antibodies and wash each well twice with 1 ml of sterile PBS.
22. Incubate the secondary antibodies for 1 h at room temperature in the dark. See Table 2 for list of antibodies. Antibodies should be diluted in PBS to concentrations listed in Table 2.
23. Aspirate the secondary antibodies and wash each well twice with 1 ml of sterile PBS.

24. Add 0.5 ml of Hoechst 3342 (1:5000 dilution in PBS) to each well to counterstain nuclei. Incubate for 10 min a room temperature in the dark.
25. Aspirate the Hoechst 3342 and ash each well twice with 1 ml of sterile PBS.
26. Image NPCs using a fluorescent microscope.

3.6 Differentiation of NPCs to Neurons

1. Warm DMEM, Neuronal Differentiation Medium (NDM) and Accutase® solution in a 37 °C water bath.
2. Aspirate NEM from passage 3 NPC cultures.
3. Add 5 ml of Accutase® solution to each plate and incubate in the CO_2 incubator for 5 min. After 5 min, gently tap the sides of the plate against a solid surface to ensure complete cell dissociation.
4. Observe under the microscope to determine if additional incubation and tapping is required.
5. Add 5 ml of warmed DMEM medium to the plate.
6. Using a 10 ml serological pipette, gently wash off the remaining attached cells.
7. Gently pipette the cell suspension up and down until all visible cells clumps are broken up.
8. Transfer the cell suspension to a 15 ml conical tube.
9. Centrifuge the tube at 200 × *g* for 5 min.
10. Aspirate the supernatant and resuspend the cells in the appropriate amount of NDM medium so that the final cell concentration is 1.0–2.0 × 10^6 cells/ml (*see* **Note 3**). Carefully pipette the cell suspension up and down 2–3 times with a 10 ml serological pipette until all cell aggregates are broken up.
11. Take 10 μl of the cell suspension to perform a cell count using the hemocytometer.
12. Dilute the cell suspension to 1.5 × 10^5 cells/ml with NDM.
13. Add 1.5 ml of cell suspension to each well of a 12-well PLO/LN coated plate.
14. Place the plate in the CO_2 incubator. Gently move the plate in several quick horizontal and vertical motions to disperse the cells evenly across the cell culture surface.
15. Change the medium every other day by aspirating the old medium and adding 2 ml of fresh NDM per well.
16. After 4 weeks in NDM cells should acquire a neuronal morphology.

3.7 Characterization of Neurons

Resultant neuronal cultures can be characterized using qPCR (Fig. 3a) and immunofluorescent staining (Fig. 3b). Neuronal cultures should express high levels of the pan-neural markers MAP2 and B3T. Neurons generated from forebrain/cortical specified NPCs should express high levels of markers such as CTIP2, CUX1, EMX1, FOXG1, SATB2, and TBR1 (blue cluster in Fig. 3a). By comparison, midbrain specified NPCs will generate neurons with a midbrain GABAergic or dopaminergic phenotype (red cluster in Fig. 3a) while neurons generated from hindbrain/spinal cord biased NPCs will express high levels of markers association with a hindbrain and spinal motor neuron phenotype (green cluster in Fig. 3a).

3.7.1 Characterization of Neurons by qPCR

1. Neurons can be characterized by qPCR analysis for pan neuronal markers and A/P-related neuronal markers.
2. Warm DMEM and Accutase® solution in a 37 °C water bath.
3. Aspirate NDM from neuronal cultures.
4. Add 0.5 ml of Accutase® solution to each well of a 12 well plate and incubate in the CO_2 incubator for 5 min. After 5 min, gently tap the sides of the plate against a solid surface to ensure complete cell dissociation.
5. Observe under the microscope to determine if additional incubation is needed.
6. Using a 1000 µl pipette, gently wash off the remaining attached cells with warmed DMEM until the plate is clear (*see* **Note 15**).
7. Gently pipette the cell solution into a 15 ml conical and spin down at 200 × *g* for 5 min.

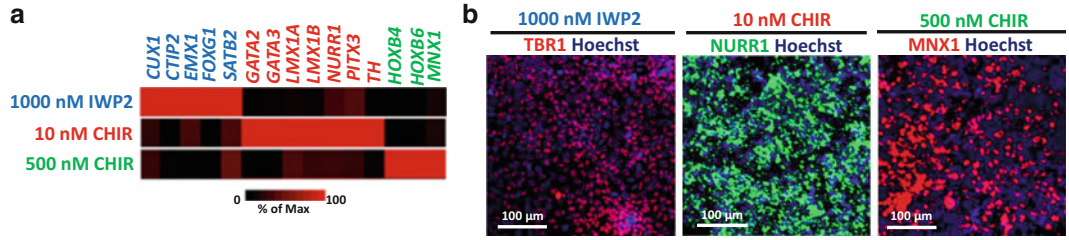

Fig. 3 Characterization of regionally specific neurons. (**a**) Gene expression analysis of neurons differentiated from NPCs generated from hPSCs in the presence of 1000 nM IWP2, 10 nM CHIR, and 500 nM CHIR. The data are displayed in a heatmap where black corresponds to minimum expression levels and red corresponds to maximum levels. For each gene analyzed, the expression levels were normalized to the sample with the highest expression level. Primers used for gene expression analysis are listed in Table 1. (**b**) Immunofluorescent analysis of neurons differentiated from NPCs generated from hPSCs in the presence of 1000 nM IWP2, 10 nM CHIR, and 500 nM CHIR. Antibodies used for immunofluorescent analysis are listed in Table 2. Figure and legend adapted with permission from [21]

