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Bioengineering tools for next-generation neural organoids

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Human stem cell–derived neural organoids were recently introduced as powerful *in vitro* 3D experimental model systems that innately undergo critical steps of organogenesis in culture and exhibit molecular, cellular, and structural features similar to the fetal human nervous system. These organoids have yielded new insights into human neurodevelopment and associated disorders. However, neural organoids have some crucial limitations that arise from the loosely controlled conditions for their development, an inability to maintain their spatial orientation in culture and a lack of technologies for taking long-term measurements on their morphology and electrical activity. Here, we review recent progress in using bioengineering methods to improve neural organoid formation and analysis by leveraging microfabrication, biomaterials, 3D printing, and flexible electrodes. We discuss how the applications of each technique can help to address critical limitations with standard neural organoid models. We conclude with a perspective on future applications of bioengineered next-generation neural organoids.

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Introduction

The uniqueness and complexity of the human nervous system poses many challenges for elucidating the intricacies of human nervous system development, uncovering the biological mechanisms underpinning neurological disorders, and formulating approaches to regenerate neural tissues after injury or degenerative diseases [1]. While animal models have proven to be indispensable for progress toward these aims, there are many questions surrounding human-specific features of neurodevelopment and disorders for which animal models are poor surrogates [2]. For example, outer radial glia cells play critical roles in the development and expansion of the human cortex [1] but are sparsely present in the mouse brain [3], and mutations resulting in microcephaly in humans often fail to produce similarly drastic effects in genetically modified mice [4,5]. To address these shortcomings, human stem cell–derived neural organoids have emerged as powerful model systems that offer researchers a window into early human nervous system development *in vitro* [2,6–8]. Neural organoids start from three-dimensional (3D) aggregates of human pluripotent stem cells that are cultured under highly specific conditions [9]—which leverage the extensive knowledge gained from studies of animal development [10] and two-dimensional (2D) human stem cell cultures [11]—that allow them to progressively advance through different developmental stages, from neuroepithelial stem cells to radial glia progenitors and finally neurons and glia, over the course of weeks or months in culture. Under these settings, organoid models make use of the inherent self-organizing capacities of pluripotent stem cells, that when combined with rational tuning of different cell signaling networks via the addition of morphogens, small molecule agonists or antagonists, can strikingly recapitulate many aspects of human neurodevelopmental [2,6–8]. Prominent among these features are the abilities to

generate spatially distinct cortical layers [12], produce different regions and subregions of the brain and spinal cord containing neurons and glia similar to those observed *in vivo* [13–16] and model certain regional interactions such as interneuron migration [17] and cross-region synaptic connectivity [15,18].

The high level of fidelity among the structure, cellular composition, and function of neural organoids and the prenatal nervous system has allowed them to be used for not only studying basic processes and mechanisms of human nervous system development, but also for modeling human disorders. Indeed, neural organoids have been used to model neurodevelopmental disorders, such as microcephaly [4,5], autism spectrum disorders [19,20], and Timothy syndrome [17,21], as well as proving useful in uncovering some early developmental features of neurodegenerative disorders, such as Huntington's disease [22], amyotrophic lateral sclerosis [23], and Alzheimer's disease [24]. Moreover, neural organoids have been applied for modeling infectious diseases that the brain is vulnerable to, such as those from Zika virus [13] and SARS-CoV2 [25,26]. Together, these foundational studies provide a paradigm for using neural organoids to study neural pathophysiology, and likely mark just the beginning of what is possible with neural organoids since this nascent field began little more than a decade ago with the creation of the first cerebral organoids [4,27,28]. Yet, notwithstanding this rapid progress, significant challenges remain for improving and analyzing organoid models to study neuropathology and its underlying mechanisms [29]. Clearing these hurdles will not only involve the refining of existing protocols but also the development of novel methods and approaches. Here, the field of bioengineering offers particularly powerful tools with vast potential to meet these needs [30].

In this review, we cover some recent advances which showcase the power of combining bioengineering methodologies with neural organoids and highlight how this synthesis can facilitate the creation of more robust organoid models, measurement techniques, and applications. We begin with a discussion of major shortcomings of recent neural organoids and their analyses. We then focus on four main bioengineering topics: microfabrication (microcontact printing and microfluidic devices), biomaterials, 3D printing, and flexible electronics. Using some recent studies as examples, we discuss how these cutting-edge bioengineering strategies are beginning to address these important limitations in existing neural organoid protocols, as well as how they can unlock new capabilities and opportunities that can be harnessed by researchers to build the next generation of neural organoids.

