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Upgrading the Physiological Relevance of Human Brain Organoids

Ashley Del Dosso[1,+], **Jean-Paul Urenda**[1,+], **Tuan Nguyen**[2], **Giorgia Quadrato**[1,2,*]

[1]Department of Stem Cell Biology and Regenerative Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA

[2]Eli and Edythe Broad CIRM Center for Regenerative Medicine and Stem Cell Research at the University of Southern California, Los Angeles, CA 90033, USA

Abstract

The recent advent of human pluripotent stem cell (PSC) derived 3D brain organoids has opened a window into aspects of human brain development that were not accessible before, allowing tractable monitoring and assessment of early developmental processes. However, their broad and effective use for modeling later stages of human brain development and disease is hampered by the lack of a stereotypic anatomical organization, which limits maturation processes dependent upon formation of unique cellular interactions and short and long-range network connectivity. Emerging methods and technologies aimed at tighter regulatory control through bioengineering approaches, along with newer unbiased organoid analysis readouts should resolve several of the current limitations. Here, we review recent advances in brain organoid generation and characterization with a focus on highlighting future directions utilizing interdisciplinary strategies that will be important for improving the physiological relevance of this model system.

Keywords

Brain organoids; Neurodevelopmental Disorders; Bioengineering; Synthetic Biology; Extracellular Matrix; Regional Patterning; Microfluidics; Synthetic Scaffolding; Lineage Tracing; Neuronal Network Activity

1. Introduction

Can an *in vitro* system recapitulate the complex processes underlying human organogenesis? An astonishing level of self-organization can emerge from 3D aggregates of human pluripotent stem cells (hPSCs) which then continuously evolve through positive and negative feedback interactions to drive the differentiation and assembly of highly organized tissue patterns. The seminal work from the laboratory of Yoshiki Sasai showed how autonomous emergence of highly ordered neural structures can emerge from embryonic stem cells (ESCs) grown in 3D aggregates (Eiraku et al., 2008, Eiraku et al., 2011). In the last decade, building on this work a variety of protocols have been established for the

*Correspondence to giorgia.quadrato@med.usc.edu.
+These authors contributed equally

generation of reductionist replicas of multiple brain regions as reviewed in (Quadrato et al., 2016, Di Lullo and Kriegstein, 2017, Quadrato and Arlotta, 2017, Paşca, 2019, Qian et al., 2019, Kelava and Lancaster, 2016). But how far can human brain development be recapitulated *in vitro*? The answer to this fundamental question is of enormous importance in determining which aspects of human brain development and disease can be currently modelled with high fidelity in organoids. Therefore, a systematic understanding of the principles guiding self-organization in these multicellular systems will enable us to harness their full potential and provide key information to overcome their limitations. Much work is still needed to address this point, but the general consensus in the field is that intrinsic self-organizing properties control cellular diversity and local tissue microarchitecture, while the number and localization of organ primordia is interdependent with external tissue cues and structural environment. For brain organoids, the current inability to precisely control both intrinsic and extrinsic cues results in lack of stereotypical anatomy and aberrant long-range connectivity that in turn affects levels of neuronal activity, cell maturation and overall physiological relevance of this model system. These limitations have affected data analysis and interpretations making classical analysis techniques underpowered (Atamian et al., 2020). Here we summarize, recent advances in brain organoid culturing and analysis techniques that have attempted to remedy some of these limitations. We discuss additional emerging technologies, spanning from cutting edge bioengineering innovations to tools for unbiased multimodal analysis that, combined with current methods, will allow for modeling and understanding aspects of human brain development and disease that are still inaccessible.

2. The Modern Art of Culturing Brain Organoids

2.1 Generating Self-Patterned and Patterned Organoids

Human brain development occurs in a tightly regulated series of events governed by long range morphogen driven gradients combined with local cell-cell and cell-extracellular matrix interactions that continuously evolve through positive and negative feedback regulation over the course of gestation. These complex and intricate regulatory networks allow for the formation of discrete structures and cellular stratification that contribute to the vast processing power of the human brain. Protocols for human brain organoid generation have taken inspiration from these *in vivo* patterning processes to recapitulate neurodevelopment in a dish. Current organoid protocols are divided into two major categories, patterned and self-patterned. Generally, in both approaches dissociated pluripotent stem cells are aggregated into 3D spheres or embryoid bodies to generate self-organizing 3D neuroectodermal structures. Self-patterned protocols utilize bFGF and retinoic acid during hPSC reaggregation to generate whole brain organoids. These protocols leverage the default bias of early ectoderm to generate dorsal forebrain fates (Espuny-Camacho et al., 2013). However, the efficiency and propensity of different human PSC lines to differentiate into ectoderm (Bock et al., 2011) and downstream cortical cell types varies widely (Quadrato et al., 2017). This leads to high variability among organoids generated within and across PSC lines. In addition, whole brain organoids contain a variety of neuronal and glial cell types from multiple brain regions and have been shown to contain a wide-breadth of non-ectodermal lineage cells as well (Lancaster et al., 2013, Lancaster et al., 2017, Quadrato

et al., 2017, Ormel et al., 2018, Camp et al., 2015). However, in whole brain organoids this high degree of cellular diversity comes at the expense of the reproducible generation of distinct cell types causing substantial organoid-to-organoid variability (Quadrato et al., 2017). The stochastic nature of cellular diversity in self-patterned organoids makes this approach not well suited for disease modeling and drug screening purposes. Therefore, patterned protocols that improve regional accuracy and cellular reproducibility have emerged as a widely used alternative in the field (Figure 1).

A unifying characteristic among most patterned protocols is an initial inhibition of the SMAD pathway (Chambers et al., 2009). The SMAD family of proteins act as signal transducers downstream of TGF-beta superfamily ligand binding to promote the generation of epidermis over neural ectoderm. Patterned protocols have generally employed the use of partial or dual SMAD inhibition through administration of small molecule inhibitors of the TGF-beta type I receptor, and/ or administration of inhibitors which target the activity of bone morphogenic proteins (BMPs). Additionally, WNT inhibition during early stages of organoid generation is often employed to repress the mesodermal lineage and in turn promote the production of anterior neuroectoderm (ten Berge et al., 2008). A number of variations of this basic patterning strategy are currently used for the generation of cortical organoids, leveraging the default bias of early ectoderm to generate dorsal forebrain fates (Kadoshima et al., 2013, Mariani et al., 2015, Paşca et al., 2015, Qian et al., 2016, Rigamonti et al., 2016, Bagley et al., 2017, Watanabe et al., 2017, Velasco et al., 2019). Despite patterning and culturing differences, cortical organoids across protocols can develop neural-tube-like neuroepithelial structures that follow a similar temporal developmental trajectory as *in vivo*. These structures give rise to a diverse range of cortical cell types as seen in the endogenous human forebrain including, inner and outer radial glia, intermediate progenitors, deep and upper layer cortical neurons, astroglia, and oligodendroglia precursor cells. The question of whether broad cell types in cortical organoids can be reproducibly generated has been addressed by Yoon et al. (2019), who were able to obtain consistent a cellular composition in cortical spheroids at different developmental stages. Further characterization of cellular diversity and reproducibility of cortical cell types generated in individual organoids, within and across lines, was thoroughly tested by Velasco et al. (2019), who demonstrated reproducible generation of all main classes of cortical progenitors and postmitotic cells of cortical organoids using a modified version of the Kadoshima et al. (2013) protocol. However, the molecular signature fidelity of cortical subtypes generated in organoids has been questioned in a recent paper that demonstrates increased metabolic stress due to *in vitro* culture conditions can alter subtype specification (Bhaduri et al., 2020). Cortical brain organoid research is rapidly evolving, and it will be exciting to follow how quickly other brain region organoid protocols improve.