8. Aspirate the supernatant and freeze the cell pellet at −80 °C or proceed directly with RNA isolation protocol in Machery Nagel kit described below.
9. Add 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol to each sample tube. Vortex vigorously.
10. Filtrate the lysate by placing a NucleoSpin® filter (purple ring) in a collection tube and applying mixture to the filter. Centrifuge for 1 min at 11,000 × *g*.
11. Discard the NucleoSpin® filter and add 350 µl ethanol (70 %) to the lysate. Mix by pipetting up and down five times.
12. For each sample take one NucleoSpin® RNA column (light blue ring) placed in a Collection Tube. Pipette the sample lysate up and down 2–3 times and load the lysate to the column. Centrifuge for 30 s at 11,000 × *g*. Following centrifugation place the column in a new collection tube (2 ml).
13. Add 350 µl Membrane Desalting Buffer (MDB) and centrifuge at 11,000 × *g* for 1 min to dry the membrane.
14. Prepare DNAse reaction mixture in a sterile 1.7 ml eppendorf tube. For each isolation add 10 µl reconstituted rDNase (*see* **Notes 16** and **17**) to 90 µl Reaction Buffer for rDNase. Mix by flicking the tube. Apply 95 µl DNase reaction mixture onto the center of the silica membrane and incubate at room temperature for 15 min.
15. Add 200 µl Buffer RAW2 to the column and centrifuge for 30 s at 11,000 × *g*. Following centrifugation place the column in a new collection tube (2ml).
16. Add 600 µl Buffer RA3 to the column and centrifuge for 30 s at 11,000 × *g*. Aspirate flow through and place column back into the collection tube (*see* **Note 18**).
17. Add 250 µl Buffer RA3 to the column and centrifuge for 2 min at 11,000 × *g*.
18. Place the column in a nuclease free collection tube (1.5 ml) and elute the RNA in 20 µl RNase-free H_2O at 11,000 × *g* for 1 min.
19. Following RNA isolation check RNA quality and concentration using a Biospec Nano. To do this, first blank the Biospec Nano by placing 1 µl of nuclease free H_2O on the pedestal and selecting "Blank."
20. Following blanking, place 1 µl of RNA on pedestal and click "Measure." Record values for concentration, 260/280, and 260/230 (*see* **Note 19**).
21. Calculate the required volume of RNA required for the reverse transcription reaction. Using the previously measured

concentration of RNA calculate the volume of RNA equivalent to 1000 ng.

22. In 0.2 µl PCR tubes prepare the following volumes for each sample: 4 µl iScript RT Supermix, 1000 ng of RNA (volume previously calculated), and nuclease free water to 20 µl volume.
23. Place the 0.2 µl PCR tubes in a thermal cycler and run for 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C.
24. Following reverse transcription cDNA may be stored at −80 °C or used directly to perform RT-qPCR.
25. Prepare the 384 well qPCR plate as follows.
26. In order to prepare a master mix for each sample of cDNA to be analyzed. First calculate the number of wells required for each sample (x) by multiplying the number of genes to be analyzed by 3 (for 3 technical replicates/sample of cDNA) (*see* **Note 20**). Add 3 to this number to account for pipetting losses ($y = x + 3$).
27. Take the number calculated in **step 27** (y) and multiply it by the following values to determine the volumes to add to a 1.7 ml Eppendorf tube in order to prepare cDNA sample master mixes (*see* **Note 21**).
    (a) TaqMan® Gene Expression Mastermix: 5(y) µl.
    (b) cDNA: 0.1(y) µl.
    (c) Nuclease free H_2O: 4.4(y) µl.
28. Add 9.5 µl of sample master mix to each well (*see* **Note 22**).
29. Add 0.5 µl of primer for a gene to each well. Primers used for qPCR analysis are listed in Table 1. Include an endogenous reference gene such as 18S (*see* **Notes 22** and **23**).
30. Once the plate is fully loaded, seal with optical cover being careful not to touch the surface of the optical cover (*see* **Note 24**).
31. Spin down the 384 well plate at 200 × *g* for 1 min.
32. Load the 384 well plate into the thermal cycler and run for 10 min at 95 °C, then 40 cycles (15 s at 95 °C, 1 min at 60 °C).
33. Following completion, use to Ct values to calculate relative fold changes in gene expression using the $2^{-\Delta\Delta CT}$ method [23].

3.7.2 Characterization of Neurons by IF Staining

1. Fix neurons by adding 1.5 ml of Fixation Buffer to each well. Incubate the cells in Fixation Buffer for 10 min at room temperature
2. Aspirate the Fixation Buffer and wash each well twice with 2 ml of sterile PBS.

