Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks

Yichen Shi, Peter Kirwan & Frederick J Livesey

Gurdon Institute, University of Cambridge, Cambridge, UK. Correspondence should be addressed to F.J.L. (rick@gurdon.cam.ac.uk).

Published online 13 September 2012; doi:10.1038/nprot.2012.116

Efficient derivation of human cerebral neocortical neural stem cells (NSCs) and functional neurons from pluripotent stem cells (PSCs) facilitates functional studies of human cerebral cortex development, disease modeling and drug discovery. Here we provide a detailed protocol for directing the differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) to all classes of cortical projection neurons. We demonstrate an 80-d, three-stage process that recapitulates cortical development, in which human PSCs (hPSCs) first differentiate to cortical stem and progenitor cells that then generate cortical projection neurons in a stereotypical temporal order before maturing to actively fire action potentials, undergo synaptogenesis and form neural circuits *in vitro*. Methods to characterize cortical neuron identity and synapse formation are described.

INTRODUCTION

Stem cell biology is commonly predicted to hold great potential for the study and treatment of neurodegenerative disease[1]. The development of technologies to generate hESCs raised the possibility of producing large numbers of defined classes of neurons for research and transplantation. More recently, the development of methods to reprogram adult somatic cells, including fibroblasts, to pluripotent cells, referred to as iPSCs[2–4], has made it possible to generate patient-specific PSCs[5]. In neurology, this approach has been used to generate *in vitro* models for a number of genetic conditions, such as spinal muscular atrophy[6] and familial dysautonomia[7]. Similar approaches are being taken to inherited and sporadic forms of a range of human neurodegenerative conditions, including motor neuron disease, Parkinson's disease and Alzheimer's disease[8–11]. In all cases, ESCs and iPSCs are being used to generate *in vitro* disease models as well as large populations of healthy neurons to explore the therapeutic potential of transplantation.

Recently developed methods to model human cerebral cortex development from ESC and iPSC[12] (collectively referred to as PSCs) enable functional studies of human cortical development and function, the production of *in vitro* human models of cortical diseases and the development of cortical implants for cell-based therapies. The primate cerebral cortex differs from that of the rodent in a number of important features: the cerebral cortex is substantially larger in primates relative to the rest of the nervous system; the size, complexity and the nature of the stem cell populations differ in primate cortex[13]; and there is also an increase in the diversity of neuronal cell types in the primate cortex[14]. Combining human genetics with methods to generate cortical circuits from patient-specific stem cells has the potential to address many of the challenges encountered when developing mouse models that accurately replicate the pathogenesis of cortical diseases, such as Alzheimer's disease, autism and schizophrenia.

Mouse ESCs have successfully been differentiated to telencephalic progenitor cells[15] and to cerebral cortex projection neurons[16], although it is not clear whether that process includes the generation of all classes of cortical stem or progenitor cells. Production of all three major stem or progenitor cell populations of the human neocortex by directed differentiation from PSCs is the key for the derivation of the full spectrum of the neocortical excitatory neurons[12]. Previously published methods for deriving human forebrain neurons from PSCs generated neuroepithelial cells in aggregate culture conditions, but failed to demonstrate the existence of secondary progenitor cells and produced primarily early-born, deep-layer cortical neuron types[17]. Those findings led to speculation that there could be limitations to stem cell differentiation or culture conditions that lead to the inability to generate all cortical cell types, perhaps because of the absence of secondary progenitor cells[18].

Experimental design

Here we provide a detailed protocol for a simple and robust culture system for the generation of both deep- and upper-layer excitatory neurons from hPSCs, which are electrically active and form functional cortical circuits. This protocol is based on the methods used in our recent *Nature Neuroscience* paper[12]. The culture system combines defined neural induction and maintenance medium with specific culture substrates, and can generate almost homogenous cultures of cortical primary stem/progenitor cells within 2 weeks of neural induction that can also be cryopreserved for future use, if desired (**Box 1**).

A key feature of this system is that both primary neuroepithelial cells and secondary cortical stem/progenitor cells are derived from hPSCs. The protocol described here is based on the previously described SMAD signaling inhibition strategy that channels differentiation toward the anterior neuroectodermal lineage[19]. In the context of dual SMAD inhibition, we found that vitamin A is crucial for the efficient induction of cortical stem/progenitor cell differentiation and subsequent cortical neurogenesis. These findings agree with previous reports that retinoids have important roles in derivation of NSCs and telencephalic glutamatergic neurons from mouse ESCs[20], and that meningeal-secreted retinoic acid (a derivative of vitamin A) is required for the cortical neurogenesis *in vivo*[21]. By using this approach, the efficiency of cortical neural induction from PSCs approaches 100% (ref. 12). As in all directed differentiation processes, there is some variation in the efficiency of cortical induction among ESC and iPSC lines. However, we have found that such variation is reduced by replacing noggin with a second small-molecule SMAD inhibitor, dorsomorphin, as has been

Box 1 | Cryostorage and thawing of cortical NSCs ● TIMING 1 d

1. Following dissociation of the culture with Accutase as described in Steps 43–47 of the main PROCEDURE, resuspend cortical stem cells at a density of 2 × 10^6 cells per ml of neural freezing medium.
2. Aliquot 1 ml of the cell suspension into each cryovial.
3. Freeze in a CoolCell freezing container at −80 °C overnight.
4. Transfer the cryovials to liquid nitrogen for long-term storage.

■ **PAUSE POINT** Cells can be stored in liquid nitrogen for up to 2 years.

Thawing NSCs

5. Partially thaw the cells in a 37 °C water bath.
6. Transfer the partially thawed NSCs to 10 volumes of room-temperature neural maintenance medium.
7. Centrifuge the cells once at 160*g* for 3 min at 18–22 °C.
8. Gently resuspend the cells in 1 ml of neural maintenance medium, and plate into poly-ornithine/laminin-coated 35-mm dishes at 50,000 cells per cm^2.
9. Resume culturing of cells at Step 51 of the main PROCEDURE.

reported by others[22]. We have successfully used the method described here on three different hESC lines, including the widely used H9 line[23], as well as iPSC lines derived from over ten individuals, with several clones per individual in many cases.

A notable feature of this process is how long it takes for all of the different classes of neurons to be generated: deep-layer, early-born projection neurons are produced first, ~2–3 weeks after the initiation of neural induction; in contrast, the last-born, layer 2 neurons are produced as late as day 90 of differentiation[12]. This time frame for cortical neurogenesis is remarkably similar to that seen in the developing human cortex *in utero*. Analysis of the generation of relative numbers of different classes of cortical projection neurons from PSCs demonstrated that approximately equal numbers of deep- and upper-layer neurons are produced in this system, and these proportions are highly reproducible across different hESC and iPSC lines[12].

