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Microinstrumentation for Brain Organoids

*Devan Patel, Saniya Shetty, Chris Acha, Itzy E. Morales Pantoja, Alice Zhao, Derosh George, and David H. Gracias**

Brain organoids are three-dimensional aggregates of self-organized differentiated stem cells that mimic the structure and function of human brain regions. Organoids bridge the gaps between conventional drug screening models such as planar mammalian cell culture, animal studies, and clinical trials. They can revolutionize the fields of developmental biology, neuroscience, toxicology, and computer engineering. Conventional microinstrumentation for conventional cellular engineering, such as planar microfluidic chips; microelectrode arrays (MEAs); and optical, magnetic, and acoustic techniques, has limitations when applied to three-dimensional (3D) organoids, primarily due to their limits with inherently two-dimensional geometry and interfacing. Hence, there is an urgent need to develop new instrumentation compatible with live cell culture techniques and with scalable 3D formats relevant to organoids. This review discusses conventional planar approaches and emerging 3D microinstrumentation necessary for advanced organoid–machine interfaces. Specifically, this article surveys recently developed microinstrumentation, including 3D printed and curved microfluidics, 3D and fast-scan optical techniques, buckling and self-folding MEAs, 3D interfaces for electrochemical measurements, and 3D spatially controllable magnetic and acoustic technologies relevant to two-way information transfer with brain organoids. This article highlights key challenges that must be addressed for robust organoid culture and reliable 3D spatiotemporal information transfer.

1. Introduction

Understanding the human brain's development, function, and diseases is a critical challenge in present-day science, engineering, and medicine.[1,2] Apart from these challenges, the mechanisms underlying the brain's ability to continuously store, retrieve, process, and learn from immense amounts of information with minimal energy consumption are still largely unknown and unrealized by modern technology.[3] Brain-inspired or non-von Neumann neuromorphic computing architectures may be required for energy-efficient and sustainable artificial intelligence and computing.[3–6] But before we can develop architectures that approach the human brain's capabilities or find treatments for neurodegenerative diseases, we must first understand their origins in the brain.

Studies involving model organisms, humans, and ex vivo human brain slices have been used extensively to model the structure of the human brain and understand the functions associated with different regions.[7] They have significantly advanced

D. Patel, C. Acha, D. George, D. H. Gracias
Department of Chemical and Biomolecular Engineering
Johns Hopkins University
Baltimore, MD 21218, USA
E-mail: dgracias@jhu.edu

S. Shetty
Department of Biomedical Engineering
Johns Hopkins University
Baltimore, MD 21218, USA

I. E. M. Pantoja
Center for Alternatives to Animal Testing (CAAT)
Department of Environmental Health and Engineering
Bloomberg School of Public Health, Johns Hopkins University
Baltimore, MD 21205, USA

A. Zhao
Department of Biology
Johns Hopkins University
Baltimore, MD 21218, USA

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D. H. Gracias
Department of Chemistry
Johns Hopkins University
Baltimore, MD 21218, USA

D. H. Gracias
Department of Materials Science and Engineering
Johns Hopkins University
Baltimore, MD 21218, USA

D. H. Gracias
Department of Oncology
Johns Hopkins University School of Medicine
Baltimore, MD 21205, USA

D. H. Gracias
Laboratory for Computational Sensing and Robotics (LCSR)
Johns Hopkins University
Baltimore, MD 21218, USA

D. H. Gracias
Sidney Kimmel Comprehensive Cancer Center (SKCCC)
Johns Hopkins School of Medicine
Baltimore, MD 21205, USA

D. H. Gracias
Center for MicroPhysiological Systems (MPS)
Johns Hopkins School of Medicine
Baltimore, MD 21205, USA

our understanding of neural processes from the Aristotelian view of the brain as a cooling organ to our modern-day understanding of complex cellular networks.[8,9] However, these approaches have inherent limitations and challenges.[10] In vivo, human studies can be invasive and have limited spatial resolution, while animal studies pose ethical challenges alongside difficulties in the transferability of results, especially for drug testing trials.[10–14] In utero studies, critical to studying brain development, involve neurosonography, magnetoencephalography, and magnetic resonance imaging. These techniques are expensive and have limited long-term accessibility for longitudinal temporal studies.[15,16] Ex vivo human brain studies with approaches such as diffusion magnetic resonance imaging (diffusion MRI), polarization microscopy, and electron microscopy have significantly advanced our understanding of the structure of the human brain but provide limited information on its function and activity.[17–19] Deciphering the connectomes in organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster* offers fascinating glimpses of the nervous system network structure and connectivity.[20,21] However, organism and animal brains are less complex and lack features found in humans.

In vitro two-dimensional (2D) mammalian cell cultures have also replicated tissue structure and function.[7] However, monolayer in vitro human neuronal culture fails to recreate the complex spatial organization, cellular heterogeneity, and connectivity of the three-dimensional (3D) human brain,[11,12,22,23] which is essential in determining physiological function.[23] Thus, the field is transitioning from planar approaches to in vitro 3D models.[24,25]

Organoids are a promising 3D in vitro model that can be created by introducing human stem cells—-adult, embryonic, or pluripotent—-to extracellular matrix (ECM) components that can bind and form 3D aggregates of self-organized cells.[7,11,12,23,26,27] Upon further introduction of specific growth factors and cofactors during culture, these stem cells can differentiate into various types, including retinal cells,[28] gastrointestinal cells,[29] renal cells,[30] and even neural cells.[11,12,23] As they develop, these 3D cultures form structures that partially recreate their corresponding organs' cellular heterogeneity, cytoarchitecture, and general function, making them powerful tools for analyzing organogenesis, disease, and pharmacokinetic phenomena.[7,26] More specifically, brain organoids advance planar cell culture and provide a scaffold to better understand the spatial complexities associated with neural structure and function.[22,23,31,32] With improved compositional and spatial recapitulation of cortical neural networks, brain organoids can enhance the fidelity and translatability of neural development studies, allowing researchers to better analyze higher-order neural phenomena.[11,12,22] However, long-term analyses require further research into infrastructure to maintain the stability and reproducibility of brain organoids.[23]

Microinstrumentation is advancing alongside cellular models to enable more reliable, reproducible, and detailed functional studies of brain organoids and the developing brain. Emerging microfluidic devices are creating microenvironments conducive to forming more mature organoids.[23] Optical technologies such as calcium imaging, two-photon microscopy, optogenetics, and light-sensitive nanoparticles for neuromodulation allow for real-time neural activity analysis in cellular models. Similarly, microelectrode arrays (MEAs), commonly used for 2D neural network studies, are being adapted for 3D studies. Recently, the field of Organoid Intelligence (OI) was proposed to investigate the use of human brain organoids for biological and neuromorphic computing.[3,33] OI will require stable and robust organoid–machine interfaces to operate over long periods, from days to months.

In this review, we discuss the emergence and broad applicability of brain organoids as neural models and survey current trends and future developments in relevant microinstrumentation (**Figure 1**).

2. Emergence of Brain Organoids as Neural Models

To fully understand the potential of brain organoids in research across fields, we first examine the role of brain organoids within the broad landscape of neural models. Planar models, including in vitro 2D mammalian cell culture and ex vivo brain slices, alongside in vivo animal models, have been widely used to reconstruct the human brain and elucidate neural phenomena.[34,35] Yet, they falter in their ability to accurately capture human neural cytoarchitecture, composition, and function. Human-derived in vitro 3D models such as spheroids, brain organoids, and assembloids build upon these limitations to offer a greater understanding of neural networks and enhanced transferability of findings. Here, we provide a brief survey and comparative analysis of the advantages and constraints posed by these neural models (**Figure 2A**).

2.1. In Vitro Stem Cell-Derived 2D Human Neural Cell Culture

Much of our current understanding of the brain is based on studies using 2D in vitro cell culture. Julius Petri, under the mentorship of Robert Koch, is credited with introducing the Petri dish, which transitioned both prokaryotic and eukaryotic 2D cell culture from liquid to solid platforms and eventually to stem cell culture.[38–42] The established 2D stem cell culture approach involves the neural lineage differentiation of induced pluripotent stem cells (iPSCs). iPSCs are human adult stem cells that have been reprogrammed into embryonic-like pluripotent stem cells. They are typically achieved in cell culture media depleted of serum (i.e., serum-free conditions).[43,44] Serum-free media allows cells to grow in an environment with well-defined biochemicals and limited confounding variables, pivotal for maintaining neural cell cultures.[42,45–47] Through applying highly specialized differentiation protocols, scientists have been able to generate homogenous populations of cells found in the central nervous system (e.g., motor and sensory neurons,[48,49] neuron rosettes,[50,51] excitatory and inhibitory neurons,[52,53] astrocytes, and oligodendrocytes).[54,55]

Due to the homogeneity of its cell populations, 2D cell culture is used for relatively simple calcium and transcriptome assays and for drug screening across fields.[48,49] It benefits from access to widely available imaging systems such as calcium and fluorescence imaging.[55,56] 2D culture enables high-throughput analyses, but it fails to recapitulate the human brain's cytoarchitecture and 3D structure. For example, the human brain consists of

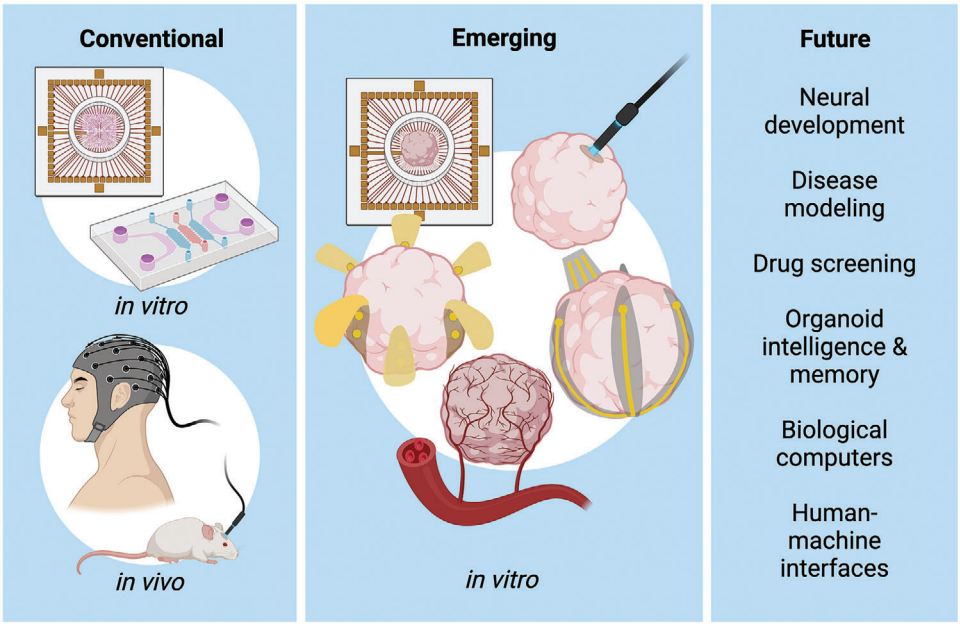

Figure 1. Progression of instrumentation in neuroscience and the advent of brain organoids. (Left panel) Conventional in vitro and in vivo techniques were primarily used to study neural interactions. (Center panel) The emergence of brain organoids paved the way for more sophisticated microinstrumentation technology like 3D microelectrode arrays (MEAs), microfluidics, optical tools, etc. (Right panel) The future of advanced microinstrumentation for brain organoids will help answer unresolved questions related to neural development and aid in disease modeling. Its applications in drug screening could reshape the medical sector. In addition, future advancements in brain organoid technology raise questions about its intelligence and memory, which could lead to Organoid Intelligence (OI), biological computers, and human–machine interface devices. Created with BioRender.com.

≈86 billion neurons and 84 billion non-neuronal cells vastly connected and concertedly working within a complex 3D ECM and microenvironment.[57] 2D cultures cannot represent these complexities, reducing their predictive power,[58,59] especially when assessing drug performance in preclinical testing.[60,61] Hence, higher-order models such as in vivo and in vitro 3D models emerge as necessary for improved recapitulation and transferability of results.

