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Generation of human induced pluripotent stem cell-derived cerebral organoids for cellular and molecular characterization

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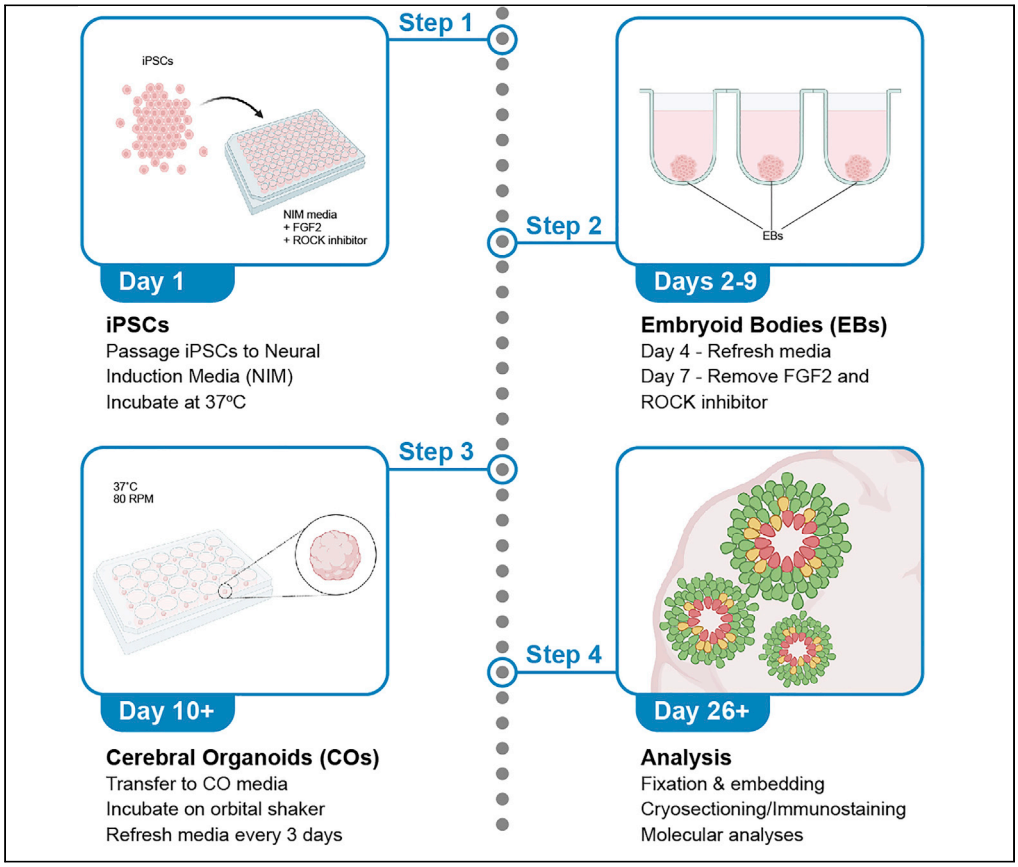

Human induced pluripotent stem cell (hiPSC)-derived cerebral organoids (COs) can serve as an *in vitro* model for studying normal and pathologic human brain development. Here, we optimized existing protocols to streamline the generation of forebrain COs from hiPSCs. We employ these COs to define the impact of disease-causing mutations on cell fate, differentiation, maturation, and morphology relevant to neurodevelopmental disorders. Although limited to forebrain CO identity, this schema requires minimal external interference and is amenable to low-throughput biochemical assays.

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Highlights

Minimally engaging protocol for human iPSC forebrain cerebral organoid (CO) generation

COs can define impact of disease-causing mutations on cell identity and differentiation

COs can model cell morphology deficits relevant to neurodevelopmental disorders

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Generation of human induced pluripotent stem cell-derived cerebral organoids for cellular and molecular characterization

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SUMMARY

Human induced pluripotent stem cell (hiPSC)-derived cerebral organoids (COs) can serve as an *in vitro* model for studying normal and pathologic human brain development. Here, we optimized existing protocols to streamline the generation of forebrain COs from hiPSCs. We employ these COs to define the impact of disease-causing mutations on cell fate, differentiation, maturation, and morphology relevant to neurodevelopmental disorders. Although limited to forebrain CO identity, this schema requires minimal external interference and is amenable to low-throughput biochemical assays.

For complete details on the use and execution of this profile, please refer to Anastasaki et al. (2020) and Wegscheid et al. (2021).

BEFORE YOU BEGIN

For additional details regarding the landmark protocol, please refer to Lancaster et al. (2013) (Lancaster et al., 2013). Prior to generation of cerebral organoids, first prepare Matrigel-coated plates or flasks to grow hiPSCs to an optimal confluency.

Preparing Matrigel-coated flasks

Timing: 1 h 45 min, protocol generates 10 coated T25 flasks

1. Thaw Matrigel (8–12 mg/mL; concentration varies depending on the lot number) on ice. Once liquid, swirl the vial to ensure that the contents are evenly dispersed, prior to aliquotting into pre-chilled 0.5 mL microcentrifuge tubes.
   a. When aliquoting, it is important to consider that the Matrigel stock must be diluted 100-fold in DMEM/F12 ("working solution") for subsequent plate coating. The total volume of the Matrigel working solution also depends on the surface area of each container. For instance, 1.5 mL of total Matrigel working solution volume is required to coat one well of a 6-well plate, 5 mL for T25 flasks, and 7 mL for 10 cm petri dishes.
   b. Matrigel aliquots can be stored at −20°C for up to 2 months and at −80°C for up to 6 months.
2. To coat ten T25 flasks, make 50 mL of Matrigel working solution: add 500 µL of Matrigel to 50 mL of pre-chilled DMEM/F-12 in a pre-chilled 50 mL falcon tube. Pipette up and down to ensure an even distribution of Matrigel.
3. Add 5 mL of the Matrigel working solution to each T25 flask. Gently agitate the flask so that the Matrigel mixture is evenly distributed along the bottom of the flask.

4. Incubate the flask at 37°C for a minimum of 1 h and a maximum of 1 week. Coated flasks are now ready for use. Troubleshooting 1
   a. The coated flasks can be stored up to 1 week in a 37°C incubator without excessive evaporation noted. Incubation at 37°C for longer periods may result in reagent evaporation and thus uneven covering of the flask surface. Proper humidity in the incubator chamber is critical to ensure slower evaporation.
   b. Alternatively, it is possible to coat the flasks or plates, seal them with parafilm and store them at 4°C for up to 2 weeks.

⚠ CRITICAL: While a liquid at 4°C, Matrigel readily solidifies at 20°C–25°C, and therefore, it is important to work on ice with pre-chilled reagents.

Growing iPSCs

Timing: Cells take approximately 10 min to passage. Grow cells to 70%–80% confluency (variable duration).

5. iPSCs are grown in mTeSR Plus on Matrigel-coated 25 cm^2 flasks and incubated at 37°C with 5% CO_2.
6. Passaging iPSCs (adapted from the manufacturer's protocol):
   a. Completely remove all media from the flask by aspiration.
   b. Wash cells with 5 mL of 1× DPBS and aspirate.
   c. Add 3 mL of ReLeSR to the flask and aspirate immediately.
   d. Incubate cells at 22°C for 5 min.
   e. Lightly tap the flasks to loosen the cells.
   f. Detach the cells from the bottom of the flask by adding 5 mL of 1× 22°C D-PBS (pH 7.0–7.3), pipetting up and down gently to release the cells.
   g. Transfer 5 mL of the cells to one 15 mL conical tube, and centrifuge for 3 min at 300×*g* at 22°C with full acceleration and deceleration.
   h. Aspirate the supernatant.
   i. Resuspend the cell pellets in 1 mL of DMEM/F-12 media, determine the cell number using either an automated cell counter or a hemocytometer, and seed the new flask at the desired cell density.

Note: A good cell density that facilitates the healthy growth of hiPSCs and allows for weekly passaging is 50,000–100,000 cells/ T25 flask.