In parallel with the lengthy period of neurogenesis in this system, synapse formation begins relatively early in culture, with physical and functional synapses observed as early as days 45–50, becoming more abundant over the subsequent weeks[12]. However, emphasizing the protracted nature of human cortical development, although functional chemical synapses are present from day 45, we have found that functional cortical neural networks only emerge in culture after 2–3 months (data not shown). Again, this mimics the timing of human cortical development *in utero*, and demonstrates that PSC-derived cortical neurons are functionally equivalent to primary cortical neurons.

To date, iPSC lines have been derived from patients with a number of diseases of the cerebral cortex, including Alzheimer's disease, Rett syndrome and schizophrenia[8,10,24,25]. In most cases, the regional identities of the neurons derived from these iPSC lines have not been well characterized in those studies, nor has the significance of modeling disease pathogenesis in the cell types typically affected by each disease been assessed. Recently, we have demonstrated that Down's syndrome PSC–derived cortical excitatory neurons faithfully reproduce the early pathogenesis of Alzheimer's disease[9]. The ability to derive disease-relevant cell types should contribute to the elucidation of the mechanisms of cell type–specific disease onset, as well as the validation of candidate drugs and therapies in the appropriate target cell types. Finally, the ability to produce functional cortical neural networks from hPSCs can facilitate tissue engineering of cortical implants.

MATERIALS

REAGENTS

- 2-Mercaptoethanol (Life Technologies, cat. no. 21985-023) **! CAUTION** 2-Mercaptoethanol is toxic; avoid inhalation, ingestion or contact with skin or mucous membranes.
- Accutase (Innovative Cell Technologies, cat. no. AT104)
- B-27 supplement (Life Technologies, cat. no. 17504-044)
- CELLstart (Life Technologies, cat. no. A10142-01)
- Dispase (Life Technologies, cat. no. 17105)
- DMEM, high glucose (Life Technologies, cat. no. 41965-039)
- DMEM/F-12, GlutaMAX (Life Technologies, cat. no. 31331-028)
- Dimethyl sulfoxide (DMSO; Sigma, cat. no. D2650)
- Fetal bovine serum (FBS; Life Technologies, cat. no. 16000-044)
- Fibroblast growth factor 2 (FGF2; PeproTech, cat. no. 100-18B)
- Gelatin (Sigma, cat. no. G9391)
- Human iPSCs or hESCs **! CAUTION** Please ensure that hESC and iPSC research is carried out in accordance with your governmental and institutional guidelines and regulations. **▲ CRITICAL** The quality of the starting PSCs directly influences the yield and quality of the cortical stem cells and neurons derived.
- Insulin (Sigma, cat. no. 19278)
- KnockOut serum replacement (KSR; Life Technologies, cat. no. 10828-028)
- L-Glutamine (Life Technologies, cat. no. 25030-024)
- Laminin (Sigma, cat. no. L2020)
- Irradiated mouse embryonic fibroblasts (MEFs; GlobalStem, cat. no. GSC-6001G)
- N-2 supplement (Life Technologies, cat. no. 17502-048)
- Neurobasal (Life Technologies, cat. no. 12348-017)
- Noggin (R&D Systems, cat. no. 3344-NG-050)
- Non-essential amino acid solution (Life Technologies, cat. no. 11140-050)
- PBS, without calcium and magnesium (Life Technologies, cat. no. 14190-094)
- Penicillin-streptomycin (Life Technologies, cat. no. 15140-122)
- Poly-L-ornithine solution (Sigma, cat. no. P4957)
- SB431542 (Tocris Bioscience, cat. no. 1614)
- Sodium pyruvate (Sigma, cat. no. S8636)
- Y-27632 dihydrochloride (ROCK-I, Tocris Bioscience, cat. no. 1254)
- Antibodies (**Table 1**)

TABLE 1 | Antibodies used in this study.

Name	Expression	Dilution	Supplier	Cat. no.
Pax6	Primary progenitor cells	1:300	Covance	PRB-278P
Foxg1	Primary progenitor cells	1:300	Abcam	ab18259
Otx1/2	Primary progenitor cells	1:300	Millipore	AB9566
Phospho-vimentin	Mitotic (M-phase) progenitor cells	1:400	MBL	D076-3S
CD133	Primary progenitor cells	1:100	Abcam	ab19898
Tbr2	Secondary progenitor cells	1:200	Abcam	ab23345
Ki67	Cycling cells	1:600	BD	550609
Tbr1	Prelate, layer 1 and deep-layer neurons	1:300	Abcam	ab31940
CTIP2	Deep-layer neurons	1:300	Abcam	ab18465
Satb2	Upper layers and some layer 5 neurons	1:100	Abcam	ab51502
Brn2	Progenitor cells and upper-layer neurons	1:400	Santa Cruz	sc-6029
Cux1	Progenitor cells and upper-layer neurons	1:300	Santa Cruz	sc-13024
VGlut1	Glutamatergic neurons	1:300	Synaptic Systems	135303
S100	Astrocytes	1:400	Dako	Z0311
GFAP	Astrocytes and progenitor cells	1:600	Abcam	ab4674
PSD-95	Glutamatergic neurons, postsynaptic density	1:400	Abcam	ab2723
Munc13	Presynaptic terminals	1:300	Synaptic Systems	126103
Synaptophysin	Presynaptic terminals	1:500	Abcam	ab68851
Homer1	Glutamatergic neurons	1:300	Synaptic Systems	160011
Nestin	Primary progenitor cells	1:300	Abcam	Ab22035
Phospho-H3	Mitotic (M-phase) progenitor cells	1:1,000	Abcam	Ab10543

- PCR primers (**Table 2**)
- Artificial cerebrospinal fluid (ACSF; REAGENT SETUP)
- Oxygen (95%), carbon dioxide (5%) mix (BOC, 131-F)
- Internal recording solution (REAGENT SETUP)
- Borosilicate glass capillaries (Harvard Apparatus, GC150F-7.5)
- Drugs for electrophysiology (see REAGENT SETUP): CNQX (Tocris, cat. no. 0190), DL-AP5 (Tocris, cat. no. 0105), tetrodotoxin (Tocris, cat. no. 1069) and 4-aminopyridine (4-AP; Tocris, cat. no. 0940)
  ! CAUTION These drugs are highly toxic. Take care when handling them and use a fume hood when making stock solutions.
- Paraformaldehyde
- Donkey serum
- Triton X-100
- TBS

EQUIPMENT

- Water bath, 37 °C
- Cell culture centrifuge
- CO_2 incubator
- CoolCell freezing box (BioCision, cat. no. BCS136)
- Glass hemocytometer
- Laminar flow hood
- Liquid nitrogen cell storage tank

Cell culture plastics

- Six-well plates (Nunc, cat. no. 140675)
- Plates (12 well; (Nunc, cat. no. 150628))
- Dishes (35 mm) (Nunc, cat. no. 153066))
- Cryovials (Greiner Bio-one, cat. no. 121280)
- Stericup vacuum filter unit (Millipore, cat. no. SCGPU05RE)
- Filter (0.22-µm pore size) (Millex-GP; Millipore, cat. no. SLGP033RS)
- Falcon centrifuge tubes
- Plastic pipettes
- Disposable syringe, 10 ml

Electrophysiology rig

- Upright fixed-stage microscope (e.g., Zeiss Axioexaminer A1) with a fluorescence unit and digital camera
- Micromanipulator (e.g., Mini25, Luigs & Neumann)
- Digital amplifier (e.g., Multiclamp 700B, Molecular Devices)
- Digital to analog converter (e.g., Digidata 1440a, Molecular Devices)
- Pipette puller (e.g., Narishige PC-10)
- Air table
- Faraday cage
- Perfusion system

REAGENT SETUP

▲ **CRITICAL** Sterilize all media with a 0.22-µm-pore filter or a Stericup vacuum filter unit.

