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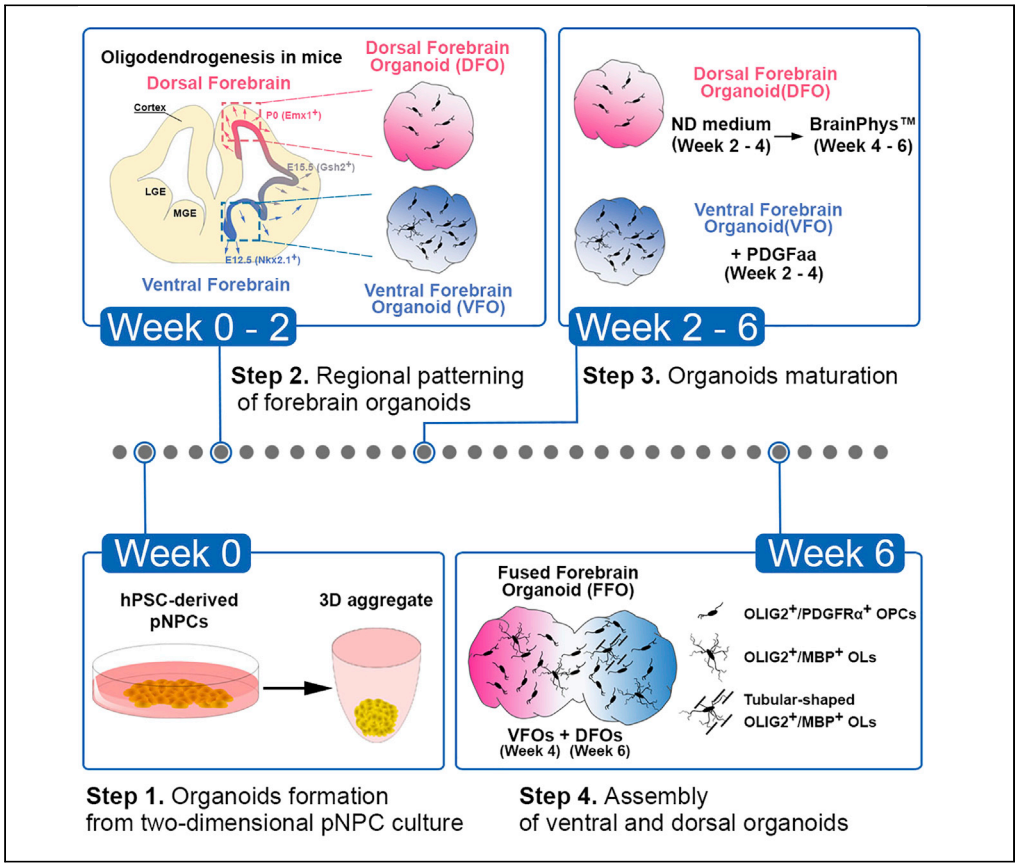
Generation of human pluripotent stem cell-derived fused organoids with oligodendroglia and myelin



Current knowledge on brain oligodendrogenesis, composed of three sequential waves from different regions, is mainly obtained from rodent studies. Oligodendroglial development and myelination in the rodent brain may not fully mirror those processes in the human brain. Here, we provide a step-by-step protocol for generating fused forebrain organoids derived from human pluripotent stem cells. These fused organoids recapitulate human oligodendroglial developments, offering innovative insights into human myelination research *in vitro*.

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Highlights

Regional determination of differentially generated forebrain organoids

Detailed methods to derive VFOs and DFOs by cost-effective, chemically defined media

OLIG2 expression exhibits distinct temporal patterns in VFOs versus DFOs

Assembling VFOs and DFOs to generate FFOs promotes oligodendroglial maturation

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Generation of human pluripotent stem cell-derived fused organoids with oligodendroglia and myelin

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SUMMARY

Current knowledge on brain oligodendrogenesis, composed of three sequential waves from different regions, is mainly obtained from rodent studies. Oligodendroglial development and myelination in the rodent brain may not fully mirror those processes in the human brain. Here, we provide a step-by-step protocol for generating fused forebrain organoids derived from human pluripotent stem cells. These fused organoids recapitulate human oligodendroglial developments, offering innovative insights into human myelination research *in vitro*.

For complete details on the use and execution of this protocol, please refer to Cameron-Curry and Le Douarin (1995), Kessaris et al. (2006), and Kim et al. (2019).

BEFORE YOU BEGIN

Prepare the needed materials before starting the cell culture and differentiation. Refer to key resources table for a complete list of materials.

Note: All procedures are performed in a Class II biological safety cabinet with standard aseptic techniques. Cells are cultured in a humidified 37°C incubator with 5% CO_2, and cerebral organoids are cultured on an orbital shaker with a speed of 85 rpm in the incubator.

Alternatives: Here, we describe the generation of myelination from forebrain organoids derived from human embryonic stem cells (hESCs), human induced pluripotent stem cells (hiPSCs), and OLIG2-GFP hPSC reporter lines (Liu et al., 2011; Xue et al., 2009).

Note: If reagents from alternative suppliers are used, you must validate the organoids for the first time.

