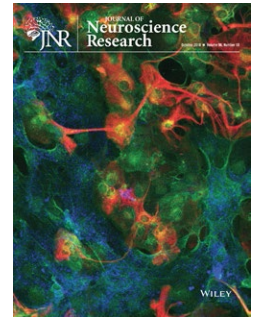


REVIEW

Advances in microfluidic *in vitro* systems for neurological disease modeling

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Abstract

Neurological disorders are the leading cause of disability and the second largest cause of death worldwide. Despite significant research efforts, neurology remains one of the most failure-prone areas of drug development. The complexity of the human brain, boundaries to examining the brain directly *in vivo*, and the significant evolutionary gap between animal models and humans, all serve to hamper translational success. Recent advances in microfluidic *in vitro* models have provided new opportunities to study human cells with enhanced physiological relevance. The ability to precisely micro-engineer cell-scale architecture, tailoring form and function, has allowed for detailed dissection of cell biology using microphysiological systems (MPS) of varying complexities from single cell systems to "Organ-on-chip" models. Simplified neuronal networks have allowed for unique insights into neuronal transport and neurogenesis, while more complex 3D heterotypic cellular models such as neurovascular unit mimetics and "Organ-on-chip" systems have enabled new understanding of metabolic coupling and blood-brain barrier transport. These systems are now being developed beyond MPS toward disease specific micro-pathophysiological systems, moving from "Organ-on-chip" to "Disease-on-chip." This review gives an outline of current state of the art in microfluidic technologies for neurological disease research, discussing the challenges and limitations while highlighting the benefits and potential of integrating technologies. We provide examples of where such toolsets have enabled novel insights and how these technologies may empower future investigation into neurological diseases.

KEYWORDS

Alzheimer's, CNS, MPS, organ-on-chip, Parkinson's, stroke

Abbreviations: α -syn, alpha-synuclein; A β , amyloid beta; BBB, blood-brain barrier; BEC, brain endothelial cells; EC, endothelial cell; ECM, extracellular matrix; iPSC, induced pluripotent stem cell; MEA, micro-electrode array; MPS, microphysiological systems; NVU, neurovascular unit; OoC, organon-chip; TEER, transepithelial/transendothelial electrical resistance; TJP, tight junction protein; WSS, wall shear stress.

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1 | INTRODUCTION

Neurological disorders are estimated to be responsible for 276 million disability-adjusted life years and 9 million deaths per year worldwide (Feigin et al., 2019). With an increasing incidence of neurological diseases over the last 25 years and an economic burden of \$789 billion per annum in the United States alone (Gooch et al., 2017), neurological disease presents a significant challenge for modern medicine. Despite significant research efforts, neurology remains one of the most failure-prone areas of drug development.

Across all disease types, as much as 90% of prospective medications entering clinical trials fail to reach approval (Perry & Lawrence, 2017). According to the 2018 Tufts Center for the Study of Drug Development Impact report, central nervous system (CNS) drugs take 20% longer to develop and 38% longer to be approved when compared to non-CNS drugs. Furthermore, CNS drugs show an increased probability of late-stage clinical trial failures (Pankevich et al., 2014). These significant scientific challenges faced by translational neuroscience resulted in a substantial drop in pharmaceutical CNS program portfolios between 2009 and 2014, with all of the top 10 leading pharmaceutical companies reducing their CNS drug development programs over this period (D. W. Choi et al., 2014). Despite a recent resurgence in interest in CNS drugs, with regulatory bodies responding to the unmet need for new CNS drugs through policies to promote innovation, along with pharmaceutical companies re-strategizing by partnering with academic institutions and small biotechnology companies, development of novel CNS therapeutics remains extremely challenging.