Cortical organoids are widely used because of their relevance to human neurodevelopmental disorders, as defects in higher order cognitive abilities predominately stem from dysregulation within this brain region. However, in the last few years patterned protocols that overcome the default forebrain bias to differentiate into other brain regions have been developed. These include methods to generate human organoids of the thalamus (Xiang et al., 2019), hypothalamus (Qian et al., 2016), hippocampus (Sakaguchi et al., 2015),

cerebellum (Muguruma et al., 2015), midbrain (Jo et al., 2016, Qian et al., 2016, Monzel et al., 2017), anterior pituitary (Ozone et al., 2016), and spinal cord (Ogura et al., 2018).

The thalamus emerges from the caudal region of the forebrain and its generation requires a delicate balance of morphogen gradients along the rostro-caudal axis. A recent thalamic organoid protocol attempted to mimic this through early insulin treatment to promote caudalization, which is later attenuated by MEK-ERK inhibition to block excessive caudalization towards a midbrain fate, and simultaneous BMP7 treatment to promote a thalamic specific fate (Xiang et al., 2019). This protocol has successfully generated distinct thalamic lineages including glutamatergic neurons and a small percentage of GABAergic and serotonergic neurons with undefined regional identity.

The hippocampus is derived from the dorsomedial telencephalon. Sakaguchi et al. (2015) recapitulated this specification through modulation of BMP4 and Wnt signaling (CHIR) to mimic the *in vivo* dorsal midline and cortical hem signaling center that is required for the generation of dorsomedial telencephalic tissue. The specific timing and duration of the morphogen treatment in this protocol allowed for the first time, generation of hippocampal granule neurons and CA3 pyramidal neurons in a 3D culture environment.

The midbrain is a centrally located ventral structure that represents the most superior portion of the brain stem. Jo et al. (2016) designed a patterning strategy for this region centered around promoting ventralization using SHH and determining the rostro-caudal positional identity through Fgf8 administration, ultimately resulting in a tissue type similar to the midbrain and capable of producing dopaminergic neurons. Qian et al. (2016) used a similar strategy with the addition of a WNT agonist to initially promote a floor plate-like identity before pushing for a midbrain specific identity to robustly generate dopaminergic neurons. An alternative approach in which neuroepithelial stem cells (NESCs) were used as the starting population for midbrain organoid generation was developed by Monzel et al. (2017). In this case the 3D NESC spheres generated were patterned with a similar approach as previous groups, using a SHH pathway agonist and a GSK3 inhibitor. These organoids contained post-mitotic dopaminergic neurons as in previous protocols, however astrocytes and functionally mature oligodendrocytes were found to be present as well.

The cerebellum is an elaborately organized hindbrain structure with several distinct progenitor zones that give rise to different neuronal populations, making the recapitulation of the structure *in vitro* complicated. Mugaruma et al. (2015), established a 3D cerebellar organoid protocol with the goal of generating Purkinje cells using FGF2, which has been shown to be a potent caudalizing factor at high concentrations. This caudalizing factor allowed for the generation of an isthmic organizer-like center, a region that expresses signaling molecules that promotes the generation of midbrain and hindbrain on either side of it. Differentiation occurs along this axis to result in a multilayered structure resembling the ventricular and rhombic lip germinal zones of early embryonic development. However, maturation of Purkinje cells to its recognizable “tree-like” structure could be achieved only following coculture with mouse cerebellar granule neurons.

The spinal cord is the most caudal portion of the central nervous system and functions to relay sensory information to and from the brain. The structural organization of the spinal cord is complex, involving the intermingling of more than twenty distinct neuronal subclasses. The generation of 3D spinal cord tissue has recently been accomplished by Ogura et al. (2018) who took inspiration from *in vivo* signaling centers pertinent to early stages of spinal cord development. This included the roof plate and floor plate which are largely responsible for producing BMP's, Wnts and SHH, respectively. By utilizing these morphogens alongside retinoic acid to promote the caudalization of the organoid the authors successfully generated several subclasses of spinal cord neurons, including GABAergic, glutamatergic and cholinergic neurons within tissue reminiscent of the dorsal, intermediate and ventral spinal cord.

The Lancaster group has also recently explored the generation of choroid plexus-like tissue in brain organoids through the addition of the patterning factors CHIR and BMP4 (Pellegrini et al., 2020). *In vivo,* the choroid plexus functions to produce cerebrospinal fluid (CSF) which contains nutrients and signaling molecules important to brain development. *In vitro,* the Lancaster group was able to induce the formation of choroid plexus-like stroma and epithelial tissue capable of forming tight junctions and producing CSF. The induction of this functional structure in brain organoid cultures may contribute to the ability to mimic a physiologically relevant microenvironment *in vitro*.

While some studies addressing the fidelity and reproducibility of cell types generated in cortical organoids at different developmental stages have been carried out, the characterization of organoids of other brain regions is still in its infancy. Many outstanding questions related to the reproducibility of cell types generated within and across cell lines, as well as, the molecular fidelity of these cells to their endogenous counterparts need to be answered before they can be reliably used for disease modeling and drug screening.

2.2. "Integrated" Organoids

Patterning protocols have successfully generated distinct regions of the brain reminiscent of their *in vivo* counterparts by taking inspiration from morphogen gradients that guide human brain development (Figure 2). Currently, morphogen treatment of organoids *in vitro* is done via bath application, which does not wholly recapitulate the tightly regulated spatiotemporal gradients that are key to proper neurodevelopment. Although cells within organoids are capable of self-generated local gradients that mimic *in vivo* patterning, the reproducibility of these local cues and the extent to which they can influence the generation of discrete structures remains unclear. Generally, these directed differentiation approaches generate a single early brain region and do not recapitulate multiple brain regions or the long-range connectivity between them, which are necessary for refinement and specification of cells within a developing human brain. Additionally, when PSCs are directed towards a neuroectoderm fate using a bath of morphogens, non-ectodermal lineage cells are excluded. Importantly, these include yolk-sac derived microglia and other cells of mesodermal origin such as endothelial cells which form the vascular network. These non-ectodermal cell types play critical roles in brain development and their absence negatively impacts human brain organoid development. However, recent publications have explored alternative methods to

incorporate non-ectodermal cells, improve cellular diversity, and enable co-development of multiple brain structures.