2.2. In Vivo, Ex Vivo, and In Silico 3D Neural Models

The complex neuronal circuit formation of the human brain is difficult to model using 2D in vitro systems; thus, researchers have relied on animal models. Notably, many crucial functions indicative of higher human cognition are absent in lower mammalian brains. However, analogous areas have been reported in non-human primate brains.[62] For example, dopaminergic neurons derived from monkey models are implanted in patients with Parkinson's disease as a cell replacement therapy to provide them with long-term relief.[63–65] Similarly, in the field of neural prosthetics, which attempts to link human brain waveforms to external mechanical devices, non-human primates play a pivotal role in procuring sensory and motor information supporting device development.[66,67] Highly efficient genome-editing techniques have further expanded the feasibility of modifying primate models for neuroscience and brain disorders.[68] Other in vivo animal models exist, such as chemically stimulated, xenograft, and genetically engineered mouse models.[69] Among those, mouse models are frequently used to model neurological disorders such as brain tumors and metastasis, through which scientists can study genetic and molecular features in detail by making genetic alterations.[70] The main advantage of 3D in vivo mouse models is the short latency between disease induction and disease development, allowing scientists to study molecular features in detail.[71] However, in addition to ethical concerns, a notable limitation of in vivo animal models is that homogenous genetic alterations fail to completely recapitulate the complex genetic, molecular, and phenotypic diversity present in humans.[69]

Meanwhile, there are ex vivo approaches where brain slices are used for mapping structure and connectivity and monitoring electrophysiological activity.[72–75] Scientists have used technologies such as diffusion MRI and transmission electron microscopy to analyze neural microstructures and study the spatial distribution of proteins and vesicles via an ex vivo approach.[76,77] Both techniques are used primarily to elucidate structure rather than assess neural function, and alternative methods are needed for probing functionality ex vivo. For example, Jang et al. generated mice brain slices with genetically encoded voltage indicators (GEVIs) and then applied GEVI imaging to identify cortical layers contributing to the most afferent activity.[78] Together, these approaches provide a more comprehensive understanding of the brain when compared to in vitro 2D studies.

With a large availability of in vivo and ex vivo data, neuroscientists worldwide have developed scientific infrastructure to map the brain and elucidate the biological mechanisms driving neural pathways and their disorders. For example, the Multiomic Human Brain Cell Atlas was set up with the intent to characterize 1500 human brain samples' molecular features,[79] while the Human Brain Project (HBP) encourages scientists to use artificial

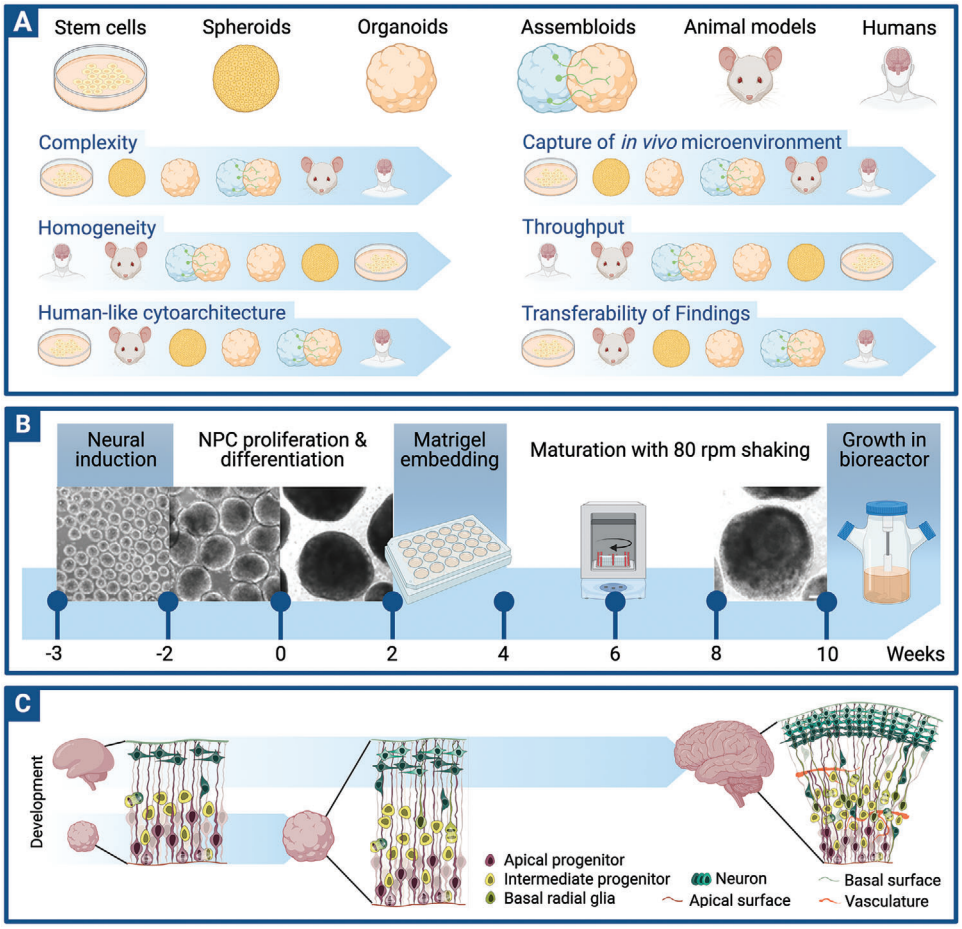

Figure 2. Brain organoids offer numerous advantages as compared to conventional neural models. A) Multiple factors influence the choice of commonly used models in neuroscience. (Left to right) The standing of these models, as depicted from least to most, aligned with the criteria, indicated with the shades of blue from light to dark, respectively. Amongst the models in consideration, organoids and organoid structures (assembloids) are a bridge between the extreme in vitro and in vivo models; they encompass the best combination of characteristics. B) Illustration of the steps in the protocol commonly used to create brain organoids. The process begins three weeks before and can extend ten weeks past organoid formation. On formation, the cells are suspended in Matrigel and are provided with nutrients supporting maturation. The process culminates in a bioreactor, where brain organoids can continue to grow to attain complete cellular diversity. Adapted with permission.[36] Copyright 2019, Elsevier. C) Schematic depicting the morphological similarities and differences between the brain organoid and the human brain. In the early stages, in vivo and in vitro cell population growth are similar in the ventricular (VZ) and subventricular zone (SVZ). In the later stages, the cortical wall is more extensively developed and larger in the human brain than the brain organoid. The brain's greater complexity in vivo is introduced through improved vasculature from the blood vessels, leading to a greater network of neurons and a larger subventricular zone. Adapted with permission.[37] Copyright 2016, Elsevier. Created with BioRender.com.

intelligence, neuroinformatics, and biocomputing to conduct advanced brain research.[80] The HBP provides a research infrastructure of the human brain upon which scientists can perform in silico experiments. EBRAINS Cellular Level Simulation Platform (EBRAINS), powered by HBP, is another platform that aims to create computational simulations and models of the human brain from in vivo and ex vivo data.[80] Specifically, EBRAINS would construct data-driven models of neurons, synapses, and other brain tissues.[81] In another effort, the Blue Brain project, led by Henry Markram, sought to unveil the mysteries of the brain by reconstructing it digitally.[82] With in silico modeling, scientists could validate experimental results using computational simulation.[83] However, bottom-up reconstruction of the brain in silico is generally met with skepticism since a scale against which success could be measured is almost non-existent. In other words, critics argue that the reconstructed model could be as mysterious as the brain, even if it works.[84] Data sourced from in vitro 3D models could better expand on this existing infrastructure to further map the brain and, ultimately, drive more accurate outcomes.

2.3. Organoids as Anatomically Relevant 3D Cell Culture Systems

Early work on organogenesis described it simply as the cultivation of germ layers outside the body; however, developments since then have vastly redefined its level of complexity.[85] Rheinwald and Green's landmark 1975 report on using human skin cells to establish a long-term tissue culture was a significant step in advancing 2D in vitro cell studies.[86] In the last few

decades, the scientific community has incorporated novel techniques like soft hydrogels, 3D scaffolding, bioprinting, stem cell differentiation, and tissue engineering for long-term 3D tissue cultures.[42,87–97] Together, these developments in cell culture have paved the way for generating a vast array of 3D structures from human skin cells through focused differentiation, intrinsic signaling, and self-assembly.[85,98]

There are two main techniques to produce 3D cultures: scaffold-free and scaffold-based.[99] In scaffold-free culture, no ECM or ECM-associated molecules are present during culture. Therefore, cells undergo 3D self-assembly to form spheroids. Alternatively, scaffold-based cultures rely on seeding cells into 3D matrices—-composed of natural or synthetic materials such as Matrigel—-to help facilitate the transfer of behavior signals, ultimately resulting in the formation of organoids.[99] Currently, biologists can generate a variety of brain organoids that mimic subregions of the human brain; the forebrain, hindbrain, hypothalamus, midbrain, and neocortex.[100–104] Various organoids from diverse cell populations can be combined to form assembloids replicating higher-order organ structures.[23]

Intrinsically, 3D brain organoids, are cultured like 2D neural cultures. iPSCs differentiate into neural stem cells and then into various nerve cell types. The typical process flow to generate a brain organoid is first generating embryoid bodies (EBs) from iPSCs in suspension, using biochemical cues such as the basic fibroblast growth factor. EBs are then subjected to neuroectoderm induction by applying specific neural induction media. Neuroectoderm tissue is then transferred to Matrigel droplets that allow further differentiation of neuroepithelial buds. Finally, the tissue is transferred to a spinning bioreactor or orbital shaker to allow for further growth and development of the organoid through nourishment and oxygenation (Figure 2B).[105,106] Together, these individual steps comprise a complete patterning protocol to generate brain organoids of a particular type. Eigenhuis et al. and Mayhew and Singhania illustrate that precise control over the patterning protocol (i.e., biochemical cues, microenvironment, media, etc.) allows for the formation of cerebral, cortical, midbrain, thalamic, and other region-specific organoids.[107,108] The ability to model various facets of the brain can significantly enhance studies of neurodevelopment and disease.

Moreover, because of their inherently 3D nature and extended development, 3D brain organoids can exhibit complex cytoarchitecture. Quadrato et al. discovered dopaminergic neurons, astroglia, neuroepithelial cells, retina, etc., in a six-month-old brain organoid.[109] 3D brain organoids also exhibit neuron and non-neuron cell interaction, commonly observed in the human brain. Upon three or more months of differentiation, brain organoids can develop cell layers, including the ventricular zone, subventricular zone, and cortical plate.[110] Together, brain organoids can recapitulate these aspects of in vivo human brain development more accurately than their 2D counterparts (Figure 2C). As a result, they can be leveraged to study infectious diseases such as COVID-19 better and to characterize their pathology at a molecular level.[111]

3D brain organoids can capture the complexity of brain architecture and the diversity of brain cell types using human cell lines. However, 3D brain organoids also come with their limitations. The major shortcoming is that they lack an adequate circulatory (i.e., vascular) system, immune system, and more developed, human-like neuroarchitecture. Moreover, 3D region-specific brain organoids are structurally small and architecturally undefined to capture the communication between different brain regions.[112] The latest advancements are focused on addressing these deficiencies by using a co-culture method to combine organoids of dorsal and ventral forebrain identities to form a dorsal-ventral axis,[113] and microinstrumentation can help accelerate developments in this regard.

3. Applications of Brain Organoids to Mimic the Human Brain

Now that we have assessed the landscape of neural models, their offerings, and shortcomings, we can discuss how brain organoids are uniquely situated for use in a broad range of studies. Specifically, the brain organoid's enhanced capture of the human brain's specializations, such as its complexity and unique structure, makes it a more representative model than others.

