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Protocol review

A review of protocols for brain organoids and applications for disease modeling

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SUMMARY

Recent breakthroughs in human stem cell technologies have enabled the generation of 3D brain organoid platforms for modeling human neurodevelopment and disease. Here, we review advances in brain organoid development, approaches for generating whole-brain or cerebral organoids and region-specific brain organoids, and their applications in disease modeling. We present a comprehensive overview of various brain organoid generation protocols, including culture steps, media, timelines, and technical considerations associated with each protocol, and highlight the advantages and disadvantages of each protocol. We also discuss the current limitations as well as increasing sophistication of brain organoid technology, and future directions for the field. These insights provide a valuable assessment of multiple commonly used brain organoid models and main considerations for investigators who are considering implementing brain organoid technologies in their laboratories.

INTRODUCTION

Studies of human brain are challenging, largely due to the complexities of the human brain and inaccessibility of primary brain tissue. Animal models have traditionally been used as model organisms for neuroscience research since human brain tissue is limited by availability and ethical considerations. However, rodents can only model the human brain to a limited extent and are not fully representative of human disease pathology, which likely contributes to the overall low success of translating findings from animal studies to the clinic. The development of human pluripotent stem cell (hPSC)-based neural models in the last two decades has provided new systems for studying human brain development and disease.[1] These models generate a variety of platforms of varying complexity, beginning with least complex monolayer cultures of neural stem cells that can be differentiated into specialized neural cell types, to more complex neural tube-like structures called rosettes, which exhibit spontaneous radial organization reminiscent of an embryonic neural tube, or the highly complex 3D spheroid and organoid models that have organ-like tissue morphology and composition of certain brain regions or contain multiple regions within a single organoid called cerebral or whole-brain organoids.[1,2] Collectively, brain organoids represent physiologically relevant 3D *in vitro* neural systems for functional modeling of human brain development and disease.[3]

The balance between complexity and heterogeneity is an important feature to consider when deciding which approach to use for a particular study. 2D cultures are simplistic models to study

cell types in isolation; however, they fail to capture complex cellular interactions.As such they have limited utility in modeling many disease-relevant neurobiological phenomenon, but at the same time they offer the advantage of homogeneity needed for robust and reliable assay readouts. 3D culture models are more physiologically relevant as they contain diverse cell types, which undergo complex spatiotemporal structural organization to mimic functional features of the brain with greater complexity than 2D models. However, their inherent heterogeneity results in issues of reproducibility. All these models have accelerated our understanding of neurobiology and led to promising findings. In this review we will focus on protocols to produce brain organoids, three-dimensional brain-like tissues.

Recent years have seen an explosion in development of brain organoid systems, from whole-brain or cerebral organoids to region specific organoids—cerebellar, pituitary, midbrain, forebrain, cortical, thalamic, and choroid plexus organoids, and more recently fused organoids or assembloids (Figure 1, Table 1). The continually expanding set of brain organoid systems has provided researchers with fantastic tools to unlock the complexities of normal and diseased brain. With the plethora of brain organoid protocols available, each consisting of multiple steps with specialized media systems and equipment, it can be a daunting task for scientists looking to adopt this emerging technology for their research.

To provide scientists a summary of the major considerations for implementing brain organoid technologies we provide an overview of the different detailed brain organoid protocols published in *STAR Protocols*, as well as the groundbreaking whole-brain protocol published by the Knoblich group (a complete list of brain organoid protocols published in STAR Protocols is available at: https://star-protocols.cell.com/search?categories = Organoids&query = brain).[44] Given the intensive nature of cell culture needed to generate these complex 3D structures, several metrics such as protocol steps, reagents and timing, special equipment, and technical expertise needed, as well as advantages and disadvantages of each protocol are discussed. Additionally, we examine protocols for fusing different brain-region specific organoids such as dorso-ventral forebrain assembloids to model oligodendroglial development and myelination, and generation of hybrid organoids by fusion of neural and mesenchymal organoids to form vascularized neural organoids. Lastly, we look at how these systems have been applied in disease modeling of glioblastoma tumors, Alzheimer's disease and traumatic brain injury, the current disadvantages of brain organoids and highlight future directions of this promising technology. Our aim in reviewing this expanding toolkit of brain organoid models is to foster their wider adoption by the scientific community.

SUMMARIES OF BRAIN ORGANOID PROTOCOLS PUBLISHED IN STAR PROTOCOLS

See Box 1 for a list of abbreviations used in Tables 2, 3, 4, and 5.

PRACTICAL CONSIDERATIONS

How to choose the right protocol for your work

The choice of the optimal protocol to generate brain organoids is largely dependent on your research question. You should validate the identity of cells generated in these organoids and document appropriate functional properties. If multiple protocols exist for generating the same regional organoid type, you should confirm the presence and function of the desired cells type(s). Typically, protocols that most closely mirror developmental paradigms produce neurons that exhibit the greatest *in vitro* maturation and functionality.[67] Other factors to consider include the length and complexity of each protocol and the availability of appropriate resources (staffing, equipment, and key reagents).

Key factors for success

It can be expensive, challenging, and time consuming to introduce the culture and directed differentiation of hPSCs to a lab. Key factors that affect the successful implementation of a brain organoid

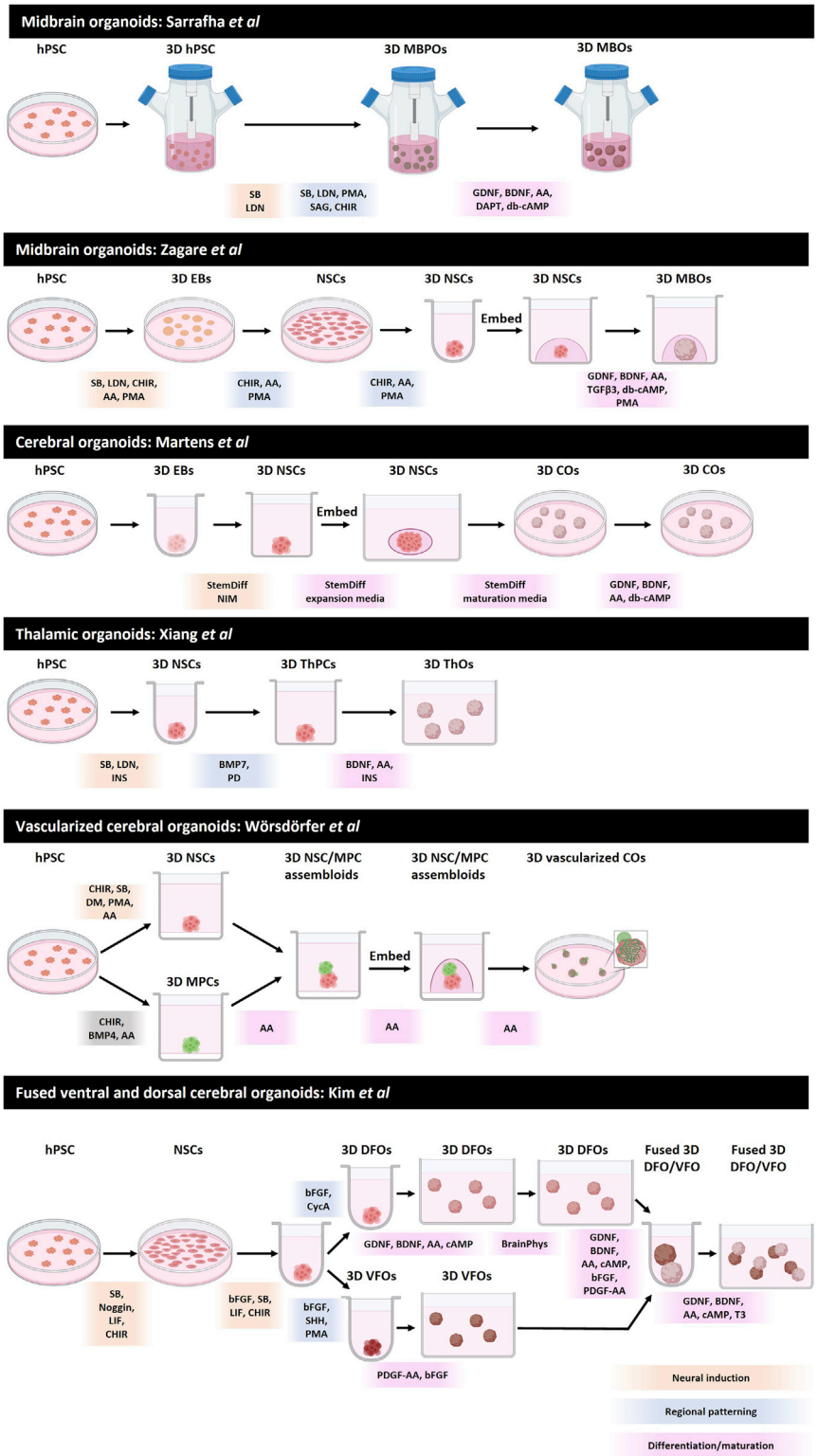
Midbrain organoids: Sarrafha et al
hPSC
3D hPSC
3D MBPOs
3D MBOs
SB
LDN
SB, LDN, PMA,
SAG, CHIR
GDNF, BDNF, AA,
DAPT, db-cAMP
Midbrain organoids: Zagare et al
hPSC
3D EBs
NSCs
3D NSCs
3D NSCs
3D MBOs
Embed
SB, LDN, CHIR,
AA, PMA
CHIR, AA,
PMA
CHIR, AA,
PMA
GDNF, BDNF, AA,
TGFβ3, db-cAMP,
PMA
Cerebral organoids: Martens et al
hPSC
3D EBs
3D NSCs
3D NSCs
3D COs
3D COs
Embed
StemDiff
NIM
StemDiff
expansion media
StemDiff
maturation media
GDNF, BDNF,
AA, db-cAMP
Thalamic organoids: Xiang et al
hPSC
3D NSCs
3D ThPCs
3D ThOs
SB, LDN,
INS
BMP7,
PD
BDNF, AA,
INS
Vascularized cerebral organoids: Wörsdörfer et al
hPSC
3D NSCs
3D NSC/MPC
assembloids
3D NSC/MPC
assembloids
3D vascularized COs
CHIR, SB,
DM, PMA,
AA
3D MPCs
CHIR,
BMP4, AA
Embed
AA
AA
AA
Fused ventral and dorsal cerebral organoids: Kim et al
hPSC
NSCs
3D DFOs
3D DFOs
3D DFOs
Fused 3D
DFO/VFO
Fused 3D
DFO/VFO
bFGF,
CycA
GDNF, BDNF, AA, cAMP
BrainPhys
GDNF,
BDNF,
AA, cAMP,
bFGF,
PDGF-AA
GDNF, BDNF,
AA, cAMP, T3
3D VFOs
3D VFOs
SB,
Noggin,
LIF,
CHIR
bFGF, SB,
LIF, CHIR
bFGF,
SHH,
PMA
PDGF-AA, bFGF
Neural induction
Regional patterning
Differentiation/maturation

Figure 1. Graphical overview of stages of brain organoid generation protocols presented in Tables 2, 3, and 4, including key stages, culture vessels, and media components

Notes: media compositions are simplified to show an overview of only key factors at each stage. Carefully review detailed media composition in protocols for exact composition, concentrations, and timings used. Depicted culture vessels indicate those described by protocol authors. Where possible, we recommend researchers use these vessels, but in some cases, alternatives may be used. Created with BioRender.com.

protocol include the availability of trained staff, and the reproducible quality of hPSCs and reagents. The availability of a shared resource/core facility that can facilitate access to high-quality undifferentiated hPSCs or provide training in their culture and differentiation can significantly alleviate this issue. Ideally, hPSCs should be obtained from a well-characterized, cryopreserved bank. The identity of banked cells must be authenticated, and hPSCs should be mycoplasma-free, contain a stable karyotype, and demonstrate tri-lineage differentiation capacity. Furthermore, the activity of growth factors and small molecules can vary from batch-to-batch and vendor-to-vendor. We recommend assessing the quality of batches of these reagents and purchasing validated lots in bulk. Although expensive, the increasing availability of commercial differentiation kits can alleviate some of the challenges associated with protocol implementation.

