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Innovations in 3-Dimensional Tissue Models of Human Brain Physiology and Diseases

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Abstract

3-dimensional (3D) laboratory tissue cultures have emerged as an alternative to traditional 2-dimensional (2D) culture systems that do not recapitulate native cell behavior. The discrepancy between *in vivo* and *in vitro* tissue-cell-molecular responses impedes understanding of human physiology in general and creates roadblocks for the discovery of therapeutic solutions. Two parallel approaches have emerged for the design of 3D culture systems. The first is biomedical engineering methodology, including bioengineered materials, bioprinting, microfluidics and bioreactors, used alone or in combination, to mimic the microenvironments of native tissues. The second approach is organoid technology, in which stem cells are exposed to chemical and/or biological cues to activate differentiation programs that are reminiscent of human (prenatal) development. This review article describes recent technological advances in engineering 3D cultures that more closely resemble the human brain. The contributions of *in vitro* 3D tissue culture systems to new insights in neurophysiology, neurological diseases and regenerative medicine are highlighted. Perspectives on designing improved tissue models of the human brain are offered, focusing on an integrative approach merging biomedical engineering tools with organoid biology.

Graphical Abstract

This review outlines current bioengineering and organoid technology approaches to developing 3-dimensional (3D) *in vitro* models of the human brain. Advances in generating representative microenvironments to observe and manipulate cellular behavior are described, with emphasis on opportunities to combine these approaches to generate advanced tissue models that are suitable for modeling human brain development, disease, and injury.

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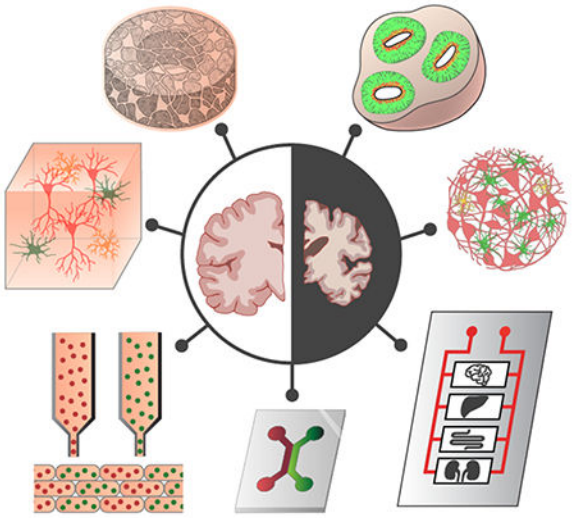

Keywords

bioengineering; organoids; stem cells; neurodevelopment; neurodegenerative and psychiatric diseases

1. Introduction

To understand human brain physiology, the human condition must be studied in a laboratory setting. Animal models, such as rodents, primates, worms and experimental systems such as yeast, have yielded great insights into development, function and diseases of the brain. However, these model systems do not fully recapitulate the complexity of the human brain related to neural circuit architecture, genetics, gene expression, cell-type diversification, lipidomics and proteomics. The cerebral cortex, the part of the brain involved in motor and sensory function and processing information, as well as associated with psychiatric diseases, is less complex in rodents in comparison to primates in general, and to humans in particular.[1–5] Evolution also had its impact on the divergence of genetic make-up of humans compared to other mammals, notably the expression of non-coding RNAs.[6–7] Species-specific gene expression patterns are found in the context of neural circuit function and specific cell-types, and they may be related to the organization of cis-regulatory elements in DNA or stress conditions such as aging.[8–15] Ultimately, they may lead to changes in interactome at the mesoscale, such as lipid profiles or the composition of the synaptic proteome.[16–17] In light of this broad spectrum of species-specific differences on the level of DNA, molecular pathways, cells and brain networks, it should not come as a surprise that the modeling in rodents of complex neurodegenerative diseases and psychiatric disorders pose challenges, in particular for drug and target discovery.[3, 5, 18] Unlike rodent models, access to living human test subjects and primates, or *ex vivo* brain slices derived thereof, is surrounded with ethical issues. Moreover, only a limited number of scientists and clinicians master the necessary expertise in, or have access to, brain imaging techniques such as non-invasive functional magnetic resonance imaging (fMRI) and electroencephalography (EEG), or minimally invasive methods such as the implantation of recording and stimulating

electrodes in the brain. These methods also suffer from low spatial and/or temporal resolution, as well as the need for large patient cohorts and consortia to identify the features of the human brain connectome related to normal aging or disease.[19–22]

The advent of stem cell engineering, both embryonic (ESC) and induced pluripotent stem cells (iPSC) has been a game changer in the study of the human brain. These advancements in cell biology made possible, for the first time, the generation of a renewable source (vis-à-vis terminal explant studies) and propagation of *in vitro* human culture models of the human brain that are scalable and available to the scientific community at large.[23] iPSCs, which are generated from healthy individuals or patient fibroblasts or their peripheral blood monocytes, have demonstrated to be excellent tools to study human neurological diseases. The literature is now replete with examples from Alzheimer's and Parkinson's disease, schizophrenia, autism, epilepsy, pain and other fields.[23] Genome engineering approaches, in particular based on clustered regularly interspaced short palindromic repeats (CRISPR) technology (other examples are Zinc-fingers and transcription activator-like effector nucleases (TALENs)), have made a similar transformative impact. Genetic variants associated with increased (or decreased) risk of disease can now be introduced, with relative ease, in the germ-line of healthy cells to replicate human disease. They can also be removed from the cells as a strategy to correct brain illnesses.[24–28] These cells may be used in traditional *in vitro* mono-cultures grown on flat, 2-dimensional (2D) surfaces, but these experimental cell systems do not replicate the complexity and biology of the human brain. Salient examples of this fact can be found in studies of gene expression signatures, extracellular matrix microenvironment, neural network firing and biomarkers of brain development and disease.[29–34] This realization has spurred the development of 3-dimensional (3D) laboratory cell and tissue models that aim to better replicate the cellular composition, microenvironment and architecture of the brain.

We will discuss two different techniques that have become popular for designing 3D brain-like tissue structures. The first is the use of organoids (grown in bioreactors or otherwise), dense multicellular tissues differentiated from stem cells following their innate developmental cues.[35–37] The other is bioengineering, a term collectively used to describe methods that make use of a bottom up strategy, involving seeding (multiple) cells (types) in biomaterial scaffolds and/or hydrogel environments, including those generated by 3-dimensional (3D) printing, and the use of bioreactors such as microfluidic devices or perfusion devices.[38–39] These two complementary approaches have matured to the stage that we now see bioengineering techniques being used in a complementary fashion to improve on organoid cultures.[40] We will conclude with future perspectives on generating more complex *in vitro* tissue models of the human brain that integrate organoid and bioengineering technology, with a vision towards high-throughput screening systems for biomarker and therapeutic discovery.

2. *Ex Vivo* and 2D *In Vitro* Platforms to Study the Brain

Much of our fundamental understanding of the brain is derived from traditional *in vitro* culture systems. Through extensive research studies of postmortem brain tissues as well as isolated cell cultures in 2D, many key features of brain physiology and mechanisms of

disease have been identified. This knowledge serves as the basis for generating 3D models of the brain, and thus we begin by reviewing the literature on traditional *in vitro* systems to provide context to the sections describing 3D brain models.

2.1. Human postmortem brain tissues

Historically, preliminary attempts to understand the structure and function of the brain, the most complex organ in the body, have relied on the use of postmortem tissues. In a classic example from the 1860's, the French physician Pierre Paul Broca found lesions in the same area of the postmortem brains of twelve patients with impaired language skills, providing the first evidence linking particular brain regions to specific functions.[41] Since then, many other key discoveries have been made by examining and identifying the hallmark anatomical changes in postmortem brains associated with patients who suffer from neurological dysfunctions such as Alzheimer's disease (AD; loss of neurons in cortex and subcortical regions) and Parkinson's disease (PD; loss of dopamine neurons in the *substantia nigra* of the midbrain). Due to advances in technological capabilities, postmortem brain studies continue to be an indispensable tool to study human brain function and disorders through the ability to observe and analyze tissue structures, cellular morphologies, and protein and gene expression.[42] As an example, the examination of AD patient brain tissues has been used to identify what are now described as the two classical molecular hallmarks of the disease - extracellular accumulation of beta-amyloid (Aβ) plaques and intracellular aggregation of hyperphosphorylated tau proteins in neurofibrillary tangles.[43] More recently, transcriptomics analysis and single cell-RNA sequencing from AD brain tissues have identified region-specific and cell-type-specific expression patterns.[43] By taking advantage of new technologies such as single-cell RNA sequencing, a reduction in excitatory neurons in the AD-gene carrier but not in unaffected family members or sporadic cases has been found, indicating differences in disease mechanisms between familiar and sporadic forms of the disease.[44] Similarly, combinations of postmortem studies detailed the molecular changes in the PD midbrain, including abnormal levels of α-synuclein production and aggregation, reduction in dopamine synthesis, increased oxidative stress, mitochondrial dysfunction, and reduction in neurotrophic factors essential for cell survival.[45] A recent study closely examined a small number of postmortem brain tissues of patients who received deep brain stimulation therapy, an approved surgical intervention alternative to medication. The results suggested recovery of mitochondria numbers and volume as underlying mechanisms of neuroprotection.[46]

In a recent and high-profile example of identifying neurodegenerative disease through examination of postmortem tissues, the study of brains subjected to repeated injuries, particularly from American football players, has led to the identification of chronic traumatic encephalopathy (CTE). Using postmortem brain tissues, researchers were able to define the distinguishable molecular feature of perivascular tau protein pathology. A postmortem study on four teenage athletes' brains during the acute and subacute (1 day – 4 months) phase post brain trauma showed microvascular damage, neuroinflammation, axonal injuries, and tau pathology – indication of early stage of CTE.[47]

Postmortem brains have similarly been used to study neurodevelopmental and neuropsychiatric disorders, such as autism spectrum disorder (ASD), schizophrenia, and bipolar disorder. These disorders are particularly challenging as the majority of cases have no known genetic cause. To begin to address this issue, hundreds of postmortem brains from ASD patients and control cohorts were recently investigated, with allelic imbalance (expression favors one allele) reported in two specific regions associated with small nucleolar RNA.[48] More recent studies examining large numbers of tissue samples and data sets have identified the association of neuroinflammation with mental illnesses, such as schizophrenia and bipolar disorder.[49–51]

As evidenced above, the precise genetics, cell types involved, and pathogenesis are not well understood for most brain disorders. Phenotype heterogeneity in presentations and confounding factors such as sex, age, medications, and environmental factors add to the difficulties in investigating brain disorders.[52]. Also limiting is the availability of postmortem brain samples. Recently, successful efforts to increase awareness for brain donations has made possible the building of networks of brain tissue repositories and databases. The NIH NeuroBioBank (NBB) networks six brain tissue repositories, with the largest one storing over two-thousand brain samples, in order to facilitate the collection and redistribution of high quality brain tissues in the United States.[53]

Despite the high level of relevance to human brain disorders, postmortem brain studies are limited in their ability to provide information on dynamic, temporal changes of disease progression. Furthermore, from the perspective of disease prevention or treatment, molecular and cellular changes may occur years prior to clinical presentation of symptoms of the decline in cognitive or motor skills[54] and researchers cannot control the genetic background or the external insults to the study objects. Thus, the need for well-controlled laboratory models are essential for probing the human brain (Figure 1).

