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Bridging molecular mechanisms and therapeutic innovations: The role of brain organoids in neurodevelopmental disorder research

Moawiah M. Naffaa[1,2]*

[1]Department of Psychology and Neuroscience, Duke University, Durham, North Carolina, United States of America

[2]Department of Cell Biology, Duke University School of Medicine, Durham, North Carolina, United States of America

***Corresponding author:** Moawiah M. Naffaa (moawiah.naffaa@duke.edu)

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Abstract

This article examines the role of brain organoids in understanding neurodevelopmental disorders (NDDs), including autism spectrum disorder, intellectual disabilities, and schizophrenia, which arise from disruptions in the complex processes of brain development. The transformative potential of human pluripotent stem cell-derived brain organoids as models for investigating the molecular mechanisms underlying NDDs and their implications for therapeutic innovation is explored. By simulating critical stages of human brain development – such as neurulation, neurogenesis, and gliogenesis – organoids provide a physiologically relevant platform to investigate cellular diversity, synaptic connectivity, and neuronal circuit formation. Advanced methodologies, including single-cell RNA sequencing and chromatin accessibility profiling, are utilized to dissect lineage-specific gene expression patterns and regulatory mechanisms within organoids. In addition, metabolic profiling and functional assessments comprehensively evaluate neuronal maturation, synaptic plasticity, and cellular interactions, addressing the limitations of traditional two-dimensional cultures. This article also examines the influence of environmental factors, such as viral infections, by utilizing organoid models to simulate host-pathogen interactions and assess their impact on neural progenitor function and cortical development. The integration of machine learning and innovative culture systems, including microfluidic and vascularized models, enhances the physiological relevance and reproducibility of brain organoid research. This review highlights the potential use of organoids in elucidating the molecular pathology of NDDs and as platforms for drug discovery and personalized therapeutic screening, ultimately bridging molecular insights with therapeutic applications and underscoring the vital role of brain organoids in advancing the understanding of NDDs and facilitating the development of targeted interventions.

Keywords: Organoids; Neurodevelopmental disorders; Human pluripotent stem cells; Single-cell RNA sequencing; Viral infections; Culture systems; Machine learning

1. Introduction

Human brain development is a highly intricate and tightly regulated process that unfolds through multiple stages, during which various cell types perform specialized functions.[1] While animal models, particularly rodents, have provided valuable insights into neurodevelopmental mechanisms, it is crucial to note that significant species-specific differences exist. These differences are evident in brain size, cytoarchitecture, and cellular composition.[2] Rodent brains are relatively small and feature smooth, lissencephalic structures, whereas human brains are larger

and exhibit complex cortical folding.[3] Notably, human brains possess unique structures such as the inner fiber layer and the outer subventricular zone (oSVZ), which harbor intermediate progenitor cells and outer radial glial cells (oRGCs).[4,5] These structures, absent from rodents, play a crucial role in cortical expansion and neurogenesis. Given these distinctions, the development of humanized model systems that accurately replicate human neurodevelopmental processes remains a critical area of research.

Primary human brain tissue, typically obtained from surgical procedures or electively terminated fetuses, serves as a valuable model for studying human neurodevelopment and its associated disorders.[6] This tissue retains the full spectrum of essential cell types, thereby enabling the investigation of human-specific mechanisms. However, its utility is constrained by several challenges, including limited availability, difficulties in maintaining long-term cultures, and variability in genetic background, which can affect experimental reproducibility.[7]

In recent years, human brain organoids have emerged as indispensable models for studying neurodevelopmental disorders (NDDs). These self-assembling, three-dimensional (3D) cultures recapitulate key functional and structural features of the fetal human brain.[8] Generated from human pluripotent stem cells (hPSCs) under specific 3D culture conditions, brain organoids develop organized structures comprising neuronal progenitors, neurons, and glial cells, mimicking the cellular diversity and cytoarchitecture of the developing brain.[9,10] They also retain hallmark features of human neurodevelopment, including extended neuroepithelial expansion, enriched populations of oRGCs, and characteristic gene expression patterns. As such, brain organoids provide an advanced model system for investigating neurodevelopmental processes and disorders.[11,12]

The advent of brain organoid technology has significantly enhanced our ability to study neurodevelopmental diseases, bridging the gap between genetic and molecular mechanisms and clinical phenotypes. Traditional animal models, while instrumental in neuroscience research, often fail to capture the full spectrum of human brain development, particularly in disorders influenced by both genetic and environmental factors, such as autism, schizophrenia, and epilepsy.[13] Brain organoids offer a physiologically relevant platform for modeling these diseases, enabling researchers to examine cellular diversity, tissue architecture, and functional activity in a human-specific context.[14]

In addition, organoid models facilitate the study of key developmental processes such as neurogenesis, progenitor differentiation, and synaptogenesis, which are not easily assessed using conventional two-dimensional (2D) cultures.[15] Advances in genetic engineering, particularly CRISPR-Cas9 technology, have enabled the generation of organoid models with specific mutations linked to NDDs.[16] These models have provided crucial insights into how genetic alterations disrupt brain development and function.

The integration of high-resolution imaging, single-cell RNA sequencing (scRNA-seq), and electrophysiological approaches has further expanded the potential of brain organoids in studying disease mechanisms. Techniques such as calcium imaging, patch-clamp recordings, and multi-electrode arrays allow for the investigation of synaptic activity and network connectivity, shedding light on neuronal dysfunction in NDDs.[7,17,18] Furthermore, these organoid-based models serve as platforms for identifying potential therapeutic strategies, including gene therapies, pharmacological interventions, and targeted modulation of signaling pathways.[19]

By offering a physiologically relevant and scalable system for studying human brain development and disease, brain organoids have transformed neurodevelopmental research. Their continued refinement will undoubtedly contribute to a deeper understanding of neurological disorders and the development of novel therapeutic strategies.

This article highlights the significance of brain organoids derived from hPSCs as advanced models that accurately replicate human brain architecture and facilitate the investigation of NDDs, effectively addressing the limitations of traditional animal models. By integrating high-resolution analyses and innovative culture techniques, this research enhances the physiological relevance of these organoids, yielding valuable insights into the cellular and molecular mechanisms underlying NDDs. Ultimately, this article highlights the transformative potential of brain organoids in unraveling the impact of genetic and environmental factors on neurodevelopment, offering deeper insights into brain development and related disorders while paving the way for innovative therapeutic strategies.

2. Orchestrating brain development: From neural tube formation to NDDs and organoid modeling

Brain development is a precisely orchestrated process involving a series of tightly regulated events that commence with neurulation. During this stage, the neural plate of the embryonic ectoderm folds and fuses to form the neural tube, which undergoes segmentation and patterning to generate all regions of the central nervous system (CNS).[20] The ventricular zone (VZ), located at the apical surface of the neural tube, harbors neuroepithelial cells (NECs), the multipotent stem cells of the nervous system.[21] Initially, NECs undergo extensive proliferation to expand the stem cell pool before differentiating into radial glial cells (RGCs),

specialized neural progenitors responsible for generating both neuronal and glial cells.[22]

RGCs exhibit a highly polarized morphology with an apically positioned soma and elongated radial processes extending to the pial surface. Initially, they divide symmetrically to amplify the progenitor pool. As corticogenesis proceeds, RGCs transition to asymmetric divisions, producing neurons and additional RGCs in a process termed direct neurogenesis.[23,24] Alternatively, asymmetric divisions generate intermediate progenitor cells in the subventricular zone (SVZ), which undergo limited proliferation before giving rise to neurons through indirect neurogenesis.[25] Another distinct progenitor population, oRGCs, emerges in the outer SVZ. Unlike RGCs, oRGCs lack apical membrane attachment but retain radial processes extending to the pial surface. They exhibit enhanced proliferative capacity, becoming the predominant progenitor type in the human brain around mid-gestation and substantially contributing to cortical neurogenesis.[26]

Neuronal differentiation and migration occur concurrently, ensuring proper integration into developing networks.[27] Dendritic spine morphogenesis and synaptogenesis establish synaptic connectivity, followed by synaptic pruning, which refines neuronal circuits throughout development and into adulthood.[28] Around mid-gestation, RGCs and oRGCs shift from neurogenesis to gliogenesis, giving rise to astrocyte progenitor cells and oligodendrocyte progenitor cells, which differentiate into astrocytes and oligodendrocytes, respectively.[29,30] These glial cells integrate into neuronal circuits, supporting synaptic function and maintenance.