Note: Cells that remain attached to the same plate for more than 10 days frequently undergo cellular senescence. Increased passage number may also reduce the ability of some cell lines to generate cerebral organoids. Once weekly hiPSC passaging is optimal.

7. Remove Matrigel from Matrigel-coated T25 flask and replace with 5–15 mL mTeSR Plus.
   a. hiPSCs of <70% confluence maintained in mTeSR Plus can be refreshed daily (5 mL/T25 for 24 h), every other day (10 mL T25 for 48 h), or every two days (15 mL/T25 for 72 h).
8. Add iPSCs to mTeSR Plus-containing flasks, and spread them evenly along the surface of the flask by moving the flask in a left-to-right and top-to-bottom motion on a flat surface.
9. Incubate hiPSCs at 37°C at 5% CO_2.
10. Monitor daily for confluence, as well as for potential contamination or non-specific differentiation.

Note: Prolonged maintenance of iPSCs in the same flask increases the likelihood of contamination. Be sure that iPSCs are not in the same flask for more than 10 days. Typical signs of

contamination include overabundance of cell death, media that is turbid in appearance, and obvious fungi/yeast growth.

Note: Non-specific differentiation of hiPSCs is possible. The morphology of these differentiated cells is easily distinguished from the healthy flattened hiPSC colonies (The International Stem Cell Banking Initiative, 2009). The use of ReLeSR is selected to specifically detach iPSCs, rather than differentiated cells, within 5 min to minimize the propagation of differentiated cells.

⚠ CRITICAL: Perform all experiments on at least two different clones of iPSCs, with at least three passages per clone, to ensure specificity and validity of phenotypes in COs. There is no upper limit to the number of passages that can be used per clone.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Monoclonal Anti-SOX2 (1:200)	Cell Signaling Technology	Cat# 4900; RRID: AB_10560516
Rabbit Monoclonal Anti-SOX2 (1:200)	Abcam	Cat# ab92494; RRID: AB_10585428
Rabbit Monoclonal Anti-NeuroD1 (1:500)	Abcam	Cat# ab205300
Mouse Monoclonal Anti-NeuroD1 (1:500)	Abcam	Cat# ab60704; RRID: AB_943491
Mouse Monoclonal Anti-NeuN (1:500)	Millipore	Cat# MAB377; RRID: AB_2298772
Rabbit Monoclonal Anti-Cleaved Caspase-3 (Asp175) (5A1E) (1:250)	Cell Signaling Technology	Cat# 9664; RRID: AB_2070042
Goat Anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 (1:200)	Thermo Fisher Scientific	Cat# A-11034; RRID: AB_2576217
Goat Anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 (1:200)	Thermo Fisher Scientific	Cat# A-11029; RRID: AB_138404
Goat Anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568 (1:200)	Thermo Fisher Scientific	Cat# A-11011; RRID: AB_143157
Goat Anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 568 (1:200)	Thermo Fisher Scientific	Cat# A-11004; RRID: AB_2534072
Rabbit Monoclonal Anti-PAX6 (1:350)	Abcam	Cat# ab19504; RRID: AB_2750924
Mouse Monoclonal Anti-OTX2 (1:200)	Thermo Fisher Scientific	Cat# MA5-15854; RRID: AB_11155193
Rabbit Polyclonal Anti-EN1 (1:25)	Thermo Fisher Scientific	Cat# PA5-14149; RRID: AB_2231168
Mouse Monoclonal Anti-GBX2 (1:50)	Lifespan Biosciences	Cat# LS-C197281
Rabbit Polyclonal Anti-EAAT1 (1:500)	Abcam	Cat# ab416; RRID: AB_304334
Mouse Monoclonal Anti-GFAP (1:500)	Abcam	Cat# ab4648; RRID: AB_449329
Mouse Monoclonal Anti-Ki67 (1:100)	Thermo Fisher Scientific	Cat# BDB556003; RRID: AB_396287
Mouse Monoclonal Anti-MAP2 (1:500)	Abcam	Cat# ab11267; RRID: AB_297885
Mouse Monoclonal Anti-TUJ-1 (1:1,000)	Abcam	Cat# ab78078; RRID: AB_2256751
Chemicals, peptides, and recombinant proteins		
Matrigel® Basement Membrane Matrix, LDEV-free	Corning	Cat# 354234
Dulbecco's Modified Eagle Media (DMEM)/F-12	Thermo Fisher Scientific	Cat# 11320033
1× DPBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat# 14190144
mTeSR™ Plus	STEMCELL™ Technologies	Cat# 100-0276
ReLeSR™	STEMCELL™ Technologies	Cat# 100-0484
Gibco™ Neurobasal™ Media	Thermo Fisher Scientific	Cat# 21103049
Antibiotic-Antimycotic Solution (100×)	Caisson Labs	Cat# ABL02-100ML
Gibco™ N-2 Supplement (100×)	Thermo Fisher Scientific	Cat# 17502048
Gibco™ B-27™ Supplement (50×), serum free	Thermo Fisher Scientific	Cat# 17504044
Humulin® R Regular Human Insulin 100 U/mL	McKesson Corporation	Cat# 00002821517

