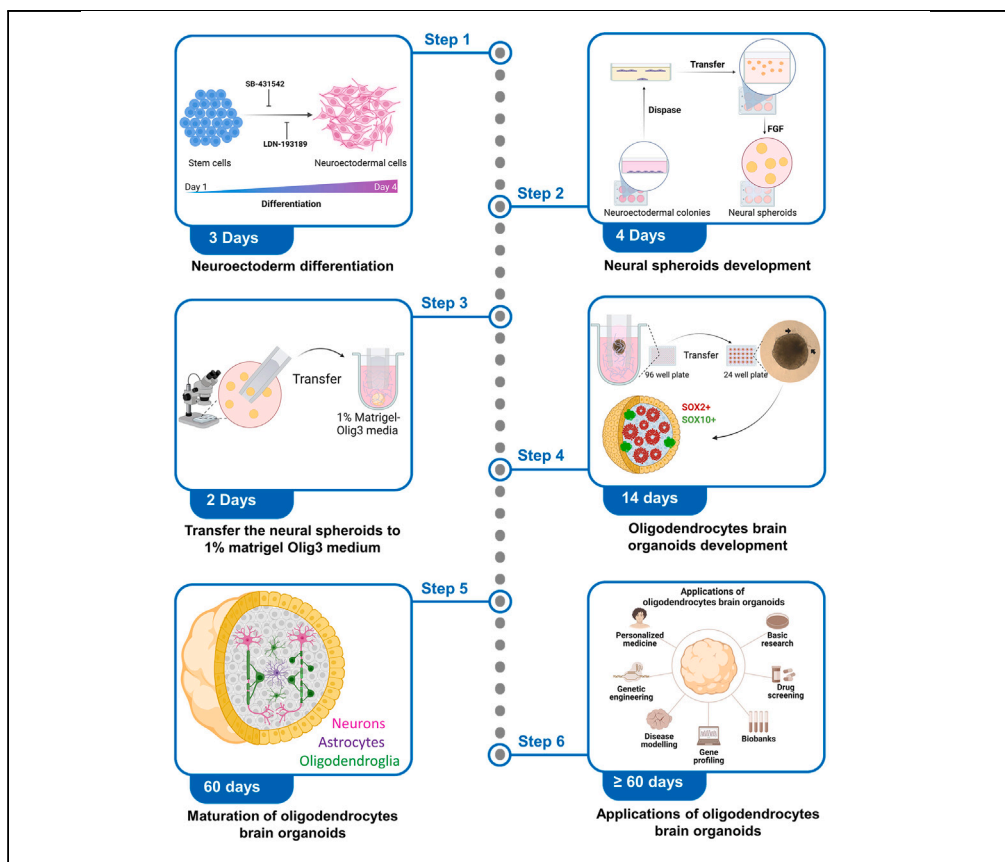


Protocol

Protocol for generating embedding-free brain organoids enriched with oligodendrocytes



In response to the scarcity of advanced *in vitro* models dedicated to human CNS white matter research, we present a protocol to generate neuroectoderm-derived embedding-free human brain organoids enriched with oligodendrocytes. We describe steps for neuroectoderm differentiation, development of neural spheroids, and their transferal to Matrigel. We then detail procedures for the development, maturation, and application of oligodendrocyte-enriched brain organoids. The presence of myelin-producing cells makes these organoids useful for studying human white matter diseases, such as leukodystrophy.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Step-by-step protocol to generate human oligodendrocyte-enriched brain organoids

Detailed steps to develop brain organoids free from embedding with Matrigel

Rapid induction of oligodendrocyte precursors over 9 days of brain organoids differentiation

Oligodendrocyte brain organoids are enriched with cortical neurons and astrocytes

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Protocol

Protocol for generating embedding-free brain organoids enriched with oligodendrocytes

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SUMMARY

In response to the scarcity of advanced *in vitro* models dedicated to human CNS white matter research, we present a protocol to generate neuroectoderm-derived embedding-free human brain organoids enriched with oligodendrocytes. We describe steps for neuroectoderm differentiation, development of neural spheroids, and their transferal to Matrigel. We then detail procedures for the development, maturation, and application of oligodendrocyte-enriched brain organoids. The presence of myelin-producing cells makes these organoids useful for studying human white matter diseases, such as leukodystrophy.

BEFORE YOU BEGIN

Before initiating any experiment, make sure to obtain all required institutional permissions and approvals, particularly when working with human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Appropriate approvals from the respective Institutional Review Board (IRB), as well as Material Transfer Agreements (MTAs) for cell lines or materials obtained from external sources must be in place. These precautions are important for ensuring that the research is conducted ethically and in compliance with regulatory guidelines. In this study, all experiments were carried out in accordance with the ethical guidelines of the University of Queensland and with the approval by the University of Queensland Human Research Ethics Committee (Approval number-2019000159).

Several methods exist for the generation of brain organoids enriched in oligodendrocytes,^{1–5} each with its unique advantages and limitations. However, the protocol described in this manuscript offers several benefits over existing methods. First, it is embedding-free protocol. Unlike many existing protocols, our method generates brain organoids without the need for embedding in Matrigel, thereby reducing complexity and potential variability in the organoid formation process, collectively increases the reproducibility of our protocol and makes it cost effective. Second, we have emphasized the rapid enrichment in oligodendrocytes as a significant advantage of our protocol. Specifically, our method has been meticulously optimized to expedite the generation of organoids



enriched with OPCs over 9 days of *in vitro* differentiation, manifesting a higher proportion of oligodendrocytes in a relatively shorter time frame.

To generating oligodendrocyte (OL)-enriched brain organoids, it is essential to prepare all necessary materials as previously described in Shaker et al.,^{6,7} and the specific differences will be highlighted in this protocol. The differences here mainly include the process of generating these oligodendrocytes brain organoids free from embedding with Matrigel. In the [key resources table](#), we provide a comprehensive list of materials and required equipment. It is essential that all procedures are performed in a biological enclosure of a Class II biohazard hood, using standard sterilized equipment. The cells and brain organoids must be cultured in a tissue culture incubator with a 37°C temperature and a 5% CO₂ atmosphere. The following protocol describes in detail how to generate oligodendrocyte enriched brain organoids from embryonic stem cells ESCs (G22 and Mel-1) and induced pluripotent stem cells iPSCs (WTC, HBSL3C, EU79) cell lines, with comparable results.

Note: if you choose to use reagents from diverse suppliers, be mindful to verify the quality of these reagents for organoid generation.

Institutional permissions

All experiments were carried out in accordance with the ethical guidelines of the University of Queensland and with the approval by the University of Queensland Human Research Ethics Committee (Approval number-2019000159).

Media preparation

⌚ Timing: 15–30 min

Our protocol requires the preparation of three different types of media. The first one is neural induction medium (see materials and equipment (Olig1)). Olig1 medium can be stored at 4°C for up to one month. The second medium is the neural expansion medium (Olig2 medium; see materials and equipment). The third medium is the differentiation and maintenance medium (Olig3 medium; see materials and equipment).

Note: It is recommended to prepare Olig2 and Olig3 medium fresh and avoid long-term storage at 4°C. Alternatively, they can be stored for long term at –20°C prior to the addition of 2-BME and insulin (semi-prepared medium). When adopting latter option, after thawing the Olig2- or Olig3 medium, freshly add 2-BME and insulin prior to feeding the organoids.