TABLE 2 | RT-PCR primers for phenotyping neural rosettes.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)
Pax6	GTGTCCAACGGATGTGTGAG	CTAGCCAGGTTGCGAAGAAC	60.0	254
Foxg1	AGGAGGGCGAGAAGAAGAAC	TCACGAAGCACTTGTTGAGG	60.0	213
Sox2	ACTTTTGTCGGAGACGGAGA	GTTCATGTGCGCGTAACTGT	59.8	253
Otx1	GCCTCCCCTTCCAGTCTTTC	GGGCAGAAACACGCCAGTTA	63.0	140
Emx1	TGACGGTTCCAGTCCGAAGT	CCAAGGACAGGTGAGCATCC	63.0	53
Sox1	TACAGCCCCATCTCCAACTC	GCTCCGACTTCACCAGAGAG	60.1	199

MEF medium MEF medium contains DMEM supplemented with 10% (vol/vol) FBS, 1 mM L-glutamine, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. Store the medium at 4 °C and use it within 2 weeks.

hPSC medium hPSC medium contains DMEM/F-12 GlutaMAX supplemented with 20% (vol/vol) KSR, 10 ng ml^{-1} FGF2, 100 µM non-essential amino acids, 100 µM 2-mercaptoethanol, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. Store the medium at 4 °C and use it within 2 weeks. ▲ **CRITICAL** The concentration of FGF2 can be elevated to 30 ng ml^{-1} in the medium to boost the recovery of thawed PSCs. Reduce the concentration to 10 ng ml^{-1} once the PSCs have reached the optimal growth rate.

MEF-conditioned hPSC medium Plate MEF cells on a gelatin-coated, six-well dish at 40,000 cells per cm^2 in 2 ml of MEF medium per well. The next day, wash the cells with warm PBS, and add 2 ml per well of hPSC medium without FGF2. Collect the medium every 24 h for up to 6 d. Filter the medium with a 0.22-µm-pore filter and store at −80 °C until use. Add 10 ng ml^{-1} FGF2 before use. The medium can be stored at −80 °C for up to 1 month; once thawed, use it within 5 d.

Neural maintenance medium This is a 1:1 mixture of N-2 and B-27-containing media. N-2 medium consists of DMEM/F-12 GlutaMAX, 1× N-2, 5 µg ml^{-1} insulin, 1 mM L-glutamine, 100 µm nonessential amino acids, 100 µM 2-mercaptoethanol, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. B-27 medium consists of Neurobasal, 1× B-27, 200 mM L-glutamine, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin. Store the medium at 4 °C and use it within 3 weeks.

Neural induction medium Neural maintenance medium is supplemented with 500 ng ml^{-1} noggin and 10 µM SB431542 or with 1 µM Dorsomorphin and 10 µM SB431542. Store the medium at 4 °C and use it within 5 d.

Neural freezing medium Neural maintenance medium is supplemented with 10% (vol/vol) DMSO and 20 ng ml^{-1} FGF2. Prepare neural freezing medium immediately before use. Do not store.

Stock Dispase solution Dissolve the nonsterile enzyme in PBS to 10 mg ml^{-1}. The stock can be stored at −80 °C for at least 1 year. Filter the stock solution with a 0.22-µm-pore filter before use.

Gelatin solution Dissolve 0.1 g of gelatin in 100 ml of distilled water and autoclave the solution. Store the solution at 4 °C and use it within 2 weeks.

ACSF for electrophysiology Make up the following solution: 125 mM NaCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose and 3 mM pyruvic acid. Bubble with 95% O_2, 5% CO_2. Adjust the pH to 7.2–7.4 with NaOH. ACSF can be stored for up to 1 week at 4 °C.

Internal recording solution Make up the following solution: 135 mM potassium gluconate, 7 mM NaCl, 10 mM HEPES, 2 mM Na_2ATP, 0.3 mM Na_2GTP and 2 mM $MgCl_2$. Adjust the pH to 7.2–7.4 with KOH. The solution can be stored for up to 1 year at −80 °C.

CNQX Make up a 5 mM stock in DMSO. Store in small aliquots at −20 °C. For the working solution, dilute to 50 µM in ACSF. ! **CAUTION** This drug is highly toxic. Take care when handling and use a fume hood when making stock solutions.

DL-AP5 Make up a 5 mM stock in distilled water. Store in small aliquots at −20 °C. For the working solution, dilute to 50 µM in ACSF. ! **CAUTION** This drug is highly toxic. Take care when handling and use a fume hood when making stock solutions.

Tetrodotoxin Make up a 100 µM stock in distilled water. Store in small aliquots at −20 °C. For the working solution, dilute to 1 µM. ! **CAUTION** This drug is highly toxic. Take care when handling and use a fume hood when making stock solutions.

4-AP Make up a 5 mM stock in distilled water. Store in small aliquots at −20 °C. For the working solution, dilute to 1 µM. ! **CAUTION** This drug is highly toxic. Take care when handling and use a fume hood when making stock solutions.

EQUIPMENT SETUP

Gelatin-coated six-well plates Add 1 ml of the gelatin solution to cover the bottom of the wells of a six-well plate and incubate it at 37 °C for 10 min. Aspirate the gelatin solution before addition of the medium.

CELLStart-coated 12-well plates Dilute CELLStart 1:50 with PBS (containing calcium and magnesium). Cover the bottom of the wells of a 12-well plate with 0.4 ml of the diluted solution. Incubate the plate at 37 °C for 2 h. Aspirate the solution before addition of the medium.

Laminin-coated 35-mm dishes Dilute the 1 mg ml^{-1} laminin stock 1:50 with PBS to a final concentration of 20 µg ml^{-1}. Cover the bottom of the dish with 1 ml of the diluted solution. Incubate at 37 °C for 4 h. Aspirate the solution before addition of the medium.

Poly-L-ornithine/laminin-coated 35-mm dishes Cover the bottom of each 35-mm dish with 1 ml of 0.01% (wt/vol) poly-L-ornithine solution. Incubate at 37 °C for 4 h. Aspirate the solution and coat with laminin as described above.