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
2-Mercaptoethanol, ≥99.0%	Millipore Sigma Company	Cat# M6250-10ML
GlutaMAX™ Supplement (100×)	Thermo Fisher Scientific	Cat# 35050061
MEM Non-Essential Amino Acids Solution (100×)	Thermo Fisher Scientific	Cat# 11140050
STEMdiff™ Neural Induction Media	STEMCELL™ Technologies	Cat# 05835
Human Recombinant basic FGF (FGF2)	STEMCELL™ Technologies	Cat# 78003.1
Y-27632 (ROCK Inhibitor)	STEMCELL™ Technologies	Cat# 72302
Human Recombinant LIF	STEMCELL™ Technologies	Cat #78055
CHIR99021	STEMCELL™ Technologies	Cat #72054
SB431541	STEMCELL™ Technologies	Cat # 72234
Dorsomorphin	STEMCELL™ Technologies	Cat #72102
Human recombinant BDNF	STEMCELL™ Technologies	Cat # 78005
Human recombinant GDNF	STEMCELL™ Technologies	Cat# 78058
Hoechst 33258, Pentahydrate (bis-Benzimide)	Thermo Fisher Scientific	Cat# H3569
RNaseZAP™	Millipore Sigma Company	Cat# R2020-250ML
Sucrose; for molecular biology, ≥99.5% (GC)	Millipore Sigma Company	Cat# S0389-1KG
Paraformaldehyde; reagent grade, crystalline	Millipore Sigma Company	Cat# P6148-1KG
Tissue-Tek® O.C.T. Compound	VWR	Cat# 25608-930
2-Methylbutane	Millipore Sigma Company	Cat# 277258-1L
2-Propanol	Millipore Sigma Company	Cat# 278475-1L
Triton™ X-100	Millipore Sigma Company	Cat# X100-500ML
Goat Serum, New Zealand Origin	Thermo Fisher Scientific	Cat# 16210072
Epredia™ Immu-Mount™	Thermo Fisher Scientific	Cat# 9990402
Critical commercial assays		
Applied Biosystems High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	Thermo Fisher Scientific	Cat# 4374966
Applied Biosystems TaqMan Fast Advanced Master Mix, no UNG	Thermo Fisher Scientific	Cat# A44359
NucleoSpin® RNA Plus (50 preps)	Takara Bio USA, Inc.	Cat# 740984.50
cAMP ELISA Kit	Enzo Life Sciences	Cat# ADI-900-067
RhoA G-LISA Activation Assay Kit (Colorimetric Format) 96 Assays	Cytoskeleton, Inc.	Cat# BK124
Experimental models: Cell lines		
BJFF.6 iPSCs	Washington University Genome Engineering and iPSC Core (GeiC)	RRID: CVCL_VU02
TGD1 hiPSCs	(Wegscheid et al., 2021)	N/A
TGD2 hiPSCs	(Wegscheid et al., 2021)	N/A
TGD2 hiPSCs	(Wegscheid et al., 2021)	N/A
aTGD hiPSCs	(Wegscheid et al., 2021)	N/A
shCTL1 hiPSCs	(Wegscheid et al., 2021)	N/A
shCTL2 hiPSCs	(Wegscheid et al., 2021)	N/A
shCTL3 hiPSCs	(Wegscheid et al., 2021)	N/A
shCTL4 hiPSCs	(Wegscheid et al., 2021)	N/A
sh*CRLF3*-1 hiPSCs	(Wegscheid et al., 2021)	N/A
sh*CRLF3*-2 hiPSCs	(Wegscheid et al., 2021)	N/A
sh*CRLF3*-4 hiPSCs	(Wegscheid et al., 2021)	N/A
sh*CRLF3*-4 hiPSCs	(Wegscheid et al., 2021)	N/A
c.1149C>A *NF1*-mutant hiPSCs	(Anastasaki et al., 2020)	N/A
c.1185+1G>A *NF1*-mutant hiPSCs	(Anastasaki et al., 2020)	N/A
c.3431-32_dupGT *NF1*-mutant hiPSCs	(Anastasaki et al., 2020)	N/A
c.5425C>T *NF1*-mutant hiPSCs	(Anastasaki et al., 2020)	N/A
c.6619C>T *NF1*-mutant hiPSCs	(Anastasaki et al., 2020)	N/A
CTL2 hiPSCs	Washington University – GeiC (Dr. Matthew B. Harms)	N/A
CTL3 hiPSCs	Washington University – GeiC (Dr. Fumihiko Urano)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Human *GAPDH* TaqMan® Gene Expression Assay FAM-MGB	Thermo Fisher Scientific	Hs02786624_g1
Human *NeuroD1* TaqMan® Gene Expression Assay FAM-MGB	Thermo Fisher Scientific	Hs01922995_s1
Human *Neurofibromin 1* TaqMan® Gene Expression Assay FAM-MGB	Thermo Fisher Scientific	Hs01035108_m1
Software and algorithms		
NanoDrop™ 2000	Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers/nanodrop-product-authentication.html
CFX Manager™ Software Version 3.1	Bio-RAD Laboratories, Inc.	Cat# 1845000
Other		
25 cm^2 Tissue Culture Flask, Vent Cap, Sterile	CELLTREAT	Cat# 229331
Costar® Ultra-Low Attachment Multiple Well Plate, U-Shaped-Bottom (96-well)	Corning	Cat# 3799
Costar® Ultra-Low Attachment Multiple Well Plate, (24-well)	Corning	Cat# 3473
6 Well Tissue Culture Plate with Lid, Individual, Sterile	CELLTREAT	Cat# 229106
EMD Millipore Stericup Sterile Vacuum Filter Units - 500 mL funnel; PES membrane; 500 mL receiver; Pore	Thermo Fisher Scientific	Cat# SCGPU10RE
Fisherbrand™ Disposable Base Molds	Thermo Fisher Scientific	Cat# 22-363-553
Microscope Slides, Diamond White Glass, 25 × 75 mm, Charged, 45° Beveled Edges, Safety Corners, WHITE Frosted	Globe Scientific, Inc.	Cat# W1354
Super HIT PAP Pen, Mini, 2.5 mm Tapered Tip	Kiyota International	Cat# K-650
NanoDrop™ 2000 Spectrophotometer	Thermo Fisher Scientific	Cat# ND-2000
CFX96 Optical Reaction Module for Real-Time PCR Systems	Bio-RAD Laboratories, Inc.	Cat# 1845096
C1000 Touch™ Thermal Cycler Chassis	Bio-RAD Laboratories, Inc.	Cat# 1841100
Eppendorf® Mastercycler® Nexus Thermal Cyclers	Millipore Sigma Company	Cat# EP6334000026
Carbon Dioxide Resistant Orbital Shaker	Fisher Scientific	Cat# 88-881-101

MATERIALS AND EQUIPMENT

Neural Induction Media (NIM)		
Reagent	Final concentration	Amount
STEMdiff™ Neural Induction Media	n/a	20 mL
human FGF2 [10 μg/mL]	4 ng/mL	8 μL
Y-27632 Rock Inhibitor [20 mM]	20 μM	20 μL
Total Volume		**20 mL**

Mix all of these components using aseptic technique. Prepare the media on the day of use. If needed, store reagents at 4°C, but do not store for more than 4 h.

Alternatives: It is possible to produce neural induction embryoid body media by supplementing neurobasal media with 1% GlutaMAX, 1% penicillin/streptomycin, 1% N2 supplement, 2% B27 supplement, and 1.25 μM dorsomorphin. Optional additions include 10 ng/mL human recombinant LIF, 3 μM CHIR99021 and 3 μM SB431542.

Organoid Media		
Reagent	Final concentration	Amount
DMEM/F-12	n/a	500 mL
Neurobasal media	n/a	500 mL
Antibiotic Antimycotic	1% (v/v)	10 mL
N2 (100×)	0.5×	5 mL
B27 (50×)	0.5×	10 mL
Humulin	100 U/mL	250 µL
2-Mercaptoethanol (50 mM)	200 nM	4 µL
GlutaMAX (100×)	1×	10 mL
Minimum Essential Media (MEM) Non-Essential Amino Acid (NEAA) 100×	0.5×	5 mL
Total	n/a	**1000 mL**

Mix, and filter them using a sterile vacuum filter with a PES membrane. Store at 4°C and use within two weeks.

⚠ CRITICAL: While 2-Mercaptoethanol is harmful, the negative effects of 2-Mercaptoethanol are alleviated using proper standard health and safety measures.

Alternatives: Although it is critical that no alternatives to this solution is used, the organoid media can be supplemented with additional neurotrophic factors, such as 20 ng/mL BDNF, 20 ng/mL GDNF and 20 ng/mL FGF2 to accelerate neuronal maturation.

30% Sucrose Solution		
Reagent	Final concentration	Amount
Sucrose	30%	30 g
D-PBS	n/a	100 mL
Total	n/a	**100 mL**

Mix the components and store at 4°C and use within three days

Alternatives: It is critical that no alternatives to these solutions are used.

Embedding Solution		
Reagent	Final concentration	Amount
Tissue-Tek O.C.T. Compound	50%	5 mL
30% Sucrose Solution	50%	5 mL
Total	n/a	**10 mL**

Mix the components a few hours prior to use and store at 22°C to reduce air bubble content of the solution. Do not store for more than 24 h.

Alternatives: It is critical that no alternatives to these solutions are used.

Permeabilization Buffer		
Reagent	Final concentration	Amount
Triton X-100	0.5%	500 µL
D-PBS	n/a	100 mL
Total	n/a	**100 mL**

Mix the components and store at 22°C up to a year.

Alternatives: It is critical that no alternatives to these solutions are used.

Blocking Solution		
Reagent	Final concentration	Amount
Goat serum	10%	10 mL
D-PBS	n/a	100 mL
Total	n/a	**100 mL**
Store at 4°C and use within one week.		

⚠ CRITICAL: Goat serum must be replaced with donkey serum when the host of the primary antibody used is goat.

Antibody Diluent Solution		
Reagent	Final concentration	Amount
Goat serum	2%	2 mL
D-PBS	n/a	100 mL
Total	n/a	**100 mL**
Store at 4°C and use within one week.		

⚠ CRITICAL: Goat serum must be replaced with donkey serum when the host of the primary antibody used is goat.

STEP-BY-STEP METHOD DETAILS

Generating embryoid bodies (EBs)

Timing: 20–30 min

The purpose of this step is to generate embryoid bodies (EBs). EBs will appear as a spherical cluster of cells with translucent borders, which do not attach to the walls of the multi-well plates.