Note: The oligodendrogenesis procedure in the fused organoids contains each step's validation process to ensure proper maintenance during the long-term culture. For qRT-PCR, we extract total RNA from organoids with RNAeasy kit (QIAGEN) to make complementary DNA with a Superscript III First-Strand kit (Invitrogen). The qRT-PCR is performed with TaqMan universal master mix and primers (Thermofisher) on an Abi 7500 Real-Time PCR system. TaqMan primers used in this study are listed in the key resources table. Experimental samples were analyzed by normalization with the expression level of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative quantification was performed by applying the 2-ΔΔCt method. For immunostaining, organoids fixed with 4%

paraformaldehyde were processed and cryo-sectioned for immunofluorescence staining. Primary antibodies are listed in the key resources table with dilution factors. Slides were mounted with the anti-fade Fluoromount-G medium containing 1,40,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Southern Biotechnology).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
B27 Supplement, minus vitamin A	Thermo Fisher	Cat#12587-010
BDNF	PeproTech	Cat#450-02
bFGF	PeproTech	Cat#100-18B
BrainPhys N2 Supplement	STEMCELL Technologies	Cat#07156
BrainPhys™ Neuronal Medium	STEMCELL Technologies	Cat#05790
CAMP	Sigma-Aldrich	Cat#D0260
CHIR99021	Stemgent	Cat#04-0004-10
Cyclopamine A (CycA)	Calbiochem	Cat#239803
DAPI Fluoromount-G	SouthernBiotech	Cat#0100-20
DMEM/F-12	HyClone	Cat#SH3002301
DPBS	Fisher Scientific	Cat#SH30028FS
GDNF	PeproTech	Cat#450-10
Human leukemia inhibitory factor (LIF)	PeproTech	Cat#300-05-25UG
L-Ascorbic acid	Sigma-Aldrich	Cat#A4403
Matrigel	Corning/VWR	Cat#354230/47743-720
N2 Supplement	Thermo Fisher	Cat#17502-048
Neurobasal Medium	Thermo Fisher	Cat#21103-049
P/S antibiotic	HyClone	Cat#SV30010
PDGF-AA	PeproTech	Cat#100-13A
Purmorphamine	Cayman Chem	Cat#10009634
SB431542	Stemgent	Cat#04-0010-05
SHH	HyClone	Cat#100-45
SM1 Neuronal Supplement	STEMCELL Technologies	Cat#05711
T3	Cayman Chem	Cat#16028
TrypLE	Thermo Fisher	Cat#12605028
Y-27632 Dihydrochloride	Tocris	Cat#1254
Oligonucleotides		
ARHGEF9	Thermo Fisher	Cat#Hs01003480_m1
DLX1	Thermo Fisher	Cat#Hs00269993_m1
EMX1	Thermo Fisher	Cat#Hs00417957_m1
GAD1	Thermo Fisher	Cat#Hs01065893_m1
GAPDH	Thermo Fisher	Cat#Hs02758991_g1
GPHN	Thermo Fisher	Cat#Hs00982840_m1
HOMER1	Thermo Fisher	Cat#Hs01029333_m1
LEF1	Thermo Fisher	Cat#Hs01547250_m1
LHX6	Thermo Fisher	Cat#Hs01030941_g1
MBP	Thermo Fisher	Cat#Hs00921945_m1
NKX-2-2	Thermo Fisher	Cat#Hs05035641_s1
OLIG2	Thermo Fisher	Cat#Hs00300164_s1
PDGFRα	Thermo Fisher	Cat#Hs00998018_m1
S100β	Thermo Fisher	Cat#Hs00389217_m1
SHANK3	Thermo Fisher	Cat#Hs01393541_m1
SLC17A6 (VGLUT2)	Thermo Fisher	Cat#Hs00220439_m1
SLC17A7 (VGLUT1)	Thermo Fisher	Cat#Hs00220404_m1
SLC6A1 (GAT1)	Thermo Fisher	Cat#Hs01104475_m1
TBR2	Thermo Fisher	Cat#Hs00232429_m1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
c-FOS (dilution 1:100)	Santa Cruz	Cat#SC-52; RRID:AB_2106783
CUX1 (dilution 1:500)	Santa Cruz	Cat#SC13024; RRID:AB_2630510
DCX (dilution 1:500)	Cell Signal	Cat#4604s; RRID:AB_561007
EMX1 (dilution 1:1000)	Sigma	Cat#HPA006421; RRID:AB_1078739
FOXG1 (dilution 1:500)<	Abcam	Cat#ab18259; RRID:AB_732415
GFAP (dilution 1:1000)	Millipore	Cat#AB5804; RRID:AB_2109645
GFP (dilution 1:1000)	Rockland	Cat#600-141-215; RRID:AB_1961516
GFP (dilution 1:500)	Thermo Fisher	Cat#MA5-15256; RRID:AB_10979281
GLS (dilution 1:250)	Abcam	Cat#ab156876; RRID:AB_2721038
Ki67 (dilution 1:400)	Cell Signal	Cat#9449; RRID:AB_2715512
Ki67 (dilution 1:200)	Thermo Fisher	Cat#MA5-14520; RRID:AB_10979488
LHX6 (dilution 1:100)	Abcam	Cat#ab22885; RRID:AB_447345
MAP2 (dilution 1:500)	Millipore	Cat#MAB3418; RRID:AB_94856
MBP (dilution 1:100)	Millipore	Cat#MAB386; RRID:AB_94975
Nestin (dilution 1:100)	Santa Cruz	Cat#SC-21249; RRID:AB_2267112
NeuN (dilution 1:100)	Millipore	Cat#MAB377; RRID:AB_2298772
NKX2.1(TTF1) (dilution 1:200)	Abcam	Cat#ab76013; RRID:AB_1310784
OLIG2 (dilution 1:1000; WB 1:2000)	PhosphoSolutions	Cat#1538; RRID:AB_2492193
PAX6 (dilution 1:400)	GeneTex	Cat#GTX11324; RRID:AB_381313
PDGFRα (dilution 1:50)	Santa Cruz	Cat#SC338; RRID:AB_631064
P-Histone H3 (dilution WB 1:1000)	Thermo Fisher	Cat#PA5–17869; RRID:AB_10984484
PSD95 (dilution 1:100)	Invitrogen	Cat#51-6900; RRID:AB_2533914
S100β (dilution 1:1000)	Sigma	Cat#S2532; AB_477499
SOX2 (dilution 1:100)	Millipore	Cat#AB5603; RRID:AB_2286686
Synapsin I (dilution 1:400)	Millipore	Cat#AB1543P; RRID:AB_90757
TBR1 (dilution 1:100)	EMD Millipore	Cat#AB2261; RRID:AB_10615497
TBR2 (dilution 1:100)	Abcam	Cat#AB23345; RRID:AB_778267
VGLUT1 (dilution 1:250)	Millipore	Cat#AB5905; RRID:AB_2301751
βIIItubulin (dilution 1:200)	Millipore	Cat#MAB1637; RRID:AB_2210524
β-tubulin (dilution WB 1:1000)	DSHB	Cat#E7; UniProt:P07437
Other		
ABI 7500 Real-Time PCR system	Thermo Fisher	Cat# 4406985
Ultra-low attachment 96-well round-bottom plates	Corning	Cat#CLS7007
Ultra-low attachment 6-well plate	Corning	Cat#CLS3471
Experimental models: cell lines		
Primitive neural progenitor cell	Kim et al., 2019	N/A
Critical commerical assays		
RNAeasy Kit	QIAGEN	Cat#74104
SuperScript III First-Strand kit	Thermo Fisher	Cat#180810400
TaqMan™ Universal PCR Master Mix	Thermo Fisher	Cat# 4305719