2.2.1 Incorporation of diverse cell types—While neuronal populations have been a longstanding focus of brain research, glial cells have also emerged as key players in the human brain during development and throughout life (Zuchero and Barres, 2015). Oligodendrocytes and astrocytes are collectively termed macroglia and together constitute most of the cell types present in the brain. Gliogenesis follows neurogenesis, beginning mid gestation in humans and continuing beyond birth; encompassing developmental stages that are difficult to recapitulate in brain organoids. Currently it is possible to generate macroglia across several brain organoid generation protocols (Paşca et al., 2015, Quadrato et al., 2017, Bhaduri et al., 2020, Velasco et al., 2019, Sloan et al., 2017) but the level of maturity and functionality that they attain remains limited. In order to model later stages of development it is critical that macroglia be present, mature and functional, an endeavor pioneered by several groups that have modified culture conditions to promote this. The promotion of oligodendrocyte maturation has been accomplished in brain organoids through modulation of the culturing media to include factors known to promote oligodendrocyte differentiation and maturation while preserving a robust neuronal population. This has been accomplished by Madhavan et al. (2018) and Marton et al. (2019) who both recorded functionally mature oligodendrocytes after 28 and 30 weeks, respectively.

As previously mentioned, patterned brain organoids lack several non-ectodermal cell types. While oligodendrocytes and astrocytes are derived from neural progenitors within the neuroectoderm, microglia are generated separately from hematopoietic stem cells in the yolk sac during early embryogenesis and migrate into the brain parenchyma during early development. This process generally begins around the fifth week of gestation in humans, just following the onset of neurogenesis (Monier et al., 2007, Kierdorf et al., 2013). As microglia take residence in the brain, they receive and provide feedback to neuronal populations influencing migration, differentiation and synaptic connectivity as well as regulating immune responses (Cunningham et al., 2013, Squarzoni et al., 2014). These microglia dependent processes have been implicated in the pathogenesis of several neurodevelopmental disorders, creating a need to incorporate this glial population into brain organoid models (Tay et al., 2017, Lenz and Nelson, 2018). While protocols that rely on undirected neuronal differentiation (Lancaster et al., 2013) have observed that microglia can innately develop within brain organoids (Ormel et al., 2018), more reproducible directed protocols that employ SMAD inhibition to force a neuroectodermal fate, do not form microglia progenitors. This has led to the development of *in vitro* protocols to generate ESC and iPSC derived microglia (Muffat et al., 2016, Abud et al., 2017, Douvaras et al., 2017, Haenseler et al., 2017, McQuade et al., 2018), which can then be integrated into brain organoid cultures. This strategy has been used to study Alzheimer's disease (Abud et al., 2017, Lin et al., 2018), basic neuro-immune interactions (Abreu et al., 2018, Muffat et al., 2018, Song et al., 2019) and to assess the transcriptomic changes upon microglia integration into brain organoids (Bejoy et al., 2019, Schmunk et al., 2020). While these studies have effectively demonstrated that exogenously derived microglia can integrate, generate an immune response, and participate in functional cross talk with neuronal

populations within brain organoids, there is still much work to be done in optimizing this system. Currently, the time point for integration of microglia into organoids differs across protocols; however, the exact timing of integration may have drastically different effects on neuronal populations. Additionally, the number of successfully integrated microglia may impart differing effects on neuronal survival and immune response, and while attempts have been made to incorporate microglia in proportions similar to what is seen *in vivo*, there is yet no standardized protocol for the timing and amount of microglia to add. Lastly, the longevity of microglia in brain organoid cultures remains unclear, with the longest recorded time point being one month (Lin et al., 2018), and a majority of other protocols using a shorter time point of one week. Much of the appeal that surrounds brain organoids is the ability to maintain cultures over extended periods of time to allow for the emergence and functional maturation of distinct neuronal subtypes. Successful long-term culture of microglia integrated brain organoids will be crucial to understanding how neuroimmune interactions shape organoid cytoarchitecture and influence the functional maturity of neuronal populations during normal and aberrant development.

Brain organoids lack vasculature and therefore undergo chronic hypoxia in the innermost regions. *In vivo,* by six gestational weeks, a capillary plexus covers the entire cerebral cortex and by eight gestational weeks the intracerebral microvasculature in the brain begins to develop in a ventral to dorsal progression, providing oxygenation, and serving to deliver nutrients and eliminate waste (Marin-Padilla, 2012). As this neurovascular network expands in complexity and magnitude, it begins to innervate deeper regions of the brain and participates in dynamic crosstalk with neuronal progenitor populations to guide proliferation, migration and differentiation (Tata and Ruhrberg, 2018). Therefore, integrating a functional vascular network into 3D brain organoids is necessary to accurately model later stages of human brain development. Recently, Ham et al. (2020) treated brain organoids at early stages with vascular endothelial growth factor (VEGF) and Wnt7b to promote the formation of vascular endothelial cells and also pericyte-like supporting cells without compromising neuronal populations. This led to the formation of open-circle vascular structures that contained CD31 positive cells and modulation of gene networks associated with brain embryogenesis in neuronal populations, indicating crosstalk between neuronal and endothelial cell populations. Cakir et al. (2019) generated human cortical organoids that displayed vascular-like structures by co-culturing hPSCs engineered to overexpress ETV2, a transcription factor known to induce endothelial cell fate, with unedited hPSCs. This resulted in vascularized cortical brain organoids that displayed blood-brain barrier-like characteristics such as expression of tight junctions and nutrient transporters. Worsdorfer et al. (2019) developed a co-culture method in which mesodermal progenitor cells integrated into brain organoid cultures produced vascular networks with typical blood vessel ultrastructure after roughly eight weeks *in vitro*. Alternatively, Pham et al. (2018) co-cultured endothelial cells with brain organoids and supplemented the media with vascular endothelial growth factor (VEGF), resulting in the emergence of robust vascular networks after roughly four weeks *in vitro*. However, these *in vitro* based systems still lack a pump and therefore an active flow of media through the vascular structures.

Finally, Xenograft models have been developed to increase cellular diversity and functional neuronal connectivity. This method was pioneered by Mansour et al. (2018) where after

several weeks of *in vitro* growth, organoids were intracerebrally grafted into a cleared cavity in the mouse cortex and allowed to grow for several more months. Upon analysis of the grafted organoids, it was revealed that they exhibited progressive neuronal differentiation and maturation, gliogenesis, integration of microglia and invasion of mouse vasculature, and outgrowth of axons that formed functional connections with the host brain. This model system will provide important insights for the development and refinement of completely *in vitro*-based approaches that achieve similar results, but in a higher throughput, human specific brain organoid model system.

2.2.2 The Fusion of Patterned Brain Organoids—To establish the formation of multiple regional identities, fusion protocols have been used, wherein organoids are individually directed toward a specific brain fate and subsequently fused together via co-culturing. This method has been utilized by several groups to combine dorsal and ventral forebrain organoids (Bagley et al., 2017, Birey et al., 2017, Xiang et al., 2017). *In vivo,* the ventral forebrain produces GABAergic inhibitory interneurons that migrate to dorsal regions of the forebrain and integrate themselves into cortical circuits during development. The groups were able to identify GABAergic inhibitory interneurons of several subtypes, including the medial ganglionic eminence derived somatostatin and parvalbumin positive interneuron subtypes and observed their migration into the dorsally patterned forebrain organoid, recapitulating what is seen i*n vivo*. While it remains largely unknown in humans, during mouse brain development there are three waves of oligodendrocyte generation during embryonic development. The first two waves are transient and originate from the ventral regions of the brain while the latter is a permanent population that is generated from more dorsal brain regions. Kim et al. (2019) fused dorsal and ventral forebrain organoids to monitor this process of oligodendrocyte development. After fusion, fifteen weeks of culture, and five weeks of T3 (a thyroid hormone to promote oligodendrocyte maturation) treatment, the authors concluded that there are indeed similar waves of ventrally and dorsally derived oligodendrocytes. This fusion method has also been employed to interrogate the reciprocal neuronal projections between thalamic and cortical organoids (Xiang et al., 2019). These studies indicate that fused organoid models can form functionally relevant connections, improving functional maturation in some cases and pave the way for future studies to investigate long-range connectivity between multiple distinct brain regions.

