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Generation and assembly of human brain region–specific three-dimensional cultures

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The ability to generate region-specific three-dimensional (3D) models to study human brain development offers great promise for understanding the nervous system in both healthy individuals and patients. In this protocol, we describe how to generate and assemble subdomain-specific forebrain spheroids, also known as brain region–specific organoids, from human pluripotent stem cells (hPSCs). We describe how to pattern the neural spheroids toward either a dorsal forebrain or a ventral forebrain fate, establishing human cortical spheroids (hCSs) and human subpallial spheroids (hSSs), respectively. We also describe how to combine the neural spheroids in vitro to assemble forebrain assembloids that recapitulate the interactions of glutamatergic and GABAergic neurons seen in vivo. Astrocytes are also present in the human forebrain–specific spheroids, and these undergo maturation when the forebrain spheroids are cultured long term. The initial generation of neural spheroids from hPSCs occurs in <1 week, with regional patterning occurring over the subsequent 5 weeks. After the maturation stage, brain region–specific spheroids are amenable to a variety of assays, including live-cell imaging, calcium dynamics, electrophysiology, cell purification, single-cell transcriptomics, and immunohistochemistry studies. Once generated, forebrain spheroids can also be matured for >24 months in culture.

Introduction

Understanding the development of the human nervous system and elucidating the mechanisms that lead to brain disorders represent some of the most challenging ongoing endeavors in neurobiology. One major obstacle is the restricted access to healthy and diseased human brain tissue for functional molecular and cellular studies[1,2]. As a result, experimental paradigms have been largely confined to animal models or in vitro cell culture systems that do not fully recapitulate the developmental, architectural, and species-specific aspects of the human brain. Thus, there is a great need for human-derived model systems that recapitulate features of human CNS development and allow for the study of these processes in both healthy and diseased conditions.

Modeling human neural development with pluripotent stem cells

The ability to reprogram human somatic cells to induced pluripotent stem cells (hiPSCs)[3–5] and subsequently differentiate these cells into neural lineages provides the opportunity to study features of human neural development in vitro[6]. In addition, these systems allow scientists to link rare, highly penetrant mutations, as well as more complex genetic events, to cellular disease phenotypes[7,8]. Initial patient-derived hiPSC models are centered on the use of adherent two-dimensional (2D) cell culture systems[9–13]. These protocols generate relatively homogeneous populations of neural types but do not capture the cellular diversity or 3D tissue architecture[14]. 3D human brain cultures were established to provide physiologically relevant models that more faithfully recapitulate the spatial organization, cellular diversity, and cell–cell interactions that are present in the developing human nervous system[15,16].

In this protocol, we describe how to generate brain region–specific spheroids from hiPSCs or human embryonic stem cells (hESCs). These region-specific spheroids or organoids are patterned to resemble the dorsal forebrain, also known as the pallium (hCSs)[17,18], or the ventral forebrain, also known as the subpallium (hSSs)[19]. The protocol consists of neural induction of 3D aggregates of hPSCs, followed by culture in the presence of a variety of small molecules to derive specific cell fates. We have previously used this protocol for the in vitro study of the development of the human cerebral cortex[17] and the maturation of glial cells into postnatal stages[20], to test infectivity in human

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neural cells of novel adeno-associated virus (AAV) subtypes[21], to model interregional cross-talk in the developing forebrain, and to identify phenotypes in a monogenic form of autism spectrum disorders[19]. Because neural spheroids are derived from reprogrammed human cells, they are an ideal system for studying disease phenotypes from specific human genetic backgrounds[18]. In addition, this platform allows for the study of later stages of human brain development, including the maturation of astrocytes[20], and can be used to derive and assemble different region-specific spheroids into 'brain assembloids', which can be used to study cell migration and neural circuit formation[19].

Features of human forebrain development

Human forebrain development proceeds through a series of stereotyped steps that are guided by specific patterning cues[22,23]. Early in embryonic development, the neural tube begins to expand and forms three primary pouches, which are anatomic structures that will eventually segregate into the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon)[24]. The maturing forebrain can be anatomically and functionally subdivided along a dorsal–ventral axis, with the dorsal region (pallium) eventually generating the bulk of the cerebral cortex and the ventral region (subpallium) comprising the lateral and medial/caudal divisions of the ganglionic eminences (LGE and MGE/CGE, respectively)[25]. These eminences are the origin of cortical interneuron populations[26]. The vast majority of GABAergic interneurons are born in the MGE or the CGE, where they also acquire specific subtype identities (e.g., parvalbumin, somatostatin, and calbindin). Having been specified in the subpallium, these interneurons begin a migratory journey to populate the developing dorsal pallium. Around gestational week (GW) 15 in humans, interneurons exit the pallial–subpallial border and enter the dorsal forebrain via tangential migration to disperse throughout the subplate, cortical plate, and marginal zones[27]. This process continues in frontal cortical regions from then onward and throughout the second year of life in humans[28]. These cortical interneurons then undergo an activity-dependent maturation process and integrate into neural circuits[29], where they contribute to the excitation-to-inhibition balance (E/I). This is a critical process in CNS development and circuit functioning. Furthermore, genetic or environmental imbalances of the E/I are thought to contribute to neuropsychiatric disorders such as epilepsy, autism spectrum disorders, and intellectual disabilities[30,31].

Neuronal production is just one of the many components of CNS development. During later gestational stages and continuing after birth in humans, neural progenitors switch from a largely neurogenic to a gliogenic fate, generating astrocytes and later oligodendrocytes[32,33]. This transition constitutes the bulk of astrogenesis, as newly born astrocyte precursor cells begin to populate the brain parenchyma and distribute throughout the CNS in a tiled distribution. Astrocytes help choreograph neural development and actively contribute to neural circuit formation by controlling synapse formation, function, and elimination. This raises the possibility that astrocyte dysfunction may also directly contribute to the pathophysiology of neuropsychiatric disorders.

Development of the method and comparison with other approaches

In recent years, a number of 3D brain tissue models have been developed[16,17,34–37], each with specific advantages and disadvantages. What is common among these methodologies is that they use the capacity of hPSCs to differentiate and self-organize in a 3D environment and to recapitulate key features of human brain development, such as the presence of ventricular-like structures surrounded by progenitor populations that differentiate into multiple cell lineages.

The method we describe here builds upon several previous advances. Inhibitors of the SMAD pathway were shown to induce rapid and fast neural differentiation of high-density hPSCs with a default dorsal forebrain fate[38]. In addition, hPSCs were shown to undergo neural differentiation following aggregation in the presence of a ROCK inhibitor in V- or U-shaped wells, followed by plating in 2D cultures several weeks later[39]. Building upon these studies, we developed an approach that involves transferring intact colonies of hPSCs to ultra-low-attachment plates and culturing these cells exclusively in suspension without an extracellular matrix or a bioreactor, in the presence of two inhibitors of the SMAD pathway to facilitate neuralization[17]. For neuralization, we have primarily used dorsomorphin (DM) and SB-4321542 (SB), but other combinations of SMAD inhibitors can be used. Our approach also uses a longer exposure to the growth factors EGF and FGF2 to allow proliferation and corticogenesis progression, followed by an 18-d exposure to BDNF and NT3 for maturation. This protocol yields self-organizing 3D cultures resembling the dorsal forebrain, which can be maintained for years, displaying cellular features observed in the postnatal brain[20]. We have

subsequently developed a complementary approach to derive ventral forebrain 3D cultures in which dual SMAD inhibition is followed by exposure to a small-molecule sonic hedgehog agonist, retinoic acid (RA), for fate specification, and allopregnanolone (AlloP), for neural maturation[19].

Overall, our method yields region-specific 3D forebrain cultures. In the years following the development of the protocol outlined here, a number of additional groups have continued to adapt the strategy of regional patterning to obtain other brain region–specific patterned structures, such as midbrain, hindbrain, or cerebellum[17,35–37,40]. In addition, and in contrast, other groups have combined the intrinsic differentiation ability and the inherent self-differentiation cues within hPSCs with extracellular matrices or bioreactors to develop organoids via an undirected approach[34]. Without specific inductive signals, this approach has the advantage of generating a variety of cell fates within and across organoids. Single-cell transcriptomic studies of undirected organoids[41] confirm the presence of mixed dorsal and ventral forebrain cell lineages, along with cells from other brain regions, such as hindbrain, midbrain, retina, and mesodermal cells. This high degree of cellular diversity may allow for future studies of human CNS diversity and mapping of genes associated with neuropsychiatric disease onto specific cell types[16]. However, given the lack of external cues, these undirected differentiation techniques tend to display a higher degree of stochasticity and may exhibit a degree of variation in cell types and maturation that can make interpreting disease phenotypes challenging.

One application of the region-specific brain spheroids is for modeling interregional interactions in CNS development[19]. In this modular approach, separately patterned ventral and dorsal forebrain spheroids are cultured together so that they fuse and generate forebrain assembloids. This enables cortical GABAergic neurons, which are specified in ventral forebrain spheroids, to migrate toward the dorsal forebrain spheroid and then to synaptically integrate into networks with cortical glutamatergic neurons. This platform allows live monitoring and genetic and pharmacological manipulation of cortical interneuron migration, a process that happens at the late stages of human gestation. Importantly, we have compared our findings with results from human primary fetal forebrain slices (GW 20). We have also demonstrated the power of this platform to identify cell-specific phenotypes and rescue strategies by deriving forebrain assembloids from patients with a rare genetic form of autism spectrum disorder and epilepsy[19]. Two other groups have subsequently adapted similar, assembly-based approaches to re-create and modulate cell migration from ventral forebrain (subpallium)-like to dorsal forebrain (pallium)-like 3D cultures[42,43]. Bagley et al.[42] used Matrigel-embedding and an orbital shaker for assembly, whereas Xiang et al.[43] implemented a spontaneous fusion approach. Each study also used a unique molecular-labeling approach to track migrating cells from one 3D culture to the other. For example, Bagley et al. used an EF1α-GFP–expressing hPSC line to track the population of migrating cells (60% of which were GABAergic cells), whereas Xiang et al. used an NKX2.1-GFP hPSC line (similar to the Dlxi1/2b::GFP enhancer we have used) to track MGE-lineage cells. These studies show that brain assembloids can reproducibly model interregional interactions and complex cellular phenotypes such as cell migration in a 3D microphysiological environment.

Applications and limitations

hPSC-derived 3D cultures can be used to address questions that are related to human brain development and disease modeling[15,44]. One particular advantage of this method is the ability to investigate human-specific phenotypes in patient-derived cultures, which may be challenging to capture in other systems. The entirety of the protocol described here is performed in ultra-low-attachment culture plates without embedding into extracellular matrices or the use of spinning bioreactors. At later stages of differentiation, cell death may be observed in hCSs and hSSs. However, our biosensor measurements demonstrate a partial pressure of oxygen that remains >60 mmHg in the center of hCSs that are 4 mm in diameter.

