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Review

Bioengineering innovations for neural organoids with enhanced fidelity and function

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SUMMARY

Neural organoids have been utilized to recapitulate different aspects of the developing nervous system. While hailed as promising experimental tools for studying human neural development and neuropathology, current neural organoids do not fully recapitulate the anatomy or microcircuitry-level functionality of the developing brain, spinal cord, or peripheral nervous system. In this review, we discuss emerging bioengineering approaches that control morphogen signals and biophysical microenvironments, which have improved the efficiency, fidelity, and utility of neural organoids. Furthermore, advancements in bioengineered tools have facilitated more sophisticated analyses of neural organoid functions and applications, including improved neural-bioelectronic interfaces and organoid-based information processing. Emerging bioethical issues associated with advanced neural organoids are also discussed. Future opportunities of neural organoid research lie in enhancing their fidelity, maturity, and complexity and expanding their applications in a scalable manner.

INTRODUCTION

Neural organoids as promising models of development and neurological disease

Due to the complexity of human brain development and the difficulty in accessing human brain tissues, animal models have been extensively utilized for studies of neural development and neuropathology. However, animal models fail to capture many human-specific genetic, cellular, and circuit features of brain development. Over the last decade, neural organoids (including brain organoids, spinal cord organoids, and peripheral neural organoids) have emerged as an exciting experimental tool for modeling human neural development and disease.[1–3] Derived from human pluripotent stem cells (hPSCs), neural organoids are 3D cellular structures that recapitulate many complex features of the developing human nervous system, including cell type heterogeneity, cellular organization, and cell-cell interactions that are necessary to enable complex patterns of neural activity to emerge.[4] More recently, regionalized neural organoids modeling various components of the nervous systems, including ganglionic eminences (GEs),[5–7] striatum,[8] hippocampus,[9] choroid plexus,[10,11] thalamus,[12] hypothalamus,[13] pituitary gland,[14] midbrain,[3,15,16] and cerebellum,[17,18] as well as spinal cord,[19,20] have been demonstrated. Particularly, cortical organoids, aiming to model the development of the human cortex, the brain region responsible for higher brain functions such as cognition and sensorimotor functions, have been developed and applied to study human-specific features and evolution.[3] Compared with rodent models, cortical organoids exhibit human-specific cell types, including outer radial glia (oRG) cells, a distinguishing feature of the mouse and human developing brains.[21] Neural organoids generated from patient-derived hPSCs harboring disease-relevant genetic variants have been utilized for modeling neurodevelopmental disorders that involve structural malformations such as microcephaly, macrocephaly, and lissencephaly,[22,23] different aspects of cell fate patterning,[24,25] as well as neuropsychiatric disorders (e.g., schizophrenia and autism spectrum disorders)[26,27] and neurodegenerative diseases (e.g., Alzheimer's disease).[28,29] Neural organoids are compatible with genetic engineering tools (e.g., genetic barcoding-based lineage tracing), advanced imaging technologies, single-cell multi-omics, and bioelectronic technologies, and thus they are becoming promising experimental tools that can offer molecular, cellular, and functional details at unparalleled spatiotemporal resolutions and even be used for functional genetic screens.[30,31]

Neural organoids have enabled the discovery of fundamental knowledge in neurogenesis, human evolution, and cellular and

molecular mechanisms of neurological diseases. For instance, studies using neural organoids have identified mammalian Target of Rapamycin (mTOR) signaling to affect the actin cytoskeleton of oRG cells, influencing their morphology, migration, and mitotic behaviors.[32] Comparative studies of cerebral organoids from humans and other primates revealed that neuroepithelial cell shape transition is a key factor contributing to human brain expansion.[33] Additionally, neural organoids with schizophrenia-associated neurexin-1 deletion showed disrupted developmental trajectories of early neural progenitors and revealed disease-specific transcriptomic signatures.[34] These examples, albeit providing a partial list, highlight neural organoids as promising experimental tools to reveal novel insights of human neural development and disease.

Limitations of current neural organoids

Despite the promising features of neural organoids and the fact that they are becoming increasingly more complex and manifest a variety of robust features, they remain rudimentary and inherently artificial compared with the natural human neural system. Furthermore, their broad utility is tempered by some important limitations, including the lack of high-fidelity cell types, limited maturation, atypical physiology, and lack of arealization. Importantly, current neural organoids are not capable of faithfully recapitulating brain anatomy, cytoarchitecture, or high-level functions.[35,36] Related to this, to date, the demonstration of complex neural network activities in neural organoids remains extremely limited.[37–40] In addition, the reproducibility of neural organoids across cell lines or differentiation protocols and from batch to batch remains suboptimal compared with conventional 2D cultures developed to obtain specific neuronal lineages. The culture and maintenance of functional neural organoids are technically challenging and expensive, hindering their broader adoption for applications that require quick turnover times or scalability. Moreover, functional electrophysiological analysis and modulation of the entire neural organoid in 3D are still difficult. Below, we examine these limitations of neural organoids in detail.

Firstly, the development of distinct regions of the brain is orchestrated and synchronized, and the formation of neuronal connections between different brain regions is critical for their development, functional interactions, and maturation. However, current neural organoid protocols cannot generate compartmentalized brain subregions in an organized, *in vivo*-like manner. Instead, neural organoids are generated either by unguided approaches with randomly distributed subregions and cell types or by guided differentiation protocols to generate region-specific neural organoids that only mimic one or two brain subregions.[41] To study regional interactions in the developing brain, assembloid approaches are utilized through physical co-culture of two or more brain region-specific organoids to enable cellular interactions between the organoids.[42] Although assembloid approaches are becoming increasingly sophisticated,[42] they still offer imperfect solutions. Organoid fusions require individual organoids to be differentiated in parallel into distinct regions, and in most cases, organoid fusion is not performed until their regional identities are fully acquired. Thus, these region-specific neural organoids might not develop in a synchronized manner as those brain subregions *in vivo*. It is also unclear how artificial assembly of different brain organoids can recapitulate the continuous, temporally choreographed interactions between different regions of the developing brain.

Secondly, suboptimal reproducibility of neural organoids presents another challenge for their broad utility. The distribution of nutrients, oxygen, and other soluble factors in 3D neural organoid cultures can be uneven, leading to differential cell growth and activity.[43] Localized changes in pH within 3D neural organoid cultures can also affect cell behavior and function. In addition, variations in cell density and arrangement within neural organoids can lead to differences in local cell-cell and cell-matrix interactions. Understanding and controlling the functions of these biochemical and biomechanical signals in neural organoid cultures are crucial for making them more consistent and reproducible. There have been recent efforts in characterizing and improving the reproducibility of neural organoid protocols.[25,44] By employing carefully tailored protocols with timely controlled supplementation of various chemical factors, neural organoid development has shown enhanced reproducibility, resulting in consistent cellular compositions from batch to batch and cell line to cell line. However, achieving reproducibility at the level of cellular organization and tissue architecture remains a significant challenge and has not been fully explored so far.

To use neural organoids as disease models, extended culture periods (>3 months) are often needed to obtain mature neurons and glia and to allow the emergence of functional electrophysiological activities in the organoids.[45] This requirement increases cost and tissue heterogeneity, lowers experimental throughput, and heightens the risks of contamination and necrosis, posing substantial barriers for broad utility of neural organoids. Certain subtypes of neurons and glia cells, such as myelinating oligodendrocytes, which insulate axons to facilitate electrical signal transmission, are still difficult to obtain with physiologically relevant quantity and morphology even under prolonged cultures.[46–48] Additionally, important cell lineages that do not originate from the ectoderm, such as the brain microvascular endothelial cells and microglial cells, cannot be directly derived using current neural organoid culture protocols. This is because nearly all neural organoid protocols begin with a constraining neural induction step, which limits the mesodermal and endodermal lineage development. Brain microvascular endothelial cells interact with pericytes and astrocytes to form a vascular network with proper barrier functions. The brain microvasculature not only provides oxygen and nutrient support important for proper brain development but also establishes a blood-brain barrier to control the transportation of chemical factors and immune cells.[49] In the developing brain, microglial cells can identify and destroy invading pathogens and help monitor nervous system health. They engulf unhealthy and unused synapses and are involved in the refinement of circuits. Dysfunction of these microglial processes is related to a variety of disease states.[50] Therefore, the absence of these important cell types in neural organoids limits their applicability for modeling certain brain functions and diseases, such as Alzheimer's disease, in which neuroinflammation plays a critical role.[51] To address these issues, various strategies have been used to reintroduce these cells into neural organoids but with limited success. For instance, vascularization of neural organoids has been attempted by transplanting them to immunodeficient rodent brains,[52,53] inducing hPSCs in neural organoids to overexpress transcription

factors related to endothelial cell differentiation,[54] or co-culturing neural organoids with vascular organoids.[55] However, a fully mature vascular network carrying blood cells spanning the entire neural organoid has not been achieved, and the properties of blood-brain and blood-cerebrospinal fluid barriers have not been fully replicated in neural organoids.[56] Together, the organized development of multiple brain regions and the integration of non-neural tissues in neural organoids are critically needed to expand their capabilities for modeling normal brain development and diseases affecting cells in multiple brain regions.