PROCEDURE

Preparing cultures of hPSCs for neural differentiation ● TIMING ~3 weeks

1| Thaw a vial of frozen irradiated MEFs in a 37 °C water bath. Resuspend the cells in 10 ml of MEF medium at room temperature (18–22 °C). Spin for 3 min at 160*g* at room temperature. Resuspend in 1.2 ml of MEF medium. Add 2 ml of MEF medium to each well of two gelatin-coated six-well plates. Add 100 µl of the resuspended MEFs to each well of each six-well plate. Rock the dishes forward and backward and from side to side for 1 min to ensure an even plating of the MEFs on the plates. Culture the cells overnight at 37 °C in 5% CO_2.

2| On the following day, remove the MEF medium and rinse the MEFs once with 37 °C PBS.

3| Add 2 ml of hPSC medium that contains 10 μM Y-27632 and 10 ng ml^{-1} FGF2 to one well of the six-well plate. Add hPSC medium to the remaining wells and use those wells for collecting MEF-conditioned hPSC medium (REAGENT SETUP).

4| Partially thaw a vial of frozen PSCs in a 37 °C water bath.

5| Transfer the partially thawed PSCs to 10 volumes of hPSC medium at room temperature.

6| Centrifuge the cells once at 160*g* for 2 min at room temperature.

7| Wash once more by repeating Steps 5 and 6.

8| Gently resuspend the cells in 100 μl of hPSC medium, and then plate the cells in solution into the MEF-coated well containing hPSC medium with 10 μM Y-27632 and 10 ng ml^{-1} FGF2 as prepared earlier. Culture overnight at 37 °C and 7% CO_2.

9| On the next day, replace the medium with fresh hPSC medium containing 10 ng ml^{-1} FGF2 but lacking Y-27632. From this point, continue to incubate the cells, changing the medium every day until PSC colonies are ~1 mm in diameter and can be seen with the naked eye (i.e., without an inverted microscope). Depending on the cell line, this can take between 4 and 6 d.

10| Once visible colonies appear, passage the PSC colonies with dispase. First add 200 μl of 37 °C stock dispase solution directly to the PSC cultures, without removing the PSC culture medium.

11| Incubate at 37 °C for up to 45 min, checking every 10 min on an inverted microscope for detachment of colonies from the underlying MEFs.

12| When the colonies are detached from the MEF layer, transfer the medium containing the colonies to a 15-ml Falcon tube.

13| Pellet the colonies by spinning for 2 min at 160*g* at room temperature. Remove and discard the supernatant.

14| Resuspend and rinse the colonies twice with 10 ml of PBS to remove the remaining dispase.
▲ **CRITICAL STEP** Dispase is not inactivated by the hPSC medium, and thus multiple washes are required to dilute any remaining enzyme. Failure to remove dispase will compromise the ability of PSC colonies to reattach when plated.

15| Resuspend the colonies in 2 ml of hPSC medium, and pipette up and down 5 or 6 times with a P1000 pipette to break the colonies into fragments containing 50 to 100 cells (**Fig. 1**).
▲ **CRITICAL STEP** The size of the colony fragments influences the yield of PSCs. If the colony fragments are too small, only small numbers of colonies will grow; if the sizes are too big, the colonies will spontaneously differentiate. See **Figure 1** for an example of optimal fragment size.

16| Plate the fragmented PSC colonies at a ratio of 1:6 (relative to the original number of culture dishes) into fresh MEF-coated dishes in hPSC medium with 10 ng ml^{-1} FGF2. Make sure that the colony fragments are distributed evenly on the plate.

17| Continue to incubate the cells for 4–6 d and change the medium every day until the PSC colonies are ~1 mm in diameter and are visible with the naked eye.

Preparing PSCs for neural induction ● TIMING ~3 weeks

18| Inspect cultures for spontaneously differentiated colonies and remove them using a P200 pipette. Differentiated colonies typically show loss of defined edges.

19| Coat one well of a 12-well cell culture plate with CELLStart for 2 h at 37 °C.

20| Isolate the PSC colonies with dispase and wash them with hPSC medium as described in Steps 10–14.

21| Resuspend the colonies in 1 ml of MEF-conditioned hPSC medium containing 10 ng ml^{-1} FGF2.

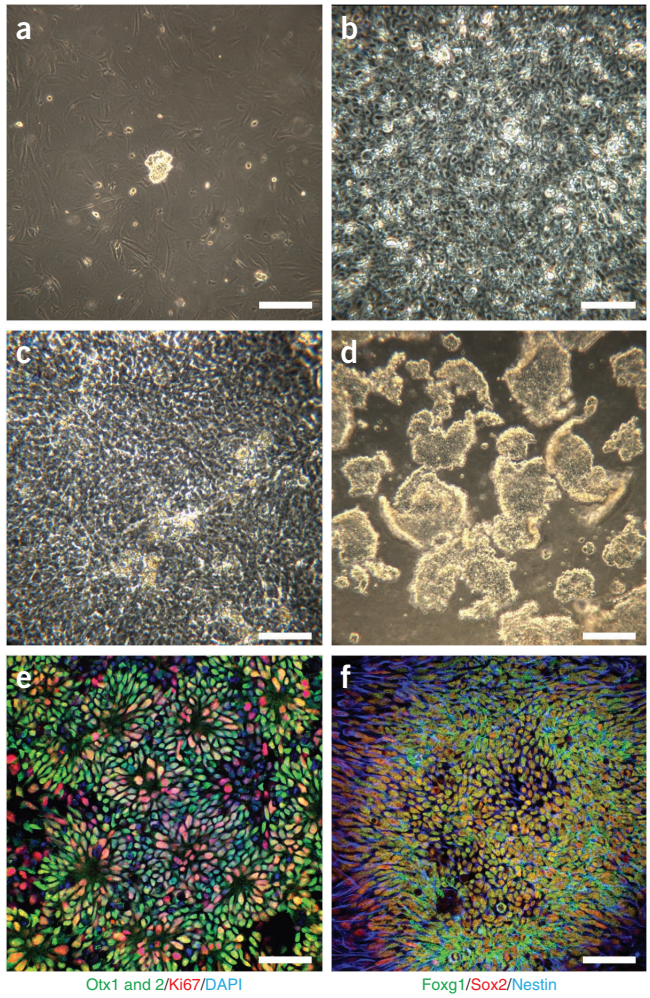

Figure 1 | Morphological changes in PSC cultures during neural induction. (**a**) Example of a PSC colony of optimal size for passaging after fragmentation, with MEF cells visible in the background. Scale bar, 100 µm. (**b**) Confluent monolayer PSCs growing on a CELLStart-coated 12-well plate ready for neural induction. Scale bar, 50 µm. (**c**) At 10 d after neural induction, a homogenous neuroepithelial layer is formed, ready for passaging onto laminin-coated 35-mm dishes. Scale bar, 50 µm. (**d**) Optimal size of neuroepithelial clumps for passaging. Scale bar, 100 µm. (**e**,**f**) Immunofluorescence staining to confirm the cortical identity of induced neural tissue—Otx1 and 2, Foxg1, Sox2 and nestin—is shown. Nuclei in **e** are stained with DAPI. Scale bars, 50 µm.