3. Guiding self-organization in brain organoids

3.1 Engineering a Pseudo-Signaling Center

During human brain development, morphogen gradients are orchestrated by signaling centers. These gradients in turn, establish cell fate and polarity necessary for proper formation of the central nervous system. As current organoid generation protocols use a bath application of morphogens and lack a true signaling center to direct cell polarity, these organoids form multiple neural tube-like rosette structures that give rise to neural progenitors. Cederquist et al. (2019) probed the use of a pseudo Sonic Hedgehog (SHH) organizing center by creating an inducible SHH-expressing hPSC line (iSHH) that was embedded at one pole of a forebrain organoid during development. They observed that a SHH gradient established by this signaling center could induce the formation of forebrain

subdivisions with *in vivo*-like topography. While SHH is a critical morphogen for the establishment of a dorsal-ventral and rostral-caudal axis during development, it acts in concert with an array of other factors to precisely pattern distinct regions. Having a model that uses a singular signaling center is a promising step forward in organoid generation but establishment of multiple synthetic signaling centers to generate interacting morphogen gradients would more closely recapitulate *in vivo* neurodevelopment.

Signaling centers can also be engineered via microfluidics. A microfluidic device contains a set of micro-channels designed to precisely control the delivery of media and/or gases to a culture chamber where nutrients can be exchanged, and wastes removed. Microfluidic devices have the potential to change patterning dynamics by facilitating orthogonal gradients of morphogens. This was demonstrated using gradients of retinoic acid, a major determinant of the rostral-caudal axis, and SAG, a ventrally secreted smoothened agonist, to mimic the combinatorial effect of these orthogonally distributed morphogens on specification of ventral lower layer motor neuron differentiation in the developing neural tube (Uzel et al., 2016). The Kirkeby lab has implemented an approach termed microfluidic-controlled stem cell regionalization (MiSTR), to model neural tube development by creating a WNT gradient during hPSC differentiation (Rifes et al., 2020). The gradient allowed cells to adopt a rostral-caudal organization and develop molecular characteristics of organizing centers, for example formation of an isthmic organizer. Collectively, these results indicate the possibility to modulate the self-organization of hPSC by extrinsically controlling the spatiotemporal formation of morphogen gradients via a microfluidic approach.

3.2 Engineering anatomical features

The lack of stereotypical anatomy found in cortical organoids hinders the ability to perform complex functional and tracing analysis. One reason hypothesized to contribute to this deficient layer separation is the limited diffusion of nutrients and oxygen at the cortical organoid core which may lead to organoids undergoing chronic hypoxia. To overcome this limitation, Qian et al. (2020) generated sliced neocortical organoids (SNOs) to bypass diffusion limitations, and showed reduced cell death and improved oxygenation in organoid cores. This method allowed organoids to maintain long-term neurogenesis, sustained progenitor expansion and separation of cortical layers. Similarly, the Lancaster group cultured sliced cortical organoids at the air-liquid-interface, termed ALI-COs (Giandomenico et al., 2019), and these organoids displayed improved neuronal survival compared to whole organoids, as well as mature morphological and functional features including formation of discrete long range axonal tracts that were able to make functional connections with mouse spinal cord explants. Discrete long-range axonal tracts have also been shown to form between cortical organoids in a static microculture device (Kirihara et al., 2019). Here, organoids were individually cultured in separate chambers, connected by a microchannel that was 9 mm in length in which axon fascicles were shown to form reciprocal connections. In this study, axonal tracts formed spontaneously, but made sporadic connections; however, a more robust system in which axons are guided will be required to establish reproducible and functional long-range connectivity between distinct brain structures. Additional engineering of hPSC using techniques such as the recently described optogenetic control of growth cone guidance in zebrafish motor neurons (Harris

et al., 2020) or through the modulation of axonal guidance cues may facilitate future studies of long range connections between distinct organoids. The generation of distinct anatomical features in brain organoids remains challenging but emerging techniques such as sliced culturing protocols that improve neuronal survival, axonal growth, and connectivity provide direction to increase feasibility. In addition, axonal connectivity can be guided via microchannels or by optogenetic control, which opens the possibility of connecting brain organoids in reproducible architectures that will anatomically and functionally mimic *in vivo* brain circuits.

3.3 Engineering biomimetic cellular microenvironments

Extrinsic cues such as morphogens have been heavily used via bath application to control and direct the differentiation of hPSCs into brain organoids. This method, while effective, remains simplistic and alone does not encompass the complexity that is required to generate and maintain discrete brain regions in a reproducible manner. The inclusion of additional external cues will allow for further refinement of the model by providing additional layers of control. The extracellular matrix (ECM) is a 3D structure composed of insoluble components secreted from a diverse range of cell types. It plays a key role in maintaining structural integrity and participates in transducing cell-cell communication. Within the brain, the ECM is highly specialized and its composition varies based upon the region and developmental time point (Krishnaswamy et al., 2019). Dysregulation associated with the ECM is becoming increasingly implicated in neurodevelopmental and neuropsychiatric disorders, creating a need to accurately model this component of the brain in 3D brain organoid cultures (Beroun et al., 2019). However, several important cell types known to contribute to ECM formation and maintenance, such as microglia and vascular endothelial cells are not present in many current brain organoid cultures. While there have been several reports of synthetic hydrogels or decellularized human fetal ECM being used as an alternative to the intrinsically produced ECM in 2D culture conditions (Barry et al., 2017, Jin et al., 2018, Cho et al., 2019, Lam et al., 2019, Galarza et al., 2020, Reginensi et al., 2020), matrigel remains the preferred ECM-like component utilized in 3D brain organoid cultures. Although extensively used, the composition of matrigel is generic and largely uncharacterized, which can lead to variability and a lack of physiologically relevant biochemical cues during brain organoid generation. Sood et al. (2019) has recently employed the use of ECM derived from fetal porcine brains in combination with silk scaffolding to better recapitulate the *in vivo* ECM micro-environment. The authors observed that inclusion of the fetal porcine ECM improved the functional maturation of neuronal populations and supported their long-term survival (Sood et al., 2019). The use of PLGA, a synthetic microfilament fiber was used to provide a basement membrane-like scaffold for self-patterned brain organoids termed microfilament-engineered cerebral organoids (enCORs) (Lancaster et al., 2017). This additional scaffolding improved radial organization and cytoarchitecture within enCORs, demonstrating the benefits that structural cues have on brain organoid development. Finally, the field of bioprinting or microcontact printing has allowed for tightly regulated patterning of cells at specified locations within a culture device, providing spatial control during differentiation (Knight et al., 2018, Haremaki et al., 2019, Fedorchak et al., 2020). This approach can also be combined with microfluidic devices to add additional levels of patterning control and mechanical flow cues to organoids. Adopting

more biologically relevant scaffolding for brain organoid cultures is another step of many that will need to be taken to improve organoid generation and the quality of data that can be extracted from them.