Current limitations for neural organoid generation and analysis

Three broad classes of neural organoid models can be defined: unguided, region-specific, and assembloids [6,7,9,31]. Region-specific neural organoid protocols use small molecule agonists and antagonists of developmental pathways (e.g. WNT, bone morphogenetic protein, and sonic hedgehog) to direct stem cell fate toward a precise region of the nervous system [13,14,27,28], while unguided cerebral organoid protocols rely on intrinsic neural differentiation and produce a stochastic assortment of cell types from multiple different brain regions within each organoid [4,8]. Assembloids are fusions of two or more region-specific organoids, intended to model interregional neural interaction [15,17,18]. While all these approaches have different strengths, their methodological commonalities result in several shared constraints that reduce their physiological properties (Table 1). These include a lack of control over initial conditions for embryoid body (EB) and neural rosette formation, homogeneous culturing conditions that do not permit environmental perturbations, such as the establishment of morphogen gradients, difficulties in regulating spatial configurations of organoids in culture for making assembloids and integrating nonneuronal cell types, and finally, limited tools for taking long-term measurements of organoid morphology and electrical activity (Figure 1a). Below we describe how some recent studies have used bioengineering methods to circumvent these limitations.

Microfabrication: microcontact printing and microfluidic devices

Current neural organoid protocols offer little control over EB formation beyond the initial number of pluripotent stem cells seeded. Instead, intrinsic mechanisms drive cellular aggregation [4,8], yielding EBs with properties that are difficult to modify (Figure 1a). These limitations account for several of the nonphysiological features of neural organoids, such as their uniformly spherical shape that differs from the elongated cylindrical shape of the neural tube and their lack of organization around a single central lumen or neural rosette. Furthermore, these environments for organoid generation do not allow morphogen gradients to be established, which are crucial for forming multiregional identity in the neural tube [10]. To exert control over these early processes in EB and organoid formation and patterning, microfabrication can be used to perform microcontact printing and build microfluidic devices (Figure 1b).

Microcontact printing uses microfabrication techniques to make stamps from polydimethylsiloxane (PDMS), a biocompatible elastomer, which are then used to deposit extracellular matrix proteins, such as those in Matrigel or Geltrex, onto nonadhesive glass or plastic substrates in custom-designed micron-scale patterns [32,33]. Cells are then seeded onto these patterned substrates, adhering only to Matrigel/Geltrex-coated regions. Experiments using circular micropatterns have shown that