1.1 | The importance of studying human cells for human disease

Animal models have provided significant insights into biological systems and are currently indispensable in the drug discovery pipeline, however, numerous important cellular and molecular differences between species should not be overlooked. In the context of the brain, there are significant species-specific differences in efflux transporters, tight junctions, and cell–cell signaling observed in brain endothelial cells (BECs, Warren et al., 2009), extensive interspecies variation between human and rodent astrocytes (including functional and morphological complexity, glutamate responses, and immune responsivity) (Chandrasekaran et al., 2016), and numerous differences noted in electrical activity (Beaulieu-Laroche et al., 2018), gene expression and morphology (Hodge et al., 2019) between homologous human and rodent neurons. As such the inclusion of human-derived cells, tissues, and patient samples is highly recommended to improve predictive power in dementia research (Vargas-Caballero et al., 2016). The reasons for the high failure rate of candidate CNS drugs are myriad, including an incomplete understanding of the exact mechanisms underlying human disease hampering model development. However, the considerable differences in human and animal model's cellular and molecular neurobiology clearly necessitates new more physiologically relevant methods to

Significance

Microfluidic technologies have enabled new opportunities across a range of scientific fields, particularly neuroscience, where the ability to organize cells to mimic brain structures *in vitro* has provided novel insights into neurobiology. Once restricted to specialist laboratories and cross-disciplinary collaborations, systems are now commercially available and becoming an important tool in neuroscientist's investigational arsenal. Microfluidic systems are already being developed into models that recapitulate aspects of neurological diseases to improve drug discovery. This review surmises the current state-of-the-art in *in vitro* neurological disease modeling and may serve as guide to those wishing to explore microfluidic toolsets to expand experimental possibilities.

study human cells, in order to more effectively explore fundamental mechanisms and improve drug discovery and translation.

1.2 | Limitations of current *in vitro* methods

In vitro systems provide the opportunity to interrogate human cell biology, however, many cell culture approaches rely on immortalized or cancerous cells grown in non-physiological environments on 2D substrates such as functionalized glass or plastics. While providing a near limitless supply of cells for high-throughput screening, transformed or immortalized cell lines often show substantial drift in their transcriptional and epigenetic profiles over repeated passaging which may confound pharmacogenomics studies (Horvath et al., 2016; Nestor et al., 2015). Furthermore, non-physiological culture conditions promote genetic adaptations, and phenotypic changes. Common basal media formulations, such as Dulbecco's modified eagle medium have been optimized for rapid cell growth, often at the expense of maintaining cell identity. High glucose concentrations and fetal serum found in these media preparations have, for example, been shown to promote dedifferentiation of primary cell types toward fetal-like phenotypes (Morris, 1962). Oxygen levels are also rarely controlled in standard cell culture setups, often leading to far greater oxygen tension than would be present in tissues, impacting upon the redox environment and cell metabolism (Tiede et al., 2011). Plastic and glass substrates furthermore provide high mechanical stiffness, in the gigapascal range, while most *in vivo* environments are on the millipascal to kilopascal scale. With a growing understanding of mechanobiology and appreciation of the influence of substrate stiffness and the extracellular matrix (ECM) on cell behavior appropriate cell culture substrates are increasingly being recognized as a major factor in shaping *in vitro* cell phenotypes (Buxboim et al., 2010; Engler et al., 2006; Rauti et al., 2020). Thus, standard macroscale cell culture often fails to recapitulate the distinct microenvironments that constitute the

normal and diseased phenotypes that occur *in vivo*. Advances in microfluidic cell culture and “organ-on-chip” technologies have in contrast, allowed for an exploration of precisely tuned *in vitro* cell culture environments with greater physiological relevance.

Modeling the complexity of the human CNS *in vitro*, is undoubtedly hugely challenging. With limited ability to experimentally manipulate and probe human brains, along with translational gaps in animal neurological disease models, advanced microfluidic cell culture systems can serve as experimental tools to provide unique insights into human cellular function in a quasi-physiological context.

2 | ADVANTAGES OF MICROFLUIDIC CELL CULTURE SYSTEMS OVER TRADITIONAL CELL CULTURE

Microfluidic technologies provide the ability to precisely engineer micron to nano-scale architecture to shape the physical and chemical microenvironment, distilling brain physiology into manageable functional units, relevant to a specific research question (e.g., neuronal circuits, blood-brain barrier (BBB) and neurovascular unit (NVU) models). Such microphysiological systems (MPS) have already provided unique insights into mechanisms underpinning brain physiology and are now being utilized to explore pathophysiological mechanisms and novel interventions in neurological diseases.