1. Grow iPSCs to 80% confluency. On day 1, passage iPSCs in ReLeSR, as indicated above (Figure 1). Troubleshooting 2
   a. The use of healthy iPSCs is crucial for the successful generation of cerebral organoids. iPSCs should be grown to a confluency of 70%–80% and should appear as flat colonies.
2. Resuspend the resulting pellet in 1 mL DMEM/F12 and calculate cell density, only counting live cells when determining the final cell density. Troubleshooting 3

3. Resuspend the respective number of iPSCs in an appropriate volume of **NIM** using the following guidelines:
   a. The generation of a single EB requires 40,000 live iPSCs/well.
   b. Each well requires a total volume of 200 µL of resuspended iPSCs in **NIM.**
   c. Calculate the total number of wells to be used, based on the number of live hiPSCs available (Step 2).
   d. Calculate the total number of iPSCs needed by multiplying 40,000 by the number of wells to be used (step 3c).

   Note: For 96 wells, 3.84 × 106 iPSCs in a total volume of 19.2 mL are needed.

   e. Calculate the volume of **NIM** needed.

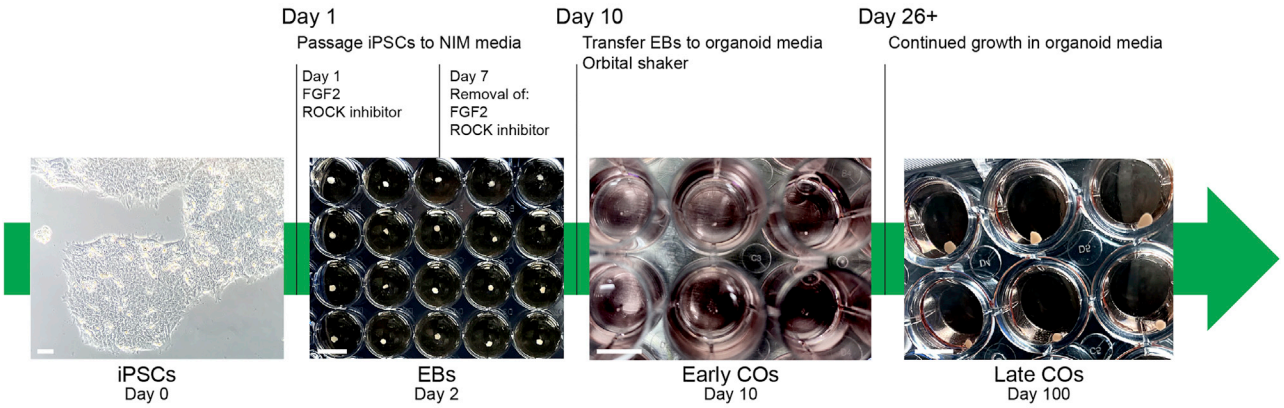

Figure 1. iPSCs are differentiated into embryoid bodies prior to forming forebrain cerebral organoids

On day 1, 40,000 iPSCs are seeded in each well of a 96-well plate and incubated in neural induction media (NIM) supplemented with FGF2 and ROCK inhibitors to allow for embryoid body (EB) differentiation. Media is refreshed on day 4. On day 7, EBs are incubated in NIM without FGF2 or ROCK inhibitors. On day 10, EBs are transferred to 24-well plates with organoid media and are placed on an orbital shaker at 80 rpms to induce cerebral organoid (CO) formation. Developing COs can be incubated in the organoid media for short- or long-term maintenance. Scale bars; iPSCs: 100 μm; all other panels: 5 mm.

4. Pipette 200 μL of hiPSC suspension per well of an ultra-low cell attachment U bottom 96-well plate.

   Optional: For the most consistent results, first transfer iPSC/NIM suspensions into a multi-channel pipette trough, and then aliquot 200 μL of this suspension into each well of an ultra-low cell attachment U bottom 96-well plate using a multichannel pipette.

5. Centrifuge the multi-well plate at 300×*g* for 3 min at 22°C with maximum acceleration and deceleration.
6. Incubate EBs at 37°C with 5% CO_2.
7. On day 2 (approximately 12–15 h after EB induction), visualize the cells in the U-bottom 96-well plates under a bright-field microscope to determine whether the successful formation of EBs has occurred (Figure 1). EBs will appear as round clusters of cells with translucent borders. EBs may also be surrounded by debris. This is normal. As long as the debris can be removed by refreshing the media at the predesignated time points, it will not affect the subsequent generation of cerebral organoids. Troubleshooting 4

Generating EBs and initiating germ layer differentiation

Timing: 10 days

The purpose of this step is to initiate neural progenitor cell differentiation of EBs.

8. On day 4, refresh the media by removing all 200 μL of **NIM** (preferably using a multi-channel pipette), without disturbing the EBs, and replacing it with 200 μL of freshly prepared NIM. Troubleshooting 5
9. Incubate EBs at 37°C with 5% CO_2.
10. On day 7, remove all 200 μL media (preferably using a multi-channel pipette), without disturbing the EBs. Replace with 200 μL of **STEMdiff Neural Induction Media** (this is the STEMCELL Technologies media without ROCK inhibitor or FGF2).
11. Incubate EBs at 37°C with 5% CO_2.

Generating forebrain cerebral organoids

Timing: minimum 7 days

The purpose of this step is to generate cerebral organoids (COs) exhibiting an optically clear neuroectoderm.

12. On day 10, transfer each EB to a single well of an ultra-low cell attachment 24-well plate. Each EB is incubated in 500 µL of **Organoid Media** per well. Troubleshooting 6

    Optional: To accelerate neuronal maturation, supplement the **Organoid Media with** 20 ng/mL of BDNF, 20 ng/mL GDNF or 20 ng/mL FGF2.

13. Incubate the ultra-low cell attachment 24-well plates containing EBs on an orbital shaker set at 80 rpms, and incubate at 37°C with 5% CO_2.

    Note: It is important to use an orbital shaker made specifically for tissue culture, because orbital shakers not designed specifically for this purpose will generate excessive heat that may negatively affect the growth of cerebral organoids.

14. Fully replace the **Organoid Media** every 3 days using a Pasteur pipette, rather than an aspirator. Observe the morphology of the growing organoids, and only propagate the growth of healthy-looking COs. Troubleshooting 7
15. Incubate COs at 37°C with 5% CO_2 on a CO_2-resistant orbital shaker (Fisher Scientific; Catalog #88-881-101).
16. It is possible to transfer COs in individual flasks (12 cm^2) for long-term culture (> 30 weeks in vitro).

    Note: COs can be maintained for at least 6 months.

    Optional: For the generation of region-specific organoids different small molecules and morphogens can be added at different points of organoid differentiation and maturation (Table 1).

Validation and analysis of resulting cerebral organoids

The purpose of these steps is to analyze cerebral organoid structure, self-organization, and cellular differentiation using cell type-specific markers.

Validation 1–Confirming the formation of ventricular zones

Timing: 3 days

The purpose of this step is to confirm the successful generation of organized ventricular zones. The following steps are modified from Sloan et al. (2018).

17. On Day 1 the COs are transferred to 1.5 mL microcentrifuge tubes, and **Organoid media** is aspirated.

    Note: 4–5 COs can be placed in the same microcentrifuge tube.

18. COs are rinsed with phosphate buffered saline (PBS), and then fixed in ice-cold **4% paraformaldehyde** (PFA) pH7.4 in PBS at 4°C for 15–20 h.

    Note: If necessary, a shortened 2-h incubation in 4% PFA is sufficient for organoid fixation.