The brain region–specific spheroids we obtain are amenable to many downstream assays and analyses (Fig. 1). They can be fixed and cryosectioned for immunohistochemistry (Step 23C) or in situ hybridization, and they can be used intact for live-cell imaging using a variety of microscopy setups (e.g., Step 23D, F). In addition, they can be easily dissociated for sorting into distinct cell populations (Step 23B), immunopanning, or single-cell transcriptomics. Forebrain spheroids resembling the dorsal or ventral part can also be fused to form assembloids[19,42,43] (Step 23A). This system captures the interactions of various region-specific neuronal and/or glial populations, including the migration and integration of GABAergic neurons into the cerebral cortex. Forebrain

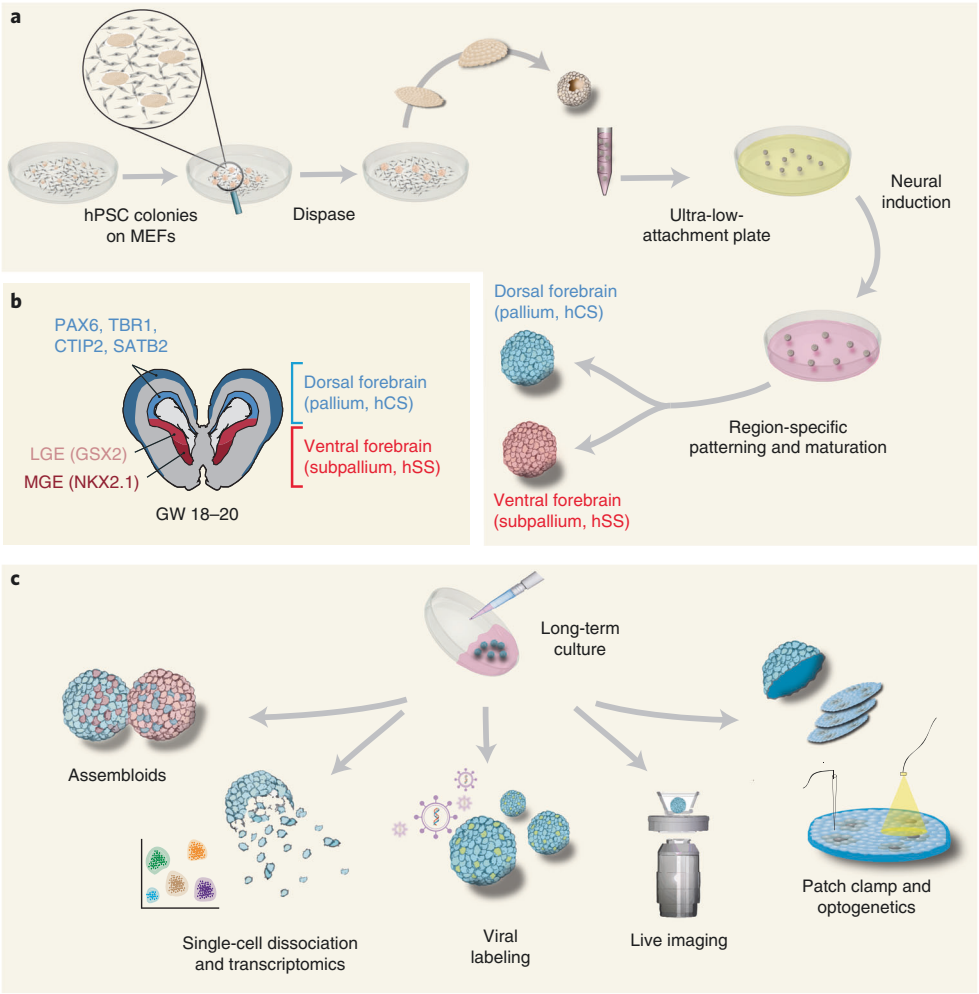

Fig. 1 | General schematic for the generation of human forebrain spheroids from hPSCs. a, Scheme illustrating the main stages of the method for the generation of dorsal (hCS) and ventral (hSS) forebrain spheroids from hPSCs. **b**, Coronal section from GW 18–20 human brain, outlining dorsal (pallial) and ventral (subpallial) markers and their domains. **c**, Examples of applications and functional assays that can be carried out using region-specific and assembled neural spheroids.

spheroids can be cryosectioned for immunostaining (Step 23C) and are amenable to physiology experiments in slices. They can also be loaded with calcium-sensitive dyes for probing neuronal activity (see ref. [19] for detailed protocols). Neural spheroids can be easily transfected at any stage with viruses carrying a variety of constructs (Box 1) and can even be transplanted with exogenous cell types such as microglia or oligodendrocytes, which engraft and migrate into the parenchyma of the 3D structure.

The system is easily scalable and therefore can be used for toxicology, CRISPR/Cas9 genome editing, and drug-screening paradigms. In addition, although the protocol described here includes growing hPSCs on MEFs, optimization to feeder-free and xeno-free conditions is currently in process and should be achievable. Perhaps most importantly, forebrain spheroids can be maintained in suspension culture for long periods of time[20] (>25 months, in our experience), which allows for the study of cell diversity and maturation as the spheroids age in vitro.

Nevertheless, although there has been unprecedented progress in the ability to differentiate hPSCs into cell types of interest and rapid advances in the ability to grow cells in 3D cultures, there are still substantial issues that must be addressed. These include the need to promote further functional maturation of neurons and glial cells, which could require more physiological medium formulations, improved extracellular matrices, or novel strategies for perfusion and grafting into mammalian tissue[16]. In addition, to capture the regional diversity of the human CNS, additional protocols will need to be developed for deriving and assembling other nervous system regions, such as the thalamus,

brainstem, or spinal cord. To capture the cellular diversity and study complex cell–cell interactions in the CNS, future organoid or assembloid models could incorporate myelinating oligodendrocytes, microglia, and vascular cell types. Variability between organoids is a barrier to disease modeling, and thus tools for controlling their internal organization, such as the use of organizer-like molecules (e.g., morphogens soaked in beads), could become powerful strategies. Developing strategies for accelerating the maturation and senescence of human neural cells will be critical to developing in vitro models of neurodegenerative disorders. Last, there is a need to be able to scale up culture systems to facilitate the running of large drug and genetic screens or studies across large cohorts of patients and controls. Over the next few years, as the field evolves, this technology promises to accelerate the study of human brain development, evolution, and disease.

Experimental design

Before beginning experiments, it is important to consider several key points that will help determine the scale and time required to perform adequately powered differentiation experiments:

- All experiments should be performed using at least three hPSC lines, with at least three separate differentiations, and using at least two to three neural spheroids per differentiation or condition.
- If validating disease phenotypes, the use of isogenic hPSC lines is essential.
- The results from hESC and hiPSC experiments should be reported separately. We have successfully generated neural spheroids from >70 hiPSC lines and hESCs such as H9.
- Some line-to-line heterogeneity (especially in early stages of differentiation) can be expected, but there should be consistency within lines and across separate differentiations of the same line.
- For intra-spheroid quantifications, at least three to five sections per spheroid should be used.

In addition, it is important to undertake the following quality-control steps during the differentiation and patterning process:

- Cultures of hPSCs, hCSs, and hSSs must be regularly checked for mycoplasma (one to two times/month).
- Genome integrity of hPSCs must be verified regularly by SNP array or comparative genomic hybridization arrays to identify de novo genomic events. If any abnormalities are noted, earlier passages of cells should be used. For differentiations, we primarily use hPSC lines between passages 10 and 40.
- The quality (size, shape, tendency to differentiate) of hPCSs must be checked daily before starting a new differentiation experiment. It is important to ensure that the properties of hPSCs are consistent across passages and before differentiations.
- Cultures should be checked regularly for bacterial/fungal infection. If the medium is found to be cloudy, it should be checked under the microscope at 40× magnification to identify moving bacteria or fungi. It is important to determine the type of contamination immediately and take appropriate measures.
- From days 0 to 6 of differentiation, newly formed neural spheroids must be checked daily for disintegrating/dying cells (the medium will be cloudy at the bottom of the plate and many floating single cells may be observed under the microscope). If cell death or spheroid disintegration exceeds expectations, it is likely that the differentiation will not progress, and it is better to discard the plates.
- The health of spheroids may vary over the culture period and be dependent on the quality of the founder hPSC colonies. Consider sampling hCSs or hSSs and carrying out periodic immunostaining to assess health. Generally, autofluorescence in the center of sectioned spheroids or detaching cells on the surface indicates cell death.
- Consider introducing quality controls for differentiation efficiency by running quantitative PCRs (qPCRs) for a panel of fate- and region-specific genes at particular time points during the differentiation (e.g., day 25).

Overview of the procedure

The procedure starts with culture and passage of hPSCs on a MEF feeder layer (Steps 1–13) (Fig. 2). When hPSC colonies reach around 1.5 mm in diameter, they are suspended in medium (Steps 14–16) and cultured in specific culture conditions that induce neuronal differentiation (Steps 17–21). Step 22 describes the specific culture conditions required to differentiate neural spheroids into hSSs or hCSs. In Step 23 of the procedure, we describe how to carry out various downstream assays. We also describe how to virally label hCSs and hSSs, which can be carried out at any stage of spheroid growth, in Box 1.