3. Permeabilize cells by adding 1.5 ml of Perm Buffer to each well. Incubate the cells in Perm Buffer for 30 min at room temperature.
4. Aspirate the Perm Buffer and wash each well twice with 2 ml of sterile PBS.
5. Add 1.5 ml of primary antibodies and incubate overnight at 4 °C. See Table 2 for list of antibodies. Antibodies should be diluted in PBS to concentrations listed in Table 2.
6. Aspirate the primary antibodies and wash each well twice with 2 ml of sterile PBS.
7. Add 1.5 ml of secondary antibodies and incubate for 1 h at room temperature in the dark. See Table 2 for list of antibodies. Antibodies should be diluted in PBS to concentrations listed in Table 2.
8. Aspirate the secondary antibodies and wash each well twice with 1 ml of sterile PBS.
9. Add 0.5 ml of Hoechst 3342 to each well to counterstain nuclei. Incubate for 10 min at room temperature in the dark.
10. Aspirate the Hoechst 3342 and wash each well twice with 1 ml of sterile PBS.
11. Image neurons using a fluorescent microscope.

4 Notes

1. Matrigel™ aliquots and working solution should be kept on ice during thawing.
2. If not used within 24 h the culture plate should be sealed with Parafilm™ to avoid evaporation of Matrigel™ working solution. Culture plates can be sealed with Parafilm™ and stored at 4 °C for up to 1 week after coating. Prior to using stored Matrigel™ coated plates, allow the plates to equilibrate at room temperature for 15 min.
3. The dynamic range of the hemacytometer is 5.0×10^5–2.0×10^6 cells/ml. If the cell suspension is not in this range additional E8 medium may need to be added to obtain an accurate cell count.
4. hPSCs can be grown and maintained in other tissue culture plate sizes (e.g., multi-well dishes). Cells should be plated in these formats at a density of approximately $9.0 \times 10^3/cm^2$. Ideal seeding densities for different hPSC lines will vary and may need to be determined empirically.
5. Aspirate Matrigel™ solution immediately prior to seeding cells. Do not allow the Matrigel™ coated surfaces to dry out.

6. Do not add ROCKi. ROCKi is only added during passaging to aid in hPSC survival.
7. If the colonies are passaged too late, then colonies will begin to display signs of differentiation. Differentiated cells develop a fibroblast-like morphology and can be removed using manual dissection techniques prior to passaging.
8. Optimal cell density for EB formation may be hPSC line dependent. When working with a new cell line determine the optimal cell density will need to be empirically determined.
9. Use caution when aspirating medium as not to disturb the EBs in suspension.
10. Do not add ROCKi. ROCKi is added on the first day of EB formation to aid cell survival.
11. Optimal concentrations for CHIR 98014 and IWP2 may be hPSC line dependent. When working with a new cell line determine optimal concentrations empirically.
12. Failure to evenly disperse the EBs throughout the cell culture surface may result in aggregation of EBs into the center of the well and hinder the ability neural rosettes to form.
13. Do not move the plate prior to 24 h to allow for sufficient time for the EBs to settle and adhere. On average, approximately 5–10 % of the EBs will be of cystic or nonuniform shape and will not attach to the Matrigel™ coated plates.
14. If not used immediately, the PLO/LN culture plates should be sealed to avoid evaporation of laminin working solution. Culture plates can be sealed with Parafilm™ and stored at −20 °C.
15. Complete dissociation from the cell culture surface may require the use of a cell lifter.
16. Lyophilized rDNase should be initially stored at 4 °C. To aliquot rDNase, add indicated volume of nuclease free H_2O indicated (on the vial) to the vial and incubate at room temperature for 1 min. Gently swirl the vial to completely dissolve rDNAse. Be careful not to mix rDNase vigorously as it is sensitive to mechanical agitation. Dispense aliquots at 10 μl and store at −20 °C.
17. rDNase is sensitive to mechanical agitation, be careful not to mix vigorously.
18. Make sure residual buffer is washed away with Buffer RA3.
19. RNA quality can be assessed by examining the A_{260}/A_{280}, a ratio between 1.8 and 2.0 is acceptable. A ratio below 1.8 could indicate protein contamination which can lower reaction efficiency in downstream applications.
20. qPCR experiments should be carried out in three technical replicates.

21. Components of the TaqMan® Gene Expression Mastermix can settle during storage, so gently mix prior to use. However, do not vortex or mix vigorously to prevent the formation of bubbles which can impact fluorescence readings.
22. It is critical to not dispense to the second stop to avoid the formation of bubbles which would interfere with fluorescence readings.
23. A reference gene is required to analyze data using the $2^{-\Delta\Delta CT}$ method. For addition information on the selection of reference genes the reader should consult previous publications [24].
24. Use the tabs delineated with perforations to handle the optical covers to prevent fingerprints on the optical cover. Use a square plastic installing tool provided with the covers to smooth down the cover, especially along the four edges at the top of the plate, to prevent evaporation in reaction wells during PCR cycles.

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