Regardless of the brain organoid type generated and differentiation method used, robust assessment of the identity of the organoids produced is critical. The identity of cells in brain organoids can be heterogeneous and exhibit significant variability from batch-to-batch and from line-to-line. We recommend establishing robust quality assessment criteria that allow unequivocal, quantitative analysis of the key cell types that should be present in specific organoids. This can be a resource intensive process but is essential for reproducible and robust data interpretation. Optimal quality analysis includes implementation at intermediate steps in addition to at the completion of the differentiation protocol to enable the rapid identification of a suboptimal batch.

Table 1. Advances in brain organoid development

Brain organoid technologies	Reference
First 3D neural organoid in the form of self-organized optic cup	Eiraku et al.[4]
First brain organoid in the form of self-patterned cerebral organoids and self-organizing cortical tissue	Kadoshima et al.[5]; Lancaster et al.[6]
Enhanced reproducibility, survival, and maturation of brain organoids	Giandomenico et al.[7]; Lancaster et al.[8]; Qian et al.[9]; Quadrato et al.[10]; Velasco et al.[11]
Increased cellular diversity in brain organoids	Madhavan et al.[12]; Marton et al.[13]; Ormel et al.[14]; Pasca et al.[15]; Quadrato et al.[10]; Sakaguchi et al.[16]; Velasco et al.[11]
Development of region-specific brain organoids: cerebellar, pituitary, midbrain, forebrain, cortical, and thalamic organoids	Birey et al.[17]; Jo et al.[18]; Muguruma et al.[19]; Ozone et al.[20]; Qian et al.[21]; Schukking et al.[22]; Trujillo et al.[23]; Xiang et al.[24]
Intracerebral transplantation to achieve vascularized brain organoids	Mansour et al.[25]; Wang et al.[26]
Functional neural circuit and oscillatory waves in brain organoids	Giandomenico et al.[7]; Trujillo et al.[23]
Axially patterned brain organoids	Cederquist et al.[27]
CNS-barrier forming brain organoids with cerebrospinal fluid production	Pellegrini et al.[28]
Organoid/microglia co-cultures to recapitulate neural-immune interactions in the brain	Abud et al.[29]; Brownjohn et al.[30]; Lin et al.[31]; Xu et al.[32]
Organoid/endothelial cell co-cultures to recapitulate neural-vascular interactions in the brain	Cakir et al.[33]; Pham et al.[34]; Shi et al.[35]; Worsdorfer et al.[36]
Organoid/tumor cell co-cultures to recapitulate brain tumor invasion	Goranci-Buzhala et al.[37]; Krieger et al.[38]; Linkous et al.[39]
Fusion of organoids (assembloids) to study brain regional interconnectivity and neural circuit formation	Andersen et al.[40]; Bagley et al.[41]; Birey et al.[17]; Miura et al.[42]; Xiang et al.[24]; Xiang et al.[43]

Box 1. Abbreviations

AA: ascorbic acid, B27-RA: B-27 supplement without vitamin A (retinyl acetate), BDNF: brain-derived neurotrophic factor, bFGF: basic fibroblast growth factor, BME: β-mercaptoethanol, BMP4: bone morphogenetic protein 4, BMP7: bone morphogenetic protein 7, cAMP: cyclic adenosine monophosphate, CHIR: CHIR99021 (glycogen synthase kinase 3 inhibitor, Wnt pathway agonist), CO: cerebral organoid, CycA: cyclopamine (smoothened receptor inhibitor, hedgehog pathway antagonist), DAPT: γ-secretase inhibitor (NOTCH pathway antagonist), db-cAMP: dibutyryl-cyclic adenosine monophosphate, DFO: dorsal forebrain organoid, DM: dorsomorphin (BMP type I receptor inhibitor, BMP pathway antagonist), DA: dopaminergic, EB: embryoid body, GDNF: glial cell line-derived neurotrophic factor, GLICO: glioma cerebral organoid, GSC: glioma stem cells, hESC: human embryonic stem cell, hPSC: human pluripotent stem cell, INS: insulin, iPSC: induced pluripotent stem cell, LDN: LDN139189 (BMP type I receptor inhibitor, BMP pathway antagonist), L-Glu: L-glutamine, LIF: leukemia inhibitory factor, MBO: midbrain organoid, MBPO: midbrain progenitor organoid, MPC: mesodermal progenitor cell, N2: N-2 supplement, NEAA: Minimum Essential Medium-non-essential amino acids, NSC: neural stem cell, PD: PD325901 (MEK inhibitor, MAPK/ERK pathway antagonist), PDOX: patient derived orthotopic xenografts, PMA: purmorphamine (smoothened receptor activator, hedgehog pathway agonist), P/S: penicillin/streptomycin, SAG: smoothened agonist (smoothened receptor activator, hedgehog pathway agonist), SB: SB431542 (TGF-β type I receptor inhibitor, TGF-β/Activin/NODAL pathway antagonist), T3: Triiodo-L-thyronine (thyroid hormone), TGFβ3: transforming growth factor-β3, ThPC: thalamic progenitor cell, ThO: thalamic organoid, PDO: patient derived organoids, ULA: ultra-low attachment, VFO: ventral forebrain organoid.

Finally, given that generation of brain organoids frequently takes several months, it is crucial to have a well-developed plan to ensure a consistent supply of validated reagents and to account for production disruptions such as microbial contamination of cultures. We recommend generating brain organoids in staged batches to enable faster recovery from unexpected loss of a single batch. Some protocols enable cryopreservation of intermediate cell types.[50] This not only facilitates more rapid recovery from production disruptions but permits generation of banks of quality-controlled cells for organoid production that can help reduce batch to batch variation.

DISADVANTAGES AND IMPROVEMENTS

Cerebral organoids model early brain development with remarkable fidelity; however, their further neuronal maturation and size is limited by interior hypoxia and cell death due to lack of vasculature. Efforts are underway to achieve *in vitro* vascularization of tissue-engineered constructs; however, until a fully functional vascular system, especially an authentic blood-brain-barrier, can be developed, scientists have achieved vascularization by transplanting brain organoids into the rodent brain, which allowed extensive growth and invasion of host blood vessels into the human organoid and resulted in greater survival of cells owing to effective blood perfusion.[25] To circumvent the problem of insufficient surface diffusion of nutrients and oxygen, scientists have applied the classic method of organotypic slice culture to organoids.[7,9] By sectioning and culturing mature cerebral organoid slices at the air-liquid interface, not only does the tissue remain healthy over an extended time but also exhibits improved neuronal maturation. Another study suggested adding BDNF to the maturation medium to obtain long-term growth and improved development.[10] Additionally, Lancaster et al.[6] improved the reproducibility of their original cerebral organoid protocol by using PLGA microfilaments as a floating scaffold to generate elongated embryoid bodies, performing short treatment with WNT activator, and adding dissolved Matrigel to maturation medium to increase reproducibility and improve forebrain tissue architecture and cortical development.[8]

Current brain organoids also lack an inherent microglia component, the resident immune cells of the brain. Given that microglia are critical for brain development and homeostasis, generation of more complex organoids integrating these cells is an area of significant interest. Scientists have utilized a co-culture approach to incorporate iPSC-derived microglia cells or primary human microglia in brain organoids to investigate the neuro-immune crosstalk that plays a critical role in either protecting or enhancing neuronal pathology.[29,31,68]

Table 2. Generation of whole-brain or cerebral organoids

Organoid type: Cerebral organoids (CO)		
Protocol title	Generation and validation of APOE knockout human iPSC-derived cerebral organoids	Generation of cerebral organoids from human pluripotent stem cells
STAR Protocols reference/ Seminal Reference	Martens et al.[45]	Lancaster and Knoblich[46]
Original publication	Zhao et al.[47]	Lancaster et al.[6]
Protocol length (before long-term culture)	~5 weeks	
Specialized equipment needed	Orbital shaker or Spinning bioreactor	
Note	Martens et al.[45] protocol uses the StemDiff Cerebral Organoid Kit available commercially from StemCell Technologies. While this media is based on published recipes,[6,46] the precise formulation is proprietary.	
Protocol stages overview	1) 2D hPSC culture, 2) EB formation, 3) Neural induction, 4) Matrigel embedding and expansion of NSCs, 5) Cerebral organoid differentiation, 6) Cerebral organoid maturation, 7) Long-term culture	
Protocol stage 1	**2D hPSC culture**	
Stage timing	1–2 h	
Culture format	Cell culture dishes	Cell culture dishes
Culture system/Culture medium	mTeSR1, hESC-qualified Matrigel, dispase clump passaging	mTeSR1, Matrigel-coated plates
Cell number/preparation for differentiation	70% confluence, Accutase to single cells, seed at 15,000 cells/well of U-bottomed 96-well ULA plate	EDTA/accutase-treatment to obtain single cell suspension, seed at 9,000 cells/well of U-bottomed 96-well ULA plate. One hPSC well will yield approximately an entire 96-well plate of EBs
Protocol stage 2	**3D EB formation**	
Stage timing	5 days	5–7 days
Culture format	U-bottomed 96-well ULA plate	
Culture medium, growth factors and small molecules	StemDiff Cerebral Organoid Kit: complete EB Formation medium + 10μM Y27632	DMEM-F12 medium + 20% knockout serum replacement + 3% ESC-quality Fetal Bovine Serum + 1 mM β-Mercaptoethanol (BME) + 1% non-essential amino acids (NEAA) + 1% Glutamax + 4 ng/mL Basic Fibroblast Growth Factor (bFGF) + 50μM Y27632
Protocol stage 3	**Neural induction**	
Stage timing	2–5 days	4–5 days
Culture format	48-well ULA plate	24-well ULA plate
Culture medium, growth factors and small molecules	StemDiff Cerebral Organoid Kit: complete Neural Induction Medium	DMEM-F12 medium + 1% N2 + 1% Glutamax + 1% NEAA + 1 μg/mL heparin
Protocol stage 4	**Matrigel embedding and expansion of neuroepithelial buds**	
Stage timing	3 days	4 days
Culture format	EBs embedded in Matrigel in 6-well ULA cell culture plate	EBs embedded in Matrigel droplets in 6-cm dish
Culture medium, growth factors and small molecules	StemDiff Cerebral Organoid Kit: complete Expansion Medium	Cerebral organoid differentiation medium without vitamin A: DMEM-F12 medium + Neurobasal medium (1:1) + 0.5x N2 + 1x B27-RA + 2.5 μg/mL INS + 50μM BME + 1% Glutamax + 1x P/S
Protocol stage 5	**CO maturation**	
Stage timing	4 weeks	
Culture format	10 cm dish on orbital shaker @ 40 rpm	Transfer embedded organoids to a 125 mL spinning bioreactor or 6 cm dish on orbital shaker @ 85 rpm
Culture medium, growth factors and small molecules	StemDiff Cerebral Organoid Kit: complete Maturation Medium	Cerebral organoid differentiation medium containing vitamin A: DMEM-F12 medium + Neurobasal medium (1:1) + 0.5x N2 + 1x B27 + 2.5 μg/mL INS + 50μM BME + 1% Glutamax + 1x P/S
Protocol stage 6	**Long-term culture**	
Stage timing	Up to 1 year	
Culture format	10 cm dish on orbital shaker @ 40 rpm	125-mL spinning bioreactor or 6 cm dish on orbital shaker @ 85 rpm