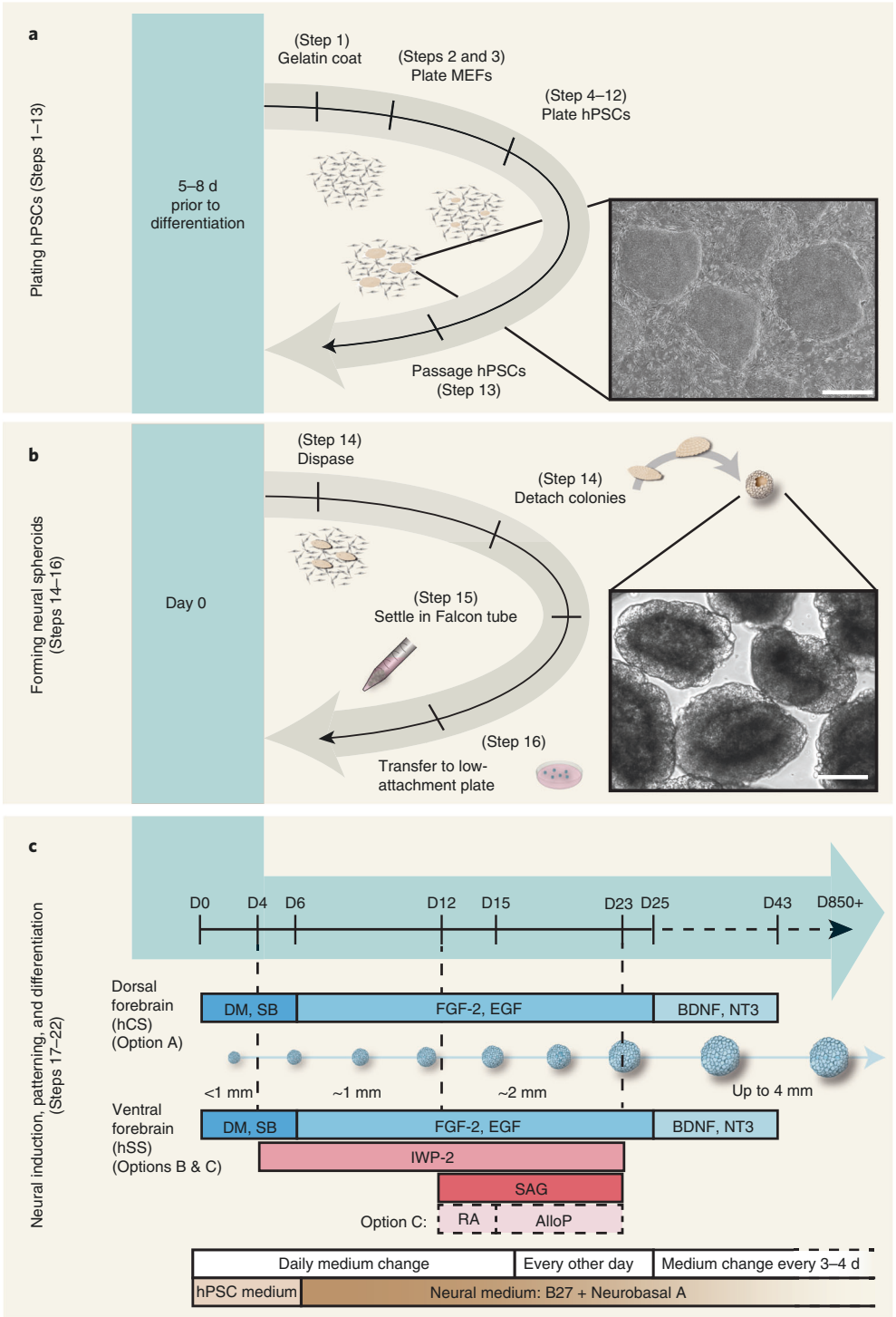

Fig. 2 | Outline of the human forebrain spheroid protocol. a, Recommendations for growing hPSC colonies before aggregation and spheroid formation. Representative image of ideal hPSC colonies either before passaging or enzymatic lifting to form spheroids. Scale bar, 600 µm. **b**, Details of how to lift hPSC colonies to form spheroids using the enzyme dispase. Representative image of colonies immediately after dispase treatment. Scale bar, 150 µm. **c**, Neural induction and patterning protocols, and the timeline for the generation of dorsal and ventral forebrain–specific 3D cultures, including hSS-ISRA (option C, which includes exposure to RA and AlloP). D, day. The results shown here are from experiments using hPSCs that conformed to institutional and federal regulations. IRB panel approval and appropriate informed consent were obtained for these studies.

Box 1 | Viral labeling of hCSs and hSSs ● Timing 6–10 d

Additional reagents and materials

- HEK 293T cells (ATCC, cat. no. CRL-3216)
- Lenti-X concentrator (Clontech, cat. no. 631232)
- Packaging plasmids (Takara, cat. no. 631275)
- Opti-MEM reduced-serum medium (Life Technologies, cat. no. 31985-062)
- Lipofectamine 2000 transfection reagent (Life Technologies, cat. no. 11668019)
- AAV-DJ-hSyn::YFP or AAV-DJ-hSyn::mCherry (Stanford Neuroscience Institue (SNI) Gene Vector and Virus Core: https://neuroscience.stanford.edu/research/programs/community-labs/neuroscience-gene-vector-and-virus-core), for labeling neurons in hCSs
- Lenti-Dlxi1/2::eGFP (from J. Rubenstein, UCSF), for labeling cortical GABAergic interneurons
- Lenti-GFAP::eGFP (from C.-L. Zhang, UT Southwestern), for labeling astrocytes in hCSs and hSSs

Additional equipment

- Parafilm (VWR, cat. no. PM-999)

Procedure

1 hCSs and/or hSSs can be fluorescently labeled using viruses before assembly. We obtain AAVs from the SNI Gene Vector and Virus Core. AAV-DJ is generally our serotype of choice. However, we have successfully used other AAV serotypes[21]. We make lentiviruses in-house using standard protocols[47]. Briefly, transfect target plasmid and third/fourth-generation packaging plasmids in HEK 293T cells using Lipofectamine 2000. After collecting the supernatant, concentrate the lentivirus by incubation with a Lenti X concentrator (at 4 °C for 30 min to overnight) followed by centrifugation (1,500*g* for 45 min at 4 °C).

2 For labeling spheroids, add the virus to a 1.5-ml microcentrifuge tube containing one to three spheroids in 250 µl of NM (Reagent setup). Agitate the tube gently and incubate overnight in an incubator at 37 °C, 5% CO_2.
▲ **CRITICAL STEP** The optimal titer for each virus must be empirically determined.

3 On the next day, add an extra 800 µl of NM to the mix and incubate for 24 h. On day 2, transfer the spheroids back to 10-cm plates. Expression is usually seen 6–10 d post exposure.
? TROUBLESHOOTING

Materials

Biological materials

- Human pluripotent stem cells (hPSCs); forebrain spheroids can be generated from cultures of either hESCs, e.g., H9, or hiPSCs. **! CAUTION** The experiments involving hPSCs in this study were approved by the Stanford University School of Medicine Institutional Review Board (IRB) and Stem Cell Research Oversight. The hPSCs used to generate the results shown in this protocol were derived at Stanford University and validated using standardized methods (e.g., pluripotency assays, genome-integrity checks), as previously described[10,45,46]. Cultures were tested and maintained mycoplasma-free. All experiments involving the use of hPSCs must conform to institutional and federal regulations, including IRB panel approval and appropriate informed consent.
- EmbryoMax primary mouse embryo fibroblasts, neo resistant, Myto-C treated (Millipore, cat. no. PMEF-N-K); alternatively, DR4 mouse embryo fibroblasts (Stanford Human Pluripotent Stem Cells Core Facility) can also be used.

Reagents

Media and supplements

- DMEM (Life Technologies, cat. no. 10313-039)
- DMEM/F12 (1:1; Life Technologies, cat. no. 11330-032)
- Neurobasal A medium (Life Technologies, cat. no. 10888022) **! CAUTION** Neurobasal A medium is extremely sensitive to changes in temperature and light. Keep refrigerated at all times.
- FBS albumin, certified (Life Technologies, cat. no. 16000-044) ▲ **CRITICAL** To avoid lot-to-lot variability biases, we recommend testing and purchasing this product in bulk.
- KnockOut serum replacement (KSR; Life Technologies, cat. no. 10828-028) ▲ **CRITICAL** To avoid lot-to-lot variability biases, we recommend testing and purchasing this product in bulk.
- B27 supplement (Life Technologies, cat. no. 12587010) ▲ **CRITICAL** Use B27 supplement without vitamin A. To avoid lot-to-lot variability biases, we recommend testing and purchasing this product in bulk.

- GlutaMAX supplement, 200 mM (Life Technologies, cat. no. 35050-061)
- MEM non-essential amino acids (NEAA) solution, 100× (Life Technologies, cat. no. 11140-050)
- Penicillin–streptomycin (Pen–Strep), 10,000 U/ml (Life Technologies, cat. no. 15070-063) ▲ **CRITICAL** Differentiations can also be performed without Pen–Strep.
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148)
- Human recombinant FGF2 (R&D Systems, cat. no. 233-FB)
- Rock inhibitor Y-27632 (Selleckchem, cat. no. S1049)
- SMAD inhibitor dorsomorphin (DM; AKA compound C; Sigma-Aldrich, cat. no. P5499)
- SMAD inhibitor SB-431542 (SB; R&D Systems/Tocris, cat. no. 1614)
- Human recombinant EGF (R&D Systems, cat. no. 236-EG)
- Wnt inhibitor (IWP-2; Selleckchem, cat. no. S7085)
- Smoothened agonist (SAG; Selleckchem, cat. no. S7779)
- Allopregnanolone (AlloP; Cayman Chemicals, cat. no. 16930)
- Retinoic acid (RA; Sigma-Aldrich, cat. no. R2625)
- Human recombinant brain-derived neurotrophic factor (BDNF; PeproTech, cat. no. 450-02)
- Human recombinant neurotrophin 3 (NT3; PeproTech, cat. no. 450-03)
- 30% (wt/vol) D(+)-Glucose (Sigma, cat. no. G7021)
- $NaHCO_3$ (Sigma, cat. no. S5761)
- EDTA (Sigma, cat. no. ED)

Cell culture reagents

- Gelatin solution bioreagent, 2% in H_2O (Sigma, cat. no. G1393)
- Dispase (Invitrogen, cat. no. 17105-041)
- Dulbecco's DPBS with calcium and magnesium (DPBS; Caisson Labs, cat. no. PBL02)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650)
- Ethyl ethanol, 200 proof (Gold Shield, cat. no. 412804) ! **CAUTION** Ethyl ethanol is flammable and should be maintained in a flame-protective cabinet.

For single-cell dissociation only

- 10× Earle's Balanced Salt Solution (EBSS; Sigma, cat. no. E7510)
- BSA (Sigma, cat. no. A4161)
- 0.4% (vol/vol) DNase, 12,500 U/ml (Worthington, cat. no. LS002007)
- L-Cysteine hydrochloride monochloride (Sigma, cat. no. C7880)
- Papain (Worthington, cat. no. LS 03126)
- Trypsin inhibitor (Worthington, cat. no. LS003086)
- Low-Ovo solution (10×) (Reagent setup)
- High-Ovo solution (10×) (Reagent setup)

For cryosectioning and immunostaining only

- 16% (vol/vol) Paraformaldehyde (PFA; Electron Microscopy Sciences, cat. no. 15710) ! **CAUTION** PFA must be handled in a safety cabinet and should be disposed of according to institutional guidelines.
- Sucrose (Sigma-Aldrich, cat. no. S9378)
- Fisher Healthcare Tissue-Plus OCT Compound, clear (Fisher Scientific, cat. no. 23-730-571)
- Triton X-100, laboratory grade (Sigma-Aldrich, cat. no. X100)
- Normal donkey serum (NDS; EMD Millipore, cat. no. S30-M)
- Primary antibodies (Table 1)
- Secondary antibodies, species-specific anti-IgG (H+L), Alexa Fluor–conjugated (Molecular Probes and Jackson Immunoresearch)
- Hoechst 33258, pentahydrate (bis-benzimide; Life Technologies, cat. no. H3569)
- Aqua-Poly/Mount (Polysciences, cat. no. 18606)

For optical clearing of intact spheroids by immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO) only

- Methanol (certified ACS; Fisher Chemical, cat. no. A412-1)
- Hydrogen peroxide, H_2O_2 (certified ACS; Fisher Chemical, cat. no. H325-100)
- Glycine (Sigma-Aldrich, cat. no. G7126)
- Goat serum, heat-inactivated (MP Biomedicals, cat. no. 092939249)
- Heparin sodium salt (Sigma-Aldrich, cat. no. 84020)

Table 1 | Markers that can be used to characterize hPSC-derived 3D spheroids.