4. Emerging techniques for the Unbiased Analysis of Organoids

4.1 Cellular and Molecular Analysis at Single Cell Resolution

Over the past decade there has been incredible momentum within the field of brain organoids that has led to the establishment and improvement of a wide variety of protocols for their generation (Figure 3). As brain organoids become more complex, it is imperative that the technology used to analyze them is appropriately matched in complexity. Classical techniques that provide transcriptional information, such as bulk RNA-seq and qPCR, have been used extensively to characterize organoids, but do not provide enough resolution to resolve the complexity of human brain development. As technology has progressed, we have achieved single-cell resolution at the functional and molecular level which has been used to characterize brain organoids. Single-cell RNA-seq (scRNA-seq) in particular has been used extensively to characterize brain organoids across protocols with the use of both droplet-based and multi-well sorting methods (Birey et al., 2017, Quadrato et al., 2017, Xiang et al., 2017, Madhavan et al., 2018, Cakir et al., 2019, Giandomenico et al., 2019, Kanton et al., 2019, Klaus et al., 2019, Marton et al., 2019, Pollen et al., 2019, Velasco et al., 2019, Xiang et al., 2019). These studies have demonstrated the cellular diversity in patterned and self-patterned brain organoid protocols (as Reviewed in Atamian et al., 2020). An *in silico* analysis has been performed to compare the various single cell datasets of differentiation protocols to the developing fetal brain (Tanaka et al., 2020). These sets of analyses showed a similarity in cellular composition across protocols; however, individual protocols displayed distinct differentiation trajectories and unique developmental gene networks were identified when comparing oranoids to fetal brains.

Several researchers have begun to probe the epigenetic landscape of cortical organoids using ATAC-seq (assay for transposase-accessible chromatin with high throughput sequencing). Trevino et al. (2020) found that after long term culture (20 months), organoids undergo dynamic chromatin state transitions that recapitulate primary brain tissue chromatin accessibility patterns suggesting organoids retain the intrinsic ability to progressively remodel their epigenetic landscape during protracted development. Single-cell ATAC-seq has also been used to identify human specific differences in chromatin accessibility between human and chimpanzee derived brain organoids (Kanton et al., 2019). This study found that a majority of the differentially accessible peaks between human and chimpanzee were specific to neural progenitor cells and neurons, suggesting a unique evolutionary divergence of these cells at the epigenetic level. Using single-nucleus ATAC-seq (snATAC-seq), comparisons of the cell type-specific chromatin landscape of cerebral organoids to primary developing cortex found organoids establish broad cell type-specific enhancer accessibility patterns similar to the developing cortex, but lack many putative regulatory elements identified in homologous primary cell types (Ziffra et al., 2019). Here, the authors utilize single-cell RNA-seq and snATAC-seq separately and perform a coembedding analysis to combine the datasets and make inferences on cell-type specific epigenetic status. These

studies highlight the need for understanding the epigenetic similarities and limitations of brain organoids to facilitate improvement of culture conditions and future disease modelling.

Brain organoids give us for the first time the opportunity to investigate the emergence of cellular diversity and lineage relationships in human tissue. With scRNA-seq, several computational approaches have been developed to infer lineage relationships based on transcriptomic high-dimensional data. A detailed comparison of these methods and guidelines for users was recently provided by Saelens et al. (2019). In brain organoids, several papers have implemented different iterations of the Monocle package to construct pseudotime trajectories (Camp et al., 2015, Marton et al., 2019, Velasco et al., 2019, Ziffra et al., 2019, Sloan et al., 2017). As these computational approaches rely on making inferences, combining single cell sequencing with lineage tracing systems would improve the robustness of future cell lineage findings and allow for direct tracing of neuronal ontogeny within organoids. Broadly speaking, lineage tracing techniques can be categorized by their tagging system. Currently cell ontogeny can be traced based on somatic mutations, reporter systems, cell barcoding, and dynamic lineage tracing (Frieda et al., 2017, Yao et al., 2017, Alemany et al., 2018, Raj et al., 2018, Spanjaard et al., 2018, McKenna and Gagnon, 2019). A detailed description of the advantages and considerations of emerging lineage tracing approaches that can be combined with scRNA-seq are reviewed by Wagner and Klein (2020). Recently, scRNA-seq has been combined with an inducible CRISPR/Cas9-based barcoding system to reconstruct dynamic lineage trees within microdissected self-patterned organoids for the first time (He et al., 2020). This elegant approach, called iTracer, integrates the sleeping beauty transposon system (Ivics et al., 1997) with an inducible iCRISPR system (Gonzalez et al., 2014). Using this approach, progenitor-neuron lineage relationships were reconstructed revealing a clonal accumulation bias of iPSCs towards distinct brain regions. We envision that similar approaches will emerge and become commonplace for the analysis of patterned organoids in healthy and disease states.

Similarly, just as lineage tracing can be leveraged to determine neuronal ontogeny, spatial transcriptomics will allow for the probing of cellular spatial relationships during human brain organoid development. Several techniques that incorporate fluorescence *in situ* hybridization have emerged to identify and spatially resolve specific transcripts at the single cell level (Chen et al., 2015, Codeluppi et al., 2018, Salmen et al., 2018, Wang et al., 2018, Eng et al., 2019). Recently, the 10X Visium platform was used to perform spatial transcriptomics of iTracer (barcoded and scarred) cells within cerebral organoids (He et al., 2020). This analysis confirmed previously discussed findings of iPSC clonal bias in distinct brain organoid regions and marks the first use of spatial transcriptomics in the brain organoid field. The future incorporation of spatial registration combined with transcriptomic and epigenomic techniques will be critical in the analysis of brain organoids as they do not reproducibly recapitulate *in vivo* stereotypic anatomy.

To date large-scale, unbiased proteomic analysis has not been performed on brain organoids at single cell resolution. With the use of proteomics, researchers will no longer have to rely upon mRNA to determine expression of cell identifying marker genes. Strategies to profile the proteome of individual cells are starting to emerge (Doerr, 2019) and will provide additional cellular readouts in healthy and diseased organoids. The addition of emerging

proteomics with current unbiased -omic modalities to brain organoid analysis will allow for the molecular, phenotypic and functional characterization of single cells. Though there are limitations to any single technique, the characterization of organoids will undoubtedly utilize complementary methods to compensate for their individual shortcomings (Figure 4). As sequencing technologies have advanced, several groups have been able to simultaneously perform multiplexing or multiple-omic assays at the single cell level. This represents a powerful new era in single cell technology wherein additional layers of information can be measured in a single experiment. This is of importance for brain organoids as the fidelity of *in vitro* cells types to endogenous tissue has been questioned at the transcriptomic, epigenetic, and spatial level. Thus, performing multiple measurements from a single cell will provide an accurate portrayal of molecular underpinnings in organoids.

