Protocol

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. Oligodendrogial 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 oligodendrogial 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 oligodendrogial maturation
Protocol
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% CO2, 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 1,4,6-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 |
| BrainPhysTM Neuronal Medium | STEMCELL Technologies | Cat#05790 |
| CAMP | Sigma-Aldrich | Cat#D0260 |
| CHIR99021 | Stemgent | Cat#04-0004-10 |
| Cycloamine 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**

| Oligonucleotide | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| 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 |
| NXX-2-2 | Thermo Fisher | Cat#Hs00535641_s1 |
| OLG2 | Thermo Fisher | Cat#Hs00300164_s1 |
| PDGFRa | Thermo Fisher | Cat#Hs0098018_m1 |
| S100i | Thermo Fisher | Cat#Hs00389217_m1 |
| SHANK2 | 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

| Antibody       | Source                  | Cat/ID#        | RRID          |
|----------------|-------------------------|----------------|---------------|
| 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 1:500)   | Rockland                | Cat#600-141-215 | RRID:AB_1961516 |
| 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#MAS-15256 | RRID:AB_10979281 |
| LHX6 (dilution 1:100)  | Abcam                   | Cat#ab22885   | RRID:AB_447345 |
| MAP2 (dilution 1:500)  | Millipore               | Cat#MA83418   | RRID:AB_94856 |
| MBP (dilution 1:100)   | Millipore               | Cat#MA8386    | RRID:AB_94975 |
| Nestin (dilution 1:100) | Santa Cruz             | Cat#SC-21249  | RRID:AB_2267112 |
| NeuN (dilution 1:100)  | Millipore               | Cat#MA8377    | 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#PAS-17869 | RRID:AB_10984484 |
| PSD95 (dilution 1:100) | Invitrogen              | Cat#S1-6900   | RRID:AB_2533914 |
| S100β (dilution 1:1000) | Sigma                   | Cat#S2532     | AB_477499 |
| SOX2 (dilution 1:100)  | Millipore               | Cat#AB5603    | RRID:AB_2286868 |
| 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 |
| βIII-tubulin (dilution WB 1:1000) | Millipore          | Cat#MAB1637   | RRID:AB_2210524 |
| b-tubulin (dilution WB 1:1000) | DSHB                | Cat#E7; UniProt:PO7437 |

#### Other

| REAGENT or RESOURCE | SOURCE | Cat/ID# | RRID |
|---------------------|--------|---------|------|
| 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

| REAGENT or RESOURCE | SOURCE | Cat/ID# | RRID |
|---------------------|--------|---------|------|
| Primitive neural progenitor cell | Kim et al., 2019 | N/A | |

#### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | Cat/ID# | RRID |
|---------------------|--------|---------|------|
| 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)

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

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

Store up to a week 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                           | 2x          | 1x          | 25 mL |
| Neurobasal Medium                   | 2x          | 1x          | 25 mL |
| N2(1x) Supplement                   | 100x        | 1x          | 500 μL|
| B27(1x) Supplement, minus vitamin A | 50x         | 1x          | 1.0 mL|
| bFGF                                | 20 μg/mL    | 20 ng/mL    | 50 μL |
| SHH                                 | 25 μg/mL    | 1x          | 500 μL|
| Pumorphamine                        | 2000x (5 mM)| 1x (5 μM)  | 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                           | 1x          | 1/2x        | 25 mL |
| Neurobasal Medium                   | 1x          | 1/2x        | 25 mL |
| N2 Supplement                       | 100x        | 1x          | 500 μL|

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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

### Recipe for 50 mL of Oligodendrocyte progenitor cell (OPC) medium (Week 2 ~ 4)

| Components                       | Stock conc. | Final conc. | 50 mL |
|----------------------------------|-------------|-------------|-------|
| DMEM/F-12                        | 1x          | 1x          | 50 mL |
| PDGF-AA                          | 10 μg/mL    | 10 ng/mL    | 50 μL |
| N2 Supplement                    | 100x        | 1x          | 500 μL|
| B27 Supplement, minus vitamin A  | 50x         | 1x          | 1.0 mL|
| bFGF                             | 20 μg/mL    | 10 ng/mL    | 25 μL |
| P/S                              | 100x        | 1x          | 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        | 1x          | 1x          | 50 mL |
| N2 Supplement                   | 100x        | 1x          | 500 μL|
| SM1 Neuronal Supplement         | 50x         | 1x          | 1.0 mL|
| P/S                             | 100x        | 1x          | 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                        | 2x          | 1x          | 25 mL |
| Neurobasal Medium                | 2x          | 1x          | 25 mL |
| N2 Supplement                    | 100x        | 1x          | 0.5 mL|
| B27 Supplement, minus vitamin A  | 50x         | 1x          | 1.0 mL|
| P/S                              | 100x        | 1x          | 500 μL|
| GDNF                             | 10 μg/mL    | 10 ng/mL    | 50 μL |
| BDNF                             | 10 μg/mL    | 10 ng/mL    | 50 μL |
| L-Ascorbic acid                  | 200 μM      | 200 nM      | 50 μL |
| cAMP                             | 10 mM       | 1.0 μM      | 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
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 pNPC 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.
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 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 Cyclopaamine 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
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 add 2.0 mL fresh 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
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

Δ 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)**

Ω 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% CO2 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.
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+ oligodendrogial 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 oligodendroglias and maturation of the human oligodendroglias with different origins are not well understood. Fused forebrain...
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

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**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, 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.
RESOURCES 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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