NDDs encompass a broad spectrum of conditions characterized by abnormal brain development, leading to cognitive, emotional, and behavioral impairments.[31] Affecting approximately 5% of children worldwide, NDDs manifest with diverse clinical features, including intellectual disability, autism spectrum disorder, and developmental delays.[32] Their etiology is multifactorial, involving intricate genetic and environmental interactions. Animal models, particularly transgenic rodents, have been instrumental in uncovering the molecular mechanisms underlying NDDs and in testing pharmacological interventions (Figure 1). However, their inherent limitations, including structural and functional differences from the human brain and limited genetic heterogeneity, often hinder translational success, underscoring the need to develop alternative models.[33,34]

hPSCs provide a powerful platform for investigating human brain development and NDD pathology. These cells, which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), exhibit remarkable self-renewal and differentiation capabilities.[35-37] iPSCs, generated by reprogramming somatic cells through transcription factors such as Oct4, Klf4, Sox2, and c-Myc, enable the study of patient-specific disease mechanisms and facilitate drug screening and gene therapy development.[38,39]

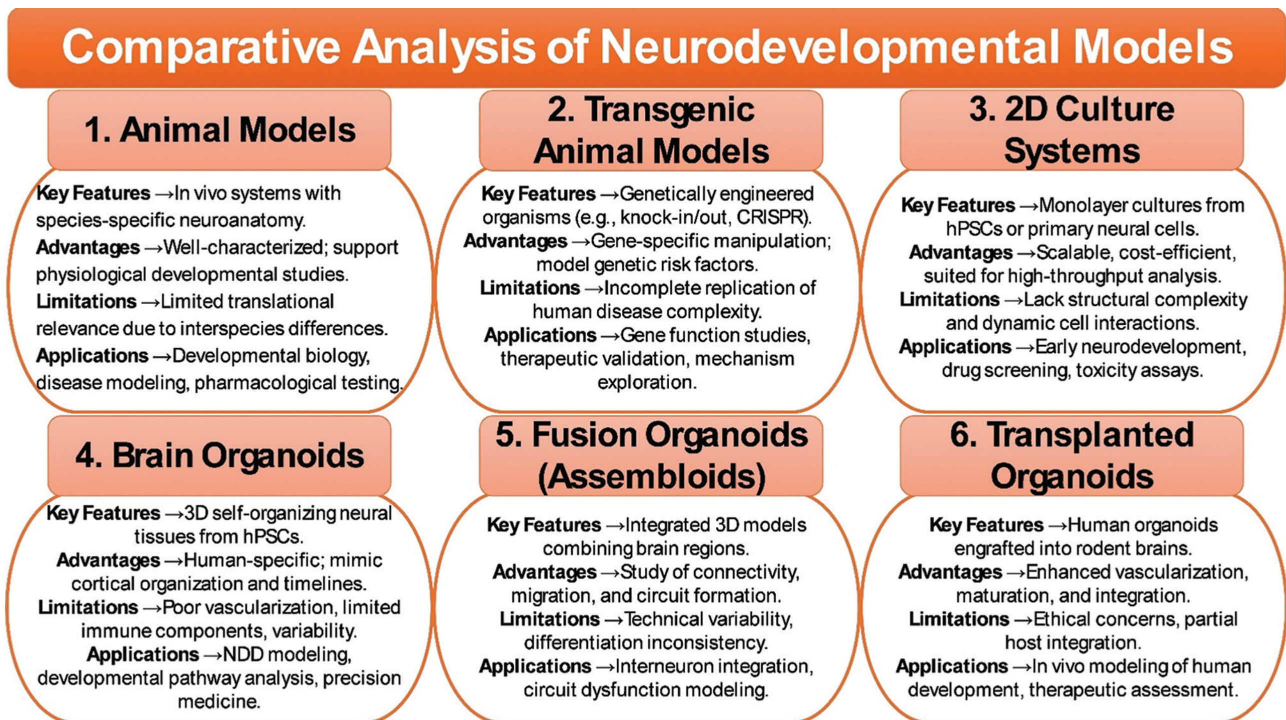

Figure 1. Comparative experimental models for human brain development and neurodevelopmental disorders

While traditional 2D monolayer cultures have been widely used for NDD research, they lack the spatial complexity necessary to replicate critical developmental processes such as cell migration, polarization, and intricate cell-cell interactions.[40]

Brain organoids represent a groundbreaking advancement in modeling human neurodevelopment, overcoming many limitations of animal models and 2D culture systems. These 3D structures recapitulate key aspects of human brain development, including cytoarchitecture, gene expression profiles, and epigenomic signatures.[8,11] Derived from hPSCs, organoids are formed by allowing cells to aggregate into embryoid bodies (EBs), which, under specific culture conditions, differentiate into neuroectodermal tissue.[41,42] Neural progenitor cells (NPCs) within these structures proliferate, expand the epithelial architecture, and sequentially transition through neurogenic and gliogenic phases.[43] The complexity and cellular diversity of brain organoids depend on the morphogens used during differentiation. Unguided protocols generate cerebral organoids containing various brain regions, while guided protocols employ specific morphogen gradients to establish distinct regional identities.[44,45] Advanced approaches, including fusion organoids (assembloids), enable the study of interregional interactions, while *in vivo* transplantation into rodent brains facilitates vascularization and neuronal circuit maturation.[14]

Compared to 2D cultures, brain organoids provide a more physiologically relevant environment by mimicking the 3D structure, temporal dynamics, and spatial organization of the developing brain,[46] which are essential for studying processes such as neurogenesis, lamination, and long-range connectivity. In contrast, 2D systems are limited by their inability to support complex tissue architecture and often fail to capture disease phenotypes that depend on multicellular or layered organization.[47]

Compared to *in vivo* animal models, organoids offer species-specific insights into human brain development and pathology. Rodent brains differ significantly from human brains in terms of size, structure, cortical organization, and gene regulatory networks. Moreover, transgenic models often do not fully replicate the polygenic or epigenetic contributions to NDDs observed in humans.[13,48]

Organoids also allow for high-throughput screening and genetic manipulation in a patient-specific context, which is often impractical or ethically constrained in animal models. Furthermore, they enable the study of early developmental processes that are inaccessible *in vivo*, such as the emergence of human-specific progenitor populations like outer radial glia.[13,48] Nevertheless, organoids also face challenges, including variability in differentiation protocols, lack of vascularization and immune components, and limited maturation. Integration with microfluidic systems, vascular scaffolds, or co-culture with microglia is under development to address these limitations.

Collectively, these innovations position brain organoids as a transformative model for investigating NDDs, offering unprecedented opportunities to elucidate disease mechanisms, personalize medicine, and accelerate therapeutic discovery.

3. Brain organoids: 3D self-organized models for neurodevelopmental research and disease modeling

Brain organoids are 3D, self-organized cellular structures generated *in vitro*, serving as powerful models for studying neurodevelopment and neural function.[49] Derived from iPSCs, these models can differentiate into specific brain regions, such as the dorsal or ventral forebrain, in response to developmental signaling factors. Alternatively, through unguided differentiation, they can give rise to diverse neural and non-neural cell lineages.[15,50]

3D organoid culture technology has significantly advanced developmental neurobiology by providing physiologically relevant models that more closely mimic human brain development than traditional animal models, which often exhibit structural, functional, and developmental differences.[51,52] Unlike 2D monolayer cultures, brain organoids replicate many characteristics of human brain tissue, including early cellular organization, cell-type diversity, and cell-cell interactions.[42,53] This structural resemblance to *in vivo* tissue enables the study of neuronal and glial interactions during early development. Under optimal differentiation conditions, brain organoids contain mature, functional neurons, and astrocytes, as confirmed by immunohistological and electrophysiological analyses. Their ability to model connectivity between different brain regions allows researchers to study network formation and synaptic activity. Given their genetic and structural compatibility with the human brain, brain organoids provide an essential platform for investigating NDDs, particularly those involving large-scale structural rearrangements and complex genetic interactions.[54] Patient-derived iPSCs enable the generation of organoids that replicate disease phenotypes, facilitating the study of congenital brain malformations and neural circuit dysfunctions.[8,55] Furthermore, organoids offer a scalable and accessible alternative to human brain tissue, allowing researchers to explore uniquely human aspects of brain formation and evolution, thus broadening our understanding of neurodevelopmental processes and disorders.[50,56]

The generation of human brain organoids from human iPSCs and human ESCs under 3D culture conditions relies on either unguided or guided differentiation methods (Table 1). Unguided differentiation exploits the intrinsic

Table 1. Comparative analysis of unguided and guided differentiation: Key characteristics and insights into cell lineage and function

Characteristic	Unguided differentiation	Guided differentiation	References
Differentiation control	Low–relies on intrinsic signaling pathways	High–controlled by specific growth factors and small molecules	42
Cellular heterogeneity	High–multiple neural and non-neural cell lineages present	Lower–more uniform cell composition	63
Reproducibility	Variable–significant batch-to-batch variation	More consistent due to directed differentiation	64
Brain region specificity	Mixed populations–various brain regions may arise randomly	Targeted differentiation into specific brain regions	65
Use in disease modeling	Limited–higher variability makes precise modeling challenging	More effective–greater control over disease-relevant features	15,42
Developmental staging	Broad–includes a range of progenitor and mature cell types	Defined–controlled development of specific neuronal subtypes	63,66
Tissue organization	Less structured–random spatial organization of cells	More structured–mimics laminar organization of the brain	42,63
Duration of maturation	Long–maturation can be slow and heterogeneous	Shorter–optimized conditions accelerate development	15,42
Microenvironment influence	Highly variable–dependent on spontaneous differentiation signals	Precisely controlled by external signaling factors	67
Applications in drug screening	Limited–high variability affects assay reproducibility	More suitable–consistent cellular composition aids in screening	68
Potential for neural circuit studies	Unpredictable–randomized cell arrangements limit functional connectivity	Higher–directed differentiation improves synaptic organization	42,69
Scalability and standardization	Challenging–high variability hampers large-scale production	More feasible–standardized protocols improve scalability	42,70

signaling potential of pluripotent stem cell aggregates, allowing spontaneous generation of brain organoids containing multiple cell lineages. When grown in a stromal gel suspension such as Matrigel, these organoids can develop into various brain regions, including the dorsal and ventral forebrain, midbrain, hindbrain, hippocampus, retina, and choroid plexus.[57,58] However, the stochastic nature of differentiation results in variability in composition and organization, posing challenges for systematic studies and reproducibility.[59] Early developmental conditions influence organoid formation, potentially leading to non-physiological cellular interactions if certain populations are selectively favored or eliminated.[60,61] To mitigate variability and enhance consistency in neural induction, researchers have introduced small molecules and fibrous microfilaments, yet the heterogeneity of unguided organoids remains a limitation in disease modeling and drug screening.[62,63]