22| Break the colonies into fragments containing ~50 to 100 cells using a P1000 pipette, as described in Step 15.

23| Plate five out of six of the total PSCs from one six-well plate (i.e., the contents of five wells) into one well of the CELLStart-coated 12-well plate (from Step 18) in 800 µl of MEF-conditioned hPSC medium containing 10 ng ml^{-1} of FGF2. Plate the contents of the remaining well into all six wells of a fresh MEF-coated, six-well plate with hPSC medium to maintain and expand the undifferentiated PSCs for future use. Ensure that the cells are evenly distributed in the wells and dishes. Allow the cells to attach overnight in the incubator.
▲ **CRITICAL STEP** Ensure that the plating density is as high as proposed here to ensure that the cells reach 100% confluence 1 d after plating.
? TROUBLESHOOTING

Neural induction ● **TIMING ~12 d**

24| Check the cells on the day after plating (day 1). If the cells have reached ~100% confluence, wash the cells once with PBS and add 1 ml of neural induction medium per well. If the cells are not 100% confluent, incubate in MEF-conditioned hPSC medium containing 10 ng ml^{-1} FGF2 until they reach 100% confluence, at which point the medium should be switched to neural induction medium.

25| Continue to incubate cells, replacing the neural induction medium every day. Closely monitor for morphological changes of the PSCs during differentiation. PSCs with large nuclei should gradually be replaced by tightly packed neuroepithelial cells with notably smaller nuclei (**Fig. 1**).
? TROUBLESHOOTING

26| Between days 8 and 12 after plating, a uniform neuroepithelial sheet should appear. At this point, collect the neuroepithelial cells using dispase. First add 100 µl of 37 °C dispase stock directly into the medium in the well of the 12-well plate.
▲ **CRITICAL STEP** Ensure that a uniform neuroepithelial cell layer has formed before passaging the cells.

27| Incubate at 37 °C for 3 min.

28| Gently break the neuroepithelial sheet into aggregates of 300 to 500 cells by slowly pipetting up and down three times with a P1000 pipette.

29| Pellet the cells by centrifugation at 160*g* for 2 min at room temperature and discard the supernatant. Resuspend the cells with 10 ml of 37 °C neural maintenance medium, pellet the cells by centrifugation at 160*g* for 2 min at room temperature and discard the supernatant. Repeat this wash.

30| Gently resuspend the cells in 200 µl of neural induction medium.

31| Plate the cells from each well of the 12-well plate into individual laminin-coated 35-mm dishes containing 2 ml of neural induction medium. Allow cells to attach in the incubator overnight and change the medium to neural maintenance medium the next day.

32| Confirm successful neural induction by reverse-transcription PCR (RT-PCR) for the expression of pluripotency genes (*Pou5f1* (also known as *Oct4*), *Nanog*). Genes should be downregulated compared with hPSCs. The cortical identity of the neural tissue formed can be validated by RT-PCR detection of a combination of genes expressed in cortical progenitor cells and by the absence of expression of other regionally expressed genes (see **Table 2** for primer details), or by immunofluorescence staining for Pax6, Foxg1 and Otx1 and Otx2 expression (**Fig. 1**). It is advisable to prepare additional wells of neural induction in parallel for these purposes. RT-PCR or immunofluorescence can be performed on cells collected from single wells.
▲ **CRITICAL STEP** The time required for complete neural induction varies among different PSC lines, but generally occurs between 8 and 12 d. The sizes of the passaged cell aggregates influence the cell fate of the primitive neuroepithelium. Generally, the smaller the cell clumps, the fewer the cortical stem and progenitor cells that are generated.

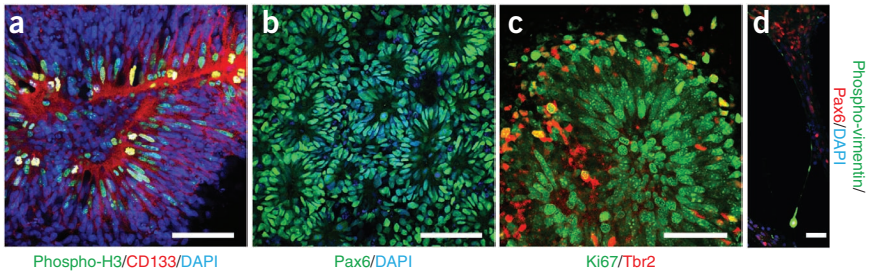

Figure 2 | Confocal microscopy images of human PSC-derived neocortical cortical stem/progenitor cells. (**a**) Example of a polarized rosette, with luminal/apical localization of CD133 (red) and many apical mitoses (phospho-histone H3, green). Scale bar, 50 µm. (**b**) Pax6$^+$ primary cortical stem/progenitor cells at day 15. Scale bar, 50 µm. (**c**) Tbr2$^+$/Ki67$^+$ secondary progenitor cells forming a basal layer around the rosette structure at day 24. Scale bar, 50 µm. (**d**) Mitotic outer radial glial cells, Pax6$^+$/phospho-vimentin$^+$, with long basal fibers but lacking an apical fiber at day 70. Nuclei in **a**,**b** and **d** are stained with DAPI (blue). Scale bar, 20 µm.

NSC expansion and differentiation ● TIMING 20 d

33| Change the medium to neural maintenance medium. Check cells daily. Neural rosette structures should be obvious when cultures are viewed with an inverted microscope around days 12–17 after neural induction (i.e., after Step 22) in neural maintenance medium. From this point, change the medium every other day.
? TROUBLESHOOTING

34| Upon appearance of rosettes, add 20 ng ml^{-1} of FGF2 for 2–4 d, which promotes the expansion of the NSCs but does not block neuronal differentiation. For examples of immunostained rosettes, showing the different populations of cortical stem/progenitor cells that make up the rosette, see **Figure 2**. After withdrawal of FGF2, around days 16–20 after neural induction, cells should be split and expanded further by passaging with dispase as described in Steps 35–40.
▲ **CRITICAL STEP** Avoid adding higher concentrations of FGF2 or longer FGF2 treatment. These can caudalize the regional identity of the NSCs, resulting in a loss of cortical identity.

35| Add 200 µl of dispase per 35-mm dish.

36| Incubate at 37 °C for 3 min.

37| Gently break the detached cell clumps into aggregates of 300 to 500 cells by slowly pipetting up and down three times with a P1000 pipette.