2.2. Organotypic slice culture from animals

In contrast to human post mortem tissues, *in vitro* organotypic slice cultures from animals allow for the control of genetics, sex, age, external insults, and reduction of confounding factors. The major advantage of *in vitro* organotypic slice cultures is that they provide investigators access to the cells while preserving native cell types, ECM, cell organization, tissue architecture, and local synaptic connections. In addition, multiple tissue samples are generated from each brain, reducing the number of animals needed. In typical organotypic slice studies, brain slices are prepared with a thickness of 100-400 μm from acutely sacrificed animals.[55] These slices can be used in acute experiments, in which they are analyzed immediately after dissection to provide information from a near-*in vivo* state, or cultured in media for longer term studies (days to months) for the purpose of studying cell behavior over time after *in vitro* stimuli. A recent study compared the level of membrane associated tau protein vs. tau protein released into the media in brain slice cultures from wild type and AD model mice over the duration of 1 month.[56] A higher level of tau protein was released from the AD slices and, unlike in wild type slices, the release did not respond to neuronal stimulation. These results suggested differences in the mechanisms in tau protein release and spreading in AD.[56]

Organotypic slices are particularly useful when the tissue structure and organization are critical to the study outcome, such as in traumatic brain injury (TBI), cancer biology or neural circuit structure and functional activity. The tearing and shearing of the soft, connected brain tissue as a consequence of TBI, is not sufficiently recapitulated by single cells cultured on conventional 2D hard plastic surface, but can be mimicked by mechanically stretching the brain slices. Complementary to *in vivo* mouse models, slice culture allows for the tracking of cell death, tissue injury, inflammation and changes in neural network activity over time.[57–58] Brain slice models of TBI also provide a useful tool for screening therapeutic strategies, and several candidates have already been identified, such as hypothermia,[59] inert gases,[60] sodium calcium exchanger blockers,[61–62] and glutamate receptor blockers.[63] Although, hypothermia has been used in the treatment for traumatic brain injury, a recent review did not identify improvement in clinical outcomes.[64]

Because tumor metastasis to the brain and brain tumor invasiveness directly correlates to poor prognosis, many researchers study tumor cell migration *in vitro*. The majority of these studies, however, utilize 2D scratch or transwell assays that capture few of the actual *in vivo* interactions between tumor cells, host cells and ECM, or the cellular heterogeneity of the cancer. Moreover, the majority of organotypic slice studies use rodent brains, and therefore they face the same drawbacks of lacking proper human-equivalent models as other brain disorders. There are slice culture models that attempt to address these critical issues by employing the implantation of human tumor cell lines in the form of single cells or spheroids on rodent brain slices. This made it possible to examine in detail the interactions between glioblastoma cells and the vasculature, identifying surface protein targets and small molecule candidates to reduce tumor invasion or migration. Further, this approach provided insight into changes in gene expression in reactive astrocytes, turning the microenvironment to become pro-metastasis.[65–67]

In any slice culture, one of the key limitations is the age-dependency of slice survival and the resulting impact on study design. Slices have high viability when prepared from embryonic animal brains, but at the same time contain immature cell and phenotype. Slices from postnatal animals provide a higher level of cellular and tissue maturation than from embryonic animals and are more resistant to the mechanical trauma from slice preparation (up to months).[68] Adult brain slices, including from animals and from human biopsy and postmortem tissues, historically have a poor survival rate, but improvements have been made by adding neurotrophic factors, the addition of cerebrospinal fluid in the medium, or media flow systems to enhance nutrient and oxygen exchange.[69–72] However, it should be noted that slice preparation induces significant mechanical trauma to the tissue, which can cause the tissue to have injury-like responses that may affect the outcome of the experiment and introduce artifacts to interpretations into brain functions.

2.3. Two-dimensional cell culture systems

Culturing dissociated cells *in vitro* offers the advantages of optical transparency, substrate consistency, easy handling, robustness, reproducibility, relatively low cost, compatibility with electrophysiology techniques and high-throughput screening. In conventional 2D culture, dissociated cells are cultured on plastic or glass surfaces coated with laminin,

fibronectin, Matrigel, poly-lysine, and/or poly-L-ornithine for adhesion.[73] In comparison to the abovementioned models, cell culture allows for controlled co-cultures with different cell types and/or cells with different genetic backgrounds (Table 1).

To date, investigating the human brain *in vitro* has been hindered by the inaccessibility to live human brain tissue and the extreme difficulty of isolating and culturing primary human neurons. Historically, rodent embryonic or postnatal neuronal culture has been used for studying neuronal functions and to model diseases. For these studies, the neurons are often genetically modified or cultured in the presence of toxins or disease-associated protein, such as Aβ for Alzheimer's disease. Given that the underlying etiologies are not well understood for many human neurodevelopmental, neuropsychiatric, and neurodegenerative disorders, there is a lack of appropriate rodent models and the subsequent results from rodent cell culture often fail to translate to the human condition. A major breakthrough towards this goal was the method of reprogramming somatic cells to iPSCs, developed by Takahashi and Yamanaka in 2006, achieved by overexpressing transcription factors Oct3/4, Sox2, Klf4, and c-Myc in fibroblasts. Since then, many protocols have been developed for the direct differentiation of iPSCs or the transdifferentiation into relevant brain cell types, including cortical neurons, striatal dopamine neurons, astrocytes, microglia, oligodendrocytes, pericytes, and brain microvasculature endothelial cells.[74–75] The use of iPSCs from individuals affected by neurological disorders presents an unprecedented tool to unveil the underlying genetics, mechanisms, and pathogenesis associated with these disorders. Within the past decade, iPSC-derived neurons have provided novel insights on the etiology and demonstrated opportunities to screen for therapeutic interventions.

Due in part to the fact that AD is the most prevalent neurodegenerative disorder, many iPSC lines have been derived from AD patients, and their healthy family members, for the study of monogenetic and sporadic forms of the disease, as well as novel candidate genetic risk factors identified in genetic analysis of patients.[75–78] Studies have shown that human iPSC (hiPSC)-derived cortical neurons carrying familial Alzheimer's disease (fAD) mutations presented elevated levels of Aβ42 – the main component of Aβ plaque, as well as pathological tau protein phenotype and neuroinflammation as seen in patients, demonstrating the utility of iPSCs as a preclinical model of the disease.[79–81] Treatment with pharmacological compounds targeting pathways related to Aβ production and cleavage reduced AD pathology, supporting the feasibility of iPSC models as *in vitro* platforms for identifying therapeutic interventions.

These approaches have been used to study aspects of other neurodegenerative disorders such as Parkinson's or Huntington diseases. One of the hallmarks of Parkinson's disease is death of midbrain dopamine neurons and the formation of α-synuclein protein aggregates. Several different types of PD models have been established, either by treating healthy iPSC-derived dopamine neurons with α-synuclein aggregates or by generating DA neurons from familial PD (fPD) and sporadic PD (sPD) patients. iPSC lines derived from rare fPD cases have helped in the validation of novel risk genes.[82] One study compared neurons derived from sPD and fPD iPSCs, interestingly finding epigenetic dysregulation in both groups with DNA methylation patterns differing from healthy dopamine neurons and hypermethylation in regions relevant to PD.[83] For the study of Huntington disease, a hereditary, monogenic,

neurodegenerative movement disorder caused by an expansion of CAG repeats in the huntingtin (HTT) gene, iPSC models have been used to identify the genetic and cellular mechanisms of disease progression. A recent study of patient iPSC-derived cortical neurons found modifications in transcriptomics, morphology, and maturation, suggesting altered corticogenesis.[84] Another study detected astrocyte dysfunction in a HD-iPSC co-culture model, suggesting contribution of glial-neuron interactions in neuronal death and the importance of studying multiple cell types in a disease model.

Since the timeline for iPSC differentiation and maturation into neurons or other cell-types follows the same trajectory as in the developing embryo, these cells are particularly useful tools to study neurodevelopmental disorders such as autism spectrum disorders (ASD). Several iPSC lines have been established from monogenic ASD-iPSC (e.g., Fragile X syndrome, Rett syndrome, Timothy syndrome, Angelman syndrome, and Phelan-McDermid syndrome) as well as from idiopathic ASD patients. Investigators use these ASD-iPSC-neurons to study cell proliferation, excitatory and inhibitory balance, synapse connectivity, neural networks, epigenetics, and proteomics.[85] Many of them showed upregulation of inhibitory neuron genes, deficits in excitatory synaptic transmission, reduction in neurite length and synaptogenesis, indicating the relevance of abnormal network formation in ASD.[85]

The development of cells and 2D cell culture models has led to tremendous discoveries in the field. Despite this insight, the main drawback of 2D cell culture remains that they are oversimplified and lack the complexity and organization of the *in vivo* microenvironment (Figure 2). As an example, the architecture of the original neural networks is lost when neurons are dissociated into single cells. Furthermore, cell density in 2D culture is typically much lower than that found *in vivo*, with cell-cell interactions limited to the XY directions. The local cellular environment is also altered in 2D as typical cell culture substrates are several orders of magnitude more rigid than brain tissues, and the dynamics of nutrient, oxygen, and secreted factor transport are much different in open media than in 3D tissues. As an example, an AD model with iPSC-neurons cultured in 2D were unable to generate Aβ plaques as efficiently as in 3D because the secreted Aβ freely diffused away.[86] Because many environmental factors can significantly impact cell behavior and thus disease mechanisms, the development of 3D tissue model systems are actively pursued.

3. 3D Brain Organoid Models

3.1. Organoids as an emerging technology for analyzing the human brain—

All methods for generating organoids rely on the innate properties of stem cells to develop and self-organize into 3-dimensional architectures that share similarities with native tissues. Stem cells are uniquely endowed with the capacity to self-renew, differentiate and mature into virtually any type of cell.[23] As such, ESCs and iPSCs are ideal substrates for bioengineering of organoids. The first organoid systems generated modelled peripheral (i.e., not the central nervous system) tissues using mouse, and then human, cells. Salient examples derive from the retina[87–88] and the anterior pituitary,[89] two tissue types composed of neural and non-neural cell-types, and the intestine.[90–92]

The first protocols for engineering brain organoids made use of embryoid bodies generated from hiPCSs and grown on mouse embryonic fibroblasts. These cells were then embedded in Matrigel droplets and cultured for prolonged time (up to 10 months) in a spinning bioreactor. The resulting cerebral organoid resembled the characteristics of the human cortex, including the multilayered structure and inner and outer sub-ventricular zones, which house progenitor cells and (outer) radial glia. This type of glia plays an important role in human evolution, driving the expansion of the human brain compared to other primates. Generated from hiPSCs, the emerging 3D systems were heterogenous, also showing characteristics of meninges, choroid plexus and retina structures.[35, 93] A related approach relied on low-adhesion plates instead of bioreactors, and embryonic stem cells rather than iPSCs.[37]

Subsequent large-scale RNA sequence analysis of thousands of single cells have expanded on the notion that organoids give rise to a broad range of cell-types, such as neuroepithelial cells, photoreceptors and dopamine neurons.[94] Gene expression, epigenetic and image analysis studies further indicate that the maturing organoids follow the normal pace of development of the human fetal brain,[95–98] adhering to species specific rules of evolution.[99–101] As such, organoids have become exciting tools to examine the evolutionary cues that shape development of the brain, in particular for studies inspired by genetic analysis comparing humans to Neanderthals, chimpanzee or other animals.[102–105]

3.2. Optimizing differentiation protocols for organoid cultures

Organoid technology is often associated with limitations related to reproducibility and heterogeneity between organoids from the same or different batches as well as necrosis. As a consequence, the implementation of organoid cultures to study brain development and function is challenging. To overcome these hurdles, several groups have developed the next generation of protocols for culturing organoids that are more homogenous. Commonly referred to as ‘directed differentiation’, these methods make use of cocktails of chemicals and trophic factors, either alone[106] or in combination with low-adhesion plates, and custom-built miniaturized bioreactors or 3D scaffold environments.[107] With these techniques, it is now possible to guide the development of iPSCs towards specific sublineages, such as forebrain, midbrain or hypothalamus.[98, 108–117] The success of these efforts in achieving more reliable organoid differentiation was demonstrated recently in the testing of multiple iPSC lines, independent differentiation, and analyzing hundreds of thousands of single cells and in 96-well plate format assays.[115, 118–119]

3.3. Organoids as a tool to study neurodevelopment, disease and evolution

The power of organoid technology for characterization of neurological disease signatures was first demonstrated in a patient population stratified based on clinical record but with no knowledge of the genetic underpinnings. Focusing on idiopathic forms of autism spectrum disorder (ASD), differences in cell proliferation, neural differentiation and synapse biology were observed in organoids of iPSC selected from patients with macrocephaly phenotypes compared to healthy controls. With ‘omics’ analyses, changes in a forkhead box G1 (FOXG1) transcription factor dependent signaling was found associated with an imbalance between gamma-aminobutyric acid (GABA)-ergic and glutamatergic neurons,[108] often

cited as one of the underlying culprits of ASD.[120] Interestingly, aberrant FOXG1 signaling is also implicated in Rett syndrome, a rare neurodevelopmental disorder mainly affecting girls.[121–123] Organoid studies link microRNA pathways to altered extracellular signal-regulated kinase (ERK) and serine/threonine-protein kinase (AKT) signaling pathways involved in neurogenesis in patients with Rett syndrome.[124]

A unique approach to the study of human (brain) development is the comparison of our genome to closely related species, such as Neanderthals and the chimpanzee. Such comparative analysis led to an intriguing organoid study of NOTCH signaling and regulators of the PI3K-AKT-mTOR pathway in radial glia, implicating the rapid evolution of the larger human cortex to loss of genome stability and ensuing neurodevelopmental disorders, such as 1q21.1 deletion and duplication syndrome.[103, 125] Related studies point to the importance of specific sets of chromatin enzymes, gene networks regulated by long non-coding RNAs and enhancers involved with brain development,[126] with a potential link to autism. [105, 126–128]

Organoids have been used to successfully study a wide range of neurological disease such as hereditary spastic paraplegia, periventricular heterotopia, a disease associated with intellectual disability and dysfunction of cadherin adhesion molecules[129] and autoimmune diseases caused by neuro-inflammation.[130] Organoid studies of neurodevelopmental diseases successfully highlighted defects in a broad range of cell-molecular functions, such as oxidative stress,[131] vesicular traffic,[132] calcium channels,[114] the expansion of DNA nucleotide repeats in the context of Huntingtin disease,[133] chromatin remodeling enzymes, [134–135] mRNA chemical modifications,[136] and lipid homeostasis.[137] A particular intriguing application that is otherwise hard to conduct in actual living humans is the real-time tracking of dynamic phenotypes in organoids, such as neuronal migration and cytoskeleton dynamics.[35, 97, 114, 138–143]

3.4. Organoids as a tool to study neurodegenerative diseases

Despite their typical fetal or early post-natal state,[95, 99, 144–145] it has been possible to successfully model phenotypes in organoids that are typically associated with older age human brains. Alterations in cortical structure, neurogenesis, cell proliferation and cell signaling related to schizophrenia, which emerges during adolescence, have been replicated in ESC and iPSC type organoids.[131, 146–147] Organoids from patients suffering from early-onset (EO) fAD or Down Syndrome (DS), and the feeding of pathogenic Aβ peptides, recapitulate the progressive neuropathology of patients, with the appearance first of Aβ and subsequently Tau pathology[148–150] and inflammatory and extracellular matrix responses. [151] Intriguingly, DS derived organoids also show an increase in subclasses of GABAergic interneurons through overexpression of OLIG2, providing one potential explanation for impaired cognitive ability of patients.[152]

Further mirroring clinical observations, the development of AD molecular pathology was shown to be delayed in organoids expressing the late-onset (LO) E4 risk variant of a gene called apolipoprotein E (APOE) relative to organoids from early-onset patients.[153] Related studies have highlighted a disease pathway shared between EO-fAD and LO-fAD involving gene networks regulated by the repressor element-1 silencing transcription factor (REST).