Guided differentiation directs aggregated pluripotent stem cells toward an ectodermal fate, promoting the formation of region-specific organoids through the use of small molecules and growth factors that induce neural differentiation.[57,71] The inhibition of the bone morphogenetic protein (BMP)/transforming growth factor-beta signaling pathway facilitates neural induction, while subsequent exposure to factors such as WNT3A, SHH, BMP7, and FGF8 enables the formation of specific brain regions.[72-74] Early studies demonstrated that ESCs could self-organize into polarized cortical tissue resembling human brain structures.[75,76] This approach was refined to develop dorsal forebrain organoids in suspension culture without requiring an extracellular matrix (ECM), yielding organoids that contain both deep and superficial cortical neurons. Over several months, these organoids mature to resemble postnatal brain structures, with rotating bioreactors further enhancing their ability to replicate key features of cortical development, including progenitor organization, neurogenesis, and glial cell layer formation.[62,77]

Guided differentiation has facilitated the generation of brain organoids representing various regions, such as the dorsal forebrain cortex, ventral forebrain, hippocampus, thalamus, hypothalamus, midbrain, and cerebellum.[78,79] These region-specific organoids are instrumental in brain assembly studies, allowing the investigation of interneuron migration, neuronal projections, and oligodendrocyte development.[15,80] While astrocytes and oligodendrocyte progenitors emerge in cortical organoids after long-term culture, mature oligodendrocytes remain challenging to establish.[81-83] Researchers have developed region-specific

organoids enriched in oligodendrocytes, astrocytes, and neurons to study myelin formation and cellular interactions.[84,85] Microglia plays a crucial role in regulating brain health through inflammatory responses, microbial phagocytosis, and synaptic pruning. Incorporating microglial cells into brain organoids provides a valuable model for studying microglia migration and response to neural damage in a 3D environment.[86,87] Compared to unguided differentiation, guided differentiation enhances reproducibility and facilitates the study of specific brain structures, advancing research in neurodevelopment, disease modeling, and regenerative medicine.

4. Deciphering cellular complexity: Integrative insights from transcriptomic, metabolic, and functional analyses in brain organoids

Brain organoids provide a versatile model for investigating human neurodevelopment and disease, integrating transcriptomic, metabolic, and functional processes.[42,88] scRNA-seq and chromatin accessibility profiling have enabled precise mapping of lineage-specific gene expression and regulatory mechanisms underlying differentiation.[89] Metabolic profiling highlights the critical balance between glycolysis and oxidative phosphorylation in neurogenesis, with metabolomic and isotope tracing approaches offering insights into cellular energy flux.[90] Functional studies leveraging electrophysiology and high-resolution imaging have advanced our understanding of neural network activity, synaptic plasticity, and circuit maturation, with techniques such as multielectrode arrays (MEAs) and genetically encoded voltage indicators (GEVIs) providing scalable assessment platforms.

4.1. Transcriptomic insights in human brain organoids

Cellular physiology is governed by the transcriptome, which orchestrates a range of biological processes essential for cellular function and development.[91] With the advent of next-generation sequencing technologies, such as bulk RNA sequencing (RNA-seq) and scRNA-seq, researchers have gained unprecedented insights into the transcriptomic profiles of various cell types, developmental stages, and disease states.[92] However, the limited availability of human brain tissue remains a significant challenge for neuroscientific research. To address this, human brain organoids have emerged as powerful models that enable the study of transcriptomic dynamics at single-cell resolution.

Human brain organoids, including cortical, thalamic, and medial ganglionic eminence models, exhibit distinct yet reproducible transcriptomic profiles that serve as reliable representations of human neurodevelopment.[12,93] A notable advancement in this field is the development of an integrated human neural organoid cell atlas, which combines multiple scRNA-seq datasets to map the primary human brain cell types. This facilitates comparative analysis across various models and disease conditions. For example, organoids at 6 months of age demonstrate increasingly complex gene expression signatures, reflecting cellular maturation and differentiation.[94,95] Advanced trajectory inference methods have provided insights into differentiation processes, such as the transformation of radial glia into mature excitatory and inhibitory neurons.[96] In addition, scRNA-seq has expanded beyond mRNA expression to include long non-coding RNAs (lncRNAs), providing deeper insights into brain development, NDDs, and neuropsychiatric conditions.[97] These evolving transcriptomic tools offer a more comprehensive understanding of cellular dynamics in both healthy and diseased states.

Despite their remarkable potential, organoid-based transcriptomics faces several challenges (Table 2). Variability in organoid culture conditions, underrepresentation of certain cell types such as inhibitory neurons, limitations of single-cell transcriptomic platforms, and difficulties in data interpretation present ongoing obstacles.[18,98] Variability in growth conditions and ECM components across laboratories can introduce inconsistencies in results. Platforms such as droplet-seq and split-seq, though effective for large-scale profiling, suffer from low sequencing depth per cell, complicating the detection of rare populations or low-expression genes.[99] Furthermore, tools like Cell Ranger, commonly used for processing scRNA-seq data, often struggle with data interpretation, especially when comparing datasets across studies.[100]

To overcome these challenges, integrative approaches and machine learning frameworks such as BOMA (Brain and Organoid Manifold Alignment) have been developed to integrate datasets and identify common developmental trajectories across human organoids and primary brain tissues.[109] Moreover, scRNA-seq alone is insufficient for fully defining cell types and lineage relationships. Combining transcriptomics with chromatin accessibility profiling, such as single-cell ATAC-seq, has proven invaluable for providing a more holistic understanding of gene regulatory networks.[110,111] This integrated approach has revealed critical regulatory mechanisms, including interactions between transcription factors and distal enhancers. However, challenges such as high noise levels, batch effects, and complexities in data integration persist.

4.2. Metabolic regulation and analytical advances in brain organoids

The brain's high metabolic demands necessitate a continuous supply of ATP to sustain cellular and neuronal activities. Disruptions in these metabolic processes are closely linked to various neurological disorders,[112,113] highlighting

Table 2. Challenges and innovations in organoid-based transcriptomics

Challenge	Description	Potential solution	Impact	Scope	Progress	References
Variability in organoid culture conditions	Differences in protocols, ECM components, and lab conditions → Inconsistent organoid development and low reproducibility	Standardize protocols and ECM composition across labs	Undermine data reproducibility and inter-study comparability	Universal framework for standardization across tissues and labs	Protocol harmonization underway; full standardization still lacking	7,60,101
Underrepresentation of specific cell types	Key populations (e.g., inhibitory neurons) are often absent → Biased transcriptomic profiles	Optimize differentiation cues and markers to enrich specific cell types	Lead to incomplete models of development and disease	Tailored differentiation protocols to ensure diverse cell representation	Some protocols (e.g., GABAergic neuron enrichment) developed; inter-study variability persists	42,102
Limitations of single-cell transcriptomic platforms	Low sequencing depth → Poor detection of rare cell types and low-abundance genes	Increase depth and integrate spatial transcriptomics or complementary methods	Missed rare populations → Limited insight into cellular heterogeneity	Deep sequencing and multi-modal profiling to improve resolution	Technologies are improving; rare cell detection remains difficult	103,104
Data interpretation challenges	High-dimensional scRNA-seq data+cross-study variation → Analytical complexity	Develop scalable computational tools and machine learning models for integration	Risk of misclassification and flawed biological inference	Algorithms to reduce noise, unify datasets, and enhance interpretability	Tools (e.g., Seurat, Cell Ranger) exist; multi-study harmonization is still evolving	105,106
Multi-omics integration challenges	Batch effects, noise, and inconsistent conditions hinder integration of omic layers	Use deep learning + Standardized protocols to reduce variability and noise	Distorted biological signals → Flawed conclusions	Robust integration frameworks and refined experimental design	Transcriptome–epigenome integration progressing; noise is still a major barrier	107,108

Abbreviation: ECM: Extracellular matrix.

the importance of accurate metabolic modeling using organoid systems. The metabolic environment within organoid cultures is influenced by factors such as culture media composition, oxygen availability, and ECM components.[114,115] Optimizing these parameters is crucial for replicating physiological conditions and ensuring the reliability of organoid-based studies.