Table 1. Generation of region-specific organoids from hiPSCs

Region specificity	Time of addition	Added factors	Container used	References
Forebrain (Dorsal)	Day 1–6	5 μM dorsomorphin, 10 μM SB431542	Low attachment multiwell plates; static	Birey et al. (2017), Sloan et al. (2018)
	Day 6–25	20 ng/mL human epidermal growth factor (EGF), 20 ng/mL human basic fibroblast growth factor (FGF2)		
	Day 26–43	20 ng/mL human brain derived neurotrophic factor (BDNF), 20 ng/mL human recombinant neurotrophin-3 (NT3)		
Forebrain (Ventral)	Day 1–5	DMEM/F12, 20% knockout serum replacement, 1% non-essential amino acids (NEAA), 0.5% GlutaMAX, 0.1 mM β-mercaptoethanol, 5 μM dorsomorphin, 10 μM SB431542	Low attachment multiwell plates; static	Birey et al. (2017), Sloan et al. (2018)
	Day 5–6	DMEM/F12, 20% knockout serum replacement, 1% NEAA, 0.5% GlutaMAX, 0.1 mM β-mercaptoethanol, 5 μM dorsomorphin, 10 μM SB431542, 5 μM IWP-2		
	Day 7–12	Neurobasal A, 2% B27, 1% GlutaMAX, 20 ng/mL EGF, 20 ng/mL FGF2		
	Day 13–14	NeurobasalA, 2% B27, 1% GlutaMAX, 20 ng/mL EGF, 20 ng/mL FGF2, 5 μM IWP-2 (Wnt inhibitor), 100 nM smoothened agonist		
	Day 13–16	NeurobasalA, 2% B27, 1% GlutaMAX, 20 ng/mL EGF, 20 ng/mL FGF2, 5 μM IWP-2 (Wnt inhibitor), 100 nM smoothened agonist, 100 nM retinoic acid (RA)		
	Day 16–26	NeurobasalA, 2% B27, 1% GlutaMAX, 20 ng/mL EGF, 20 ng/mL FGF2, 5 μM IWP-2 (Wnt inhibitor), 100 nM smoothened agonist, 100 nM allopregnanolone		
	Day 26–43	Neurobasal A, 2% B27, 1% GlutaMAX, 20 ng/mL BDNF, 20 ng/mL NT3		
	Day 44+	NeurobasalA, 2% B27, 1% GlutaMAX,		
Forebrain (Hypothalamus)	Day 1–3	2.5 μM LDN-193189, 3 μM SB431542, 450 μM 1-thioglycerol	Low attachment multiwell plates; static	Qian et al. (2016), Qian et al. (2018)
	Day 3–6	10 ng/mL Wnt-3A, 20 ng/mL human recombinant sonic hedgehog (SHH), 2 μM purmorphamine		
	Day 6+	10 ng/mL FGF2, 10 ng/mL human recombinant ciliary neurotrophic factor (CTNF)	Low cell attachment plates – orbital shaker	
Forebrain (Thalamus)	Day 1	100 nM LDN-193189, 10 μM SB431542, 4 μg/mL insulin , 50 μM ROCK inhibitor, 5% fetal bovine serum (FBS)	Low attachmentmultiwellplates; static	Xiang et al. (2019)
	Day 2	100 nM LDN-193189, 10 μM SB431542, 4 μg/mL insulin , 50 μM ROCK inhibitor		
	Day 4	100 nM LDN-193189, 10 μM SB431542, 4 μg/mL insulin		
	Day 9	DMEM/F12, 0.15 (w/v) dextrose, 100 μM β-mercaptoethanol, 1% N2, 2% B27, 30 ng/mL human recombinant bone morphogenetic protein 7 (BMP7), 1 μM PD0325901	Low cell attachment plates – orbital shaker	
	Day 16	20 ng/mL BDNF, 200 μM ascorbic acid, 20 ng/mL FGF2		

(*Continued on next page*)

Table 1. *Continued*

Region specificity	Time of addition	Added factors	Container used	References
Midbrain	Day 1–4	100 nM LDN-193189, 10 SB431542, 100 ng/mL SHH, 2 μM purmorphamine, 100 ng/mL human recombinant fibroblast growth factor 8 (FGF-8)	Low attachmentmultiwell plates; static	Kim et al. (2019), Qian et al. (2016), Jo et al. (2016)
	Day 5	100 nM LDN-193189, 3 μM CHIR99021, 100 ng/mL SHH, 2 μM purmorphamine, 100 ng/mL FGF-8		
	Day 7	100 nM LDN-193189, 3 μM CHIR99021		
	Day 14	20 ng/mL BNDF, 20 ng/mL human recombinant glial-derived neurotrophic factor (GDNF), 0.2 mM ascorbic acid, 1 ng/mL human recombinant transforming growth factor beta (TGFβ), 0.5 mM cyclic AMP (cAMP)	Low cell attachment plates – orbital shaker	
Hindbrain (Brainstem)	Day 1	1 mM dorsomorphin, 10 μM SB431542, 10 μM transferrin, 5 mg/L insulin, 0.063 mg/L progesterone	Low attachment multiwell plates; static	Eura et al. (2020)
	Day 9–12	20 ng/mL FGF2, 10 μM transferrin, 5 mg/L insulin, 0.063 mg/L progesterone		
	Day 13–18	20 ng/mL FGF2, 20 ng/mL EGF		
	Day 19–25	ascorbic acid, cAMP, 20 ng/mL BDNF, 20 ng/mL GDNF, 20 ng/mL NT3		
Hindbrain (Cerebellum)	Day 1	iPSCs passaged to containers pre-coated with matrigel supplemented with 1% bovine serum albumin	Matrigel plates - static	Valiulahi et al. (2021)
	Day 1	Knockout serum replacement medium	Low attachment multiwell plates; static	
	Day 5	25% N2 medium, 2 μM purmorphamine, 2 μM RA		
	Day 7	50% N2, 2 μM purmorphamine, 2 μM RA		
	Day 9	75% N2, 2 μM purmorphamine, 2 μM RA		
	Day 10–13	20 ng/mL BDNF, 2 μM purmorphamine, 2 μM RA	Low cell attachment plates – orbital shaker	
	Day 14+	20 ng/mL BDNF		

19. On Day 2, after fixation, the **4% PFA** is removed, and the COs are gently washed three times for 15 min each in PBS pH7.6 at 22°C.
    a. Stopping point. The fixed COs can be stored in PBS for up to 1 week at 4°C.
20. Gently remove the PBS and replace with 1.5 mL of **30% sucrose solution.**
21. Incubate COs in 30% sucrose solution at 4°C until the COs no longer float.
    a. This step can be performed for a minimum of 5 h or 15–20 h at 4°C.
22. On Day 3, prepare the **Embedding solution** using a P1000 pipette tip to mix by gently pipetting up and down.
23. Aspirate the sucrose solution and apply 500 μL **Embedding solution,** avoiding the introduction of bubbles.
24. Fill an appropriate container with a layer of dry ice and spread evenly such that the molds rest flat on top of the ice. Add 2-methylbutane or 2-propanol to barely cover the dry ice.

    Note: Wait for the 2-methylbutane or 2-propanol to stop boiling prior to proceeding. The slurry will be very cold. Avoid direct contact to prevent injury. Wear appropriate personal protective equipment at all times.

    Note: Liquid nitrogen should not be used for this step, as the freezing will be faster.

25. Fill disposable plastic 15 × 15 × 5 mm base molds (Fisherbrand Cat # 22363553) three quarters of the way up with **Embedding solution**, avoiding bubble introduction, and place on a flat bench surface (not on dry ice).

26. Transfer COs to the center of each mold using a P1000 pipette tip in which the tip has been cut with scissors or a razor blade.
27. Use fine forceps or a P200 pipette tip to position the COs in the desired arrangement and orientation, taking care that each CO does not touch other tissues or the edges of the mold.

Note: Up to six COs can be arranged in a 15 × 15 × 5 mm mold.

28. Freeze the tissues by placing the molds directly on the bed of the dry ice/2-methylbutane or 2-propanol slurry, and wait until they are completely frozen.

Note: As the **Embedding solution** freezes, it changes colors. It turns from a clear solution to an opaque white solid. The freezing process takes 1–2 min.

29. Wrap the molds individually in aluminum foil and store indefinitely at −80°C.

Note: A minimum freezing period of 15 h at −80°C is preferred for optimal cryosectioning results.

30. On Day 4, thaw the mold with the frozen COs for 30 min in a cryostat chamber set to −20°C.
31. Remove the block from the mold and place it on the specimen stage using Tissue-Tek O.C.T. Compound.
32. Retrieve 10 μm sections, placing two sections per slide.