[154] In one of the first forays in using organoids to profile responses to therapeutic drugs, treatment with beta and gamma-secretase inhibitors reduced amyloid and tau phenotypes associated with Alzheimer's disease.[148]

In PD, midbrain organoids from patients expressing the G2019S mutation in the leucine rich repeat kinase 2 (LRRK2), one of the prominent genetic causes of the disease, faithfully reproduced hallmarks of the disease, such as reduced dopamine neuron content, an increase in the pathogenic alpha-synuclein protein and changes in gene expression that implicate the lysosome.[154] In an interesting contrast with conventional 2D cultures, only mid-brain style organoids produce neuromelanin *in vitro*, a biomarker for aging and PD that is produced by the *substantia nigra*, the part of the brain affected early in PD.[31]

3.5. The impact of environmental insults and infection on human biology in brain organoids

Brain organoids are also useful tools for examining brain function and related pathology that does not depend on a genetic component per se. Organoids have been used to model changes in neurodevelopment caused by hypoxia (and rescue thereof)[155–156] and exposure to trace elements,[157] anti-cancer agents,[158] substances of abuse,[159–161] therapeutic drugs,[162] and natural products that purportedly improve brain function.[163] Brain organoids have similarly proven to be a useful tool in elucidating the pathology underlying prion[164] and viral infection, such as Herpes Simplex,[165] Dengue,[166] and, in particular, Zika virus (see below). Studies indicate that Japanese encephalitis virus appears to have a preference for astrocytes and neuroprogenitor cells, in particular outer radial glial cells, inducing cell death, and immune responses when telencephalic organoids mature.[167]

In the face of the global public health crises that ensued after the re-emergence of the virus in the Western world in 2015, organoids quickly became a popular model to study Zika virus (ZIKV) related pathology. Organoid models were instrumental in finding determinants of viral tropism and identifying how the virus may negatively impact brain development by impeding neurogenesis and cortical layer development processes.[101, 109, 168–172] An interesting connection between ZIKV infection and the genesis of other brain pathologies such as schizophrenia and intellectual disability was established, pointing to changes in the DNA methylome of ZIKV-infected neuroprogenitors, neurons and astrocytes with associated alterations in gene expression.[173] Organoids have also been used in the search for therapeutic solutions for Zika virus, leading to the discovery of key players in cellular entry of ZIKV[174–175] and small molecules with potential clinical benefits.[175–179] ZIKV triggers the endogenous RNAi-based anti-viral response, and broad-spectrum antibiotics such as enoxacin, which stimulates RNAi, abolishes viral infections and the ensuing microcephalic morphology of cerebral organoids.[180]

3.6. Modeling interactions between different cell-subtypes and different brain regions

The circuitry of the brain is complex. Connections form between local and distant sub-networks, involving the communication between many neuronal subtypes and support cells.[1–2] The functional maturation of developing organoids is now well established through computational, fluorescence imaging, and electrophysiology techniques.[181–182] Recent

data suggests that the activity patterns emerging in organoids are more irregular in time and space than conventional 2D-cultures, in a way more akin to that of human brain.[33]

It is possible to generate functional organoids and use them as a source to generate, isolate and study a broad range of cell subtypes, and to model the interactions between different types of cells, including cancer cells,[183–185] tissue elements such as the blood-brain barrier,[186] or even disparate brain regions. Protocols for glutamatergic, midbrain and dopaminergic neurons, inhibitory neurons, astrocytes and oligodendrocytes, thalamic neuron and organoid models have been published.[31, 98, 108, 110, 114, 143, 145, 175, 187–190] Long-term (20 month) cultivation of organoids created an environment supportive of the transition of astrocytes from fetal to a more mature stage.[145] Microglia-like cells, the brain resident immune cells that are derived from the mesoderm lineage, were successfully grafted onto organoids.[191] Recent literature suggest that it is possible that functional microglia arise naturally in brain organoids, which are ectoderm derived, possibly from a small pool of mesodermal cells.[192] When derived from Alzheimer's patients carrying defined early-onset mutations, microglia exacerbated AD phenotypes when grafted onto organoid cultures.[153] Furthermore, within a single organoid, developing cells may specialize into regions resembling the make-up of specific subregions of the brain. Organoids can be fused to create 3D structures that emulate connections between ventral-rostral and dorsal areas of the cortex and with the medial ganglionic eminence and thalamus.[110–111, 114, 143, 187, 193] In one of the first steps towards regenerative medicine applications, human organoids grafted in mouse brains showed enhanced functional maturation and integration with the host tissue compared to the implantation of neuroprogenitor cells.[194–195]

4. Engineering Approaches to 3D Brain Models

In order to address the limitations of the systems described earlier, microfluidics and bioreactor systems are increasingly being used as enabling technologies for precise manipulation of the cellular environment (Table 2). These microscale systems, now commonly referred to as "tissue-on-a-chip," are used to recapitulate the physiological function of tissues *in vitro*, with noteworthy success in creating tissue models as diverse as the human lung,[196–197] intestine[198] and liver,[199] among others. Recently, these systems have incorporated multiple tissue types into microphysiological systems (MPS), or "body-on-a-chip" devices, comprised of individual chambers that mimic the physiological function of different tissues all connected to mimic inter-tissue communication.[200–201] Here we focus on brain-specific microfluidic systems, but a thorough examination of all advances in tissue- and body-on-a-chip systems can be found expertly reviewed elsewhere.[202–203]

4.1. Microfluidic culture devices

The production of a tissue-on-a-chip system requires control over pattern and flow in a microscale environment.[202] Borrowing concepts from the photolithographic etching methods used to make computer chips, the innovative use of elastomeric materials such as poly(dimethylsiloxane) (PDMS) allowed for the design and fabrication of devices with micro- to nano-scale control of feature shapes and sizes.[204] Networks of microfluidic channels and/or wells designed using computer-assisted design (CAD) software are used to

create photomasks which are used to generate a master pattern etched into a photosensitive material (photoresist). This master pattern is then used as a mold for casting liquid PDMS, curing the polymer, and peeling the resultant replica with a negative relief from the master. When oxidized in oxygen plasma, the surface of the replica PDMS can be sealed using other materials, typically PDMS (patterned or slabs) or glass.[204] This soft lithography approach creates hollow channels for the perfusion of fluids, with applications in controlling fluid flow and cell patterning in biological systems, which offer advantages as compared to traditional 2D cultures. These devices can also be integrated with microsensors to provide feedback on culture conditions, including tissue barrier functions. To date, microfluidic brain-on-a-chip systems have been designed to model the blood-brain barrier, neural differentiation and brain development, and brain cancer.[205]

4.2. Blood-brain barrier-on-a-chip

Barrier functions at tissue interfaces can be modeled and modulated by seeding cells in different compartments or channels of a microfluidic chip. By incorporating a porous membrane between two microfluidic compartments and seeding with the appropriate cell types, the microfluidic components can be used to control barrier function biochemically or mechanically through exposure to fluid shear stress. The fidelity of the *in vitro* model to *in vivo* physiology is governed by the ability to replicate key characteristics of the BBB including tight junctions between endothelial cells, co-culture with astrocytes, and integration of fluid shear stress as well as functional outcomes such as high electrical resistance and selective permeability.[206] Early models of the blood-brain barrier (BBB) used murine cells such as mouse endothelial and astrocyte cell lines seeded on both sides of a porous membrane to establish a micro-BBB that mimics the cerebrovascular environment including expression of tight junction component zonula occludens-1 (ZO-1) and increased transendothelial electrical resistance (TEER) in response to fluid flow. Experimental measures such as a transient drop in TEER in response to histamine exposure as well as selective permeability to fluorescent tracers as a function of molecular size were used to evaluate barrier function.[206] In another model system, tight junction molecules were shown to be upregulated by culturing an immortalized rat brain endothelial cell line (RBE4) with a perfusate of astrocyte conditioned medium in a synthetic BBB model.[207]

While these studies demonstrate the utility of murine cell models of the BBB, they do not wholly recapitulate human biology. Similar results were observed using human cell lines, as a microfluidic system was used to study the barrier function of immortalized human brain endothelial cells (hCMEC/D3), demonstrating that shear stress increased TEER, while the addition of tumor necrosis factor alpha (TNF-α) had the opposite effect.[208] This further demonstrated the importance of a shear force component in mimicking barrier function in BBB models, particularly over prior approaches using human cells that were largely focused on static transwells. A hybrid model using rat cortical neurons and astrocytes, along with human endothelial cells, was used to form a 3D neurovascular microfluidic model of the BBB.[209] These systems demonstrated cell-type specific markers, endothelial barrier and selective permeability, and also showed neural functionality and could be used in the future as a platform for assessing therapeutic effects on neurons as well as BBB permeability.

As in murine models, the contribution of multiple cell types (endothelial cells, pericytes, smooth muscle cells, astrocytes, microglia, and neurons) is critical for BBB formation. The increased availability of hiPSCs, including differentiation protocols for the various cell types, has facilitated the development of human cell-based models. This has been accomplished by interfacing human brain-derived microvascular endothelial cells (BMECs) with primary human astrocytes and pericytes to provide critical intercellular communication for the establishment and maintenance of the high level barrier function of the *in vivo* human BBB.[210] The further incorporation of neurons, differentiated from hiPSCs, into a microfluidic BBB system provides a mechanism for investigating drug effects on neuronal function in addition to drug permeability and effects on barrier function.[211] More recent efforts have sought to create a fully iPSC-derived BBB model using hiPSC-derived BMECs and neural cells (neurons and astrocytes) that can be personalized to detect individual variability in BBB function.[212]

Importantly, these systems can be used to examine the delivery of therapeutics across the BBB. By co-culturing BMECs derived from hiPSCs with rat primary astrocytes, the drug permeability of large molecules (fluorescein isothiocyanate (FITC)-dextrans) and model drugs (caffeine, cimetidine, and doxorubicin) were measured and correlated with *in vivo* data.[213] Recent efforts have focused on developing high-throughput microfluidic platforms using human immortalized cell lines of brain microvascular endothelial cells, pericytes, and astrocytes suitable for drug screening applications.[214] These systems represent a promising alternative to current options for preclinical studies such as *in vitro* rodent or human cell culture or *in vivo* animal models.[215] In fact, microfluidic systems mimicking the BBB are now being used to probe disease mechanisms, such as the role of BBB dysfunction in AD and may be a platform for screening new drugs.[216] The development of microfluidic platforms, combined with the advancement in stem cell technology, will continue to improve understanding of the BBB and neurodegenerative disorders, with the ultimate goal of discovering effective therapies.[217]