Lastly, to fully achieve the potential of neural organoids for fundamental and translational applications, systems for measuring and modulating neuronal electrophysiological activities in 3D neural organoids with high spatial and temporal resolutions are highly desired.[37,57] Although systems such as 2D multi-electrode arrays (MEAs) and 3D shank probes have been successfully used to study neural activity and connectivity in neural organoids,[58] they are not capable of providing measurements over the entire neural organoid topology in a non-biased fashion over a long term. These bioelectronic systems also have not yet demonstrated a closed-loop feedback control to enable real time, dynamic interaction between the systems and the biological processes being studied in neural organoids. Bioelectronic systems that can serve as chronically stable, high-performance electronic recording and stimulation interfaces to neural organoids, with a cellular-level resolution across macroscopic areas, are of great interest to future studies involving neural organoids.

Bioengineering tools for advanced neural organoids

Various bioengineering tools have been incorporated into routine neural organoid culture practice, including microwell arrays[59] for controllable and reproducible generation of initial cell clusters and spinning bioreactors/orbital shakers to enhance oxygen and nutrient exchange for long-term culture.[60] We anticipate that future bioengineering innovations will provide enabling tools for the development of advanced neural organoids, making them more reproducible, controllable, *in vivo*-like, and convenient for experimental measurements and perturbations and even for large-scale screening applications. These advanced neural organoids should display proper cellular and tissue-scale features of the developing human brain, including highly specified and organized cell types, functional circuit formation and activity, and interactions between central nervous system (CNS)-resident neuronal and non-neuronal cell types, thus offering useful experimental tools to study human brain development and disease. In this review, after a brief survey of state-of-the-art current neural organoid systems, we focus on recent bioengineering technological developments that have been implemented to advance neural organoid research. We also discuss the emerging bioethical issues of such bioengineered neural organoids and offer our perspectives.

STATUS OF CURRENT NEURAL ORGANOIDS

Cerebral and region-specific neural organoids

The first neural organoids were developed by unguided differentiation of free-floating embryoid bodies (EBs) derived from hPSCs.[61] Matrigel embedding and agitation promoted neural expansion and morphogenesis, leading to the development of cerebral organoids through an unguided or minimally guided approach without exogenous growth factors.[1] These cerebral organoids contain various brain regions and thus allow spontaneous interactions to occur. However, as the self-organization process is difficult to control, and not all brain regions can be reproducibly produced, it is often challenging to perform systematic or quantitative analyses using these cerebral organoids.

Inspired by the knowledge of rodent CNS development and 2D neural differentiation of hPSCs, morphogens or small molecules like hedgehog agonist smoothened agonist (SAG) were introduced in neural organoid cultures to derive region-specific neural organoids.[62] Since then, neural organoids representing key features of almost all major brain regions and the spinal cord have been developed. Figure 1 summarizes the key signaling events involved in the derivation of each region-specific neural organoid. Generally speaking, the differentiation process of region-specific neural organoids begins with EB formation[61] followed by neural induction under Bone Morphogenetic Protein (BMP) and transforming growth factor β (TGF-β) inhibition conditions.[63] These culture steps lead to the formation of 3D neuroepithelial tissues containing variable numbers of neural rosette structures. Some recent protocols bypass the EB formation step by aggregating small fragments of 2D neuroepithelium sheets to achieve single-rosette cortical organoids.[64] These 3D neuroepithelial structures are then patterned with specific regional identities by modulating caudalizing, dorsalizing, and/or ventralizing signals involving BMP, Wingless-related integration site (WNT), fibroblast growth factor (FGF), retinoic acid (RA), and sonic hedgehog (SHH) signals. Following regionalization, neural organoids are further cultured in maturation medium containing various neurotrophic factors for long-term development. Compared with 2D neuronal differentiation of hPSCs, guided neural organoid differentiation generally takes longer time and leads to more complex cellular composition and lineage diversification that mimic *in vivo* development of targeted specific brain regions.

Transplantation of neural organoids

Transplanting neural organoids into host organisms, such as neonatal or adult rodent brains, can significantly improve their maturity, promote functional vascularization, and enable integration with host tissues.[53,65] Unlike dissociated neural progenitor cells with poor engraftment rates, neural organoids exhibit robust survival, axonal outgrowth, and functional integration with rodent hosts. For example, human cortical organoids transplanted into the primary somatosensory cortex of early postnatal immunodeficient rats demonstrated advanced maturation features, including increased cell size, dendrite length, spontaneous firing rate, and expression of activity-regulated genes.[52] Additionally, cortical organoids transplanted into injured visual cortex of adult rats partially responded to visual stimulation, highlighting their functional integration with host neuronal networks and potential therapeutic applications of cortical organoids for cortical injuries.[66] Another notable use of xenotransplanted neural organoids is to study neuro-immune interactions.[67] It has been shown that forebrain organoids populated with human microglia progenitors support microglial maturation and injury responses after

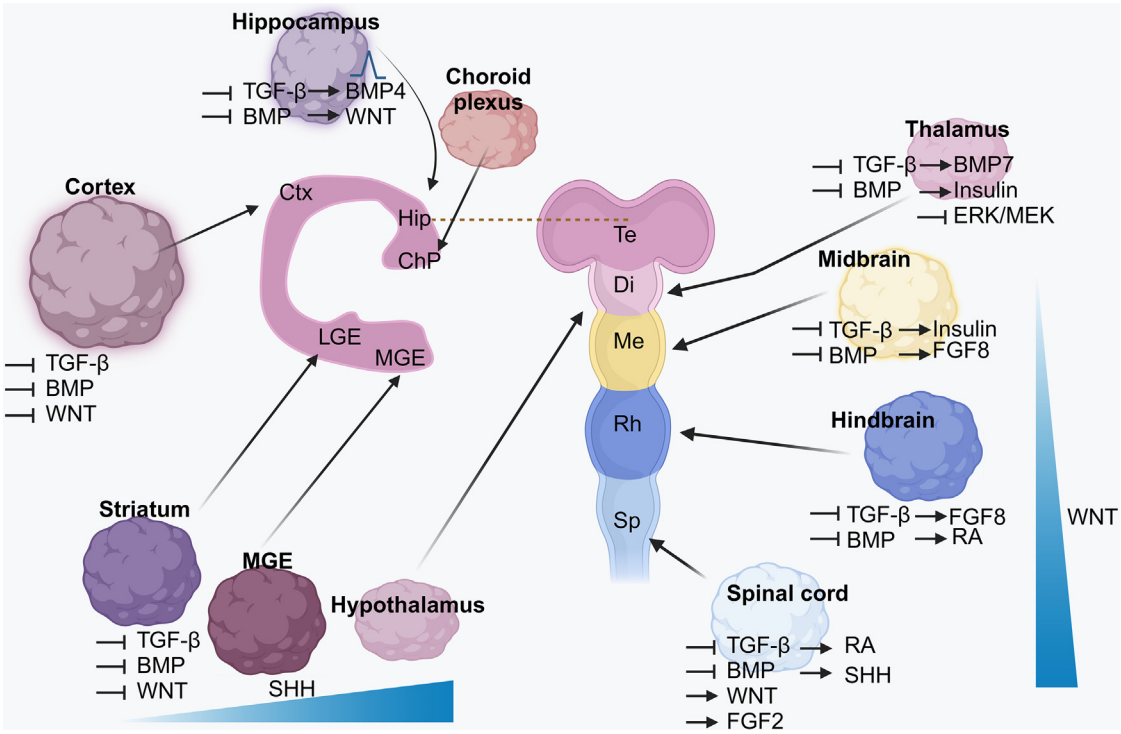

Figure 1. Schematics summarizing key signaling pathways for patterning region-specific neural organoids after EB formation

Dual Smad inhibitions are used to induce (dorsal) ectoderm fate. Caudalization is induced by insulin, FGFs, WNT, and/or RA. Ventralization is induced by fine-tuning SHH activities by a combination of SAG, purmorphamine, and/or recombinant SHH. BMP4 and WNT pulses induce dorsal fate. Abbreviations: Ctx, cortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; TE, telencephalon; Hip, hippocampus; ChP, choroid plexus; DI, diencephalon; Me, mesencephalon; Rh, rhombencephalon; Sp, spinal cord; SHH, sonic hedgehog; RA, retinoid acid. Created in BioRender. Sun, Y. (2025) https://BioRender.com/u26f604.

transplantation into the mouse brain. Moreover, transplanting cortical organoids with enriched glial cells into the retrosplenial cortex of immunodeficient mice facilitated astrogliogenesis.[68] Astrocytes within the transplanted organoids exhibited improved maturation and advanced morphological features, including the formation of extended long processes to the vasculature and perivascular astrocytic endfeet. The transplanted organoids also featured the specification of cortical layer-specific subclasses of astrocytes and heterogeneous responses to pro-inflammatory factors across different astrocyte subpopulations.

BIOENGINEERING ENHANCED NEURAL ORGANOIDS

Broader applications of neural organoids in disease modeling and drug discovery require further development and optimization of neural organoid cultures to enhance their relevance to human physiology and pathology, robustness, scalability and multiplexing capability, manufacturability, and compatibility of real-time monitoring and longitudinal data collection. We believe that bioengineering tools hold great potential to enhance these important features of neural organoid cultures, ultimately promoting their applications in drug discovery and screening practice. In this section, we discuss various bioengineering tools that can precisely modulate organoid culture microenvironments to make neural organoids more robust, predictive, and aligned with human physiology.