* For dilution of antibodies, western blotting is specifically marked as WB, and others are for immunostaining.

MATERIALS AND EQUIPMENT

Recipe for 50 mL of Primitive Neural Progenitor Cell (pNPC) medium (Week −1 ~ 0)

Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	1×	1/2×	25 mL
Neurobasal Medium	1×	1/2×	25 mL
N2 Supplement	100×	1×	500 μL

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Continued

Components	Stock conc.	Final conc.	50 mL
B27 Supplement, minus vitamin A	50×	1×	1.0 mL
bFGF	20 ug/mL	20 ng/mL	50 μL
P/S	100×	1×	500 μL
SB431542	10 mM	2 uM	10 μL
Human leukemia inhibitory factor (LIF)	10 ug/mL	10 ng/mL	50 μL
CHIR99021	3 mM	3 uM	50 μL

Store up to 2 weeks at 4°C.

Recipe for 50 mL of Neural Progenitor Cell (NPC) medium for spheroid formation

Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	1×	$^1/_2$×	25 mL
Neurobasal Media	1×	$^1/_2$×	25 mL
N2 Supplement	100×	1×	500 μL
B27 Supplement, minus vitamin A	50×	1×	1.0 mL
bFGF	20 ug/mL	20 ng/mL	50 μL
P/S Antibiotic	100×	1×	500 μL

Store up to 2 weeks at 4°C.

Recipe for 50 mL of Ventralization medium (NPC + SHH + Pumorphamine; Week 0 ~ 2)

Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	2×	1×	25 mL
Neurobasal Medium	2×	1×	25 mL
N2(1×) Supplement	100×	1×	500 μL
B27(1×) Supplement, minus vitamin A	50×	1×	1.0 mL
bFGF	20 μg/mL	20 ng/mL	50 μL
P/S	100×	1×	500 μL
SHH	25 μg/mL	50 ng/mL	100 μL
Purmorphamine	2000× (2 mM)	1× (1 μM)	25 μL
Y-27632 dihydrochloride	1000× (10 M)	1× (10 mM)	50 μL

Store up to a week at 4°C.

Recipe for 50 mL of Dorsalization medium (NPC + CycA; Week 0 ~ 2)

Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	2×	1×	25 mL
Neurobasal Medium	2×	1×	25 mL
N2 Supplement	100×	1×	500 μL
B27 Supplement, minus vitamin A	50×	1×	1.0 mL
bFGF	20 μg/mL	20 ng/mL	50 μL
Cyclopamine A (CycA)	1000× (5 mM)	1× (5 μM)	50 μL
P/S	100×	1×	500 μL
Y-27632 dihydrochloride	1000× (10 M)	1× (10 mM)	50 μL

Store up to a week at 4°C.

Recipe for 50 mL of Neuronal Differentiation (ND) medium (Week 2 ~ 4)

Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	1×	$^1/_2$×	25 mL
Neurobasal Medium	1×	$^1/_2$×	25 mL
N2 Supplement	100×	1×	0.5 mL

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Continued			
Components	Stock conc.	Final conc.	50 mL
B27 Supplement, minus vitamin A	50×	1×	1.0 mL
P/S	100×	1×	500 µL
GDNF	10 µg/mL	10 ng/mL	50 µL
BDNF	10 µg/mL	10 ng/mL	50 µL
cAMP	10 mM	1.0 µM	5.0 µL
L-Ascorbic Acid	200 µM	200 nM	50 µL
Store up to a week at 4°C.			

Recipe for 50 mL of Oligodendrocyte progenitor cell (OPC) medium (Week 2 ~ 4)			
Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	1×	1×	50 mL
PDGF-AA	10 ug/mL	10 ng/mL	50 µL
N2 Supplement	100×	1×	500 µL
B27 Supplement, minus vitamin A	50×	1×	1.0 mL
bFGF	20 ug/mL	10 ng/mL	25 µL
P/S	100×	1×	500 µL
Store up to 2 weeks at 4°C.			

Recipe for 50 mL of BrainPhys Neuronal Medium (Week 4 ~ 6)			
Components	Stock conc.	Final conc.	50 mL
BrainPhysNeuronal Medium	1×	1×	50 mL
N2 Supplement	100×	1×	500 µL
SM1 Neuronal Supplement	50×	1×	1.0 mL
P/S	100×	1×	500 µL
Store up to 2 weeks at 4°C.			