⚠ **CRITICAL:** if the medium contains 2-BME and/or insulin, do not freeze it, as these reagents lose potency once frozen.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
SOX1 (1:150)	R&D Systems	AF2018
SOX2 (ICC [1:300], whole mount [1:150])	Cell Signaling Technology	D9B8N
SOX10 (ICC [1:300], whole mount [1:150])	Cell Signaling Technology	89356
OLIG2 (1:300)	In Vitro Technologies	RDSAF2418SP
GFAP (1:300)	Thermo Fisher Scientific	71-1500

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
MBP (1:300)	Cell Signaling Technology	78896
NANOG (1:300)	Cell Signaling Technology	4893
N-CADHERIN (ICC [1:300], whole mount [1:150])	Cell Signaling Technology	14215
OCT4 (1:300)	Bio-Rad	mca5683
CTIP2 (1:300)	Abcam	ab18465
SATB2 (1:300)	Abcam	Ab51502
ZO-1 (1:150)	Thermo Fisher Scientific	33-9100
HOXC9 (1:150)	DSHB	5B5-2-s
Hoechst (1:1,000)	Abcam	AB228551-5ML

Chemicals, peptides, and recombinant proteins

Matrigel hESC-qualified matrix	Corning	354277
Neurobasal medium	Invitrogen	21103049
DMEM-F12	Thermo Fisher Scientific	11320033
GlutaMAX supplement	Thermo Fisher Scientific	35050061
N-2 supplement	Thermo Fisher Scientific	17502001
B27 supplement without vitamin A	Invitrogen	12587-010
MEM non-essential amino acids solution (100x)	Thermo Fisher Scientific	11140050
Penicillin/Streptomycin (P/S)	Invitrogen	15140122
T3	Sigma-Aldrich Pty Ltd.	T2877-250MG
NT3	Capsugel	450-03-100UG
Human IGF-I (1 mg)	Capsugel	100-11-1000
Human HGF	Capsugel	100-39H-100
FGF	In Vitro Technologies Pty Ltd.	RDS233FB01M
Biotin	Sigma-Aldrich Pty Ltd.	B4639-1G
PDGF-AA	Capsugel	100-13A-100UG
CAMP	Sigma	D0627
SB-431542	Merck	US1616464-5MG
LDN193189	Sigma	SML0559-5MG
2-Mercaptoethanol	Thermo Fisher Scientific	21985023
Insulin	Sigma-Aldrich Pty Ltd.	I9278-5ML
ReLeSR	STEMCELL Technologies	100-0484
PBS	Thermo Fisher Scientific	20012027
HBSS	Thermo Fisher Scientific	88284
Dispase	Thermo Fisher Scientific	17105041
mTeSR Plus	STEMCELL Technologies	100-0276

Software and Algorithms

GraphPad Prism	https://www.graphpad.com/features
Adobe Illustrator	https://www.adobe.com/products/illustrator.html
Fiji	https://fiji.sc/
BeeNet v1	https://honeycombbio.zendesk.com/hc/en-us/articles/4408704149147-BeeNet-v1-1-X-Installation-Video
Seurat software	https://satijalab.org/seurat/

Experimental models: Cell lines

Human ESCs	G22, Mel-1	A13777
Human iPSCs	WTC, EU79, HBSL3i	EU79 ⁹ and HBSL3i were generated in-house in the Wolvetang lab; WTC is a kind gift of Bruce Conklin

Other

Ultra-low attachment 24-well plate	In Vitro Technologies Pty Ltd.	COR3473
U-bottom ultra-low attachment plates, 96 well	Sigma	CLS7007-24EA
Ultra-low attachment plates, 6 well	Sigma	CLS7007-24EA
6-Well plate	Sigma-Aldrich Pty Ltd.	CLS3516-50EA
Scissors and forceps	N/A	

MATERIALS AND EQUIPMENT

Neural induction medium (Olig1)			
No.	Medium components	Concentration/Stock	100 mL volume
1	DMEM F12	10× 500 mL	47.45 mL
2	N2 Supplement 5 mL (100×)	Supplemented at 1%	1 mL
3	B 27 Supplement 10 mL	Supplemented at 2%	2 mL
4	MEM Non-Essential Amino Acids Solution (100×)	Supplemented at 1%	1 mL
5	Penicillin-Streptomycin (10,000 U/mL)	Supplemented at 1%	1 mL
6	2-Mercaptoethanol k(1000×)	Supplemented at 0.1%	0.1 mL

Note: Avoid medium freezing if already added 2-mercaptoethanol to the medium.

Note: The medium is stable for 2–3 weeks at 4°C and for 6 months in –20°C.

Expansion medium (Olig2)			
No.	Medium components	Concentration/Stock	100 mL volume
1	DMEM F12 Nutrient mix	10× 500 mL	95.44 mL
2	B27 without vitamin A	Supplemented at 2%	2 mL
3	N2	Supplemented at 1%	1 mL
4	MEM Non-Essential Amino Acids Solution (100×)	Supplemented at 1%	1 mL
5	Penicillin-Streptomycin (10,000 U/mL)	Supplemented at 1%	1 mL
6	10 ng/mL PDGF-AA	50 µg/mL	20 µL
7	10 ng/mL IGF	100 µg/mL	10 µL
8	10 ng/mL NT3	50 µg/mL	20 µL
9	60 ng/mL T3	600 µg/mL	10 µL
10	100 ng/mL biotin	1 mg/mL	10 µL
11	10 µM cAMP	100 mM	1 µL
12	10 ng/mL HGF	50 µg/mL	20 µL
13	2-Mercaptoethanol 50 mL (1000×)	Supplemented at 0.1%	0.035 mL

Note: Avoid medium freezing if already added 2-mercaptoethanol to the medium.

Note: The medium is stable for 2–3 weeks at 4°C and for 6 months in –20°C.

△ **CRITICAL:** Please use B27 supplement without Vitamin A.

Differentiation and maturation medium (Olig3)			
No.	Olig3 medium components	Concentration/Stock	100 mL volume
1	DMEM F12 Nutrient mix 10× 500 mL	1:1 ratio of DMEM/F12 and Neurobasal medium	47.67 mL
	Neurobasal Medium		47.67 mL
2	B27 without vitamin A	Supplemented at 1%	1 mL
3	N2	Supplemented at 0.5%	0.5 mL
4	MEM Non-Essential Amino Acids	Supplemented at 1%	1 mL
5	GlutaMAX	Supplemented at 1%	1 mL
6	Pen/Strep	Supplemented at 1%	1 mL
7	10 ng/mL PDGF-AA	50 µg/mL	20 µL
8	10 ng/mL IGF	100 µg/mL	10 µL
9	10 ng/mL NT3	50 µg/mL	20 µL
10	60 ng/mL T3	600 µg/mL	10 µL
11	100 ng/mL biotin	1 mg/mL	10 µL

(Continued on next page)

<i>Continued</i>			
No.	Olig3 medium components	Concentration/Stock	100 mL volume
12	10 μ M cAMP	100 mM	1 μ L
13	10 ng/mL HGF	50 μ g/mL	20 μ L
14	2-Mercaptoethanol (1000 \times)	0.35 μ L/mL	35 μ L
15	Insulin	9.5–11.5 mg/mL	25 μ L

Note: Avoid medium freezing if already added 2-mercaptoethanol and Insulin to the medium.

Note: The medium is stable for 2–3 weeks at 4°C and for 6 months in –20°C.

△ CRITICAL: Please use B27 supplement without Vitamin A.

STEP-BY-STEP METHOD DETAILS

Neuroectodermal (NECT) colonies induction (days 1–3)

⌚ **Timing:** 3 days

Before initiating the neuroectodermal induction (NECT, See [Figure 1A](#)), prepare the experimental environment by culturing the stem cells on human embryonic stem cell (hESC)-qualified Matrigel, using the following steps.