4.3. Platforms for enhancing brain organoid and neural spheroid culture

Engineering tools and techniques have similarly been used to overcome the limitations of brain organoid and neural spheroid cultures (e.g., reproducibility, heterogeneity, and necrosis). In contrast to traditional well plates, these systems offer spatiotemporal control of culture conditions, which can be used to enhance cell viability and to direct neural tissue engineering.[218] With the help of microfluidic and microwell arrays, researchers have uncovered mechanisms of neural differentiation, brain development, and neurological disease. Early studies used microfluidic systems to maintain a gradient of cytokines to control differentiation of neural progenitors derived from human embryonic stem cells into neurons.[219] More recent work has focused on immobilizing embryoid bodies (EBs) within an extracellular matrix (e.g., Matrigel) in a microfluidic channel and cultured under static or perfused conditions to induce differentiation into brain organoids.[220] These systems have been used to model neurodevelopmental disorders such as fetal brain dysfunction due to prenatal nicotine exposure, demonstrating premature neuronal differentiation and disrupted cortical development as a function of nicotine exposure.[160] Other studies of brain

development have used microfabricated compartments to model the physics of brain wrinkling during the development of human brain organoids.[221]

In addition to using microfluidic chips to control the stem cell environment, microwell arrays have been engineered to control sphere size and shape and aid in homogeneous formation of 3D neural spheroids tissues. Neural spheroids derived from cell lines, primary rodent brain cells, or human stem cell-derived neural cells have been used to recapitulate key features of the brain such as cellular microenvironment and diversity.[222–225] However, because typical 3D neural spheroid fabrication methods such as hanging drop or spinner flask culture do not provide adequate size control and reproducibility, non-adhesive molds, made of agarose or PDMS, or ultra-low attachment plates are used to allow cellular self-assembly into spheroids in a controlled manner.[226–227] Using a micromold technique to control size and composition, spheroids prepared from primary rat cortical cells demonstrated formation of synaptic connections between neurons, electrical activity, brain-native ECM, and brain-like mechanical properties.[227] Similarly, neurospheres comprised of rat cortical neurons cultured in microwell arrays demonstrated controllable size distribution, enhanced viability, neural architecture and network formation, as well as decreased cell viability upon exposure to Aβ, a hallmark of AD.[228] Additional physiological relevance was achieved by using a concave microarray system and osmotic pump to culture neurospheroids under continuous low flow rates to mimic the interstitial fluid responsible for delivering nutrients and clearing waste in brain tissue *in vivo*. In addition to investigating flow effects in static versus dynamic cultures on neural differentiation and network formation, neurospheroids were cultured with Aβ to examine neurotoxicity in an *in vitro* model of AD.[229]

One of the critical remaining technical obstacles for organoid and spheroid cultures remains overcoming limitations of the distribution of oxygen and nutrients as organoids develop into larger and more mature tissues. The high cell density, in combination with the absence of active perfusion, can lead to a necrotic core in larger (>500 μm) spheroids or organoids. Miniaturized spinning bioreactors were 3D printed and used to optimize forebrain organoid cultures derived from human iPSCs.[109] These systems provided enhanced cell viability compared to stationary cultures and demonstrated cortical neurogenesis. Exposure of the cultured organoids to Zika virus led to characteristic features of microencephaly, offering potential utility of the bioreactor platform for disease modeling of brain development with subsequent compound testing during drug screening.[109] Another future option is to implement microfluidic perfusion networks to drive fluid flow or to grow organoids directly onto microfluidic channels seeded with endothelial cells to mimic vasculature.[230]

4.4. Chip-based models of neurodegenerative disease

While the use of brain organoid-on-a-chip systems for studying neurodegenerative diseases is in the early stages of development, other neural cultures have used microfluidic systems to help model Alzheimer's and other CNS diseases due to their ability to control fluid delivery, incorporate micro-chambers for protein accumulation, and establish gradients to study chemotaxis.[231] Compared to traditional 2D cultures, these are more representative systems for studying specific pathologies of neurodegenerative diseases such as propagation of Aβ or

tau protein through neuronal networks, chemotaxis of microglia, and/or synapse activity. Compartmentalized microchannels separating soma from axons have demonstrated early-stage AD pathological events in response to Aβ application such as transmission of Aβ to cell bodies via axons,[232] impaired axonal transport of brain-derived neurotrophic factor (BDNF)[233] or synaptic dysfunction in a cortico-hippocampal neuronal network.[234] In addition to Aβ transport, microfluidic platforms can be used to study other AD pathologies such as the accumulation of microglia near Aβ plaques by observing directional migration of human microglial in response to concentrations of monomer and oligomers.[235]

Mechanisms behind other hallmarks of AD pathology, such as the formation of tau protein aggregates, have been similarly been studied using microfluidic systems. As with Aβ, a microfluidic device with separate somatodendritic and axonal compartments was used to demonstrate that human wild type tau protein could be transferred between primary rat neuronal cells.[236] Using a tripartite microfluidic chamber device, it was further shown that tau aggregate formation could be induced in downstream neurons through cell to cell propagation as well as through extracellular medium.[237] More recently, 3D tissue models have been developed that incorporate multiple human cell types (neurons, astrocytes, and microglia) and hallmarks of AD (Aβ aggregation, phosphorylated tau accumulation, and glial cell activation), thereby offering a more complete representation of neurodegeneration and neuroinflammation in AD.[238] By incorporating many of the advances described in prior work, this microfluidic 3D triculture advances understanding of pathogenic mechanisms, particularly as it relates to glial activation and inflammatory processes.

Similar approaches have also been applied to the study of other neurodegenerative disorders such as Parkinson's disease by examining the role of alpha-synuclein (aSyn) spreading and aggregation as well as neuroinflammation in disease pathology. A microfluidic system was used to study intercellular communication between human neuroglioma cells and murine microglial cells by controlling the fluid exchange of soluble factors between two isolated cell culture chambers.[239] More recent efforts have focused on using patient-derived iPSCs to generate midbrain-specific dopaminergic neurons to study PD in 3D microfluidic devices. 3D cultures of neurons carrying the LRRK2-G2019S mutation demonstrated time-dependent dopaminergic degeneration as well as the preceding mitochondrial abnormalities. Interestingly, treatment with LRRK2 inhibitor 2 rescued dopaminergic phenotype, suggesting that this system may be useful for drug screening purposes.[240] High-content imaging systems utilizing automated fluorescent microscopy and analysis algorithms allow for real-time assessment of these cultures with other recent efforts aimed at constructing fully automated workstations for differentiating PD patient-derived stem cells into dopaminergic neurons, with cultures maintained for over 100 days and monitored for electrophysiological activity in real-time.[241]

4.5. Brain cancer-on-a-chip

Microfluidic technologies have been used in brain cancer research for diagnostic applications such as circulating tumor cell isolation and biomarker detection, as well as to create *in vitro* models to study drug efficacy and tumor progression.[242] As with models of neurodevelopment or neurodegeneration, brain cancer-on-a-chip models rely on precise

control of cellular microenvironment and capability for real-time outputs (e.g., high-resolution imaging, biochemical detection) to understand disease pathology. To date, researchers have focused on controlling variations in extracellular matrix (ECM) composition and mechanics, vascular integration, and cellular interactions as a means of studying glioblastoma multiforme (GBM) tumor progression. As an example, single cell microchips can be used to quantitate the distance dependence of signaling between two GBM cancer cells, providing insight into the cell-cell interactions that may govern diffuse nature of GBM tumor architecture.[243] Tumor progression can be predicted using a microfluidic platform for single-cell proteomics of human glioblastoma cell lines and brain tumor specimens.[244] For understanding tumor metastasis, microfabricated polyacrylamide channels of defined wall stiffness and width were used to study the effects of matrix composition and geometry on tumor cell migration, with narrow channels enhancing migration.[245] Other microfluidic cell culture devices have been developed to investigate the role of hypoxia in maintaining a stem cell niche in glioblastoma spheroids[246] or to examine clinical hallmarks of glioblastoma such as the formation of migratory hypercellular regions induced by oxygen and nutrient depletion by controlling the flow of medium using microfluidics.[247] Each of these systems demonstrate the utility of 3D microfluidic systems by providing insights due to the enhanced control over cell environment and experimental variables that are not otherwise achievable using standard 2D cultures.

Recent microfluidic models have examined the role of vascular cells in mediating glioma stem cell invasion. Using a microchannel co-culture system of human endothelial cells and glioma stem cells (GSC) separated by a collagen gel, migration of the glioma stem cells was promoted by endothelial cells through the genetic upregulation of cell-ECM adhesion-associated proteins and down-regulation of cell-cell adhesion proteins.[248] A more representative 3D microfluidic model of GSC and vascular cell interactions was established with a microvascular network in a fibrin hydrogel prior to introducing patient-derived GSCs in Matrigel to the microfluidic device. Using a receptor antagonist, this system demonstrated enhanced migration of GSCs in the presence of endothelial cells via the chemokine (CXC motif) ligand 12/chemokine (CXC motif) receptor 4 axis (CXCL12-CXCR4) signaling pathway.[249]

Brain cancer-on-a-chip platforms are often utilized as more representative model systems for preclinical drug screening as compared to traditional monolayer cultures due to their ability to recapitulate drug pharmacokinetics in a 3D environment. Brain cancer spheroids, cultured from glioblastoma cells within microwells in a 3D chip, were treated with anti-cancer drugs, pitavastatin and irinotecan, as a combinatorial treatment at varied concentrations in a high-throughput manner to determine the optimal treatment to reduce cell viability.[250] A modified version of this chip was able culture primary GBM cancer cells derived from primary human brain tumors and treat with combinations of chemotherapy drugs, temozolmide and bevacizumab to determine the optimal concentrations for personalized medicine.[251] Importantly, since these microchips are prepared from a polymer that blocks non-specific protein adsorption, poly(ethylene) glycol diacrylate (PEGDA), this system avoids the pitfalls of PDMS-based microfluidics which can adsorb hydrophobic molecules, an important consideration for drug screening applications. [250–251] In addition to drug efficacy, other personalized approaches to drug screening have included predicting drug

resistivity of GBM cell lines by measuring mRNA markers of temozolmide resistance[252] as well as determining the drug sensitivity of live slice cultures to chemotherapies in a dose-dependent manner.[253]

4.6. Technology and systems integration in microfluidic platforms

The development of advanced *in vitro* 3D models of human brain physiology and disease through brain-on-a-chip, tissue engineering or other approaches, has necessitated the integration of technologies to monitor real-time changes in cellular microenvironment and activity, particularly in response to pharmaceutical intervention. As outlined in many of the systems described above, interfacing microfluidic platforms with optical, electrical and biochemical capabilities is critical to identifying biological changes in real time. Due to the optical clarity of PDMS and other polymers used in microfluidic systems, conventional microscopy techniques (bright field, fluorescence, phase contrast) have been used to observe cells and tissues in these devices. As microfluidic devices have trended towards thicker 3D tissues, the emergence of scanning-based imaging techniques such as confocal microscopy have allowed for increased depth resolution.[254] Future integration of light sheet microscopy in microdevices may allow for even greater spatial and temporal resolution, with lower phototoxicity than confocal microscopy.[255] While standard microscopes are still the most frequently used, continued development of compact microscope-on-a-chip imaging systems will reduce costs and increase capabilities for automation and portability.[256] Future integration of smartphones, utilizing their high-resolution complementary metal oxide semiconductor (CMOS) cameras, offers another alternative for optical biosensors,[257] with proof-of-concept already demonstrated in a microfluidic chip system.[258]

For neurological systems, the ability to monitor electrical signaling in real-time is critically important for understanding information processing in neural circuits. For microfluidic systems, this is typically achieved through the integration of multi-electrode arrays (MEA) that are capable of recording electrical activity with high spatial and temporal resolution. Because these are recorded non-invasively, repeated measurements can be achieved over time in either brain slices or tissue culture preparations.[259] Based on the design of the microfluidic system and MEA, research can range from investigating intracellular dynamics in a single axon[260] to propagation between neuronal sub-populations (e.g., cortical-thalmic) in a co-culture system.[261] Although not part of a microfluidic chip, MEAs have also shown utility in drug screening applications such as the evaluation of neurotoxic compounds.[262–265] Importantly, electrical recordings by MEAs can be combined with optical methods for a more comprehensive assessment of neuronal function. As an example, by loading fluorescent calcium sensitive dye (Fluo-4) in the sample medium, simultaneous recordings of extracellular calcium and electrical traces can be resolved to show a propagating wave in response to electrical or chemical stimulation of murine brain slices.[266] Though not explicitly for brain organoids, the recent development of a non-invasive, automated platform for physical, biochemical and optical monitoring represents the latest achievement in real-time multisensory feedback in a microfluidic device.[267]

In addition to advances in the hardware for MEA and imaging techniques, protein and genetic engineering approaches, such as genetically encoded calcium indicators (GECIs) or

optogenetic techniques, are increasingly being used as alternative methods to voltage-sensitive fluorescent dyes to observe, analyze, and control neuronal activity. The development of a high affinity, green fluorescent protein (GFP)-based calcium (Ca^{2+}) probe (GCaMP) allows for chronic imaging and quantification of changes in intracellular calcium concentration as a function of firing action potentials and synaptic input.[268–269] By transfecting neurons with GCaMP and culturing them in 3D hydrogel matrices, multi-planar images provide spatial specificity and the functional activity of the developed 3D neural networks, particularly in response to drug input.[270–271] Additional fluorescent protein-based probes such as red GECIs (RCaMP) provide the option of using different colors within the same cell or to distinguish cell populations (e.g., neurons and astrocytes) and may be integrated with optogenetic approaches.[272]