Marker	Temporal expression	Spatial expression and specificity	Antibody (optimal dilution)
SOX2	Throughout differentiation	Present in pluripotent stem cells, neuronal progenitors and astrocytes in both hCSs and hSSs	Cell Signaling Technologies, SOX2 (D6D9), cat. no. 3579S (1:500)
PAX6	From ~day 20	Early neuroectoderm progenitors and dorsal pallium progenitors	DSHB, cat. no. PAX6 (1:250)
TBR2	From ~day 50	Intermediate progenitors	Abcam, cat. no. ab757520 (1:300)
HOPX	From ~day 50	Outer radial glia	Santa Cruz, cat. no. sc-398703 (1:500)
TBR1	From ~day 50	Deep-layer neurons in hCSs	Abcam, cat. no. ab31940 (1:500)
CTIP2	From ~day 50	Deep-layer neurons in hCSs, but also expressed in hSSs neural cells	[25B6] Abcam, cat. no. ab18465 (1: 300)
SATB2	From ~day 100	Superficial-layer neurons in hCSs	[SATBA4B10] Abcam, cat. no. ab51502 (1:400)
NKX2.1	From ~day 25	Ventral forebrain progenitors in hSSs	Santa Cruz, cat. no. sc-13040 (1:200)
GAD67	From ~day 50	GABAergic neurons in hSSs; sporadic expression in hCSs	Millipore, cat. no. MAB5406 (1: 1,000)
GABA	From ~day 50	GABAergic neurons in hSSs; some hCS cells can also express it transiently	Sigma-Aldrich, cat. no. A2052 (1:1,000)
SST	From ~day 50	Interneuron subtype in hSSs	Millipore, cat. no. MAB354 (1:200)
PV	From ~day 200	Interneuron subtype in hSSs	Swant, cat. no. PV27 (1:6,000), Millipore, cat. no. MAB1572 (1:1,000)
CR	From ~day 50	Interneuron subtype in hSSs; sporadic expression in hCSs	Swant, cat. no. CR7697 (1:1,000)
CB	From ~day 50	Interneuron subtype in hSSs; sporadic expression in hCSs	Swant, cat. no. CB38 (1:1,000)
NEUN	From ~day 75	Neurons in both hCSs and hSSs	Millipore, cat. no. MAB377 (1:500)
MAP2	From ~day 30	Neurons in both hCSs and hSSs	Synaptic Systems, cat. no. 188004 (1:10,000)
GFAP	From ~day 50	Astrocytes in both hCSs and hSSs; also marks radial glia	DAKO, cat. no. Z0334 (1:1,000)

- Tween-20 (Sigma-Aldrich, cat. no. P1379)
- Tetrahydrofuran, anhydrous (THF; Sigma-Aldrich, cat. no. 186562)
- Dichloromethane (Sigma-Aldrich, cat. no. 270997)
- Benzyl ether (Sigma-Aldrich, cat. no. 108014)
- Anti-GFP (GeneText, GTX113617)
- RS Hughes RTV108, 2.8-oz. silicone sealant (Fisher Scientific, cat. no. NC0380109)

For calcium imaging only
- Fluo-4 acetoxymethyl ester (Fluo-4AM; Invitrogen, F14201)

Equipment
- Primaria cell culture dish, 10 cm (BD Falcon, cat. no. 353803)
- Sterile plastic tubes, 15 ml and 50 ml (Corning, cat. no. 430791 and 430829)
- Ultra-low-attachment plates (Corning, cat. no. 3262 (10 cm), 3261 (6 cm), 3471 (six well), and 3473 (24 well))
- Fisherbrand premium microcentrifuge tubes, natural, 1.5 ml (Fisher Scientific, cat. no. 05-408-129)
  ▲ **CRITICAL** Autoclave the tubes before use.
- Sterile plastic pipettes (Corning, cat. no. 356543 (5 ml), 356551 (10 ml), 356525 (25 ml), and 356550 (50 ml))
- Sterile tips (MBP ART, cat. no. 2779-HR (P1000), 2769-HR (P200), and 2749-HR (P20))
- Spray bottle (VWR, cat. no. 23609-182)
- Air-jacketed 37 °C and 5% CO_2 incubator (VWR, cat. no. 10810-902)
- Biological safety cabinet (Labconco, cat. no. 97000-862)
- Water bath (VWR, cat. no. 89501-460)
- Culture microscope (Olympus, cat. no. CKX41)
- EVOS FL Cell Imaging System (Life Technologies, model no. AMF4300)
- Keyence fluorescence microscope (Keyence, model BZ-X710)

- Confocal microscope (Leica, model no. TCS SP8)
- Stainless-steel surgical blade, sterile, no. 10 (Medicon, cat. no. 01.22.10)
- Sterile cell strainer (Corning, cat. no. 352340)
- Petri dishes (non–tissue culture treated), 6 cm (Corning, product no. 430589)
- Hemocytometer (VWR, cat. no. 15170-208)
- Nylon, 0.22-μm nylon syringe filter (Celltreat, cat. no. 229775)
- Centrifuge, 5702 series (Eppendorf, cat. no. 022628102)
- Falcon tubes (50 ml; Fisher, cat. no. 14-432-22)
- 5% CO_2 tank with plastic hosing leading to culture hood (VWR, cat. no. 89068)

For assembloid formation only
- Sterile 1.5-ml microcentrifuge tubes (Thomas, cat. no. 1218A70)
- Scissors (Fisher, cat. no. 08-951-20) and/or razor blades (Fisher, cat. no. 12-640)

For cryosectioning and immunostaining only
- Microslides, Superfrost Plus (VWR, cat. no. 48311-703)
- Tissue Path Disposable Base Molds—15 × 15 × 5 mm (Fisher Scientific, cat. no. 22-363-553)
- Humidified chamber (we use a homemade chamber made from a slide box and wet tissue paper)
- Elite PAP pen (Diagnostic BioSystems, cat. no. K039)
- Cover glasses, rectangular (Fisherbrand, cat. no. 22-266882)
- Cryostat (Leica, cat. no. CM1860)
- Sable brushes, no. 1, 1.5 mm width × 9.5 mm length (Ted Pella, cat. no. 11812)

For optical clearing of intact spheroids by iDISCO only
- 4-ml E-C borosilicate glass screw thread vials with TFE-lined caps (Wheaton, cat. no. 03-343-6C)
- Rectangular cover glasses (Fisher Scientific, cat. no. 22-266973)

For live imaging of cell migration and calcium dynamics only
- Environmental chamber for confocal microscope (Okolab (CO_2 unit: CO_2-O_2 UNIT-BL (0-20; 1-95); temp chamber: cage incubator, T unit))
- Glass-bottom plates (Corning, cat. no. 4580)

Reagent setup

Reconstitution and storage of growth factor and chemical stock solutions

Resuspend FGF2, EGF, BDNF, and NT3 growth factors in sterile 0.1% BSA and DPBS to the desired stock concentration. Stock solution concentrations are as follows: FGF2 stock solution, 20 μg/ml; use at 10 ng/ml for hPSCs (1:2,000) and 20 ng/ml for neural differentiation (1:1,000); EGF, BDNF and NT3 stock solutions, 20 μg/ml; use at 20 ng/ml (1:1,000). Resuspend 25 mg of DM in 12.52 ml of sterile dimethyl sulfoxide (DMSO) to obtain a 5 mM stock solution; use at a final concentration of 5 μM (1:1,000). Resuspend 10 mg of SB in 2.60 ml of 100% (vol/vol) ethanol to obtain a 10 mM stock solution; use at a final concentration of 10 μM (1:1,000). Resuspend 10 mg of IWP-2 in 4.29 ml of sterile DMSO to obtain a 5 mM stock solution; use at a final concentration of 5 μM (1:1,000). Resuspend 5 mg of SAG in 94.96 ml of sterile DMSO to obtain a 100 μM stock solution; use at a final concentration of 100 nM (1:1,000). Resuspend 5 mg of AlloP in 156.99 ml of sterile DMSO to obtain a 100 μM stock solution; use at a final concentration of 100 nM (1:1,000). Resuspend 50 mg of RA in 166.42 ml of sterile DMSO to obtain a 1 mM stock solution; further dilute the stock solution to obtain a 100 μM solution; use at a final concentration of 100 nM (1:1,000). Prepare aliquots of stock solutions and store at −80 °C for up to 1 year. Once thawed, stocks can be kept at 4 °C for up to 2 weeks.

General media guidance

Various media are required; compositions are as indicated below for each specific medium. When ready for use, add aliquots of required media to 50-ml sterile tubes and prewarm in a water bath at 37 °C for <20 min. Avoid cycles of refrigeration and warming. ▲ **CRITICAL** Add growth factors and small molecules immediately before use and only after prewarming aliquoted media.

MEF medium (for use in Steps 1 and 2). Prepare the medium as detailed in the table below. This medium can be stored for 1–2 weeks at 4 °C.

Composition	Volume (500 ml)	Final concentration
DMEM (high glucose)	445 ml	
FBS	50 ml	10% (vol/vol)
GlutaMAX	5 ml	1% (vol/vol)
NEAA	5 ml	1% (vol/vol)

hPSC medium (for use in Steps 5–16). Prepare the medium as detailed in the table below. This medium can be stored for 1–2 weeks at 4 °C in the absence of growth factors. Add growth factor (FGF2) just before use.

Composition	Volume (500 ml)	Final concentration	Comments
DMEM/F12	392.5 ml		
KSR	100 ml	20% (vol/vol)	
NEAA	5 ml	1% (vol/vol)	
GlutaMAX	2.5 ml	0.5% (vol/vol)	
2-Mercaptoethanol	3.4 µl	0.1 mM	
FGF2 (20 µg/ml stock)	250 µl	10 ng/ml	Add just before use

Neural induction medium (for use in Steps 17–23, on days 0–5 of differentiation). Prepare the medium as detailed in the table below. This medium can be stored for 1 week at 4 °C in the absence of growth factors. Add growth factors (DM, SB, and IWP-2) just before use.

Composition	Volume (500 ml)	Final concentration	Comments
DMEM/F12	486.5 ml		
KSR	100 ml	20% (vol/vol)	
NEAA	5 ml	1% (vol/vol)	
GlutaMAX	2.5 ml	0.5% (vol/vol)	
Penicillin–streptomycin	5 ml	1% (vol/vol)	
2-Mercaptoethanol	3.4 µl	0.1 mM	
DM	500 µl	5 µM	Add just before use
SB	500 µl	10 µM	Add just before use
IWP-2	500 µl	5 µM	Only required for differentiation to hSS conditions (Step 23B) from day 4 onward. Add just before use

Neural differentiation medium (NM; for use in Steps 22 and 23, on day 6 and onward of differentiation). Prepare the medium as detailed in the table below. The stock can be stored for up to 1 week at 4 °C; add growth factors just before use if required for the culture.

Composition	Volume (~500 ml)	Final concentration	Comments
Neurobasal A medium	480 ml		
B27 supplement	10 ml	2% (vol/vol)	
GlutaMAX	5 ml	1% (vol/vol)	
Penicillin–streptomycin	5 ml	1% (vol/vol)	
EGF (20 µg/ml stock)	500 µl	20 ng/ml	For hSSs and hCSs. Only from day 6 to 25. Add just before use
FGF2 (20 µg/ml stock)	500 µl	20 ng/ml	For hSSs and hCSs. Only from day 6 to 25. Add just before use
BDNF (20 µg/ml stock)	500 µl	20 ng/ml	For hSSs and hCSs. Only from day 25 to 43. Add just before use

Table continued

(continued)

Composition	Volume (~500 ml)	Final concentration	Comments
NT3 (20 µg/ml stock)	500 µl	20 ng/ml	For hSSs and hCSs. Only from day 25 to 43. Add just before use
IWP-2 (5 mM stock)	500 µl	5 µM	Only for hSS conditions from day 6 to 24. Add just before use
SAG (100 µM stock)	500 µl	100 nM	Only for hSS conditions from day 12 to 24. Add just before use
AlloP (100 µM stock)	500 µl	100 nM	Only for hSS conditions from day 15 to 24. Add just before use
RA (100 µM stock)	500 µl	100 nM	Only for hSS conditions from day 12 to 15. Add just before use

Enzyme stock solution (required for dissociation of cells into a single-cell suspension)
Prepare a stock solution as detailed in the table below. The stock can be stored for 2–3 months at 4 °C.