1. Prepare the basement membrane matrix by thawing a vial of 100% Matrigel on ice.
2. Dilute the Matrigel for coating at a ratio of 1:50 in a standard DMEM/F12 medium.
3. Add 1 mL of DMEM/F12 medium with Matrigel per well of a 6-well plate.
 - a. Evenly spread across the well.
 - b. Let it coat the floor of the well at room temperature for an estimated 1-h or 30 min in the tissue culture incubator at 37°C temperature and 5% CO₂.
4. Once the well coating is done.
 - a. Carefully remove all the coating materials and avoid touching the surface of the coated wells.
 - b. Passage the stem cells using ReLeSR as described by STEMCELL Technologies.
5. Plate the stem cells into these Matrigel coated wells at a density between 20%–30% confluency per well. To achieve this density, transfer 60% confluent stem cell colonies from one well of a 6-well plate into three wells of another 6-well plate.
6. Maintain the stem cells colonies for one day in mTeSR Plus medium prior to the differentiation.
7. Colony Assessment.
 - a. On the day of planned differentiation, inspect the stem cell colonies using a bright-field microscope at a magnification between 4 \times to 10 \times . It is best to photograph your colonies using phase optics for your records (e.g., See [Figure 1A](#)).
 - b. Ensure that the colonies show no signs of differentiation. A healthy iPSC colony should display a compact edge and contain cells with a large nucleus, minimal cytoplasm, and visible nucleoli ([Figure 1A](#), day 0), and should express the pluripotent markers NANOG and OCT4 ([Figure 1B](#))
8. Prepare Olig1 medium by combining the reagents listed in neural induction medium recipe and allow it to reach room temperature prior to use.
9. Remove the mTeSR Plus medium from the cultured plate with stem cells and add 2 mL of Olig1 medium to each well of a 6-well plate.
10. Add the dual SMAD inhibitors, SB-431542 (10 μ M) and LDN 193189 (0.1 μ M) to each well containing Olig1 medium. Alternatively, add these inhibitors at appropriate concentrations to the required volume of Olig1 medium before adding it to the cells.

Note: To ensure even distribution of the inhibitors, gently rotate the plate or invert the tube containing the medium and the SMAD inhibitors 3–4 times.

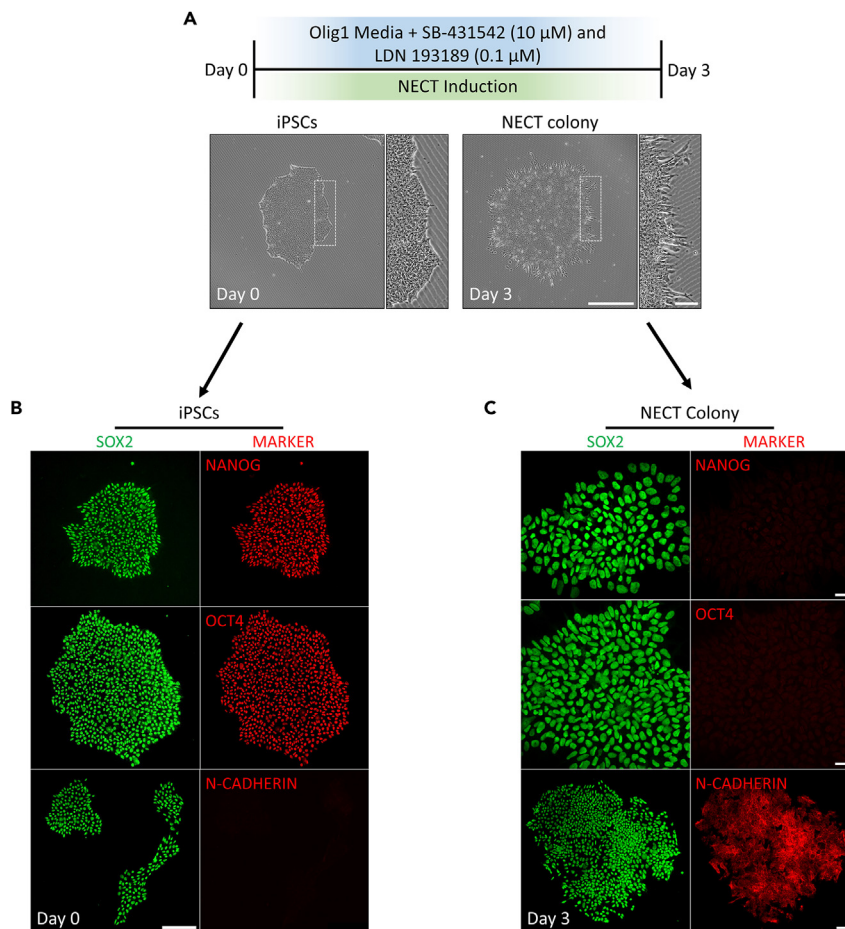


Figure 1. Characterization of iPSCs and neuroectodermal (NECT) colonies using immunocytochemistry

(A) Schematic diagram representing the protocol used to generate NECT colonies from stem cells. Representative bright-field-phase images showing the morphology of stem cells and NECT colonies *in vitro* are shown. Dotted white boxes indicate zoomed in regions. Scale bar = 500 μ m, zoomed images scale bar = 100 μ m.

(B) Immunocytochemistry staining of stem cell colonies marked with SOX2 (Green), NANOG (Red), OCT4 (Red), and N-CADHERIN (RED). Scale bar = 100 μ m.

(C) Immunocytochemistry staining of NECT colonies marked with SOX2 (Green), NANOG (Red), OCT4 (Red), and N-CADHERIN (RED). Scale bar = 50 μ m.

- For the next 2 days, add fresh Olig1 medium containing 10 μ M of SB-431542 and 0.1 μ M of LDN 193189 daily.

Note: Differentiated iPSC colonies towards neuroectodermal lineage should have a distinct shape compared to iPSC colonies, particularly at the colony's external borders. Namely, at day 3 one should see in these regions, a loss of cell contacts with fine cell membrane extensions (i.e., presence of filopodia and lamellipodia; Figure 1A, day 3). During this period (day 0–3), neuroectodermal colonies should lose pluripotent markers and express markers of neural cells, such as N-CADHERIN (Figures 1B and 1C).

Generation/development of neural spheroids enriched in oligodendrocyte precursors (OPCs) (days 4–7)

⌚ Timing: 4 days

This phase of the protocol focuses on the preparation and enrichment of neural spheroids with oligodendrocyte precursors (OPCs). The following steps promote differentiation towards the oligodendrocytes lineage, fostering the proliferation of newly generated OPCs.

12. Begin the procedure by preparing Olig2 medium using the reagents listed in Olig2 recipe and allow the medium to reach room temperature.
13. Colony detachment and Dispase treatment (Figure 2).
 - a. Remove Olig1 medium from each well of your 6 well plate and wash once with HBSS to ensure the total removal of Olig1 medium.

Note: Ensure a complete removal of the Olig1 medium as it may potentially disrupt the enzymatic activity of Dispase, which could result in a failure to properly detach neuroectodermal colonies.

- b. Remove the HBSS and add 1 mL of Dispase of 2.4 units/mL to detach NECT colonies.
 - c. Subject the tissue culture well to incubation at 37°C for a period ranging from 20 min to a maximum of 30 min and periodically monitor for colony detachment.

Note: It's possible for smaller colonies to detach within the initial 20 min. After a 30-min period, all NECT colonies should be detached, and any colonies still attached should be discarded. Only detached colonies should be used for the next steps.

14. NECT colonies handling (Figure 2).
 - a. After the Dispase incubation and detachment of the colonies, add 1 mL of Olig2 medium to each tissue culture well to halt the enzymatic activity of Dispase.
 - b. Use a wide-bore (cut) sterilized P1000 pipette tip (modified) to transfer the colonies into a 15 mL tube.
 - c. Allow the colonies to settle at the bottom of the tube by gravity, a process expected to take about 1 min (inspect by eye).
 - d. After the colonies have settled, cautiously remove the supernatant using a standard P1000 pipette tip and replacing it with 1 mL of fresh Olig2 medium. This step should be repeated three times to ensure a complete removal of Dispase from around the colonies.

Note: The complete removal of Dispase is crucial, as any remaining Dispase may hinder the formation of neural spheroids and lead to significant cell death.

Note: To make the washing step cost effective, you can perform these washes using DMEM/F12 medium and only use the Olig2 medium on the last wash since Olig2 is significantly more expensive as compared to DMEM/F12 medium.

15. Colony Suspension and Seeding (Figure 2).
 - a. After the cell aggregates are sufficiently washed, resuspend them in 3 mL of Olig2 medium. This medium contains a cocktail of growth factors and small molecules that included thyroid hormone T3, neurotrophin NT3, hepatocytes growth factor (HGF), insulin growth factor (IGF), and platelet derived growth factor (PDGF-AA) to promote differentiation toward the oligodendroglial lineage and to foster the proliferation of newly generated OPCs.
 - b. Place the resuspended colonies in a single well of a 6-well plate and add 40 ng/mL of bFGF (Figure 3B).