A range of analytical techniques have been developed to assess organoid metabolism. Colorimetric assays, mitochondrial function tests, and metabolomic profiling provide critical insights into cellular energy states and metabolic dynamics.[7,116] Advanced methodologies, including targeted and untargeted metabolomics coupled with imaging mass spectrometry, allow for spatially resolved analysis of metabolic alterations.[117,118] These approaches are instrumental in elucidating both normal brain development and the metabolic dysregulation associated with neurological diseases.

Given the complexity of metabolic processes in organoids, precise analytical tools are required to capture dynamic biochemical changes (Figure 2). Microscopy-based assays enable real-time visualization of mitochondrial function and oxidative stress,[119,120] while high-throughput plate-based assays, such as Seahorse analysis, facilitate detailed metabolic profiling of glycolysis and mitochondrial respiration.[121,122] However, these methods are sensitive to factors such as organoid size and culture conditions, necessitating standardized experimental protocols.

Emerging technologies are expanding the scope of metabolic research in organoids. Nanoparticle-based assays utilizing oxygen-sensitive phosphorescent probes map intra-organoid oxygen gradients, providing insights into metabolic adaptations under hypoxic conditions.[120,123,124] Isotope tracing with stable isotope labeling and mass spectrometry allows for precise tracking of metabolic fluxes,

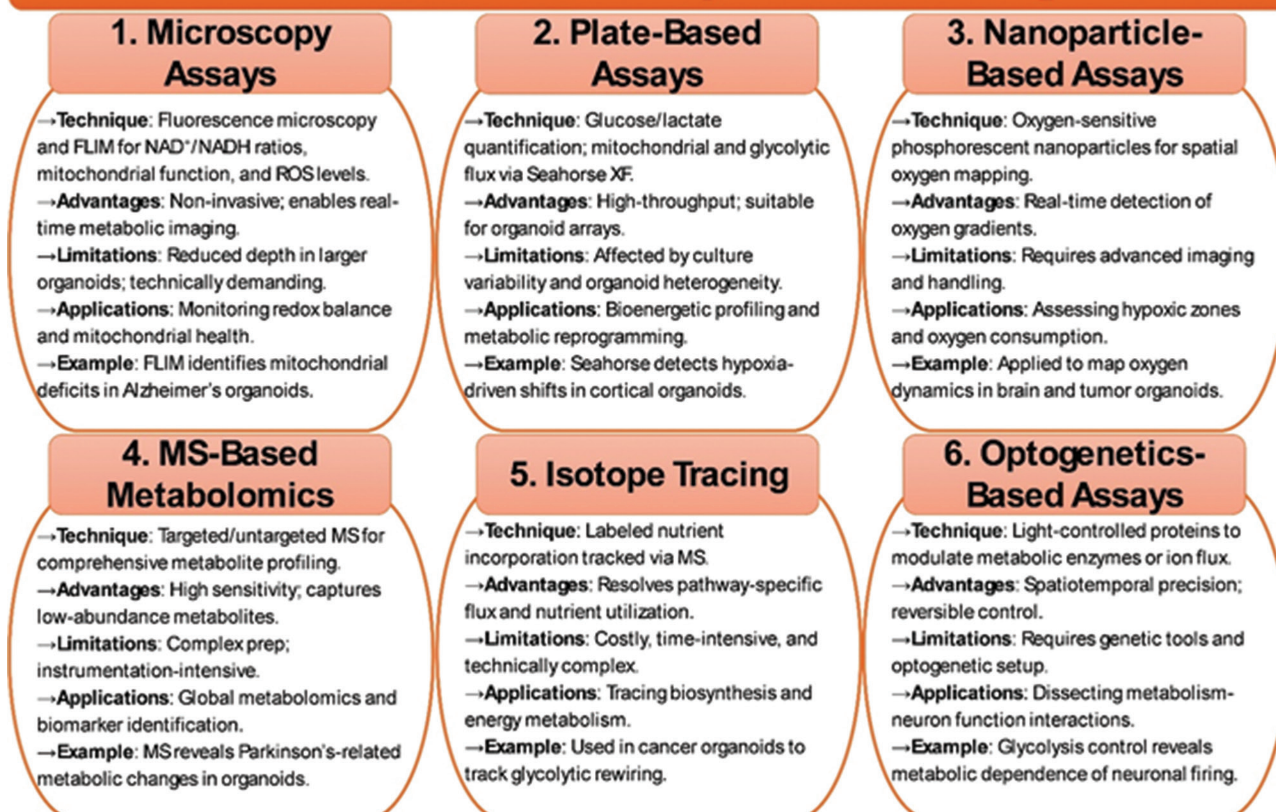

Figure 2. Innovative metabolic assays for brain organoids
Abbreviations: FLIM: Fluorescence lifetime imaging microscopy; MS: Mass spectrometry; ROS: Reactive oxygen species.

offering a deeper understanding of energy utilization and biosynthetic pathways.[125-127] Optogenetics-based metabolic control further enhances the ability to modulate and study metabolic activity with spatial and temporal precision.[128,129] These advancements hold great potential for investigating disease mechanisms and developing novel therapeutic strategies in organoid models.

Metabolomics, both targeted and untargeted, provides a comprehensive view of the metabolic landscape within organoids.[130] Although metabolomics in brain organoids is still an emerging field, it offers valuable data on key metabolic pathways such as glycolysis, fatty acid metabolism, and oxidative phosphorylation.[131] Integrating transcriptomic data with metabolomic analysis enriches the understanding of the metabolic state within organoid models. One promising development is the application of imaging mass spectrometry to map lipid distributions within human brain organoids,[132] offering new insights into cellular metabolic processes. However, challenges remain, such as the technical difficulties of spatial metabolomics and the integration of these data with transcriptomic analyses of metabolic enzymes.

Several factors complicate metabolic studies in organoids (Table 3). Oxygen and nutrient diffusion present significant challenges, particularly in larger organoids, where insufficient oxygen and nutrient availability can lead to hypoxic conditions and core nutrient deprivation, skewing metabolic assessments.[133,134] Cellular heterogeneity further complicates analysis, as neural progenitors primarily rely on glycolysis, whereas postmitotic neurons depend on oxidative phosphorylation.[135] In addition, hyperglycemic culture media can induce glucose-related stress, increasing reactive oxygen species production and disrupting neuronal differentiation.[7,136] Variations in glucose and oxygen concentrations across studies contribute to inconsistencies in metabolic outcomes. Furthermore, transcriptomic data alone do not fully capture metabolic function, particularly enzyme activity, which is critical for understanding metabolic processes. Enzyme activity needs to be studied in conjunction with transcriptomic analyses to provide a complete metabolic profile.[137]

Addressing these challenges requires optimizing culture conditions, refining metabolic assessment protocols, and fostering interdisciplinary collaboration. By overcoming

Table 3. Challenges in metabolic studies of brain organoids

Challenge	Description	Impact	Potential solutions	References
Oxygen and nutrient diffusion	Larger organoids → limited diffusion → hypoxia and necrosis in core	Core cell death/altered states → skewed metabolic data	↓ Organoid size, ↑ media optimization, ↑ perfusion systems	7,133
Cellular heterogeneity	Diverse cell types → varying metabolic demands	Mixed metabolic signals → difficult interpretation	Isolate cell populations or design cell type-specific models	60,138,139
Glucose-induced stress	Hyperglycemic media → oxidative stress, ↑ ROS, impaired neuronal differentiation	Alter metabolic profiles → inconsistent outcomes	Refine glucose levels, monitor ROS to reduce stress	7,140
Enzyme activity	Transcriptomics≠enzyme function → lack of functional metabolic insight	Enzymatic activity unmeasured → incomplete metabolic understanding	Combine transcriptomics with enzyme activity assays	7,60,141
Spatial metabolomics	Imaging mass spectrometry → technically complex; hard to integrate with transcriptomics	Poor spatial resolution of metabolic data	Develop improved spatial profiling and integration tools.	132,137

Abbreviation: ROS: Reactive oxygen species.

these hurdles, researchers can enhance the physiological relevance of brain organoids, improving their utility for studying human brain development and neurological diseases.

4.3. Advanced functional imaging and electrophysiology in brain organoids: Unraveling neural circuit maturation and disease mechanisms

Brain organoids serve as *in vitro* models for NDDs, replicating key aspects of human brain development.[15,52] Their functional activity is assessed using patch-clamp electrophysiology, calcium imaging, MEAs, and voltage imaging, each offering unique insights into neuronal maturation and disease mechanisms (Table 4).[7,142]

Patch-clamp electrophysiology remains the gold standard for single-cell analysis, providing precise control over membrane potential and detailed measurements of synaptic responses.[17,163] This technique can be applied to whole mount, sliced, or dissociated organoids, each with distinct advantages and limitations. Whole-mount organoids offer a comprehensive view of neuronal activity but are limited to surface neurons,[143] while sliced and dissociated organoids provide access to deeper structures at the expense of 3D integrity.[146] Patch-seq, which integrates patch-clamp with scRNA-seq, has further advanced the understanding of molecular mechanisms underlying neuronal activity.[164] However, patch-clamp remains labor-intensive, requires specialized training, and has limited throughput.