Composition	Volume (200 ml)	Final concentration
ddH_2O	170.4 ml	
10× EBSS	20 ml	1×
30% D(+)-Glucose	2.4 ml	0.46% (vol/vol)
1 M $NaHCO_3$	5.2 ml	26 mM
50 mM EDTA	2 ml	0.5 mM

Inhibitor stock solution (required for dissociation of cells into a single-cell suspension)
Prepare a stock solution as detailed in the table below. The stock can be stored for 2–3 months at 4 °C.

Composition	Volume (500 ml)	Final concentration
ddH_2O	431 ml	
10× EBSS	50 ml	1×
30% D(+)-Glucose	6 ml	0.46% (vol/vol)
1 M $NaHCO_3$	13 ml	25 mM

Dispase solution
Reconstitute dispase in hPSC medium (without growth factors) to a stock concentration of 1.75 mg/ml. Prepare aliquots of the stock solution and store at –20 °C for up to 1 year. For hPSC passaging, thaw the dispase and dilute in hPSC medium to a concentration of 0.875 mg/ml. For lifting hPSC colonies for sphere formation, dilute the stock solution to 0.35 mg/ml in hPSC medium and warm in a water bath at 37 °C for no more than 10 min. The working dispase solution can be kept at 4 °C for up to 1 week.

Low-Ovo solution (10×; required only for single-cell dissociation)
Add 3 g of BSA to 150 ml of DPBS and mix well. Add 3 g of trypsin inhibitor and mix to dissolve. Adjust the pH to 7.4; this requires the addition of ~1 ml of 1 N NaOH. When completely dissolved, bring to a final volume of 200 ml with DPBS and filter through a 0.22-µm filter. Prepare 1.0-ml aliquots and store at –20 °C for up to 6 months.

High-Ovo solution (10×; required only for single-cell dissociation)
Add 6 g of BSA to 150 ml of DPBS. Add 6 g of trypsin inhibitor and mix to dissolve. Adjust the pH to 7.4; this requires the addition of at least 1.5 ml of 1 N NaOH. If necessary, add NaOH until the solution is no longer too acidic. Bring to a final volume of 200 ml with DPBS. When completely

dissolved, filter through a 0.22-μm filter. Prepare 1.0-ml aliquots and store at −20 °C for up to 6 months.

Paraformaldehyde
Prepare 4% (vol/vol) paraformaldehyde (PFA) by mixing 10 ml of 16% (vol/vol) PFA with 30 ml of deionized (DI) water, and store at 4 °C for up to 1 week. **! CAUTION** PFA must be handled in a safety cabinet and should be disposed of according to institutional guidelines.

Embedding solution
Prepare a 30% (wt/vol) sucrose solution by mixing 30 g of sucrose with DPBS to a final volume of 100 ml. Store the 30% (wt/vol) sucrose solution at 4 °C for up to 2 weeks. Add 5 ml of OCT to 5 ml of 30% (wt/vol) sucrose to make a 1:1 embedding solution. Shake the solution vigorously until the OCT and sucrose have mixed together. Leave the mixed solution overnight at 4 °C to allow bubbles formed during the mixing process to disappear.

Blocking solution for immunostaining
Prepare a blocking solution for immunostaining by mixing 10% (vol/vol) normal donkey serum with 0.3% (vol/vol) Triton X-100 in DPBS. Keep this solution at 4 °C for up to 1 week.

Equipment setup

CO_2 tank
The 5% CO_2 tank setup should have plastic hosing leading to the culture hood and can include multiple three-way stops to split the hosing into multiple lines. To bubble CO_2 through solutions, attach a syringe filter to the end of the hosing, and then attach the broken end of a 2-ml pipette and place the tip into the liquid. During dissociation, place a syringe filter on the end of the plastic tubing and set the filter tip into a small hole cut into the lid of the Petri dish (without contacting the solution) so that CO_2 can be blown over the digesting solution. If this setup is not available, solutions can also be equilibrated in the incubator.

Procedure

MEF feeder seeding and initiation of hPSC cultures ● Timing 2 h

1 Coat 10-cm cell culture dishes with 0.1% gelatin for at least 1 h at room temperature (RT) (~24 °C).
2 Thaw one vial of MEFs (containing ~5×10^6 cells) by placing it in a water bath at 37 °C until it is 80% thawed (and for no longer than 2 min). Briefly spin the cells (at 200*g* for 4 min at RT) and plate at a density of 1–2×10^6 cells per 10-cm plate.
3 The next day, assess feeder quality before use; this will determine the quality of the hPSCs. Once the MEFs are ready for plating hPSCs (after ~2 h), wash the culture plates at least once with fresh DPBS.

Passaging hPSCs on the MEF feeder layer ● Timing 4–7 d

4 Remove hPSC medium from the refrigerator, aliquot the required amount into a centrifuge tube (~12 ml per 10-cm plate), wrap the container's cap with Parafilm to prevent contamination when putting it into the water bath, and warm in a water bath for up to 20 min.
5 Remove the hPSC plate from the incubator and look at it under a microscope. While under the microscope, mark the differentiated areas on the bottom of the plate, using a marker. Differentiated colonies may appear to have a hole or a pit in the center of the colony or a border of differentiated cells (Fig. 3a).
6 After wiping with a paper towel sprayed with 70% (vol/vol) ethyl alcohol, transfer the plates to the cell culture hood. Then use a pipette tip to carefully remove demarcated differentiated colonies from the plate.
7 Aspirate the medium and add 5 ml of dispase (0.875 mg/ml in hPSC medium) to each culture plate, and place in the cell culture incubator for 7 min or until hPSC colonies have lifted.
8 Transfer the suspended cells from a single plate to a 50-ml centrifuge tube. Use 5 ml of hPSC medium with 10 ng/ml FGF2 and 10 μM Y-27632 to wash each 10-cm plate. Perform this step three times, adding the medium to the appropriate 50-ml centrifuge tube after each wash.
9 Gently swirl the 50-ml centrifuge tubes to suspend the harvested cells in 20 ml of hPSC medium.
10 Transfer the hPSC medium and suspended cells to a freshly prepared feeder plate (from Step 3).

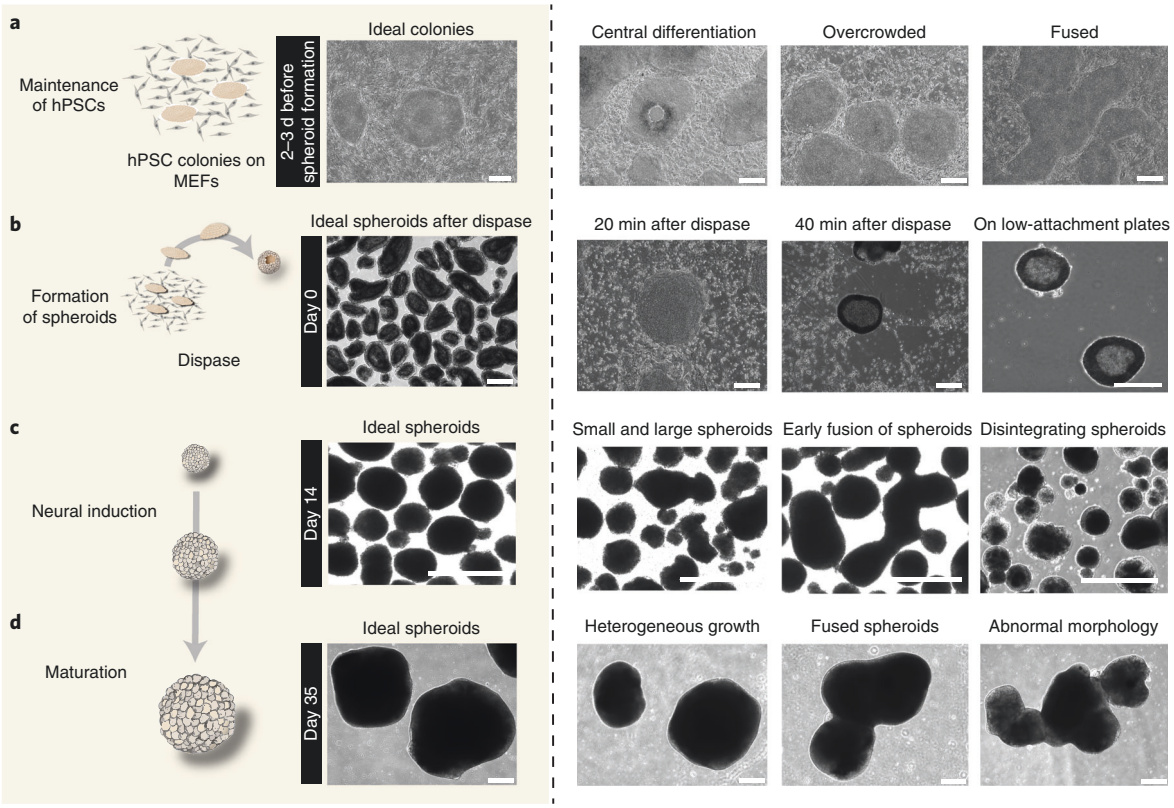

Fig. 3 | Images of forebrain spheroid formation. a–d, Images of colonies and spheroids growing optimally (left-hand images) and suboptimally (right-hand images) are shown. **a**, Representative images of hiPSC colonies grown on MEFs. Note that ideal colonies are large and sparsely distributed, without evidence of central differentiation. Scale bars, 500 μm. **b**, Representative images of spheroid formation during and immediately after dispase treatment. Note that intact colonies are separated without dislodging the layer of MEFs. Scale bars, 500 μm. **c**, Representative images of ideal and abnormal neural spheroids during the neural induction period (days 3–14). Scale bars, 500 μm. **d**, Representative images of ideal and abnormal neural spheroids during the maturation phase (after day 25). Note the accelerated growth in size of neural spheroids at this stage. Scale bars, 500 μm. The results shown here are from experiments using hPSCs that conformed to institutional and federal regulations. IRB panel approval and appropriate informed consent were obtained for these studies.

11 Gently swirl the plate and slide the plate in a back-and-forth motion on the shelf of the incubator to evenly disperse the cells.

12 Incubate the cells in the cell culture incubator. Leave the cells undisturbed for the first day after passaging, and replace the medium every day thereafter for 5–7 d. To replace the medium, warm aliquoted fresh hPSC medium in a water bath for up to 10 min at 37 °C, aspirate old medium from the plates, and add 12 ml of fresh medium per 10-cm culture plate.
▲ **CRITICAL STEP** Replace hPSC culture medium at approximately the same time each day.

13 Monitor the hPSC colonies to determine when they are ready for spheroid formation. This will usually be ~6–7 d from the last passage (depending on the hPSC line); colonies should measure ~1.5 mm in diameter. See Fig. 3a for examples of hPSC colonies of appropriate, and inappropriate, morphology. When cultures are ready, proceed to the next step. Alternatively, continue to passage hPSCs every 5–7 d (depending on the growth rate of each line) by repeating Steps 1–12; however, bear in mind that, ideally, hPSCs should not be kept beyond passage 40.
▲ **CRITICAL STEP** It is essential for the colonies to be large in order to generate spheroids (i.e., just before the time at which colonies would be passaged, or 1–2 d later). If the colonies are too small, allow more time for growth before subsequent steps. Avoid using hPSC colonies that touch each other because they will detach as large clusters together with the MEF layer. Avoid hPSC colonies that have grown too large and that are differentiating in the center.

Suspending hPSC colonies to form spheroids ● Timing 2–3 h

14 Remove the hPSC media from the culture plates. Detach hPSC colonies by incubating them with 0.35 mg/ml dispase (dissolved in hPSC medium; 5 ml per plate) for 30–45 min at 37 °C, 5% CO_2.