38| Pellet the cells by centrifugation at 160*g* for 2 min at room temperature and discard the supernatant. Resuspend the cells with 10 ml of 37 °C neural maintenance medium, pellet the cells by centrifugation at 160*g* for 2 min at room temperature and discard the supernatant. Repeat this wash.

39| Gently resuspend the cells in neural maintenance medium.

40| Plate the cells at a ratio of a single 35-mm dish into two or three laminin-coated 35-mm dishes and allow the cells to attach in the incubator overnight. Replace the medium the next day.

41| Maintain the cells for a further 4–6 d. Cells can be expanded further at this point by repeating Steps 35–40.

42| Between days 20 and 30, substantial neurogenesis should occur. When neurons first begin to accumulate at the outside of the rosettes, cells should be passaged using Accutase.

43| Add 0.5 ml of Accutase per 35-mm dish.

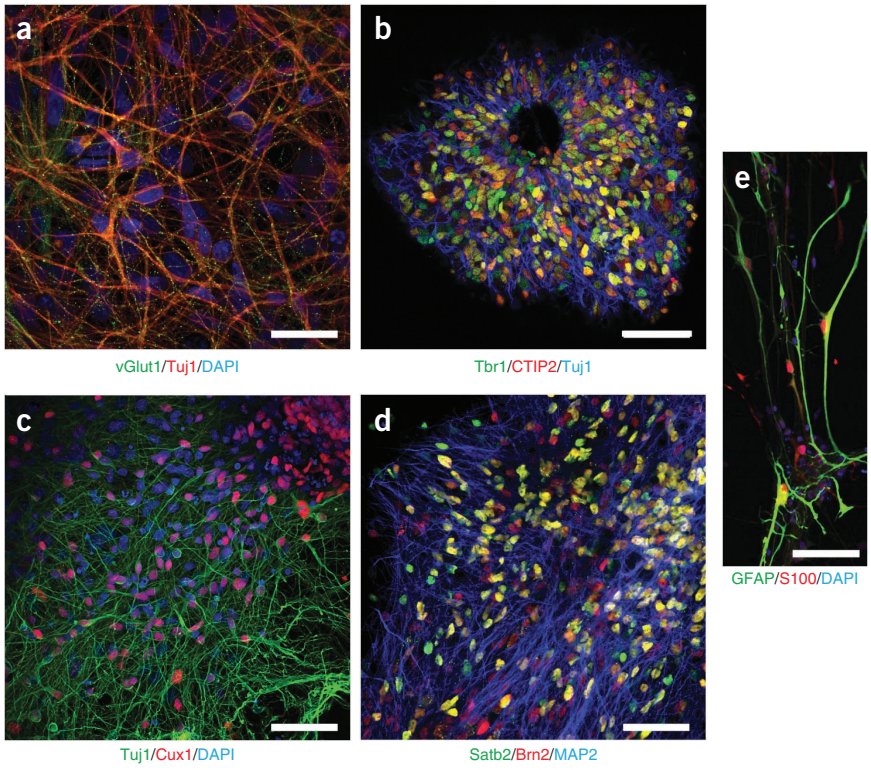

Figure 3 | Confocal microscopy images of immunofluorescence staining for different classes of excitatory neurons and astrocytes derived from human PSCs. (**a**) Confirmation of the glutamatergic identity of neurons generated with this approach by immunostaining for the vesicular glutamate transporter 1 (vGlut1, green punctate staining) and neuron-specific tubulin (Tuj1, red). Scale bar, 25 µm. (**b**) Tbr1$^+$ and CTIP2$^+$ deep-layer neurons at day 40. Scale bar, 50 µm. (**c**) Cux1-expressing upper-layer neurons at day 70 that also express neuron-specific tubulin, Tuj1. Scale bar, 50 µm. (**d**) Satb2$^+$ and Brn2$^+$ upper-layer neurons at day 80. Scale bar, 50 µm. (**e**) S100$^+$ and GFAP$^+$ astrocytes at day 90. Nuclei in **a**,**c** and **e** are stained with DAPI. Scale bar, 50 µm.

44| Incubate at 37 °C for 5 min.

45| Pipette the cells up and down 3 or 4 times in the Accutase solution to dissociate the cell clumps into a single-cell suspension.

46| Dilute Accutase with 4 volumes of neural maintenance medium; centrifuge at 160*g* for 5 min at room temperature to collect the cells.

47| Discard the supernatant and resuspend the cell pellet in neural maintenance medium. Plate cells into laminin-coated 35-mm dishes at 50,000 cells per cm^2 and incubate overnight.
■ **PAUSE POINT** If desired, cells can be cryopreserved as described in **Box 1**.

48| Replace the medium on the day after plating and continue to incubate the cells.

49| Change the culture medium every other day.

50| On days 27–31 after initial plating, passage the culture using Accutase at a ratio of 1:4 onto laminin-coated 35-mm dishes as described in Steps 43–48. Passaging the cells after day 35, at which point the proportion of neurons is becoming relatively high, is not recommended. As neurons are fragile, particularly mature neurons, their survival rate after passage is low.
? TROUBLESHOOTING

51| Continue to culture cells for up to another 50–60 d after the last passage, with medium changes every second day. If you wish to characterize the cells further, proceed to Step 52 and follow the appropriate option at your preferred time point.

Characterization of cortical neurons derived from PSCs

52| To characterize the electrical properties of PSC-derived cortical neurons, perform whole-cell patch-clamp recordings as described in option A. We recommend repeating option A weekly to monitor the electrical maturation of the neurons and synaptogenesis. To investigate the appearance of synapses by microscopy, follow option B. Synaptic proteins should be visible after day 30 after neural induction.

The identity and proportions of different types of cortical projection neurons can be defined by their combinatorial expression of a core set of transcription factors: Tbr1, CTIP2, Cux1, Satb2 and Brn2. Early-born, deep-layer cortical neurons can be observed as early as day 20 of differentiation, defined by the expression of both Tbr1 and CTIP2, and the absence of Brn2 and Cux1 expression, detected by antibody staining of fixed cultures (**Fig. 3**). Later-born, upper-layer neurons can be detected after day 70, defined by the expression of Satb2, Brn2 or Cux1, and the absence of Tbr1 and CTIP2 protein expression (**Fig. 3**).

(A) Whole-cell patch-clamp recordings to characterize the electrical properties of PSC-derived cortical neurons

(i) Take a 35-mm dish of cells and remove the medium.

(ii) Cut the walls of the dish down to ~5 mm in height using a soldering iron.