Elucidating the molecular and cellular mechanisms that govern human brain development is crucial to understanding human cognition, developmental disorders, and cognitive decline during neurodegeneration. Through the use of human brain organoids, we can model the early and late stages of development since, depending on patterning protocols, organoids can recapitulate the forebrain, midbrain, dorsal-ventral axis, telencephalon, hippocampus, and cerebellum.[105,114–117]

Cognition is hypothesized to be strongly associated with brain size and topography. While a large brain is undoubtedly a hallmark of humans, size is only one aspect of the evolution of our brain's capacity for intelligence. An elephant's brain is three times larger than the average human's and contains about three times as many neurons (≈260 billion). Like humans, most of their neurons are tiny and reside in the cerebellum; however, within the cerebral cortex of the elephant brain, the number of larger neurons, with their expansive axonal projections, is only one-third of those found in humans.[118,119]

The evolutionary expansion of the human cerebral cortex is one of the most remarkable differences between humans and other mammals.[114,118,120,121] The cerebral cortex occupies 80% of the total brain volume in humans while it occupies ≈40% in mice,[121] with higher cerebral cortex volumes correlating with higher-order thinking.[122] Although the main stages of cortical development are conserved in all mammals, cortical size has been disproportionately expanded in humans.[114,123]

Comparative studies of human and non-human primates on cortical network circuitry show that the critical cortical change during evolution was an increase in the number, rather than the size, of neural circuits.[118,121] The difference in the expansion and duration of corticogenesis, including the mechanism of cortical folding generating deeper and richer convolutions, a process known as gyrification, has provided an evolutionary advantage for humans in creating a more compact yet highly interconnected brain with superior cognitive abilities.[124,125] With the emergence of 3D cell culture, it is paramount that human brain models, such as brain organoids, accurately represent these key characteristics. The following sections discuss how brain organoids have been applied in studies across numerous focus areas.

3.1. Embryonic Neurodevelopment and Disease

Brain organoids have been used as an in vitro model in studying neurodevelopment and neurological disorders due to their improved recapitulation of the human brain. For example, human cerebral organoids can model fetal brain development during the first trimester of pregnancy, positioning them as an excellent tool for studying embryonic neurodevelopment.[126] Here, brain organoids emerge as a powerful tool enabling direct, real-time access to in utero phenomena in ways that in vivo clinical studies cannot. For instance, brain organoids have been used to study autism spectrum disorders (ASDs) induced by in utero complications, such as prenatal exposure to recreational drugs and chemicals or maternal diabetes.[126,127] Mariani et al. generated telencephalic organoids to model mid-stage development of the fetal cortex from iPSCs derived from patients with ASDs.[128] Although the etiology behind ASDs is not entirely elucidated, Mariani et al. later found that GABAergic inhibitory neurons are overproduced in the organoids.[128] Findings from such in vitro brain organoid studies can help uncover the mechanisms underlying such neurodevelopmental disorders. These mechanisms can be targeted to support the development of effective therapeutics and preventative approaches.

Further, brain organoids, with their unique ability to model neuroarchitecture and composition in the fetal cortex, also serve as excellent tools in understanding the pathophysiology and neurological effects of infectious diseases. For instance, Cugola et al. and Garcez et al. studied the effects of the Zika virus on the fetal cortex using human iPSC-derived brain organoids. Their findings suggest that exposure of brain organoids to the Zika virus yielded diminished cortical neuron populations, cortical thickness, and overall organoid growth in addition to abnormality in the neurosphere.[129,130] These effects confirm that fetal exposure to the Zika virus may lead to incomplete neurological development, such as the well-observed microcephaly or other neurological disorders. In another study using human forebrain organoids, Xu et al. identified several small molecules that either inhibit Zika infection or suppress infection-induced caspase-3 activities.[131] Thus, brain organoids have been used to both identify the effects of the Zika virus on the developing fetus and identify potential therapeutics to diminish these effects.

Brain organoids are also being used to study the cellular pathways and neurotrophic properties of COVID-19. Ng et al. used human brain organoids to validate the function of an angiotensin converting enzyme 2 receptor in facilitating viral particle entry into host cells.[132] They illustrate that COVID-19 is more likely to invade mature neurons than neural progenitor cells.[132] Studies like these, where access to fetal brains is ethically plagued, help cement the potential of brain organoids in advancing scientific findings and, further, developing therapeutics.

While investigating the application of brain organoids to neurodevelopment, scientists are also concerned with the methodological improvements in the culture of brain organoids. Many investigations of neurodevelopment and disease require systematic, long-term cultivation of brain organoids. Generally, brain organoids can be cultured for a different time range: from days to months.[115,133] Long-term culture would enhance the maturation of brain organoids and, more precisely, recapitulate the development of the human brain.[34] Matsui et al. generated cerebral organoids induced from H9 human embryonic stem cells cultivated for six months, leading to mature oligodendrocytes and excitatory neurons.[133] However, studies have also shown that long-term cultivation can induce cell death due to limited access to oxygen and nutrients.[134] More specifically, present-day organoids' lack of microvasculature systems to circulate oxygen and nutrients hinders their growth, leading to undesired differentiation in regions with poor supply.[105,135,136] The complexity of the brain organoid compared to other organoid types makes microvasculature crucial to support its long-term growth and viability.

3.2. Drug and Toxicity Screening

Brain organoids can significantly accelerate drug screening by overcoming conventional animal studies' cost, ethical and human translatability challenges.[137] Toxicity and bioavailability studies in in vivo animal models are the gold standards used to define drug tolerability and efficacy before clinical trials. However, there are fundamental issues regarding the accuracy and transferability of findings from such studies due to critical differences in neural organization and anatomical complexity between model organisms and humans. As a result, in vitro brain organoids have emerged as a viable alternative since they can be human-derived and more accurately represent the human brain. Brain organoids also lend themselves to higher throughput, accelerating therapeutic development while reducing overall cost.

Patient-derived organoids (PDOs) have become an increasingly prevalent tool used in drug screening. PDOs can predict a patient's response and sensitivity to potential drug candidates and thus improve their treatment outcomes.[138] Specifically, brain organoids derived from patients have been used in drug-screening processes for a variety of neurological disorders. Park et al. used 1300 organoids derived from 11 individuals to generate a high-content drug screening platform for Alzheimer's disease.[139] After applying blood-brain barrier-permeable drugs, the quantification of hallmark proteins was assessed to determine the extent of pathogenesis.[139] In another study, Groveman et al. developed a drug screening model for Creutzfeldt-Jakob disease, a fatal neurodegenerative disease marked by the formation of prions—-misfolded proteins that transmit their misfolded conformation to other variants and, thus, induce cell death—-using infected iPSC-derived human cerebral organoids.[140] These studies show that leveraging brain organoids for drug screening provides efficient preclinical efficacy models and can narrow the gap between translational research and clinical trials.

Brain organoids have also been applied to toxicity screening. Neurotoxicity, which refers to the direct or indirect effect of exogenous substances on the nervous system, can directly influence neural cells or metabolic processes.[141] The fetal brain is especially vulnerable to such exogenous substances because its blood-brain barrier is not fully formed.[142] The emergence of brain organoids provides a potential model for assessing the effects of toxins such as cadmium (Cd), ethanol, and nicotine during fetal brain development. Huang et al. exposed cerebral organoids to cadmium chloride and concluded that Cd exposure induced neuronal apoptosis, inhibition of proliferation of neuronal progenitor cells, strong activation of mineral absorption in the brain, and impaired cilium development.[143] Similarly,

maternal alcohol exposure led to apoptosis in neurons and astrocytes, disruption of mitochondria cristae, decreased intensity of mitochondrial matrix, and disorganized cytoskeleton in cerebral organoids.[144] In another study, Wang et al. experimented with the effect of nicotine exposure on a human brain organoid-on-a-chip (OoC) model. They discovered that prenatal nicotine exposure led to premature neuronal differentiation, disruption of brain regionalization, and abnormal neurite outgrowth.[145] Each of these studies captures the brain organoids' power in assessing the effects of toxins on fetal neurodevelopment.

Although human brain organoids are now extensively used for drug and toxicity screening,[146] there are still limitations present. First, most brain organoids are only used as tools to indicate toxicity in the fetal brain due to their limited maturity. As a result, more mature brain organoids exhibiting increased cellular diversity are required to model the effects of toxicants on adult brains more accurately.[147] Second, most existing organogenesis protocols rely on inherent self-organization rather than a prescribed spatial arrangement, resulting in substantial heterogeneity between organoids. This heterogeneity could stem from differences in the culture media, genetics of the donor iPSCs, and fluctuating shear forces during growth in bioreactors. Brain organoids specifically display varying dimensions of EBs due to variability in progenitor cell growth.[146] These factors introduce confounding variables for large-scale toxicity and drug screening, which can hinder the significance of findings. Lastly, their inability to truly capture the brain's neuronal pathways due to a lack of supportive vasculature limits brain organoids' current applications in screening. Therefore, there is a need for the development of new engineering methods coupled with robust automation to precisely control cellular arrangement for generating organoids with reproducible characteristics. With these advancements, brain organoids can enable widespread screening of drugs and toxins before use, significantly help understand their side effects, and reduce potential risks.[148]

3.3. Computational Networks and Neuromorphic Computing

Besides enabling direct access to neural phenomena in real-time to study neurodevelopment, disease, and therapeutic effects, brain organoids are also ushering in a new era in computing. In biological neural networks, a synapse's strength determines the efficiency and effectiveness of signal transmission; however, in modern artificial neural networks, numerical weights emulate such synaptic connections, enabling complex pattern recognition in silico. Artificial intelligence (AI) based on such machine learning models has already evolved, as demonstrated by GPT-4's strategic and advanced reasoning abilities, albeit at the expense of energy-inefficient computation primarily due to data transfer between processor and memory.[149,150] Yet, the brain dramatically outperforms the current in silico state-of-the-art by computing and storing information in an indistinguishable manner, enabling a highly efficient, in-memory, parallel, and analog computation, all within its three-pound jelly mass of about 86 billion neurons.[3] Motivated by this energy-efficient computing, many leading companies in silicon-based technologies have successfully demonstrated complementary metal-oxide semiconductor (CMOS)-based neuromorphic computing with improved efficiency.[151,152] Alternatively, analog computation using materials like memristors is also gaining momentum, especially as deep-learning accelerators.[153–162] However, unlike a brain, these in material computing paradigms have neither error-tolerant, self-sufficient, self-evolving connections nor actual three-dimensionality, making the architectures less adaptable, versatile, and powerful than a brain. Brain organoids are the closest in vitro replicates of the human brain that enable the field to achieve this highly coveted feat of efficient, in-memory, and parallel computation. A new field of OI has been proposed to examine this possibility further.[3]

While brain organoids have not achieved the clearly defined, six-layered cortical structure of the neocortex, which encodes human cognitive abilities, several researchers have been able to develop organoids with cortex-like structures.[36,163,164] Qian et al. reported forebrain organoids expressing layer-specific markers for each of the six layers,[165] demonstrating that forebrain organoids can recapitulate aspects of the human gene expression profile of the neocortex.[166] Although this gene expression profile elicits a resemblance in topography, its similarity to the functional connectivity of the human brain remains unknown. In advancing this knowledge, researchers are studying the electrophysiological properties of brain organoids.[36,163,167–169] These studies are essential for uncovering the principles that govern learning and memory. Trujillo et al. have shown oscillatory dynamics that mimic the bursting phenomenon in the preterm neonatal period of a human brain as observed by electroencephalography (EEG).[36] Similarly, Zafeiriou et al. demonstrated that the electrophysiological behavior of brain organoids mimics early network activity in humans.[170] The study further showed evidence for long-term potentiation, a process involving persistent strengthening of synapses that leads to a long-lasting increase in signal transmission between neurons and is the basis of memory. These studies illustrate that the potential to investigate early stages of cognition as the neuron circuitry formation emerges is feasible.

Interfacing organoids with digital systems to harness their innate computational power has great potential to investigate how inputs can be efficiently processed to produce a desired output. DeMarse et al. and Kagan et al. were able to interface and train a monolayer of neurons to respond to specific tasks.[171,172] Trujillo et al. explored the potential for cortical organoids to perform like biological computers by instructing a robot to navigate its environment using AI to decode the electrophysiological activity from the organoid's neural oscillations.[36] Important questions, such as: i) how the inputs were processed, ii) how the stimulation affected network dynamics, and iii) how this was reflected at the functional level, remain unanswered. Nevertheless, these studies demonstrated the feasibility of harnessing the natural intelligence and intrinsic ability of 2D neuronal cultures and 3D brain organoids. They represent the early steps in developing neuromorphic computing architectures that harness the power and efficiency of the human brain.