The emergence of optogenetics, starting from the use of a light-sensitive ion channel protein (Channelrhodopsin-2, ChR2), as a means of optically controlling the activity of neuronal populations has provided researchers with another tool to activate or inhibit the activity of specific neurons non-invasively in culture with high temporal and spatial resolution.[273–275] While the majority of development is focused on *in vivo* applications, such as neural probes for brain stimulation, microfluidic systems have used optogenetics and calcium imaging to observe synaptic communication *in vitro*.[276] 3D *in vitro* tissue models have demonstrated the ability to record changes in fluorescent intensity upon photostimulation of neural cells that are transfected with both a ChR2 and a GECI and cultured in a collagen gel on a CMOS chip.[277] As optogenetic tools continue to be developed, additional methods of cellular control and/or control of small molecule delivery in 3D systems are available for researchers to study different mechanisms of neuromodulation.[278] As a key example, the ability to combine optogenetic actuators (channelrhodopsins) with archaerhodopsin-based voltage indicators in hiPSC-derived neurons allows for non-invasive, all-optical electrophysiology that is well-suited for high-throughput drug screening.[279]

5. Materials-Based Approaches to 3D Brain Models

Whether in a chip-based or stand-alone system, the choice of materials for a 3D brain model is multifaceted, with considerations for biocompatibility, mechanics, porosity and imaging compatibility, among the key concerns. Neural cells must be able to proliferate, differentiate, and form viable networks within materials that also need to provide the appropriate porosity, structure and mechanical support over time. Additional biophysical cues such as electrical conductivity and architectural patterning are increasingly being integrated into material designs to guide cell growth, development and organization.[280] In addition to support structure, materials must also incorporate domains for cell attachment as well as appropriate bioactive signaling molecules (e.g., immobilized growth factors or chemical functional groups). By carefully designing and engineering the appropriate biochemical and biophysical cues, researchers can direct cellular behavior by recapitulating the natural cellular microenvironment in a 3D brain model.[281] For many researchers, particularly in the organoid field, Matrigel is the chosen support material as it offers a complex mixture of structural, functional and signaling molecules suitable for 3D culture, but remains a poorly defined and heterogeneous mixture of extracellular matrix proteins that is subject to batch-to-batch variability.[282] To address these deficiencies, a variety of material-based

approaches have been used to bioengineer 3D scaffolds using a bottom-up approach to support *in vitro* neural tissue development and disease modeling.

5.1. Natural hydrogel systems

Hydrogels are commonly used in bioengineered neuronal culture systems as their chemical and mechanical properties can be tailored to resemble those naturally occurring in brain.[283] Derived from natural or synthetic polymers, they are cross-linked to form hydrated networks of tunable mechanical stiffness and may be functionalized with bioactive components.[283] In contrast to Matrigel systems, hydrogels prepared from fully-defined source materials can be formulated with specific control over ECM components and signaling cues, using *in vivo* conditions as a guide. Because brain ECM is composed of a mixture of proteoglycans, hyaluronic acid, tenascins, collagens, fibronectin, vitronectin, and laminin,[284] combinations of these materials have been used as a starting point to engineer hydrogel scaffolds for neural tissue engineering.[282] Importantly, hydrogels must be formulated to mimic the mechanical properties of the brain (elastic modulus of <1 kPa), providing an appropriately soft matrix to direct neuronal cell behavior.[285] This is typically achieved by preparing hydrogels that are cross-linked with sufficiently low density to achieve this mechanical stiffness while also allowing for exchange of oxygen and soluble factors to support neuronal culture.

Due to its wide availability, biocompatibility, and biodegradability, as well as its well-documented historical use as a hydrogel scaffold for tissue engineering, the most commonly used natural biomaterial for neural cell culture applications is collagen type I. It has been demonstrated that rodent-derived neural stem and progenitor cells (NSPCs) in type I collagen gels can expand and differentiate into neurons with functional synapse and neuronal circuit formation.[286] These gels have also shown the ability to support the differentiation into multiple cell types (neurons, astrocytes, and oligodendrocytes) under optimal conditions of cell density and collagen concentration.[287] In order to further increase relevancy to native brain tissue, additional natural polymers such as hyaluronan have been combined with collagen to enhance neuronal differentiation of NSPCs.[288] Similarly, the incorporation of soluble growth factors in engineered hydrogels have been used to direct cell proliferation, migration, and fate. Using a layer-by-layer bioprinting approach, collagen-fibrin composite hydrogels were used to promote the migration of neural stem cells toward hydrogel layers containing vascular endothelial growth factor (VEGF).[289] Combinations of neurotrophic factors such as nerve growth factor (NGF), glial cell-line derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) have been explored in dorsal root ganglion (DRG)-seeded collagen gels to optimize neurite outgrowth.[290] Mechanical cues such as gradients of stiffness in 3D collagen gels or bio-acoustic levitation in fibrin gels have also been utilized to drive neurite growth or generate multilayered 3D brain-like constructs from hESC-derived neuroprogenitors, respectively.[291–292]

Due to the prevalence of hyaluronic acid (HA) in native brain tissue, this polymer has been extensively studied as a substrate for central neural tissue engineering.[293] However, since unmodified HA does not support cell attachment, HA hydrogels must be modified with cell

adhesion molecules or blended with other ECM components such as collagen.[294] As an example, a Nogo-66 receptor antagonist was used to modify HA hydrogels to improve the neuronal adherence and survival of DRGs *in vitro* as well as induce neurite outgrowth.[295] HA-poly-D-lysine (PDL) copolymer hydrogels have also been used to control neuronal cell adhesion and network formation based on HA:PDL ratios.[296] Similarly, ECM components such as laminin or fibronectin- or laminin-derived peptides (RGD, IKVAV) have been used to facilitate cell attachment to HA.[294] A combination of HA, collagen, and laminin was used as a mimic of natural ECM composition to support the co-culture of Schwann cells and neurons in 3D hydrogels for 2 weeks, offering a potential platform for studying mechanisms of interactions between the two cell types with applications in nerve regeneration.[297] Interestingly, as with collagen hydrogels, mechanical properties of HA gels influenced neural progenitor cell (NPC) differentiation towards neuronal subtype in softer hydrogels versus an astrocytic subtype in stiffer hydrogels.[298]

In addition to HA, other naturally-derived polysaccharides such as alginate, agarose, chitosan, and composites thereof have been used as substrates for 3D neural tissue engineering. As with HA, these polymers are inert biomaterials that can be tailored for 3D neural cell culture by varying composition and crosslink density. These polymers are similarly non-adhesive for cultured neurons and must incorporate cell attachment factors, although there is some evidence that unmodified soft gels can support neurite growth.[299] As an example, alginate hydrogels functionalized with cell-specific attachment ligands (laminin, IKVAV, or RGD) were used to study co-cultures of neurons, glia and endothelial cells, observing neural functions such as gap junction formation, synaptic vesicle release, and electrophysiological activity.[300] Another approach to incorporating cell binding domains has been to mix in collagen fibrils, with this collagen/alginate blended hydrogel used to culture neurons generated from human iPSCs.[301] Other researchers have used defined mixtures of nutrients in agarose and alginate hydrogels to generate 3D multi-layered scaffolds that mimic cortical layers using primary rat cortical neurons in a microfluidic device.[302]

Though the vast majority of neuronal tissue engineering has been achieved using rodent-derived neurons, combinatorial studies on matrix composition and architecture have sought to provide a more comprehensive understanding of how cellular microenvironment (ECM and signaling molecules) can influence cell fate in 3D cultures. This includes efforts to use these parameters to direct human stem cell differentiation toward neural and glial lineages in natural polymers such as Matrigel, collagen type I, gelatin, or HA.[303] As the field progresses towards high-throughput, human cell-based neuronal models for developmental/disease biology, regenerative medicine, or drug/toxicity screening applications, researchers aim to overcome limitations on reproducibility and culture duration that is inherent for natural materials. One approach is the use of novel biomaterials such as silk fibroin protein to generate hydrogels with tunable stiffness in the range of native neural tissues, capability for functionalization with growth factors or ECM molecules, as well as the ability to maintain structural integrity for much longer duration (months to years) than typical hydrogel materials.[304] Others approaches center around the development of synthetic materials, including polymers and self-assembling peptides, to address these concerns.

5.2. Synthetic hydrogel systems

In contrast to naturally-derived polymers which offer physiological relevance but also greater variability in chemical composition, synthetic hydrogels derived from polymeric materials such as polyethylene glycol (PEG) and methacrylates, among others, offer the advantage of tight control over physical and mechanical properties based on chemistry and polymerization.[283] However, these hydrogels are generally biologically inert and with minimal cell adhesion and protein adsorption to support tissue formation.[305] This requires chemical modifications to the polymer hydrogels for neural tissue engineering applications that are similar to those used for polysaccharide-derived hydrogels (e.g., RGD, IKVAV) or mixtures with other ECM proteins (e.g., collagen). As with chemically-defined natural hydrogels, the use of synthetic polymers is an alternative to Matrigel, replacing the inherent variability of a poorly defined mixture of bioactive components with a well-defined and controlled matrix for neural tissue engineering applications.

To date, PEG is the most extensively used synthetic polymer for neural cell culture in 3D hydrogels. Preliminary studies incorporated rat neurons in photopolymerizable PEG hydrogels, modified with poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) to control degradation rate and supplemented with basic fibroblast growth factor 2 (bFGF-2) to support cell growth.[306] These tissues demonstrated neural tissue development and functionality, with the time-scale of neurite outgrowth a function of gel degradation rate. In another study, peripheral nerve regeneration was enhanced by covalently attaching cell adhesion ligands (RGD, IKVAV, and YIGSR) to PEG hydrogels.[307] Adhesion ligands conjugated to PEG hydrogels have similarly been used to study human iPSC-derived NPCs attachment and migration induced by astrocytes.[308] Recent advancement in PEG hydrogels has been achieved by incorporating various functional groups, including polymer-peptide conjugates, for cell attachment, signaling, and cleavage sites to tune physical and mechanical properties. Using star-shaped PEG (starPEG) and heparin peptide conjugates, hydrogel compositions were optimized to embed human vascular endothelial cells and DRGs and stimulated to control cell phenotype (angiogenic or neurite outgrowth).[309]

Synthetic polymers have been used to modify natural polymers, preserving the beneficial properties of the natural material while offering additional functionality. One notable example is the synthesis of methacrylamide chitosan (MAC), a synthetic polymer that can be modified with peptides for cell adhesion and neurite outgrowth, formed into a transparent gel via chemical crosslinking, and seeded on the surface with rat neurons.[310] This polymer has subsequently been modified to enhance oxygen diffusion within the hydrogel through the incorporation of D-mannitol[311] or conjugation of perfluorocarbons to MAC[312] to enhance the neuronal differentiation of rat neural stem cells (NSCs)/progenitor cells (NPCs) in 3D. Methacrylate has also been used to modify hyaluronic-acid, with researchers using this material to create layered 3D hydrogels of variable mechanical properties to study the differentiation and migration of human iPSC-derived NPCs.[308]

Synthetic, self-assembling peptides (SAPs) have been developed as a new class of biologically-inspired materials for neuronal culture. Oligopeptides of varied sequences of arginine-alanine-aspartate (RAD) are able to self-assemble under physiological conditions and support neuronal cell attachment, neurite outgrowth, and synapse formation,[313] with

the most commonly used peptide, $RADA_{16}$-I, commercially available as PuraMatrix. Neural stem cell proliferation, migration, and differentiation may be further promoted by modifying these SAPs with a functional cell adhesion motif ($RADA_{16}$-IKVAV) in a 3D hydrogel scaffold using both rodent and human cells.[314–316] Despite these successes, these formulations suffer from low pH impacting cell viability. To address this issue, oppositely charged SAPs with different cell adhesion epitopes ($RADA_{16}$-IKVAV and $RADA_{16}$-RGD) were combined to form a 3D hydrogel at neutral pH that was capable of encapsulating NPCs/NSCs and differentiating into neurons and astrocytes.[317] Because SAPs often suffer from poor mechanical properties, linear or branched $(LDLK)_3$ SAPs were recently used as an alternative to form hydrogels of controlled mechanical properties, demonstrating that neuronal differentiation is enhanced when scaffold stiffness is comparable to that of the native brain.[318–319] These constructs have also demonstrated the ability to support *in vitro*, serum-free differentiation of human NSCs into GABAergic, glutamatergic, and cholinergic neurons that give rise to the formation of electrically active networks.[320]

5.3. Bioprinting

The emergence of bioprinting as a technique to control the spatial distribution of cells, biomaterials, and signaling molecules offers new possibilities for building 3D neural tissues *in vitro* using a bottom-up approach. Using hydrogel-based bioinks to precisely arrange cells in 3D, heterogeneous tissues can be composed with the appropriate physiological cues and resultant functionality that is similar to that of native brain tissue. This approach further improves reproducibility in model development and potential for automation, critical elements downstream high-throughput screening applications.[222]

Many of the hydrogel materials described above have been investigated as potential bioinks for neural tissue engineering applications, with the choice dependent on a number of considerations including the printing technique (e.g., inkjet, extrusion-based, laser-guided), cytocompatibility, curing time and mechanical properties, among others.[321–322] In one of the first examples of bioprinting neurons, inkjet printing was used to pattern rat hippocampal and cortical cells in a single layer, with these cells demonstrating electrophysiological activity. Researchers in this study used the same technique to fabricate multi-layer constructs of human NT2 neurons and fibrin gels via a layer-by-layer printing approach to create 3D neural sheets.[323] A similar approach using multilayered collagen type I gels and rat embryonic neurons and astrocytes was used to form multicellular constructs with defined spatial patterning to examine cell-to-cell interactions.[324] Layer-by-layer inkjet printing has also been used to study cell migration through the controlled spatial distribution of VEGF-containing fibrin gel and murine NSCs within a 3D collagen scaffolds.[289] Subsequent work has focused on exploring tunable polymers as inks for 3D bioprinting such as thermoresponsive polyurethane hydrogels[325] or stereolithography-based printing of functionalized materials such as dopamine-modified gelatin methacrylate[326] to improve NSC proliferation and differentiation.