Calcium imaging enables non-invasive, high-throughput monitoring of neuronal network activity using genetically encoded calcium indicators (GECIs) such as GCaMP.[165] This method provides valuable insights into synaptic plasticity and network maturation, particularly when combined with optogenetics for precise neuronal control.[166] Despite its advantages for long-term studies, calcium imaging has slow kinetics that limit the resolution of rapid neuronal events, necessitating the development of faster alternatives such as voltage imaging.

Voltage imaging provides real-time monitoring of neuronal membrane potential across networks using voltage-sensitive dyes (VSDs) and GEVIs.[154,167] VSDs offer high-speed, network-wide activity measurements but can be invasive and susceptible to dye bleaching,[168] whereas GEVIs provide non-invasive, long-term expression in specific neuronal populations but have slow kinetics and limited spatial resolution.[167,169,170] These approaches enhance electrophysiological studies, dynamic neuronal activity analysis, and network dynamics investigations.

MEAs facilitate high-throughput, long-term monitoring of extracellular voltage fluctuations, capturing network-wide electrical events in real-time.[171] 2D MEAs are effective for studying network dynamics but lack the complexity of 3D systems.[172] Advances in 3D MEA technology enhance spatial and temporal resolution, allowing for more comprehensive analysis of neuronal network formation and activity in organoids.[151] Integration with optogenetics enables targeted neuronal stimulation, improving the study of specific cell types in neural circuits.[152] Traditional MEA systems, initially designed for 2D cultures, face challenges in fully capturing 3D organoid complexity, prompting the development of specialized platforms for improved electrode placement and recording.

Fluorescence imaging techniques such as whole-organoid and live-cell imaging enable non-invasive, long-term observation of cellular and network activity.[157,158] Whole-organoid imaging captures morphological and functional changes but are limited to surface measurements, while live-cell imaging provides high temporal resolution for tracking dynamic processes such as migration and differentiation, though it requires advanced microscopy and can cause phototoxicity.[173] *In vivo* imaging, particularly two-photon microscopy, allows for high-resolution, deep-

Table 4. Comparative overview of functional imaging and electrophysiological techniques in brain organoids

Technique	Preparation	Advantages → Limitations	Resolution	Throughput	Applications	References
Patch-clamp	Whole-mount organoids	Whole-organoid activity view → Limited to surface neurons	High (single-cell)	Low	Single-neuron activity, synaptic responses, maturation	143,144
	Sliced organoids	Preserves 3D structure → May disrupt networks, limited depth access	Moderate	Low	Activity in structured tissue, reduced resolution	145,146
	Dissociated organoids	Deep neuron access → Loss of 3D network architecture	Moderate	Low	Single-cell studies, simplified access	147,148
Calcium imaging	GECIs	Long-term, neuron-specific expression → Slow kinetics, limited fast event resolution	Moderate (network-level)	High	Network activity, plasticity, disease models	62,149
	GECIs + Optogenetics	Precise neuronal control → Limited temporal resolution	Moderate	Moderate	Network manipulation, disease modeling	129
MEAs	2D MEA	High-throughput, non-invasive → Lacks 3D complexity	Low (network-level)	Very high	Screening, pharmacology, network dynamics	142,150
	3D MEA	Higher spatiotemporal resolution → Requires complex setup	High (3D)	Moderate	3D networks, development, behavior studies	151,152
Voltage imaging	VSDs	Real-time membrane potential tracking → Invasive, limited penetration, bleaching	High (network-wide)	Moderate	Fast network dynamics, electrophysiology	153,154
	GEVIs	Long-term, non-invasive tracking → Limited fast spike detection	Moderate	High	Regional activity, network monitoring	155,156
Fluorescence imaging	Whole organoid	Non-invasive, whole-organoid view → Poor deep structure resolution	Moderate	High	Long-term tracking, morphological studies	157-159
	Live-cell imaging	High temporal tracking → Requires advanced setups, risk of phototoxicity	High	Moderate	Cell migration, differentiation, interaction	157,160
In vivo imaging	Two-photon microscopy	Deep tissue imaging, high resolution → Costly, complex, limited organoid size	High	Low	Deep activity mapping, synaptic analysis	161,162

Abbreviations: GECIs: Genetically encoded calcium indicators; GEVIs: Genetically encoded voltage indicators; MEA: Multielectrode array; VSDs: Voltage-sensitive dyes.

tissue imaging of neuronal networks but are expensive and complex, primarily applicable to animal models or specific organoid sizes.[162,174]

These techniques collectively provide a powerful toolkit for investigating neuronal circuits and NDDs. Ongoing advancements in 3D-compatible platforms and imaging technologies continue to refine their applications, expanding their potential for studying human brain function and disease.

5. Advancing brain organoid analysis: Standardized markers, imaging techniques, and the methodological challenges

Brain organoids serve as essential models for studying the 3D development of the human brain. Their comprehensive evaluation requires rigorous analysis of architectural organization and the spatial distribution of diverse cell populations. Histological assessments using lineage-specific markers confirm that brain organoids recapitulate fundamental aspects of cortical cytoarchitecture,[175] though quantitative data remain limited. A thorough examination of current methodologies for assessing cellular composition leads to the proposal of a standardized framework aimed at enhancing rigor and reproducibility in organoid-based research.

5.1. Harnessing cell type-specific markers for enhanced analysis of brain organoids

Brain organoids serve as sophisticated *in vitro* platforms that emulate key aspects of human brain development, enabling the study of neurogenesis, lineage specification, and disease pathogenesis under controlled conditions.[52,56] A critical aspect of organoid analysis is the application of well-characterized, cell type–specific markers, originally

developed for *in vivo* studies of brain development (Figure 3). These markers are essential for identifying the developmental stage and functional identity of various neural and glial populations within organoids. During early developmental phases, NPCs dominate and are typically localized in the VZ and SVZ.[176] VZ progenitors' express transcription factors such as PAX6, which governs neural stem cell identity and early fate decisions, and SOX2, which maintains pluripotency and self-renewal capacity.[177] SVZ progenitors are marked by EOMES (TBR2), a key regulator of intermediate progenitor proliferation and neuronal commitment.[177] In addition, migrating and differentiating neuroblasts express doublecortin (DCX), which is indispensable for early cortical lamination.[178] These spatial and temporal markers enhance the resolution of developmental mapping, thereby improving the precision and reproducibility of cellular characterization. Despite their utility, organoid systems face significant challenges in fully recapitulating the spatial and temporal fidelity of *in vivo* differentiation processes. Structural heterogeneity is particularly evident in early neuroepithelial rosettes, and cortical lamination often diverges from canonical *in vivo* patterns, complicating direct comparisons.[10,179-181] Moreover, even among organoids derived from the same hPSC line, intrinsic developmental variability hinders the reproducibility of results.

As organoids advance toward maturation, the emergence of distinct neuronal and glial subtypes can be assessed using a repertoire of layer- and lineage-specific markers. Deep-layer cortical neurons (Layers V–VI) are identified by the expression of TBR1, CTIP2 (BCL11B), and FOXP2, which collectively contribute to corticothalamic and corticospinal connectivity.[182] In contrast, upper-layer neurons (Layers II–IV) express SATB2 and CUX1, both of which regulate callosal projection development and intracortical connectivity. General neuronal maturation is marked by RBFOX3 (NeuN) and TUBB3 (βIII-tubulin), indicative of axonal development and neuronal identity.[182] These markers enable the delineation of neuronal maturation stages and the establishment of cortical layer identities. In parallel, glial populations emerge, including astrocytes expressing ALDH1L1 and GFAP, which reflect metabolic and structural support functions.[183] Oligodendrocyte lineage cells are identified by OLIG2 and MBP, both essential for myelination, while microglia are marked by Iba1, denoting their immunological roles.[184] However, challenges persist in achieving robust and functionally complete glial diversification within current organoid models, thereby limiting their translational fidelity. The complexity of developmental trajectories necessitates the use of multiple markers for accurate cell type identification. Furthermore, the lack of standardized protocols across