4. Key Microinstrumentation for Brain Organoids

Developments in biological culture techniques have ultimately positioned brain organoids as a promising new model for studying the human brain. However, accurate and reliable studies of brain organoids can only be carried out with sophisticated

microinstrumentation suited for their more complex developmental necessities, composition, and spatial geometry. Here, we discuss microfluidic, optical, electrical, magnetic, and acoustic technologies that are being applied to enhance the offerings of brain organoids, along with their capabilities and limitations (**Table 1**).

4.1. Microfluidics

As we have shown, precise control over a developing brain organoid's microenvironment is paramount to advancing studies of neurodevelopment and disease as well as in drug and toxicity screening applications. Such precise control can be achieved by interfacing organoids with tiny vessels or channels via microfluidic devices. These devices scale down the macroscopic cell culture process onto a microfabricated chip architecture with channels, wells, and other components that allow for precise spatiotemporal control and modeling of the in vivo microenvironment.[145,176,177,208] Integrated microfluidic devices offer the possibility of creating "OoC" or even more complex "Organ-on-Chip" systems. Since cells cultured in microfluidic devices are exposed to a much smaller environment than those typically cultured in plates, their microenvironment is more susceptible to shifts in chemical concentration, pH, temperature, and other physicochemical properties. Therefore, the fabrication process and material selection for microfluidic devices are paramount. Generally, microfluidic devices are fabricated using lithographic patterning and are made of biocompatible,[176,177,178,208] bioinert materials like polydimethylsiloxane (PDMS),[145,176,178] SU-8,[176] silicon, or glass.[177] However, polymer-based devices such as those fabricated with PDMS could affect the microenvironment due to their ability to absorb, retain, and release small, lipophilic species.[177,209] More recently, 3D printing, layering, and self-folding have been utilized to create a variety of complex microfluidic devices that are important for interfacing the inherently 3D organoid.[210–215] Microfluidics are used primarily for two purposes; to mimic vascularization, and to control and modulate the heterogeneous environment around the developing and functioning organoid.

4.1.1. Integration of Organoids and Microfluidics for Organoid-on-Chip (OoC) Devices

OoC devices have emerged as powerful tools in tissue engineering and regenerative medicine since they can mimic the complexity of tissue architecture and functions more accurately than 2D cultures. OoC technology arose from integrating organoids with microfluidic devices, which enhance the simulation of physiological conditions such as blood flow, formation of morphogen gradients, and mechanical forces.[216] OoC and more complex Organ-on-Chip devices are distinct yet complementary and aim to emulate the intricacies of human organs in a lab setting. OoC systems are crafted based on our understanding of human organs, aiming to create synthetic environments where cellular conditions are meticulously regulated.[208] On these devices, organoids can naturally evolve from stem cells, harnessing their inherent developmental processes to form structures and functions that mirror their natural counterparts within the body.[216]

The fabrication of OoC devices generally commences with using soft lithography and a suite of microfabrication techniques. These methods create microfluidic channels and chambers into biocompatible substances, such as PDMS. Subsequently, the integration of organoids takes place, initiating with the strategic placement of stem cells or organ progenitor cells into the fabricated chambers. Within these confines, cells can differentiate and mature into organoids under controlled conditions. Further, a scaffold or an extracellular matrix is integrated, serving as the critical underpinning for the organoids' 3D structure and development. A crucial step is incorporating dynamic flow and applying mechanical forces to mimic shear stress and strain upon the organoids, replicating the essential mechanical stimuli intrinsic to certain organ functions.[217]

OoC technology offers advantages that underscore its potential to revolutionize biological research and drug development. One of its primary benefits is generating human-relevant data, which proves more applicable than what is obtained from animal models or 2D cultures.[218] As a result, reliance on animal testing can be minimized by leveraging OoC devices, aligning with ethical research practices.[219] In addition to generating more relevant data, OoC's capacity to emulate human diseases in vitro opens new avenues for disease study. For example, Wang et al. developed a brain OoC system to study the effects of prenatal nicotine exposure on embryonic brain development, depicting that it caused premature differentiation and cell death.[145] Similarly, Karzbrun et al. leveraged an OoC device to visualize the formation of surface wrinkles during neurodevelopment (i.e., gyrification), which is responsible for the human brain's comparatively superior cognitive abilities.[220] Studies like these can be performed more accurately and reliably because of the precise microenvironmental control during in vitro organogenesis offered by OoC devices. One day, these devices could fully automate the cell culture process, further reducing variation in organogenesis from batch to batch.[208] Overall, the chip architecture, and its small scale, lend themselves to more rapid, high-throughput organogenesis and pharmacokinetic and pharmacodynamic analyses, positioning them as powerful tools for drug discovery.[178,208]

Despite these benefits, there are notable drawbacks. The complexity of fabricating these systems demands advanced technology and specialized expertise, posing a barrier to entry for some researchers. Standardization remains a challenge, as ensuring consistent organoid growth and responses is difficult, which is critical for reproducible research outcomes.[221] Furthermore, the costs associated with setting up and maintaining these systems can be prohibitive. Also, while they adeptly create microenvironments, they do not replicate full organ scale, which can limit the scope of some functional studies.

With advances in automation, higher throughput capabilities and broader adoption in the biomedical research community is expected. Continued interdisciplinary collaboration will be vital, with input from cell biologists, materials scientists, engineers, and clinicians, to overcome the current drawbacks and fully realize the potential of OoC technology.

4.1.2. Vascularization of Brain Organoids

The absence of vasculature hinders the longitudinal development of brain organoids, as their development relies

Table 1. Summary of key conventional and emerging microinstrumentation for brain organoids.

	Technology	Attributes	Challenges	Future developments	Representative references
Microfluidic	Vascularized brain organoids	Biochemical exchange needed for viability and organogenesis.	Lack of biocompatibility of materials for long term culture and planarity of conventional microfluidics.	3D printing, sacrificial materials.	Mansour et al. (2018)[173] Pham et al. (2018)[174] Zhang et al. (2021)[175]
	Spatiotemporal control of the brain organoid's microenvironment	Control of fluid flow, shear stress and patterning of chemical gradients to mimic microphysiological environment.	Limited spatial resolution in 3D.	3D microphysiological systems, self-folding microfluidics.	Jamal et al. (2011)[176] Demers et al. (2016)[177] Kim et al. (2019)[178]
Optical	Calcium imaging	Fluorescent indicators for monitoring spatial and temporal brain activity.	Limited temporal resolution and depth penetration. High cost and limited accessibility. Phototoxicity and photobleaching.	Miniaturized imaging systems, multi-photon techniques, improved fluorescent probes.	Tsien (1980)[179] Qian et al. (2016)[165] Grienberger and Konnerth (2012)[180] Mansour et al. (2018)[173]
	Optogenetics	Light-sensitive biomolecules to monitor and study their signals in a neuronal circuit.	Optical access in 3D, artifacts.	Advances in microscopy, computational methods, automation.	Fenno et al. (2011)[181] Deisseroth (2015)[182] Shiri et al. (2019)[183]
	Optical neuromodulation using nanoparticles	Tuning nanoparticles through size and bioconjugation to manipulate the activities of cellular networks by altering the electrical potential of the cell membranes.	Cost, toxicity, and instability of nanoparticles.	Nanoparticle synthesis and tailored bioconjugation.	Acarón Ledesma et al. (2019)[184] Wang et al. (2023)[185]
Electrical	Patch clamps	Electrical activity of individual cells typically using a glass micropipette.	High equipment cost and training, technical complexity, low throughput, cell damage.	Miniaturized and arrayed probes, automation.	Hodgkin and Huxley (1952)[186] Neher and Sakmann (1976)[187] Hamill et al. (1981)[188] Aerts et al. (2014)[189]
	2D–MEAs	Electrode arrays to measure electrical activity.	2D planar form factor, modulus mismatch, external to organoid.	Flexible, soft, 3D and penetrating arrays. Wireless operation, adaptive interfaces, energy harvesting, machine learning integration, closed-loop systems, and further transparency.	Thomas et al. (1972)[190] Gross (1977)[191] Trujillo et al. (2019)[36] Lee and Someya (2019)[192]
	3D–MEAs	Three-dimensional microelectrode arrays in the form of mesh, buckled or shell interfaces.	Complex fabrication and high cost, reproducibility, 3D data analysis and interpretation.	Improved fabrication protocols, enhanced throughput, and standardization. Improved electrical modeling and analyses.	Liu et al. (2019)[193] Steinmetz et al. (2021)[194] Park et al. (2022)[195] Huang et al. (2022)[169]
	Electrochemical monitoring	Monitoring oxidation and reduction states and concentrations of neurochemicals.	Electrode fouling, limited selectivity and specificity, background noise and signal stability.	Advances in electrodes and coatings, miniaturization, electrochemical recording interfaces and instrumentation.	Kissinger et al. (1973)[196] Ou et al. (2019)[197] Jill Venton and Cao (2020)[198] Zanetti et al. (2021)[199]
Magnetic and acoustic	Magnetic	Neurostimulation via electromagnetic induction.	Resolution, tissue magnetic susceptibility heterogeneities, miniaturization challenges.	3D RF systems, improved mechanistic understanding of RF neurostimulation.	Barker et al. (1985)[200] Anacleto et al. (2016)[201] Cash et al. (2021)[202] Signorelli et al. (2022)[203]
	Acoustic	Use of surface or internal pressure waves for mechanical activation, heating, or the stimulation of mechanosensitive ion channels.	Limited mechanistic understanding of neuromodulation using acoustic waves. Potential for tissue damage	Improved and miniaturized acoustic transducers.	Chen et al. (2015)[204] Cai et al. (2020)[205] Ao et al. (2021)[206] Rufo et al. (2022)[207]

heavily on precise environmental cues, optimal oxygenation, and nutrition.[173,174,178,208,222] In the human body, cells typically reside within 200 microns of a blood vessel.[223–226] Vasculature provides a highway system for circulating oxygen, vital nutrients, signaling molecules, waste, and other critical elements across tissue via convective transport—-or distribution via bulk fluid flow. In the absence of vasculature, nutrients deposited at the periphery of tissue slowly penetrate toward the center, causing central cells to receive fractional nutrients compared to peripheral cells, resulting in a necrotic core. Thus, some means of circulation and a more biomimetic microenvironment for cellular maturation are essential for growing brain organoids.

Microfluidic devices improve organogenesis by simulating vasculature in vitro, offering a more reproducible, high-throughput alternative to techniques like xenotransplantation and cellular co-culture. Xenotransplantation involves the trans-species transfer of living cells and is one methodology adopted to develop microvasculature in human brain organoids.[173] Mansour et al. illustrated that the implantation of cerebral organoids into rodent hosts led to microvasculature integration between the host and the organoid, and improved cellular differentiation and maturation within the organoid itself.[173] As a result, xenotransplantation emerges as a means to further mature organoids possibly used for patient-centered cell therapies. However, it poses some risks since the organoid's development is not in a regulated environment. As such, it is difficult to discern what effects the host organism has on the organoid's development, especially if there is significant integration between the two entities. Further, xenotransplantation also lends itself to increased variability and is not a high-throughput approach to brain organoid maturation.

Another approach to developing microvasculature in brain organoids is co-culturing them with endothelial cells (ECs). Pham et al. attempted to recreate the microenvironment favoring vascularization of the developing central nervous system during human embryonic corticogenesis. After forming brain organoids in vitro from iPSCs derived from a patient, they co-cultured them with ECs derived from the same patient introduced externally in Matrigel. Further, Pham et al. exposed the ECs to vascular endothelial growth factor, promoting the formation of tubular structures, namely vessels, within the matrix and partially into the periphery of the organoids within 54 days.[174] While this technique introduced vasculature into the periphery of the organoid, penetration into the core occurred after the transplantation of the organoids into mice. Shi et al. took a similar approach but used human umbilical vein endothelial cells (HUVECs) rather than iPSC-derived ECs. Their approach produced vascularization in as early as 42 days and recapitulated cell fates during embryonic cortical development, but the vasculature remained on the periphery before transplantation in mice.[222] Ultimately, both methods pose similar concerns to those addressed above with xenotransplantation and only expand the growth limit associated with in vitro organogenesis rather than eliminate it.