Enhancing structural fidelity using external morphogen gradients

The challenges in developing faithful neural organoids arise from the intrinsic complexity of brain structures. Along the anteroposterior (A-P) axis, the neural tube is patterned into three primary vesicles and subsequently five secondary vesicles, including the prosencephalon (forebrain), which soon divides into diencephalon and telencephalon; the mesencephalon (midbrain); and the rhombencephalon (hindbrain), which subdivides into the metencephalon (giving rise to the pons and cerebellum) and myelencephalon (developing into the medulla oblongata), followed by the spinal cord (Figure 2A).[69] An important concept from developmental biology is that the embryonic field is compartmentalized, and cells within distinct compartments, often defined morphologically, are lineage-restricted and do not intermingle with cells from adjacent compartments.[70] Animal studies suggest that the global A-P axis of the neural tube is defined by caudalizing signals involving WNT, FGFs, and RA[71] (Figure 2A). Following global A-P patterning, local secondary signaling centers or organizers (the anterior neural ridge, or ANR,[72] for the telencephalon; the zona limitans intrathalamica, or ZLI, for the diencephalon; and the isthmic organizer, or isthmic organizer (IsO), for the midbrain and hindbrain) emerge in the neural tube, and these signaling centers often develop at the boundaries of adjacent neural compartments (Figure 2A). An important role of these local secondary organizers is to emanate additional

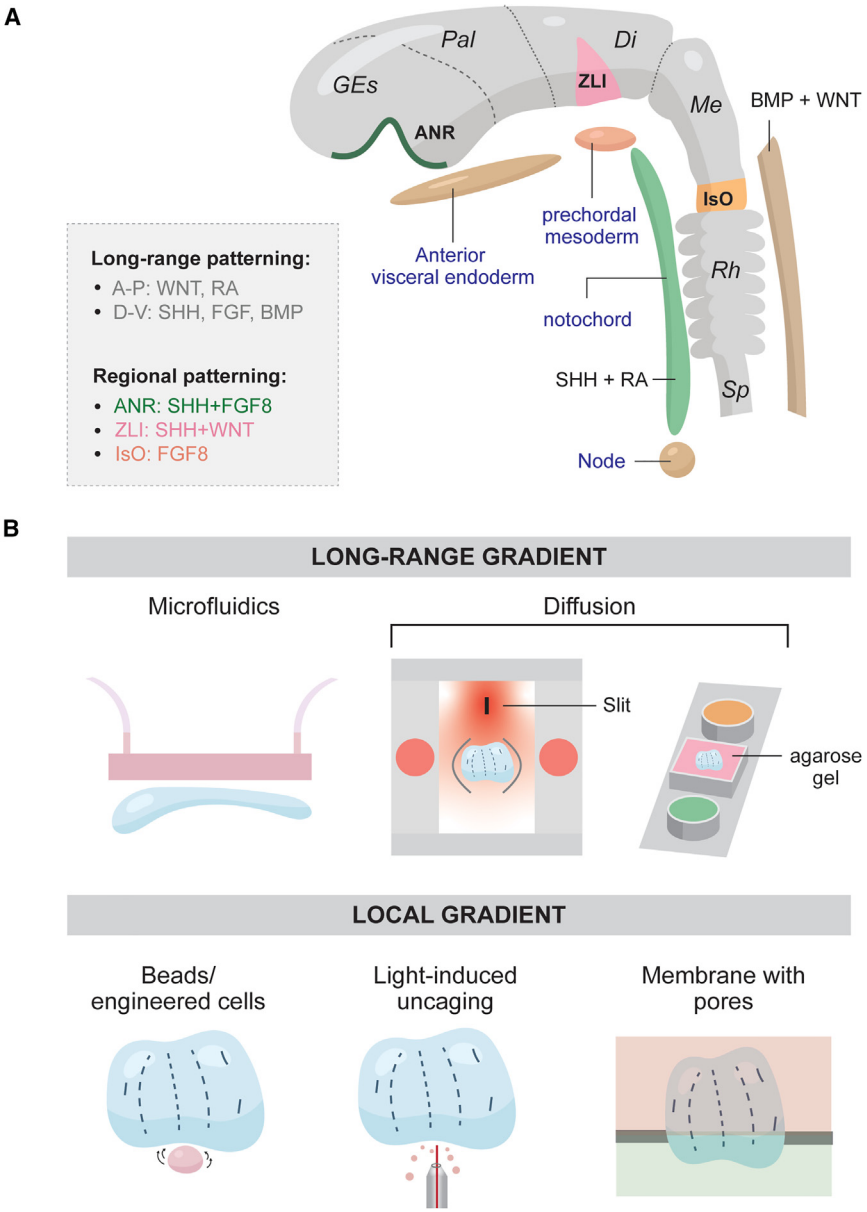

Figure 2. Morphogen gradients that pattern the neural tube *in vivo* and engineering approaches to create concentration gradients *in vitro*

(A) Schematics showing long-range and regional signals that pattern the neural tube. Abbreviations: GEs, ganglionic eminences; Pal, pallium; ANR, anterior neural ridge; ZLI, zona limitans intrathalamica; Di, diencephalon; Me, mesencephalon; IsO, isthmic organizer; Rh, rhombencephalon; Sp, spinal cord.

(B) Engineering strategies to generate long-range and local gradients to derive neural organoids.

endogenous signaling factors to refine and specify regional identities of each compartment of the neural tube.[73] These organizers secrete signaling molecules such as FGF8, SHH, and WNT.[74] Dorsoventral (D-V) patterning of the neural tube is more clearly defined through classic developmental biology studies (Figure 2A). In the telencephalon, D-V patterning is initiated by SHH secreted by the prechordal plate, which induces an SHH-expressing medial GE (MGE).[75] Similarly, SHH in the ANR, ZLI, and floor plate (induced by the notochord) and BMP in the roof plate drive D-V patterning along the neural tube.

As discussed above, patterning in the CNS is initiated through inductive interactions between neural ectoderm and non-neural ectoderm tissues, followed by regional organizers emerging in the neuroectoderm, both of which create complex morphogen gradients to establish a coordinate system to specify lineage identities in the neural tube. By contrast, during neural organoid derivation, exogenous chemical signals that are supposed to mimic the inductive patterning signals emanated from non-neural ectoderm tissues are provided uniformly in culture medium. Without graded/localized patterning signals provided in their cultures, current neural organoids lack well-defined A-P or D-V axes. Therefore, establishing precisely controlled exogenous morphogen gradients is crucial for achieving patterned brain regions and *in vivo*-like organizations in neural organoids. Generating concentration gradients of soluble factors *in vitro* has been achieved using various methods, including local delivery vehicles and microfluidic gradients based on either source-sink diffusion or active fluidic mixing. Readers are directed to recent excellent reviews on this topic for further details.[76–78] Here, we review their applications in neural organoids and discuss their design principles and limitations (Figure 2B).

Long-range microfluidic gradients

Strategies based on laminar flow mixing have been widely used for establishing stable gradients of chemical signals.[79] A linear concentration gradient of CHIR99021 generated by serial dilution in microfluidic devices was sufficient to induce A-P patterning of a thin strip of neuroectoderm tissue derived from hPSCs.[80] After 14 days of culture, the neuroectoderm tissue displayed patterned regions with identities of telencephalon, diencephalon, midbrain, and hindbrain against the WNT signal gradient. Importantly, changing the steepness of the WNT signal gradient shifted the relative spatial distribution of each region, indicating that neural patterning along the A-P axis depends on WNT concentration thresholds rather than its gradient steepness. Genes associated with WNT signaling inhibition, such as *HESX1* and *LHX5*, were shown to express in the forebrain region, supporting self-augmented, autonomous WNT inhibition through endogenous mechanisms for guiding the default anterior forebrain fate in the neuroectoderm tissue. Combining the WNT gradient with uniform SHH signal activation appeared to promote ventralization of the cells toward

ventral/floor plate fates. For example, NKX2.1^{+} MGE region appeared in areas exposed to low exogenous WNT signal, and floor plate marker FOXA2 expressed in the midbrain-like region. These data lend support to the neural patterning theory that A-P and D-V patterning signals might exert their functions independently.