4.2 Functional Assessment of Neural Activity in Brain Organoids

Refinement of culture conditions has allowed for the protracted development of organoids and opened the door to understanding the functional physiological properties of neurons within organoids. Dendritic spines and structurally defined synaptic connections have been identified in both patterned and self-patterned protocols suggesting that 3D cultures favor the development of structural features characteristic of mature neurons. Given the presence of these mature neuronal populations, it is unsurprising that brain organoids can develop spontaneous neural network activity as evidenced by calcium fluxes as well as intracellular and extracellular recordings (Mariani et al., 2015, Muguruma et al., 2015, Paşca et al., 2015, Sakaguchi et al., 2015, Jo et al., 2016, Qian et al., 2016, Birey et al., 2017, Quadrato et al., 2017, Cakir et al., 2019, Marton et al., 2019, Song et al., 2019, Qian et al., 2020). Whole-cell patch-clamp, voltage clamp, and current clamp have been utilized extensively to characterize neurons in organoids, typically in sliced cultures or organoid edges where functional connectivity between neurons has been detected (Mariani et al., 2015, Muguruma et al., 2015, Paşca et al., 2015, Sakaguchi et al., 2015, Qian et al., 2016, Birey et al., 2017, Xiang et al., 2017, Marton et al., 2019, Smits et al., 2019, Song et al., 2019, Xiang et al., 2019, Qian et al., 2020). Though intracellular patch-clamp provides important information on the electrical properties and functional connectivity of neurons, it is not a high throughput approach, which is necessary to comprehensively characterize the complex neuronal network behaviour for the large number of neuronal subtypes present in organoids. The standard microelectrode arrays (MEAs) approach for extracellular recordings of *in vitro* assays uses planar electrodes arranged in grids. While these 2D MEAs are useful for detecting spikes in mono-layers and have been used extensively in the organoid field, they are limited to capturing the activity of neurons in the outer layers of the organoid (Giandomenico et al., 2019, Schmunk et al., 2020). High density silicon microelectrodes are an emerging technology that allows for insertion of probes into 3D tissue and can provide single cell resolution by leveraging the simultaneous recordings of each ‘spike’, across multiple spatially distributed channels, to improve the clustering of ‘spikes’ that originate from the same neuron. Simultaneously, it is also possible to record network burst activity indicated by recordings of neurons in multiple probes that fire coordinated bursts of action potentials in a temporal manner. In Quadrato et al (2017) this technology has been successfully used to characterize network behaviour in 8 months old self-patterning brain organoids. However, this technology is difficult to implement for high throughput

experiments that require simultaneous recording of multiple organoids. Several studies have used calcium indicators to record transient calcium waves that arise during depolarization (Lancaster et al., 2013, Sakaguchi et al., 2015, Birey et al., 2017, Lancaster et al., 2017, Xiang et al., 2017, Park et al., 2018, Kim et al., 2019, Xiang et al., 2019, Samarasinghe et al., 2019). Calcium imaging is more amenable to large scale studies as compared to MEA technology and represents a powerful tool for the extraction of temporal information on neuronal firing patterns. However, currently there is no effective method to monitor the activity of a large number of neurons over long periods of time in organoids although this has been accomplished *in vivo*. Typically, imaging is performed at the organoid surface, yet with two-photon and light-sheet microscopy it is possible to acquire data from deeper organoid layers and eventually perform 4D imaging (Chen et al., 2014, Schoneberg et al., 2018, Lavagnino et al., 2016). Lineage tracking of early formed embryoid bodies (EBs) was recently performed using 4D light-sheet microscopy to visualize how single nuclei give rise to spatially restricted daughter cells, which only migrate short distances from parent cells (He et al., 2020). The development of miniscopes to perform long-term calcium imaging in freely moving rodents, suggest the exciting possibility that similar approaches could be adapted to perform much needed long-term recordings of neuronal activity in organoids (Gonzalez et al., 2019). The major drawback compared to other techniques result from the decaying dynamics of the calcium indicators and resolving signal over noise, which becomes increasingly difficult to distinguish at single-cell resolution as neuronal firing rates increase. A powerful yet low-throughput technique that enables correlation of transcriptomic and functional data at the single cell level to simultaneously combine functional and molecular profiling of neurons is Patch-seq (Cadwell et al., 2016, van den Hurk et al., 2018), which combines whole-cell patch-clamp recordings with scRNA-seq and morphological characterization. This recently developed method, in which a single neuron can be targeted by a microelectrode, recorded for its electrical function and thereafter aspired and prepared for sequencing, is critical for investigating how electrophysiological properties in neurons correlate to cell subtype specific transcriptomes. Application of this powerful technique to the analysis of brain organoids will provide the field with much needed understanding of the molecular determinants of human neuronal diversity and it will facilitate and improve the classification of neural cell types across different stages of neural development in a human cellular context. This knowledge will also be pivotal for the cell-type specific characterization of disease states.

4.3 Tracing Neural Connections in Brain Organoids

The wiring diagram of distinct or fused organoids has not been traced so far. Here we review established and emerging techniques that could be used to elucidate connectivity in this complex system. The traditional gold standard of electron microscopy provides a high-resolution view of cellular structural features including the connection points between neurons, synapses. Additionally, the use of scanning electron microscopy (SEM) on ultra-thin brain sections has allowed large volumes of brain tissue (several cubic millimeters) to be reconstructed (Witvliet et al., 2020, Hildebrand et al., 2017), and has been used for 3D reconstructions of synapses and dendritic spines in brain organoids (Quadrato et al., 2017). However, this technique is not well suited for high-throughput studies or to trace multiple long-range connections. Additionally, EM-based techniques such as SEM are performed on

fixed tissue, limiting their use in a multimodal analysis with functional assays and genetic manipulations.

Viral-based retrograde (rabies, pseudorabies) and anterograde (HSV-1 strain H129) trans-synaptic tracers have been extensively used to elucidate connectivity in mammalian model systems (Dix et al., 1983, Strack et al., 1989, Zemanick et al., 1991, Ugolini, 1995, Aston-Jones and Card, 2000). Though initially developed using wild-type replication-competent viruses, these tracers have been genetically modified to improve their neurotropism, specificity, and safety. Though neurotropic viral tracers have been extensively used in neurobiology, recent evidence suggests that different retrograde viral strains have biased neurotropism and altered degrees of neurotoxicity (Sun et al., 2019). It is currently unclear how widespread this bias in tropism is among neurotropic viruses. As viral tracers result in neurotoxicity that increases with exposure time, the technique is difficult to implement with additional functional analyses. Currently efforts to reduce viral-mediated cytotoxicity are being made which can potentially allow the technique to be used in conjunction with other downstream analysis (Chatterjee et al., 2018).

Another set of tracing techniques being developed are the highly amenable ligand-induced intramembrane proteolytic cleavage-based systems (Huang et al., 2016, Morsut et al., 2016, Inagaki et al., 2012, Jagadish et al., 2014, Huang et al., 2017). These tracing approaches utilize ‘sender’ cells expressing an artificial ligand to bind and activate ‘receiver’ cells expressing an artificial receptor to perform trans-synaptic tracing. A detailed description of this mechanism and the various methods of implementation for synaptic tracing are reviewed in (Lee et al., 2017). To date, studies using this system have primarily been done in 2D cultures and model systems such as Drosophila. With additional engineering in hPSCs, these systems allow the capability of ligand-receptor induced trans-cellular expression of a transgene allowing for easily combining tracing with additional analyses. One potential caveat to using a ligand-induced intramembrane proteolytic cleavage-based system is the considerable amount of optimization required to ensure there is minimal leakiness of expression/activation of the receiver cells. The background signaling must be kept minimal to ensure the error of making a false-positive is kept low. Both viral-based and synthetic engineering-based tracing systems have the potential to be combined to assess circuit connectivity in organoids as well as in conjunction with spatial transcriptomics. This combination will allow for interrogation of dynamic cell-cell interactions with single cell resolution.