Note: If a large number of colonies have been detached, consider dividing them across multiple wells of a 6-well plate to prevent spheroids fusion. Monitor these fusions over the next 24 h.

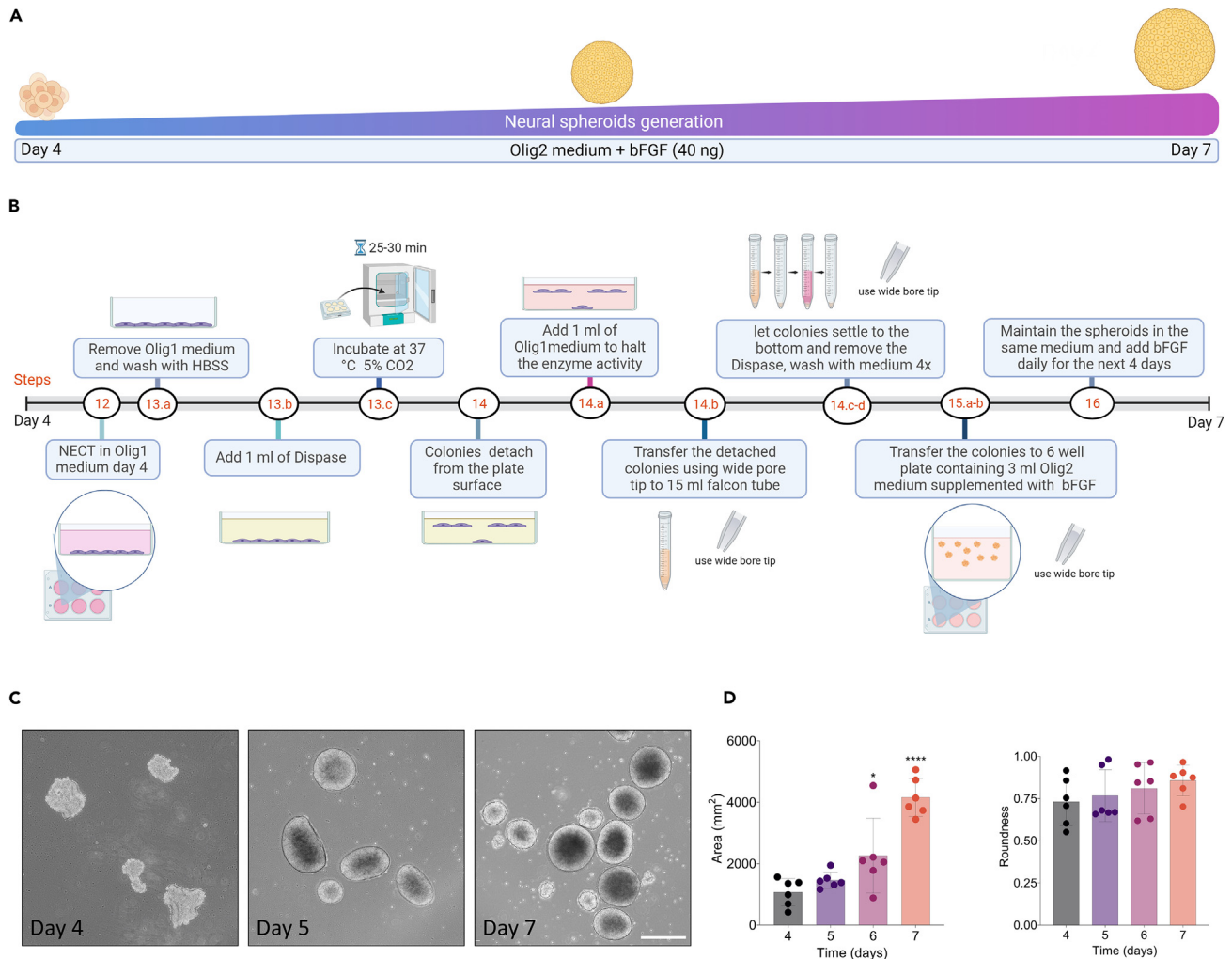


Figure 2. Schematic diagram showing the process of neural spheroids generation enriched with oligodendrocyte progenitor cells from day 4 to day 7 *in vitro* differentiation

Created with BioRender.com.

(A) Schematic diagram illustrates generation and characterization of neural spheroids.

(B) Schematic diagram showing the protocol used to culture the neural spheroids.

(C) Bright field images showing the progressive changes in morphology of neural spheroids during 4 days of proliferation. Scale bar = 400 μm .

(D) Bar graphs showing the quantification of neural spheroids area and roundness over 4 days of *in vitro* differentiation. Data are shown as the mean \pm standard deviation; * $p < 0.05$, **** $p < 0.0001$ via one-way ANOVA.

16. Maintain the spheroids in the same medium for the subsequent 3–4 days.
 - a. Supplement them with 40 ng/mL of bFGF daily to induce the proliferation of NECT cells and the self-organization activity that accompanies the neuroepithelia expansion (Figure 3B).

Note: If the spheroids are plated at a higher density, a yellowing of the medium may occur, signaling a need to replace it every two days with fresh medium and 40 ng/mL of bFGF. To circumvent this issue, it's advised to keep a lower number of spheroids in each well.

Note: Between days 3 and 4, niches of SOX10+ cells, the precursors of oligodendrocytes, may begin to emerge. Use immunostaining procedures, to confirm their presence in organoids sections or intact (whole mount) organoids.

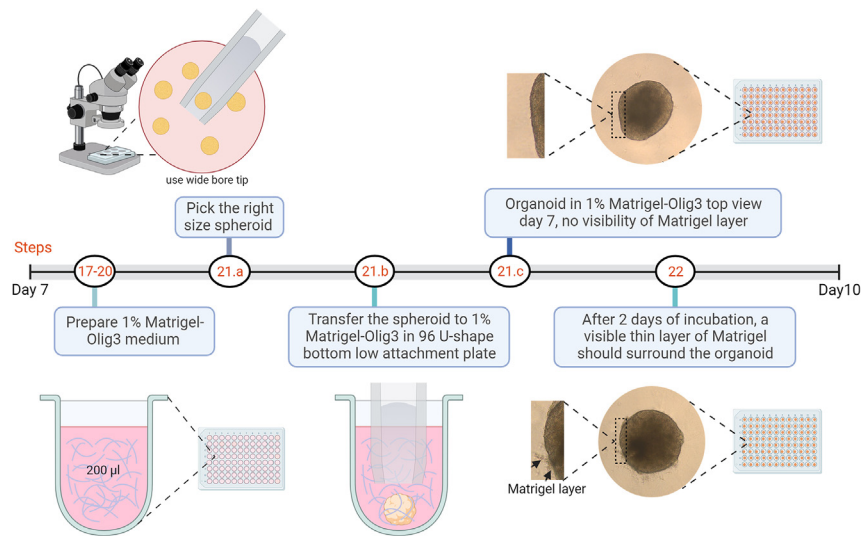


Figure 3. Schematic diagram representing the detailed protocol of generating brain organoids enriched with oligodendrocyte precursors from day 7 to day 10

Created with BioRender.com.

Generation of brain organoids enriched in OPCs (day 8–10/11)

⌚ Timing: 3 days

This phase is dedicated to the process of generating brain organoids enriched with OPCs.

17. Begin the procedure by preparing Olig3 medium using the reagents listed in Olig3 recipe and allow the medium to reach room temperature before use.
18. Thaw a vial or aliquot of 100% Matrigel on ice.
19. Calculation and preparation of Matrigel and Olig3.
 - a. Calculate the required amounts of Olig3 medium and Matrigel to achieve 1% Matrigel. For instance, for 10 wells of 96 well plate, you will need 2.1 mL of Olig3 medium (including an extra 100 μ L for pipetting error) and 21 μ L of Matrigel.
 - b. Add the calculated amount of Matrigel to Olig3 medium in a 15 mL tube and mix well by inverting the tube vertically 4–5 times.
20. Add 200 μ L of 1% Matrigel-Olig3 medium to each well of U-bottom low-attachment 96 well plate.
21. Selecting and transferring neural spheroids (Figure 3).
 - a. Use the stereomicroscope and select neural spheroids of similar size (around 500 μ m) from the 6-well plate.
 - b. Use modified (cut tip) or wide bore pipette tip of 100 μ L or 200 μ L and carefully transfer neural spheroid one by one to each well of a 96-well plate (U-bottom low attachment).