(iii) Perfuse the cells with room-temperature ACSF.
(iv) Use a pipette puller to make borosilicate glass pipettes with a resistance of 5–15 mΩ.
(v) Fill the glass pipette to about one-quarter of its capacity with a 0.22-µm-filtered intracellular recording solution.
(vi) Perform a whole-cell patch; note the membrane potential.
(vii) Switch to zero current mode to check for spontaneous action potential firing and spontaneous synaptic potentials.
(viii) Check for sodium and potassium membrane currents by performing families of step depolarizations from a holding potential of −80 mV to +40 mV in voltage clamp mode. Sodium and potassium currents can be blocked using 1 µM of tetrodotoxin and 4-AP, respectively.
(ix) Check for action potential firing in current clamp mode by performing stepwise current injections from −20pA to +60pA. See **Figure 4** for examples of typical recordings at different stages of differentiation.
(x) Check for spontaneous mini excitatory postsynaptic currents by recording at a holding potential of −70 mV in voltage clamp mode. Apply a 1 kHz low-pass Gaussian filter to visualize currents. α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) currents, if present, can be blocked with 50 µM CNQX. Similarly, use 50 µM DL-AP5 to block *N*-methyl-D-aspartic acid (NMDA) currents if present.

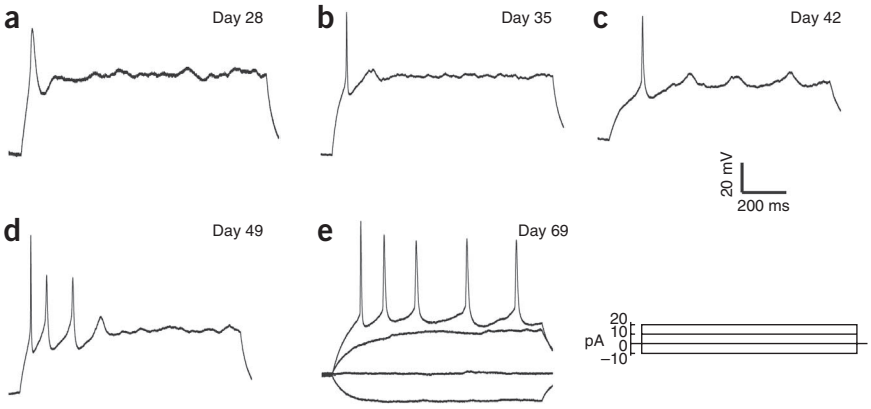

Figure 4 | Example of single-neuron electrophysiological properties at different stages of terminal differentiation. (**a**–**e**) Typical single-cell, patch-clamp recordings from neurons at a range of ages following cortical differentiation. Young neurons, as shown in **a**–**c**, fire a single action potential following current injection. As neurons mature, they progress from firing a short burst of action potentials in response to current injection (**d**) to sustained action potential firing (**e**).

(B) Microscopy to investigate the appearance of synapses
(i) Fix cells in culture in 4% (wt/vol) paraformaldehyde for 10 min. Remove the paraformaldehyde.
(ii) Perform three 5-min washes with TBS and permeabilize the cells by washing three times for 5 min each with 0.3% (vol/vol) Triton X-100 in TBS.
(iii) Block for 1 h with 5% (vol/vol) donkey serum in 0.3% (vol/vol) Triton X-100–TBS.
(iv) Stain with primary antibodies to MAP2, PSD95, Homer1 and synaptophysin (**Table 1**) diluted in blocking solution at 4 °C overnight.
(v) Look for the juxtaposition of presynaptic proteins such as synaptophysin and Munc13-1 with postsynaptic density components such as PSD95 and Homer1. For optimal spatial resolution of the localization of these proteins, use a super-resolution microscope as used in our original report[12]. See **Figure 5** for examples of results obtained.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible solutions
23	Lots of carry-over of MEFs	Check the concentration of dispase
		Collect the detached colonies only
		Avoid flushing the feeder plate with the medium to immobilize the colonies
25	No uniform neuroepithelial sheet is formed	Check the concentration and activity of the SMAD signaling inhibitors
		Were cells fully confluent before starting neural induction?
		Check for genome changes in the starting PSCs (translocations, ploidy changes, copy number differences)
		Check for infection in the PSC line or in MEFs (e.g., mycoplasma)

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible solutions
	The neuroepithelial sheet fragments before replating	Passage the cells promptly when the neuroepithelium appears
		Avoid leaving the cells in neural induction medium for too long
33	No neural rosettes are formed	Keep the sizes of the neuroepithelial clumps above 300 cells
		Avoid dissociating the neuroepithelium too harshly
50	Very few cortical neurons	Avoid adding FGF2 at a concentration >20 ng ml^{-1} or treating with FGF2 for longer than 4 d
		Use fresh B-27
		Use fresh neural maintenance medium

● TIMING

Expanding the hPCS from frozen stocks (Steps 1–17) and preparing them for neural induction cells for neural induction (Steps 18–23) takes ~3 weeks. The neural induction stage (Steps 24–32) takes about 12 d. Expansion of the NSCs and subsequent neural differentiation (Steps 33–50) takes a further 20 d. The neuronal cultures can then be maintained, if desired, for over 100 d.

ANTICIPITATED RESULTS

The *in vitro* cortical neurogenesis system described here typically generates almost pure cultures of primary cortical stem/progenitor cells from PSCs within 15 d of neural induction. The duration and efficiency of the neural induction depend on the particular PSC lines used, although in all the lines that we have studied to date this step is reached within 8–12 d. It should be emphasized that this is the critical step in generating cortical projection neurons from hPSCs: if efficient cortical induction is achieved, subsequent neuronal differentiation is robust and reproducible. The cortical identity of the stem/progenitor cells can be characterized by their coexpression of Pax6, Foxg1, Emx1, Otx1 and vimentin, and apical localization of CD133 (**Figs. 1** and **2**). Neural differentiation will be accompanied by the loss of expression of pluripotency genes, such as *Pou5f1* (*Oct4*) and *Nanog*, which should be confirmed by RT-PCR or immunofluorescence staining. Addition of another BMP signaling inhibitor, dorsomorphin, is useful for neural differentiation of lines that do not undergo efficient neural induction with noggin and SB431542, as has been previously described[22].

The primary cortical progenitor cells generated from PSCs subsequently give rise to all populations of secondary intermediate progenitor cells (**Fig. 2**). When setting up the process, the presence of secondary cortical progenitor cells can be confirmed by immunostaining and confocal microscopy, as shown in **Figure 2**. Intermediate or basal progenitor cells can be defined by coexpression of Tbr2 and Ki67 (to distinguish cycling stem/progenitor cells from postmitotic neurons), and lack of expression of Pax6. Typically, $Tbr2^+/Ki67^+$ cells are found at the periphery of each neural rosette, constitute around 10–15% of the cycling cells in each rosette and make up half of the $Tbr2^+$ population, the other half being newly born, postmitotic neurons[12]. Outer SVZ (oSVZ) cells are more challenging to characterize, because of the absence of definitive, cell-specific markers. However, the presence of oSVZ cells can be confirmed by immunostaining for Pax6 and phospho-vimentin, which labels the basal processes of secondary, oSVZ progenitor cells in M-phase (**Fig. 2**). In this case, oSVZ cells should also lack an apical process (**Fig. 3**). At this stage, neural rosettes can be passaged as single cells and cryopreserved for future use. Replating of dissociated rosette cells leads to reformation of rosettes and robust, reproducible cortical neurogenesis.