Check the status of the detachment after 20 min and then every 5 min thereafter. Different lines will require different durations of dispase treatment. Plates can be gently moved (back and forth) at the end of the incubation period to facilitate the detachment of curled hPSC colonies from the feeders. Almost all feeders should remain attached to the 10-cm plate, whereas the hPSC colonies should come off. Intact colonies should curl up and lift off the plate (Fig. 3b).

15 Gently transfer the floating colonies with a pre-wet 25-ml pipette to a 50-ml Falcon tube. Once the colonies settle at the bottom of the tube, aspirate the media and gently wash the colonies two times with 20 ml of warm hPSC medium (without growth factors).
▲ CRITICAL STEP Do not centrifuge. Let the colonies settle at the bottom of the Falcon tube.
? TROUBLESHOOTING

16 Gently resuspend 30–50 suspended colonies in 12 ml of neural induction medium (the medium should also contain 5 μM DM, 10 μM SB, and 10 μM Y-27632) and transfer to a 10-cm ultra-low-attachment plate. Denote this day as day 0. Place the plate in the incubator at 37 °C, 5% CO_2 for 48 h. Keep the spheroids in the incubator on day 1 without any medium change to allow the spheroids a day to recover.
! CAUTION It is important to distribute the spheroids evenly once the plate is placed in the incubator to avoid clustering and fusion of colonies at the center of the plate.

Neural induction ● Timing 6 d

▲ CRITICAL Minimize the overall time that the spheroids are kept outside of the incubator at all stages of differentiation.

17 (Day 2) After ~48 h of incubation, i.e., on day 2, replace the medium with 12 ml of neural induction medium (with DM and SB but without Y-27632). To change the medium, gently transfer the spheroids to a 50-ml Falcon tube and carefully aspirate the medium once they settle. Incubate the cells for a further 24 h.

18 (Day 3) Replace the medium as described in Step 17 and incubate the cells for a further 24 h.

19 (Day 4) If you plan to differentiate hSSs (when you reach Step 22B, C), replace the medium as described in Step 17, but include 5 μM IWP-2 in the medium in addition. If you plan to generate hCSs, replace the medium as before. Incubate the cells for a further 24 h.
▲ CRITICAL STEP IWP-2 should not be thawed at RT, as it will precipitate. Frozen aliquots should be placed directly into a water bath before use.

20 (Day 5) Replace the medium as described in Step 19. Incubate the cells for a further 24 h.

Patterning and differentiation ● Timing up to 37 d

21 From day 6 onward, replace the medium daily with NM in place of the neural induction medium. Use NM supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF. In addition, include 5 μm IWP-2 in the medium if you are generating hSSs. Change the medium daily on days 6–11. At day 6, neural spheroids should be ~0.3–0.5 mm in diameter (Fig. 3c). The medium can be changed as described in Step 17 or by gently tilting the plate (allowing the spheroids to settle) and removing the remaining medium.

22 From day 12 onward, the medium requirements for the generation of hCSs and hSSs deviate further. To generate hCSs, follow option A (see Fig. 2c for a detailed protocol timeline). To generate hSSs, follow option B (see Fig. 2c for a detailed protocol timeline). To generate hSSs with more activity visible by calcium imaging[19] (hSS-ISRAs), follow option C. After neural spheroids grow larger (>2–3 mm in diameter), no more than 30 spheroids should be maintained per 10-cm plate.
▲ CRITICAL STEP Neural spheroids tend to fuse to each other as they get larger (Fig. 3d). When changing the medium, be sure to separate them by either pipetting up and down gently with a 10-ml pipette or by using a P10 pipette tip to separate them when they are bigger. If spheroids fuse during later time points (after ~day 50), it may greatly compromise the outcome.
? TROUBLESHOOTING

(A) **Generation of hCSs**

(i) From days 12 to 15, continue to replace medium daily with NM supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF.

(ii) From day 16 onward, change the medium every other day until day 25. Continue to use NM supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF.

(iii) From days 25 to 43, replace FGF2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3. Perform medium changes with 14–15 ml of supplemented NM per 10-cm plate every 2–3 d.

(B) **Generation of hSSs**

(i) From days 12 to 23, supplement the NM (already containing 20 ng/ml FGF2, 20 ng/ml EGF, and 5 μm IWP-2) with 100 nM SAG. Perform medium changes with 14–15 ml of supplemented NM per 10-cm plate every day. On day 24, replace medium with NM without additional growth factors.

▲ **CRITICAL STEP** Note that early hSSs can be smaller in diameter in comparison with early hCSs during 12–20 d of differentiation.

(ii) From days 25 to 43, replace FGF2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3. Perform medium changes with 14–15 ml of supplemented NM per 10-cm plate every 2–3 d.

(C) **Generation of hSS-ISRAs**

(i) On days 12, 13, and 14, supplement the NM (with IWP-2) with 100 nM SAG. Perform medium changes with 14–15 ml of supplemented NM per 10-cm plate every day.

(ii) From days 12 to 15, add 100 nM RA, and from days 15 to 23, add 100 nM AlloP (in addition to the IWP-2) to the neural induction medium (Fig. 2c). On day 24, replace medium with NM without additional growth factors.

! **CAUTION** RA is air- and light-sensitive. Minimize light exposure.

(iii) From days 25 to 43, replace FGF2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3. Perform medium changes with 14–15 ml of supplemented NM per 10-cm plate every 2–3 d.

Maintenance and long-term culture ● Timing indefinite

23 From day 43 onward, perform media changes with 17–18 ml of NM without growth factors every 4 d. Using careful sterile technique, spheroids can be maintained for many months in culture, if desired (Fig. 3d). Carry out any additional procedures at time points determined by your experiment. Examples of additional procedures that can be carried out on the spheroids include the generation of forebrain assembloids (option A), dissociation into a single-cell suspension in preparation for further assays (option B), cryosectioning and immunolabeling (option C), calcium imaging (option D), and optical clearing by iDISCO (option E). Bear in mind that if you wish to assemble forebrain spheroids (option A), this procedure is most successful if the combined size of the assembled spheroids is <6 mm (between days 60 and 90 of culture).

? **TROUBLESHOOTING**

(A) **Generation of forebrain assembloids using hCSs and hSSs ● Timing 3–7 d**

(i) For assembly of forebrain spheroids, transfer one hCS and one hSS to a 1.5-ml microcentrifuge tube that is resting in a standard microtube rack (Fig. 4f). Denote the day of assembly as day 0. The size of the spheroids to be assembled should not exceed 3 mm, as they are challenging to fuse once they are too large.

▲ **CRITICAL STEP** Once in the tube, make sure that there are no bubbles underneath the spheroids and that they rest next to each other in contact at the bottom of the tube and are not stacked up vertically.

(ii) Incubate the spheroids for at least 3 d in 1 ml of NM, completely replacing the medium on day 2.

! **CAUTION** Because the assembly interface is fragile at this stage, perform the medium change in the tube very carefully.

? **TROUBLESHOOTING**

(iii) Check that fusion is complete. Fusion is considered complete when the two spheroids are inseparable with gentle shaking of the Eppendorf tube (Fig. 4g). After fusion, use a P1000 pipette with the tip cut off for a larger bore opening to transfer the assembled hCS–hSSs back to the ultra-low-attachment 10-cm plates. If fusion is carried out between days 60 and 90, when this process is most efficient, hCS–hSS assembly is usually complete after 3–4 d. Earlier time points are also permissive. Assembly of hCS–hSSs at later stages of differentiation (after day 90) may take longer (up to 7 d).

(B) **Dissociation into a single-cell suspension ● Timing 2–4 h**

(i) Add enzyme stock solution (use 10 ml per dissociation dish, which should correspond to about 15 early-stage spheroids or seven late-stage spheroids, plus an extra 2 ml) to a 50-ml