(*Continued on next page*)

Table 2. *Continued*

Culture medium	DMEM-F12 medium + Neurobasal medium (1:1) + 0.5x B27-RA + 0.5x N2 + 1x Glutamax + 1x Sodium pyruvate + 200 ng/mL AA + 1x NEAA + 500 ng/mL dcAMP + 1x P/S + 10 ng/mL BDNF + 10 ng/mL GDNF	Cerebral organoid differentiation medium containing vitamin A
Analysis & summary		
Marker analysis of organoid cellular identity in protocol and original publication	D28 cerebral organoids: neural progenitor cells (SOX2), dorsal region marker (PAX6), ventral region marker (NKX2.1), intermediate progenitors (TBR2), deep cortical layer neurons (CTIP2), early neurons (TUJ1), astrocytes (GFAP); D84 cerebral organoids: superficial cortical layer neurons (SATB2), deep cortical layer neurons (CTIP2), astrocytes (GFAP, S100β)	1–2-month-old COs: progenitors (SOX2), neurons (Tuj1 or DCX), forebrain (Foxg1), choroid plexus (TTR), hippocampus (Prox1 and Fzd9), mitotic radial glia (P-vim), cortical layer neurons (CTIP2 and SATB2)
Functional analysis of organoids	Assessment of the expression of mature neuronal markers (MAP2, CTIP, SATB2) pre- and post-synaptic markers (synaptophysin and PSD95) by immunoblot. No direct assessment of organoid function (e.g., electrophysiology).	Calcium imaging (in original publication)
Additional protocols described in STAR Protocols paper	Guide RNA design, cloning oligos into px459 plasmid, human iPSC electroporation, genotyping to identify gene edited clones, neural marker immunostaining, gene knockout efficiency analysis by RT-qPCR	N/A
Protocol applications	Modeling aspects of human brain and assessment of gene function in brain development and disease	
Protocol advantages	Provides complete workflow for generating iPSC clones containing targeted INDELs for knockout studies, as well as protocols for quality assessment of targeted clones. Commercially sourced differentiation media simplifies organoid production and improves consistency. Simple, optimized workflow with access to technical support from differentiation kit vendor.	Provides in-depth and easy to follow protocol for generation and analysis of 3D cerebral organoids from hPSCs. Highlighted critical steps, provided representative images of optimal and suboptimal organoids at various stages, and compared with alternative methods
Protocol limitations	CO are not regionally restricted, containing tissue resembling multiple brain regions. These tissues develop stochastically and are highly heterogeneous, making COs challenging to use in some applications. Commercially sourced media adds cost to the protocol. Neither protocol nor original publication describe functional assessment of generated organoids	
Technical expertise needed[a]	Proficiency with undifferentiated hPSC culture is essential. Some experience with organoid production and manipulation is helpful but not necessary	Proficiency with undifferentiated hPSC culture is essential. Proficiency with organoid production and manipulation is useful
Additional protocols described	Guide RNA design, cloning oligos into px459 plasmid, human iPSC electroporation, genotyping to identify gene edited clones, neural marker immunostaining, gene knockout efficiency analysis by RT-qPCR	Cryosectioning and immunostaining of COs
Conclusion	These protocols are based on the seminal work of Lancaster et al., 2013 and organoids produced using this method have been widely used and extensively validated. The use of consistent commercially sourced media in Martens et al., 2021 protocol and associated technical support from the vendor is a significant advantage making this protocol an excellent choice for novices where "whole-brain" organoids are appropriate	

NB: in addition to protocol-specific details outlined below, there are many additional factors to consider when implementing an organoid generation protocol. These are reviewed in "Practical Considerations".

[a]Proficiency in the culture of high-quality undifferentiated hPSCs is essential for the success of any differentiation protocol and is thus a prerequisite for implementing all brain organoid protocols described. All organoid generation protocols described in this review can be implemented by skilled technical staff. However, differentiation can be highly variable from line-to-line, warranting optimization of the protocol for each line. Furthermore, the steps involved in some protocols are more complex than others (e.g., organoid fusion). Therefore, here we provide our assessment of the ideal level of technical expertise needed to successfully implement this protocol.

Since the initial establishment of brain organoid protocols, subsequent studies have further modified protocols to generate organoids with more specific brain regional identities using a combination of specific growth factors and signaling molecules or inhibitors resembling the *in vivo* regional brain developmental programs, as well as models with increased cellular diversity (Table 1). These directed differentiation protocols.[11,15] generate organoids with greater reproducibility, addressing the issue of morphological and cellular variability seen with the organoids generated *via* self-patterning, non-guided differentiation process.

Table 3. Generation of region-specific brain organoids

Organoid type: midbrain dopaminergic organoids (MBO)	
Protocol title	High-throughput generation of midbrain dopaminergic neuron organoids from reporter human pluripotent stem cells.
STAR Protocols reference	Sarrafha et al.[48]
Original publication	Ahfeldt et al.[49]
Protocol length (before long-term culture)	~6 weeks
Specialized equipment needed	Magnetic stir plate, spinner flasks
Protocol stages overview	1) 2D hPSC culture, 2) 3D hPSC culture, 3) Generation of 3D midbrain progenitor organoids, 4) Differentiation to 3D dopaminergic-neuron organoids, 5) Long-term culture
Protocol stage 1	**2D hPSC culture**
Stage timing	Feeder-free maintenance culture
Culture format	Cell culture dishes
Culture system/Culture medium	StemFlex (ThermoFisher) + Geltrex (ThermoFisher), Accutase clump passaging
Cell number/preparation for differentiation	Four 10-cm dishes in monolayer used to seed spinner flasks @ ~40x10e6 per flask
Protocol stage 2	**3D hPSC culture**
Stage timing	6 days
Culture format	Spinner flask
Culture system/Culture medium	StemFlex (ThermoFisher)
Protocol stage 3	**Generation of 3D midbrain progenitor organoids (MBPOs)**
Stage timing	12 days
Culture format	Spinner flask
Culture medium, growth factors and small molecules	D0-1: DMEM-F12 medium + 1x Glutamax + 1x B27 Supplement minus vitamin A (B27-RA) + 1x N-2 supplement (N2) + 100 nM LDN193189 (LDN) + 10μM SB431542 (SB) D2-3: DMEM-F12 medium + 1x Glutamax + 1x B27-RA + 1x N2 + 100nM LDN + 10μM SB + 2μM Purmorphamine (PMA) + 1μM Smoothened Agonist (SAG) D4-7: DMEM-F12 medium + 1x Glutamax + 1x B27-RA + 1x N2 + 100nM LDN + 10μM SB + 2μM PMA + 1μM SAG + 1.5μM CHIR99021 (CHIR) D8-11: DMEM-F12 medium + 1x Glutamax + 1x B27-RA + 1x N2 + 100nM LDN + 1.5μM CHIR
Protocol stage 4	**Differentiation to 3D MBOs**
Stage timing	24 days
Culture format	Spinner flask
Culture medium, growth factors and small molecules	D12-35: DMEM-F12 medium + 1x Glutamax + 1x B27-RA + 1x N2 + 20 ng/mL Brain-derived neurotrophic factor (BDNF) + 20 ng/mL Glial cell line-derived neurotrophic factor (GDNF) + 0.2mM Ascorbic Acid (AA) + 10μM DAPT + 0.1mM Dibutyryl-Cyclic Adenosine Monophosphate (db-cAMP)
Protocol stage 5	**Long-term culture**
Stage timing	Up to 200 days
Culture format	Spinner flask
Culture medium, growth factors and small molecules	D36+: DMEM-F12 medium + 1x Glutamax + 1x B27-RA + 1x N2 + 20 ng/mL BDNF + 20 ng/mL GDNF + 0.2mM AA
Analysis & summary	
Cell types generated	D15: midbrain progenitor organoids: dopaminergic (DA) neurons (TH), midbrain progenitors (FoxA2, LMX1A); D20 midbrain progenitor organoids: TH midbrain neurons (TH, TUBB3); D30 dopaminergic neuron organoids: DA neurons (TH, TUBB3, NURR1, GIRK2); D80: midbrain DA neuron organoid: astrocytes (GFAP); D200: midbrain DA neuron organoid: (TH, Melanin)
Additional information	Midbrain organoids can be plated on PLO-LN plates to generate DA-neuron outgrowths, or dissociated to single cells and plated on human astrocytes
Functional analysis of organoids	Whole patch clamp recordings showing voltage-gated sodium and potassium currents, and evoked and spontaneous action potential generation of isolated TH-neurons from midbrain organoids. Live Ca2+ imaging showing spontaneous tetrodotoxin-sensitive activity
Protocol applications	Analysis of human midbrain development; identification of pathophysiological mechanisms in disease affecting midbrain (e.g., Parkinson's disease)
Protocol advantages	Protocol facilitates scalability and high throughput midbrain organoid production
Protocol limitations	Expensive relative to other midbrain organoid protocols due to large volume of media/growth factors used for differentiation. However, protocol can be scaled down to 6-well ultra-low attachment plates on orbital shaker

(*Continued on next page*)