There have been significant efforts in developing different microfluidic systems to achieve diffusion-driven, simultaneous or sequential, orthogonal gradients of soluble signals in 3D hydrogel-based cell culture systems.[81,82] In one such system, a microfluidic platform was designed to combine a 3D hydrogel-filled culture chamber with the ability to generate orthogonal linear chemical gradients within the hydrogel region.[83] Importantly, this microfluidic platform was utilized to demonstrate the combinatorial effect of RA and SHH, which are orthogonally distributed *in vivo*. More recently, another microfluidic device design was demonstrated to achieve diffusion-driven, simultaneous, orthogonal linear concentration gradients for axial patterning of neuroepithelial structures embedded in hydrogels along both A-P and D-V axes.[84] An important feature of this microfluidic device design was that it allowed the formation of hPSC colonies with defined shapes (such as spherical or cylindrical shapes) and differentiated them into lumenal neuroepithelial structures.[84] After superimposing two orthogonal but independent morphogen gradients for axial patterning, these neuroepithelial structures were shown to recapitulate several crucial aspects of neural patterning in the brain and spinal cord regions and along both A-P and D-V axes.[84] Using this microfluidic system, D-V patterned microfluidic forebrain-like structures with spatially segregated dorsal and ventral regions and layered apicobasal cellular organizations that mimicked the development of the human forebrain pallium and subpallium, respectively, were achieved.[84] Thus, this microfluidics-based neural organoid model provided not only 3D luminal tissue architecture at the early stage but also the *in vivo*-like spatiotemporal cell differentiation and organization after initial patterning. Instead of patterning single neural tube-like tissues, similar orthogonal gradients of WNT and SHH signals have been shown to pattern an ensemble of neural organoids in distinct locations within the gradients. These organoids, collectively, demonstrated extensive cell diversity resembling that of human fetal brain tissues.[85]

Diffusion-based gradient generators can be utilized for providing more localized morphogen gradients to pattern spherical organoids. For example, a passive diffusion-based device was used to pattern 2D neuroepithelial sheets dorsoventrally using an SHH agonist purmorphamine gradient.[86] This device was further applied to pattern forebrain organoids.[87] It was found that an exponential purmorphamine concentration gradient range of 0.5–1 μM generated by the passive diffusion device induced patterned GE organoids with dorsal and ventral GEs regions within the same organoid. These findings support the notion that a single SHH gradient is sufficient to pattern the forebrain region, with cell fate depending on a concentration threshold of SHH or its agonist.

Despite their useful applications in generating controllable and long-range chemical gradients, microfluidic systems have some notable limitations for neural organoid cultures. Continuous pumping is often necessary for prolonged microfluidic tissue cultures, which could increase the complexity of experimental setup and culture process. Long-term culture of neural organoids can be restricted by the spatial constraint of microfluidic topologies, and retrieving organoids from microfluidic devices is not necessarily convenient. Furthermore, serial dilution used in microfluidic systems typically leads to shallow linear gradients, which are not ideal for patterning spatially approximate brain regions, such as striatum and thalamus. Another important concern of using microfluidics for neural organoid research is their manufacturing complexity and limited user-friendliness and organoid culture throughput. Many microfluidic devices remain challenging in integrating multiple detection modalities and are complex to operate, requiring expertise in microfabrication and fluid and device handling. Thus, integrating microfluidic systems into the workflows of existing neural organoid studies will require careful design and adaptation, which can be time-consuming and require significant re-engineering of existing protocols and processes.

Engineered local delivery of morphogens

A major limitation using diffusion-based gradients for patterning neural organoids is its relatively low spatial resolution as well as a lack of dynamic control of morphogen signals. Several strategies have been developed to provide regionally restricted local morphogen gradients. Morphogen-soaked beads and morphogen-secreting cell clusters (either explants or genetically engineered cells) have been successfully employed as local sources of morphogens in a few neural organoid studies. Agarose-based Affi-Gel beads soaked with BMP4 and WNT agonist CHIR99021 have been used to locally activate BMP and WNT pathways, respectively, and induce dorsal/posterior fates in neural organoids in a concentration-dependent manner.[88] Alternatively, engineered cells secreting morphogens can be used as artificial signaling centers. SHH-expressing cells could effectively induce MGE fate in forebrain organoids using a doxycycline-inducible system.[24] Moreover, cell aggregates expressing FGF8 could induce rostral identity in cortical organoids, and using this approach, rostrocaudal patterning was achieved in elongated assembloids.[89] Compared with bead-based approaches, genetically engineered cells provide a more sustained supply of desirable morphogens and better temporal control, even though the morphogen concentration profiles are less predictable. For both methods, the amount and spatiotemporal distribution of diffusible factors cannot be precisely controlled, and supplementing multiple morphogens at different desirable locations for simultaneous patterning of neural organoids along orthogonal axes is challenging.

Another potential solution is to use light to locally and precisely control morphogen concentrations using so-called "caged" compounds. In general, caged compounds result from coupling biomolecules with a protecting group via a photo-cleavable bond. The protecting group is normally conjugated on a functionally critical part of the target molecule to inactivate the compound ("caging"). Light exposure activates the caged compound by removing the protecting group without damaging the compound. Toward spatiotemporal control of SHH activity *in vitro*, a photo-caged SHH pathway activator, nitroveratryloxycarbonyl (NVOC)-SAG, was developed.[90,91] NVOC-SAG was generated by coupling SHH agonist SAG with a photo-protecting group (NVOC). NVOC-SAG did not show bioactivity without light irradiation. Upon UV light irradiation, SAG was released from

NVOC-SAG to activate the SHH pathway in cells at the irradiated region. By applying the NVOC-SAG system under local light irradiations, ventralization of local, irradiated region in forebrain organoids was achieved.[90] With a similar strategy, a caged hedgehog antagonist NVOC-SANT (SANT-75 is a Smoothened inhibitor) was shown to locally inhibit hedgehog signaling based on confined UV light irradiation.[92] While light-dependent activation of caged molecules is effective for local control of cell differentiation in neural organoids, diffusion rates of uncaged compounds are often too large as compared with cell differentiation kinetics, which makes it difficult to maintain concentration gradients of activated molecules for a duration long enough to affect local cell differentiation. In future studies, hydrogels and other materials that can control the release rate and diffusion kinetics of soluble signals could be applied in conjunction with caged bioactive molecules to enhance the effectiveness of this approach.

Local and acute activation of cell differentiation programs in neural organoids could also be achieved using optogenetic methods.[93] Although such light-sensitive hPSCs need to be generated through genetic engineering tools prior to neural organoid culture, optogenetic differentiation has a notable advantage of providing sustained effects after light exposure. In addition to cell fate patterning, morphology of neural organoids can also be modulated by light through optogenetic modulation of intracellular signaling events. For example, photo-induced binding of split and engineered Shroom3 protein can regulate epithelial morphogenesis of neural organoids upon light exposure.[94] Whether optogenetic approaches could be utilized for controlling multiple and orthogonal signaling events simultaneously in organoid patterning remains to be demonstrated.

Another approach to achieve asymmetric chemical stimulations of neural organoids is to place them on an engineered membrane with a small pore at the membrane center. Like transwell membrane, this membrane separates two solution pools. Thus, when organoids are placed directly at the pore on the membrane, only a portion of the organoids is exposed to the medium condition on the bottom, whereas the rest of the organoids are submerged in a distinct medium condition that contains a different set of soluble factors.[95] Although this method can physically constrain exposure of morphogens on limited areas of neural organoids, it remains to be fully explored for this method to achieve patterned neural organoids containing different subdomains with *in vivo*-like tissue organizations.

Further considerations and challenges

Notably, morphogen gradients can be imposed not only by spatial concentration variation but also by temporal modulation. Changing durations of morphogen treatment can have significant effects on cell behaviors and functions, comparable to those through modulated spatial morphogen concentration gradients.[96] For example, a temporal gradient (gradually decreasing concentration over time) of TGF-β in culture medium within 6 days led to enhanced cortical fates of neural organoids.[97] These findings underscore the importance of considering both spatial and temporal domains in gradient construction for advanced neural organoid models. Importantly, multiplex screening platforms or gradient generation devices have been used to systematically analyzed the effects of timing, concentration, and morphogen combinations during the regional patterning step of neural organoid cultures on cell fate patterning and lineage diversification in the organoids, which provides a guideline for designing new organoid differentiation strategies.[98,99]

Additionally, the precision of patterning can be affected by post-translational modification,[100] the presence of morphogen-binding extracellular matrices (ECMs),[101,102] and autocrine/paracrine signals that depend on endogenous cellular states. Therefore, the final presentation of effective morphogen distribution depends not only on exogenous morphogen or chemical agonist/antagonist gradients provided but also on local microenvironment and intrinsic cellular properties that modify the concentration, functionality, and diffusivity of these molecules. Controlling these microenvironmental cues and endogenous cellular activities presents a challenge to achieve desirable spatial cell fate patterning.

While global A-P and D-V patterning can be achieved by establishing exogenous morphogen gradients, more refined, subregional patterning within each brain segment remains a challenge. Introducing biomimicry secondary organizers (ANR for the telencephalon, ZLI for the diencephalon, and IsO for midbrain-hindbrain) will be a promising next step for finetuned patterning of brain organoids with distinct regional identities. This effort might also be useful for applying cortical organoids to study arealization during human cortical development, an area of intense interest. We envision that the combination of long-range gradient generation tools (e.g., microfluidics) with local gradient generation methods (e.g., optogenetics and morphogen-soaked beads), alongside innovations in chemical delivery and controlled release strategies, will achieve more precise patterning in neural organoids.

Regulating organoid morphogenesis and fate patterning by tissue geometry

Morphogens and genetic programs are insufficient in fully defining cell fate patterning during development. Embryogenesis and organogenesis are brought about by changes in cell and tissue shape, which are regulated by mechanical forces and insoluble ECM. These biomechanical cues crosstalk with biochemical signals to instruct tissue self-organization.[103,104] Cells can directly respond to mechanical cues through various mechanotransduction pathways to regulate gene expression profiles and cell polarity.[105] On the other hand, gene expression changes also lead to modification of tissue mechanical properties and geometry.[106,107] Therefore, controlling biomechanical signals and tissue geometry is an important aspect of current efforts in achieving properly patterned neural organoids.