Improvements in printing techniques and bioink material properties have supported the fabrication of more complex structures, such as cortical layers, that mimic the native brain. Through the use of a bioink (gellan gum-RGD) that forms a porous structure suitable for

oxygen and nutrient exchange, primary rat neural cells were printed in a layered, brain-like cortical construct.[327] More recently, human NSCs and iPSCs in a polysaccharide-based (alginate, carboxymethyl-chitosan, agarose) bioink have been used to print mini-tissues that differentiate into neurons, form neural networks, and demonstrate electrical activity. Using an extrusion-based printing technique, cells are co-printed with the bioink before gelation to encapsulate them in a 3D matrix for *in situ* expansion and differentiation into functional neurons and neuroglia.[328–329]

5.4. Solid porous scaffolds

While hydrogel-based techniques offer the benefit of soft mechanical properties for neurite outgrowth, they generally lack the ability for long-term culture (greater than 1 month) due to fast degradation rates, uncontrolled contraction, or the presence of necrotic cores. As an alternative, solid porous scaffolds prepared from synthetic or natural polymers offer mechanical and morphological stability and pore interconnectivity to facilitate nutrient transport required in long-term neural tissue models. Furthermore, these scaffolds may be modified with topographical cues for cell attachment and directed neurite outgrowth.[222].

As an example, researchers have used polystyrene as a synthetic polymer for generating porous scaffolds for 3D culture of human neural progenitor cells. Scaffolds were prepared by a gaseous salt leaching method where polystyrene is dissolved in chloroform, mixed with salt particles of known size, and cast into wells before curing in an oven.[330] When using these scaffolds to culture human neural progenitors, the cells more accurately emulated the gene expression profiles of *in vivo* surrogates (3D neurospheres) in comparison to standard 2D culture.[331] Porous polystyrene scaffolds cultured with primary rodent neural cells also demonstrated more representative calcium channel functionality versus standard 2D cultures in comparison to freshly dissected intact tissues.[332] More recently, graphene foam has been pursued as a biocompatible and conductive scaffold for 3D neural culture that represents an alternative to the inert, non-biodegradable polymers such as polystyrene. These scaffolds, prepared via a chemical vapor deposition method, showed that they can enhance NSC differentiation into neurons/astrocytes as well as serve as a conductive platform for electrical stimulation.[333] As an alternative to porous scaffolds, electrospinning techniques have been used to prepare fibrous scaffolds from synthetic polymers that can be functionalized to support neural cell culture and neurite outgrowth. However, because standard methods typically produce dense fiber meshes that function more as a 2D surface, recent efforts have used an array of needles as a collector to form lower density electrospun fiber scaffolds that support 3D culture of human NPCs and their formation of highly integrated, functional networks.[334]

Similar to synthetic polymers, multiple methods have been used to create porous or fibrous 3D scaffolds from natural polymers. Freeze-drying, in particular, has been commonly used to form porous scaffolds from biological materials such as collagen or HA. The freeze drying process can be manipulated to control the mechanical properties and to determine pore size of collagen-glycosaminoglycan scaffolds.[335–336] Collagen and HA were mixed at different ratios, freeze dried, and cross-linked to tailor the resultant scaffolds to have moduli and porosity similar to those of native brain tissue.[337] This provides the appropriate

conditions to support neural progenitor cells by allowing for the exchange of nutrients, the outgrowth of neurites, or the infiltration of cells in *in vivo* applications.[338] Collagen-based scaffolds can be tailored to include cell adhesion molecules as in the modification of 3D freeze-dried collagen matrices with IKVAV and/or RGD peptide to enhance the attachment of rat DRGs.[339] Cryogels from gelatin-laminin were used as porous scaffolds for differentiating human cord blood-derived stem cells into artificial neural tissue with mature networks of neurons and glia, suitable for implantation in rat brain tissue or high-throughput personalized medicine applications.[340] Recently, porous silk scaffolds have been used in combination with collagen hydrogels to create functional brain-like cortical tissue, where the compartmentalization of neuronal cell bodies and axons recapitulates grey and white matter.[341] This combination of porous scaffold and hydrogel matrix was able to mimic the mechanics of the native rodent brain while allowing for a robust neural network formation and electrophysiological activity. The composite system (silk and collagen type I) was further used as a model of traumatic brain injury, monitoring changes in cellular damage, biochemical signaling, and electrical activity following a controlled impact.[341] These versatile systems have also demonstrated utility as engineered neural tissue implants for replacing large brain cavities.[342]

5.5. Materials-based models of neurological disorders, damage and dysfunction

In addition to supporting 3D cultures to study neurite outgrowth and neural network formation, materials-based approaches have been utilized to create models of neurological disease. To date, work has primarily focused on the development of 3D *in vitro* models of Alzheimer's disease and glioblastoma as well as platforms for studying traumatic brain injury or neurotoxicity. By controlling material properties as well as cell type and genetic composition, particularly using human iPSCs or neural progenitors, key features of the disease may be recapitulated and used to understand mechanisms and/or to screen therapeutic interventions.

Initial 3D models of Alzheimer's disease have sought to recapitulate the pathological hallmarks of the disease, Aβ accumulation and plaque formation as well as aggregation of phosphorylated tau protein. In one example, fAD mutations in Aβ precursor protein (APP) and presenilin 1 (PS1) genes were overexpressed in human NPCs. These cells were cultured in Matrigel as a 3D support matrix, which limited diffusion and accelerated β deposition and tau protein aggregation. This model system recapitulated, for the first time, these key features of AD in a 3D model and was subsequently used to screen beta- or gamma-secretase inhibitors to decrease Aβ pathology.[86] In another study, Aβ aggregates were added to conventional 2D cultures and 3D cultures of human stem cell-derived neurons in a PuraMatrix hydrogel. Cellular response to the Aβ aggregates, as mediated by p21-activated kinase, was only observed in the 3D cultures demonstrating the critical role of the 3D environment in modeling AD mechanisms.[343] Subsequent 3D models of AD have used collagen or PEG hydrogels to study Aβ-induced toxicity of neural cell lines or plasticity loss of human neural stem cells, respectively.[344–345] Others have used a composite silk scaffold-collagen gel system to support long-term (>8 month) 3D human neural network models using iPSCs derived from healthy individuals and Alzheimer's disease patients, offering a potential platform for studying development, plasticity, and degeneration over

time.[346] The continued development of 3D models that incorporate multiple cell types (neurons, astrocytes, microglia, and oligodendrocytes) will be critical in understanding the mechanisms of neurodegeneration and neuroinflammation that are key features of neurodegenerative disorders such as AD or PD.[347]

In addition to AD, the development of *in vitro* 3D brain cancer models has received a significant amount of attention, with specific focus on materials-based approaches to mimic the tumor microenvironment *in vitro* that drives glioblastoma (GBM) progression. Currently used bioengineered models are primarily based on the encapsulation of glioblastoma cells in 3D biomaterial microenvironments that recapitulate the hypoxic conditions observed *in vivo* and allow for cell migration within the scaffold.[348] Through the use of natural ECM-derived materials, primarily HA, researchers are able to mimic the composition and structure of the native brain. In fact, glioma cell invasion into HA hydrogels has been shown to be mediated by controlling the stiffness of HA-methacrylate hydrogels and functionalization with RGD peptides for cell adhesion.[349] One of the major drawbacks of using these systems is that by increasing polymer concentration or cross-linking density, biochemical signaling is affected. Others have sought to independently control matrix stiffness using synthetic polymer-based hydrogels such as polyacrylamide or PEG to isolate the effects of matrix stiffness and confinement on human glioma cell migration.[245, 350] In addition to hydrogels, solid porous scaffolds of chitosan and HA have been developed to study glioblastoma microenvironment, demonstrating enhanced invasiveness of human GBM cells and chemotherapy drug resistance.[351] Using porous silk scaffolds infused with ECM hydrogels as a brain-mimicking microenvironment, researchers have further showed that pediatric and adult brain tumor progression is mediated via ECM-dependent alterations in transcriptomic and metabolic profiles.[352] Additional spatial control may be achieved by using bioprinting approaches to control the deposition of different cells types as well as ECM and signaling molecules in complex 3D models of the tumor microenvironment.[353] As an example, a 3D brain tumor model was developed from glioma stem cells bioprinted in a gelatin/alginate/fibrinogen hydrogel. Similar to conditions found *in vivo*, these cells formed spheroids, remodeled ECM to occupy a larger space, and demonstrated chemotherapy drug resistance.[354] Spatial control of cells, matrix, signaling molecules, and oxygen content may also be controlled using microfluidic mixing as demonstrated in a HA-gelatin-methacrylate hydrogel brain tumor model.[355] More recently, bioprinting techniques have been used to create 3D mini-brains that include GBM cells as well as macrophages to study cellular interactions and therapeutic control of those interactions.[356] With these 3D systems, the goal is to not only understand the mechanisms of tumor progression, but also serve as a platform for preclinical screening of therapeutics and/or radiotherapy.[357] Using GBM cell lines seeded in a 3D Matrigel-coated polystyrene scaffold, researchers showed that the *in vitro* cellular response to radiotherapy in combination with clinically-used agents (temozolomide, bevacizumab, erlotinib) strongly correlated with clinical observations.[358]

In addition to studying neurological disease, recent work has sought to develop preclinical models for studying traumatic brain injury or neurotoxicity. These models are used correlate clinical observations with *in vitro* results to better understand mechanisms and/or to more accurately predict *in vivo* outcomes than traditional 2D cultures or animal models. For traumatic brain injury, collagen gels seeded with rat cortical neurons were subjected to

compressive deformation, providing insight on how neuron viability and function is dependent on strain magnitude and rate.[359] Others have used a weight drop method to inflict mechanical damage in a 3D model of cortical brain-like tissue formed from a silk-collagen composite scaffold, observing enhanced glutamate release and transient hyperactivity that is consistent with *in vivo* results.[341] For neurotoxicity studies, 3D *in vitro* models using human cells represent an alternative to animal models that may be more predictive of chemical safety and reduce the number of drug failures in clinical trials. Recently, human embryonic stem cell-derived neural progenitor cells, endothelial cells, mesenchymal stem cells, and microglia/macrophage precursors formed neural tissue constructs following seeding on top of PEG hydrogels functionalized with biodegradable peptide cross-links and RGD peptides to promote cell adhesion. Leveraging the reproducible and controllable intracellular interactions achievable via this method, these constructs were then used for neurotoxicity screening *in vitro* by treating the tissues with toxic and non-toxic chemicals, collecting RNA sequencing data, and then using machine learning to build a predictive model capable of correctly predicting the toxicity of additional compounds.[360]

6. Future Perspectives for 3D Brain Models

Over the past decade, there has been tremendous progress in the development of 3D models of the human brain. Through technological advances and innovation in the key components of engineered tissues, cells, scaffolds, and signaling molecules, otherwise known as the tissue engineering triad, researchers can now create models that offer greater fidelity to neurological development and disease. These models offer the ability to better understand human physiology and the potential to identify therapeutic or regenerative medicine approaches to treat disorders of the human brain. As outlined in this review, there have been two generally parallel approaches to creating 3D brain culture systems, organoid technology and bioengineering methodologies. The fundamental difference between these approaches is that organoid technology is largely a cell-driven, bottom-up approach whereas the bioengineering methodology is a materials-templating bottom-up approach for generating 3D brain models. Looking forward, we see tremendous potential for integrating these two approaches, leveraging the strengths of each. Furthermore, we see potential for integrating other tools and techniques such as microfluidics and bioreactors for improving cell culture duration or electrode arrays and imaging modalities for non-invasive monitoring of the models in real time. Lastly, as the potential for 3D models as a screening tool is currently limited by reproducibility issues, we highlight prospective solutions for generating high-throughput 3D brain models for biomarker or drug screening applications (Figure 3).

