May 27, 2020

Generation and assembly of human brain region-specific organoids

Steven A. Sloan[1], Jimena Anderson[1], Anca M. Pașca[2], Fikri Birey[1], Sergiu Pasca[1]

[1]Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA, USA.,

[2]Division of Neonatology, Department of Pediatrics, Stanford University, Stanford, CA, USA.

Neurodegeneration Method Development Community
Tech. support email: **ndcn-help@chanzuckerberg.com**

Sergiu Pasca

ABSTRACT

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. 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 humanderived model systems that recapitulate features of human CNS development and allow for the study of these processes in both healthy and diseased conditions.

EXTERNAL LINK

https://www.nature.com/articles/s41596-018-0032-7#article-info

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Sloan, S.A., Andersen, J., Pașca, A.M. et al. Generation and assembly of human brain region–specific three-dimensional cultures. *Nat Protoc* **13**, 2062–2085 (2018). https://doi.org/10.1038/s41596-018-0032-7

GUIDELINES

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).

MATERIALS TEXT

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, genomeintegrity checks). Cultures were tested and maintained mycoplasmafree. 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)
- NaHCO3 (Sigma, cat. no. S5761)
- EDTA (Sigma, cat. no. ED)

Cell culture reagents

- Gelatin solution bioreagent, 2% in H2O (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, H2O2 (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)
- 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% CO2 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% CO2 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 (CO2 unit: CO2-O2 UNIT-BL (0-20; 1-95); temp chamber: cage

incubator, T unit))

- Glass-bottom plates (Corning, cat. no. 4580)

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)

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 Milimolar (mM)** stock solution; use at a final concentration of **5 Micromolar (µM)** (1:1,000).
- Resuspend **10 mg** of **SB** in **2.6 ml** of 100% (vol/vol) ethanol to obtain a **10 Milimolar (mM)** stock solution; use at a final concentration of **10 Micromolar (µM)** (1:1,000).
- Resuspend **10 mg** of **IWP-2** in **4.29 ml** of sterile DMSO to obtain a **5 Milimolar (mM)** stock solution; use at a final concentration of **5 Micromolar (µM)** (1:1,000).
- Resuspend **5 mg** of **SAG** in **94.96 ml** of sterile DMSO to obtain a **100 Micromolar (µM)** stock solution; use at a final concentration of **100 Nanomolar (nM)** (1:1,000).
- Resuspend **5 mg** of **AlloP** in **156.99 ml** of sterile DMSO to obtain a **100 Micromolar (µM)** stock solution; use at a final concentration of **100 Nanomolar (nM)** (1:1,000).
- Resuspend **50 mg** of **RA** in **166.42 ml** of sterile DMSO to obtain a **1 Milimolar (mM)** stock solution; further dilute the stock solution to obtain a **100 Micromolar (µM)** ; use at a final concentration of **100 Nanomolar (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.

Media Preperation

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.

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)

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

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

hPSC medium (for use in Steps 5–16). Prepare the medium as detailed in the table above. 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	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 induction medium (for use in Steps 17–20, on days 0–5 of differentiation). Prepare the medium as detailed in the table above. 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
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

Neural differentiation medium (NM; for use in Steps 21 to 26, on day 6 and onward of differentiation). Prepare the medium as detailed in the table above. The stock can be stored for up to 1 week at 4 °C; add growth factors just before use if required for the culture.

Enzyme stock solution (required for dissociation of cells into a single-cell suspension)

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

Prepare a stock solution as detailed in the table above. The stock can be stored for 2–3 months at 4 °C.

Inhibitor stock solution (required for dissociation of cells into a single-cell suspension)

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

Prepare a stock solution as detailed in the table above. The stock can be stored for 2–3 months at 4 °C.

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% CO2 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 CO2 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 CO2 can be blown over the digesting solution.
- If this setup is not available, solutions can also be equilibrated in the incubator.

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards.

Ethyl ethanol is flammable and should be maintained in a flame-protective cabinet.

PFA must be handled in a safety cabinet and should be disposed of according to institutional guidelines.

BEFORE STARTING

See "Materials" for reagent setup.

MEF feeder seeding and initiation of hPSC cultures

2h

1 Coat 10-cm cell culture dishes with 0.1% gelatin for at least **01:00:00** at **Room temperature** .

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 **00:02:00**).

3 Briefly spin the cells at **200 x g** for **00:04:00** at **Room temperature** and plate at a density of $1–2 \times 10^6$ cells per 10-cm plate.

4 The next day, assess feeder quality before use; this will determine the quality of the hPSCs.

4.1 Once the MEFs are ready for plating hPSCs (after **02:00:00**), wash the culture plates at least once with fresh DPBS.

Passaging hPSCs on the MEF feeder layer

5 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 **00:20:00** .

6 Remove the hPSC plate from the incubator and look at it under a microscope.

6.1 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. (See Fig. 3a of the original publication).

7 After wiping with a paper towel sprayed with 70% (vol/vol) ethyl alcohol, transfer the plates to the cell culture hood.

7.1 Use a pipette tip to carefully remove demarcated differentiated colonies from the plate.

8 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 **00:07:00** or until hPSC colonies have lifted.

9 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.