Note: COs grown for fewer than 4 weeks in **Organoid media** will contain organized ventricular zones. These zones appear closest to the edges of the COs. Confirm the presence of these zones under a light microscope. CO specimens that have been grown for more than 4 weeks may not retain these organized zones.

Note: A minimum of 50 sections should be retained from each CO to ensure a representative depiction of all structures present. Only the sections containing the structures of interest need to be further analyzed.

Note: The use of Frosted 25 × 75 × 1 mm Beveled edge charged glass microscope slides is preferred (Cat #1354W Globe Scientific).

33. Allow slides to dry at 22°C for 2–5 h.
    a. Minimum drying time is 2 h, while maximum drying time is 15 h.
34. Store the dry slides at −80°C indefinitely.
    a. Short-term storage at −20°C is acceptable.
35. On Day 5, prepare for immunofluorescent staining of the CO cryosections. Allow slides to thaw at 22°C for 5–10 min.

⚠ CRITICAL: Perform all immunostaining steps in a humidified chamber to prevent the tissues from drying out.

36. Wash each slide with 1xD-PBS to remove excess **Embedding solution**.
37. Using a hydrophobic PAP pen, draw a circle around the tissues, ensuring that the PAP pen does not touch the sectioned COs.
38. Place slides in a humidified chamber, and permeabilize the COs for 5 min by adding 100 μL (or appropriate volume that sufficiently covers all COs) of **Permeabilization Buffer** within the boundaries of the PAP pen.
39. Aspirate the **Permeabilization Buffer** and wash once with 1× PBS.
40. Block the COs for 1 h at 22°C with **Blocking solution.**

41. Incubate COs with the appropriate primary antibodies (key resources table) diluted in **Antibody Diluent Solution** 15–20 h at 4°C.

Primary Antibodies	
Cell type marker	Primary antibody
Stem cells	SOX2
Neurons	NeuroD1
Neurons	TUJ-1
Neurons	MAP2

42. On Day 6, aspirate the primary antibodies and wash once with 1× D-PBS.
43. Incubate COs with appropriate fluorescently tagged secondary antibodies diluted at 1:200 in **Antibody Diluent Solution** for 1 h at 22°C.

⚠ CRITICAL: After adding the secondary antibody, protect the tissue from ultraviolet light.

44. Aspirate antibodies and wash 3 times with 1× D-PBS.

Note: Ensure that optimal antibody development has been achieved prior to counterstaining

45. Add Hoechst solution diluted 1:5,000 in 1× D-PBS for 5 min at 22°C.

Alternatives: DAPI mounting solution can be used as an alternative nuclear stain.

46. Aspirate Hoechst and wash three times with 1× D-PBS, taking care to aspirate all of the D-PBS between each wash.
47. Add a single drop of Immu-Mount™ to the center of each slide, and seal the COs by placing an appropriately sized coverslip on top of the mounting media using forceps.

Alternatives: optically clear mounting reagents can be used in combination with adhesive paint to seal the edges of the coverslips.

48. The mounted slides can be readily imaged using a fluorescent microscope (Figure 2) and stored short term (maximum 2 weeks) at 4°C or long-term (maximum 2 years) at −20°C.

⚠ CRITICAL: If ventricular zones are not observed at 2 weeks in vitro, CO generation has not been successfully induced.

Validation 2–Confirmation of CO regional identity

Timing: 1–2 days

The purpose of this step is to confirm the successful generation of a forebrain cerebral organoids by assessing the expression of forebrain, midbrain, and hindbrain markers.

49. Perform immunohistochemistry on fixed, embedded, and cryosectioned COs as described in Steps 35–48 using appropriate primary antibodies (key resources table).
    a. Forebrain identity antibodies: PAX6, OTX2
    b. Midbrain identity antibodies: OTX2, EN1
    c. Hindbrain identity antibodies: GBX2
50. COs should be immunopositive for PAX6, OTX2 and EN1, but negative for hindbrain identity marker expression (Figure 2).

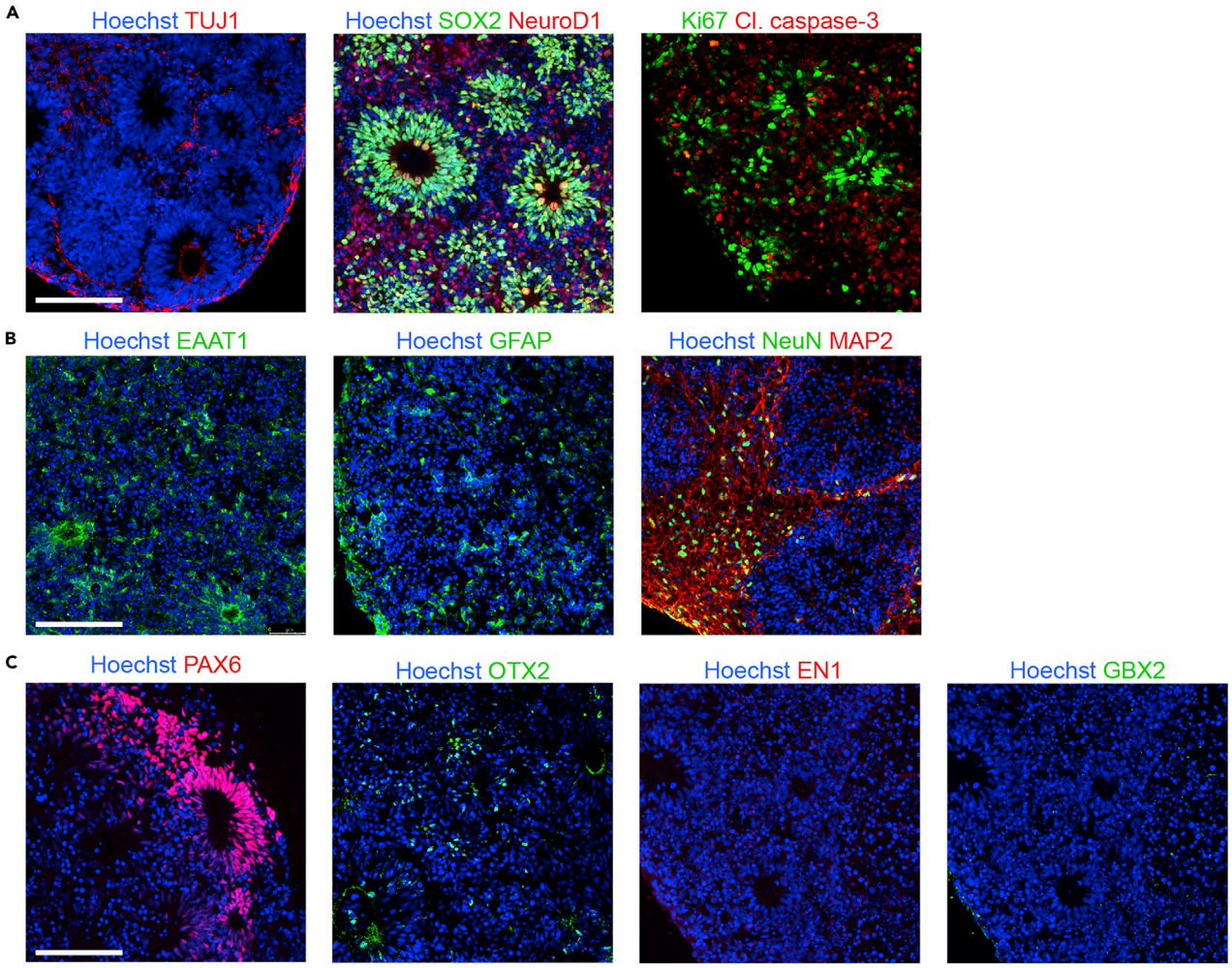

Figure 2. Immunofluorescent analysis of cerebral organoids

(A) 26 days *in vitro* (DIV) COs have ventricular and subventricular zones immunopositive for differentiated neurons (TUJ1$^+$ cells), neural stem cells (SOX2$^+$ cells; green), immature neurons (NeuroD1$^+$ cells; red), proliferative (Ki67$^+$ cells; green), and apoptotic (cleaved caspase 3$^+$ cells; red) cells. An elevated number of cleaved caspase-3+ cells may indicate increased neurosis in larger hCOs.