5. Concluding Remarks

Human brain organoids have revolutionized the field of developmental neurobiology by providing a tractable system to interrogate human specific aspects of neurodevelopmental processes in normal and diseased states. While the use of inhibitors of the WNT and SMAD pathways has improved cortical organoid-to-organoid reproducibility, their use might be problematic. Core components or modulators of WNT and SMAD signaling are causative of neurodevelopmental disorders (NDDs) (Kumar et al., 2019) and their inhibition by patterning molecules may affect disease phenotypes and drug screening. In addition, external patterning might negatively affect the survival and differentiation of other cell

types, including microglial cells and endothelial cells that have recently been successfully integrated into brain organoids. Thus, alternative strategies to increase brain organoid reproducibility that do not interfere with key signaling pathways must be established for organoids to accurately model disease. The confluence of other disciplines including, tissue engineering and synthetic biology onto brain organoid generation will be required to achieve reproducibility in the absence of patterning, and surmount the obstacles surrounding the ability of organoids to achieve more mature cellular states. Tissue engineering approaches will be key to establish a controlled microenvironment and for engineering reproducible anatomical features. In addition, instructing organoid maturation through synthetic control of gene regulatory networks that govern cellular maturation across multiple lineages is an interesting concept for brain organoids and has already been successfully implemented in liver organoids (Velazquez et al., 2020). Improving brain organoids may also depend on biophysical mechanisms as kidney organoids showed improved vascularization and maturation when subjected to the mechanical shear stress forces within a microfluidics device that mimics fluid flow during *in vivo* development (Homan et al., 2019). Pursuing this logic, culturing 'integrated' organoids that contain vasculature-like structures within a fluidic device may aid in the maturation of the vascular network and neural populations. Recently, extracellular recordings of primary neuronal cultures have been performed as they differentiate and develop within a microfluidic device (Lam et al., 2019), thus demonstrating how the integration of tissue engineering approaches could be exploited to perform much needed functional longitudinal analyses of the organoids as they develop. As organoid generation protocols continue to be improved and refined, it is important that the technology used to analyze them is equipped to extract finely resolved data that encompasses the full potential and limitations of the system. Finally, we envision that the multidisciplinary integration of the emergent technologies highlighted in this review will inform the further improvement of organoid generation protocols in a continuous cycle of growth that will eventually result in "upgraded" brain organoids capable of more accurately modeling complex developmental processes.

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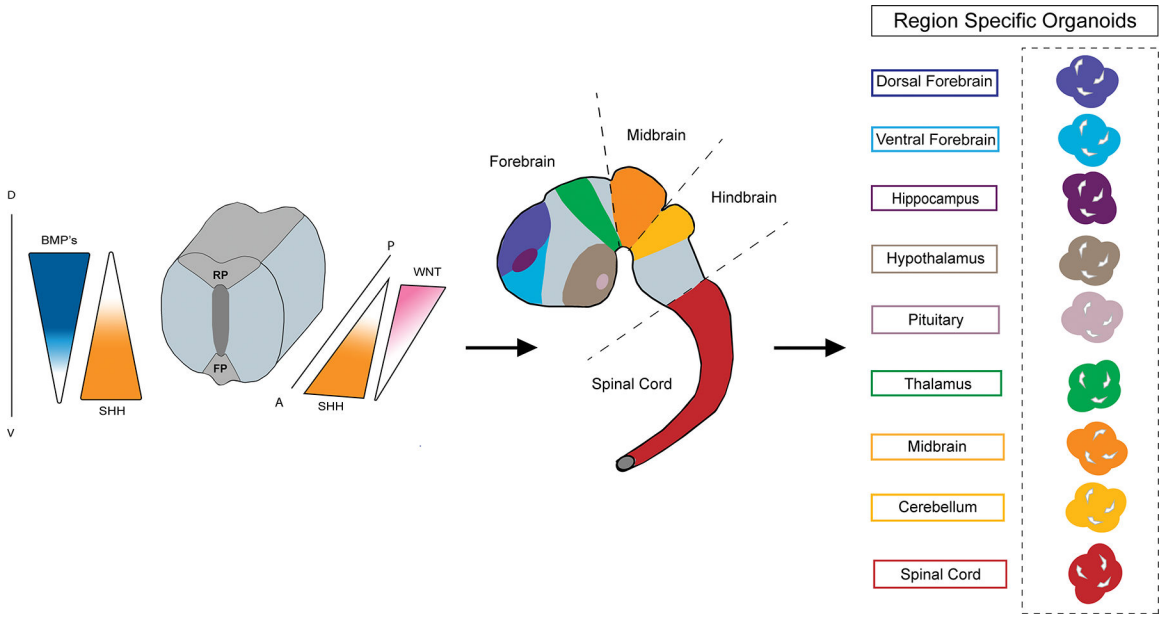

Figure 1. Development-inspired strategies to recapitulate early human brain regionalization.

Morphogen gradients within the neural tube are critical in determining the rostro-caudal and dorsal-ventral axis of the developing brain and contribute to the formation of discrete regionalization. Several key signaling centers present in the neural tube including the Roof Plate (RP) and Floor Plate (FP) are a prominent source of BMP (bone morphogenic protein), WNT, SHH (sonic hedgehog), RA (retinoic acid) and FGFs (fibroblast growth factors) respectively. Patterning strategies have allowed for the establishment of protocols resembling distinct brain regions including the dorsal and ventral forebrain, hippocampus, hypothalamus, anterior pituitary, thalamus, midbrain, cerebellum, midbrain, spinal cord and choroid plexus. The bottom panel is a simplified description of the patterning strategies leveraged to create the region-specific brain organoids previously mentioned. The red asterisk indicates that a variation of SMAD inhibition (TGFb with or without BMP inhibition and or WNT inhibition) was employed to direct towards an initial neuroectodermal fate.