Tissue geometry can regulate the reaction-diffusion process of morphogens and induce differential mechanical status within tissue compartments, both of which contribute to cell fate patterning. In 2D, confining hPSCs in circular adhesive islands of certain sizes leads to spatial patterning of cell fates associated with neuroepithelial, neural crest, and epidermal lineages,[108–110] suggesting a connection between tissue geometry and neural plate patterning during neural induction. This tissue geometry-mediated neural patterning is likely regulated by reaction-diffusion of key morphogens (such as BMP4) and/or the differential mechanical forces induced by geometrical

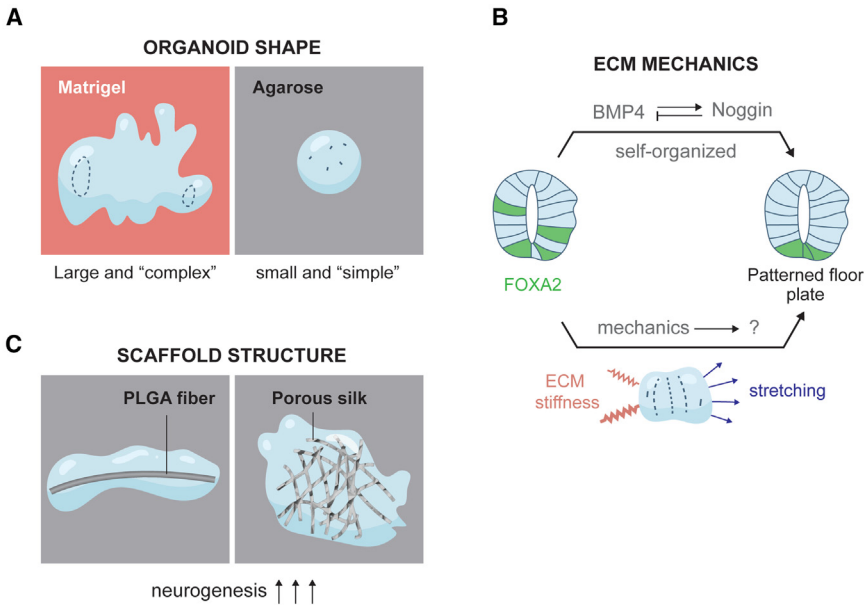

Figure 3. Biophysical cues that regulate neural organoid development

(A) Organoid shape mediated by physical constraint correlates with development.

(B) Single PLGA fiber or porous scaffold, such as silk, supports organoid growth with different morphology.

(C) The FOXA2+ floor plate patterning appears spontaneously through self-organization and can be facilitated by proper ECM mechanics and mechanical stretching.

confinements.[108,109,111] For 3D organoids, their size and surface topology were also found to be important indicators of proper maturation (Figure 3A). Cerebral organoids with more complex surface morphology, compared with those with smooth and spherical topologies, showed temporal cell fate progression that mimics brain development better.[112] Changing organoid morphology alone, either by dissociation and reaggregation or by embedding organoids in agarose gels, was sufficient to disrupt normal developmental trajectories.[112] It remains inconclusive, however, whether cell differentiation is directly regulated by morphological cues such as curvature and local mechanical forces, or indirectly through the effects of these biomechanical cues on tuning local morphogen distributions. Nevertheless, these observations are consistent with a common notion that certain organoid culture factors, such as initial cell number and cluster size, Matrigel embedding, and orbital shaker rotation speed, are not trivial and should be optimized as they significantly influence organoid morphology. Thus, using bioengineering tools to precisely regulate the size, curvature, and aspect ratio of neural organoids may enhance neurogenesis in the organoids and their reproducibility.

Controlling ECM in organoid culture

ECM is another important insoluble factor that is often not properly controlled in neural organoid cultures. ECM not only provides a physical scaffold to support cell adhesion and tissue organization but also regulates cell signaling through various surface receptors and cytoskeletal proteins. ECM in the brain is produced by meningeal fibroblasts forming pial basement membrane,[113] neural progenitor cells during early neural development,[114] and later, astrocytes.[115] ECM of the adult brain is composed mainly of proteoglycans (e.g., hyaluronan and chondroitin sulfate proteoglycans), glycoproteins (e.g., laminin, tenascin, and fibronectin), and collagens, which differ from laminin-rich Matrigel. Despite its common use in neural organoid culture, Matrigel, extracted from the murine Engelbreth-Holm-Swarm sarcoma, is known for its batch-to-batch variability and high cost.

Functionally, ECM can regulate cell proliferation, fate decision, and morphogenesis during neural development through their capabilities to bind receptors such as integrins and various morphogens.[114] For example, perlecan, a proteoglycan, binds SHH[116] and FGF2[117] and thus influences their signaling activities and diffusion, as well as downstream neurogenesis and cell fate patterning. Loss of perlecan led to a lengthened G1 phase of the cell cycle, likely due to a disruption of FGF2-mediated G1/S transition.[118] In the perlecan-deficient mouse embryos, SHH activity in the ventral forebrain was reduced drastically, leading to a reduction in size of the ventral telencephalon and a microcephalic phenotype.[119] In addition, laminins were found to promote neural progenitor proliferation through integrin binding, which in turn activates downstream signaling pathways including mitogen-activated protein kinase (MAPK) signaling.[120] ECM also influences cell shape, polarity, and migration, which collectively regulate neurogenesis and morphogenesis of the nervous system. For example, the polarity and migration of apical radial glial cells (aRGCs) are regulated by integrin-mediated signaling. Integrin β1 is required for attachment of both apical[121] and basal[122] processes of aRGCs. Mutation of laminin γ1, while not affecting cell shape and polarity, randomized the orientation of the cleavage plane and disrupted the interkinetic nuclear migration mediated by focal adhesion kinase (FAK) activities.[123]

All the abovementioned studies are from 2D cell cultures or mouse models, and the specific impact of each ECM component on human neural organoid development is still not fully characterized. Exogenous ECM molecules have been included in some neural organoid protocols at various culture stages and concentrations, while others do not include any exogenous ECM. Interestingly, Matrigel, but not its main components laminin-111 or collagen IV, enhanced neural rosette formation and led to less rounded morphology in neural organoids, with polarized ECM distribution at organoid outer surfaces.[124] Exposure to Matrigel also increased optic cup differentiation but did not significantly affect late-stage excitatory/inhibitory neuron differentiation.[124] This observation suggests the importance of undefined unique physical or biochemical properties of Matrigel in controlling neural organoid development. Omitting Matrigel in cerebral organoid cultures led to "simpler" organoid morphology and a significant increase in interneurons.[112] Mixing decellularized human brain ECM with Matrigel enhanced neurogenesis

and functional neuronal maturation, together with an increased radial glial cell population and basement membrane protein secretion in unguided cerebral organoids.[125] Notably, ECM protein expression profiles in current neural organoids do not closely resemble that of human fetal brain organoids (FeBOs) derived from fetal brain tissues.[126] Resuspending FeBOs into single cells and allowing the cells to reform 3D aggregates led to small neurospheres with significantly different ECM secretion profiles and poor cytoarchitecture, highlighting the functional link between ECM presentation, cell fate patterning, and tissue geometry. Altogether, these studies support that endogenous ECM production is often insufficient in promoting neural organoid development, and adding exogenous ECM may benefit organoid growth. Understanding how different ECM molecules regulate cell fate decisions in neural organoids requires systematic characterization.

In addition to ECM composition, mechanical properties of ECM can also influence neuronal differentiation, patterning, and maturation.[127–129] Matrigel exhibits mechanical properties similar to brain ECM gel, with a storage modulus of about 10^2 Pa and a loss modulus about 10 Pa.[125] Mechanical properties of Matrigel can be tuned by forming a Matrigel-Alginate interpenetrating network.[130] Under midbrain differentiation conditions, stiffer Matrigel-Alginate matrices led to decreases in organoid size and rosette number, although cell fate bias was marginally affected.[130] Notably, mechanical cues have been shown to directly regulate stem cell differentiation.[105] For example, mechanically stretched ectoderm lineage cells are more likely to differentiate into neural crest cells rather than neuroepithelium through activating mechanosensitive BMP signaling.[109] Even when cell fate is not overall biased, the distribution of different cell types in neural organoids could be influenced under mechanical stimulations (Figure 3B). For example, when stretched during their development, a greater proportion of human neural tube organoids embedded in polyethylene glycol (PEG)-based hydrogels showed the formation of a patterned floor plate region, likely through mechanisms involving planar cell polarity and reduced compressive forces due to tissue growth.[131] Self-organized floor plate patterning was still observable, albeit at a lower frequency, in neural tube organoids embedded in unstretched hydrogels, possibly through a BMP4-dependent competition and sorting mechanism.[20,132]

Various synthetic ECM scaffolds with tunable biomechanical and biochemical properties have been applied to neural organoid studies (Figure 3C). Poly(lactide-co-glycolide) copolymer (PLGA) microfilaments were used as a scaffold that generated EBs elongated along the length of individual microfilament.[133] Intriguingly, this change in organoid aspect ratio significantly reduced meso-endoderm differentiation and improved neurogenesis with a bias toward forebrain fate under an unguided differentiation protocol.[133] Long-term culture of the PLGA-containing neural organoids together with diluted Matrigel led to more robust cortical development, featuring radially aligned radial glia cells and properly formed cortical plate.[133] Under a midbrain differentiation condition, neural organoids cultured with planar-engineered fibronectin matrix supported by PLGA lattices showed enhanced choroid plexus differentiation.[134] In another study, 3D silk polymers functionalized with laminin-111 were utilized for neural organoid culture.[135] This synthetic scaffold provided porous structures to enhance oxygen delivery, leading to improved neuronal maturation in neural organoids.[135] A recent study further developed vascular-inspired, 3D-printed meshed tubular channel networks to reduce necrosis and hypoxia in midbrain organoids, thereby promoting neural maturation and activities.[136] Various scaffold properties, such as functionalization, porosity, and mechanical properties, have clear impacts on cell fate patterning, cytoarchitecture, and morphogenesis in neural organoid development.[137] However, detailed characterizations of the impact of individual scaffold properties on neural organoid development remain to be fully explored.