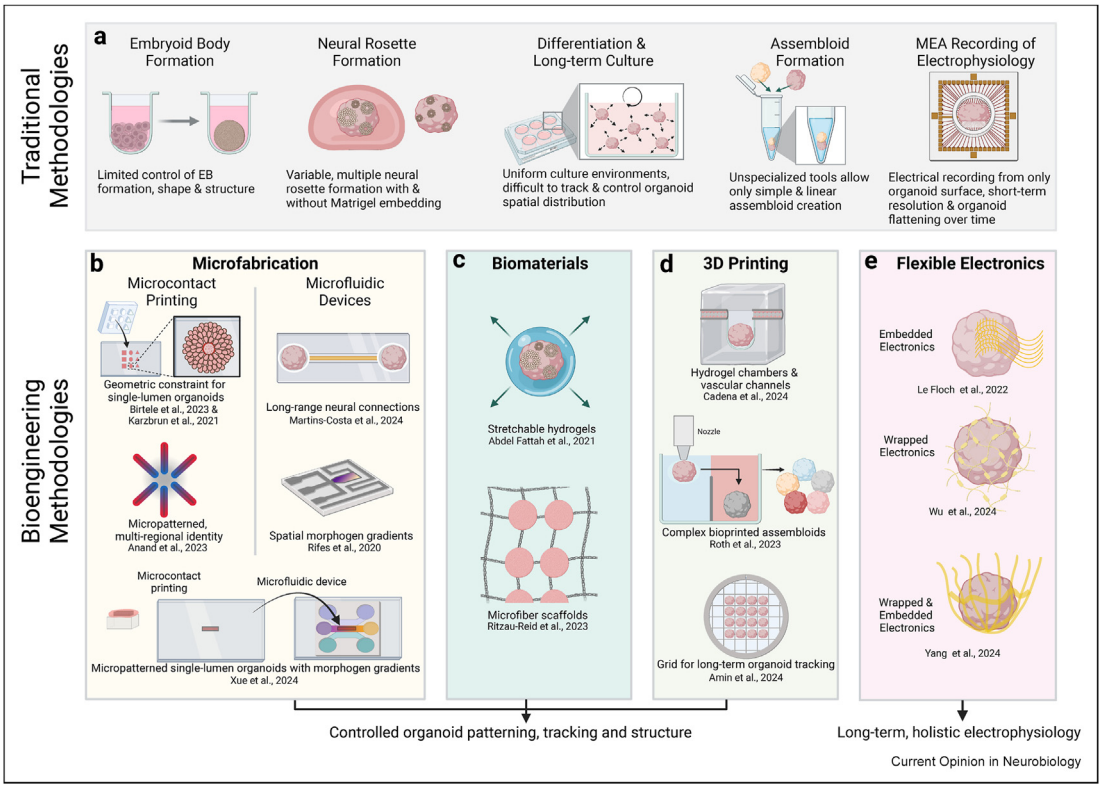
Table 1

Bioengineering tools for addressing limitations of neural organoid generation and their analyses. The traditional limitations of standard protocols for neural organoid culture are indicated along with the particular applications that each bioengineering method is relevant for. Reference numbers are indicated for studies that have address limitations in each application area, and brief descriptions of the solution methods are given.

Traditional limitations	Application	Microfabrication (microcontact printing and microfluidic devices)	Biomaterials	3D printing	Flexible electronics
Initial conditions	Control over EB/ organoid formation and shape	- PDMS stamps to make poly(ethylene glycol) brushes on glass coated with gold and titanium [34,35] - PDMS stamps to deposit Matrigel/Geltrex patterns onto glass coverslips [36,37,39] - PDMS stamps on glass coverslips to protect regions from poly(L-lysine)/poly(ethylene glycol) treatment, followed by laminin treatment [38]	- Microfiber-based scaffolds [52,54] - Flat polycaprolactone sheet scaffolds [53]		
	Control over lumen/neural rosette number and size	- Geometric constraint of cells on micropatterns [34–39]	- Synthetic hydrogels [48] - Matrigel supplementation with additional components [49,50] - Mechanical stretching of hydrogel [51] - Tunable microfiber scaffolds [54]		
	Morphogen gradients	- Tunable micropattern grouping and orientation [36,37] - Microfluidic device with serpentine mixers and continuous flow [44] - Microfluidic device with passive diffusion across a Geltrex channel [39]			
Organoid spatial control	Assembloid formation	- Microfluidic device with adjustable wells [43]		- Custom PDMS wells [58] - Magnetic lifting and placement of organoids [62]	
	Integrating nonneural cell types			- Cell-permeable scaffolds [59] - Cell seeding channels adjacent to organoid culture area [60,61]	
Long-term measurements	Organoid morphology			- Continuous culture platform [55,56] - Grid insert for well plate [57]	
	Neural connections	- Long-distance channels connecting organoids growing in microfluidic devices [40–42]			- Well-shaped arrays [68] - Arrays piercing organoid interior [69]

(*continued on next page*)

Figure 1



Current bioengineering approaches advancing neural organoid technology. (a) Traditional methodologies for generating, culturing, and analyzing neural organoids, along with the associated shortcomings and constraints of each process. (**b**) Microfabrication can be used for microcontact printing to make geometrically constrained single lumen organoids (e.g. Birtele et al. [35] and Karzbrun et al. [38]), the creation of microfluidic devices for studying long-range neural connections (Martins-Costa et al. [42]) and the generation of morphogen gradients for multiregional neural patterning via microfluidic devices (Rifes et al. [44]), microcontact printing (Anand et al. [37]) or a combined approach (Xue et al. [39]). (**c**) Biomaterials such as Matrigel are often used in standard organoid culturing protocols; however, stretchable synthetic hydrogels can aid in neural rosette formation and cellular fate acquisition (Abdel Fattah et al. [51]), and alternative biomaterials such as fibrous scaffolds can alter EB formation and tune neural rosette development (Ritzau-Reid et al. [54]). (**d**) 3D printing can produce hydrogel chambers for organoids with vascular channels seeded with endothelial cells (Cadena et al. [61]), control the positions of organoids for complex assembloid formation (Roth et al. [62]) and make gridded well plate adaptors that allow tracking of organoids during long-term culture (Amin et al. [57]). (**e**) Flexible electronics that can embed within (e.g. Le Floch et al. [70]) and wrap around (e.g. Wu et al. [72]) organoids, or both (Yang et al. [68]), to enable neural activity to be measured at greater resolution and for a greater duration across a greater portion of the organoid surface than what is possible with MEAs. EB, embryoid body; MEA, multielectrode array.