Table 3. *Continued*

Technical expertise needed[a]	Proficiency with undifferentiated hPSC culture is essential. Proficiency with organoid production and manipulation is useful
Additional protocols described in STAR Protocols paper	Reporter iPSC line generation by CRISPR/Cas
Conclusion	Protocol generates MBOs that express midbrain markers and contain DA neurons exhibiting electrophysiological activity and astrocytes. However, the Zagare protocol described below appears to generate organoids containing more DA neurons (>60% at D30) than this protocol (plateau at ~30% by D40. MBOs generated with this method contain astrocytes, but no oligodendrocyte formation is described. This protocol facilitates significant scale up using spinner flasks (although increased media consumption will increase cost), however the protocol is also amenable to scale down in 6-well plates. We recommend implementing and optimizing this protocol in 6-well plates before scaling up. Technically, this protocol will present few difficulties to users with experience culturing and differentiating hPSCs, especially those familiar with organoid production.
Organoid type: MBO	
Protocol title	A robust protocol for the generation of human midbrain organoids
STAR protocols reference	Zagare et al.[50]
Original publication	Monzel et al.[51]
Protocol length (before long-term culture)	~4 weeks (cryopreserved NSCs), ~10 weeks (new derivation)
Specialized equipment needed	Multichannel pipet (optimally), horizontal shaker
Protocol stages overview	1) 2D hPSC culture, 2) 3D embryoid body (EB) formation 3) 2D NSC culture, 4) Generation of 3D NSCs, 5) Embedding 3D NSCs in Geltrex, 6) Differentiation to 3D midbrain organoids, 7) Long-term culture
Protocol stage 1	**2D hPSC culture** (Reinhardt et al.[52])
Stage timing	Feeder-dependent maintenance culture
Culture format	Cell culture dishes
Culture system/culture medium	DMEM-F12 medium + 20% knockout serum replacement + 1 mM BME + 1% non-essential amino acids (NEAA), 1% P/S + 1% L-Glu + 5 ng/mL Basic Fibroblast Growth Factor (bFGF). Inactivated MEFs substrate. Collagenase IV for routine passaging and EB generation.
Protocol stage 2	**3D EB generation** (Reinhardt et al.[52])
Stage timing	6 days
Culture format	10 cm petri dishes
Culture system/culture medium	D1-2: DMEM-F12 medium+ 20% knockout serum replacement + 1 mM BME + 1% non-essential amino acids (NEAA), 1% P/S + 1% L-Glu + 1μM Dorsomorphin (DM) + 3μM CHIR + 0.5μM PMA D3-4: DMEM/F12 medium + Neurobasal medium (1:1) + 2mM L-Glu + 0.5x N2 + 1x B27-RA + 1x P/S + 1μM Dorsomorphin (DM) + 3μM CHIR + 0.5μM PMA D5-6: DMEM-F12 medium + Neurobasal medium + 2mM L-Glu + 0.5x N2 + 1x B27-RA + 1x P/S + 0.5μM PMA + 150μM AA. EBs triturated to break into smaller pieces.
Protocol stage 3	**2D NSC culture**
Stage timing	2 months (new derivation), 2 weeks (cryopreserved NSCs)
Culture format	Cell culture dishes
Culture medium, growth factors and small molecules	DMEM/HAM's F12 medium + Neurobasal medium (1:1) + 2mM Glutamax + 1x Penicillin/Streptomycin (P/S) + 0.5x N2 + 1x B27-RA + 3μM CHIR + 0.75μM PMA + 150μM AA. Matrigel or Geltrex-coated plates. Single cells prepared by Accutase passaging
Cell number/preparation	9,000 NSCs required per well of a 96-well ultra-low attachment (ULA) plate
Protocol stage 4	**Generation of 3D NSCs**
Stage timing	6 days
Culture format	96-well U-bottomed ULA (D0-5), 24-well cell culture plate (D6-7)
Culture medium, growth factors and small molecules	N2B27 maintenance medium: DMEM/HAM's F12 medium + Neurobasal medium (1:1) + 2mM Glutamax + 0.5x N2 + 1x B27-RA + 1x P/S
Protocol stage 5	**Embedding 3D NSCs in Geltrex**
Stage timing	2 days
Culture format	24-well cell culture plate, embedded in Geltrex
Culture medium, growth factors and small molecules	N2B27 maintenance medium
Protocol stage 6	**Differentiation to 3D MBOs**
Stage timing	6 days

(*Continued on next page*)

Table 3. *Continued*

Culture format	24-well cell culture plate, embedded in Geltrex; plate placed on horizontal shaker (80 rpm) for remainder of protocol starting on d14
Culture medium, growth factors and small molecules	N2B27 maintenance medium + 10 ng/mL BDNF + 10 ng/mL GDNF + 200μM AA + 1 ng/mL Transforming Growth Factor-β3 (TGFβ3) + 500μM db-cAMP + 1μM PMA
Protocol stage 7	**Long-term culture**
Stage timing	Up to 1 year
Culture format	24-well cell culture plate, embedded in Geltrex
Culture medium, growth factors and small molecules	N2B27 maintenance medium + 10 ng/mL BDNF + 10 ng/mL GDNF + 200μM AA + 1 ng/mL TGFβ3 + 500μM db-cAMP
Analysis & summary	
Marker analysis of organoid cellular identity in protocol and original publication	D0: NSCs (NESTIN, SOX2, PAX6); D30 midbrain organoids: DA neurons (TUJ1B, TH, NURR1); D44 midbrain organoids: oligodendrocytes (O4); D60 midbrain organoids: DA neurons (FOXA2, LMX1A, TH, NURR1, DAT, DDC), astrocytes (S100b, GFAP); D149 DA neurons: neuromelanin granules (Fontana Masson staining)
Functional analysis of organoids	Synaptic connections determined by detection of SYP-positive pre-synapses directly contacting PSD95-positive post-synapses by immunoanalysis; Spontaneous electrophysiological activity detected by Ca2+ imaging and multi-electrode array analysis; dopamine production confirmed by immunoanalysis; Oligodendrocyte function demonstrated by ensheathment of TUJ1-positive neurites by myelin sheets of CNPase+/MBP + oligodendrocytes
Protocol applications	Analysis of human midbrain development; identification of pathophysiological mechanisms in disease affecting midbrain (e.g., Parkinson's disease)
Protocol advantages	NSCs can be cryopreserved and thawed for organoid production, enhancing consistency if using quality-controlled banks of cryopreserved NSCs. Oligodendrocytes co-develop with neurons.
Protocol limitations	Utilizes undefined hPSC culture media and feeders. Requires production of NSCs from hPSCs (~2 months) or prior production and cryopreservation of NSCs.[52] Requires time-consuming embedding 3D NSC spheres in semi-solid matrix (Geltrex).
Technical expertise needed[a]	Proficiency with undifferentiated hPSC culture is essential. Some experience with organoid production and manipulation is helpful but not necessary
Additional protocols described in STAR Protocols paper	Derivation of neural stem cells (NSCs)
Conclusion	Protocol describes generation of MBOs that express midbrain markers and contain DA neurons exhibiting electrophysiological activity. This protocol generates organoids containing a higher percentage of DA neurons (>60% at D30) than the Sarrafha protocol (plateau at ~30% by D40). MBOs contain both astrocytes and oligodendrocytes and are amenable to long-term culture. Although this protocol requires production of NSCs, these can be cryopreserved and thawed for organoid production, facilitating increased reproducibility as well as organoid generation without a need to consistently culture hPSCs. Technically, this protocol will present few difficulties to users with experience culturing and differentiating hPSCs, especially those familiar with organoid production. However, the undefined nature of the hPSC culture system described for generating NSCs may make implementation of this protocol somewhat more challenging
Organoid type: thalamic organoids (ThOs)	
Protocol title	Generation of Regionally Specified Human Brain Organoids Resembling Thalamus Development
STAR Protocols reference	Xiang et al.[53]
Original publication	Xiang et al.[24]
Protocol length (before long-term culture)	16 days
Specialized equipment needed	Orbital shaker
Protocol stages overview	1) 2D hPSC culture, 2) Neural induction, 3) Thalamic patterning, 4) Neural maturation and long-term culture
Protocol stage 1	**2D hPSC culture**
Stage timing	Feeder-free maintenance culture
Culture format	Cell culture dishes
Culture system/Culture medium	mTeSR1, Matrigel, dispase clump passaging
Cell number/preparation for differentiation	9,000 cells per well of a ULA 96-well U-bottomed plate. Single cells prepared by Accutase
Protocol stage 2	**Neural induction**
Stage timing	8 days
Culture format	EB formation in ULA 96-well U-bottomed plate

(*Continued on next page*)

Table 3. *Continued*

Culture medium, growth factors and small molecules	DMEM-F12 medium + 15% Knockout serum replacement + 1x NEAA+ 1x Glutamax + 100μM BME + 100nM LDN + 10μM SB+ 4 μg/mL Insulin (INS)
Protocol stage 3	**Thalamic patterning**
Stage timing	8 days
Culture format	Transfer EBs to ULA 24-well plate and incubate on an orbital shaker
Culture medium, growth factors and small molecules	DMEM-F12 medium + 0.15% (w/v) Dextrose + 100μM BME + 1% N2 + 2% B27-RA + 30 ng/mL Bone Morphogenetic Protein 7 (BMP7) + 1μM PD325901 (PD)
Protocol stage 4	**Neural maturation and long-term culture**
Stage timing	Up to 1 year
Culture format	6-well plate on orbital shaker
Culture medium, growth factors and small molecules	DMEM-F12 medium + Neurobasal medium (1:1) + 0.5% N2 + 1% B27 + NEAA + 1% Glutamax + 1% P/S+ 0.025% INS + 50μM BME + 20 ng/mL BDNF + 200μM AA
Analysis & Summary	
Marker analysis of organoid cellular identity in protocol and original publication	D60 thalamic organoids: caudal forebrain (OTX2); ventral thalamus (DBX1); thalamus marginal zone (GBX2); thalamus (TCF7L2). D89: Astrocytes (GFAP, EAAT1). scRNA-seq analysis identified cell types human fetal thalamus cell types
Functional analysis of organoids	Spontaneous action potential production detected by whole-cell patch clamp analysis and Ca2+ imaging (GCaMP6s); and synapse formation by immunoanalysis demonstrating adjacency of presynaptic marker SYP and postsynaptic marker PSD95. Axonal projections to and from fused COs
Protocol applications	Analysis of thalamus development and disease; mechanisms of axon targeting and synapse formation (following fusion with cortical organoids)
Protocol advantages	Thalamic organoids can be fused with other region-specific brain organoids to model axonal connections and function of neural circuits between the thalamus and different regions in the brain.
Protocol limitations	Challenging to generate specific thalamic nuclei
Technical expertise needed[a]	Proficiency with undifferentiated hPSC culture is essential. Some experience with organoid production and manipulation is helpful but not necessary
Conclusion	Protocol generates thalamic organoids containing fetal thalamic cell types and electrophysiologically active thalamic neurons. Technically, the production of thalamic organoids is straightforward, although fusion with other organoid types is more challenging

[a]Proficiency in the culture of high-quality undifferentiated hPSCs is essential for the success of any differentiation protocol and is thus a prerequisite for implementing all brain organoid protocols described. All organoid generation protocols described in this review can be implemented by skilled technical staff. However, differentiation can be highly variable from line-to-line, warranting optimization of the protocol for each line. Furthermore, the steps involved in some protocols are more complex than others (e.g., organoid fusion). Therefore, here we provide our assessment of the ideal level of technical expertise needed to successfully implement this protocol.