We still lack a definitive route for providing neural organoids under long-term cultures with the necessary scaffolding. The size of neural organoids increases drastically over time, which requires scaffolds that can dynamically "grow" with the organoids without physically constraining them or disrupting their structural integrity. Additionally, the spatial organization of ECM is crucial for promoting the development of key structural features of neural development, such as cortical layering and lumen formation and maintenance. To introduce exogenous ECM properly in neural organoid cultures, a detailed developmental mapping of the "matrisome" in developing brain tissues will be desirable. We envision that advanced biofabrication tools, such as microfluidics and bioprinting, can facilitate the control of neural organoid geometry, and synthetic ECM with desirable mechanical properties, forms, and tunable bioactive molecules will enable spatiotemporal controls of extracellular signals to further guide neural organoid development.

Modeling inter-regional connections and neural circuits with organoids

Interaction between adjacent regions of the brain can be modeled with innovative methods described in the above sections. However, it is still challenging to model long-range interaction between regions that are separated apart within the brain, although it is critical for different regions to be connected with axons traveling through the white matter for the brain to function properly.

Neural assembloids have been developed to study interactions between specific brain regions, as well as between various brain regions and non-neural tissues that connect with the brain, such as muscle, vasculature, and glioblastoma.[42,63,138] Assembloids have been demonstrated as useful systems for studying dorsal migration of cortical inhibitory interneurons and their integration with cortical layers[7] as well as directional axonal projection between different human brain subregions.[8] Some emerging applications of assembloids include studying axon guidance using midline assembloids composed of floor plate organoids and spinal cord organoids[139] and constructing a putative corticostriatal-thalamic-cortical loop circuit by assembling four region-specific organoids representing these different circuity components.[140] From an engineering perspective, while early assembloid systems relied on manual fusion of different organoids, bioengineering tools such as 3D-printed molds[140] and magnetized 3D bioprinter[141] have been employed to better control the spatial arrangement and organization of neural organoids, including linear or loop configurations.

Although assembloids can successfully model interactions between different brain regions, axonal tracts that connect

distant brain regions are underrepresented in current assembloids. Bioengineering strategies have been utilized to facilitate modeling of long-range projection of axon bundles in the brain.[142–146] For instance, cerebral tracts that connect cortex regions, including the corpus callosum, can be modeled by connecting two cerebral organoids placed in separate microfluidic chambers connected by a microfluidic channel, with axon bundles grown and assembled in the channel.[142,143] The axons can be isolated from the tissues for downstream biochemical analyses,[146–150] and axon degeneration can be examined using fluorescence microscopy conveniently.[146,151] The geometrical arrangement of the connected organoids, or "connectoids," ensures physical separations of the organoids while they are still connected with sufficiently long axon bundles.[152] Importantly, the microfluidic device holding the connectoids can be designed and aligned to locate neural organoids on MEAs for electrophysiological assessment of functional network properties,[142] with axonal conductance velocity directly monitored with MEAs.[153] In recent studies, networks of multiple neural tissues have been reported using the connectoid approach.[154] Constructing and modeling macroscopic or mesoscopic networks of neural organoids could provide novel topological organizations of neural networks, which could provide an innovative bioengineering way for building functional circuits.

Innovations in microfluidics, biofabrication including 3D bioprinting,[155] and robotic approaches will further increase the precision, complexity, and scalability of engineering approaches and enhance their applications for accurate modeling of intra- and inter-brain region interactions.

BIOENGINEERING ENHANCED ORGANOID ANALYSIS/ FUNCTION

Neural-bioelectronic interfaces

Electrophysiological activities of single neurons and neuron-glia networks are essential functional features of the brain. Firing patterns of neurons are distinct for different neuron subtypes and can provide rich information of their network activities.[156] Correlating large-scale neural recording with behavior testing is essential for understanding the fundamental mechanisms of brain functions.[157] Additionally, electrophysiological studies of neural networks in the brain have been commonly used to assess neurological diseases and evaluate drug effects.[158] Notably, some levels of similarity between the electrophysiological network activity patterns of neural organoids and the human preterm neonatal electroencephalogram have been reported,[37] supporting the potential application of neural organoids for studying the electrophysiological features of brain development and disease.

Tools used for *in vivo* electrophysiological characterizations have been adapted for neural organoids. Currently, given its convenience and robustness, calcium imaging based on permeable (e.g., X-Rhod-1) or genetically encoded (e.g., GCaMPs) calcium indicator has been widely used as a proxy to assess network activities in neural organoids. High-resolution imaging of neural organoids could reveal cellular-level activities, whereas low-magnification imaging would reveal ensemble network activities in neural organoids. High magnification, wide-field microscopes would further allow cellular-level network analysis across entire neural organoids. Nonetheless, imaging large organoids remains challenging with conventional confocal microscopy due to deep-tissue diffraction, and the temporal resolution of calcium imaging is insufficient for detailed characterization of neuronal electrophysiology. Given the phototoxicity of fluorescent indicators, it is challenging to use them to perform long-term and continuous recording to track the dynamics of network activity. Other advanced microscopy tools, such as ElectroChromic Optical Recording, quantum diamond microscopy, and interferometric microscopy, have also been developed for label-free optical electrophysiology.[159] These approaches, however, are primarily developed for single-neuron applications, necessitating future work to expand their applications for whole neural organoid studies.

Compared with optical methods, direct electrical recording provides a label-free method for tracking electrophysiological activities with high spatiotemporal resolutions. However, conventional electrical recording techniques have significant limitations when applied to neural organoids. For example, patch-clamp, while providing detailed single-neuron characterization,[8,16,54,142] is not scalable for studying network activities and has limited neuron accessibility. Shank probes, such as Neuropixels, provide deep-tissue access in neural organoids.[160] Some probes have been integrated with microLED arrays for optogenetic stimulations[161] or made with soft hydrogels for better tissue integration.[162] However, these probes are primarily designed for *in vivo* testing and are too bulky and thus could affect structural integrity of neural organoids. Furthermore, shank probes still only access a small region of neural organoids confined to the insertion plane (Figure 4). By contrast, planar MEAs offer scalability, which has enabled recording of neural network activities on the basal side of neural organoids recently.[37,38,40,163] Conventional MEAs are typically equipped with 10–100 planar electrodes per well. Simultaneous recording from MEA electrodes could reveal network dynamics, including synchronicity, action potential propagation, and oscillation, in neural organoids.[142] Each MEA electrode can record action potentials of nearby neurons as spikes and an ensemble of network activity as slower waves called local field potential (LFP). The voltage changes sensed by MEA electrodes are digitized and sampled by electronics and computers at a typical rate of 5–20 kHz depending on data bit depth and number of electrodes to accommodate the large data stream. Complex signal mixture on each electrode can be analyzed to separate spikes and LFPs by Fourier transform and frequency band separation. High-frequency signals (>300 Hz) contain mostly spikes of action potentials, which can be further separated into signals from multiple neurons (single unit) by trace shapes using spike sorting algorithms. Low-frequency signals contain LFPs that could be further separated into different frequency bands.[142] High-density (HD) MEAs based on Complementary Metal-Oxide-Semiconductor (CMOS) sensor array are increasingly used recently.[163] The reduced electrode size and significantly increased density of electrodes allow the detection of more action potential spikes of neurons than LFPs, enabling the study of network structures and activity dynamics within neural organoids. Network activity patterns of spike timings and firing sequences can be characterized with various indexes, including burstiness, synchrony, criticality,[164]

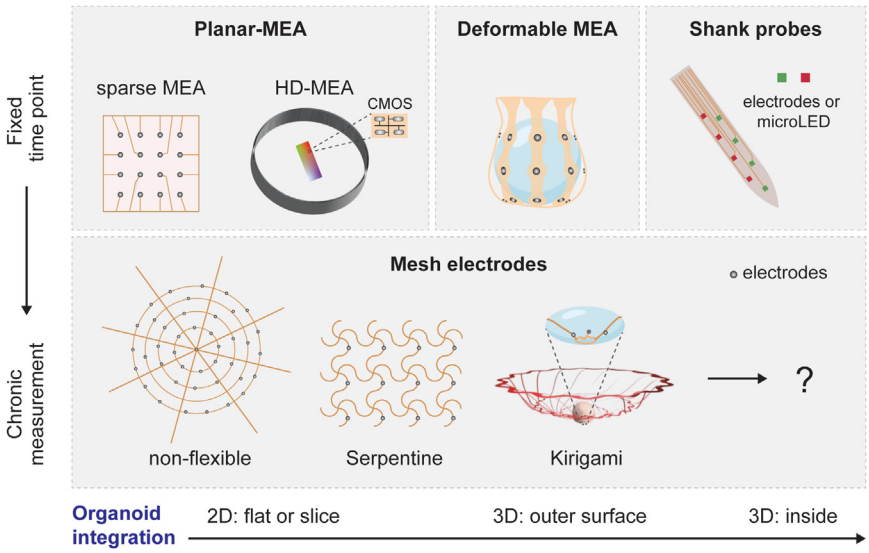

Figure 4. Current bioelectronics for recording and stimulation in neural organoids

Some systems, such as MEA and shank probes, are more suitable for short-term measurement, while mesh electrodes are suitable for long-term observations. The form of integration of organoids with bioelectronics also depends on the design of the electrodes.

neuronal avalanche size distribution, functional connectivity, and network topology using graph theoretical metrics.[165] Notably, due to a lack of data from fetal brain tissues, it remains completely unknown how recordings from neural organoids correlate to fetal brain activities.