Recipe for 50 mL of Oligodendrocyte (OL) medium (Week 6 ~)			
Components	Stock conc.	Final conc.	50 mL
DMEM/F-12	2×	1×	25 mL
Neurobasal Medium	2×	1×	25 mL
N2 Supplement	100×	1×	0.5 mL
B27 Supplement, minus vitamin A	50×	1×	1.0 mL
P/S	100×	1×	500 µL
GDNF	10 ug/mL	10 ng/mL	50 µL
BDNF	10 ug/mL	10 ng/mL	50 µL
L-Ascorbic acid	200 uM	200 nM	50 µL
cAMP	10 mM	1.0 uM	5.0 µL
T3	60 µg/mL	10 ng/mL	8.3 µL
Store up to a week at 4°C.			

STEP-BY-STEP METHOD DETAILS

This protocol describes all steps of organoid culture from purified primitive neural progenitor cells (pNPCs) 2D culture to fused forebrain organoids (Figure 1). Our protocol relies on the fusion of dorsal and ventral forebrain organoids, which provides opportunities to examine the differentiation, migration, and maturation of oligodendrocytes in the organoids. To avoid non-CNS tissue and reduce variability among individual organoid generation, we used pNPCs as the starting cell population to generate forebrain organoids. pNPCs were derived from either hPSCs or hiPSCs between passages (P) 20 and 45 using a small molecule-based protocol (Chen et al., 2016; Li et al., 2011). The

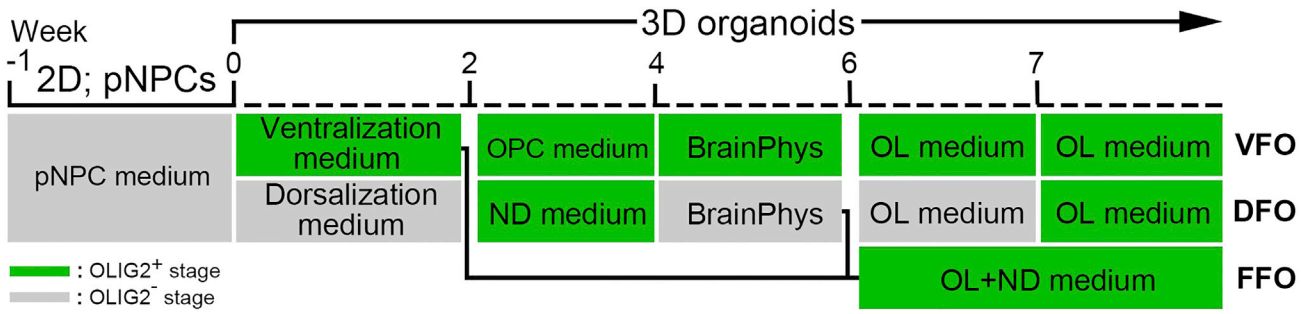

Figure 1. Timeline for the fused organoids formation

A schematic procedure for deriving region-specific forebrain organoids from pNPCs by stage-specific medium changes. The stages after week 0 are color-coded based on the expression of GFP:OLIG2.

harvested pNPCs were stored in liquid nitrogen for long-term storage, and cells were used no more than P5.

Thawing pNPCs (week –1)

Timing: ~ 30 min

This step provides a detailed procedure for thawing pNPCs.

Prepare the pNPC medium before starting this step

1. Warm pNPC medium in 37°C water bath (~ 20 min) beforehand.
2. Move Matrigel-coated 6-well plates from the incubator and place them in the hood.
3. Remove cryovial of pNPCs from liquid nitrogen and thaw 1–2 min in 37°C water bath.
4. Transfer contents of the cryovial to 15 mL tube, and then add 5 mL of pNPC medium dropwise to cells.
5. Spin at 500G for 4 min, aspirate supernatant, and resuspend the cell pellet in 2 mL pNPCs medium.
6. Aspirate Matrigel from one well of a 6-well plate and add the 2 mL pNPC suspension to the well.
7. Culture these pNPCs with every two days of media change until you have sufficient numbers to have the desired cell density of $3*10^6$ pNPCs per well. Cell number of the full confluence of pNPCs in one 6-well plate will be around $1*10^7$.

Note: At week 0, we recommend immunostaining with an antibody specific of PAX6 (GeneTex, 1:400) to verify the human neuroectodermal cell fate (see key resources table for the antibody).

Organoid formation from two-dimensional pNPC culture (week 0)

Timing: 2 days

This step provides a detailed procedure for producing uniform organoids from the two-dimensional culture of pNPCs (Figure 2A).

Prepare the NPC medium before starting this step

8. Aspirate pNPC medium and wash the well with 2 mL of PBS, then aspirate the PBS.
9. Pipet 2 mL of TrypLE into each well of pNPC.

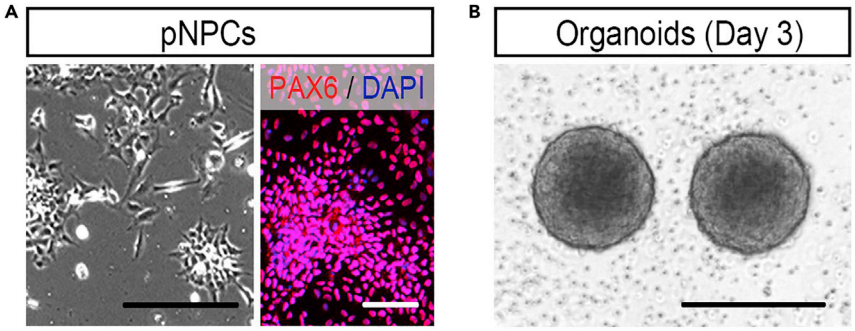

Figure 2. Organoids formation from pNPCs

(A) Representative bright-field and fluorescence images of pNPCs, which were positive for PAX6 staining. Scale bars, 100 μm.