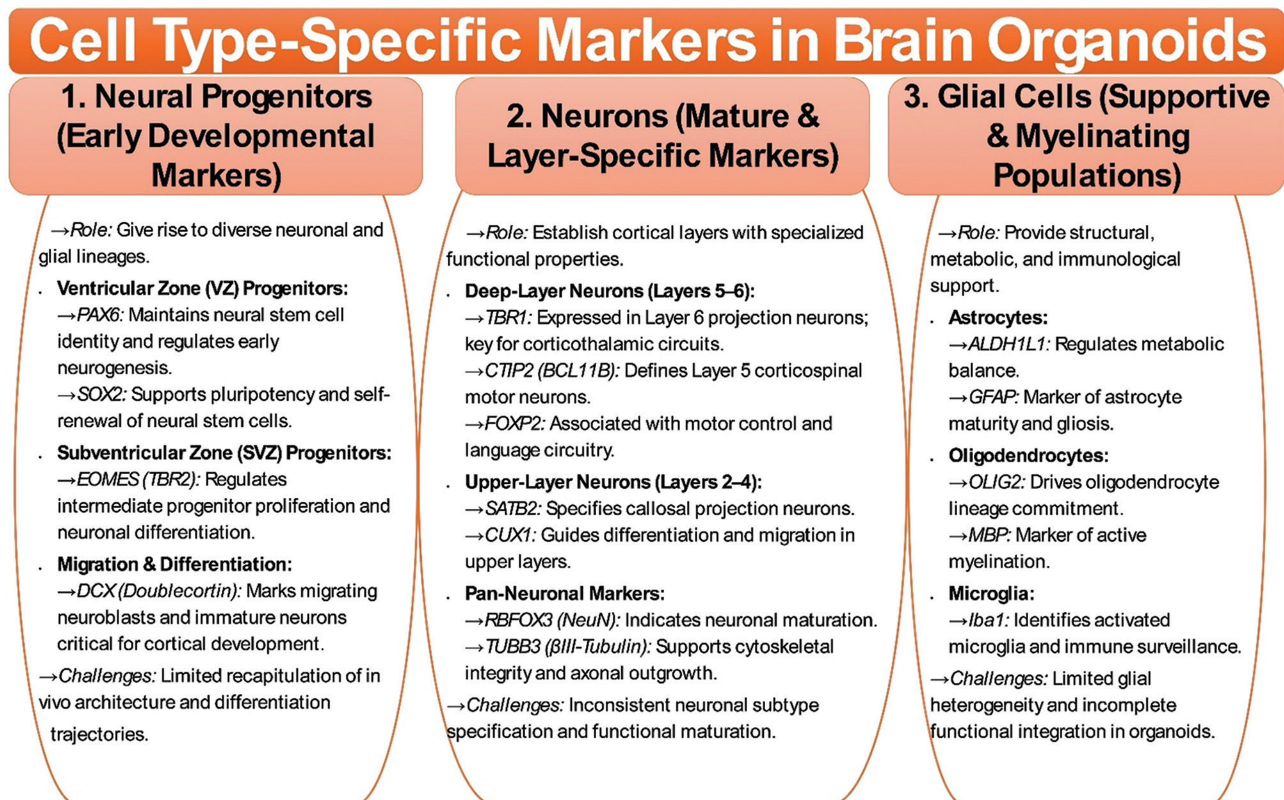

Figure 3. Cell type-specific markers in brain organoids

laboratories continues to impede reproducibility and constrains the comparability of findings between studies.

5.2. Optical clearing techniques and their applications in high-resolution imaging of brain organoids

Optical clearing techniques, combined with high-resolution microscopy methods such as confocal and light sheet microscopy, have emerged as powerful tools for 3D visualization. These methods preserve tissue integrity while enhancing imaging depth, enabling more accurate morphological assessments (Table 5). Various clearing techniques offer distinct advantages: ClearT2 minimizes distortion and is particularly suitable for deep tissue imaging, making it ideal for neuroscience and developmental biology studies.[185] ScaleSQ induces tissue expansion, which facilitates higher-resolution imaging, although this expansion can complicate volumetric analyses.[186] SeeDB enhances resolution for thin samples but may alter cellular arrangements, complicating interpretations of cellular structures.[187] The choice of clearing method depends on study objectives, with each presenting unique strengths and trade-offs.

Despite these advancements, challenges persist in high-resolution imaging. Large datasets require substantial computational power for processing and analysis, and chromatic aberrations can distort reconstructed 3D models. Segmentation of cellular structures is further complicated

Table 5. Comparative exploration of tissue clearing techniques

Method	Key feature	Advantages	Limitations	Applications	Optimal tissue type	Use with organoids	References
ClearT2	Preserves structural integrity	Ideal for deep tissue imaging without distortion	May require longer processing times	Neuroscience, developmental biology	Brain, muscle, and other solid organs	Suitable for imaging organoids	188,189
ScaleSQ	Induces tissue expansion	Enables higher-resolution imaging	Expansion can complicate imaging analysis	3D imaging, vascular studies	Soft tissues, including brain and vascular tissue	Applicable for organoid studies	190
SeeDB	Tissue shrinkage	Enhances resolution for thin samples	Shrinkage may alter cellular arrangement	Histology, cellular morphology analysis	Thin slices of various tissues	Compatible with thin organoid slices	187
Organic solvent-based	Effective deep tissue clearing	Suitable for dense or lipid-rich tissues	Potential toxicity and prolonged processing times	Cancer research, lipid studies	Lipid-rich tissues, including fat and brain tissue	Possible, but requires careful handling	191
CUBIC	Minimal distortion	High transparency and preservation of morphology	Time-consuming multiple steps	Imaging whole organs and tissues	Various tissues, including brain and kidney	Effective for organoid imaging	192
PARS	Enzyme-assisted clearing	Preserves proteins and nucleic acids	Limited to specific tissue types	Molecular biology, protein localization studies	Fixed or frozen tissues with complex structures	Limited use; mostly for specific types	193
TDE	Dehydration followed by expansion	Allows for detailed imaging at high resolutions	Requires careful handling to prevent damage	Developmental studies, organoid research	Various types of organoids and cultured cells	Highly suitable for organoid applications	194
ClearMap	Combines clearing and imaging	Simultaneous imaging and clearing	Limited to certain imaging modalities	*In vivo* imaging, dynamic studies	Tissues amenable to dynamic imaging	Usable for dynamic studies with organoids	195
iDISCO	Immunolabeling combined with clearing	Retains antigenicity while providing transparency	Potential loss of cellular detail during processing	Developmental biology, immunological studies	Embryonic and adult tissues	Effective for immunolabeling organoids	196,197
3DISCO	3D imaging capability	Facilitates visualization of entire organs	Complexity of protocol and cost	Organ and large tissue studies	Whole organs like brain, liver, and heart	Suitable for whole organoid imaging	198

Abbreviation: TDE: Tissue dehydration and expansion.

by necrotic regions, which confound volumetric analyses and cell counting.[199] Addressing these issues requires advances in computational image analysis, such as machine learning-based segmentation tools, to automate cell type and structural feature identification. Standardized labeling protocols and tissue segmentation methodologies must also be developed to improve reproducibility and ensure consistent data interpretation across studies.

Integrating robust cellular markers, standardized imaging techniques, and computational tools will facilitate reproducible and precise brain organoid analysis. Refinement of these methodologies is essential for leveraging organoids as reliable models of human brain development and disease modeling. Techniques such as tissue dehydration and expansion offer detailed imaging at high resolutions and are particularly suitable for various types of organoids and cultured cells.[194] CUBIC stands out for its minimal distortion and high transparency, enabling effective imaging of organoids while preserving morphology.[192] In addition, iDISCO combines immunolabeling with clearing, allowing for the retention of antigenicity, which is vital for developmental biology and immunological studies involving organoids.[196,197] However, the complexity of protocols, particularly for methods like 3DISCO, which facilitates the visualization of entire organs, can pose challenges in both cost and implementation.[198]

To optimize the use of these techniques, careful consideration of their limitations is necessary. For example, organic solvent-based methods are effective for dense or lipid-rich tissues but may involve toxicity and extended processing times.[191] Meanwhile, PARS (enzyme-assisted clearing), although preserving proteins and nucleic acids, is limited to specific tissue types.[193] Therefore, a comprehensive understanding of each technique's capabilities and constraints is essential for advancing research in brain organoid studies.

5.3. Current limitations in brain organoid models: Reproducibility, microenvironment, and ethical challenges

Despite significant methodological advances, brain organoid systems face a range of persistent challenges that hinder their full translational and experimental utility. One of the most pressing concerns is the evaluation of reproducibility across different laboratories. Variability in protocols, cell lines, culture conditions, and analysis techniques often results in divergent phenotypes and outcomes, complicating the comparison and integration of findings across studies.[51,79]

Batch-to-batch variability is another major issue, even when using the same hPSC line.[7] Subtle differences in media composition, passage number, or even incubator humidity can lead to significant variations in organoid morphology and cellular composition.[200] This intrinsic variability limits the robustness of disease modeling and the predictive value of drug screening efforts.

Furthermore, organoids often lack key components of the *in vivo* brain microenvironment, including vasculature, the blood–brain barrier (BBB), and resident immune cells such as perivascular macrophages.[201-203] The absence of these features compromises nutrient and oxygen diffusion, limits the maturation of certain cell types, and fails to recapitulate neurovascular interactions critical to brain physiology and pathology. Although recent bioengineering approaches aim to incorporate vascular networks and BBB-like structures, these systems are still in early development and are not yet widely adopted.

Ethical considerations also continue to evolve as brain organoid models become more complex.[204,205] Questions regarding the potential for sentience, the extent of functional connectivity, and the use of human-derived stem cells pose unique bioethical challenges.[206,207] As organoids approach higher levels of structural and functional sophistication, the scientific community must establish clear ethical guidelines to ensure responsible use of this technology.

Addressing these challenges will require a concerted effort to standardize protocols, develop quality control benchmarks, and integrate missing physiological elements. Continued dialogue around the ethical implications is equally critical for the responsible advancement of organoid-based neuroscience.