Existence of functional neural networks required for producing complex brainwaves in organoids is a significant advance in brain organoid technology and could have huge implications for studying neuronal processes in normal vs disease state.[23] Moreover, the approach of fusing brain region-specific organoids expands the applicability of organoids to study more brain disorders by allowing studies of brain regional interconnectivity and neural circuit formation in a dish.[69]

FUTURE PERSPECTIVES

The advent of brain organoid technology has heralded a new era for human brain biology and neurological disease modeling that would not have been possible little more than a decade ago. Brain organoids provide a powerful human-specific platform to study mechanisms of normal brain development, interrogate complex disease phenotypes of various neurological disorders, and facilitate the discovery of novel neurotherapeutics. Organoids are chronologically relevant models to study brain development for their ability to be maintained for long periods of time in culture to achieve maturation milestones necessary for investigating late-stage processes such as gliogenesis, axonal myelination, neuronal migration, and connectivity, which have been previously difficult to study *in vitro*. Additionally, brain organoids have been used as experimental models to understand the pathological effects of viruses on the central nervous system, such as Zika[21] and more recently SARS-CoV-2.[70] Brain organoids have also been used for performing comparative studies in other primates to understand the evolution of human brain.[71,72]

Table 4. Generation of fused organoids (assembloids)

Assembloid type: Fusion of neural and mesenchymal organoids to form vascularized neural organoids		
Protocol title	Generation of Vascularized Neural Organoids by Co-culturing with Mesodermal Progenitor Cells	
STAR protocols reference	Worsdorfer et al.[54]	
Original publication	Worsdorfer et al.[36]	
Protocol length (before long-term culture)	~4 weeks	
Specialized equipment needed	2D rocking plate	
Protocol stages overview	1) hPSC culture, 2) Generation of neuro-mesenchymal organoids, 3) Assembly of Neural and Mesenchymal aggregates, 4) Culture neuromesenchymal organoids for vascular network formation, 5) Maturation of vascularized neuromesenchymal organoids, 6) Long-term culture	
Protocol stage 1	**2D hPSC culture**	
Stage timing	Feeder-free maintenance culture + 3 days	
Culture format	Cell culture dishes	
Culture system/Culture medium	StemMACS iPSC Brew Medium, Matrigel, Accutase single cell passaging	
Cell number/preparation for differentiation	4,000 cells/well of 6-well plate. hPSCs should be at ~80% confluency before seeding organoid formation.	
Protocol stage 2	**Generation of neuro-mesenchymal organoids**	
	Neural organoids	Mesenchymal organoids
Stage timing	8 days	4 days
Timing note	The timing of neural and mesenchymal organoid induction should be coordinated to be completed on the same day	
Culture format	Agarose-coated F-bottom 96-well plate (agarose to prevent attachment)	
Culture medium, growth factors and small molecules	D1-2: DMEM-F12 medium + Neurobasal medium (1:1) + 1x B27-RA + 1x N2 + 2mM L-Glu + 3μM CHIR + 10μM SB + 1μM DM + 0.5μM PMA D3-5: DMEM-F12 medium + Neurobasal medium (1:1) + 1x B27-RA+ 1x N2 + 2mM L-Glu + 3μM CHIR + 62.5 μg/mL AA + 0.5μM PMA D6-7 Neural differentiation medium: DMEM-F12 medium + Neurobasal medium (1:1) + 1x B27-RA+ 1x N2 + 2mM L-Glu + 62.5 μg/mL AA + 1x P/S	Advanced DMEM-F12 medium + 2mM L-Glu + 62.5 μg/mL AA + 10μM CHIR + 25 ng/mL Bone Morphogenetic Protein 4 (BMP4)
Protocol stage 3	**Assembly of Neural and Mesenchymal aggregates**	
Stage timing	2 days	
Assembly approach	Manually transfer a single d4 mesodermal organoid into each well of an F-bottom 96-well plate already containing a d7 neural organoid. Incubate for 2 days before embedding in Matrigel in stage 4	
Culture format	Agarose-coated F-bottom 96-well plate (agarose to prevent attachment)	
Culture medium, growth factors and small molecules	Neural differentiation medium	
Protocol stage 4	**Embed neuromesenchymal organoids for vascular network formation**	
Stage timing	20 days	
Culture format	Assembloids embedded in Matrigel in 96-well plate D9. Transferred to 10cm dish on day 10	
Culture medium, growth factors and small molecules	Neural differentiation medium	
Protocol stage 5	**Maturation of vascularized neuromesenchymal organoids**	
Stage timing	Up to 210 days	
Culture format	10 cm dish on 2D rocking plate	
Culture medium, growth factors and small molecules	Neural differentiation medium	
Analysis & Summary		
Marker analysis of organoid cellular identity in protocol and original publication	D20 assembloids: neurons (TUJ1, MAP2), endothelial cells (CD31), basement membrane (COL I, COL IV), peri-endothelial cells (SMA); D210 vascularized neural organoids: neuroepithelial cells (SOX1, Nestin), neurons (MAP2), radial glia cells or astrocytes (GFAP), microglia-like cells (IBA1)	
Functional analysis of organoids	*In vitro* differentiation of mesodermal progenitors to smooth muscle and endothelial cells. Formation of vasculature containing endothelial cell-cell junctions, a collagen IV-positive basement membrane and peri-endothelial cells.	
Protocol applications	Facilitates analysis of role of vascularization and potentially other stromal cell types in neural development	
Protocol advantages	Additional organoid complexity *via* incorporation of vascularity more closely mimics human brain cellular composition. Mesodermal organoids provide a source of additional stromal cell types including microglia-like cells. Mesodermal organoids can be mixed with tumor cells to generate vascularized tumor models and form connections with existing blood vessels when fused with chick chorion allantois membrane.	

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Table 4. *Continued*

Protocol limitations	Regional identity of neural organoids is not described. Variable and only partial vascularization of neural components in assembloids. Vasculature that forms is immature	
Technical expertise needed[a]	Proficiency with undifferentiated hPSC culture is essential. Proficiency with organoid production and manipulation is useful	
Additional protocols described in STAR Protocols paper	Paraffin sectioning and histological analysis, H&E staining, immunofluorescence analysis, tissue clearing for microscopic analysis	
Conclusion	Protocol generates partially vascularized organoids with undefined neural identity, but the additional cellular complexity achieved by fusing neural and mesodermal organoids may be useful for studying the role of specific mesenchymal cell types in early stages of brain development and development of neurovasculature. Technically, this protocol will present few difficulties to users with experience culturing hPSCs and with organoid production	
Assembloid type: Fusion of dorsal and ventral forebrain organoids to model oligodendroglial development and myelination		
Protocol title	Generation of human Pluripotent stem cell-derived fused organoids with oligodendroglia and myelin	
STAR Protocols reference	Kim and Jiang[55]	
Original publication	Kim et al.[56]	
Brain organoid type(s)	Ventral (VFO) and dorsal (DFO) forebrain organoids	
Protocol length (before long-term culture)	12 weeks	
Specialized equipment needed	Orbital shaker	
Protocol stages overview	1) hPSC culture, 2) Generation and culture of NSCs, 3) Organoid formation from NSCs, 4) Generation of ventral and dorsal forebrain organoids, 5) Oligodendroglial and neuronal differentiation, 6) Neuronal maturation of DFOs, 7) Assembly of VFOs and DFOs	
Protocol stage 1	**hPSC culture** (Chen et al.[57])	
Stage timing	Feeder-free maintenance culture	
Culture format	Cell Culture dishes	
Culture system/Culture medium	mTeSR1, Matrigel, dispase clump passaging	
Protocol stage 2	**Generation and culture of NSCs** (Chen et al.[57])	
Stage timing	4 weeks	
Culture format	EB formation in ULA plate, Rosette formation in cell culture dishes, ULA dishes, NSC formation in cell culture dishes	
Culture medium, growth factors and small molecules	D1-7 (neural induction): DMEM-F12 medium + 1x N2 + 5μM SB + 50 ng/mL Noggin D8-14 (rosette formation): DMEM-F12 medium + 1x N2 + 1 μg/mL Laminin D15-D28 (NSC expansion): DMEM-F12 medium + Neurobasal medium (1:1) + 1x N2 + 1x B27-RA + 20 ng/mL bFGF + 10 ng/mL Leukemia Inhibitory Factor (LIF) + 3μM CHIR + 2μM SB + 10μM Y27632	
Cell number/preparation for differentiation	9,000 NSCs, prepared by TrypLE passaging to single cells, plated per well of a 96-well ULA plate	
Protocol stage 3	**Cerebral organoid formation from NSCs**	
Stage timing	4 days	
Culture format	96-well ULA plate (2 days), 6-well ULA plate on an orbital shaker (2 days)	
Culture medium, growth factors and small molecules	DMEM-F12 medium + Neurobasal medium (1:1) + 1x N2 + 1x B27-RA + 20 ng/mL bFGF + 10 ng/mL LIF + 3μM CHIR 99021 + 2μM SB + 1x P/S	
Protocol stage 4	**Generation of regionally patterned forebrain organoids**	
	Ventral forebrain organoids (VFOs)	Dorsal forebrain organoids (DFOs)
Stage timing	2 weeks	2 weeks. DFOs produced at the end of this stage are fused with VFOs produced in stage 6
Culture format	6-well ULA plate on an orbital shaker	6-well ULA plate on an orbital shaker
Culture medium, growth factors and small molecules	DMEM-F12 medium + Neurobasal medium (1:1) + 1x N2 + 1x B27-RA + 20 ng/mL bFGF + 50 ng/mL SHH + 1 μM PMA + 10μM Y27632 + 1x P/S	DMEM-F12 medium + Neurobasal medium (1:1) + 1x N2 + 1x B27-RA + 20 ng/mL bFGF + 5μM Cyclopamine A (CycA) 10μM Y27632 + 1x P/S
Protocol stage 5	**Oligodendroglial differentiation**	**Neuronal differentiation**
Stage timing	2 weeks	2 weeks
Culture format	6-well ULA plate on an orbital shaker	6-well ULA plate on an orbital shaker
Culture medium, growth factors and small molecules	OPC medium: DMEM-F12 medium + 1x N2 + 1x B27-RA + 10 ng/mL PDGF-AA + 10 ng/mL bFGF + 1x P/S	ND medium: DMEM-F12 medium + Neurobasal medium (1:1) + 1x N2 + 1x B27-RA + 10 ng/mL GDNF + 10 ng/mL BDNF + 1μM cAMP + 200nM AA+ 1x P/S
Protocol stage 6	**Neuronal maturation of DFOs**	
Stage timing	2 weeks. DFOs produced at the end of this step are fused with VFOs produced in stage 4	
Culture format	6-well ULA plate on an orbital shaker	