Note: Avoid using flat-bottom low attachment 96 well plate as it does not allow optimal intermingling of neural spheroids with the Matrigel containing medium.

22. After 2 days of incubation in 1% Matrigel-Olig3 medium, the organoids should be intermingled with Matrigel, and a thin layer of Matrigel surrounding the organoid can be observed (Figure 3).
23. Alternatively, if the interaction between organoids and Matrigel isn't clear, the incubation in the 1% Matrigel-Olig3 medium can be extended for additional day.

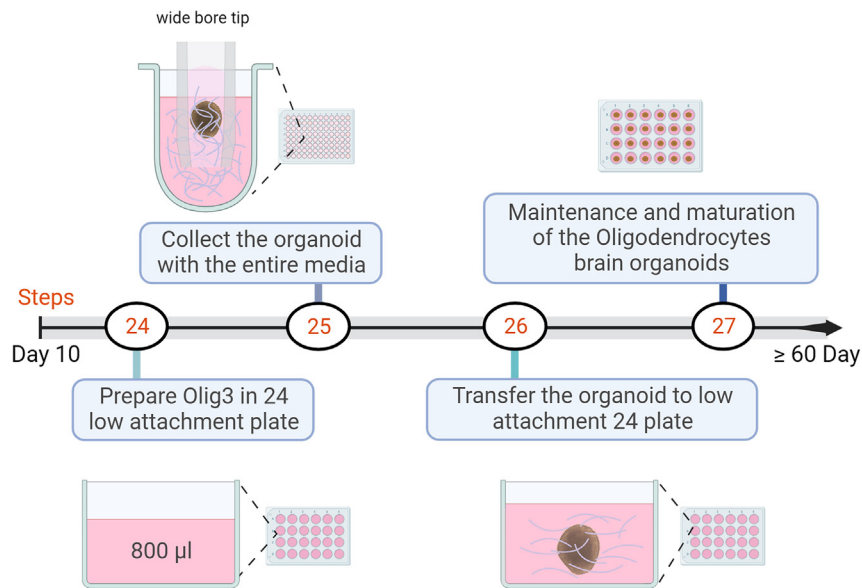


Figure 4. Schematic diagram representing the detailed protocol of generating brain organoids enriched with oligodendrocyte precursors from day 8 to day 10 or 11

Created with [BioRender.com](https://www.biorender.com).

Differentiation and maturation of brain organoid enriched oligodendrocytes (day 11/12–60)

⌚ Timing: ≥ 60 days

This segment of the protocol focuses on the extended differentiation and maturation of the brain organoid, specifically enriching it with mature oligodendrocytes.

24. Prepare 24 well low-attachment plates containing 800 μ L of Olig3 medium per well (Figure 4).
25. Collect the organoid with the entire medium using a wide bore tip.
26. Transfer each organoid to a new 24-well low-attachment plate containing Olig3 medium to make 1 mL total volume.

Note: Always handle cells with care during the transfer to avoid damaging the organoids.

⚠ CRITICAL: There is a high probability of organoids fusing when culturing multiple organoids in the same well.

27. Maintenance and maturation of the oligodendrocytes brain Organoids
 - a. Maintain the differentiated oligodendrocyte brain organoids in Olig3 medium for more than six weeks to achieve mature oligodendrocytes.
 - b. Replace 0.5 mL of medium with 0.5 mL of fresh Olig3 medium in each well every 3 days during the first week.
 - c. After the first week of differentiation, medium can be changed every 2 days.

⚠ CRITICAL: As organoid development progresses, nutrient consumption, and metabolic activity increases. Hence, it is recommended to visually inspect the medium for yellowing before the regular medium change (every 2–3 days). This is a crucial indicator for increasing the frequency of medium replacement.

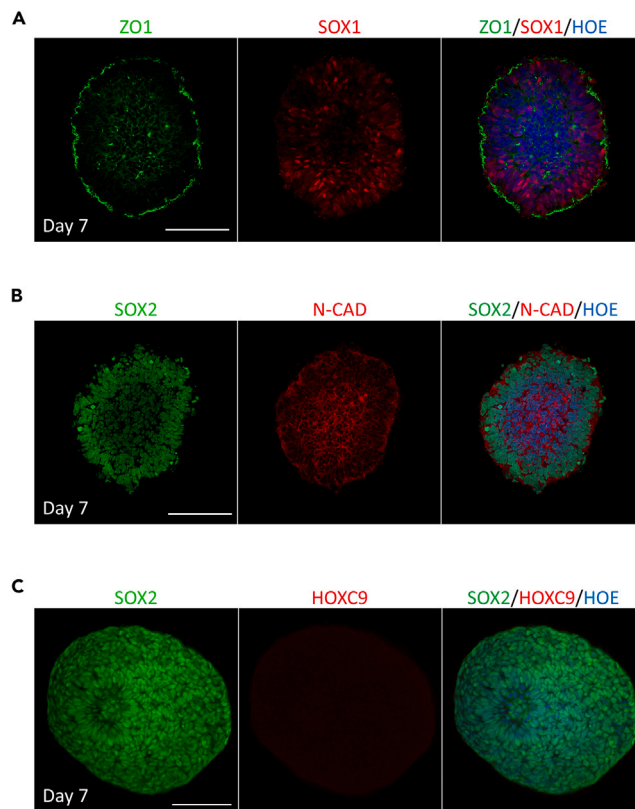


Figure 5. Characterization of neural spheroids at day 7 of *in vitro* differentiation

(A) Whole-mount immunostaining of neural spheroids stained with ZO1 (green) and SOX1 (red). All tissues were counterstained with Hoechst 33342 (blue). Scale bar = 100 μ m.

(B) Whole-mount immunostaining of neural spheroids stained with SOX2 (green) and N-CADHERIN (red). All tissues were counterstained with Hoechst 33342 (blue). Scale bar = 100 μ m.

(C) Whole-mount immunostaining of neural spheroids stained with SOX2 (green) and caudal spinal cord marker HOXC9 (red). All tissues were counterstained with Hoechst 33342 (blue). Scale bar = 100 μ m.

EXPECTED OUTCOMES

The primary outcome of the above protocol is the production of oligodendrocyte-enriched brain organoids, which are complex 3D *in vitro* models suited for human disease modeling.⁶ We have established a simplified and reproducible protocol to generate brain organoids enriched with oligodendrocytes. The steps include a fine-tuned medium cocktail of small molecules, allowing the progressive oligodendrocyte specification and maturation in addition to neurons and astrocytes. This model has applications for *in vitro* modeling of human white matter diseases such as hypomyelination and leukodystrophy.⁷

Organoids derived using the presented protocol contain multiple brain cell types, closely mimicking the human *in vivo* brain niche. At day 9 of neural expansion, the neural spheroids exhibit neural formation of rosettes and lack caudal identity (Figure 5). ZO1 labels the integrity of tight junctions at the apical side and reveals organized epithelial-like structures within the organoids. While the presence of SOX1 indicates a pool of early neural progenitors (Figure 5A), and SOX2 marks the definitive form of these proliferative progenitors within the organoid (Figure 5B). Additionally, we used HOXC9 as an indicator for anterior-posterior patterning, emphasizing the segmental rostral identity and regional differentiation within the neural tissue (Figure 5C). Finally, N-Cadherin (NCAD) staining demonstrates cell-cell adhesion of neural cells (Figure 5B). Collectively, these markers offer a multi-dimensional validation of our organoid model, confirming their rostral identity and that it closely mimics

complex human brain tissue both in cellular composition and organization as we previously demonstrated.^{8–10} At day 9 of differentiation, organoids exhibit neural tube folding (Figure 6A, white stars) as recently reported,¹¹ and the emergence of SOX10⁺ cells in niches can be observed (Figure 6B).