single neural rosette EBs robustly form on geometries between 150 and 250 μm in diameter [34–39], with organoids differentiated toward posterior identities such as the spinal cord favoring diameters on the smaller end of this range [34,36,37]. With the ability to reliably form single-rosette structures, several groups have applied these micropatterned organoids to uncover new features of human neurodevelopment. Micropatterned forebrain organoids provided a high-throughput platform for monitoring early radial glia organization within rosettes and helped reveal an adverse role for autism-associated *SYNGAP1* mutations on this process [35]. In another study, caudal neural tube organoids with hindbrain, spinal cord and tail bud regions were made through an iterative process that used machine learning to design an optimized micropattern configuration for reliably generating these complex multiregional organoids [37]. They also demonstrated that the WNT and FGF signaling pathways cooperate to promote axial extension, but that secreted WNT inhibitors at the anterior poles of the organoids are essential for unidirectional growth [37]. Finally, micropatterning was used to create a neural tube organoid model that undergoes *in vivo*–like folding morphogenesis, with an initially flat, neural plate-like inner neural tissue folding, while surrounded by nonneural ectoderm, and tuning the width of their rectangular micropatterns could alter the mechanics of neural tube folding [38]. The unique insights gained from these studies showcase the power of tunable spatial control of EB and organoid formation through microcontact printing, which would not be possible to accomplish with traditional methods of generating neural organoids. Moving forward, micropatterns will need to be optimized for long-term

organoid culture while maintaining a single neural rosette structure, and additional applications could be unlocked through combining micropatterning with other techniques, such as microfluidics.

Microfluidic devices are typically made as PDMS replica molds from silicon wafers patterned with micron-scale structures using photolithography, which enables fast prototyping and very fine control over device design and dimensions [32]. This approach can create engineered environments that are tailored toward applications such as studying neural connections [40—42], forming assembloids [43], or culturing organoids under laminar flow [24]. Recently, a microfluidic device was developed to model long-range axon projections where brain organoids placed in different reservoirs of a microfluidic device but connected via a thin channel 150 μm in height and width but 7 mm in length could form dense long-range tracts of axons between one another, similar to those in the corpus callosum that connect different brain hemispheres [42]. In this model, it was shown that mutations in the *ARID1B* gene lead to impaired corpus callosum development, as observed in patients, by altering the chromatin conformation around genes important for SATB2$^+$ neurons to form long-distance projections, resulting in fewer numbers of them [42]. Here, microfluidics provided an indispensable tool for elucidating the downstream consequences of *ARID1B* haploinsufficiency by not only creating an *in vitro* model of the corpus callosum but also by enabling long-range projections to be maintained and analyzing using retroviral tracing and various imaging techniques, which could be repurposed for studying other long-distance connections, such as cortico-spinal tracts.

Beyond their ability to provide unique environments for establishing long-range connections between neural organoids, another useful feature of microfluidic devices is their ability to create chemical gradients [32]. A "Christmas tree"-style microfluidic gradient generator [32] was used to pattern 2D cultures of stem cells along a WNT gradient, mimicking the rostrocaudal patterning of the neural tube [44]. Although this approach showed the power of microfluidic gradients, the complex device construction, need for syringe pumps, ability to form gradients in only one direction and applicability only to 2D rather than 3D stem cell cultures limited the utility of this method. A novel organoid model of the human neural tube resolved these issues via designing a microfluidic device that could apply both rostral-caudal and dorsal-ventral morphogen gradients without the need for complex fluidics by establishing orthogonal gradients across a Geltrex-filled channel [39]. Gradients were applied to cylindrical EBs created through microcontact printing using Geltrex-coated PDMS stamps with rectangular patterns, where cells transition from a 2D sheet into 3D tissues with a single central lumen after the center channel of the microfluidic device is loaded with Geltrex. These EBs were then exposed to caudalizing, ventralizing, and dorsalizing morphogen gradients, resulting in neural tube organoids containing both dorsal and ventral forebrain, midbrain, hindbrain, and spinal cord regions all correctly oriented relative to one another, with major regional neural progenitor cell types and some neurons, thereby representing one of the most advanced *in vitro* models of the human neural tube to date. Thus, microfabrication offers unprecedented control over the organoid environment, creating new organoid models that would not be permissible using traditional culturing methods. Applying microcontact printing and microfluidic devices to continue creating new multiregional or multi-subregional neural organoids will be an important next step, particularly for more complex structures, such as cortical organoids with arealization.