Various microfluidic networks have been engineered to mimic vascularization, including those created by soft lithography, bioprinting, molding, and self-assembly.[227–230] Modeling the topography of the hierarchically branched vasculature providing metabolic support and sustaining niche cellular environments is a crucial challenge to overcome within in vitro systems; this challenge would likely require the integration of engineering- and natural-based approaches.[230–233] For example, integrating co-culture and brain organoids onto photopatterned or 3D-printed microfluidic devices can develop vasculature like Pham et al. and Shi et al. without the need for xenotransplantation (**Figure 3**A). Bioprinting can bridge both approaches; it enables the precise deposition of cells, biomaterials, and growth factors to create complex vascular networks within brain organoids. Salmon et al. demonstrated that custom-designed 3D-printed microfluidics can be layered with ECs and pericytes, essential for blood vessel formation. In this hybrid of natural and synthetic materials, a perfusable complex network of vasculature formed, sprouted, and penetrated the core of the brain organoids.[234] Another biomanufacturing method for creating perfusable, complex vasculature is Sacrificial Writing Into Functional Tissue (SWIFT), developed by Skylar-Scott et al. They suspended organoids in a mold with an ECM, centrifuged it to create a compact living tissue-like matrix, and then patterned it with a sacrificial ink via embedded 3D printing. The hierarchical vascular network is revealed upon removal of the ink, and the tissue can be perfused for extended periods.[235]

While hierarchical branching is arguably nature's best design for reaching tissue perfusion at the microscale, other designs, such as grids of synthetic micro-capillary networks, can replicate cellular perfusion within 200 µm. Grebenyuk et al. used this approach to generate a microfluidic platform capable of perfusing thick, 3D tissue constructs via a 3D-printable two-photon-polymerizable soft hydrogel. In this platform, hundreds of brain organoids collected in a solution of Matrigel were placed between the grids. After a week of growth and differentiation, the organoids filled the entirety of the platform, and the ensuing tissue was then perfused for extended periods. This system resulted in rapid neuronal differentiation, higher cell viability, and less cellular stress.[239]

Microfluidics has been fundamental in bringing brain organoids closer to relevant human physiology for disease study and can even facilitate the exploration of their learning capacity.[3,233,240,241] However, improvements are still required before this goal can be realized, especially concerning fabrication methods and control strategies. Currently, the primary fabrication methods supporting vascularization include 3D printing and self-folding.[242] Two-photon lithography approaches have been successful in perfusing up to millimeter-scale brain organoids.[239] Yet, these same two-photon approaches may not support the perfusion of larger scale organoids due to limitations in print volume, especially for high-resolution printing and print time.[243] Therefore, strategies like combining digital light processing printing and two-photon lithography could become relevant.[244] On the other hand, self-folding approaches can support robust perfusion networks, though they have not yet been implemented in brain organoids.[176,242] These systems must leverage the inverse design of the perfusion network based on the final target morphology, which may become increasingly complex at larger scales or for assembloids.[245] Here, materials selection is also paramount since photoinitiators that are detrimental to cell culture are required for cross-linking the polymers used in fabrication.[246] As a result, we recommend pretreatment of the self-folding network as well as a detailed biocompatibility study. With either approach, perfusion control is

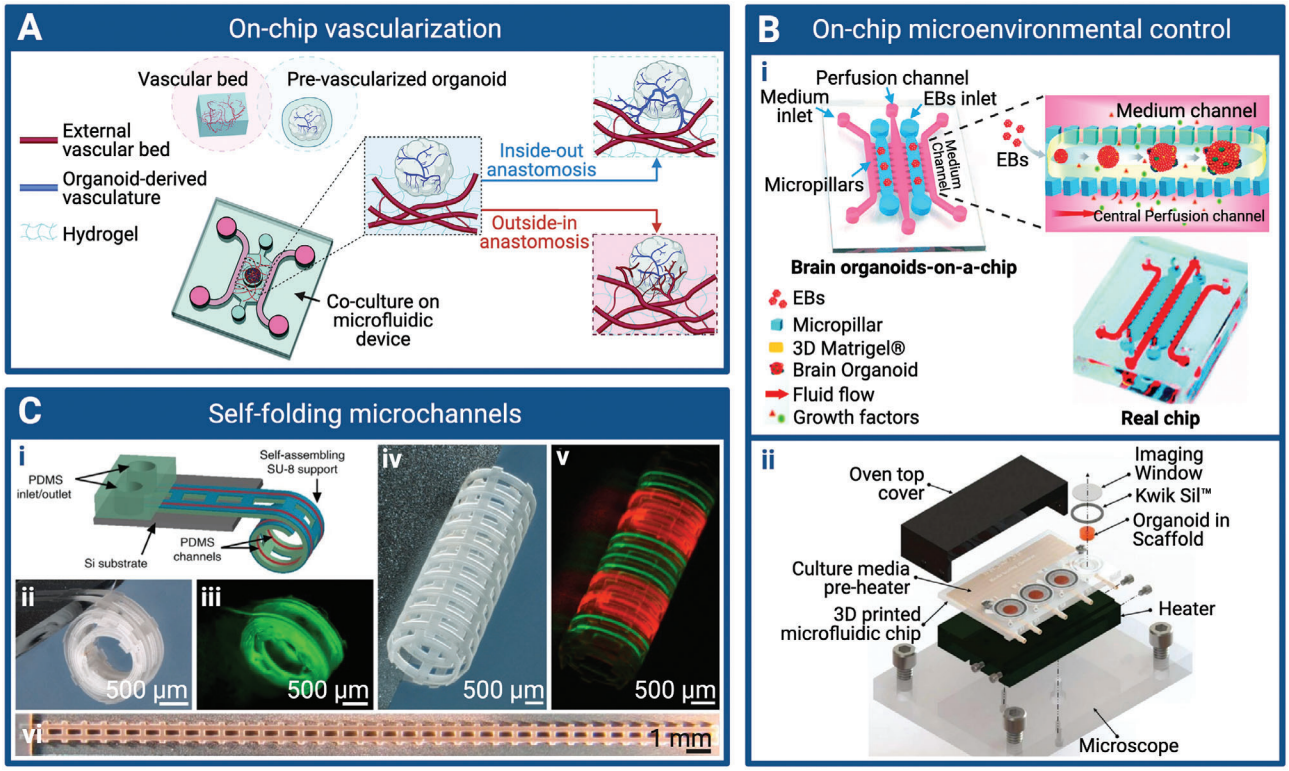

Figure 3. Advances in microfluidics for brain organoid proliferation. A) Illustration showing in vitro co-culture of pre-vascularized organoids with capillary beds containing endothelial and supporting cells, supporting better anastomosis and organoid growth. Adapted with permission.[175] Copyright 2021, Royal Society of Chemistry. B-i) Schematic of a microfluidic device containing Matrigel to culture embryoid bodies (EBs) with precise microenvironmental control through continuous media perfusion using intricate channel networks. Adapted with permission.[236,237] Copyright 2022, American Chemical Society. Copyright 2018, Royal Society of Chemistry. ii) Image of a 3D printed bioreactor for organoid growth that can provide microenvironmental control and non-invasive imaging techniques for observing cortical changes and disease modeling. Adapted with permission.[238] Copyright 2021, AIP Publishing. C) A self-folding dynamic microfluidic device made of PDMS and SU-8 could be used for precise microenvironmental control during organoid growth. i) Schematic of the device. ii) Bright-field image and iii) channel with flowing fluorescent green (fluorescein) depicting flow in a curved geometry within the single channel. iv) Bright-field image and v) image showing the flow of two fluorescent dyes, fluorescein (green) and rhodamine (red), depicting dual channel flow in a curved geometry. vi) Bright-field image of the fluidic multilayer's 3.5 cm long precursor (as fabricated) section. Adapted with permission.[176] Copyright 2011, Springer Nature. Created with BioRender.com.

necessary to avoid the harmful effects of a high shear flow on cellular viability.[247] We anticipate developing a robust control framework, which will require flow modeling and interfacing the microfluidic device with a precision pump outfitted with this control strategy. With further developments to these approaches, we envision microfluidics as a primary conduit for creating larger brain organoids with increasing biological complexity and advanced vasculature that can further our understanding of how the human brain works under homeostasis and disease.

4.1.3. Spatiotemporal Control of the Brain Organoid's Microenvironment

In addition to mimicking vasculature, microfluidics can simulate the microphysiological environment surrounding brain organoids. Specifically, they can be utilized to control hydrodynamic shear stresses, fluid flows, gradients (concentration, pH, etc.), chemical signaling events, and other dynamic microenvironments that occur in vivo,[178,208] enabling brain organoid models to become more anatomically relevant, reliable, and predictive as compared to monolayer culture and animal models (Figure 3B).[178,248,249]

Microfluidic instrumentation for brain organoids can draw inspiration from various complex gradient-generating fluidic devices created and applied to monolayer neuronal culture.[250,251] Dertinger et al. showed how laminin gradients could orient axonal specification.[252] Elsewhere, researchers have shown how controlled microfluidic environments can direct chemotaxis, adhesion, and differentiation of neuronal cells.[251,253,254]

With an OoC platform, developing brain organoids can be exposed to precisely controlled hydrodynamic shear stresses or physicochemical gradients, attenuating variability and simulating more realistic features driving neurodevelopment.[255] For example, Demers et al. fabricated a microfluidic device capable of simultaneously producing orthogonal morphogen gradients similar to those found during neural tube development.[177] As a result, they were able to capture the 3D cellular patterning associated with neural tube development in vitro with astounding similarity to the process that occurs in vivo.[177]

Dynamic, self-assembling microfluidic devices have even been created with the possibility of being used to simulate 3D circulatory systems. For example, Jamal et al. developed a reversibly self-folding microfluidic device using conventional lithography with PDMS and SU-8 (Figure 3C).[176] Integrating metal electrodes within the patterns could produce a robust, dynamic 3D architecture for brain organoid development and electrophysiological characterization. Using other 3D microfluidic platforms with controlled size and porosity, it is possible to create dynamic chemical displays that can be utilized to generate well-controlled spatiotemporal patterns guided by finite element transport simulations.[256–258] These 3D curving and folding devices can couple microfluidic vascularization with electrical and even optical technologies for integrated characterization.

4.2. Optical Microinstrumentation

Developments in optical microinstrumentation have allowed scientists to peer into the neuron to directly see and even control the chemical gradients responsible for neural signal transduction. Various nanoparticles and optical probes have been developed for neuromodulation, and leading approaches of relevance to optical microinstrumentation are discussed below.