Whole-mount 3D reconstruction clearly shows multiple niches of SOX2⁺ cells were emerged (Figure 6C). The appearance of SOX10⁺ cells at day 9 of differentiation is a clear indicator of the onset of oligodendrocyte lineage specification within the developing organoids. SOX10 is a transcription factor critical for the development of OPCs. Its presence at this stage signifies that the oligodendrogenesis process is initiated within the organoids. This may enable researchers to generate and study the biology of early OPCs when using this 9-day protocol. Interestingly, these organoids still express a large proportion of rosettes SOX2⁺ cells at day 21 (Figure 6D, white arrow), suggesting that proliferation of neural progenitors is ongoing at this stage (Figure 7A). At day 120 of differentiation, organoids exhibit a substantial population of astrocytes and mature myelinating oligodendrocytes, as demonstrated by the presence of GFAP (Figure 7B) and myelin basic protein (MBP), respectively (Figure 7C). Interestingly, we observed early and late stages of MBP⁺ cells as marked by MBP staining (Figures 7D and 7E). We used anti-oligodendrocyte transcription factor (OLIG2 antibody) as a pan-molecular marker that labels oligodendroglial cells and found a large number of OLIG2⁺ cells in these oligodendrocyte brain organoids at day 120 (Figure 7F). We further confirmed the presence of cortical CTIP2 and SATB2 positive cortical neurons in these organoids (Figure 8). CTIP2 and SATB2 are neuronal transcription factors that can be used to identify subtypes of cortical

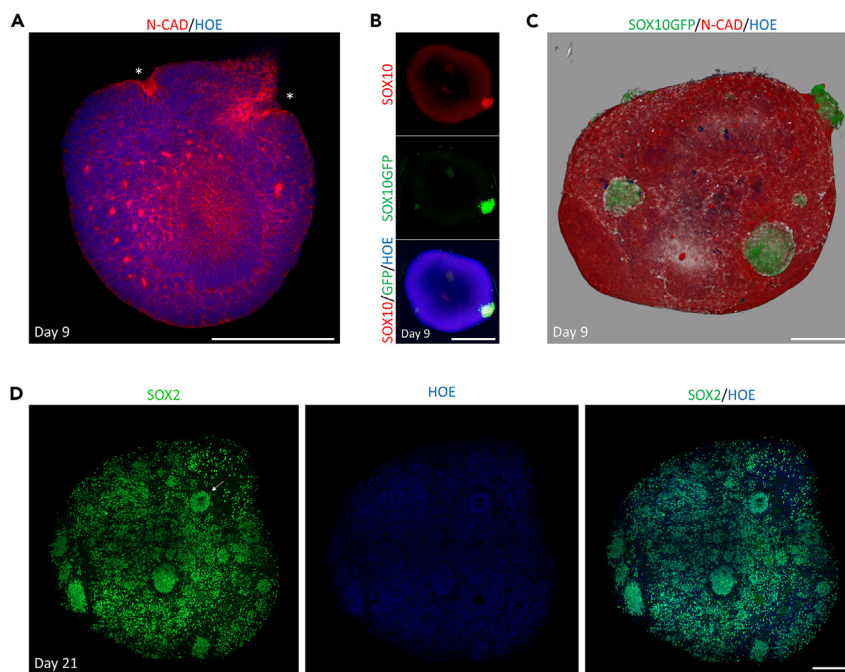


Figure 6. Characterization of differentiating neural spheroids *in vitro*

(A) Whole-mount immunostaining of differentiated neural spheroids at day 9 stained with N-CADHERIN (red) showing the neural rosettes and neural tube folding (white stars). All organoids were counterstained with Hoechst 33342 (blue). Scale bar = 50 μ m.

(B) Whole-mount immunostaining of neural spheroids derived from WTC iPSCs-SOX10GFP reporter line, tissue was stained with SOX10 (red) and GFP (green). All organoids were counterstained with Hoechst 33342 (blue). Scale bar = 50 μ m.

(C) Whole-mount 3D reconstruction images of day 9 neural spheroids derived from WTC iPSCs-SOX10GFP reporter line. The neural spheroid was immunostained with N-CADHERIN (red) and GFP (green). All organoids were counterstained with Hoechst 33342 (blue). Scale bar = 200 μ m.

(D) Whole-mount staining for SOX2 (green) of oligodendrocyte brain organoids at day 21 of *in vitro* differentiation. Organoids were counterstained with Hoechst 33342 (blue). White arrow indicates a neural rosette. Scale bar = 200 μ m.

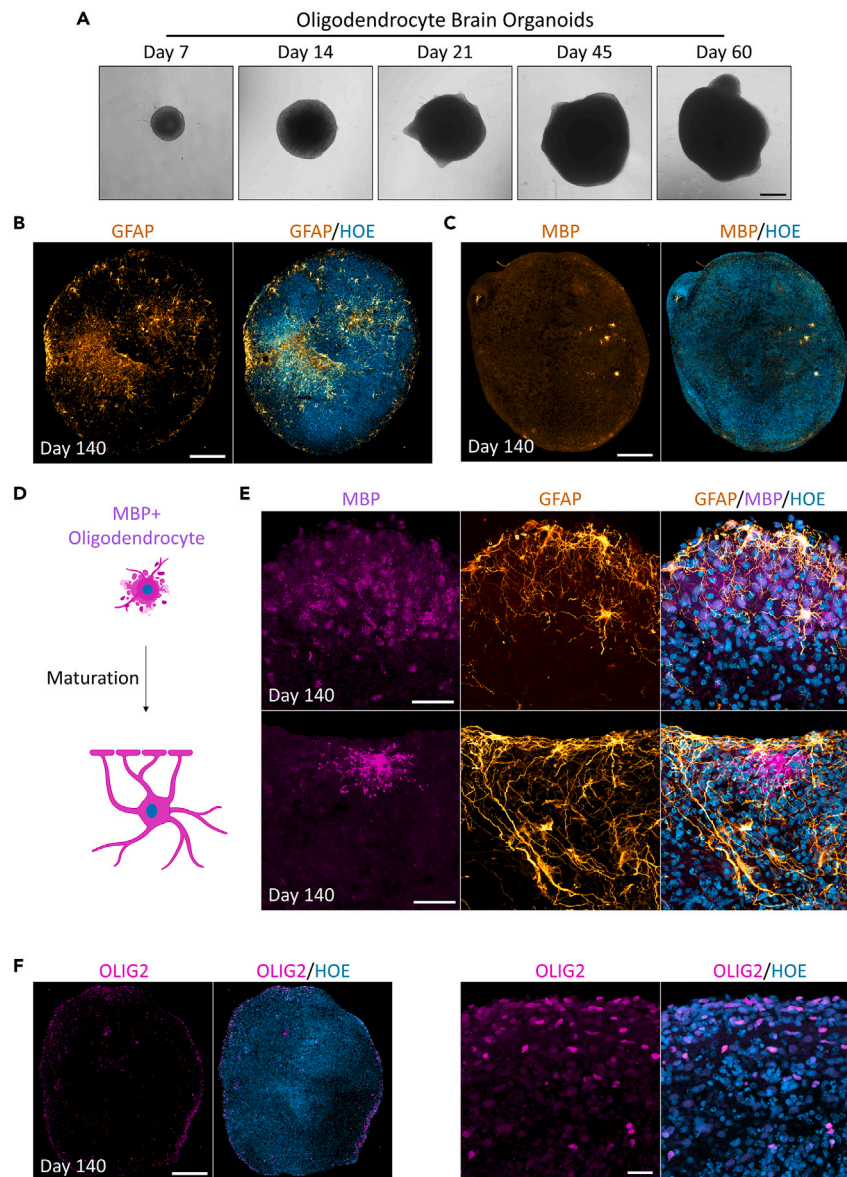


Figure 7. Characterization of oligodendrocyte brain organoids

(A) Bright-field images showing the developmental stages of oligodendrocytes brain organoids overtime *in vitro*. Scale bar = 400 μm .

(B) Analysis of immunostaining of organoid sections at day 140 showing expression of astrocyte marker GFAP (gold). The section was counterstained with Hoechst 33342 (cyan). Scale bar = 200 μm .