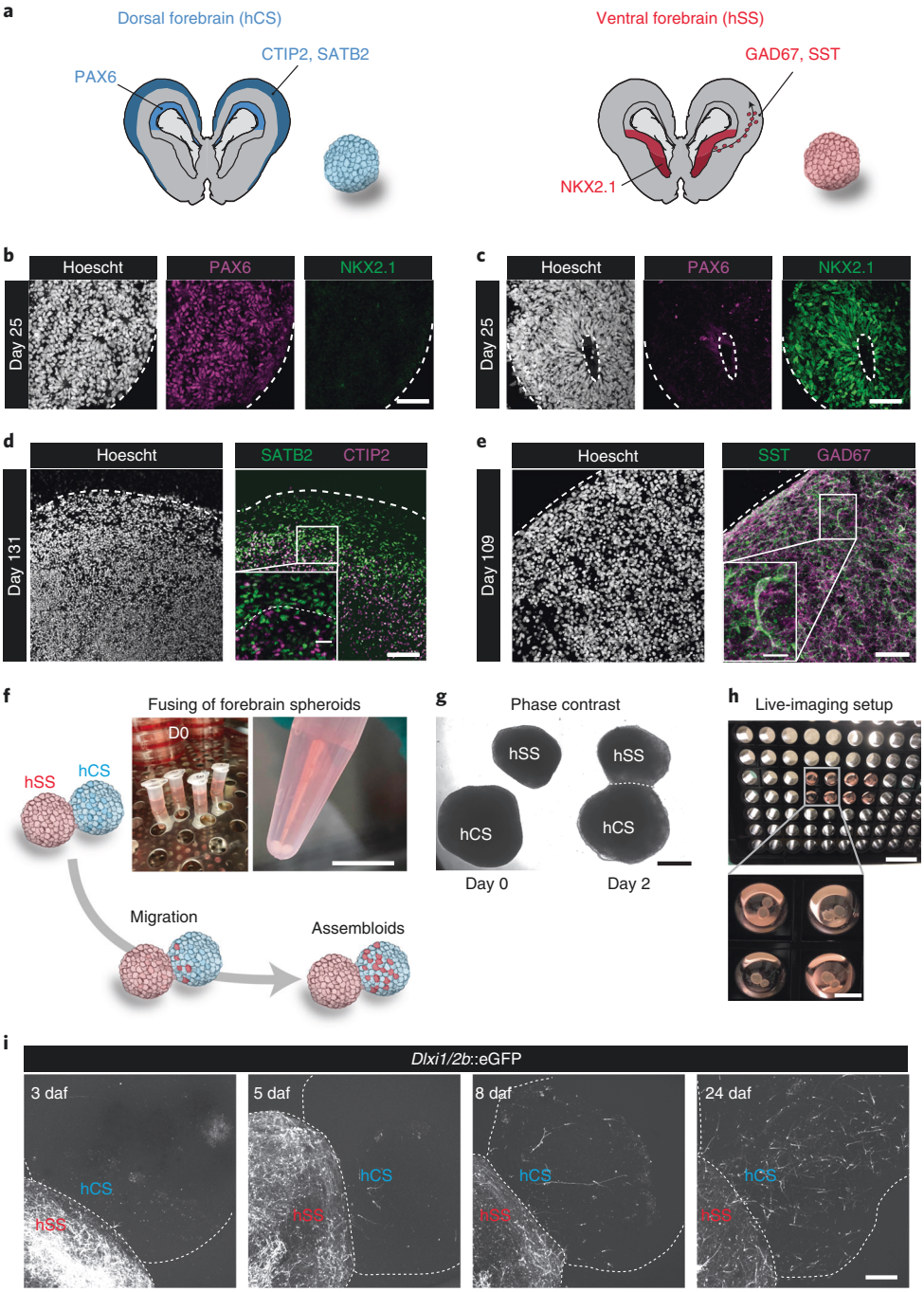

Fig. 4 | Expression of region-specific markers and fusion of dorsal and ventral neural spheroids into assembloids. **a**, Scheme illustrating expression patterns of dorsal and ventral forebrain markers in a coronal slice of GW 18–20 human fetal brain. **b**,**c**, Dorsal forebrain marker expression (PAX6, magenta) and ventral forebrain marker expression (NKX2.1, green) in pallial (**b**; hCS) and subpallial (**c**; hSS) spheroids at day 25 of differentiation. Scale bars, 50 µm. **d**, Pattern of expression of cortical markers in hCS at day 131 of differentiation in vitro. Scale bar, 100 µm. The inset shows the expression pattern of CTIP2 (also known as BLC11B; magenta) and SATB2 (green). Scale bar, 50 µm. **e**, Pattern of expression of ventral forebrain markers in hSS at day 109 of differentiation. Scale bar, 50 µm. The inset shows details of GABAergic neuron (SST, green; GAD67, magenta). Inset scale bar, 12 µm. **f**, Scheme and photographs showing how spheroids are fused in the bottom of a 1.5-ml Eppendorf tube on day 0 of forebrain assembloid formation. Eppendorf tubes are held upright in a standard microtube rack. Inset scale bar, 1 cm. **g**, Phase-contrast image of forebrain assembloids at day 0 and at day 2. Scale bar, 500 µm. **h**, Images following transfer of forebrain assembloids to a 96-well plate for live imaging. Scale bar, 4 cm; inset scale bar, 1 cm. **i**, Use of forebrain assembloids to monitor migration of virally labeled (lentivirus, Dlxi1/2b::eGFP) GABAergic neurons from hSS into hCS throughout the first 24 d after fusion (daf). Scale bar, 200 µm. The results shown here are from experiments involving the use of hPSCs that conformed to institutional and federal regulations. IRB panel approval and appropriate informed consent were obtained for these studies.

Falcon tube, and bubble 5% CO_2 through the solution at RT until the solution turns from red to orange.

(ii) Once warmed and orange in color, add papain to the enzyme stock (for neural spheroids up to day 200, use 30 U/ml; after that differentiation stage, use 50 U/ml).

(iii) Add 0.0036–0.0042 g of L-cysteine per 20 ml of enzyme stock solution and put the solution into a 34–37 °C water bath. Warm the solution mixture for at least 15 min before proceeding to the digestion (Step 23B(vi)). In the meantime, proceed to the next step.

(iv) Transfer the spheroids to a 6-cm Petri dish, remove any residual medium, and use a no. 10 scalpel blade to chop the spheroids into <1-mm^3 pieces. Finer pieces are preferable (this will require at least 30 s of chopping).

(v) Use 2 ml of enzyme stock solution to wet a 0.22-μm filter, and then filter the 10 ml of enzyme stock solution into each Petri plate containing the finely chopped spheroids. Add 200 μl of DNase to each Petri plate and swirl the dish to mix.

(vi) For the papain digestion, put the Petri dishes on a 34 °C heat block on top of a wet paper towel. Attach a hose from a CO_2 tank to a 0.22-μm syringe filter and place the filter tip into a small hole cut into a lid of the Petri dish to flow 5% CO_2 over the enzyme stock solution containing tissue pieces.

▲ **CRITICAL STEP** The filter tip should not touch the solution to ensure that gas is not bubbling into the liquid but rather blowing over the top.

(vii) Gently shake the Petri plate every 15 min. Digest for 70–100 min. The digestion time should be increased with the age of spheroids to a maximum of 100 min for spheroids older than 250 d. During the digestion proceed with the next step.

(viii) During digestion, prepare low-Ovo and high-Ovo solutions by diluting 10× low-Ovo and high-Ovo solutions in inhibitor stock solution to make 1× final concentrations of each. Trituration will require ~10 ml of low-Ovo solution and 1 ml of high-Ovo solution per dissociation dish.

(ix) After digestion, transfer the enzyme solution with tissue pieces to a 15-ml Falcon tube. Add 4–5 ml of low-Ovo solution. Spin at 100*g* for 2 min at RT until tissue chunks settle to the bottom. Aspirate and discard the supernatant.

(x) Add 1 ml of low-Ovo solution to the tube, and use a P1000 pipette to triturate 10–20 times. The solution should become cloudy. Be careful to avoid introducing bubbles; do not lift the pipette out of the solution during trituration. After the first round of trituration, allow chunks to settle to the bottom, and then transfer single cells (supernatant) to a new tube.

(xi) Repeat the trituration process with additional 1-ml aliquots of low-Ovo solution and 10–20 triturations each time. It may take two to three rounds until tissue chunks are dissolved. With each successive round, be more aggressive with trituration.

(xii) Once all tissue chunks have dissolved, use a P1000 pipette to carefully layer 1 ml of high-Ovo solution under the single-cell suspension. This should lead to a clear layer of liquid beneath a cloudy cell suspension.

(xiii) Spin the cells down through the high-Ovo solution at 100*g* for 5 min at RT. Aspirate and discard the supernatant. A cell pellet should be visible at the bottom of the Falcon tube.

(xiv) Resuspend the cell pellet in the medium of choice. Single-cell suspensions can be plated for cell culture, time-lapse imaging, calcium imaging, or immunohistochemistry, or can then be immunopanned or FACS-separated.

(C) **Cryosectioning and immunostaining** ● **Timing 3–5 d**

(i) At the time point of choice, transfer the spheroids to a 1.5-ml microcentrifuge tube, using a cut P1000 tip. Gently remove the medium and briefly wash with 1 ml of DPBS. Remove the DPBS and add 500 μl of cold 4% (vol/vol) PFA. Leave the spheres to fix for at least 30 min at 4 °C. The optimal fixation time varies depending on the antibody being used, and the age and size of the spheres. We thus recommend fixation trials be undertaken to optimize the optimal fixation required for specific antibody stainings; e.g., for membrane antigens, we have found that fixation for shorter periods of time (~2 h) is optimal, whereas for nuclear antigens, optimal results are seen after a longer fixation (up to overnight).

▲ **CRITICAL STEP** Use the same fixation and embedding protocol when quantifying across hCSs or hSSs derived from multiple hPSC lines and differentiations. Variation in fixation and the sample-processing protocol can lead to variability in antibody staining.

(ii) After fixation, gently remove PFA and perform three 15-min washes with DPBS at RT.

(iii) Add 1 ml of 30% (wt/vol) sucrose to facilitate cryoprotection. Leave the spheres in this solution at 4 °C until they sink (~24–48 h). During this time, prepare the embedding solution (1:1, OCT/30% (wt/vol) sucrose; see the 'Reagent setup' section).

(iv) To embed spheres, use square disposable molds that have been filled with embedding solution.

▲ **CRITICAL STEP** To avoid creating bubbles while filling the mold, use a P1000 pipette, the tip of which has been slightly cut, to widen the opening.

(v) Place the spheres into a mold using a P1000 cut pipette tip. Place four to ten spheroids into the mold, always making sure to mark their locations. If the spheres move while embedding, use a P20 tip to gently move them around the embedding solution.

▲ **CRITICAL STEP** Avoid carrying excess 30% (wt/vol) sucrose when transferring the spheres.

(vi) Place the mold on ice for 20 min and allow the spheres to sink to the bottom.

(vii) Snap-freeze the spheres by placing the mold directly on dry ice. Once completely frozen, store the molds at −80 °C.

■ **PAUSE POINT** Frozen molds can be stored indefinitely at −80 °C until ready to cryosection.

(viii) When ready for sectioning, remove the mold from the −80 °C freezer and allow it to increase in temperature to −20 °C by placing it in the cryostat chamber for ~30 min before sectioning.

(ix) Remove the block from the mold, use OCT to paste it onto a specimen stage, and section using standard techniques. Use a brush to prevent crumbling of the sections. We recommend that hCSs or hSSs be cryosectioned at a 10- to 15-µm thickness. Thicker sections are difficult to quantify because of the overlapping nuclei and high density of cells, especially in proliferative areas. However, if using a confocal microscope, thicker sections may be adequate.

(x) Collect the sections on Superfrost Plus slides.

■ **PAUSE POINT** Slides can be stored for up to 1 year at −80 °C until ready to use.

(xi) For immunostaining, remove the slide from the −80 °C freezer and leave at RT to thaw for 5–10 min.

(xii) Wash the slide once with DPBS to remove OCT/sucrose.

(xiii) Remove the DPBS and use a hydrophobic PAP pen to draw a circle around the sections. Let the ink dry and wash the slide with DPBS again to remove excess OCT. From this step onward, add all solutions within the circle.

(xiv) Block for 1 h at RT with 10% NDS, 0.3% (vol/vol) Triton X-100, and DPBS. Note that the optimal concentration of NDS and Triton X-100 may vary according to the antigen of interest.

▲ **CRITICAL STEP** Perform immunostaining steps in a humidified chamber to prevent sections from drying out.

(xv) Add primary antibodies diluted in blocking solution at 4 °C overnight. Some examples of antibodies and concentrations that we have used successfully are listed in Table 1.

▲ **CRITICAL STEP** Some antibodies might need longer incubation periods; therefore, further optimization may be required.

(xvi) Perform three 15-min washes at RT with DPBS.

(xvii) Add appropriate secondary antibodies (we use species-specific anti-IgG (H+L)–Alexa Fluor–conjugated antibodies, diluted between 1:500 and 1:2,000) diluted in blocking solution for 1 h at RT.

▲ **CRITICAL STEP** Protect the sections from light during this and the following steps.

(xviii) Perform three 15-min washes at RT with DPBS.

(xix) Add Hoechst solution (1/10,000 in DPBS) for 5–10 min at RT.

(xx) Remove excess Hoechst solution by washing with DPBS.

(xxi) Remove the DPBS, let the sections air-dry for 5–10 min, and mount by adding one drop of Aquamount solution on top of the sections and placing a cover glass on top.

(xxii) Use a fluorescence microscope to visualize the immunostained sections. See Fig. 4 for representative immunostainings of hCSs and hSSs with dorsal- and ventral-specific domain markers.

▲ **CRITICAL STEP** Ideally, cryosections for quantification should be sampled throughout the thickness of the spheroid. Avoid quantification of cryo-sections that are not at least

50 µm apart (five cryo-sections apart, if cutting at 10 µm) to prevent sampling the same cells. Avoid extreme edges of the hCSs or hSSs, as they are not likely to be representative, and important cell types might be missed that are usually present only in the deeper parts of the spheroids.

(D) **Calcium imaging** ● **Timing 2–6 h**

(i) Incubate intact spheroids with 10 µM Fluo-4 diluted in NM for 30 min at 37 °C and 5% CO_2, followed by a 15-min wash.

(ii) Using an appropriate imaging system, record spontaneous calcium activity for several minutes (one frame every 8–10 s) in one 10-µm *z*-stack plane. Maintain the spheroids in NM during the recordings. We do not perfuse during calcium-imaging sessions, in order to minimize drift.

(iii) Export fluorescence intensity (F) as mean gray values in the ImageJ software (https://imagej.nih.gov/ij/). Signal decay should be controlled by subtracting the mean fluorescence of the background (F_b). To estimate changes in intracellular calcium, ΔF is computed as $(F_{ell}\text{-}F_b)/F_0$, where F_0 represents the minimum F value per cell across the entire duration of recording from which F_b was subtracted.