Biomaterials

Interactions between neural organoids and their extracellular environment can have dramatic effects on their cellular state, cytoarchitecture, development, and maturation [45]. Matrigel is often used in protocols for generating neural organoids [4,13,28], where it enhances early neural rosette formation [46]. However, Matrigel's nonhuman origin, loosely defined components and limited tunability [47] are impediments for understanding the roles of biomechanical forces on neural organoid development and pose challenges for engineering organoid formation and cellular fate. The use of tunable human-based substrates or fully synthetic hydrogels have the potential to better mimic the natural environment present during human brain development, which could result in neural organoids with *in vivo*—like structural properties and enhanced neural maturation, while also offering the potential to be used for regenerative medicine applications in clinical settings [47] (Figure 1c). Some studies have made fully synthetic hydrogels [48] or hybrid hydrogels that are Matrigel-based but also contain other components, such as extracellular matrix proteins from human brain samples [49] or alginate [50], which in the case of the former, lead to larger cerebral organoid ventricular zones and increased neurogenesis, and in the case of the latter, enabled hydrogels with tunable stiffnesses. Using these tunable alginate hydrogels, it was shown that stiffer matrices lead to a reduction in the size of midbrain organoid ventricular zones as well as total organoid size [50]. A similar matrix-induced growth reduction was found in neural tube organoids embedded in poly(-ethylene) glycol hydrogels, but a mechanical device capable of stretching the hydrogels embedded with organoids to relieve these compressive forces not only resulted in greater organoid size but also lead to alterations in cellular fate patterning, with stretched organoids efficiently forming FOXA2$^+$ floor plate regions [51]. Finally, biomaterials have also been used to help guide the process of EB and neural rosette formation

[52–54], two processes that are only loosely controlled in traditional neural organoid protocols (Figure 1a). Seeding stem cells onto microfilaments made from poly(lactide-co-glycolide) copolymer led to the formation of oblong EBs that improved forebrain specification and cortical plate structure [52]. Additionally, engineered polycaprolactone microfiber scaffolds can allow high-throughput EB generation on a single scaffold and modulate neural rosette formation, where different fiber angles and spacing can control the number and size of lumens within each organoid [54]. In the future, biomaterials with properties tightly matching those of the developing brain may be able to dynamically tune their mechanical properties to adapt to organoid development during different stages of neurogenesis, potentially enhancing neural organoid structure, maturation, regional identity, and clinical translatability, while also allowing new kinds of experimental perturbations [47].

3D printing

3D printing can be used to create devices or directly manipulate cells for the purpose of controlling the spatial positions of neural organoids in culture and for increasing their cellular complexity (Figure 1d). In standard protocols, organoids are often grown in small groups, as opposed to in isolation, within well plates or miniature bioreactors where perturbations from plate shakers, stir rods, or even frequent media changes make it difficult to keep track of individual organoids and control their locations. Building on work for making mini-bioreactors using 3D printed components [13], continuous culture systems were recently 3D printed that allow individual organoids to be monitored over time [55,56]. Still, the number of components and the need for specialized equipment for these studies make their general adoption difficult. A more minimal approach for enabling individual organoid measurements throughout long-term culture was devised with a grid made from 3D printed polylactic acid as an insert for a standard 6-well plate [57]. Finally, PDMS wells made from 3D printed substrates could be used for guiding the fusion of individual organoids to make three-part assembloids [58].