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Table 4. *Continued*

Culture medium, growth factors and small molecules	BrainPhys neuronal medium
Note	BrainPhys medium is available commercially from StemCell Technologies. While this media is based on published recipes,[58] the precise formulation is proprietary
Protocol stage 7	**Assembly of VFOs and DFOs**
Stage timing	2 days (week 2 VFOs and week 6 DFOs are used for assembly)
Assembly approach	Manually transfer a single VFO and a single DFO into each well of a 96-well ULA round-bottomed plate. Incubate for 2h without agitation and then 8h with hourly gentle trituration of media without disturbing organoids. Transfer to 6 well ULA plate for remainder of protocol
Culture format	96-well ULA round-bottom plate for fusion and transferred to 6 well ULA plate on orbital shaker
Culture medium, growth factors and small molecules	1:1 ratio of OPC and ND medium
Protocol stage 8	**Myelination and maturation**
Stage timing	Up to 6 weeks
Culture format	6 well ULA plate on orbital shaker
Culture medium, growth factors and small molecules	OL medium: DMEM-F12 medium + Neurobasal medium (1:1) + 1x N2 + 1x B27-RA + 1x P/S + 10 ng/mL GDNF + 10 ng/mL BDNF + 1μM cAMP + 200nM AA + 10 ng/mL Triiodo-L-thyronine (T3) Feed fused organoids with a 1:1 ratio of OL and ND medium
Analysis & Summary	
Marker analysis of organoid cellular identity in protocol and original publication	D0 NSCs (PAX6). Week 2 ventral forebrain (NKX2.1, NKX2.2, DLX1, LHX6) and dorsal forebrain (PAX6, EMX1, TBR2). Week 4 oligodendrocytes (OLIG2), astrocytes (S100β), neurons (βIII-TUBULIN). Week 6 DFOs (c-FOS, SYNAPSIN 1, PSD-95). Week 9 oligodendrocytes (PDGFR, MBP), excitatory post-synaptic components (HOMER1, SHANK3), inhibitory post-synaptic components (ARHGEF9, GPHN)
Functional analysis of organoids	Spontaneous action potential production in functional neurons and glia detected by Ca2+ imaging. Myelination of neurons by electron microscopy
Protocol applications	Study of the mechanisms of human oligodendrogenesis, migration and myelination in ventral and dorsal forebrain. Study of diseases of defective myelination
Protocol advantages	NSCs can be cryopreserved and thawed for organoid production, enhancing consistency if using quality-controlled banks and avoiding the need for continual culture of undifferentiated hPSCs. The use of VFOs and DFOs may permit analysis of the first 2 oligodendrogenic waves identified in mouse brain. Analysis of fused VFOs and DFOs permits analysis of the third wave of oligodendrogenesis.
Protocol limitations	Most oligodendrocytes do not form compact myelin sheaths in organoids. Necrotic core forms in fused organoids
Technical expertise needed[a]	Proficiency with undifferentiated hPSC culture is essential. Proficiency with organoid production and manipulation is useful
Conclusion	Protocol generates forebrain organoids that recapitulate oligodendrogenesis, promote oligodendrocyte maturation, and demonstrate functional myelination. Technically, this protocol will present few difficulties to users with experience culturing hPSCs and with organoid production

[a]Proficiency in the culture of high-quality undifferentiated hPSCs is essential for the success of any differentiation protocol and is thus a prerequisite for implementing all brain organoid protocols described. All organoid generation protocols described in this review can be implemented by skilled technical staff. However, differentiation can be highly variable from line-to-line, warranting optimization of the protocol for each line. Furthermore, the steps involved in some protocols are more complex than others (e.g., organoid fusion). Therefore, here we provide our assessment of the ideal level of technical expertise needed to successfully implement this protocol.

Researchers now have access to patient-specific human brain models, which presents a useful and clinically relevant strategy to study brain disorders. As we look to the future, by combining brain organoid and genome editing technologies, disease-associated mutations can be introduced or corrected in patient-derived iPSCs, and subsequently be differentiated into brain organoids for functional analyses and small molecule screening. Moreover, single cell sequencing and protein analysis tools can be applied to organoids to decipher disease mechanisms of neurological disorders. These advances highlight the exciting prospects for their application towards drug discovery, personalized medicine and possibly cell therapy. The biological questions that can be applied to brain organoids will only increase as we gain deeper insights into experimentally manipulating and scaling these mini-tissues and make rapid progress towards generating high-quality and reproducible models as well as developing more specialized model subtypes. As brain organoid

Table 5. Application of brain organoids in disease modeling

Glioblastoma (GBM) tumors			
	Oudin et al.[59]	Gamboa et al.[60]	Linkous and Fine, 2020[61]
Model type	1. Patient derived organoids (PDO) 2. Patient derived orthotopic xenografts (PDOX)	1. Glioma stem cells (GSC) 2. PDO 3. PDOX	Glioma cerebral organoid (GLICO)
Original publication(s)	Golebiewska et al.[62]	Chadwick et al.[63]	Linkous et al.[39] Pine et al.[64]
Methods details			
• Tumor tissue processing	Mechanical	Mechanical + Enzymatic	Mechanical + Enzymatic
• Post tissue processing	Small tissue fragments	Single cell suspension	Single cell suspension to derive GSCs
• Culture media	With serum	Serum free + growth factors	Serum free + growth factors
• Model establishment	PDO – Agar coated flasks PDOX – Intracranial transplantation	PDO – Matrigel droplet	GLICO – Co-culture GSCs with cerebral organoids
Phenotypes observed	Recapitulate histological, genetic, epigenetic, and transcriptomic features of patient tumors	Recapitulate key expression profiles and tumor cell phenotypes seen in GBM patients	Recapitulate invasive behavior (tumor microtubes), molecular heterogeneity and transcriptional cellular states found in primary GBM tumors
Tumor formation timeline	PDO – 7–14 days PDOX – up to16 months	GSC – 7 days PDO – 2–12 weeks PDOX – 2–12 weeks	GLICO – 2–14 days
Additional protocols described in STAR Protocols paper provided	Intracranial implantation, MRI imaging of tumor growth *in vivo*, PDOX tumor processing and purification of human tumor cells	RNA extraction, RNA sequencing, qPCR, IF, IHC, drug synergy assays	Development of cerebral organoids from hESCs
Technical expertise needed[a]	Knowledge of advanced stem cell/organoid culture techniques; training/certification to work with patient specimens; mouse skull anatomy, intracranial implantation procedure and animal xenograft studies (for Oudin and Gamboa protocols)		
Specialized equipment needed	Surgical instruments and reagents, stereotactic apparatus with drill and automated injector pump, MRI machine or IVIS system	Orbital shaker	
Advantages	These protocols provide clinically relevant 3D glioma models to interrogate key aspects of glioma biology and preclinical drug testing in a patient-specific manner. GLICO and PDOX models allow for assessing the cross talk of tumor with its microenvironment. GSCs and PDOs offer the advantage of cryopreservation for downstream applications.		
Limitations	These protocols are limited by the quality and quantity of tumor tissue, and rate of success varies between patients. Necrotic or less proliferative tumor fragments may yield organoids of poor quality, therefore close coordination with neurosurgeons/neuropathologists for providing biopsies with high tumor content (preferably proliferative in nature) is necessary. Additionally, optimal tissue storage upon surgical removal and immediate transfer to the tissue processing facility should be prearranged to keep the tissue viable. Processing fresh tissue increases the likelihood of establishment of successful organoids.		
Alzheimer's disease (AD)			
	Martens et al.[45]		
Model type	Cerebral organoid		
Original publication	Zhao et al.[47]		
Method details	Timeline	Steps	
	1 week	gRNA/Cas9 cloning	
	1–2 weeks	iPSC electroporation	
	3–4 weeks	Single cell clone isolation and expansion	
	3–4 weeks	Positive clone selection and characterization	
	3–4 months	$APOE^{-/-}$ iPSC-derived cerebral organoid culture (covered in detail in Table 2)	
	2 weeks	Analyses of $APOE^{-/-}$ COs	
Phenotypes observed	$APOE^{-/-}$ COs show greater apoptosis and synaptic loss and have increased levels of Aβ and phosphorylated tau.		

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Table 5. *Continued*

Additional protocols described in STAR Protocols paper provided	Design of gRNAs; PCR analysis of iPSC clones, iPSC characterization - karyotyping analysis, immunostaining of pluripotency markers and differentiation into 3 germ layers; immunostaining for neural markers for CO validation, RT-qPCR and western blotting for validation of APOE$^{-/-}$ COs	
Technical expertise needed[a]	Knowledge of CRISPR/Cas9 technology and advanced stem cell/organoid culture techniques	
Specialized equipment needed	Thermocycler, Invitrogen Neon™ transfection system or similar system for electroporation, gel electrophoresis equipment, Cytovision GSL-120 scanner, orbital shaker, iCycler thermocycler or similar RT-qPCR system, western blotting equipment	
Advantages	Provides a useful protocol for CRISPR-Cas9-based genetic editing of human iPSCs and generating iPSC-derived cerebral organoids to study gene function and consequent mechanisms in disease pathology	
Limitations	Using iPSCs for cerebral organoid production can be challenging, especially for beginners as the differentiation can be highly variable between iPSC lines used, thus warranting optimization of the protocol for each line.	
Traumatic brain injury (TBI)		
	Ramirez et al.[65]	
Model type	Cerebral organoid	
Original publication	Ramirez et al.[66]	
Method details	Timeline	Steps
• Fibroblast reprogramming to iPSCs	80 days	Fibroblast culture Reprogramming iPSC characterization
• Generation of COs	50 days and up	EB formation EB induction EB embedding Maturation
• Skull preparation	4–7 days	Skull cleaning Skull craniotomy and sealing Gas sterilization
• Phantom brain preparation	20 min	Gelatin and agarose mixture Skull filling with the phantom brain
• Controlled cortical impact (CCI)	2 h	Stereotaxic frame sterilization Positioning COs CCI procedure CO recovery and analysis
Phenotypes observed	CCI induced apoptosis in COs and recapitulated pathological features of TBI, including neuronal damage, neuron loss, and astrogliosis.	
Additional protocols described in STAR Protocols paper provided	Freezing, immunostaining and alkaline phosphatase staining of iPSCs; Formalin fixation, paraffin embedding and immunostaining of COs	
Technical expertise needed[a]	Knowledge of advanced stem cell/organoid culture techniques, mouse skull anatomy, stereotaxic frames, vernier scale, CCI procedures	
Specialized equipment needed	Orbital shaker, CCI device, H_2O_2 steamer	
Advantages	CO-based model of TBI is a useful system to understand TBI-induced brain damage when compared to 2D-systems and animal models. Spatial organization and cellular heterogeneity of COs allow the study of functional consequences of disease-associated gene variants and drug screening in a more realistic setting.	
Limitations	This protocol uses agarose-based polymer in an open skull. However, using closed skull and/or transplanting COs in live mouse brain will be more suitable to model TBI. Moreover, COs in their current form lack cellular and structural features to accurately model TBI pathology.	

[a]Proficiency in the culture of high-quality undifferentiated hPSCs is essential for the success of any differentiation protocol and is thus a prerequisite for implementing all brain organoid protocols described. All organoid generation protocols described in this review can be implemented by skilled technical staff. However, differentiation can be highly variable from line-to-line, warranting optimization of the protocol for each line. Furthermore, the steps involved in some protocols are more complex than others (e.g., organoid fusion). Therefore, here we provide our assessment of the ideal level of technical expertise needed to successfully implement this protocol.

protocols evolve to produce more complex and functional human neural tissues, ethical considerations related to consciousness and the treatment of research animals transplanted with human brain tissues, will become more acute.[73]

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

REFERENCES

1. Benito-Kwiecinski, S., and Lancaster, M.A. (2020). Brain organoids: human neurodevelopment in a dish. Cold Spring Harb Perspect Biol *12*, a035709. https://doi.org/10.1101/cshperspect.a035709.

2. Sidhaye, J., and Knoblich, J.A. (2020). Brain organoids: an ensemble of bioassays to investigate human neurodevelopment and disease. Cell Death Differ. *28*, 52–67. https://doi.org/10.1038/s41418-020-0566-4.

3. Qian, X., Song, H., and Ming, G.L. (2019). Brain organoids: advances, applications and challenges. Development *146*, dev166074. https://doi.org/10.1242/dev.166074.

4. Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T., and Sasai, Y. (2011). Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature *472*, 51–56. https://doi.org/10.1038/nature09941.

5. Kadoshima, T., Sakaguchi, H., Nakano, T., Soen, M., Ando, S., Eiraku, M., and Sasai, Y. (2013). Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. Proc. Natl. Acad. Sci. USA *110*, 20284–20289. https://doi.org/10.1073/pnas.1315710110.

6. Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature *501*, 373–379. https://doi.org/10.1038/nature12517.

7. Giandomenico, S.L., Mierau, S.B., Gibbons, G.M., Wenger, L.M.D., Masullo, L., Sit, T., Sutcliffe, M., Boulanger, J., Tripodi, M., Derivery, E., et al. (2019). Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. Nat. Neurosci. *22*, 669–679. https://doi.org/10.1038/s41593-019-0350-2.

8. Lancaster, M.A., Corsini, N.S., Wolfinger, S., Gustafson, E.H., Phillips, A.W., Burkard, T.R., Otani, T., Livesey, F.J., and Knoblich, J.A. (2017). Guided self-organization and cortical plate formation in human brain organoids. Nat. Biotechnol. *35*, 659–666. https://doi.org/10.1038/nbt.3906.

9. Qian, X., Su, Y., Adam, C.D., Deutschmann, A.U., Pather, S.R., Goldberg, E.M., Su, K., Li, S., Lu, L., Jacob, F., et al. (2020). Sliced human cortical organoids for modeling Distinct cortical layer formation. Cell Stem Cell *26*, 766–781.e9. https://doi.org/10.1016/j.stem.2020.02.002.

10. Quadrato, G., Nguyen, T., Macosko, E.Z., Sherwood, J.L., Min Yang, S., Berger, D.R., Maria, N., Scholvin, J., Goldman, M., Kinney, J.P., et al. (2017). Cell diversity and network dynamics in photosensitive human brain organoids. Nature *545*, 48–53. https://doi.org/10.1038/nature22047.

11. Velasco, S., Kedaigle, A.J., Simmons, S.K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., Regev, A., et al. (2019). Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. Nature *570*, 523–527. https://doi.org/10.1038/s41586-019-1289-x.

12. 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. https://doi.org/10.1038/s41592-018-0081-4.

13. Marton, R.M., Miura, Y., Sloan, S.A., Li, Q., Revah, O., Levy, R.J., Huguenard, J.R., and Pașca, S.P. (2019). Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. Nat. Neurosci. *22*, 484–491. https://doi.org/10.1038/s41593-018-0316-9.

14. Ormel, P.R., Vieira de Sa, R., van Bodegraven, E.J., Karst, H., Harschnitz, O., Sneeboer, M.A.M., Johansen, L.E., van Dijk, R.E., Scheefhals, N., Berdenis van Berlekom, A., et al. (2018). Microglia innately develop within cerebral organoids. Nat. Commun. *9*, 4167. https://doi.org/10.1038/s41467-018-06684-2.

15. Pasca, A.M., Sloan, S.A., Clarke, L.E., Tian, Y., Makinson, C.D., Huber, N., Kim, C.H., Park, J.Y., O'Rourke, N.A., Nguyen, K.D., et al. (2015). Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. Nat. Methods *12*, 671–678. https://doi.org/10.1038/nmeth.3415.

16. Sakaguchi, H., Kadoshima, T., Soen, M., Narii, N., Ishida, Y., Ohgushi, M., Takahashi, J., Eiraku, M., and Sasai, Y. (2015). Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. Nat. Commun. *6*, 8896. https://doi.org/10.1038/ncomms9896.

17. Birey, F., Andersen, J., Makinson, C.D., Islam, S., Wei, W., Huber, N., Fan, H.C., Metzler, K.R.C., Panagiotakos, G., Thom, N., et al. (2017). Assembly of functionally integrated human forebrain spheroids. Nature *545*, 54–59. https://doi.org/10.1038/nature22330.

18. Jo, J., Xiao, Y., Sun, A., Cukuroglu, E., Tran, H.D., Goke, J., Tan, Z., Saw, T., Tan, C.P., Lokman, H., et al. (2016). Midbrain-like organoids from human pluripotent stem cells contain functional dopaminergic and Neuromelanin-producing neurons. Cell Stem Cell *19*, 248–257. https://doi.org/10.1016/j.stem.2016.07.005.

19. Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K., and Sasai, Y. (2015). Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. Cell Rep. *10*, 537–550. https://doi.org/10.1016/j.celrep.2014.12.051.

20. Ozone, C., Suga, H., Eiraku, M., Kadoshima, T., Yonemura, S., Takata, N., Oiso, Y., Tsuji, T., and Sasai, Y. (2016). Functional anterior pituitary generated in self-organizing culture of human embryonic stem cells. Nat. Commun. *7*, 10351. https://doi.org/10.1038/ncomms10351.

21. Qian, X., Nguyen, H., Song, M., Hadiono, C., Ogden, S., Hammack, C., Yao, B., Hamersky, G., Jacob, F., Zhong, C., et al. (2016). Brain-region-specific organoids using mini-bioreactors for modeling ZIKV Exposure. Cell *165*, 1238–1254. https://doi.org/10.1016/j.cell.2016.04.032.

22. Schukking, M., Miranda, H.C., Trujillo, C.A., Negraes, P.D., and Muotri, A.R. (2018). Direct generation of human cortical organoids from primary cells. Stem Cells Dev *27*, 1549–1556. https://doi.org/10.1089/scd.2018.0112.

23. Trujillo, C.A., Gao, R., Negraes, P.D., Gu, J., Buchanan, J., Preissl, S., Wang, A., Wu, W., Haddad, G.G., Chaim, I.A., et al. (2019). Complex Oscillatory Waves emerging from cortical organoids model early human brain network development. Cell Stem Cell *25*, 558–569.e7. https://doi.org/10.1016/j.stem.2019.08.002.

24. Xiang, Y., Tanaka, Y., Cakir, B., Patterson, B., Kim, K.Y., Sun, P., Kang, Y.J., Zhong, M., Liu, X., Patra, P., et al. (2019). hESC-derived thalamic organoids form Reciprocal Projections when fused with cortical organoids. Cell Stem Cell *24*, 487–497.e7. https://doi.org/10.1016/j.stem.2018.12.015.

25. Mansour, A.A., Goncalves, J.T., Bloyd, C.W., Li, H., Fernandes, S., Quang, D., Johnston, S., Parylak, S.L., Jin, X., and Gage, F.H. (2018). An in vivo model of functional and vascularized human brain organoids. Nat. Biotechnol. *36*, 432–441. https://doi.org/10.1038/nbt.4127.

26. Wang, Z., Wang, S., Xu, T., Hong, C., Cheng, M., Zhu, P., Lin, J., Su, D., and Miao, C. (2020). Cerebral organoids transplantation improves neurological motor function in rat brain injury. CNS Neurosci. Ther. *26*, 682–697. https://doi.org/10.1111/cns.13286.

27. Cederquist, G.Y., Asciolla, J.J., Tchieu, J., Walsh, R.M., Cornacchia, D., Resh, M.D., and Studer, L. (2019). Specification of positional identity in forebrain organoids. Nat. Biotechnol. *37*, 436–444. https://doi.org/10.1038/s41587-019-0085-3.

28. Pellegrini, L., Bonfio, C., Chadwick, J., Begum, F., Skehel, M., and Lancaster, M.A. (2020). Human CNS barrier-forming organoids with cerebrospinal fluid production. Science *369*, eaaz5626. https://doi.org/10.1126/science.aaz5626.

29. Abud, E.M., Ramirez, R.N., Martinez, E.S., Healy, L.M., Nguyen, C.H., Newman, S.A., Yeromin, A.V., Scarfone, V.M., Marsh, S.E., Fimbres, C., et al. (2017). iPSC-derived human microglia-like cells to study neurological diseases. Neuron *94*, 278–293.e9. https://doi.org/10.1016/j.neuron.2017.03.042.

30. Brownjohn, P.W., Smith, J., Solanki, R., Lohmann, E., Houlden, H., Hardy, J., Dietmann, S., and Livesey, F.J. (2018). Functional studies of Missense TREM2 mutations in human stem cell-derived microglia. Stem Cell Rep. *10*, 1294–1307. https://doi.org/10.1016/j.stemcr.2018.03.003.

31. Lin, Y.T., Seo, J., Gao, F., Feldman, H.M., Wen, H.L., Penney, J., Cam, H.P., Gjoneska, E., Raja, W.K., Cheng, J., et al. (2018). APOE4 Causes Widespread molecular and cellular Alterations associated with Alzheimer's disease phenotypes in human iPSC-derived brain cell types. Neuron *98*, 1141–1154.e7. https://doi.org/10.1016/j.neuron.2018.05.008.

32. Xu, R., Boreland, A.J., Li, X., Erickson, C., Jin, M., Atkins, C., Pang, Z.P., Daniels, B.P., and Jiang, P. (2021). Developing human pluripotent stem cell-based cerebral organoids with a controllable microglia ratio for modeling brain development and pathology. Stem Cell Rep. *16*, 1923–1937. https://doi.org/10.1016/j.stemcr.2021.06.011.

33. 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. https://doi.org/10.1038/s41592-019-0586-5.

34. Pham, M.T., Pollock, K.M., Rose, M.D., Cary, W.A., Stewart, H.R., Zhou, P., Nolta, J.A., and Waldau, B. (2018). Generation of human vascularized brain organoids. Neuroreport *29*, 588–593. https://doi.org/10.1097/wnr.0000000000001014.

35. Shi, Y., Sun, L., Wang, M., Liu, J., Zhong, S., Li, R., Li, P., Guo, L., Fang, A., Chen, R., et al. (2020). Vascularized human cortical organoids (vOrganoids) model cortical development in vivo. PLoS Biol. *18*, e3000705. https://doi.org/10.1371/journal.pbio.3000705.

36. Worsdorfer, P., Dalda, N., Kern, A., Kruger, S., Wagner, N., Kwok, C.K., Henke, E., and Ergun, S. (2019). Generation of complex human organoid models including vascular networks by incorporation of mesodermal progenitor cells. Sci. Rep. *9*, 15663. https://doi.org/10.1038/s41598-019-52204-7.

37. Goranci-Buzhala, G., Mariappan, A., Gabriel, E., Ramani, A., Ricci-Vitiani, L., Buccarelli, M., D'Alessandris, Q.G., Pallini, R., and Gopalakrishnan, J. (2020). Rapid and Efficient invasion assay of glioblastoma in human brain organoids. Cell Rep. *31*, 107738. https://doi.org/10.1016/j.celrep.2020.107738.

38. Krieger, T.G., Tirier, S.M., Park, J., Jechow, K., Eisemann, T., Peterziel, H., Angel, P., Eils, R., and Conrad, C. (2020). Modeling glioblastoma invasion using human brain organoids and single-cell transcriptomics. Neuro Oncol. *22*, 1138–1149. https://doi.org/10.1093/neuonc/noaa091.

39. Linkous, A., Balamatsias, D., Snuderl, M., Edwards, L., Miyaguchi, K., Milner, T., Reich, B., Cohen-Gould, L., Storaska, A., Nakayama, Y., et al. (2019). Modeling patient-derived glioblastoma with cerebral organoids. Cell Rep. *26*, 3203–3211.e5. https://doi.org/10.1016/j.celrep.2019.02.063.