4.2.1. Calcium Imaging and Two-Photon Microscopy

Calcium ion flux generates versatile signals in the nervous system. Therefore, calcium imaging, a technology to quantitatively measure and visualize intracellular calcium ion flux (location, amplitude, timescale, etc.), has become an essential approach in neuroscience.[180] Calcium imaging has been applied to studies from the subcellular to the neural network level, including brain organoid studies.[165] The technical advances of calcium imaging involve two parallel processes: developing calcium indicators and appropriate instrumentation.[259]

Tsien et al. developed a series of fluorescent calcium indicators and buffers based on hybridizing highly calcium-selective chelators.[179] Fura-2, in particular, has become popular in the field, as it provides a more quantitative calcium measurement than other indicators.[260] Many calcium indicators have been developed, including fluo-4 and the Oregon Green BAPTA.[261] The development of genetically encoded calcium indicators marked another milestone that enabled calcium measurements with a high signal-to-noise ratio in transgenic cells and animals.[262,263]

Concurrent with the development of calcium indicators, technological advances in optical instrumentation have also enabled high spatial-temporal resolution calcium imaging. Among those advances, applying two-photon microscopy in the nervous system represents a significant breakthrough.[180,264] These advances position calcium imaging as a powerful toolbox for neuroscience research. Calcium imaging has been adapted to brain organoids with the emergence and rapid growth of the brain organoid field.[106] The spontaneous and synchronized activity of neural networks from brain organoids can be examined with calcium imaging mapping.[265] Mansour et al. have used this approach to illustrate that brain organoids grafted in mice display synchronized neural activity and advanced-stage embryonic maturity.[173] Similarly, Shi et al. leveraged calcium imaging to demonstrate neuronal functional maturity in vascularized brain organoids resulting from a co-culture of human iPSCs with HUVECs.[222] Challenges of applying two-photon and related optical microscopies relevant to brain organoids include speed, axial or lateral resolution, and excitation depth.[266–268]

4.2.2. Optogenetics

Recent developments in optogenetics provide another approach to studying brain organoids.[183] As its name suggests, optogenetics combines optical and genetic tools to manipulate cellular systems.[269,270] It leverages photoreceptive proteins introduced into target cells through viral vectors or genetic manipulation to control the flow of ions across cell membranes with the introduction of incident light.[183,270]

Deisseroth is among the pioneers of optogenetics who built upon previous studies of microorganisms which uncovered the proton pumps bacteriorhodopsin and halorhodopsin;[271] proton channel channelrhodopsin-1 (ChR-1);[272] and the selective cation channel channelrhodopsin-2 (ChR-2),[273] which Nagel et al. identified as having the potential to photically control membrane depolarization.[273] These membrane proteins are microbial opsins, or light-sensitive proteins, which control the transmembrane flow of cations in response to photoactivation, effectively producing electrical current from light.[181,182,274] Bacteriorhodopsin and halorhodopsin can hyperpolarize a membrane, inhibiting a neuron's firing rate of action potentials; conversely, ChR-1 and ChR-2 can depolarize a membrane, exciting a neuron's firing rate of action potentials.[182] Deisseroth and colleagues leveraged the photoreceptive properties of these membrane proteins, as well as other engineered variants,[181] introduced them into nerve cells, and developed methods to non-destructively deliver light within an organism's brain to directly control and, therefore, study neural circuitry in vivo.[182,274] The same techniques can be applied to study the neural networks within brain organoids in vitro.[270]

Optogenetics is advancing to enhance efficiency, rapidity, and responsivity.[182,274] Overall, optogenetic techniques allow for enhanced spatiotemporal control over neural circuitry, which is critical for studying neurological disorders, neurodevelopment, and learning. In that respect, 3D multimodal interrogation of brain organoids by combining optogenetic stimulation and GEVI monitoring, such as Optopatch[275,276] or ArcLight,[277] could enable single-cell spatial resolution as well as millisecond-scale temporal resolution in studying neurological development and disorders, albeit requiring complex data analysis. Although previous studies on 2D cultures incorporated this method to probe iPSC-based models of amyotrophic lateral sclerosis to gain mechanistic insights into the disease,[278,279] a similar optogenetic study on brain organoid models could be more revealing since they can recapitulate the human brain more accurately. Besides understanding the etiologies of neurological disorders, such combined stimulation-recording explorations having single-cell resolution will be critical in understanding neurodevelopment, the interplay of neural networks and sensory stimuli,[109] and even the learning process.[280] Specifically, from an OI perspective, such a high-resolution study of synaptogenesis and synaptic plasticity in

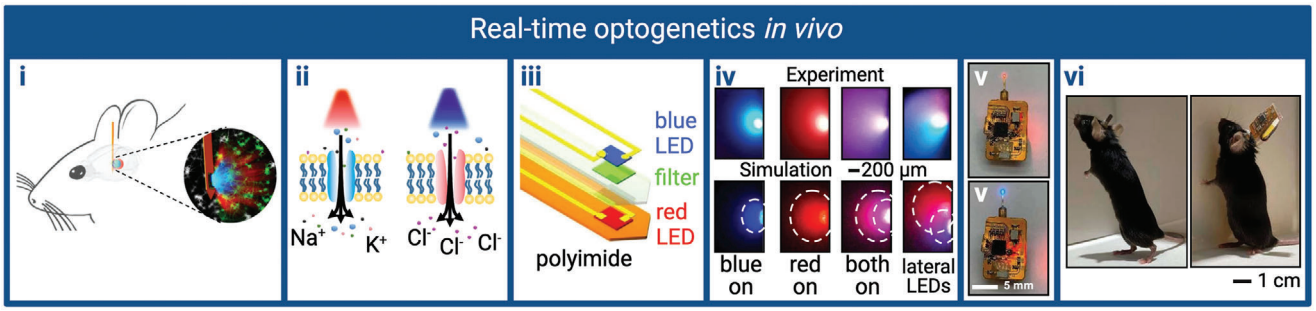

Figure 4. Optogenetics for in vivo studies of neural activity. i) Image of the LED probe insertion point in a mouse brain. ii) Schematic of the probe functionality depicted at the cellular level, where the red-light controls cation channels for membrane depolarization and blue light controls anion channels for membrane hyperpolarization. iii) Sketch of the flexible three-layered dual-color polyimide (PI) LED probe, made of thin-film red and blue micro-LEDs placed on the PI material with a thin-film selective filter for specific transmission and reflection. iv) Images from both experimental runs in brain tissue (Top panel) and simulated runs in brain phantom (Bottom panel) are highlighted in the first three columns. Each column shows the colocalized emission patterns within the same region for different conditions—blue on, red on, or both. Column four depicts the results from a lateral stack of the device micro-LEDs displaying minimal overlap between the emissions and a larger footprint than the superior vertical device assembly. v) Images of an integrated independent remote control circuit module capable of setting up a wireless system for the probe, making in vivo studies easier. vi) Images showing the probe placement on the mice and the wireless circuit setup mounted onto the mice. Adapted with permission.[288] Copyright 2022, Springer Nature. Created with BioRender.com.

organoids as a function of age and stimulation could help design organoids optimized for learning and develop pathways to teach brain organoids efficiently.

4.2.3. Optical Neuromodulation Using Nanoparticles

In recent years, with the development of new bottom-up methods for synthesizing nanoparticles of different sizes, shapes and surface morphologies, it has become possible to couple optical fields for neuromodulation at small scales and in a transient manner.[184,185,281] There are several mechanisms for neuromodulation, such as photothermal, photoacoustic, photocapacitive, and photoelectrochemical. By appropriate choice of nanoparticle (e.g., gold, carbon, or silicon nanostructures), bioconjugation, and size, it is possible to tune the nanoparticle-light coupling across wavelengths ranging from near-infrared to visible to modulate cell and organoid properties.[184] This tunable wavelength range is essential as brain organoids become larger since light has different absorption depths into tissue.

The basic principle of photothermal neuromodulation is that temperature changes can alter the electrical potential of cell membranes and the activities of cellular networks. As such, photothermal neuromodulation represents a non-genetic means of optical neuromodulation.[282,283] Plasmonic nanoparticle effects provide a convenient means for transient heating; they can be modulated by shape, size, texture, and geometry.[284,285] The mechanisms of photothermal coupling can be capacitive or coupled to temperature-responsive biomolecules such as transient receptor potential ion channels.[286]

Concurrent with the modes for optical neuromodulation are microinstrumentation for light emission and detection. Microsystems such as micro-LEDs and microphotodetectors have been developed, as demonstrated in an in vivo study using compliant ultrathin-integrated optical microsystems (**Figure 4**).[287,288] While much focus in such systems has been on in vivo implementation, soft, flexible, and highly integrated systems are particularly relevant to brain organoids.[195,289,290]

4.3. Electrical Microinstrumentation

The electrical responses of electrogenic cells, like neurons, are of great interest in revealing cell-to-cell communication and network activity in both physiological and pathological conditions. In neurons, electrical signals are driven by ions, and they travel from the soma to the axon terminal and eventually across synapses to the dendrites of neighboring nerve cells. Astonishingly, each neuron in the brain can form connections with thousands of neurons in its vicinity, making it extremely difficult to interrogate the ≈86 billion neurons within a human brain. The discovery of EEG in the early 1900s provided a means to understand the collective electrophysiology of the neurons in the brain. This non-invasive method enabled the detection of certain neurological disorders and stresses experienced by patients via monitoring abnormalities in neuronal patterns.[291] Although EEG is suitable for investigating human brain development by measuring the neuronal activity of the fetal, neonatal, and postnatal human brain, the obtained signals are limited to peripheral cortical electrical activity measured from outside the skull.[291] In contrast, electrocorticography (ECoG), an invasive intracranial electrophysiological characterization method that uses an array of electrodes placed directly on the brain's surface, offers a higher spatiotemporal resolution and better signal-to-noise ratio.[292] Besides EEG and ECoG, various in vivo and in vitro systems have been developed for understanding electrophysiology, including patch clamps, 2D and 3D MEAs, and microelectrochemical recording devices. Translating these technologies to the realm of submillimeter brain organoids is an ongoing effort,[293–295] and we survey several of these technologies below.

4.3.1. Patch Clamps

In their Nobel prize-winning study of an individual squid giant axon using inserted electrodes, Alan Hodgkin and Andrew

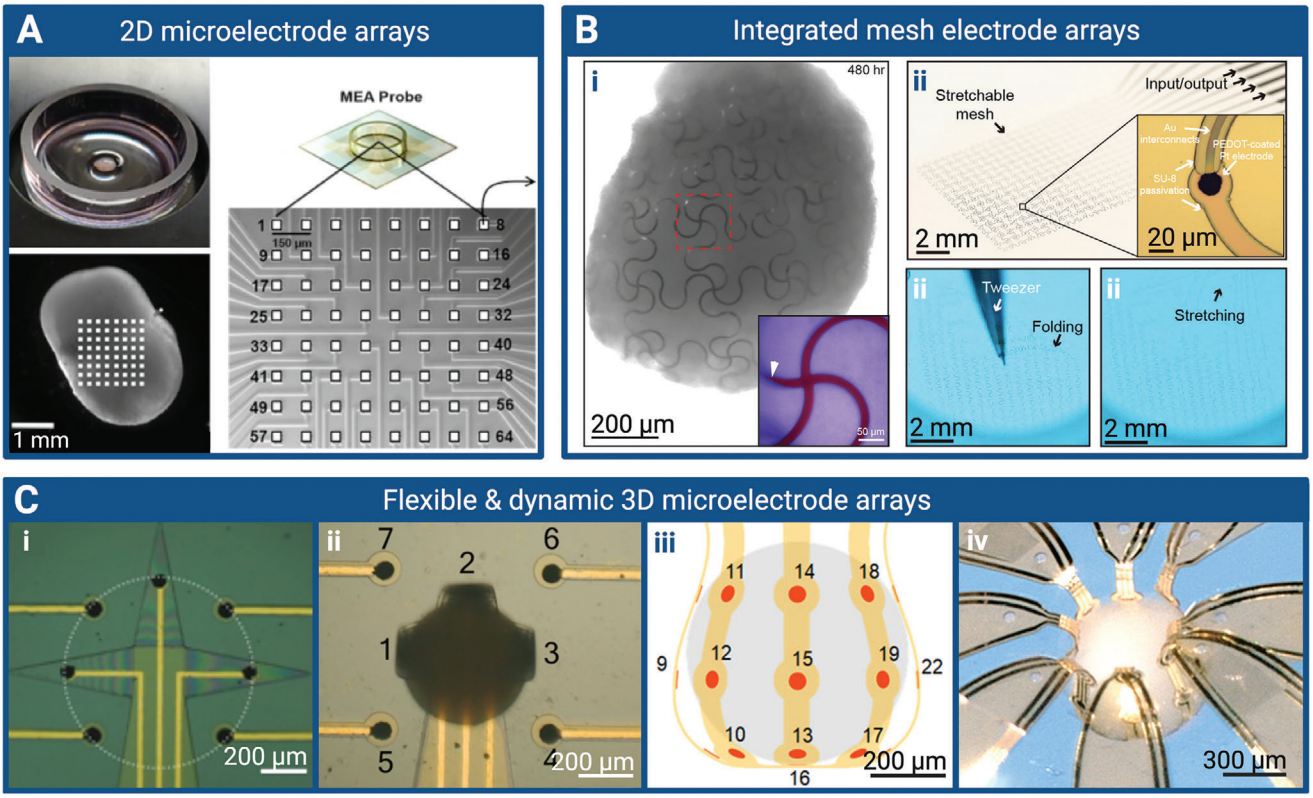

Figure 5. 2D and 3D electrical microinstrumentation for brain organoids. A) Image of a conventional 2D MEA plate consisting of a 64-microelectrode planar grid that can be used to measure spontaneous electrical activity. Adapted with permission.[314] Copyright 2020, Elsevier. B-i) Microscopy image displaying the seamless integration and wrapping of a stretchable nanoelectronic mesh around a spherical 3D organoid undergoing organogenesis. The inset shows a zoomed-in view of the mesh's uniform distribution. ii) Image of a stretchable nanoelectronic mesh. The inset depicts a close-up of one of its platinum electrodes coated with a conducting polymer, poly(3,4-ethylenedioxythiophene). Two different mesh states can be seen when folded with a tweezer and stretched. Adapted with permission.[315] Copyright 2019, American Chemical Society. C) Optical image of i) as fabricated and ii) self-folded 3D MEA shell that combines 2D and 3D electrodes for spatiotemporal electrophysiological studies of brain organoids. Adapted with permission.[169] Copyright 2022, American Association for the Advancement of Science. iii) Illustration of contact points across the brain organoid surface for a 3D multifunctional mesoscale framework. iv) Image of a brain organoid enclosed within this 3D multifunctional mesoscale framework with 25 microelectrodes for optimal design and low-force contact at the interface. Adapted with permission.[316] Copyright 2021, American Association for the Advancement of Science. Created with BioRender.com.