(B) 45DIV COs comprise EAAT1$^+$ and GFAP$^+$ astrocytes, as well as late immature neuron markers [NeuN (green) and MAP2 (red)].

(C) COs generated following this protocol are immunopositive for forebrain marker PAX6 and forebrain/midbrain marker OTX2, but not midbrain marker EN1 or hindbrain (GBX2) markers. All scale bars are 100 μm.

Analysis 1–Assessment of gene expression

Timing: 1–2 days

The purpose of this step is to confirm the expression profile of cell- and tissue-specific genes by quantitative real-time PCR for genotyping purposes, quantitative confirmation of cell composition, as well as discovery efforts.

51. Transfer a single CO in a 1.5 mL microcentrifuge tube, centrifuge at 300×*g* for 3 min at 22°C and aspirate the supernatant.

Note: Isolated COs can be snap frozen and stored at −80°C for future use or processed immediately for RNA extraction.

Note: Snap freezing of COs methods include placing microcentrifuge tubes in liquid nitrogen for a minimum of 5 s, on dry ice for a minimum of 30 s or in a −80°C freezer for a minimum of 5 min.

⚠ CRITICAL: Prior to starting the RNA isolation protocol, clean all surfaces with RNaseZAP™ and exclusively use RNase-free microcentrifuge tubes and RNase-free low barrier tips to minimize RNase contamination.

52. Isolate RNA from COs. The following RNA extraction was adapted from the NucleoSpin® RNA Plus RNA Isolation protocol. While the following describes the RNA isolation using the commercially available NucleoSpin® RNA Plus Kit, other commercially available RNA isolation kits and methodologies, such as TRIzol, can also be used.
    a. Homogenize the CO pellet in 350 μL of lysis buffer as per the manufacturer's instructions using a sterile needle (BD PrecisionGlide™ Needle, 26G × 5/8) to break apart the CO by trituration.

Alternatives: Homogenization can be alternatively achieved by CO grinding with a mortar and pestle or repeated trituration with a pipette, ensuring complete lysis of the CO tissues.

    b. Continue with the RNA isolation protocol as specified by the manufacturer.
    c. Quantify the RNA yield using a Thermo Scientific NanoDrop 2000 UV-Vis Spectrophotometer.

Alternatives: Any nucleic acid quantification method can be used as an alternate.

53. Synthesize cDNA from the total RNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Cat# 4374966) according to the manufacturer's instructions.
54. After the completion of cDNA production, add 50 μL of Nuclease-free water to each sample to dilute the cDNA.
    a. Stopping Point: Diluted cDNA can be stored at −20°C for at least 1 month.
55. Perform real-time quantitative PCR using 2 μL of the diluted cDNA, appropriate FAM-MGB-conjugated Taqman primers and Applied Biosystems TaqMan Fast Advanced Master Mix, no UNG (Thermo Fisher Scientific, Cat# A44359), according to the manufacturer's instructions. This step will determine the relative enrichment of specific cell types (e.g., neural progenitor cells, neurons, or astrocytes) or a particular gene(s) of interest.

Note: The relative expression of *NF1* is assayed with a human *NF1* TaqMan® FAM-MGB-conjugated primer, while human *SOX2, NeuroD1* and *ALDH1L1* TaqMan® FAM-MGB-conjugated primers are used to determine the relative enrichment of neural progenitor cells, neurons and astrocytes.

Note: For a detailed primer list, refer to the key resources table.

56. Relative expression of gene(s) is calculated using ΔΔCt method using human GAPDH as the reference gene.

Analysis 2–Assessment of cell proliferation, death and differentiation within Cos

Timing: 1–2 days

The purpose of this step is to define the cellular composition and attributes of COs by assaying for cell type-specific markers of neural progenitor cells (NPCs), neurons, and astrocytes, as well as assaying for cell proliferation, cell death, and maturation.

57. Perform immunohistochemistry on fixed, embedded, and cryosectioned COs as described in steps 35–48 using the appropriate primary antibodies (key resources table; Figure 2).

Cell type/biological process marker	Primary antibody
NPCs	SOX2
Early immature neurons	NeuroD1
	TUJ1
Late immature neurons	MAP2
Mature neurons	NeuN
Astrocytes	GFAP
	EAAT1
Proliferation	Ki-67
Apoptosis	Cleaved Caspase-3*

*Note: An increased number of cleaved caspase-3$^+$ cells may reflect increased neurosis in larger hCOs.

Analysis 3–Assessment of protein levels and activity

Timing: 1–3 days

The purpose of this step is to derive intact, functional proteins for ELISA-based studies (key resources table).

58. Collect COs (4–5 per 1.5 mL microcentrifuge tube), centrifuge at 300×*g* for 3 min at 22°C with maximum acceleration and deceleration, and aspirate the supernatant.
59. Snap-freeze tissues using liquid nitrogen or dry ice and store at −80°C.
    a. Stopping point: tissues may be stored at −80°C for future use.
60. Extract protein using either 150 μL 0.1 M HCl containing protease inhibitors or a specific manufacturer's recommended lysis buffer.
61. Determine the extracted protein concentrations using a protein determination assay (e.g., Pierce Bicinchoninic acid assay).

Note: it is necessary to pool multiple COs, preferably more than 3, in order to obtain sufficient protein for immunoassays.

62. Normalize protein concentrations to load equal amounts of protein across samples in each ELISA well, as per the manufacturer's instructions.
63. Perform ELISA assays according to the manufacturer's instructions.

EXPECTED OUTCOMES

This protocol describes the generation of forebrain cerebral organoids from human induced pluripotent stem cells. The successful generation of COs from certain iPSC lines requires induction of EBs from early passage number iPSCs (<15). After 15 h of plating, successful generation of EBs is characterized by the formation of a large sphere of cells with clear borders. Within the next few days, EBs steadily increase in size to around 500–600 μm, begin to brighten and exhibit smooth edges. Upon transferring EBs to CO growth conditions, COs will show a clear, radially organized, and translucent neuroectoderm. In other cases, the resulting CO is not always uniformly spherical, and may appear to have budding regions of optically clear ectoderm that is not radially organized. While not ideal, these organoids will similarly develop regions of neuroectoderm tissue. The resulting organoids will have regions of proliferating NPCs (SOX2$^+$ cells) in the ventricular zones within 16 days *in vitro*, and regions of early (NeuroD1$^+$ and TUJI$^+$ cells) and late stage (MAP2$^+$ cells) immature neurons migrating from the periventricular zone by 16 days *in vitro*. An advantage of this protocol is that

the successful completion of each of the early EB and CO induction steps is easily verified using a brightfield microscope. Later stages of CO development necessitate imaging of cryosections.

LIMITATIONS

The protocol described herein has been successful for generating COs from more than 15 independent iPSC lines derived from both male and female primary renal epithelial cells, peripheral blood mononuclear cells, and skin fibroblasts harboring large genomic deletions on chromosome 17q, *NF1* point mutations or no known germline mutations, as well as from iPSCs engineered with CRISPR/Cas9 to harbor different *NF1* point mutations on the same isogenic background (Anastasaki et al., 2020; Wegscheid et al., 2021). However, it is possible that, similarly to our previous report (Anastasaki et al., 2020), specific germline mutation(s) prevent the formation of EBs or COs. Moreover, although prolonged CO maintenance under the described conditions could induce oligodendrocyte development, the current protocol has not been optimized for oligodendrocyte differentiation.

TROUBLESHOOTING

Problem 1

Matrigel does not cover entire surface of well or flask (before you begin; step 4).

Potential solution

Drying out of the Matrigel can be avoided by ensuring proper humidity in the 37°C incubator. Other potential solutions include an increase in the volume of Matrigel used to cover the surface of the container, a decrease incubation time at 37°C and proper sealing of the container to avoid evaporation.