During neurogenesis, early-born deep-layer neurons ($Tbr1^+$ and/or $CTIP2^+$) begin to be produced from around day 20, but only become a major population around day 35 (**Fig. 3**). Considerable numbers of late-born upper-layer neurons ($Satb2^+$, $Cux1^+$ and $Brn2^+$ neurons) are seen after day 70 (**Fig. 3**). Generally, astrocytes are detected after day 45, and their proportion increases over time (**Fig. 3**). Typically, approximately equal proportions of deep- and upper-layer neurons are generated over the 3-month culture period, as we have found when studying the differentiation of two different hESC and four independent iPSC cell lines[12]. The cortical identity of the neurons generated by this process should be confirmed by combinatorial transcription factor expression and by expression of proteins specific to excitatory glutamatergic neurons (vGlut1, PSD-95). There should be clearly separate populations of deep-layer $Tbr1^+/CTIP2^+$ neurons and $Brn2^+/Cux1^+/Satb2^+$ upper-layer neurons, and all should express vGlut1. Finally, functional confirmation of excitatory, glutamatergic identity can be made using single-cell, patch-clamp recordings of spontaneous and evoked excitatory postsynaptic potentials[12].

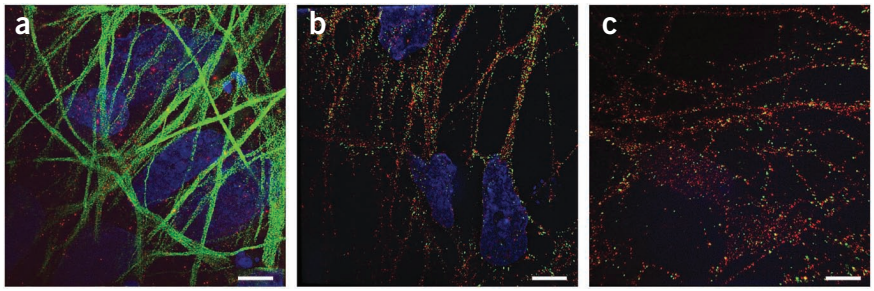

Figure 5 | Physical synapses visualized by super-resolution microscopy. (**a**) Super-resolution microscopy images of dendrites (MAP2, green) showing localization of foci of the excitatory synapse–specific PSD95 protein (red). (**b**,**c**) Physical synapses (arrows in all images) can be identified by juxtaposition of presynaptic and postsynaptic protein complexes. The examples shown are Homer and Munc13 (**b**), and PSD95 and synaptophysin (**c**). Nuclei are stained with DAPI (blue). Scale bars, 5 μm.

The maturation of electrical properties of postmitotic cortical neurons, most notably their rate of spontaneous action potential firing, takes several weeks in culture (**Fig. 4**). Before day 40, spontaneously firing/active neurons are uncommon in culture, becoming more common over time, with neurons in cultures aged day 80 and above showing complex patterns of spontaneous activity that can be detected by patch-clamp recordings of single neurons or visualized by calcium imaging (**Fig. 4**). Physical synapses are common from day 28, as visualized by immunofluorescence staining and super-resolution microscopy (**Fig. 5**). However, functional glutamatergic synapses are rarely detected before day 30, becoming common after day 50 in culture.

ACKNOWLEDGMENTS We thank all members of the Livesey group for feedback on the practical details of the protocol reported here. Y.S. was supported by a Biotechnology and Biological Sciences Research Council Dorothy Hodgkin Studentship. P.K. was supported by the University of Cambridge/Wellcome Trust PhD Programme in Developmental Biology. This research benefits from core support to the Gurdon Institute from the Wellcome Trust and Cancer Research UK and grants to F.J.L. from the Wellcome Trust and Alzheimer's Research UK.

AUTHOR CONTRIBUTIONS All authors contributed to the writing, editing and testing of this protocol.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nprot.2012.116. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Lindvall, O. & Kokaia, Z. Stem cells in human neurodegenerative disorders—time for clinical translation? *J. Clin. Invest.* **120**, 29–40 (2010).
2. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676 (2006).
3. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
4. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
5. Park, I.H. *et al.* Disease-specific induced pluripotent stem cells. *Cell* **134**, 877–886 (2008).
6. Ebert, A.D. *et al.* Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* **457**, 277–280 (2009).
7. Lee, G. *et al.* Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* **461**, 402–406 (2009).
8. Yagi, T. *et al.* Modeling familial Alzheimer's disease with induced pluripotent stem cells. *Hum. Mol. Genet.* **20**, 4530–4539 (2011).
9. Shi, Y. *et al.* A human stem cell model of early Alzheimer's disease pathology in Down's syndrome. *Sci. Transl. Med.* **4**, 124ra29 (2012).
10. Israel, M.A. *et al.* Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. *Nature* **482**, 216–220 (2012).
11. Devine, M.J. *et al.* Parkinson's disease induced pluripotent stem cells with triplication of the α-synuclein locus. *Nat. Commun.* **2**, 440 (2011).
12. Shi, Y., Kirwan, P., Smith, J., Robinson, H.P. & Livesey, F.J. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat. Neurosci.* **15**, 477–486 (2012).
13. Rakic, P. Evolution of the neocortex: a perspective from developmental biology. *Nat. Rev. Neurosci.* **10**, 724–735 (2009).
14. Hill, R.S. & Walsh, C.A. Molecular insights into human brain evolution. *Nature* **437**, 64–67 (2005).
15. Watanabe, K. *et al.* Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat. Neurosci.* **8**, 288–296 (2005).
16. Gaspard, N. *et al.* An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature* **455**, 351–357 (2008).
17. Eiraku, M. *et al.* Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* **3**, 519–532 (2008).
18. Au, E. & Fishell, G. Cortex shatters the glass ceiling. *Cell Stem Cell* **3**, 472–474 (2008).
19. Chambers, S.M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat. Biotechnol.* **27**, 275–280 (2009).
20. Bibel, M. *et al.* Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nat. Neurosci.* **7**, 1003–1009 (2004).
21. Siegenthaler, J.A. *et al.* Retinoic acid from the meninges regulates cortical neuron generation. *Cell* **139**, 597–609 (2009).
22. Kim, D.S. *et al.* Robust enhancement of neural differentiation from human ES and iPS cells regardless of their innate difference in differentiation propensity. *Stem Cell Rev.* **6**, 270–281 (2010).
23. Amit, M. *et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* **227**, 271–278 (2000).
24. Marchetto, M.C. *et al.* A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell* **143**, 527–539 (2010).
25. Brennand, K.J. *et al.* Modelling schizophrenia using human induced pluripotent stem cells. *Nature* **473**, 221–225 (2011).