Huxleyexplored the ionic mechanism of the action potential for the first time.[296] This approach inspired Bert Sakmann and Erwin Neher, in yet another Nobel prize-winning study, to invent the first patch-clamp method to resolve single ion channel currents in a frog muscle cell.[297] This method of measuring the current flowing through a patch of cell membrane isolated by a pipette is still widely used for the electrophysiological characterization of neurons,[298–300] including in brain organoids.[31,301,302] Cortical organoids were initially monitored for their action potential using the patch clamp method.[163] Although case-specific patch clamp configurations (e.g., voltage clamp, current clamp, cell-attached, inside-out, whole-cell, perforated, and outside-out configuration) facilitate a deeper understanding of the whole cell and ion channels within a cell,[188,189,300,303,304] monitoring inter-neuronal communication and functional connectivity mapping are ill-suited to this method. Therefore, to cover the entire repertoire of brain organoid electrophysiology, one must rely on other recording techniques, such as MEAs, in addition to the patch clamp method.

4.3.2. 2D MEAs

MEAs consist of electrical recording sites either in direct contact with or proximal to neuronal culture that facilitate minimally invasive,[190,191,305] multiplexed, and long-term extracellular neural recording;[306–308] these qualities make it particularly suitable for monitoring brain organoid electrophysiology. Currently, MEAs are arranged in 2D grids on the surface of a plate and contain at least 16 to 512 electrodes to optimize the number of contact points between the surface and the 3D organoid (**Figure 5**A).[192,309] Despite being limited to this geometry, each electrode monitors either a single cell or a whole network of cells. This configuration provides data processing at a higher temporal resolution than calcium imaging. Unlike patch clamps, which only fail to capture spatial recording, 2D MEAs offer interconnectivity between neural regions of the whole brain organoid rather than single neural cells.[310–313] Many studies that explored neural plasticity relevant to organoid-based neuromorphic computing and organoid-computer interfacing also incorporated 2D MEAs.[36] Trujillo et al. developed a dynamic cellular model

during the maturation of cortical organoids using 2D MEAs.[36] For an accurate human neocortex model, Trujillo et al. discovered glutamatergic and GABAergic (i.e., excitatory and inhibitory, respectively) signaling is required to maintain long-term oscillatory neuronal firing, establishing a novel framework for brain organoid electrophysiology.

2D MEAs can facilitate not just recording but also stimulation. Kagan et al. integrated embryonic rodent and human-iPSC neurons on a high-density CMOS multielectrode array to harness the multi-processing power of living neurons.[172,317] In this study, learning in a simulated gameplay environment is proven possible through the adaptive internal processing of each neuron. Stimulation allows for developing a response between hardware (i.e., 2D MEAs) and biological tissue (i.e., neurons) within this closed-loop learning environment, relying on information feedback.[318] Although learning was proven through neuronal cultures, the monolayer architecture of single cells proves limiting when addressing intelligence. More recently, Cai et al. have demonstrated brain-inspired hardware that emulates the structure of a biological brain. By applying spatiotemporal electrical stimulation, Cai et al. showcased unsupervised learning by reshaping organoid plasticity.[319]

OI computing can attempt to bridge the gap between cognition, sentience, and consciousness with its complex neuronal network interconnected with real-world sensor and output devices. However, 3D MEAs are needed to tap into the full potential of the superior neuronal network of 3D brain organoids.

4.3.3. 3D MEAs

Materials for MEAs remain an active field of research focusing on the electrodes having specific properties, including mechanical characteristics comparable to those of cells[192,309] and biocompatibility to avoid unwanted host response.[192,320] Flexible 2D MEAs offer great potential for biological tissue; however, mapping the 3D topography of a human brain or brain organoid at a lab scale continues to prove challenging. By leveraging polymers and ultra-thin structures, 3D MEAs can provide a soft, flexible shape to encapsulate spherical brain organoids. Electrodes embedded within this encapsulating surface are often made of platinum, gold, or iridium with an additional coating of porous materials such as nanoporous platinum black or conducting polymers.[193,195,321,322] These coatings increase the electrode's effective surface area, reducing its overall impedance. Further, an advantageous outcome of having a high porosity at the electrode is the enhanced charge injection capacity, making these coating materials inherently good for stimulation and recording.[195] Mesh electronics have demonstrated groundbreaking potential in organogenesis and seamlessly integrate with developing tissue during cardiac organogenesis.[315] We believe a similar approach can be applied to neural organogenesis. Further, Le Floch et al. developed stretchable mesh nanoelectronics that match the mechanical properties of brain organoids and seamlessly integrate into the organoid (Figure 5B).[323] Elsewhere, Huang et al. have reported self-folding shell MEAs for brain organoids.[169] These shells resemble mini EEG caps capable of encapsulating and recording organoids ranging in size to about 500 µm (Figure 5C). Self-folding shells have been shown to record down to the few- to single-cell scale, as demonstrated with electrical recordings on cardiomyocytes.[324] The attractive features of these shells are their soft leaflets and capacity for on-chip integration, biocompatibility, tunability in size, and compatibility with planar electronics to enable varying geometries and electrode densities. Along with shells, alternate geometries, such as cylindrical MEA rolls, can also be used for stimulation and recording.[325] For example, buckled MEA systems derived from transfer printing on pre-stretched elastomeric substrates have been utilized to create buckled 3D neural interfaces with an array of 25 microelectrodes (Figure 5C).[316]

Instead of enclosing the organoids, another approach is to have electrodes inside them. For example, Soscia et al. have developed a thin-film 3D flexible MEA that non-invasively interrogates a 3D culture of neurons.[326] In that respect, electrodes penetrating the brain/brain organoid are also categorized as 3D MEAs. High-quality signals can and have been recorded previously with 3D rigid electrodes, Utah arrays,[327,328] and traditional silicon probes.[329] However, a significant challenge in assessing long-term neural electrophysiology is the ability to record signals from the same neurons over days, weeks, and even months.[330,331] Neuropixels (NP) 2.0, penetrable high-density electrode arrays based on CMOS fabrication technology, addresses these challenges alongside open-source software for motion correction and spike sorting.[194,332] Including a head stage, the system weighs about 1.1 g, which is ideal for implementing and recording a moving mouse with programmable switches, enabling recording in less than 1 s.[194] Translating this technology to brain organoids can facilitate long-term studies, especially those related to learning and memory associated with OI. Both enclosing and embedding approaches are evolving rapidly, with enclosed structures incorporating multifunctional electrodes capable of simultaneously performing electrophysiological recording and electrochemical sensing.[316] More functionalities, such as microfluidics, within these organoid-encapsulated structures are expected soon. Current efforts to increase the flexibility of the electrode mesh result in ultrathin meshes for in vivo monitoring, with some using boron nitride and graphene as insulators and electrodes, respectively.[333] Translation of such methods to organoids is also imminent.

4.3.4. Electrochemical Monitoring

Since the brain transmits signals using small molecules (e.g., neurotransmitters and neuromodulators), electrochemical methods provide a unique set of tools to monitor their real-time effects within brain organoid models. Unlike animal models, organoids derived from humans can recapitulate various neurological pathologies more accurately. For instance, midbrain organoids are an encouraging platform for understanding the pathogenesis and progression of Parkinson's disease.[199] One could get significant insight into such diseases by monitoring neurotransmitters, making electrochemical sensing a precious tool. While dopamine is considered a biomarker for Parkinson's disease, acetylcholine is critical in many memory disorders, such as Alzheimer's disease, as is glutamate for studying sleeping disorders and addiction.[197] It would be logical to extend the current practices for detecting these chemicals used in the more

mature field of in vivo monitoring to the emerging area of brain organoids. Early in vivo detection can be traced back to Kissinger et al., who successfully identified a peak in their electrochemical study corresponding to dopamine.[196] Subsequent studies focused on improving electrode materials and electrochemical detection methods, allowing the measurement of catecholamines, monoamines, and metabolites with high sensitivity, selectivity, and spatiotemporal resolution.[334] Direct in vivo detection of electroactive species, such as dopamine, norepinephrine, and serotonin, is possible with a fast-scan cyclic voltammetry.[198,335] In contrast, other neurotransmitters, such as acetylcholine and glutamate, are detected indirectly with the help of enzyme- or aptamer-based electrochemical sensors.[336,337] A challenge in detecting electroactive neurotransmitters is interference from other electroactive chemicals, such as ascorbic acid, in the sensing environment.[338,339] Typically, researchers address these issues by selecting appropriate electrodes with enhanced charge transfer and electrocatalytic behavior,[337,340–344] or by chemically altering them with anion-impermeable materials.[345,346]

In contrast to in vivo studies, only a few studies have been reported on the electrochemical characterization of brain organoids, which can be attributed to their small size, dilution of the electroactive chemicals in the media, and lack of readily available 3D electrode brain organoid interfaces. In one study, electrochemical monitoring of various neurotransmitters within midbrain organoids in the context of drug screening showed their importance in understanding Parkinson's disease.[199] To detect micromolar concentrations of neurotransmitters relevant to organoids, investigators implemented a redox cycling amplification, where two electrodes were placed within the diffusion layer of each other to introduce repetitive redox reactions to amplify the effect of the analyte.[347] Elsewhere, a 3D electrochemical microsensor that wraps around the organoid and measures the concentration of oxygen in culture media near the location of interest has been reported by Park et al.[316] Such three-dimensional architecture offers a spatial mapping that is absent in conventional 2D electrodes and will be crucial in designing future sensors for brain organoids. Recent developments in real-time measurement in vivo by combining microdialysis aspects and electrochemical sensing could be a prospective sensing method for brain organoids.[333] However, submillimeter size, spherical shape, and a need for microfluidic channels complicate the effort to build similar electrochemical sensors for brain organoids. Moreover, unlike neurotransmitters like dopamine and norepinephrine that are secreted out to the extracellular region, excitatory neurotransmitters such as glutamate and acetylcholine and the inhibitory neurotransmitter gamma-aminobutyric acid are confined to synapses, making it somewhat challenging to monitor them real-time.[197] As a result, developing electrochemical sensors with 3D spatiotemporal mapping capability at small-size scales that overcome these challenges would be transformative for brain organoid studies.