A major limitation of planar MEAs is their incompatibility with 3D morphology of neural organoids. Establishing intimate contacts between organoids and MEAs requires protocol optimization. Nonetheless, only a small portion of surface area of neural organoids can directly interface with planar MEA electrodes. Long-term culture of neural organoids on MEAs is also challenging, often leading to a loss of organoid structural properties. To address these challenges faced by MEAs, there is a growing interest in developing advanced neural-bioelectronic interfaces for high-resolution, chronic measurements of neural organoids in 3D (Figure 4). One strategy involves integrating MEAs on compliant substrates to cover lateral surface areas of neural organoids.[166,167] However, this strategy could lead to the substrates constraining neural organoid growth, prohibiting long-term recording. Recently, mesh electrodes with brain tissue-level softness and cell-level ribbon size have emerged as a promising solution for 3D chronic recording.[168] Generally, these mesh systems utilize microfabricated metal electrodes connected by metal interconnects covered by rigid polymer insulators (polyimide or SU-8). The mesh electrodes have been designed with different geometries to integrate with neural organoids. In one design, a non-deformable, spiderweb-like suspending mesh supports the attachment of large neural organoids, with electrode positions remaining stable as organoids grow.[169] However, prolonged culture of neural organoids on the mesh electrodes led to a disk-like morphology of the organoids. Furthermore, loose integration between the mesh support and neural organoids prevented the use of orbital shaking, which is necessary for long-term neural organoid culture. Such mesh can also be designed to be deformable to withstand large strains resulting from neural organoid growth, including a serpentine mesh network free floating in culture medium.[170] While this serpentine mesh can be expanded significantly, its ultraflexible planar design makes it difficult to integrate with large spherical organoids. As a result, hPSCs were seeded directly on the mesh at the very beginning and formed "flat" neural organoids. More recently, to prevent organoid flattening, deformable, basket-like mesh electronics inspired by kirigami were developed, with carefully designed latches connecting concentric ring structures in the mesh to accommodate large deformation resulting from neural organoid growth.[171] Cortical organoids or cortical-striatal assembloids were cultured on the kirigami electronics, and they maintained a circular morphology for at least 44 days.

To fully capture spatially defined neural activities within neural organoids, in addition to the ongoing effort in creating 3D bioelectronics that can probe the entire organoid surface and inside the organoids, it is equally important to combine these tools with advanced neural organoids that exhibit compartmentalized brain subregions with *in vivo*-like organization and inter-regional interactions. Given the relatively small number of electrodes available so far, the complex structure of neural organoids with multiple ventricle-like neural rosettes makes it difficult to resolve network activities. Using neural organoids containing single neural rosettes may improve the spatial resolution of the recordings.[64,172,173]

For future advanced neural-bioelectronic interfaces, attention should be paid to material choices, electrode density, mesh geometry, and protocols for their integration with neural organoids. These factors directly affect measurement stability, signal-to-noise ratio (SNR), and spatial coverage of recording. To date, it is still difficult to measure network activities inside neural organoids, preventing studies of inter-regional connectivity within neural organoids. The number of electrodes in all current mesh systems is relatively low (<100), limiting the spatial resolution of recording to single-unit activities due to the small number of available electrodes for spike sorting. The SNR is often low, with noise levels at tens of μV vs. 30–100 μV signals. This low SNR often resulted from the high impedance of planar metal electrodes (10^5 Ω) even with surface modification. To address this issue, one solution was proposed to use novel materials such as graphene nanotransistor[174] or liquid metals[175] as electrodes. Additionally, there is limited capability for current neural-bioelectronic interfaces to perform local stimulations and simultaneously record responses in a closed-loop manner. Local stimulation of neural organoids can be achieved by electrical stimulation or optogenetic stimulation. Electrical stimulation can be triggered to any electrodes that interface neural

organoids. Stimulation amplitude and frequency can be modified to achieve efficient stimulation of neural organoids. Optogenetic stimulation or inhibition can be achieved when neural organoids are generated from genetically modified hPSCs harboring proteins containing a light-sensitive domain coupled to biological function. Optogenetic inhibition can be utilized to demonstrate functional connectivity in regions of interest by locally inhibiting the activity transmission using light. Optogenetic activation of neural organoids is suitable for precise control without large stimulation artifacts. Stimulation can trigger short- or long-term changes of activity patterns in neural organoids after repeated stimulations, which is important for understanding their network properties.

Organoid-based information processing

The organoid-electronic interfaces lay the hardware foundation for future organoid-machine interfaces. It has been proposed that the intrinsic neuroplasticity of neural organoids can be utilized for information processing,[176] which is an advanced version of neuron-based computing frameworks based on "biological neural networks" (BNNs).[177] Artificial intelligence (AI) is revolutionizing fields such as industry, medicine, and education. This advancement has been primarily driven by the rise of artificial neural networks (ANNs) and machine learning, which utilize extensive real-world datasets processed through silicon-based computing hardware. Nonetheless, current AI hardware faces significant limitations, including excessive heat production, prolonged training time, and high energy consumption.[178] These constraints impede the scalability, speed, and efficiency of AI models. As silicon computing hardware approaches its theoretical limits, diverging from "Moore's law," and contends with the "von Neumann bottleneck"—the physical separation between data storage and processing units—a transformative shift in hardware technology is critical for the continuous progress of AI development.

Advancing AI hardware could be inspired by the intricate structure and functionality of the human brain, which contains complex neural networks with approximately 200 billion neurons interconnected by about 125 trillion synapses.[179,180] The human brain's remarkable computing efficiency makes it an ideal model for AI hardware. While a typical human brain operates on around 20 W, current AI hardware requires about 8 million watts to power a comparable ANN.[178] Additionally, the human brain achieves high-precision computing with minimal energy consumption and seamlessly integrates data storage and processing, naturally circumventing issues associated with the von Neumann bottleneck. Consequently, there have been pioneering efforts in developing high-efficiency, cost-effective neuromorphic chips, such as memristors.[181] These neuromorphic chips have already found applications in various domains, but there is still a significant need to enhance the performance of current silicon-based neuromorphic computing chips and systems.

It is foreseeable that in the near future, there will be rapid advances in BNN in terms of complexity, connectivity, and neuroplasticity. Such BNN may open a new avenue for AI computing with unique features, including low energy consumption and fast adaptation. Recently, neural organoids were used as a novel biological neuromorphic system for reservoir computing, termed "Brainoware."[182] Reservoir computing is a framework for training recurrent neural networks, where the recurrent layer, or reservoir, remains fixed (or adaptive in the Brainoware) and only the readout weights are trained. After feeding the reservoir as a "black box" with input information, a simple readout layer (e.g., linear regression) is trained to read and map the state of the reservoir to the desired output for predictions or classifications. In this context of Brainoware, the complexity and adaptivity of the organoid neural networks as an adaptive reservoir enhance task training. Specifically, the Brainoware system used HD MEAs to interface with neural organoids for local stimulations and simultaneous recording. Real-world tasks such as speech recognition and nonlinear equation prediction were demonstrated using Brainoware. Interestingly, these studies revealed that the functional connectivity of neural organoids could adapt in an unsupervised manner, resulting in performance improvements over time for tasks previously solved by non-adaptive reservoir computing algorithms implemented *in silico*.

Although the number of neurons and synapses in neural organoids is only a tiny portion of the brain, the use of neural networks in neural organoids for reservoir computing may lead to significant breakthroughs in AI tasks requiring adaptability and precision. By further replicating the brain's efficiency and adaptability, neural organoids can inspire new architectures and algorithms for innovating neuromorphic computing systems and enhancing AI models. Another promising application of neural organoids is to facilitate the development of the brain-machine interface (BMI). Neural organoids may share the same "grammar" of human brains for neural communication and modulation, highlighting the potential of using them to develop advanced interfaces and communications. This can facilitate better integration between machines and the human brain and enhance the design and functionality of neuroprosthetics. However, challenges remain in developing standardized neural organoids for widespread application and maintaining their activities for long-term and stable usage. There are also potential ethical concerns of sentience and intelligence in neural organoid studies, discussed in detail in the following section. Despite these hurdles, the information processing ability of neural organoids holds immense potential to transform various fields with innovative solutions and applications, driving significant advancements in technology and medicine.