(C) Immunostaining of organoid sections on day 140 showing expression of the mature myelinating oligodendrocyte marker MBP (gold). The section was counterstained with Hoechst 33342 (cyan). Scale bar = 200 μm .

(D) Schematic diagram showing the different morphologies of MBP positive cells across organoid developmental stages.

(E) Examples of organoids sections at day 140 double immune-stained for astrocyte marker GFAP (gold) and the mature myelinating oligodendrocyte marker MBP (pink). All sections were counterstained with Hoechst 33342 (cyan). Scale bar = 25 μm .

(F) Immunostaining of organoids sections on day 120 showing expression of the pan-oligodendroglia marker OLIG2 (pink). The sections were counterstained with Hoechst 33342 (cyan). Scale bar = 200 μm . Zoomed images scale bar = 20 μm .

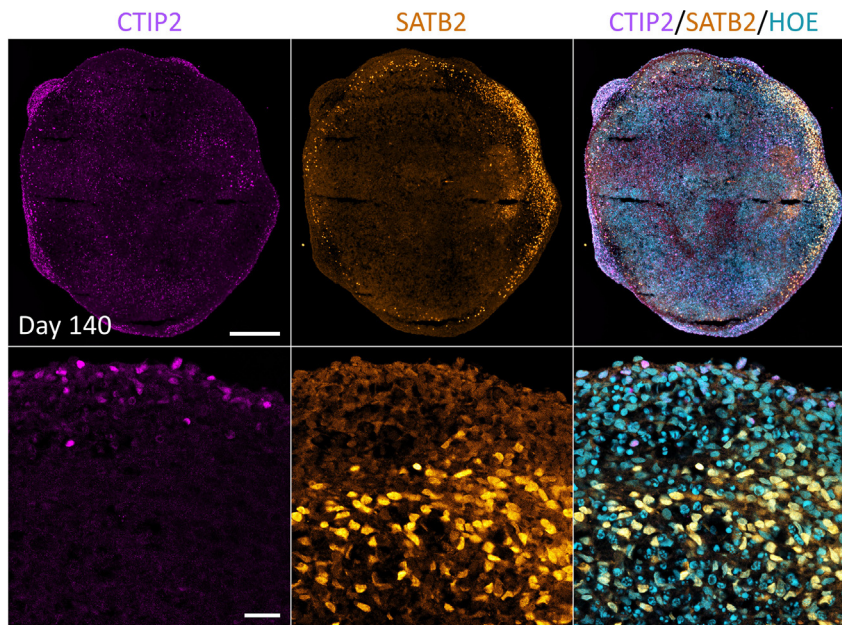


Figure 8. Cortical Neuronal Characterization of oligodendrocyte-enriched brain organoids

Immunostaining of oligodendrocyte-enriched brain organoids sections at days 140 reveals cortical layers V neurons marked by CTIP2 (pink), and layer IV neurons marked by SATB2 (gold), respectively. All sections were counterstained with Hoechst 33342 cyan). Scale bar = 200 μm . Zoomed images scale bar = 25 μm .

neurons, and as development proceeds distinct classes of projecting neurons are being specified.¹⁰ Importantly, we noted that with our embedding free protocol described here, the development of a necrotic core is rarely observed (e.g., Figures 6, 7, and 8). This is in contrast to conventionally generated oligodendrocytes enriched brain organoids that often develop a necrotic core in the center of the organoid⁶ following months of *in vitro* culture.

To compare the generated oligodendrocytes to those developed *in-vivo* in the human fetal brain, we performed single-cell RNA-sequencing of the oligodendrocyte brain organoid derived from HBSL3i using the HIVE platform. The generated fastq files were processed using BeeNet and Seurat v4.1.4 (<https://satijalab.org/seurat/>), yielding 13 clusters for 1028 cells that passed filtering criteria (Figure 9A). Cluster 9 (46 cells) expressed oligodendrocyte markers such as *PDGFRA*, *OLIG1*, and *OLIG2* with some cells expressing mature oligodendrocyte markers *APOD* and *BCAS1* (Figure 9B). This cluster was integrated using the “IntegrateData()” function into the oligodendrocyte population identified from fetal brain samples across gestational weeks 9 through 28.¹² Re-clustering into 4 new clusters that largely matched the oligodendrocyte sub-populations from the original study showed that oligodendrocytes in brain organoids overlapped with Oligo_3 population (Figure 9C). Furthermore, marker genes for each of the five cell populations identified using “FindAllMarkers()” function based on the cutoff “ $p_{\text{val_adj}} < 0.05$ & $\text{abs}(\text{avg_log2FC}) > 0.25$ ” also cluster these OPCs with Oligo_3 (Figure 9D). In conclusion, our protocol generates oligodendrocyte-enriched brain organoids that are highly reproducible in terms of size as well as cellular and molecular characteristics closely comparable to the developing human fetal oligodendrocytes, which is crucial for developing a reliable human-specific *in vitro* model for disease modeling and drug screening.

LIMITATIONS

Despite recent developments, the various organoid production techniques still need to be further optimized to recapitulate the cellular composition of the developing human brain reliably and consistently. The main limitation of the current protocol is that other brain cells such as microglia are not co-produced within the organoid, as they are formed from a non-neural lineage (i.e., outside

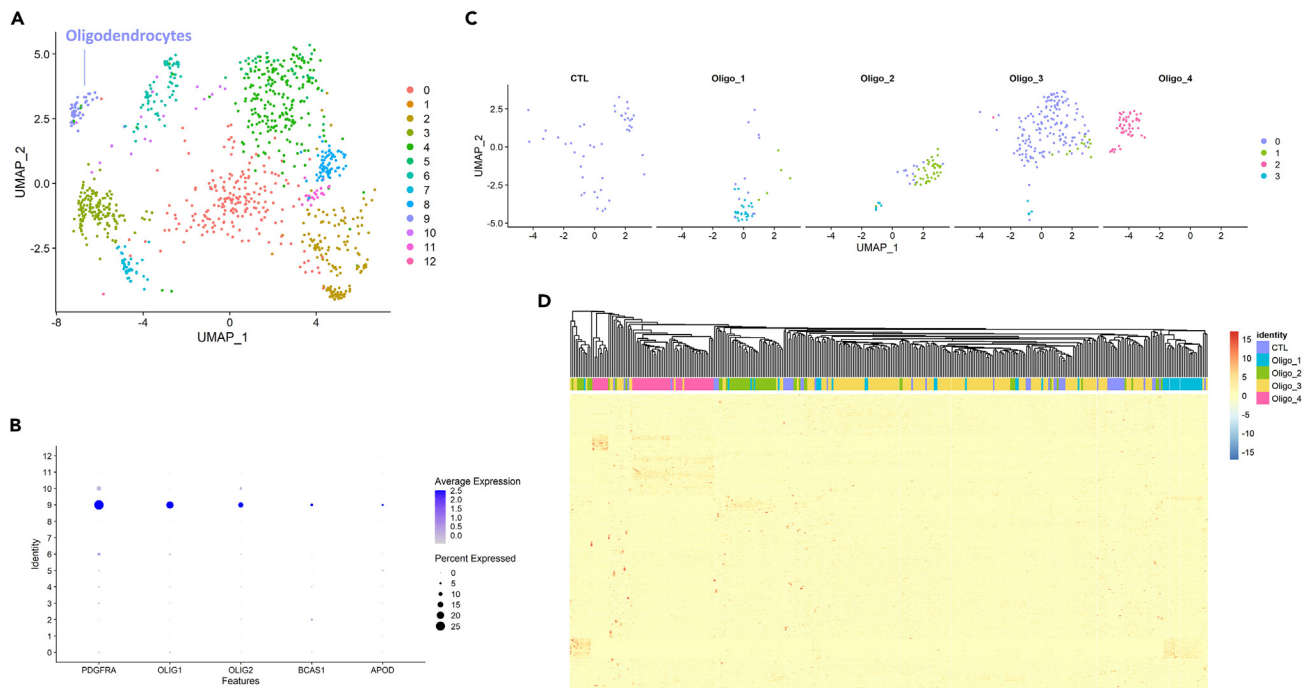


Figure 9. Comparison of oligodendrocytes in brain organoids with fetal brain OPCs/oligodendrocytes