(B) Organoids formed from pNPCs at day 3; scale bar, 200 μm.

10. Place the plate in the incubator for 4 min.
11. Tap the plate and see if the pNPC colonies have completely detached from the wells.
12. Neutralize TrypLE with 2 mL of DMEM/F12 medium. Move the contents into 15 mL conical tubes.
13. Centrifuge the cells for 4 min at 500 *g*.
14. Aspirate supernatant from the 15 mL conical tube.
15. After cell number calculation, place the cells into the low-attachment 96-well round-bottom plate at a density of 9,000 cells to develop uniform organoids with 250 μl of pNPC medium.
16. Centrifuge the plate for 4 min at 500 *g*.
17. Return cells to the incubator and do not disturb the plate for 48 h.
18. Two days later, transfer six aggregates per well of a 6-well plate with widened pipette tips to minimize mechanical stress and damage.
19. Place the plate on the orbital shaker in the incubator, set, and keep at 85 rpm.

Regional patterning of forebrain organoids (week 0–2)

Timing: 2 weeks

This step provides a detailed procedure for patterning and developing the spheroids to generate ventral or dorsal forebrain organoids (Figure 3). After spheroids' passaging into the low attachment well, start the long-term culturing on an orbital shaker.

For the two weeks of patterning culture, the medium will be changed completely every day. In this process, the medium will be the NPC-based medium supplemented with either 5 μM Cyclopamine A (CycA) for dorsalization or sonic hedgehog (SHH; 50 ng/mL) and purmorphamine (Pur; 1 μM) for ventralization.

Prepare the ventralization and dorsalization media before starting this step

Working volume: 2.0 mL per well of 6-well plate

Do not culture more than five organoids in each well of a 6-well plate.

Culture on an orbital shaker at 85 RPM.

Medium change: every day

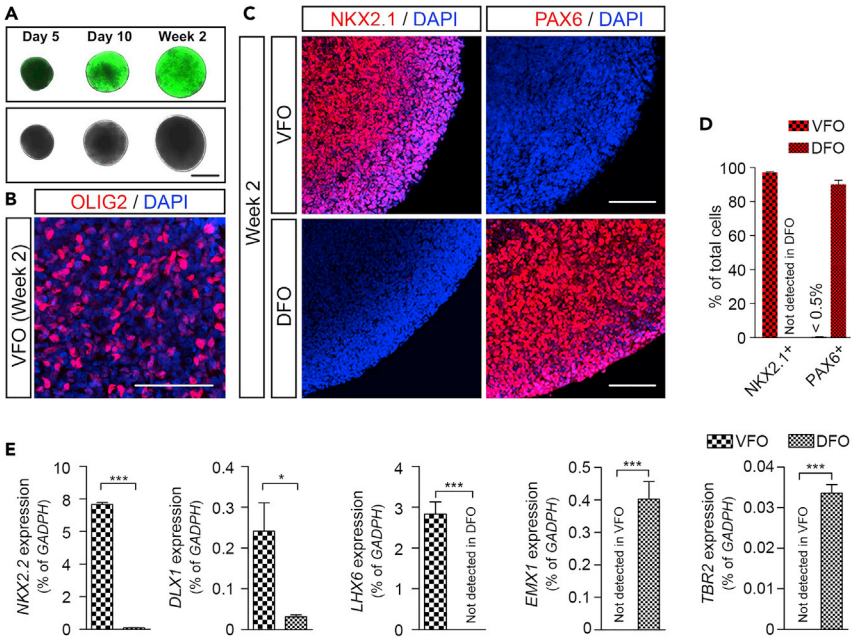

Figure 3. Expression of temporal and regional markers in patterned forebrain organoids

(A) Bright-field and fluorescence images showing the formation of VFO (top) and DFO (bottom) from OLIG2-GFP hPSC reporter lines during the period of regional patterning. Scale bars, 500 μm.

(B) Representative image of OLIG2^{+} cells in the VFOs at week 2; scale bar, 100 μm.

(C) Representative images of NKX2.1- and PAX6-expressing cells in week 2 VFOs and DFOs; scale bar, 100 μm.

(D) Quantification of NKX2.1-, and PAX6-expressing cells in week 2 VFOs or DFOs (n = 4 organoids from two hPSC lines).

(E) qRT-PCR results showing the expression of *NKX2.2*, *DLX1*, *LHX6*, *EMX1*, and *TBR2* in week 2 VFOs and DFOs (n = 3 independent experiments). Student's t test: *p < 0.05 and ***p < 0.001.

20. Gently swirl plates so that spheroids will collect into the center of the well.
21. Set aside a sterile 1.5 mL centrifuge tube for each well. Collect spheroids with a P1000 pipette. Cut the tip using a sterile scissor to create a wider opening to minimize mechanical stress.
22. Aspirate the medium remaining in the wells and then add 2.0 mL of each patterning medium to the wells. Note that excessive medium can cause unintended organoid fusion during agitated culture.
23. Wait till the spheroids sink in the bottom of the Eppendorf tube; remove the supernatant medium with a P1000 pipette without affecting the spheroids.
24. Gently pipet 500 μL fresh medium supplemented with either CycA for dorsalization or SHH/Pur for ventralization from the well and gently add it to the Eppendorf tube to suspend spheroids.
25. While free-floating, collect and transfer the spheroids back into their respective well. Repeat this if organoids remain in the Eppendorf tube.
26. Place the plate on the orbital shaker in the incubator, set, and keep at 85 rpm.