Problem 2

iPSCs remain attached to the flask after ReLeSR treatment (step 1).

Potential solution

It is possible that the 5 min incubation time is inadequate to detach iPSCs from the flask. Therefore, to ensure that the iPSCs properly detach from the bottom of the flask, increase the incubation time or incubate them at 37°C. Take care not to detach the differentiated cells.

Problem 3

Inadequate detection of dead cells (step 2).

Potential solution

Mix 10 μL cell mix with 10 μL Trypan blue, and resuspend cells thoroughly. Place 10 μL of cell mix in a hemocytometer or automated cell counter slide. Dead cells will appear Trypan blue positive or darker under light microscopy.

Problem 4

Sub-optimal EB formation (step 7). See examples in Figure 3.

Potential solution

EBs will form by 15 h. There are a number of reasons why EBs may not form at this step. The first possible explanation is that the iPSCs used to generate EBs were not healthy. It is important that the iPSCs are not overconfluent at this step, as it will negatively affect their ability to generate EBs. Prior to generating EBs, check the health of the iPSCs under the microscope. Healthy iPSCs should grow as healthy, flat colonies that have plenty of space between them, and should have minimal number of cells growing vertically (on top of each other). Another potential solution to ensure the successful generation of EBs is to minimize the time iPSCs are left at 22°C before EB seeding. Time left at 22°C in excess of 30 min will negatively impact their survival and ability to form EBs.

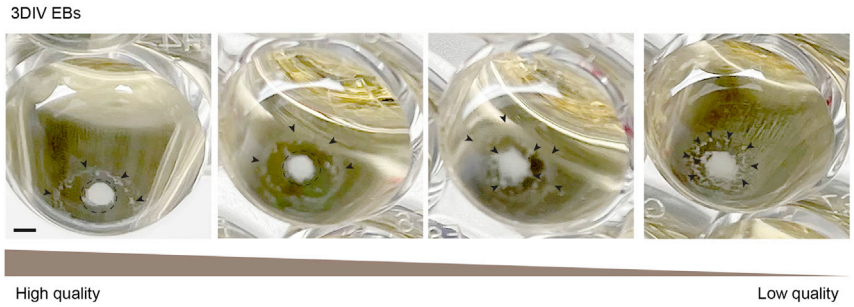

Figure 3. Illustration of high quality (left) and lower quality (right) EBs at 3 days *in vitro* (DIV) EBs

A halo of cellular debris is visible in most wells with EBs (left) and is normal. A smooth, spherical EB core without blebbing or increased cellular debris (black arrows) is required for EB survival and subsequent successful CO generation. Scale bar: 1 mm.

Additionally, observe EB growth daily. If the solid body of the EB is no longer visible and multiple smaller clusters of cells are apparent, the EB formation has halted, and new EBs will need to be initiated. If the EB appears flattened or dissociates into a monolayer of cells upon media removal, EB induction has not been successful, and new EBs need to be initiated.

Problem 5

EBs dissociate upon transfer into 24-well plates (step 8).

Potential solution

Cut a 200 μL pipette tip with sterile scissors to generate an opening approximately 1 mm in diameter. It is important that the opening of the pipette tip be at the optimal size to allow for EB transfer with minimal disruption. If the opening is too large, it will be difficult to pick up the EB. If the opening is too narrow, the EB will be damaged. See example in Figure 4.

Problem 6

The cerebral organoids do not appear smooth (step 12). See example in Figure 5.

Potential solution

A representative sample of the COs should be assayed histologically to ensure proper tissue development. If the organoids do not seem to be generating self-organized proliferative zones, new COs will need to be generated.

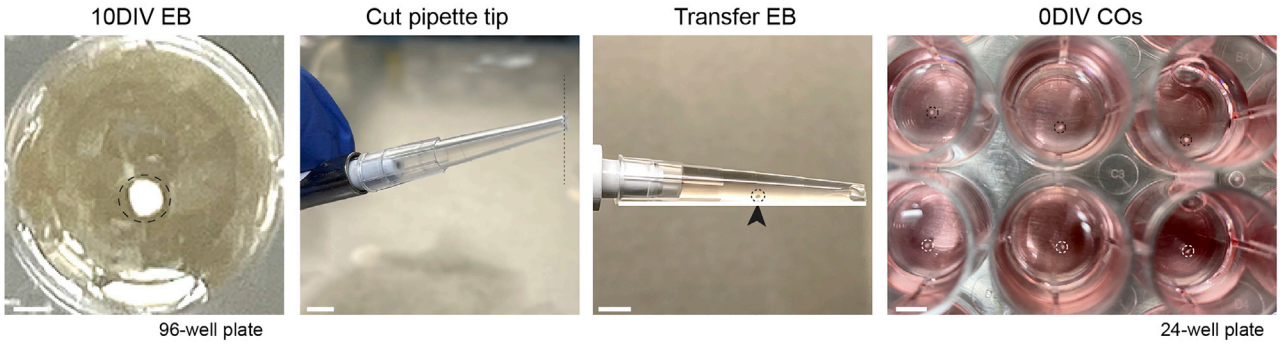

Figure 4. Representative images of EBs (dotted circles) being transferred from a well of a 96-well plate (left) to a well of a 24-well plate (right) to initiate CO differentiation

It is critical that EBs are not damaged during the transfer process. Cutting the end of the pipette tip should not disrupt the structural integrity of the EBs. Scale bars; left panel: 1 mm; all other panels: 5 mm.

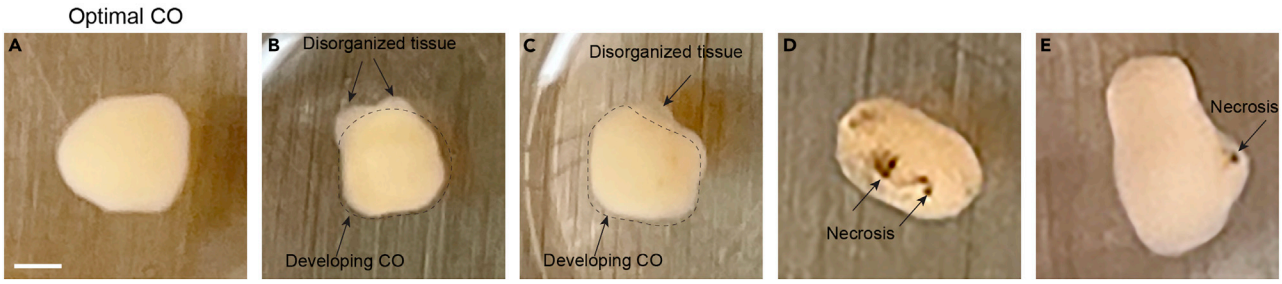

Figure 5. Representative images of 5 week *in vitro* COs

(A) Optimally developed COs will appear smooth, round in shape, and exhibit a white/off-white color (A).

(B–E) Changes in shape (B, C; blebbing) or color (D, E; black necrotic tissue) indicate defects in organoid generation. If blebbing or scalloping occurs at the outer perimeter of the CO, it is usually an indicator of premature cell death. Black spots indicate necrotic tissue. Scale bar: 1 mm.

Problem 7

COs do not form organized structures (step 14).

Potential solution

A lack of CO organization is often caused by inefficient CO induction, resulting either from premature transfer of EBs into **Organoid media** or because the reagents used to make the media were not fresh. To prevent this, ensure that each EB is at least 400 μm in diameter prior to CO induction and that the **Organoid Media** is supplemented with freshly prepared aliquots of supplements.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Corina Anastasaki, PhD; korinaanastasaki@wustl.edu

Materials availability

The study did not generate any unique reagents.

Data and code availability

This study did not generate any datasets or code.

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AUTHOR CONTRIBUTIONS

C.A., A.F.W., and A.C. wrote the manuscript. C.A., A.F.W., and M.L.S. generated the figures. C.A. and D.H.G. edited the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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