(A) UMAP of single-cell RNA-sequencing of oligodendrocyte brain organoids showing the oligodendrocyte population (cluster 9) in the generated brain organoids.
 (B) Dot plot showing the expression of selected markers of oligodendrocytes. Dot size represents the percentage of clusters expressing a specific marker, while the intensity of color indicates the average expression level for that gene, in that cluster.
 (C) Integration of cluster 9 with fetal brain OPCs and oligodendrocyte clusters the generated cells (marked as CTL) with Oligo_3 forming the new cluster 0, while Oligo_1, Oligo_2, and Oligo_4 form clusters 3, 1, and 2 respectively.
 (D) Heatmap based on marker genes for each of the five populations (CTL, Oligo_1, Oligo_2, Oligo_3, Oligo_4) reveals similar overall gene expression of CTL and Oligo_3.

the developing CNS). Microglia can however be separately produced and can seed brain organoids via co-culture if desired.¹³ Additionally, while our protocol captures the oligodendrocyte lineage’s transcriptional dynamics through single-cell RNA-sequencing, it currently lacks functional assessments like MEA recordings and electrophysiological studies, which are vital for understanding the functional maturity of these cells. As for the integration of functioning vasculature around and/or within brain organoids, this issue remains a significant challenge.¹⁴

TROUBLESHOOTING

Problem 1

Incomplete thawing of Matrigel can affect its consistency and functionality (related to step 1).

Potential solution

- Ensure that the Matrigel is thoroughly thawed on ice before using.

Problem 2

Failure to detach neuroectodermal colonies with Dispase (related to step 13).

Potential solution

- Make sure to wash thoroughly with HBSS to remove all traces of Olig1 medium, as it could interfere with Dispase activity.

- Also, monitor the incubation period closely and discard any colonies that do not detach within 30 min.

Problem 3

Death of cells or failure to form neuroectodermal spheroids (related to step 14).

Potential solution

- Ensure complete removal of Dispase after incubation, as any remaining Dispase may cause cell death or hinder spheroid formation.

Problem 4

Yellowing of the medium during neural spheroid expansion (related to step 16).

Potential solution

- This could indicate that the spheroids are plated at too high a density. Try to keep a lower number of spheroids in each well and consider changing the medium more frequently.

Problem 5

Absence of a clear layer of Matrigel around the organoids (related to step 22 and 23).

Potential solution

- Extend the incubation period for an extra day to allow more time for the Matrigel to form a layer around the organoids.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mohammed R. Shaker (m.shaker@uq.edu.au).

Materials availability

All data reported in this paper will be shared by the [lead contact](#) upon request.

Data and code availability

This paper does not report original code.

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AUTHOR CONTRIBUTIONS

B.A. conceptualized the study, designed the experiments, and implemented, analyzed, and wrote the protocol; M.B.M. designed the experiments, provided data acquisition, and implemented the protocol; S.D.M., P.G., and M.A. performed additional experiments; P.G.N. supervised the study;

and E.J.W. and M.R.S. conceived and supervised the study, interpreted results, and wrote the manuscript. The final version of the manuscript was approved by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Madhavan, M., Nevin, Z.S., Shick, H.E., Garrison, E., Clarkson-Paredes, C., Karl, M., Clayton, B.L.L., Factor, D.C., Allan, K.C., Barbar, L., et al. (2018). Induction of myelinating oligodendrocytes in human cortical spheroids. *Nat. Methods* *15*, 700–706.
- Marton, R.M., Miura, Y., Sloan, S.A., Li, Q., Revah, O., Levy, R.J., Huguenard, J.R., and Pasca, S.P. (2019). Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. *Nat. Neurosci.* *22*, 484–491.
- Kim, H., Xu, R., Padmashri, R., Dunaevsky, A., Liu, Y., Dreyfus, C.F., and Jiang, P. (2019). Pluripotent stem cell-derived cerebral organoids reveal human oligodendrogenesis with dorsal and ventral origins. *Stem Cell Rep.* *12*, 890–905.
- Yun, W., Kim, I.Y., Song, G., and You, S. (2020). Rapid induction of gliogenesis in OLIG2 and NKX2.2-expressing progenitors-derived spheroids. *Stem Cells Transl. Med.* *9*, 1643–1650.
- James, O.G., Selvaraj, B.T., Magnani, D., Burr, K., Connick, P., Barton, S.K., Vasistha, N.A., Hampton, D.W., Story, D., Smigiel, R., et al. (2021). iPSC-derived myelinoids to study myelin biology of humans. *Dev. Cell* *56*, 1346–1358.e6.
- Shaker, M.R., Pietrogrande, G., Martin, S., Lee, J.-H., Sun, W., and Wolvetang, E.J. (2021). Rapid and Efficient Generation of Myelinating Human Oligodendrocytes in Organoids. *Front. Cell. Neurosci.* *15*, 631548. <https://doi.org/10.3389/fncel.2021.631548>.
- Shaker, M.R., Kahtan, A., Prasad, R., Lee, J.-H., Pietrogrande, G., Leeson, H.C., Sun, W., Wolvetang, E.J., and Slonchak, A. (2022). Neural Epidermal Growth Factor-Like Protein 2 Is Expressed in Human Oligodendroglial Cell Types. *Front. Cell Dev. Biol.* *10*, 803061. <https://doi.org/10.3389/fcell.2022.803061>.
- Briggs, J.A., Sun, J., Shepherd, J., Ovchinnikov, D.A., Chung, T.-L., Nayler, S.P., Kao, L.-P., Morrow, C.A., Thakar, N.Y., Soo, S.-Y., et al. (2013). Integration-Free Induced Pluripotent Stem Cells Model Genetic and Neural Developmental Features of Down Syndrome Etiology. *Stem Cell.* *31*, 467–478. <https://doi.org/10.1002/stem.1297>.
- Shaker, M.R., Aguado, J., Chaggar, H.K., and Wolvetang, E.J. (2021). Klotho inhibits neuronal senescence in human brain organoids. *NPJ Aging Mech. Dis.* *7*, 18. <https://doi.org/10.1038/s41514-021-00070-x>.
- Shaker, M.R., Slonchak, A., Al-mhanawi, B., Morrison, S.D., Sng, J.D.J., Cooper-White, J., Khromykh, A., and Wolvetang, E.J. (2023). Choroid Plexus Defects in Down Syndrome Brain Organoids Enhance Neurotropism of SARS-CoV-2. Preprint at bioRxiv. <https://doi.org/10.1101/2023.06.12.544552>.
- Lee, J.-H., Shin, H., Shaker, M.R., Kim, H.J., Park, S.-H., Kim, J.H., Lee, N., Kang, M., Cho, S., Kwak, T.H., et al. (2022). Production of human spinal-cord organoids recapitulating neural-tube morphogenesis. *Nat. Biomed. Eng.* *6*, 435–448. <https://doi.org/10.1038/s41551-022-00868-4>.
- Fan, X., Fu, Y., Zhou, X., Sun, L., Yang, M., Wang, M., Chen, R., Wu, Q., Yong, J., Dong, J., et al. (2020). Single-cell transcriptome analysis reveals cell lineage specification in temporal-spatial patterns in human cortical development. *Sci. Adv.* *6*, eaaz2978. <https://doi.org/10.1126/sciadv.aaz2978>.
- Schafer, S.T., Mansour, A.A., Schlachetzki, J.C.M., Pena, M., Ghassemzadeh, S., Mitchell, L., Mar, A., Quang, D., Stumpf, S., Ortiz, I.S., et al. (2023). An in vivo neuroimmune organoid model to study human microglia phenotypes. *Cell* *186*, 2111–2126.e20. <https://doi.org/10.1016/j.cell.2023.04.022>.
- Cakir, B., Xiang, Y., Tanaka, Y., Kural, M.H., Parent, M., Kang, Y.-J., Chapeton, K., Patterson, B., Yuan, Y., He, C.-S., et al. (2019). Engineering of human brain organoids with a functional vascular-like system. *Nat. Methods* *16*, 1169–1175.