Note: At week 2, we recommend immunostaining with an antibody specific for NKX2.1 (Abcam, 1:200) to verify the ventral forebrain regional identity. Alternatively, qPCR with primers for *NKX2.2*, *DLX1*, and *LHX6* can confirm the ventral identity. Whereas dorsal forebrain regional identity can be confirmed by an antibody specific for PAX6 (GeneTex, 1:400) and primers for *EMX1* and *TBR2* at week 2 (see key resources table for antibodies and primers)

Oligodendroglial and neuronal differentiation in organoids (week 2–4)

Timing: 2 weeks

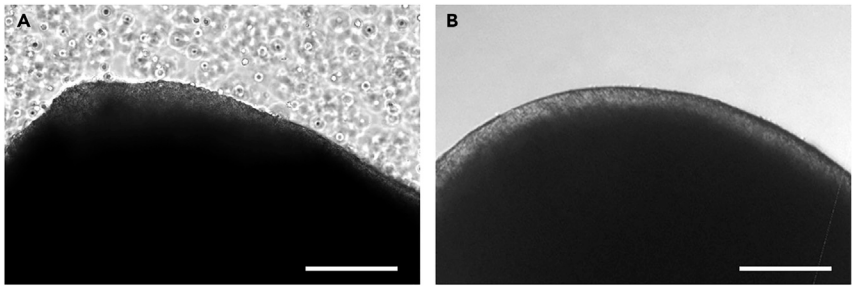

Figure 4. Representative example showing bright-field images of organoids
(A) Representative of an unhealthy organoid with debris in the medium. Scale bar, 100 μm.
(B) Representative of a healthy organoid with clear surface. Scale bar, 100 μm.

This step provides a detailed procedure for oligodendrogenesis and neuronal differentiation in the patterned forebrain organoids. After two weeks of patterning, the organoid's size reaches between 0.5 – 0.7 mm. *For the first week, the 2 mL of medium per well of the 6-well plate will be half-changed every two days.*

Note: Prepare the OPC and ND media before starting this step.

Working volume: 2.0 mL per well of the 6-well plate

Do not culture more than five organoids in one well of a 6-well plate.

Culture on an orbital shaker at 85 RPM.

Medium change: every other day.

27. Gradually transfer the organoids to either the ND medium or OPC medium. Please refer to the section of regional patterning for forebrain organoids for detailed medium change. The 2 mL of medium will be half-changed for a week and entirely changed for the second week every other day.
    Ventral organoids → OPC media
    Dorsal organoids → ND media

⚠ CRITICAL: We strongly recommend checking dorsal organoids frequently in the process of neuronal differentiation. Healthy organoids exhibit bright and clear surfaces without debris under the microscope. Unhealthy organoids, which can be identified by either the increasing debris over time in medium or organoids gradually turning darker, may compromise the rest of the procedure, including oligodendrogenesis (Figure 4).

Note: At week 4, oligodendroglial lineage cells can be identified by nuclear localization of OLIG2 using an antibody specific for OLIG2 (Phosphosolutions, 1:1000). Immunostaining with βIIIT (Millipore, 1:200) and S100β (Sigma, 1:1000) antibodies can be utilized for identifying neuronal or astroglial lineage, respectively (see key resources table for antibodies).

Neuronal maturation in organoids (week 4–6)

Timing: 2 weeks

For fusion, this step of neuronal maturation applies only to week 6 DFO but not to the VFO at week 2, the time point that VFOs are used for fusion. In the fused organoids, neuronal maturation and activity

in DFO achieved by this step can influence oligodendrogenesis and myelination. For unfused organoids, MBP signals, a marker for mature oligodendrocytes, can be detected in VFOs that had undergone this full neuronal maturation process.

Prepare the BrainPhys neuronal medium before starting this step.

Working volume: 2.0 mL per well of the 6-well plate

Do not culture more than five organoids in one well of a 6-well plate.

Culture on an orbital shaker at 85 RPM.

Medium change: every four days.

28. Gradually transfer the organoids to BrainPhys neuronal medium. The 2 mL of medium will be half-changed for a week and fully changed for the second week every four days.

Note: At week 6, neuronal activity and maturation in organoids can be measured by immunostaining with c-Fos antibody (Santa Cruz, 1:100) and synaptic markers such as Synapsin 1 (Millipore, 1:400) and PSD-95 (Invitrogen, 1:100) (see key resources table for antibodies).

Note: A dorsal organoid that had undergone full neuronal maturation with BrainPhys is used with a patterned ventral organoid for the next fusion step. Assembly of VFOs at later stages of differentiation will take longer (2–3 days) along with increased cell death.

⚠ CRITICAL: Minimize the impact of medium composition transition by a half-changing medium. Avoid cell death/breaking of the organoids.

Assembly of ventral and dorsal organoids (week 4 and 6 for VFO and DFO, respectively)

Timing: ~ 2 days

This step provides a detailed procedure for fusing forebrain organoids using a spontaneous fusion method (Figure 5).

Use P200 pipette with a widened tip using sterile scissors to create a wider opening.

Mix the ND and OPC medium at a 1:1 ratio for this fusion step.

29. Transfer two organoids into a well of the low-attachment 96-well round-bottom plate.
30. Place the plate in a humidified 37°C with 5% CO^2 incubator.
31. Leave them in static condition for 2 h without agitation.
32. After 2 h, circulate medium two times by gentle pipetting up and down every h for 8 h without touching organoids.
33. 8 h later, transfer the fused organoids into a low attachment 6-well plate.
34. Place the fused organoids in a 6-well plate on an orbital shaker with a speed of 85 rpm.