As described in previous sections, organoids have proven to be an effective developmental model of the human brain with additional utility as a tool for studying neurological function or modeling neurodegenerative, psychiatric and infectious disease. The drawbacks associated with organoid cultures, including issues with scalability, reproducibility, heterogeneity and longevity due to necrotic cores, remain active areas of pursuit in the field. While microfluidic or bioreactor approaches have most commonly been used to improve the reproducibility of organoid cultures with regard to overall size distribution and neural cell viability and differentiation, a less explored option is the use of materials-based approaches to improving brain organoid cultures. In one example, bioengineered materials were used to

help developing organoids adopt 3D architectures similar to the native brain as culturing embryoid bodies on floating scaffolds of synthetic polymer (PLGA) filaments caused self-organization in an elongated morphology and the formation of a polarized cortical plate and radial units.[40] Others have used defined, modular synthetic matrices composed of PEG-based engineered hydrogels to understand the role of biochemical and biophysical cues in regulating early neurogenesis in organoid cultures derived from mouse ESCs. By controlling the mechanical stiffness, degradability, ECM components, and soluble factors of the engineered hydrogels, they were able to identify the specific material properties that promoted neuroepithelial differentiation, apical-basal polarity and dorsal-ventral patterning, while also generating more homogenous cultures in these defined synthetic matrices in comparison to ESC cultures in Matrigel.[361] These pioneering studies demonstrate the great potential for integrating organoid and bioengineering technology to support brain organoid cultures, but with significant insight to be gained by studying bioengineering approaches used in other *in vitro* organoid-based tissue models.

To date, engineered materials for supporting organoid cultures have mainly focused on solutions for intestinal organoid tissue models. Through these studies, the importance of providing a reproducible, chemically-defined matrix with controllable mechanical properties and defined signaling cues for specific stem cell niches has been established. The knowledge gained from these studies can be transferred to guide the optimization of materials for 3D brain organoid culture.[362] Among the key goals of an engineered material for this application would be the spatiotemporal delivery of biochemical cues to direct neural differentiation. In fact, placing morphogen gradients within organoids has been shown to facilitate tissue self-organization.[363–364] This could be further optimized through the patterning of a bioengineered scaffold with growth factors known to direct neural progenitor differentiation to specific lineages. Cell migration and self-organization within the organoid could similarly be directed by controlling the spatial organization of cell-adhesive ligands, biochemical cues (including gradients), and/or mechanical properties.[362]

Controlling these aspects of cell-material interactions have been explored using synthetic or biological materials that otherwise do not possess cell instructive cues, notably HA or silk fibroin, and using the materials as a blank slate to design cell-matrix interfaces.[365–366] Additional spatial control may be achieved through continued improvement in 3D printing technology such as the printing of gradients of signaling molecules to direct neural stem cell differentiation.[367] Other recent efforts using biocompatible electroconductive materials, such as graphene or polypyrrole, offer another potential option for guiding the formation of neural networks in a 3D system through the use of electrical signaling.[366] This includes combinations of these materials to create novel matrices for neural tissue engineering applications.[368–369] In each of these examples, the engineered materials would serve to improve on the commonly used matrices, which are often undefined (e.g., Matrigel) or with limited complexity (i.e., build of a single polymer, such as collagen). Such an approach would offer the potential to improve the consistency of organoid cultures through the use of tunable and reproducible matrices that also provide the versatility of incorporating multiple signaling cues (biochemical, mechanical, spatial, electrical) as points of control.

To address the formation of necrotic cores during 3D brain tissue development, a key limitation of organoid cultures, engineering approaches may be used to control scaffold architecture to allow the proper exchange of oxygen, nutrients and waste products. One approach has been to expose cerebral organoids in liquid culture to an air interface which augments the survival and axonal outgrowth of neurons, presumably through an increased nutrient supply.[370] For a more sophisticated bioengineering approach, porous scaffolds have been used to support 3D neural tissue models, but the continued development of new fabrication methods using computer-aided design and 3D printing offer unprecedented levels of control for preparing porous scaffolds.[38, 371] Specialized architecture may be used to design suitable implants for regenerative medicine purposes *in vivo* or to support the long-term *in vitro* culture of neural tissue engineered constructs, which may be critical for the examination of neurodegenerative diseases such as AD or PD. In addition to scaffold porosity, the other commonly used engineering approaches to support chronic cultures is the use of microfluidic or bioreactor systems. Bioengineered scaffolds, particularly hydrogels, are often limited in culture duration due to concerns over relatively fast degradation rate or restricted oxygen/nutrient diffusion for dense cultures. Early bioreactor systems such as the rotary wall vessel bioreactor have been utilized to extend grow cortex-like tissue constructs without necrotic cores in collagen gels.[372] More recently, microfluidic or microarray technologies have been used, but many cultures are still short-term (less than 1 month). We expect the continued development of bioreactor systems to support longer-term culture will help in recapitulating the pathophysiology of neurological disorders.[205] Furthermore, given the recent interest in the association of brain disease with dysfunction in other physiological systems (e.g., the gut-brain axis), the incorporation of multiple systems in multi-compartment chips or bioreactors will help identify the impact of peripheral tissues in brain neurodegeneration.[373]

The combinatorial nature of neurological function and dysfunction has necessitated innovations in the ability to model interactions between different cell subtypes, brain regions, and other tissues. Brain tissue models with higher complexity have been achieved by fusing different types of spheroids together to study neural circuitry that spans multiple brain regions, such as cortical-hippocampal[374] or dorsal-ventral.[114] Furthermore, as described above, there are now published protocols for differentiating iPSCs into glutamatergic, midbrain and dopaminergic neurons, inhibitory neurons, astrocytes and oligodendrocytes. Through the use of bioengineering approaches, cells can be combined into 3D scaffolds or patterned networks to form circuit connections that can be used to examine functional communication between neuronal subtypes and support cells.[375–376] Furthermore, through the use of cells isolated from healthy or patients afflicted with the disease, researchers can probe mechanisms of circuit dysfunction at the scale of actual tissues in the native 3D environment, and examine genetic or therapeutic approaches to rescuing activity. Another pressing issue in the development of 3D brain models is the use of iPSCs as representative models of neurodegenerative disease. iPSCs have been ‘aged’ through genetic and chemical approaches interfering with specific pathways, such as mitochondria or DNA replication, and by performing direct differentiation of aged fibroblasts to neurons, however these approaches remains artificial, reduce the aging phenotype to a single factor.[377–387]

For each of the technologies described, one of the main goals is to improve the reproducibility of 3D brain models for biomarker or drug screening purposes. In considering systems that will be useful for this application, some key issues include ease of fabrication and use and confounding factors such as non-specific adsorption of therapeutics to materials used in the system. As an example of a reproducible scaffold system, researchers used an automated process to incorporate 3D polystyrene scaffolds into standard 96-well plates and were able to create a high-throughput capable platform for culturing neural stem cells and evaluating outputs in growth, gene expression, and functionality via a calcium assay.[330, 388] Other material-based approaches for high-throughput applications will similarly need to demonstrate the capability for automated production of reproducible scaffolds. Another consideration for drug screening applications, particularly for microfluidic or chip-based approaches, is the non-specific adsorption of therapeutics. Because hydrophobic molecules adsorb to PDMS, alternative materials such as silk, polyurethane or others have been proposed as the starting material for microfluidic systems. As an example, polyurethane elastomers demonstrated similar beneficial optical and mechanical properties to PDMS but offered improved resistance to adsorption of a hydrophobic small molecule dye.[389] Other researchers used poly(ethylene) glycol diacrylate (PEGDA), a synthetic polymer that blocks non-specific protein adsorption, to develop a brain cancer chip for high-throughput drug screening. Using this system to culture glioblastoma cells in a 3D brain cancer model, they were able to examine combinatorial treatments of chemotherapy drugs pitavastatin and irinotecan.[250] Subsequent systems should similarly seek to achieve a similar ease of use and applicability in developing a commercially and clinically promising high-throughput platform.

Another important consideration for commercial and clinical applicability is the integration of technological innovations in electrical or optical interfaces for monitoring 3D brain tissue models in real-time for high-throughput screening applications. While automated fluorescence image-based methods for quantifying brain tissue to classify cell types (neurons, astrocytes, microglia, and endothelial cells) and analyze their associations in cellular networks have been developed,[390] the use of label-free methods for real-time monitoring of cells is generally preferred. Using MEA-based impedance spectroscopy methods, researchers were able to monitor cellular pathology in real-time through the degradation of neurites in response to tau hyperphosphorylation in slice cultures.[391] However, because MEAs generally offer a 2-dimensional measurement of a 3D tissue, recent efforts have sought to use optical methods such as multiphoton microscopy for non-contact and non-invasive functional imaging of bioengineered neural tissues.[392] Wide-volume imaging demonstrated single cell resolution of neuronal activity via the expression of a calcium sensor (GCaMP6s). By using single and layered co-cultures of hippocampal and cortical neurons, researchers were able to examine cell-type specific and interactive neural network activity.[270] Optogenetic techniques are similarly being optimized non-invasive stimulation and recording of electrophysiological activity. The further optimization of technologies such as these for volumetric functional imaging with high resolution will continue to drive neuroscience discovery, particularly for high-throughput screening applications.

7. Conclusion

The emergence of 3-dimensional (3D) *in vitro* models of the human brain as an alternative to traditional 2-dimensonal (2D) culture systems offers a more representative environment to observe and manipulate cellular behavior. To date, organoid technology or bioengineering approaches have been the primary tools and techniques used to prepare these 3D brain models. Though only in existence for a few years, organoids have proven their utility as a tool to model human development, function, disease and evolution, with early examples of therapeutic applications in neurological and infectious disease and cancer biology. Despite this success, issues with reproducibility, heterogeneity, scalability and necrosis continue to plague these cultures given that their formation and development are largely driven by self-assembly. Bioengineering methodology, on the other hand, has been used to mimic microenvironments of the brain via cell-instructive hydrogels or scaffolding systems and/or connect with other tissues through the use of chip-based microfluidic systems and bioreactors. While these approaches provide for a greater level of control and reproducibility, they often lack the inherent complexity and physiological characteristics of the native microenvironment. Given the state of the field, there is a clear opportunity to combine these approaches to generate advanced 3D *in vitro* models with greater fidelity to the human brain and enhanced utility in disease modeling, biomarker identification, drug screening, and regenerative medicine. Through the convergence of interdisciplinary fields including biology, neuroscience, biomedical engineering and materials science, among others, we expect that future research will lead to better 3D models of the human brain that are suitable for high-throughput screening and compatible with technology for real-time functional monitoring. Through these efforts, researchers may finally be able to address the current lack of treatment options and offer solutions for patients currently suffering from neurological disease.