ETHICAL CONSIDERATIONS IN NEURAL ORGANOID ENGINEERING

As neural organoid research progresses, it is important to keep track of important bioethical considerations along the way. Most of these bioethical issues will be familiar to those working in biomedical fields that utilize human tissues: cell and tissue procurement and donor consent, research oversight, translational delivery, and animal research.[183] One key factor to monitor is whether researchers and institutional stem cell review committees can substantiate that the development of newer, more complex neural organoid systems remains consistent with the known wishes of original cell line donors.

A good example of this is likely to arise if researchers pursue the prospect of neural organoid-based information processing systems. Currently, human neural organoids are generated after informed consent by cell line donors, through broad consent for

general biomedical research uses of donors' cells, or through the use of anonymized cells without informed consent (which itself is ethically controversial despite being permissible from a regulatory standpoint). Importantly, it should not be assumed that either of these current routes for the procurement of human biomaterials to generate neural organoids is appropriate for commercial or applicational organoid-based information processing. This is because the use of neural organoids for information processing applications was previously unimagined by donors, policymakers, and regulators. Human biomaterials donated initially for biomedical research purposes do not necessarily meet informed consent requirements for other applications that lie outside of biomedicine, such as AI computing. This disconnection in donor intent raises the possibility that a separate consent process calibrated specifically for the generation and use of human neural organoids for organoid-based information processing may be required.

Alongside these important considerations, the most contentious issue to date is the concern raised by some commentators that human neural organoids might one day exhibit signs of consciousness *in vitro*.[184] There appears, however, to be very little reason to believe this is a realistic possibility for the foreseeable future. Without the aid of new technologies and much further advances in the types of bioengineering approaches outlined previously, human neural organoids will continue to lack the size, architecture, integrated organization, inputs and outputs, and full complement of relevant cell types to support the minimal physical conditions necessary to support human consciousness. Not only are human neural organoids nowhere close to espousing consciousness, but they are also nowhere close to mimicking the developmental stages at which basic sentience—i.e., the ability to feel pain and pleasure sensations—arises in fetuses, which is believed to occur after 24 gestational weeks. In comparison, almost all neural organoid protocols give rise to cells with a maturation status similar to cells observed in the second-trimester cortex when cultured up to 450 days.[185] Even with extended culture of neural organoids beyond postnatal stages,[186] due to the lack of sensory inputs in current organoid models, they are unlikely to match human postnatal developmental stages in terms of biological properties.

Much of the concern about emergent consciousness in human neural organoids may be grounded in a simplistic assumption that the innate self-organizing capabilities of neural organoids are sufficient to enable this possibility alone. But given all the bioengineering innovations yet to be realized to make neural organoids more *in vivo*-like, complex, and functionally mature, as have been extensively explained above, neither consciousness nor sentience is something that could appear on its own without significant technological innovations and efforts aimed precisely at realizing either outcome.

It should be emphasized that advances in human neural organoid research will require close collaborations between neuroscientists and bioengineers. Such interdisciplinary partnerships will create opportunities for new bioethical approaches in the neural organoid research space. For instance, due to the significant engineering assistance necessary for progress in the field, it would be advantageous for research teams to incorporate the ethics of design engineering into the mix of bioethical considerations for their work.[187] The ethics of design bioengineering begin with the recognition that engineering itself is a value-laden, goal-oriented activity and that, as such, a range of possible values, including ethical values, can inform the choices engineers make in the design of their constructs, in this case, human neural organoid systems. Since design trade-offs will not be value-neutral decisions, interdisciplinary research teams will have the chance to clarify which values (including social and ethical values) should guide the final design and purposes of their organoid systems. By employing a transparent decision-making process motivated by design engineering ethics, a team might conclude that it would be prudent to design neural organoids to answer exactly the research question at hand, without adding any unnecessary biological complexity that might raise public concerns or confusion about the models' capabilities beyond their scientifically relevant features. As the neural organoid field matures, so too might the ethics of design engineering develop in tandem during the research development process for interdisciplinary teams.

CONCLUSIONS AND PERSPECTIVES

Ever since the first emergence of neural organoids for studying brain development and disease, it has been proposed that advanced neural organoids featuring "spatiotemporally controlled patterning" would be able to model complex brain functions.[188] Although we are still not there yet, novel bioengineering tools have significantly improved the patterning accuracy and physiological relevance of neural organoids. The ability to better control morphogen gradients, ECM, and tissue geometry, and the development of novel bioelectronics will be the key aspects for establishing and studying advanced neural organoids. Here we highlight a few future research directions.

Expand applications of neural organoids

Neural organoids have already demonstrated impactful applications beyond modeling development and diseases.[41] Neural organoids have led to findings in revealing evolutionary features of human brains, molecular and cellular processes underlying various neurodevelopmental and neuropsychiatric disorders, and infectious diseases such as Zika and SARS-CoV-2. Compared with 2D culture models, neural organoids are particularly useful in pinpointing the cell types that are mostly influenced by pathological cues given the cell diversity. A prominent example is the discovery that Zika virus infection causes microcephalic phenotype via radial glia progenitor depletion,[3,189,190] which further led to targeted drug development.[191,192]

One promising future direction is to expand the applications of neural organoids for disease modeling[193] and screening systems for environmental toxins and substance use.[194–196] Such important translational applications of neural organoids will require the research community to work together to standardize protocols, reduce batch-to-batch variability, improve assay throughput and reduce cost, and develop real-time analysis modules.[197] The framework guiding cellular manufacturing needs to be expanded to define proper critical quality attributes (CQAs) and critical process parameters (CPPs) for organoid biomanufacturing. Additionally, the availability of high-quality electrophysiological activity dataset of neurons and neural networks during neural organoid development will lead

to new insights into organoid-based information processing strategies.

Improve maturation and complexity of neural organoids

Lack of maturation is one of the most widely discussed issues in neural organoids. Neurons generated in neural organoids are mostly prenatal,[185] and *in vivo*-like network activities have rarely been observed in them. While many cortical organoid studies have focused on evaluating excitatory signals, the formation of the most basic functional unit, such as cortical columns, has not been demonstrated in these organoid models. The formation of meaningful feedback loops for information processing in the brain requires inputs from various inhibitory neurons. It remains a great challenge to obtain sufficient numbers of functional inhibitory neurons and synapses in neural organoids.[198] In addition, microglia-mediated synaptic pruning promotes neural maturation in the brain, while current microglia-neural organoid co-culture models remain rudimentary.[199,200]

Developing creative protocols to accelerate neuronal differentiation/maturation in neural organoids remains a critical effort. The most promising results so far are from neural organoids transplanted into rodent brains, suggesting that a naive brain-like microenvironment, including vascularization and various microenvironment factors, may be beneficial for neural maturation in neural organoids. High-throughput tools and synthetic microenvironments that mimic brain microenvironments may help identify key factors and facilitate neural organoid maturation in a xenofree condition. Developing novel strategies to deliver nutrients and growth factors into the core of neural organoids, such as slicing organoids,[160] may promote neural organoid maturation. Another promising direction is to develop effective cryopreservation protocols and bank neural organoids at various stages for later uses.[201]

Another important limitation in current neural organoids is the lack of non-ectoderm lineage cells. Developing co-development models that include the CNS and other tissues such as vasculature, muscles, gut, and immune cells will greatly expand the applications of neural organoids in disease modeling and drug testing. Complementary to co-culture and assembloid approaches, such integrated neural organoid models have been explored using microfluidics-based organoids-on-a-chip systems.[202]

Alternative strategies to neural organoids

Depending on the specific questions researchers are interested in studying, neural organoids may not always be the most optimal experimental model to consider. Recent advances in stem cell-based embryo models[203–205] have revealed the exciting possibility of understanding brain development in the presence of other germ layer lineages. However, current embryo models are still limited to early-stage development. Alternatively, biofabrication approaches may be used to produce 3D neural tissues with cells derived in 2D. For example, bioprinted hPSC-derived cortical neural progenitor cells, differentiated neurons, and astrocytes as horizontal bands were fabricated using hyaluronic acid/fibrinogen-based bioink.[206] This approach reduces maturation time, increases control over cell density and cell types, and allows for constructing microcircuitry as desired, at the cost of losing cell diversity and polarity that often emerge spontaneously during neural organoid derivation.

As Emily Dickinson famously wrote, "If your nerve deny you, go above your nerve"[207]—we need to courageously explore and never settle. Neural organoid research still faces many challenges, but with close collaborations between bioengineers and neuroscientists, bioengineered neural organoids are poised to become a game-changer in the neural organoid field, important for uncovering the mysteries of the human brain and disease.

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DECLARATION OF INTERESTS

I.H., G.-l.M., and J.F. are advisory board members of *Cell Stem Cell*. The University of Michigan, Ann Arbor has filed a patent application describing microfluidic devices and methods for the development of neural tube-like tissues and neural spheroids (PCT/US2021/058090), with J.F. as a co-inventor. The University of Massachusetts, Amherst has filed a patent application describing passive diffusion device for patterning neural organoids (US patent no. 18/625,271), with Y.S. as a co-inventor.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT4o in order to check grammar and improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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