4.4. Magnetic and Acoustic Microinstrumentation

Recent advances in nanoparticle synthesis have greatly enhanced the use of magnetic and acoustic fields for neuromodulation.[203,348] Early work on magnetic stimulation dates back to the 19th century, followed by landmark studies by Barker and colleagues, who reported peripheral nerve stimulation and stimulation of limb movements by the placement of a magnetic coil on the scalp.[200,349,350] Magnetic fields can penetrate tissue, enabling relatively non-invasive stimulation and modulation. Magnetic stimulation in the form of transcranial magnetic stimulation (TMS) has been widely utilized in clinical medicine to investigate conduction pathways non-invasively. It is an FDA-approved treatment for various psychiatric or neurological conditions.[202,351,352] As is well known since the time of Faraday, alternating magnetic fields can induce electric currents in conductors; alternating magnetic fields can also interact with spins. In the brain, which is a conducting medium, low or high-frequency repetitive TMS can modulate neuronal and cortical excitability. However, the location, depth, and mechanisms of neuromodulation are still being debated.[353,354] Current challenges with magnetic stimulation, especially as it relates to organoids, are resolution, tissue magnetic susceptibility heterogeneities, and the availability of small-scale magnetic field generating systems that are compatible with cell culture; these challenges are being addressed by 3D integrated, self-folding, and 3D printed magnetic microsystems.[201,355–360]

Magnetic beads and structures have been widely utilized to manipulate and characterize cells.[361,362] In recent years, with the discovery of new bottom-up synthesis methods for nanoparticles and their unique size-dependent properties, such as superparamagnetism, they have been widely incorporated within cell culture. For example, magnetic nanoparticles have been introduced into cells for magnetic levitation and the generation of 3D culture models.[363] These magnetic 3D cell culture methods are attractive for the assembly and manipulation of cellular spheroids, which may be necessary for differentiating and forming networks of brain organoids.[364] It has been argued that magnetic levitation can speed up spheroid formation, does not need artificial protein substrates, and that introducing magnetic nanoparticles does not affect cell proliferation or metabolism.[365] Apart from spheroids, magnetite particles have been incorporated in multilayer cell sheets and rolled up into tubular shapes, highlighting the ability of magnetic fields for complex spatial manipulation.[366]

Elsewhere, with the aid of nanoparticles, magnetic fields can also be coupled to mechano- or thermosensitive ion channels for remote control of cellular behaviors.[184,367,368] Of relevance to brain organoids, the incorporation of magnetic nanoparticles and the use of permanent magnets or multi-axes magnetic coils could be necessary for actuation, differentiation, and network formation (**Figure 6A**).[369–372] Magnetic nanoparticles can be conjugated with receptors or antibodies to different cellular moieties, such as integrin receptors or ion channels, and magnetic fields can then be utilized to apply forces and torques to trigger cellular signaling.

Magnetic fields can also be utilized with nanoparticles such as ferromagnetic iron oxide (Fe_3O_4) for local heating (hyperthermia), which has been widely used for imaging and treating cancers, including brain tumors.[373–375] It is conceivable that magnetic microinstrumentation could be utilized to create localized lesions or to investigate treatment protocols in organoid brain cancer models.[376]

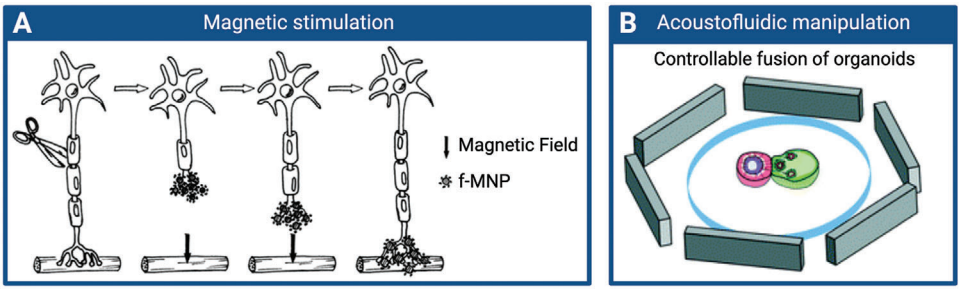

Figure 6. Neuromodulation using acoustic and magnetic fields. A) Schematic depicting axonal growth from an injured site on a nerve cell using magnetic fields and functionalized magnetic nanoparticles (f-MNPs) to assist with nerve regeneration. The tensile forces generated using f-MNPs under the influence of a magnetic field can guide the neurite in the desired directional orientation. Thus, it is capable of axonal regeneration from the proximal end to the distal end. Adapted with permission.[371] Copyright 2014, Elsevier. B) An acoustofluidic device for the controlled fusion of organoids to model physiological processes. The green portion depicts a human forebrain organoid, while the red sphere is a midbrain organoid. By varying the acoustic fields, the alignment and orientation of neuroepithelial buds within the organoids could be controlled to model the development of mesocortical pathways. Adapted with permission.[206] Copyright 2021, Royal Society of Chemistry. Created with BioRender.com.

Acoustic fields can generate surface or bulk pressure waves causing heating, mechanical disruption, or activation of mechanosensitive ion channels with a rich history in mechanobiology and biomedicine.[207] There are a variety of technologies to generate acoustic fields, typically by high-frequency vibration of a membrane using electromagnetic fields or the piezoelectric effect. These technologies are well-developed for biomedicine (e.g., cardiac pacemakers, cochlear implants) and energy harvesting. Transcranial ultrasound stimulation has been shown to alter brain function, from forming therapeutic brain lesions via ablation to brain stimulation therapy to neuromodulation.[377,378] Acoustic fields can also be used to manipulate brain organoids using spatially oriented piezoelectric transducers.[379,380] Of note, many piezoelectric transducers can be adapted with flexible or fiber optic forms with a variety of materials, including those composed of biodegradable materials such as poly(L-lactic acid) nanofibers.[381,382]

For example, Ao et al. have demonstrated acoustofluidic manipulation of organoids and controllable fusion of human forebrain and midbrain organoids; these complex organoid models may be necessary for investigating dopaminergic pathways and neurological diseases (Figure 6B).[206,207] Of relevance to brain organoid research, the non-invasive manipulation of brain organoids with different differentiation will be critical for reverse engineering human brain networks and OI. Biotunable acoustic nodes have been developed to assemble spheroids at the bottom of cell culture chambers.[204] Increasing the resolution of ultrasound techniques below one millimeter remains a challenge for in vivo systems but could be overcome for in vitro brain organoid stimulation and manipulation.[383] For example, Cai et al. recently reported trapping and immobilizing cell spheroids with diameters ranging from 20–300 µm using digital acoustofluidics.[205]

5. Future Perspectives

Brain organoids represent an attractive model system and a vital bridge between monolayer neuronal culture and in vivo studies. They overcome challenges associated with other existing approaches, which can be summarized by several key advantages. They can be mass-produced using human-derived stem cells with cellular heterogeneity; for example, brain organoids can already be created with neurons, astrocytes, and oligodendrocytes. Unlike planar culture, brain organoids mimic some of the connectivity of real brains due to their 3D nature. However, we note there is still a long way to go to enable the appropriate differentiation and complexity of organoids to mimic real human brains accurately. As Smirnova et al. have pointed out, typically sized brain organoids created thus far are about three-millionths of the size of a human brain with an estimated 800 MB of storage.[3] In the future, we envision that microfluidic and other approaches for vascularization could result in larger brain organoids. This fluidic microinstrumentation also offers the potential to control spatiotemporal biochemical environments, allowing the differentiation of various parts of an organoid into different functional areas of the human brain. In the future, we anticipate the need for protocols to culture organoids with reproducibility and standardization regarding size, complexity, and cellular heterogeneity. Standardization and benchmarking are critical for drug or toxicity screening and OI. At present, organoids are created in liquid culture on shakers, and the differentiation of organoids in smart microwells or on-chip could aid in this regard.

Apart from in vitro applications, in the future, brain organoids made from patients' cells (autologous) might later be transplanted to repair areas in a patient's body where their existing repair mechanisms are unresponsive.[384] These transplanted organoids could successfully integrate and effectively reconstruct brain tissue. As discussed previously, transplantation of human brain organoids in rats has already been demonstrated,[385] but transplantation in humans presents significant challenges regarding infection, immune rejection, and ethics.

We note several ethical considerations in this research area, and there are notable reviews and commentaries on the subject.[386–388] Human brain organoids overcome several ethical challenges with animal testing, but they present many ethical challenges mainly related to experimentation with human-derived tissue.[389] Animal experimentation is among the most controversial subjects in scientific research. To stay within the confines of the law and to foster respect for animals being bred for testing, the three Rs (replacement, reduction, and refinement) are a globally accepted policy.[390,391] Key ethical challenges with human brain organoids include donor consent, ownership, intellectual property rights, morality, and social norms.[392,393] These

considerations will become more critical as larger and more complex organoids are developed, as these might have the capacity to experience pain or consciousness. Trujillo et al. have already reported neural electrical behavior in organoids that highlights their likeliness to EEG of preterm humans.[36]

Regarding microinstrumentation, the significant challenges are the fabrication and integration of complex microfluidic, optical, and electrical networks in living tissue. The 3D nature of brain organoids will necessitate modification of present instrumentation from inherently 2D planar geometries to 3D formats. A holy grail in brain sciences is the ability to stimulate and communicate with every brain cell, which presents a considerable challenge, and trade-offs may be necessary. For example, "bed of nails" type sensors can penetrate the interior of organoids but are invasive, while shell MEAs are less invasive but can record only from the periphery of organoids. In terms of optical microinstrumentation, additional advancements in fluorescence imaging, such as lattice light sheets, have enabled large volumes of four-dimensional (4D) data to be acquired in these 3D cultures, thus introducing the need for big data analytics into the field of biology.[394] Consequently, conventional techniques of analysis that have been used for 2D data sets will need to be replaced by algorithms and machine learning tools to glean the essential information from the gigabytes of 4D data points.[394] Furthermore, self-folding, buckled, origami-inspired, and 3D-printed MEA shells and microfluidic devices can enable stimulation and recording from larger fractions of cells within the organoids, offering the possibility for high data transfer.[213,395–400] We envision that with appropriate microinstrumentation, brain organoids can be utilized to rapidly screen toxins and drugs, brain–machine interfaces, and biological analogs of microprocessors for neuromorphic computing. Also, there is a possibility of creating hybrid brain organoids with embedded nanoparticles for remote, noninvasive control of activity and function using optical, magnetic, or acoustic fields. Radiofrequency and acoustic neuromodulation of brain organoids could enable action at a distance and facilitate wireless and remote-controlled neuroelectronic devices.

6. Conclusions

As described in the review, brain organoids have emerged as a powerful tool to unravel the mysteries of human neurodevelopment and cognition. They offer researchers a means to investigate the origin of neurodegenerative diseases and screen drugs or toxins in vitro. These models could also enable novel brain–machine interfaces for OI leading to new generations of biological computers. Great strides are being made in genetic manipulation and tissue engineering to advance brain organoid models. However, alongside advances in biological manipulation, 3D microinstrumentation is needed for manipulation and spatiotemporal control and to enable anatomically accurate models. Anatomically accurate brain organoid models have the potential to revolutionize treatment for neurodegenerative diseases and facilitate new neuroinspired computing platforms. 3D microinstrumentation includes developing new neurocompatible materials, nanoprobes, and coatings. Also needed is the development of miniaturized and integrated fluidic, electrical, optical, magnetic, and acoustic platforms to create larger organoids, enable long-term maturation and spatiotemporal differentiation, and ultimately to develop and interrogate more anatomically accurate brain models. We anticipate that advances in brain organoid biology, microinstrumentation, computational methods, and ethics will lead to a widespread impact of broad cross-disciplinary relevance to science, engineering, and medicine.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

microfluidics, microphysiological systems, neuromodulation, neuromorphic computing, organoid intelligence

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Devan Patel is an M.S.E. candidate in Chemical and Biomolecular Engineering at Johns Hopkins University (JHU). He has great interest in developing microdevices for next-generation, high-throughput technologies. He is involved in research on self-folding 3D microdevices for assessing organoid intelligence. Devan received his B.S. in Chemical and Biomolecular Engineering from JHU.

Saniya Shetty is an M.S.E. graduate in Biomedical Engineering at Johns Hopkins University (JHU). She is working in the field of microfluidic devices and is passionate about translating technology from education and prototyping stages into commercial products. She has a keen interest in the growing capabilities of wearable microdevices and their potential for continuous health monitoring. Saniya received her B.S. in Chemical and Biomolecular Engineering from Iowa State University.

David Gracias has been on the faculty at Johns Hopkins University (JHU) for the last 20 years. He is internationally recognized for developing 3D, integrated micro and nanodevices using a variety of patterning, self-folding and self-assembly approaches. He has co-authored over 200 technical publications and holds 36 issued US patents. Prof. Gracias received his Ph.D. from UC Berkeley and did postdoctoral research at Harvard University before starting his independent laboratory at JHU.