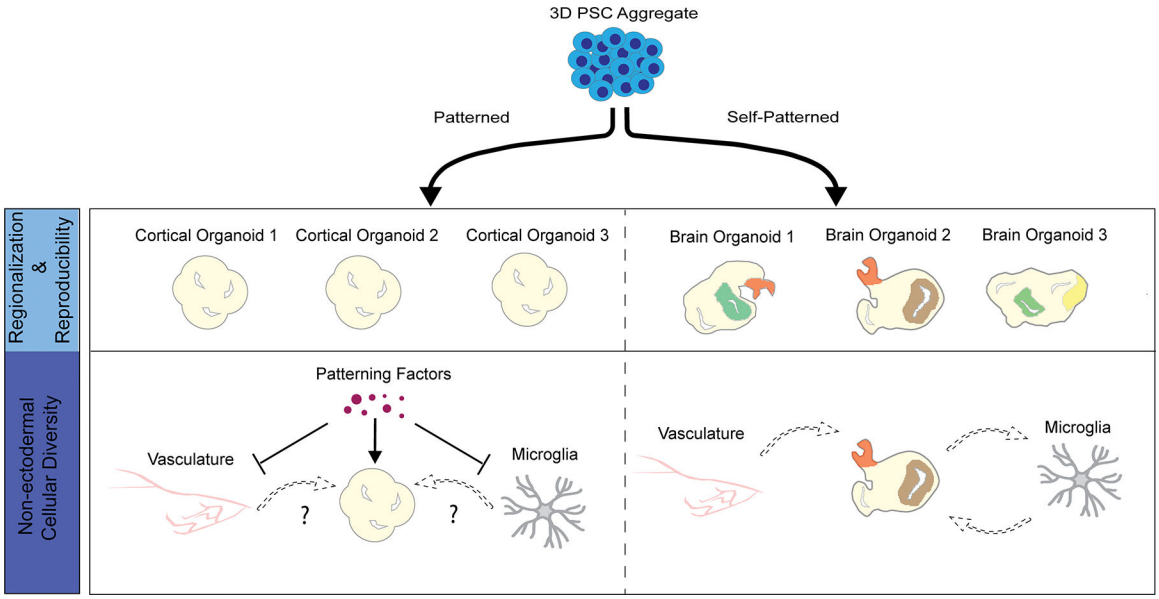

Figure 2. Comparison of patterned and self-patterning approaches in generating human brain organoids.

Protocols rely on either the use of morphogen driven patterning strategies to generate organoids reminiscent of a specific brain region, or the innate ability of aggregated pluripotent stem cells (PSC's) to establish self-patterning morphogen gradients that drives neuroectodermal identity and produce organoids reminiscent of the whole brain. While organoids patterned towards the cortex have been shown to reproducibly generate cortical neurons in similar ratios, self-patterned whole brain organoids contain diverse but heterogeneous brain regions (top panels). For example, in patterned cortical organoids, all non-ectodermal cell types including microglia are omitted. In addition, the heavy patterning employed at early stages of organoid generation in these protocols may affect the integration of microglial and vascular cells into these cultures at this stage. Self-patterned brain organoids, on the other hand, allow for the spontaneous generation of microglial populations and due to the lack of exogenous morphogens, permit integration of non-ectodermal cell types (bottom panels).

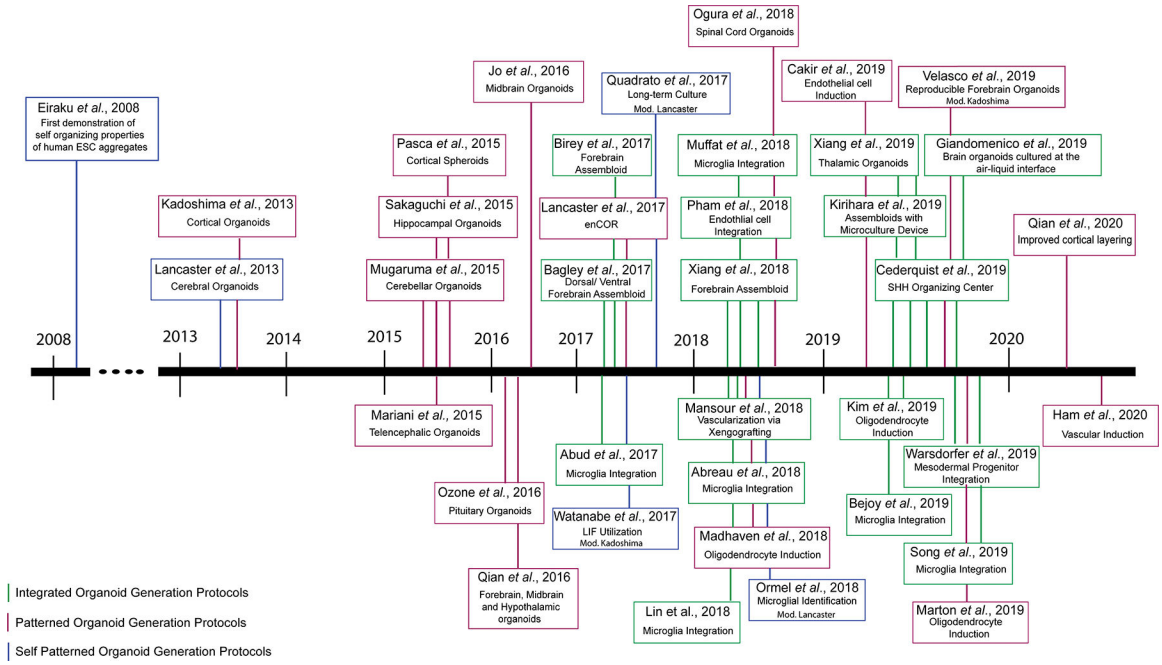

Figure 3. Protocols that have impacted the human brain organoid field over the past decade.

The use of brain organoids as a model system has gained incredible momentum over the past decade, leading to the establishment of diverse strategies to generate more complex organoids, and here we have classified as integrated (multiregional, multilineage, or including an artificial signaling center; green), patterned (magenta) or self-patterned (blue).

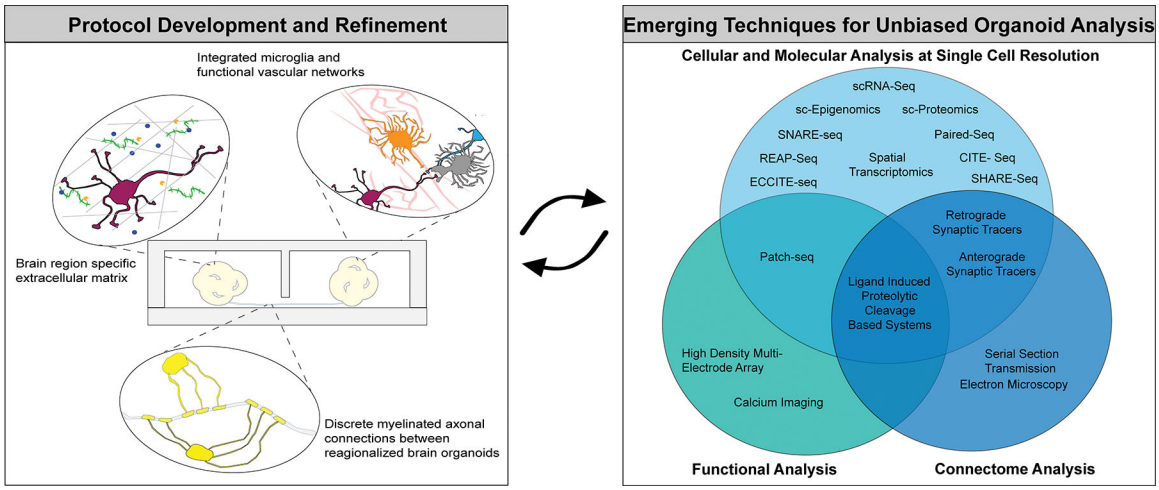

Figure 4. Upgrading the Physiological Relevance of Human Brain Organoids.

Multidisciplinary integration of emerging technologies to improve the generation (Panel 1) and characterization (Panel 2) of human brain organoids.