In general, attempts to introduce non-neuroectodermal cell types—such as microglia and vascular endothelial cells—into neural organoids have limited control of how these cells interact with organoids. To help address this issue, several recent studies have used 3D printed devices. For example, immune cell infiltration into the brain during aging has been modeled using 3D printed cell-permeable scaffolds loaded with monocytes and placed in contact with cortical organoids [59]. Additionally, vascular channels seeded with endothelial cells were incorporated into the channels of 3D printed microfluidic channels [60] and hydrogel-based chambers [61], with the possibility of fluid flow through the vascular networks. Lastly, for increasing the cellular complexity of assembloids, coating organoids in hydrogels seeded with iron oxide nanoparticles allowed them to be magnetically lifted and placed precisely in space, thereby enabling three or more organoids to be arrayed linearly or in complex configurations such as rings and pyramids [62].

Future applications of 3D printing will likely involve additional efforts to increase neural organoid size and maturity. It is interesting to speculate that combining 3D printed fluidic devices, such as those discussed above, with recently reported brain and blood vessel organoid assembloids [63], could lead to better neurovascular models with perfusable vascular networks integrated throughout the entire organoid [64], potentially enabling longer organoid culture times with smaller necrotic cores and more mature neurons. Further, the use of neural organoids as 3D bioprinting substrates could be used to create macroscopic tissues as has recently been done with intestinal organoids [65].

Flexible electronics

Electrophysiology approaches for long-term monitoring of neuronal activity within neural organoids are desirable to monitor the continuous development of neuronal properties and circuitry. Standard multielectrode array (MEA) plates take recordings only at their surface, which is in limited contact with the spherical neural organoids, therefore capturing only a fraction of their total electrical activity [66] (Figure 1a). Further, the tendency of neural organoids to gradually lose their 3D shape and flatten out over the MEA's flat surface can interfere with their development and organization [67,68], effectively rendering this a terminal measurement. Newly devised flexible electrodes are allowing more readings to be taken across the entirety of organoids (Figure 1e), while also better preserving organoid 3D cytoarchitecture [68–73].

Approaches for obtaining long-term electrophysiological measurements within the organoid interior have engineered cantilever microelectrodes that pierce through more than 100 μm of tissue [69] and soft nanoelectronic meshes that are directly incorporated into organoids during the initial stages of 3D cell aggregation [70]. Other studies have focused on less invasive methods such as flexible electrodes capable of enveloping neural organoids [68,71–73] or integrating into them during growth [68]. Electrodes that naturally wrap around neural organoids are at the forefront of these approaches. For example, autonomously folding SU-8 photoresist coatings were incorporated into electrodes [71], and soft polyurethane/thermoplastic polyurethane meshes with conductive liquid metal–polymer layers were designed to blanket hippocampal organoids [72]. Finally, a design of spiraling electrode arrays that both deform around organoids and integrate into them over time allowed

long-term electrophysiological measurements from individual organoids and assembloids for more than 100 days and captured drug- and disease-induced changes in neural firing rates [68]. Thus, flexible electrodes enable holistic and long-term measurements to be performed without compromising the organoid structure, thereby addressing a key limitation in recording organoid electrical activity with MEAs. Moving forward, innovative flexible electrodes such as these can potentially be used to uncover subtle and protracted changes in electrophysiology that occur during development and disease that cannot be obtained with traditional methods as well as enable measurements of long-range neural connections that are emerging in advanced neural organoid models [12,15].

Future directions

As neural organoid technologies advance, it is expected that new studies will increasingly utilize bioengineering methodologies as well as new tools and techniques. We also anticipate that many advances will be made by combining these methods in novel ways, building upon work that has already combined microfluidics with methods such as microcontact printing [39], MEA [41], and 3D printing [60], and even incorporating emerging fields like synthetic biology, which has the potential to further refine our ability to control and perturb neural organoids through engineered genetic and cellular circuits [74]. As neural organoid models become progressively better, it is likely that they will be integrated with organoids from other tissues or organs, such as nonneural tissues, to create advanced 'organoids-on-a-chip' [75] and multiorgan microphysiological systems. It will also be critical to use the latest genome editing and CRISPR screening techniques [19,20] to make full use of these next-generation neural organoids. Finally, high-throughput generation and screening of neural organoids will likely be important in the future to take advantage of machine learning techniques. Together, these approaches could expand the use of neural organoids beyond academic research and into the biopharmaceutical industry to help develop future therapeutics.

Declaration of competing interest

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

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Data availability

No data was used for the research described in the article.

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* of special interest
** of outstanding interest

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