6. Engineering advances in brain organoid culture: Innovations in static, microfluidic, and vascularized systems

The development of human brain organoid culture techniques has made significant strides through the integration of various engineering approaches, such as static culture, microfluidic systems, and vascularization strategies (Table 6). These advancements have reduced structural heterogeneity, enhanced neuronal tissue maturation, and improved functional outcomes. Recent methodologies in brain organoid culture offer notable advantages and limitations, with promising future applications.

Long-term static culture systems maintain cells under physiological conditions without active nutrient replenishment or waste removal.[220] Neural-inducing molecules drive self-organization, leading to the formation of spheroids that differentiate into cortical neurons and non-reactive astrocytes.[221] Low-adsorption culture plates and U- or V-bottom perforated plates promote 3D self-assembly. Embedding organoids in Matrigel and transferring them to rotating bioreactors further enhances oxygen diffusion and nutrient distribution. However, challenges such as necrotic core formation due to inadequate

Table 6. Innovative culture strategies for neural organoids

Culture method	Key features	Advantages	Limitations	Applications	Challenges	References
Static culture systems	Self-organizing spheroids using neural-inducing molecules	Simple, cost-effective, scalable	Limited nutrient exchange, necrotic core formation	Neural differentiation studies, disease modeling	Inconsistent maturation, limited long-term viability	208,209
Rotating bioreactors	Spheroids embedded in Matrigel with dynamic mixing	Improve nutrient diffusion, enhances maturation	Still prone to necrotic cores, scalability limitations	Drug testing, neurodevelopmental studies	Require specialized equipment, variability in results	210,211
Organotypic slice cultures	Sliced organoids cultured at gas-liquid interface	Enhanced oxygenation, reduced hypoxia	Potential structural disruption, contamination risks	Axonal growth studies, electrophysiological recordings	Difficult to maintain long-term viability	146,212
Microfluidic static culture	PDMS microcolumn arrays for uniform organoid generation	Reduces variability, integrated differentiation	Limited oxygenation in larger organoids	High-throughput screening, disease modeling	Requires microfabrication expertise	135,213
Microfluidic dynamic culture	Continuous perfusion systems	Enhanced nutrient exchange, high-throughput potential	Requires specialized equipment	Drug screening, neuronal connectivity analysis	Maintenance complexity, cost	214,215
In vitro vascularization	Co-culture with endothelial cells or vascular progenitors	Mimics *in vivo* vasculature, improves maturation	Complexity in maintaining vascular networks	Ischemia modeling, blood-brain barrier studies	Standardization issues, vascular regression risk	216-218
In vivo vascularization	Transplantation into animal models	Full vascular integration, improved functional maturation	Ethical concerns, host-dependent variability	Humanized models, stroke and neurodegeneration studies	Ethical considerations, interspecies differences	201,219
Hybrid approaches	Combining multiple methods (e.g., microfluidics+ vascularization)	Maximize physiological relevance, overcomes individual limitations	Increased experimental complexity, high cost	Personalized medicine, precision drug testing	Require multi-disciplinary expertise	60,201

Abbreviation: PDMS: Polydimethylsiloxane.

perfusion, restricted nutrient exchange, and limited gas diffusion remain prevalent.[210] Organotypic brain slice cultures provide an alternative by improving oxygenation and reducing hypoxia-induced necrosis. Using a gas–liquid interface technique, mature organoids are embedded in agarose, sliced, and cultured, promoting neuronal survival, axonal outgrowth, and synaptic integration.[222] Thick axon bundles exhibit diverse morphologies, and subcortical projections integrate with mouse spinal cord outgrowths, triggering muscle contractions.[223,224] A forebrain organoid sectioning approach preserves cortical structure, reduces necrotic regions, and maintains neurogenesis, yet slicing procedures pose risks of contamination and structural disruption, particularly in the VZ and SVZ.[225]

Microfluidic co-culture systems represent a cutting-edge advancement, closely mimicking *in vivo* environments.[226] Static microfluidic culture simplifies organoid formation, integrating neural induction and differentiation within a single vessel.[217] Microcolumn arrays aggregate single cells into EBs, generating uniformly sized brain organoids with reduced batch-to-batch variability and necrosis.[135,227] Dynamic microfluidic systems enhance culture conditions through continuous nutrient infusion and waste removal. Pump-based designs connected to peristaltic pumps increase oxygen availability and promote dopaminergic neuron differentiation, while hydrostatic pressure-driven approaches facilitate fluid flow without external pumps, enhancing neural progenitor differentiation, synapse formation, and high-throughput screening applications.[228,229]

Despite these advances, current models lack functional vasculature, limiting their size and maturation due to insufficient oxygen and nutrient delivery. Orbital shakers improve surface oxygenation,[230] while organotypic slice cultures enhance deeper oxygen penetration, though at the cost of 3D structural integrity.[231] Efforts to achieve

vascularization focus on *in vitro* and *in vivo* strategies. *In vitro* approaches involve co-culturing brain organoids with vascular cells, embedding them in ECM gels with endothelial cells, or integrating human umbilical vein endothelial cells, fostering spontaneous vascular network formation.[201,216] Vascularized brain organoids exhibit reduced expression of hypoxia markers, increased size, and enhanced survival, highlighting their potential to model neurodevelopmental processes and disease pathologies.[52,232] These advancements pave the way for more physiologically relevant brain organoid models suitable for translational applications.

7. Integrating molecular mechanisms and therapeutic strategies in NDDs through organoid models

A comprehensive understanding of the molecular and cellular mechanisms underlying neurodevelopmental and neuropsychiatric disorders is essential for advancing targeted therapeutic interventions. Disorders such as autism spectrum disorder, Timothy syndrome, and schizophrenia display distinct yet overlapping neuropathological features, including synaptic dysfunction, aberrant neuronal migration, and disrupted cortical development.[233] The application of advanced experimental methodologies – such as sc-RNA seq, electrophysiological recordings, and CRISPR gene editing – has significantly expanded our insights into these complex conditions. Furthermore, the identification of disorder-specific therapeutic targets highlights the pathophysiological diversity of these disorders, with strategies ranging from FOXG1 knockdown in autism spectrum disorder to the utilization of mTOR inhibitors in tuberous sclerosis complex,[234,235] underscoring the translational relevance of these findings.

Organoid models have emerged as powerful tools for studying NDDs, providing unprecedented opportunities to investigate the intricate cellular and molecular abnormalities associated with these conditions (Figure 4). Derived from patient-specific iPSCs, organoids replicate key aspects of human brain development, enabling researchers to observe disease phenotypes in a 3D context that closely mimics *in vivo* conditions.[10,52] In the case of autism spectrum disorder, organoid models have revealed increased inhibitory synapse formation and aberrant progenitor proliferation, particularly within the prefrontal cortex and hippocampus.[67,236-238] These findings suggest that altered synaptic dynamics may contribute to the characteristic cognitive and behavioral features observed in affected individuals.

Similarly, organoid studies in Timothy syndrome have demonstrated disorganized interneuron migration, a consequence of CaV1.2 dysfunction.[239-241] This cellular disorganization reflects the neuroanatomical anomalies observed in patients and provides a platform for investigating the underlying mechanisms of the disorder. Research on Down syndrome has further elucidated the effects of genetic mutations on brain development, showing an overproduction of $OLIG2^{+}$ progenitors coupled with diminished cortical neurogenesis (Figure 4).[242-245] These insights offer valuable mechanistic explanations for the cognitive deficits commonly associated with Down syndrome.

The impact of these genetic alterations extends beyond individual neuronal populations, influencing broader neural circuits and structures within the brain. For instance, alterations in the limbic system are prominent in schizophrenia,[246-248] while disruptions in the cerebellum are observed in Angelman syndrome (Figure 4).[249-251] The ability of organoid models to capture the complexity of NDDs at both cellular and systems levels underscores their significance in advancing mechanistic investigations.

Moreover, the integration of multi-omics technologies, functional imaging, and gene-editing tools into organoid research has further enhanced the resolution at which molecular and cellular dysfunctions can be studied. For example, modulation of the WNT/β-catenin signaling pathway has shown therapeutic potential in autism spectrum disorder,[252,253] providing a promising avenue for targeted intervention. Similarly, gene therapy approaches targeting MECP2 offer hope for treating Rett syndrome.[254,255] Pharmacological strategies, such as mGluR5 inhibitors for Fragile X syndrome and NMDA receptor modulators for schizophrenia, exemplify the specificity required for effective therapeutic development (Figure 4).[256,257]

Despite these advances, several critical challenges remain in the development and application of patient-specific organoid models. One major issue is the variability in differentiation potential among iPSC lines derived from different patients, which can affect the reproducibility and interpretation of experimental results.[258] Furthermore, the heterogeneity in genetic background, epigenetic modifications, and reprogramming artifacts introduces additional complexity when comparing phenotypic outcomes across samples.[259,260] Standardization of organoid protocols remains limited, with differences in culture media, growth factors, and timeline of maturation significantly impacting the cellular composition and developmental trajectory of the organoids.[261]

Another significant limitation is the incomplete maturation and lack of vascularization in most brain organoids, which constrains their ability to fully recapitulate *in vivo*-like neuronal circuitries and long-range connectivity.[201,232] These structural deficits hinder the study of later stages of development and neurodegeneration, as well as pharmacokinetic modeling of therapeutic compounds.