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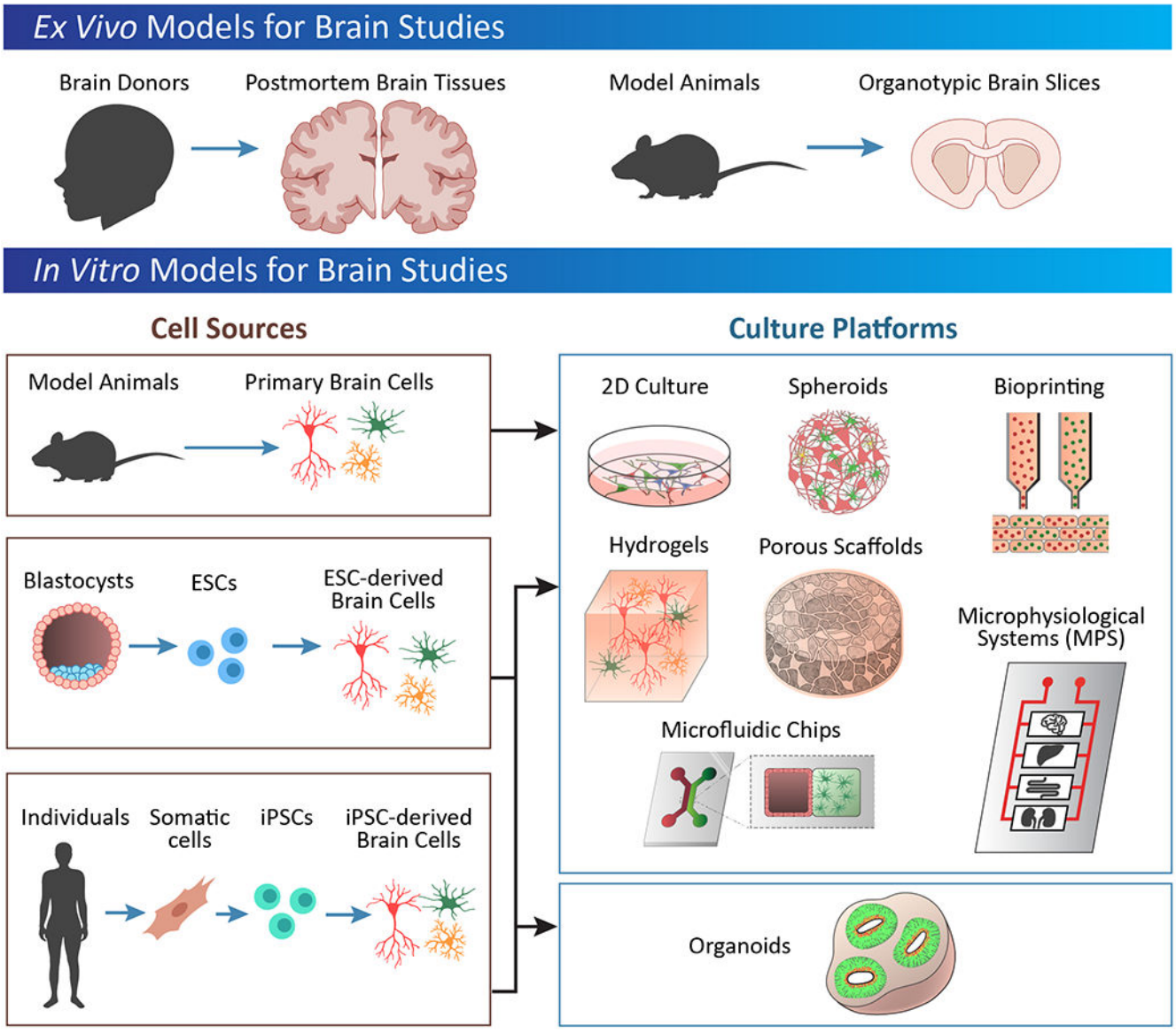

Figure 1.
Overview of commonly used model systems for studying the brain.

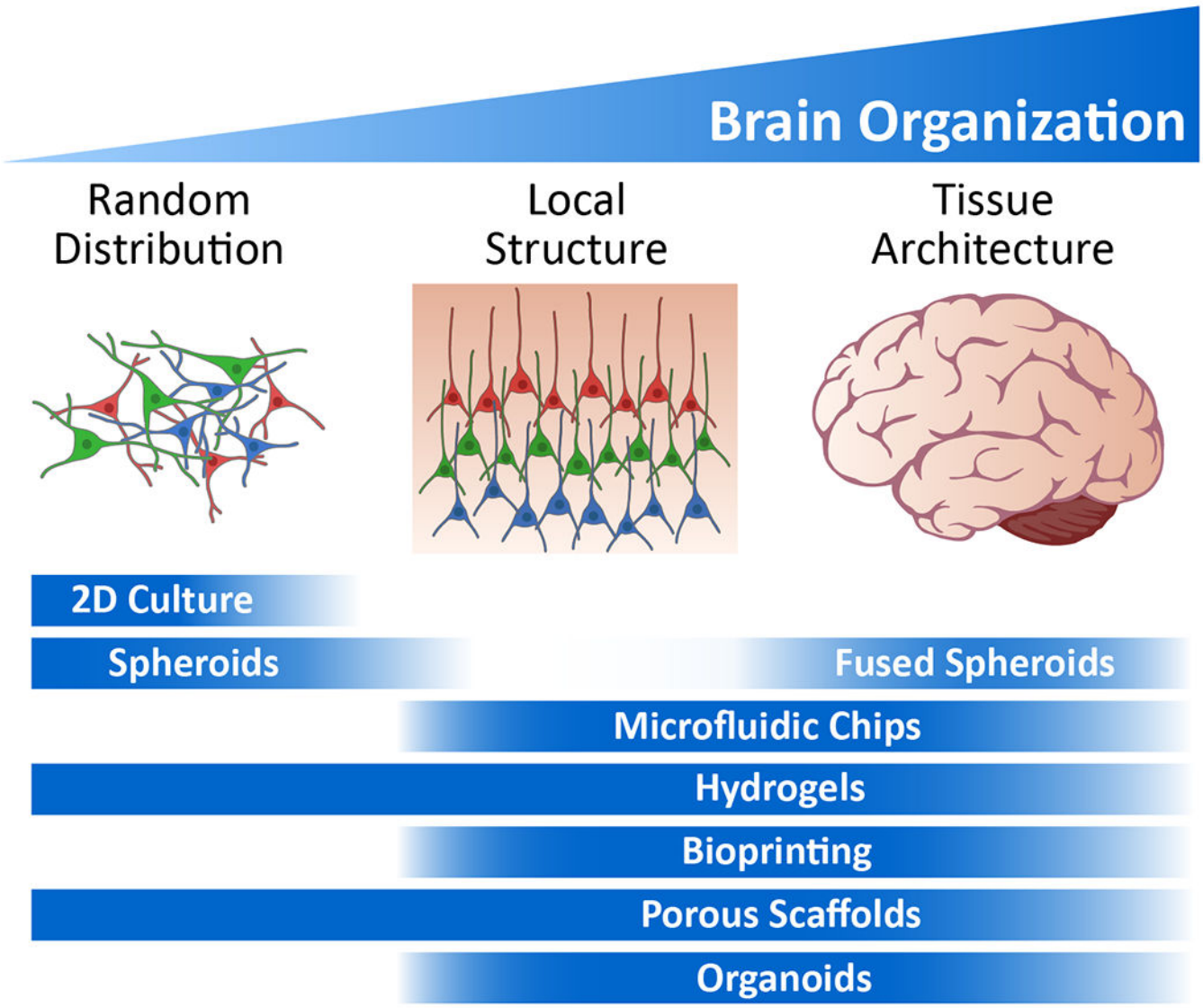

Figure 2.
Overview of the applicability of *in vitro* platforms to emulate brain physiology at different levels of brain organization. Bars represent the optimal application of each technology.

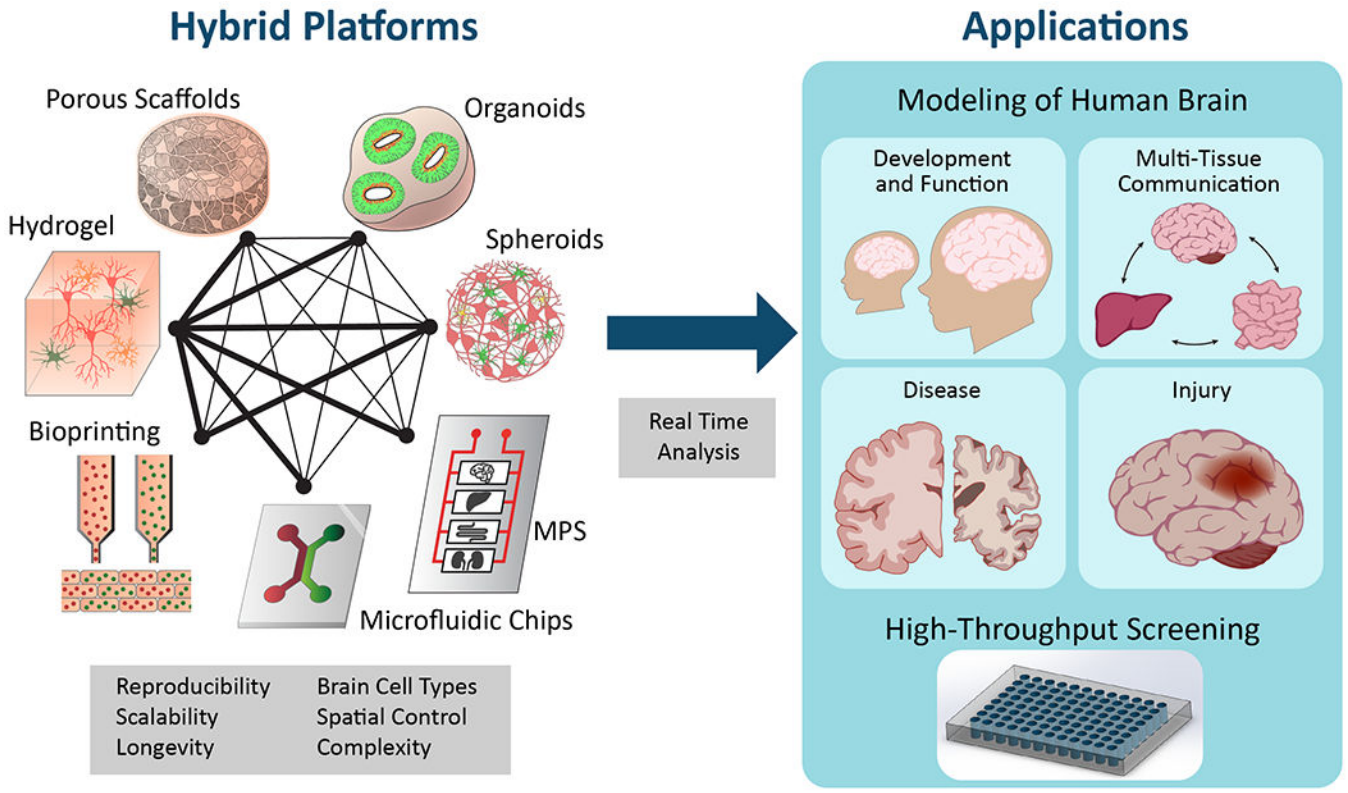

Figure 3.

Proposed integration of technology platforms for optimizing 3D tissue systems for modeling human brain physiology and high-throughput screening. Thicker lines between platforms indicate current areas of investigation whereas thinner lines show areas for further consideration. Gray boxes identify some of the key stem cell and bioengineering technology parameters to be addressed.

Table 1.

Summary of strengths and limitations of *ex vivo* and 2D *in vitro* platforms for brain studies.

	Strengths	Limitations
Human Postmortem Tissues[41–54]	Complexity of brain preserved, including cell composition and spatial organization	Tissue not living, no evaluation of function or development over time
	Identification of disease markers	Limited reproducibility or scalability for high-throughput screening
	Imaging of brain structure	
Rodent Slice Cultures[55–72]	Complexity of brain preserved, including cell composition and spatial organization	Limited capability for evaluating function or development over time
	Analysis of neural network anatomy and function	Limited reproducibility or scalability
		Not suitable for high-throughput screening
2D Cell Culture[73–86]	Reproducible and scalable: high-throughput screening	Limited culture longevity, particularly for primary cells as opposed to stem cells
	Suitable for non-invasive imaging and electrophysiological recordings	Low complexity, not representative of native cell environment
	Modeling of human biology through stem cell technology	

Table 2.

Summary of strengths and limitations of 3D *in vitro* platforms for brain studies.

	Strengths	Limitations
Organoids[35–37, 87–195]	Complexity of brain achieved through cell-guided tissue organization	Limited control of cell composition and spatial organization
	Models of neurodevelopment, neurodegenerative disease, injury, infection, evolution, etc.	Necrotic core limits tissue size
	Long-term culture capability and establishment of functional networks	Low reproducibility and capability for high-throughput screening
Microfluidic Chips[196–253]	Control of spatial organization and delivery of nutrients/growth factors via flow	Not suitable for long-term cultures to evaluate function or development
	Capability for multi-tissue systems	Limited capability for high-throughput screening applications
	Integration with imaging or electrophysiological modalities	Binding of drugs and biomolecules to polymer surfaces
Hydrogels[283–320, 344–345, 348–350, 359–361]	Ease of preparation for reproducible and scalable cultures	Limited culture longevity due to hydrogel degradation
	Suitable for non-invasive imaging and electrophysiological recordings	Variable extracellular matrix composition
	Some control of spatial organization and size of tissues	
Bioprinting[289, 321–329, 353–356]	Control of spatial organization and size of tissues	Requires optimization of cells and solution printing parameters
	Suitable for non-invasive imaging and electrophysiological recordings	Limited culture longevity due to hydrogel degradation
		Limited capability for high-throughput screening applications
Porous Scaffolds[330–342, 346, 351–352, 358]	Long-term culture capability of larger scale tissue models	Challenging to scale-up for high-throughput screening applications
	Greater control of tissue mechanics, particularly useful for injury models	Matrix needs to provide appropriate cellular microenvironment
	Suitable for non-invasive imaging and multi-tissue constructs	