9.1 Perform this step three times, adding the medium to the appropriate 50-ml centrifuge tube after each wash.

10 Gently swirl the 50-ml centrifuge tubes to suspend the harvested cells in **20 ml** of hPSC medium.

11 Transfer the hPSC medium and suspended cells to a freshly prepared feeder plate (from Step 4).

12 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.

13 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.

Critical Step: Replace hPSC culture medium at approximately the same time each day.

13.1 To replace the medium, warm aliquoted fresh hPSC medium in a water bath for up to **00:10:00** at **37 °C**, aspirate old medium from the plates, and add **12 ml** of fresh medium per 10-cm culture plate.

14 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 in original publication for examples of hPSC colonies of appropriate, and inappropriate, morphology.

14.1 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 1–13; 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

15 Remove the hPSC media from the culture plates. Detach hPSC colonies by incubating them with 0.35 mg/ml dispase[3h] (dissolved in hPSC medium; **5 ml** per plate) for **00:30:00** to **00:45:00** at **37 °C**, 5% CO_2.

Check the status of the detachment after **00:30:00** and then every **00:05:00** thereafter. Different

Check the status of the detachment after **00:20:00** and then every **00:05:00** 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 (see Fig. 3b in original publication).

16 Gently transfer the floating colonies with a pre-wet 25-ml pipette to a 50-ml Falcon tube.

16.1 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.

17 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-lowattachment plate.

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.

17.1 Denote this day as **day 0**. Place the plate in the incubator at **37 °C** , 5% CO2 for **48:00:00** . Keep the spheroids in the incubator on **day 1** without any medium change to allow the spheroids a day to recover.

Neural induction

18 **Critical:** Minimize the overall time that the spheroids are kept outside of the incubator at all stages of differentiation.

(Day 2)

After **48:00:00** 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).

18.1 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 **24:00:00** .

19 (Day 3)

Replace the medium as described in Step 18 and incubate the cells for a further **24:00:00** .

20 (Day 4)

If you plan to differentiate hSSs (when you reach Step 22, Step 24), replace the medium as described in Step 18,

but include [M]**5 Micromolar (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 **24:00:00** .

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.

Patterning and differentiation

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.

22 Change the medium daily on **days 6–11**. At day 6, neural spheroids should be ~0.3–0.5 mm in diameter (Fig. 3c of original publication).

The medium can be changed as described in Step 18 or by gently tilting the plate (allowing the spheroids to settle) and removing the remaining medium.

23 From **day 12** onward, the medium requirements for the generation of hCSs and hSSs deviate further.

For generation of **hCSs** go to step 23.

For generation of **hSSs** go to step 24.

For generation of **hSS-ISRAs** go to step 25.

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 of original publication). 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.

Generation of hCSs

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

24.1 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.

24.2 From days **25 to 43**, replace FGF2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3.

Perform medium changes with **14 ml** - **15 ml** of supplemented NM per 10-cm plate every 2–3

d.

Go to step 27

Generation of hSSs

25 From **days 12 to 23**, supplement the NM (already containing 20 ng/ml FGF2, 20 ng/ml EGF, and 5 µm IWP-2) with **[M]100 Nanomolar (nM)** SAG.

Perform medium changes with **14 ml** - **15 ml** of supplemented NM per 10-cm plate every day.

Critical Step: Note that early hSSs can be smaller in diameter in comparison with early hCSs during 12–20 d of differentiation

25.1 On **day 24**, replace medium with NM without additional growth factors.

25.2 From **days 25 to 43**, replace FGF2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3.

Perform medium changes with **14 ml** - **15 ml** of supplemented NM per 10-cm plate every 2–3 d.

Go to Step 26.

Generation of hSS-ISRAs

26 On **days 12, 13, and 14**, supplement the NM (with IWP-2) with 100 nM SAG.

Perform medium changes with **14 ml** - **15 ml** of supplemented NM per 10-cm plate every day.

26.1 From **days 12 to 15**, add **[M]100 Nanomolar (nM)** RA (in addition to the IWP-2) to the neural induction medium (Fig. 2c of original publication).

Caution: RA is air- and light-sensitive. Minimize light exposure.

26.2 From days **15 to 23**, add **[M]100 Nanomolar (nM)** AlloP (in addition to the IWP-2) to the neural induction medium (Fig. 2c of original publication).

26.3 On **day 24**, replace medium with NM without additional growth factors.

26.4 From **days 25 to 43**, replace FGF2 and EGF with 20 ng/ml BDNF and 20 ng/ml NT3.

Perform medium changes with **14 ml** - **15 ml** of supplemented NM per 10-cm plate every 2–3 d.

Maintenance and long-term culture

27 From **day 43** onward, perform media changes with **17 ml** - **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 of original publication).

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:
Step 27 includes a Step case.
Generation of forebrain assembloids w/ hCSs + hSSs
Dissociation into singe-cell suspension
Cryosectioning and immunostaining
Calcium imaging
Optical clearing of intact spheroids by iDISCO

step case

Generation of forebrain assembloids w/ hCSs + hSSs

28 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 of original publication).

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.

29 Incubate the spheroids for at least **42:00:00** 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.

30 Check that fusion is complete.

Fusion is considered complete when the two spheroids are inseparable with gentle shaking of the Eppendorf tube (Fig. 4g of original publication).

30.1 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).