40. Andersen, J., Revah, O., Miura, Y., Thom, N., Amin, N.D., Kelley, K.W., Singh, M., Chen, X., Thete, M.V., Walczak, E.M., et al. (2020). Generation of functional human 3D cortico-motor assembloids. Cell *183*, 1913–1929.e26. https://doi.org/10.1016/j.cell.2020.11.017.

41. Bagley, J.A., Reumann, D., Bian, S., Levi-Strauss, J., and Knoblich, J.A. (2017). Fused cerebral organoids model interactions between brain regions. Nat. Methods *14*, 743–751. https://doi.org/10.1038/nmeth.4304.

42. Miura, Y., Li, M.Y., Birey, F., Ikeda, K., Revah, O., Thete, M.V., Park, J.Y., Puno, A., Lee, S.H., Porteus, M.H., and Pașca, S.P. (2020). Generation of human striatal organoids and cortico-striatal assembloids from human pluripotent stem cells. Nat. Biotechnol. *38*, 1421–1430. https://doi.org/10.1038/s41587-020-00763-w.

43. Xiang, Y., Tanaka, Y., Patterson, B., Kang, Y.J., Govindaiah, G., Roselaar, N., Cakir, B., Kim, K.Y., Lombroso, A.P., Hwang, S.M., et al. (2017). Fusion of regionally specified hPSC-derived organoids models human brain development and Interneuron migration. Cell Stem Cell *21*, 383–398.e7. https://doi.org/10.1016/j.stem.2017.07.007.

44. Lancaster, M.A., and Knoblich, J.A. (2014). Organogenesis in a dish: modeling development and disease using organoid technologies. Science *345*, 1247125. https://doi.org/10.1126/science.1247125.

45. Martens, Y.A., Xu, S., Tait, R., Li, G., Zhao, X.C., Lu, W., Liu, C.C., Kanekiyo, T., Bu, G., and Zhao, J. (2021). Generation and validation of APOE knockout human iPSC-derived cerebral organoids. STAR Protoc *2*, 100571. https://doi.org/10.1016/j.xpro.2021.100571.

46. Lancaster, M.A., and Knoblich, J.A. (2014). Generation of cerebral organoids from human pluripotent stem cells. Nat. Protoc. *9*, 2329–2340. https://doi.org/10.1038/nprot.2014.158.

47. Zhao, J., Fu, Y., Yamazaki, Y., Ren, Y., Davis, M.D., Liu, C.C., Lu, W., Wang, X., Chen, K., Cherukuri, Y., et al. (2020). APOE4 exacerbates synapse loss and neurodegeneration in Alzheimer's disease patient iPSC-derived cerebral organoids. Nat. Commun. *11*, 5540. https://doi.org/10.1038/s41467-020-19264-0.

48. Sarrafha, L., Parfitt, G.M., Reyes, R., Goldman, C., Coccia, E., Kareva, T., and Ahfeldt, T. (2021). High-throughput generation of midbrain dopaminergic neuron organoids from reporter human pluripotent stem cells. STAR Protoc *2*, 100463. https://doi.org/10.1016/j.xpro.2021.100463.

49. Ahfeldt, T., Ordureau, A., Bell, C., Sarrafha, L., Sun, C., Piccinotti, S., Grass, T., Parfitt, G.M., Paulo, J.A., Yanagawa, F., et al. (2020). Pathogenic pathways in early-Onset Autosomal Recessive Parkinson's disease discovered using Isogenic human dopaminergic neurons. Stem Cell Rep. *14*, 75–90. https://doi.org/10.1016/j.stemcr.2019.12.005.

50. Zagare, A., Gobin, M., Monzel, A.S., and Schwamborn, J.C. (2021). A robust protocol for the generation of human midbrain organoids. STAR Protoc *2*, 100524. https://doi.org/10.1016/j.xpro.2021.100524.

51. Monzel, A.S., Smits, L.M., Hemmer, K., Hachi, S., Moreno, E.L., van Wuellen, T., Jarazo, J., Walter, J., Bruggemann, I., Boussaad, I., et al. (2017). Derivation of human midbrain-specific organoids from Neuroepithelial stem cells. Stem Cell Rep. *8*, 1144–1154. https://doi.org/10.1016/j.stemcr.2017.03.010.

52. Reinhardt, P., Glatza, M., Hemmer, K., Tsytsyura, Y., Thiel, C.S., Hoing, S., Moritz, S., Parga, J.A., Wagner, L., Bruder, J.M., et al. (2013). Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling. PLoS One *8*, e59252. https://doi.org/10.1371/journal.pone.0059252.

53. Xiang, Y., Cakir, B., and Park, I.H. (2020). Generation of regionally specified human brain organoids resembling Thalamus development. STAR Protoc *1*, 100001. https://doi.org/10.1016/j.xpro.2019.100001.

54. Worsdorfer, P., Rockel, A., Alt, Y., Kern, A., and Ergun, S. (2020). Generation of vascularized neural organoids by Co-culturing with mesodermal progenitor cells. STAR Protoc *1*, 100041. https://doi.org/10.1016/j.xpro.2020.100041.

55. Kim, H., and Jiang, P. (2021). Generation of human pluripotent stem cell-derived fused organoids with oligodendroglia and myelin. STAR Protoc *2*, 100443. https://doi.org/10.1016/j.xpro.2021.100443.

56. 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. https://doi.org/10.1016/j.stemcr.2019.04.011.

57. Chen, C., Kim, W.Y., and Jiang, P. (2016). Humanized neuronal chimeric mouse brain generated by neonatally engrafted human iPSC-derived primitive neural progenitor cells. JCI Insight *1*, e88632. https://doi.org/10.1172/jci.insight.88632.

58. Bardy, C., van den Hurk, M., Eames, T., Marchand, C., Hernandez, R.V., Kellogg, M., Gorris, M., Galet, B., Palomares, V., Brown, J., et al. (2015). Neuronal medium that supports basic synaptic functions and activity of human neurons in vitro. Proc. Natl. Acad. Sci. USA *112*, E2725–E2734. https://doi.org/10.1073/pnas.1504393112.

59. Oudin, A., Baus, V., Barthelemy, V., Fabian, C., Klein, E., Dieterle, M., Wantz, M., Hau, A.C., Dording, C., Bernard, A., et al. (2021). Protocol for derivation of organoids and patient-derived orthotopic xenografts from glioma patient tumors. STAR Protoc *2*, 100534. https://doi.org/10.1016/j.xpro.2021.100534.

60. Gamboa, C.M., Jara, K., Pamarthy, S., Liu, L., Aiken, R., Xiong, Z., Danish, S., and Sabaawy, H.E. (2021). Generation of glioblastoma patient-derived organoids and mouse brain orthotopic xenografts for drug screening. STAR Protoc *2*, 100345. https://doi.org/10.1016/j.xpro.2021.100345.

61. Linkous, A., and Fine, H.A. (2020). Generating patient-derived gliomas within cerebral organoids. STAR Protoc *1*, 100008. https://doi.org/10.1016/j.xpro.2019.100008.

62. Golebiewska, A., Hau, A.C., Oudin, A., Stieber, D., Yabo, Y.A., Baus, V., Barthelemy, V., Klein, E., Bougnaud, S., Keunen, O., et al. (2020). Patient-derived organoids and orthotopic xenografts of primary and recurrent gliomas represent relevant patient avatars for precision oncology. Acta Neuropathol. *140*, 919–949. https://doi.org/10.1007/s00401-020-02226-7.

63. Chadwick, M., Yang, C., Liu, L., Gamboa, C.M., Jara, K., Lee, H., and Sabaawy, H.E. (2020). Rapid processing and drug Evaluation in glioblastoma patient-derived organoid models

with 4D bioprinted arrays. iScience *23*, 101365. https://doi.org/10.1016/j.isci.2020.101365.

64. Pine, A.R., Cirigliano, S.M., Nicholson, J.G., Hu, Y., Linkous, A., Miyaguchi, K., Edwards, L., Singhania, R., Schwartz, T.H., Ramakrishna, R., et al. (2020). Tumor Microenvironment is critical for the Maintenance of cellular states Found in primary glioblastomas. Cancer Discov. *10*, 964–979. https://doi.org/10.1158/2159-8290.CD-20-0057.

65. Ramirez, S., Mukherjee, A., Sepulveda, S.E., Gherardelli, C., Becerra-Calixto, A., Bravo-Vasquez, N., and Soto, C. (2021). Protocol for controlled cortical impact in human cerebral organoids to model traumatic brain injury. STAR Protoc *2*, 100987. https://doi.org/10.1016/j.xpro.2021.100987.

66. Ramirez, S., Mukherjee, A., Sepulveda, S., Becerra-Calixto, A., Bravo-Vasquez, N., Gherardelli, C., Chavez, M., and Soto, C. (2021). Modeling Traumatic Brain Injury in Human Cerebral Organoids. Cells *10*. https://doi.org/10.3390/cells10102683.

67. Kriks, S., Shim, J.W., Piao, J., Ganat, Y.M., Wakeman, D.R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature *480*, 547–551. https://doi.org/10.1038/nature10648.

68. Popova, G., Soliman, S.S., Kim, C.N., Keefe, M.G., Hennick, K.M., Jain, S., Li, T., Tejera, D., Shin, D., Chhun, B.B., et al. (2021). Human microglia states are conserved across experimental models and regulate neural stem cell responses in chimeric organoids. Cell Stem Cell *28*, 2153–2166.e6. https://doi.org/10.1016/j.stem.2021.08.015.

69. Pasca, S.P. (2019). Assembling human brain organoids. Science *363*, 126–127. https://doi.org/10.1126/science.aau5729.

70. Pellegrini, L., Albecka, A., Mallery, D.L., Kellner, M.J., Paul, D., Carter, A.P., James, L.C., and Lancaster, M.A. (2020). SARS-CoV-2 Infects the brain choroid plexus and disrupts the blood-CSF barrier in human brain organoids. Cell Stem Cell *27*, 951–961.e5. https://doi.org/10.1016/j.stem.2020.10.001.

71. Kanton, S., Boyle, M.J., He, Z., Santel, M., Weigert, A., Sanchis-Calleja, F., Guijarro, P., Sidow, L., Fleck, J.S., Han, D., et al. (2019). Organoid single-cell genomic atlas uncovers human-specific features of brain development. Nature *574*, 418–422. https://doi.org/10.1038/s41586-019-1654-9.

72. Benito-Kwiecinski, S., Giandomenico, S.L., Sutcliffe, M., Riis, E.S., Freire-Pritchett, P., Kelava, I., Wunderlich, S., Martin, U., Wray, G.A., McDole, K., and Lancaster, M.A. (2021). An early cell shape transition drives evolutionary expansion of the human forebrain. Cell *184*, 2084–2102.e19. https://doi.org/10.1016/j.cell.2021.02.050.

73. Sawai, T., Sakaguchi, H., Thomas, E., Takahashi, J., and Fujita, M. (2019). The ethics of cerebral organoid research: Being conscious of consciousness. Stem Cell Rep. *13*, 440–447. https://doi.org/10.1016/j.stemcr.2019.08.003.