⚠ CRITICAL: Without circulation by gentle pipetting, cell death can occur, especially from ventral organoids.

Optional: If the organoids have not been firmly attached, the organoids can be maintained up to 18 h in the low attachment 96-well plate.

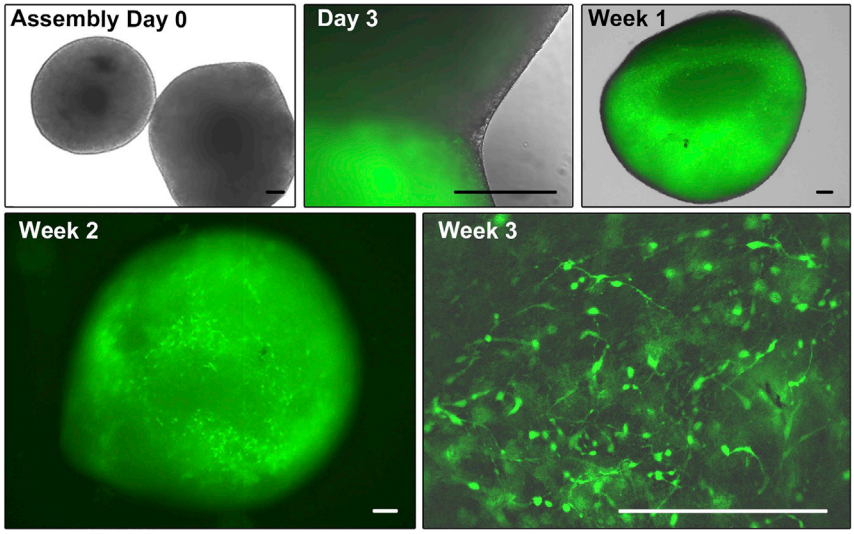

Figure 5. Representative images showing the organoids during the fusion process
The images correspond to the indicated fusion procedural step up to 3 weeks, in which organoids undergo dramatic changes in shape; scale bar, 100 μm.

Myelination and maturation

Timing: ~2 months

Two days after fusion, the organoids will be maintained in OL/ND media for long-term culture.

35. Switch media from ND/OPC media to OL/ND media by gradually changing half of the medium every other day for the first week. Maintain these organoids with media change every four days afterward.

Note: During this period, fused organoids will become round-shaped with a diameter of 1.2–2 mm. They remain steady in size at a week after fusion.

Note: At week 9, PDGFRα$^+$ and/or MBP$^+$ oligodendroglial lineages can be confirmed using immunostaining with specific antibodies for PDGFRα (Santa Cruz, 1:50) and MBP (Millipore, 1:100; Figure 6). Additionally, enhanced neuronal network during the long-term culture can be measured by the expression of genes for both inhibitory and excitatory postsynaptic machinery, such as *HOMER1* and *SHANK3* that respectively encode excitatory postsynaptic components and *ARHGEF9* and *GPHN* that respectively encode inhibitory postsynaptic components. Please refer to (see key resources table for primers). For complete details on the use of primers and outcomes, please refer to (Kim et al., 2019).

EXPECTED OUTCOMES

Current knowledge on oligodendrogenesis in the brain is mainly obtained from studies in rodents (Kessaris et al., 2006; Klämbt, 2009; Winkler et al., 2018). Although human oligodendroglial cells have been efficiently derived from hPSCs and characterized in both two-dimensional and three-dimensional systems(Goldman and Kuypers, 2015; Madhavan et al., 2018; Marton et al., 2019; Pamies et al., 2017), developmental origins of these human oligodendroglia and maturation of the human oligodendroglia with different origins are not well understood. Fused forebrain