Figure 4. Mapping neurodevelopmental disorders: Genetic causes → Organoid research outcomes
Abbreviations: EEG: Electroencephalography; fMRI: Functional magnetic resonance imaging; RNA-seq: RNA sequencing.

In the context of personalized medicine, integrating patient-derived organoid data with clinical phenotypes remains a substantial challenge. High-throughput screening of therapeutic compounds in organoids is still in its infancy and faces issues such as scalability, cost, and the need for robust computational tools to analyze multidimensional datasets.[262] Ethical concerns related to the generation and manipulation of human brain-like tissues also demand ongoing discussion and regulation, particularly as these models gain complexity.[263]

To advance the translational potential of organoid-based research, several critical hurdles must be addressed as priorities, including the development of standardized differentiation protocols, the incorporation of vascular and immune system components, and the integration of machine learning algorithms to unify genomic, transcriptomic, and phenotypic data for individualized therapeutic predictions. In addition, the establishment of collaborative frameworks that connect clinical cohorts with organoid biobanks holds promise for enhancing the precision and translational relevance of disease modeling and therapeutic discovery.

The convergence of genetic, molecular, and cellular insights through organoid modeling is reshaping our understanding of neurodevelopmental and neuropsychiatric disorders. These models not only facilitate the investigation of disease pathophysiology but also enable the identification and preclinical validation of novel, mechanism-based therapeutic strategies tailored to the complex biology of these conditions.

8. Unraveling the impact of viral infections on NDDs: Insights from 3D organoid models

Environmental factors, particularly viral infections, significantly increase the risk of NDDs such as microcephaly, autism spectrum disorder, and schizophrenia. While the placenta acts as a protective barrier, vertical transmission can still occur.[264] Maternal immune activation triggered by infections can impact fetal development at any stage of pregnancy.[265] Viral infections during gestation have been associated with various congenital neurological defects; however, the mechanisms by which pathogens cross the placental barrier remain poorly understood, primarily due to the lack of model systems that accurately replicate human brain development while integrating maternal factors. The advent of 3D organoid models has significantly advanced research on maternal and fetal viral infections, providing deeper insights into their effects on brain development.[266,267]

The "TORCH" acronym originally referred to *Toxoplasma gondii*, rubella virus, human cytomegalovirus, and herpes simplex virus, but was later expanded to include additional pathogens, sometimes referred to as "STORCH" to incorporate syphilis. Emerging infectious agents, such as the Zika virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), continue to reshape this classification.[268] TORCH pathogens are known to cause a range of congenital abnormalities, including heart malformations, eye defects, pneumonia, brain calcifications, and microcephaly, affecting multiple organ systems.[269] They are also linked to intrauterine growth restriction, miscarriages, and stillbirths, primarily transmitted to the fetus through vertical transmission, either transplacental or during birth.

Viral infections during fetal development significantly contribute to various neurological deficits, including microcephaly, encephalitis, seizures, and cognitive impairments (Figure 5).[270] These infections disrupt critical developmental processes by impairing neural progenitor function, inducing neuroinflammation, and altering synaptic connectivity.[271,272] For example, *Toxoplasma gondii* induces encephalitis and microcephaly, resulting in long-term cognitive impairments and developmental delays due to its impact on neurogenesis and microglial activation.[273] The rubella virus disrupts neuronal migration and synaptic connections, leading to congenital defects such as deafness and motor delays.[274] These manifestations underscore the need to understand how specific pathogens influence fetal brain development.

3D brain organoid models have revolutionized the study of viral neuropathogenesis by enabling researchers to accurately simulate infection dynamics and cellular responses.[266] For instance, Zika virus infection in organoids demonstrates significant disruption in NPC proliferation and cortical formation, causing microcephaly and other developmental defects observed in affected infants.[275] Human cytomegalovirus alters neuronal differentiation and cortical layer formation, leading to cognitive deficits effectively modeled in organoid systems.[276] The herpes simplex virus disrupts synaptic function and neuronal proliferation, offering insights into the mechanisms underlying developmental delays.[277] In addition, enterovirus 71 (EV71) infection results in acute flaccid paralysis and encephalitis, with organoids revealing how EV71 induces neuronal damage and disrupts neurogenesis through neuroinflammatory pathways.[278,279]

Chikungunya virus also causes neuroinflammation and cognitive impairment, with organoids elucidating how this inflammation affects neural precursor cells and normal brain development.[280] Similarly, West Nile virus impacts neuronal maturation and synaptic plasticity,[281] while influenza virus affects neurogenesis, both resulting in long-term cognitive impairments and motor dysfunction.[282] The neurodevelopmental implications of coronavirus disease 2019 (COVID-19; caused by SARS-CoV-2) are still emerging, but organoid models are proving essential for understanding its potential effects on neural differentiation and cognitive function.[283]

Overall, 3D organoid models serve as a crucial platform for dissecting the cellular and molecular mechanisms underlying the effects of these viral pathogens on brain development. By providing insights into host-pathogen interactions and the specific disruptions caused by various viruses, these models enhance our understanding of congenital viral infections and their long-term consequences, ultimately guiding potential therapeutic interventions to mitigate their impact on future generations.

Figure 5. Viral pathogen invasion and its impact on brain development in 3D organoid models

Abbreviations: COVID-19: Coronavirus disease 2019; EV71: Enterovirus 71; HCMV: Human cytomegalovirus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; RV: Rubella virus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; WNV: West Nile virus.

The development of the cerebral cortex relies on the interplay of various cell types, each impacted differently by viral infections. Ventricular radial glial cells (vRGCs) are crucial during early neurogenesis, undergoing symmetrical expansion and asymmetric division to generate oRGCs and intermediate progenitor cells. These cell types are preferentially targeted by viruses such as Zika virus, human cytomegalovirus, and herpes simplex virus.[284] Key markers, including Pax6, Nestin, Sox2, and vimentin, highlight the importance of vRGCs in cortical expansion and neurogenesis. As development progresses, oRGCs act as neuronal progenitors and scaffolds for neuron migration, remaining susceptible to the same viral infections.[285] Important markers for oRGCs, such as Pax6, Sox2, GLAST, and GFAP, underscore their role in cortical folding and neuronal placement. Although intermediate progenitor cells are not explicitly indicated as targets for viral infection, they are essential for facilitating neuronal differentiation during this stage, characterized by markers such as Tbr2 (Eomes) and NeuroD1.[286,287]

As neurogenesis transitions to post-neurogenesis, migrating neurons, vital for establishing cortical layering, move from the oSVZ through the intermediate zone to the cortical plate, indicated by markers such as DCX and βIII-tubulin (Tuj1). Mature cortical neurons, responsible for forming synaptic connections and neural circuits, are primarily affected by rabies virus and herpes simplex virus, marked by NeuN, MAP2, and synapsin.[288,289] Astrocytes are influenced by rabies virus and herpes simplex virus during the gliogenesis phase, marked by GFAP, S100β, and ALDH1L1.[289] Microglia engages in immune surveillance and synaptic pruning throughout development, indicated by Iba1, CD11b, and TMEM119, although they are not explicitly linked to specific viral infections.[290] Endothelial cells play a crucial role from early development, identified by CD31, VE-cadherin, and GLUT1.[291] Oligodendrocytes are affected during late neurodevelopment, marked by Olig2, MBP, and PLP, which are vital for rapid signal transmission.[292,293] Finally, ependymal cells line the ventricles and regulate cerebrospinal fluid, contributing to homeostasis during late development, indicated by FoxJ1,

S100β, and vimentin.[294] Understanding the preferential viral infections of these various cell types throughout distinct developmental stages is essential for elucidating how viral pathogens disrupt cortical development and contribute to neurological deficits.

9. Conclusion

Brain development is a finely regulated process that, when disrupted, can lead to NDDs such as autism spectrum disorder, schizophrenia, and intellectual disabilities. While transgenic animal models have provided valuable insights, their limitations necessitate alternative approaches, such as hPSC-derived brain organoids. These 3D models have significantly advanced our understanding of neurogenesis, neuronal circuit formation, and disease pathology, offering a physiologically relevant system for studying both genetic and environmental influences on brain development.

Despite their promise, brain organoid models face challenges, including culture variability, lack of vasculature, and limitations in replicating complex brain functions. Addressing these issues requires standardization in differentiation protocols, improved microfluidic and vascularization techniques, and integrative methodologies incorporating machine learning, optogenetics, and multimodal data analysis. Moreover, refining imaging techniques and computational tools will enhance reproducibility and structural fidelity, ensuring more accurate modeling of human brain development.

Future research should focus on improving the physiological relevance of brain organoids by optimizing culture conditions, integrating immune and vascular components, and refining functional assessments using advanced electrophysiological and metabolic profiling tools. Key questions remain, including how to fully capture the complexity of neural network formation and how to translate organoid-based findings into effective therapeutic interventions. Expanding the use of patient-specific organoid models for drug screening and precision medicine holds great potential for advancing treatments for neurodevelopmental and neuropsychiatric disorders. By bridging the gap between molecular pathology and translational applications, brain organoid research will continue to drive breakthroughs in neuroscience, offering novel insights into both fundamental brain biology and disease mechanisms.

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