(E) **Optical clearing of intact spheroids by iDISCO** ● **Timing 12 d**

(i) Fix the spheroids with 4% (vol/vol) PFA for 3 h at 4 °C. Note that this incubation time was optimized for 50- to 150-d-old hCSs, hSSs, or forebrain assembloids. However, given that the size of the spheroids after this age does not substantially change, this fixation time is probably suitable for older spheroids as well. If using younger spheroids (up to 25 d old), incubation times may be shortened. Keep in mind, however, that iDISCO clearing tends to result in a shrinking of tissue, so using early spheroids may make handling challenging.

(ii) Perform three 15-min-long washes at RT with DPBS.

(iii) Transfer the spheroids to 4-ml glass vials.

(iv) Dehydrate the spheroids with a day-long methanol (MeOH) dilution series at RT (20%, 40%, 60%, 80%, and 100% MeOH/DPBS, 30 min per step).

(v) Incubate the spheroids in 5% H_2O_2/95% MeOH overnight at 4 °C.

(vi) The following day, rehydrate with a reverse MeOH dilution series (80% MeOH/DPBS, 80%, 60%, 40%, 20%, 0%, and 0% MeOH/0.2% Triton X-100/DPBS, 30 min per step).

(vii) Incubate the spheroids overnight in 0.2% (vol/vol) Triton X-100, 20% DMSO, and 0.3 M glycine/DPBS at 37 °C.

(viii) Block the spheroids with 0.2% (vol/vol) Triton X-100, 10% DMSO, and 6% goat serum/DPBS at 37 °C for 2 d.

▲ **CRITICAL STEP** Perform this and all the following steps in closed tubes filled to the top to prevent oxidation of tissue.

(ix) Incubate the spheroids with 0.2% Tween 20 for 1 h.

(x) Incubate the spheroids in 10 µg/ml heparin/DPBS (PTwH) for 1 h.

(xi) Incubate the spheroids with the desired primary antibodies (e.g., anti-GFAP, 1:1,000 dilution; anti-GFP, 1:1,000; and anti-SOX2, 1:500) for 2 d in PTwH with 5% DMSO and 3% goat serum at 37 °C.

(xii) Undertake a day-long wash series with PTwH (30-min to 1-h steps for at least 5 h: 10 min: PTwH; 15 min: PTwH; 30 min: PTwH; 1 h: PTwH; and 2 h: PTwH).

(xiii) Dilute the secondary antibodies in PTwH and 3% goat serum and incubate for an additional 2 d at 37 °C.

(xiv) Wash off excess secondary antibody by washing for 2 h with PTwH. Repeat three times so spheroids have been washed for at least 8 h.

(xv) Incubate the spheroids in 50% THF/H_2O overnight at RT.

(xvi) Perform a three-step THF series (1 h 80%/H_2O, 1 h 100% THF, 1 h 100% 1016 THF) at RT.

(xvii) Clear the spheroids by incubating in benzyl ether. Neural spheroids should be cleared within only a few minutes of incubation.

(xviii) Image cleared tissue. When imaging cleared tissue, it is critical to use a chamber that is resistant to the organic solvents used in Step 23E(xv, xvi). For this purpose, we 3D-printed a chamber using semi-clear acrylic-type plastic from ProJet at the Stanford 3D Printing Core and followed the instructions that can be found at https://idisco.info/idisco-protocol/. If using this chamber, use silicone sealant to fix a rectangular coverslip on the bottom of the 3D-printed chamber. When spheres are cleared, add them to the chamber with benzyl ether.

▲ CRITICAL STEP Make sure the chamber is completely sealed to the cover glass to avoid any benzyl ether leaking onto the microscope. We recommend visiting https://idisco.info/ for additional troubleshooting tips.

(F) **Live imaging of cell migration and calcium dynamics** ● Timing 2–24 h

(i) Transfer a fused hCS–hSS to a well of a 96-well plate (glass-bottom plate) in 200 µl of neural medium. Owing to the limited amount of medium that can be included in a 96-well plate, avoid putting more than one brain assembloid into a well.

(ii) Place a 96-well plate into an inverted microscope with a motorized stage under environmentally controlled conditions (37 °C, 5% CO_2).

(iii) Maintain the assembled spheroids in an environmentally controlled chamber for 30–60 min before imaging to let them acclimatize and settle. Movement in the room should be kept to a minimum.

(iv) Set up the imaging positions so that up to ~8 fields with *z* stacks (corresponding to eight assembled hCS–hSSs) can be imaged in a sequential manner, with each position or frame being imaged at a rate of 15–20 min per frame. Our *z* stacks normally span a depth of 50–150 µm and have a step size of 1.5–2.5 µm. We have found that hCS–hSSs can be imaged for up to ~18 h, after which time imaging must be paused, so the medium should be carefully changed before continuing imaging. On our live-imaging system (Leica SP8 confocal microscope), we use <10% laser intensity per laser line with the 10× dry objective to avoid photobleaching and phototoxicity when imaging four to eight fields at a time. The laser power density should be empirically determined in other imaging systems.

(v) If spheroids drift and this impacts image analysis, correct the images for drift computationally. We have found that the image registration plug-ins from ImageJ (e.g., linear stack alignment with SIFT) are generally sufficient to correct for minor-to-moderate drift. However, we discard sessions with major drift due to handling. Occasional drifting of spheroids may occur, as they are not embedded during imaging sessions.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason (s)	Solution
15	Spheroids do not form	Differentiated hPSC colonies	Always check hPSC quality before use
		MEF quality is inadequate	Always try to acquire MEFs in bulk and first test their quality with a small lot
		hPSC colonies are too small	Allow hPSC colonies to grow larger and do not use until the optimal day for splitting
		High density of hPSC colonies, leading to overcrowding	Colonies should be sparsely distributed on the 10-cm plates; if colonies are in contact with each other, they tend to detach as larger clusters together with feeders
		Mycoplasma or other contamination	Perform mycoplasma testing regularly
22 and 23	Spheroids stick to each other	Too many neural spheroids per plate. Spheroids grow quickly once EGF and FGF2 are added to the medium	Transfer some spheres to a new or a bigger low-attachment plate. If already attached, separate them by gently pipetting up and down, using a 10-ml pipette when spheroids are young (up to day 25) or two P20 pipette tips when they are older
	Spheroids are too small or disintegrate	Differentiated hPSC colonies were used	See solutions for Step 15
		MEFs attached to the spheroids after dispase treatment	Titrate dispase treatment to ensure that MEFs remain attached to the plate
		hPSC colonies were too small	See solutions for Step 15
		Excessive pipetting	Be gentle when handling spheroids at all times
		Excessive centrifugation	Be gentle when handling spheroids at all times
		Mycoplasma or other contamination	See solutions for Step 15
23A(ii)	Unable to assemble hCSs and hSSs	hCSs or hSSs have differentiated for too long	Try to fuse smaller (<3 mm) or early-stage spheroids (days 30–90). After day 90, the success rate decreases, although older spheroids (up to day 150) have been successfully fused. Make sure that spheroids remain in

Table continued

Table 2 (continued)

Step	Problem	Possible reason (s)	Solution
			contact in the Eppendorf tube with minimal disturbance or shaking during the first several days of fusion
23	Cultures became contaminated	Inadequate sterile technique or contamination when transferring between tubes. With very-long-term cultures, it is important to minimize the time cells are outside the incubator and to maintain high-level sterile technique	Improve sterile technique Wear lab coats Use 70% (vol/vol) ethanol to spray your hands and anything that goes into the safety hood Autoclave all nonsterile equipment Change HEPA filters in incubators regularly Autoclean the incubators regularly Identify sources of contamination in the incubator or cell culture room Clean the water bath in the incubators and in the cell culture room on a weekly basis
	Medium turns acidic overnight	Too many spheroids per plate	In the initial stages of differentiation, 12–13 ml of medium per 10-cm plate is sufficient. As spheroids grow, use 15–18 ml of medium per plate. Fewer than 30 spheroids per 10-cm plate are recommended
Box 1, step 3	Sparse or no viral labeling following transfection	Viral titer was too low Insufficient time between infection and fluorescent protein expression Incorrect serotype	Make sure that viral titers are sufficient Wait for at least 1 week before looking for viral gene expression. Ensure that during transfection, spheroids are incubated in a high concentration of virus overnight Use human DJ serotype for AAV

Timing

Steps 1–3, MEF feeder seeding and initiation of hPSC: 2 h
Steps 4–13, culture of hPSCs on the MEF feeder layer: 4–7 d, 1 h per day
Steps 14–16, suspending hPSC colonies to form spheroids: 2–3 h, day 0
Steps 17–20, neural induction: 6 d, days 1–6, 1 h per day
Steps 21 and 22, patterning and differentiation: up to 37 d, days 7–43, 30 min per medium change
Step 23, maintenance and long-term culture: days 44–850+, 30 min per media change, every 4 d
Step 23A, generation of forebrain assembloids using hCSs and hSSs: 3–7 d
Step 23B, dissociation into a single-cell suspension: 2–4 h
Step 23C, cryosectioning and immunostaining: 3–5 d
Step 23D, calcium imaging: 2–6 h
Step 23E, optical clearing of intact hCSs or hSSs by iDISCO: 12 d
Step 23F, live imaging of cell migration and calcium imaging: 2–24 h
Box 1, viral labeling of hCSs and hSSs: 6–10 d

Anticipated results

Successful differentiations should yield ~20–40 mature spheroids per 10-cm plate of hPSCs. When hPSC colonies are ready for formation of spheroids, they should not be touching each other and should have no evidence of central differentiation (Figs. 2a and 3a). After dispase treatment, detachment of hPSC colonies should occur in <45 min (Fig. 3b). Floating spheroids will be visible to the eye. Throughout neural differentiation and maturation, spheroids should grow in size up to 4 mm in diameter (Fig. 2c; see also Paşca et al.[17]), and should be uniform in distribution across the same hPSC line. They should not be fused or touching each other in the ultra-low-attachment plate (Fig. 3c,d). At early stages (e.g., day 25), hCSs should express the forebrain markers FOXG1 and PAX6 and then exhibit proliferative zones with ventricular-like and subventricular-like domains that are surrounded by a cortical plate–like region (Fig. 4a,b,d). hSSs should express FOXG1 and the ventral forebrain maker NKX2.1. By day 60, hSSs should contain GAD67- and SST-immunopositive neurons (Fig. 4a,c,e). Astrogenesis should start after day 80–90 and continue over the next several months[20]. For forebrain assembloids, migration of GABAergic neurons into hCSs should occur 1–3 weeks post fusion (Fig. 4f–g). Migrating interneurons should disperse underneath the surface and throughout the parenchyma of the hCSs, and then integrate into the network (Fig. 4i).

Examples of hCS or hSS experiments using calcium imaging, live imaging, and iDISCO can be found in Figure 4 of Pașca et al.[17], Figures 2 and 3 of Birey et al.[19], and Figure 2 and Extended Figure 10 of Birey et al.[19], respectively.

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Author contributions

S.A.S., J.A., A.M.P., and F.B. collected data and contributed to the optimizations of the protocols. S.A.S. and S.P.P. wrote the manuscript with input from all authors. S.P.P. supervised this work.

Competing interests

The authors declare no competing interests.

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