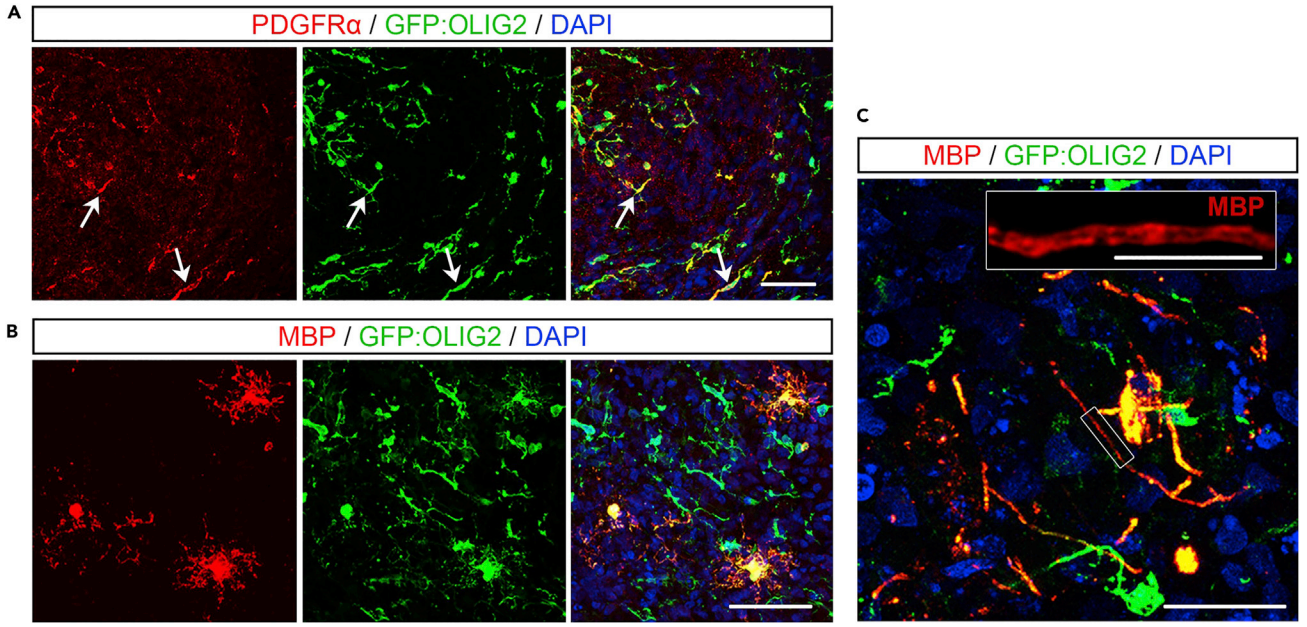

Figure 6. Oligodendroglial maturation in fused organoids after long-term culture

(A) Representatives of PDGFRα$^+$ oligodendroglial cells. Scale bar, 100 μm.

(B) Representatives of MBP$^+$ OLs. Scale bar, 100 μm.

(C) Representatives of tubular-shaped MBP$^+$ OLs. Scale bar Scale bars, 20 μm in the original and images and 10 μm in the enlarged images.

organoids formed by ventral- and dorsal forebrain organoids have a significant advantage by recapitulating human oligodendrogenesis in different regions of the brain.

This protocol should result in fused forebrain organoids with MBP$^+$ cells and myelination. The organoids usually display a diameter of approximately 1.2 to 2.0 mm after fusion and are visible with the naked eye. Afterward, the organoid size does not increase. Due to their size, the apoptotic core is also observed.

Brain regional specification can be easily monitored using OLIG2-GFP cell lines during the patterning process under the epifluorescence microscope. We routinely obtain OLIG2$^+$ cells from ventral organoids a week after the treatment of SHH and Pur. In line with this, the regional specification can also be assessed by qRT-PCR with region-specific primers listed in the key resources table, such as markers for ventral forebrain, *NKX2.2*, *DLX1*, and *LHX6*, and for dorsal forebrain, *EMX1*, and *TBR2* (Figure 3E).

At the end of this protocol, the organoids are ready for immunostaining of oligodendroglial lineage markers, such as MBP and PDGFRα listed in the key resources table. In our experience, it takes two months from the step of aggregates formation for ventral organoids and three weeks for fused organoids to develop MBP$^+$ mature oligodendrocytes (Figures 6A and 6B). Except for the necrotic inner core of organoids, MBP signals are evenly distributed without a noticeable pattern. The fused organoids can be maintained in OL medium up to 6 weeks after fusion.

LIMITATIONS

Using two OLIG2-GFP knockin hPSCs lines (hESCs and HiPSCs) and ND2.0 hiPSCs, this protocol is robustly reproducible and delivers similar-sized fused organoids with a dense population of OLIG2$^+$ cells, which will, later on, give rise to PDGFRα$^+$ or MBP$^+$ cells. Successful generation of cerebral organoids with mature oligodendrocytes is dependent upon the maintenance of healthy

organoids, especially during the fusion process. We occasionally observed tubular-shaped MBP signals and myelinated axons in fused organoids two months after long-term culture (Figure 6C). However, most of the MBP^+ OL do not form very compact myelin sheaths in organoids.

TROUBLESHOOTING

Problem 1

Forebrain regional patterning efficiency is low (step 26). At week two, after patterning, using markers for ventral forebrain, *NKX2.2*, *DLX1,* and *LHX6*, the efficiency of ventral patterning can be evaluated, whereas markers for dorsal forebrain, *EMX1,* and *TBR2* are predominantly restricted to dorsal forebrain organoids. After the proper patterning process, *NKX2.2* and *LHX6* should be undetectable in dorsal forebrain organoids by qRT-PCR. In contrast, *EMX1* and *TBR2* are predominantly expressed by dorsal forebrain organoids (Figure 3E).

Potential solutions

The regional patterning efficiency can be impacted by the quality of starting cells. The quality of starting cells should be carefully controlled before differentiation. This protocol uses pNPCs, derived from either hESCs or hiPSCs, within P5. The low efficiency of patterning also could arise from the inappropriate condition of morphogens. Therefore, it is recommended to use fresh morphogens every time.

Problem 2

There are black crystallizations in ventral organoids during forebrain patterning (step 24).

Potential solutions

This could arise from a high concentration of purmorphamine. Make sure that the final concentration of purmorphamine is 1 μM.

Problem 3

While maintaining organoids on an orbital shaker with 85 rpm, there is an unintended organoids fusion (steps 19, 22, and 27).

Potential solutions

Because the shaker speed is optimized for 2 mL in a well of 6-well plate, the different medium volumes can cause the accumulation of organoids in the center. Ensure that there is no accumulation of organoids on the shaker after changing the medium.

Problem 4

Cerebral organoids are disintegrated upon transfer/manipulation (step 33).

Potential solutions

Ensure the medium is prepared freshly with the correct concentration. Cut tips using a sterile scissor to create a wider opening to minimize mechanical stress.

Problem 5

During neuronal differentiation for DFOs, organoids are getting darker and generate cell debris in the medium (step 27).

Potential solutions

Dramatic changes in medium composition from dorsalization to ND medium can cause cell death in DFOs. Gradually transfer the organoids to the ND medium half-changed for a week and entirely changed for the second week every other day. Moreover, do not use bigger organoids formed by unintended organoid fusion during agitated culture to minimize organoids' necrotic core.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, peng.jiang@rutgers.edu.

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

H.K. and P.J. conceived the project and wrote the protocol. H.K. designed, performed, and analyzed the experimental protocol. P.J. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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