Microfluidic systems offer several advantages over traditional macroscale cell culture (for in depth reviews see (Halldorsson et al., 2015) and (Tehranirokh et al., 2013)). The ability to shape physical and chemical microenvironments with a high level of precision has allowed for new possibilities to mimic physiological environments and to manipulate and integrate different cell types (Figure 1).

2.1 | Directing cells through microphysiological architecture

Microfluidic devices provide a physical architecture at the cellular and subcellular scale, which may be used to direct, pattern or confine cells for the precise investigation of cell to cell interactions, paracrine signaling, biomolecular analysis, automated image analysis and axonal guidance and trafficking.

Geometries tailored for precise positioning of cells can be used to study contact-based signaling such as stem cell differentiation, myelination, immune responses, and synaptic activity. This is exemplified by the work of Frimat et al. in developing a single cell culture or co-culture microfluidic device for studying juxtacrine signaling (Frimat et al., 2011) (Figure 1b). By creating physical cell traps on the cellular scale and taking advantage of the predictable paths of fluid flows to direct cells, Frimat et al. achieved automated sequential positioning of cells with a 99% arraying efficiency and ~70% pairing of heterotypic cells in close proximity to allow connexon interaction. This ability to create cell-scale architecture and precisely define physical barriers is especially powerful in enabling a more faithful representation of the spatial organization and structure of the CNS.

Taylor et al. engineered a microfluidic multicomponent device that aligned the growth of neurites from a somal compartment to a neurite compartment through the use of subcellular sized axon guidance channels, thus creating a tool to manipulate neuronal microenvironments in a highly precise manner (Taylor et al., 2003). Developments on this device format have since been used to direct *in vitro* neuronal connectivity and create simplified circuits to enable numerous biological insights as discussed later. While microfluidic device geometries can provide direct physical barriers to cells, they can also be used to direct flows, deposition of molecules, surface substrate patterning, and gel formation. For instance microfluidic devices can be used to create miniature stamps or templates for patterning of surface adhesion molecules or deposition of cells on glass coverslips for customized cell patterning (Chiu et al., 2000) (Figure 1c). Channel geometries can also pattern three-dimensional (3D) gels, taking advantage of surface tension to retain gels within set boundaries (Huang et al., 2009) (Sharma et al., 2019), or even to assemble and align fibrous structures of hydrogels using flows to dictate the nanogel structure and guide subsequent cell growth (Kim et al., 2017).

2.2 | Precise control of flow and chemical environment

By taking advantage of the unique physics of flow at the microscale, microfluidics can provide precise control of shear stress experienced by cells, along with chemical gradients, to mimic dynamic environments found *in vivo*. Microfluidic control of fluidic shear has enabled new insights into mechanotransduction, for example revealing physical forces as a pleiotropic modulator of the endothelial cell phenotype, influencing BEC, tight junction expression, and transcellular permeability (Brown et al., 2019). Since laminar flow prevails in most microfluidic flow regimes, mixing is largely dictated by molecular diffusion, providing the possibility for spatial and temporal chemical environments within the same cell culture platform for localized chemical stimulation even to the scale of a single cell (Horayama et al., 2016). Kim et al. demonstrate the power of this capability, creating a fully programmable and automated cell culture array to test the pairwise combination of drugs at the different concentrations on cell cultures grown in parallel (Kim et al., 2012) (Figure 1a), helping to address the challenge of scaling screening processes with combinatorial treatments where the availability of cells is limited, such as with primary cells. In the context of the CNS microfluidic cell culture devices have enabled production of chemical gradients to provide insights into microglial chemotaxis (Cho et al., 2013), endothelial migration, angiogenesis, and vasculogenesis (Barkefors et al., 2008; Kuzmic et al., 2019) and neurite outgrowth and guidance mechanisms (Romano et al., 2015). Microfluidic control of chemical milieu has also been extended to the gaseous microenvironment, such as oxygen levels and other bioactive gaseous compounds such as nitric oxide, carbon monoxide, and hydrogen sulphide (Wu et al., 2018). Spatiotemporal chemical control has also been achieved at sub-millisecond resolution and femtoliter volumes, allowing for the investigation of extremely fast reactions such as precise ligand

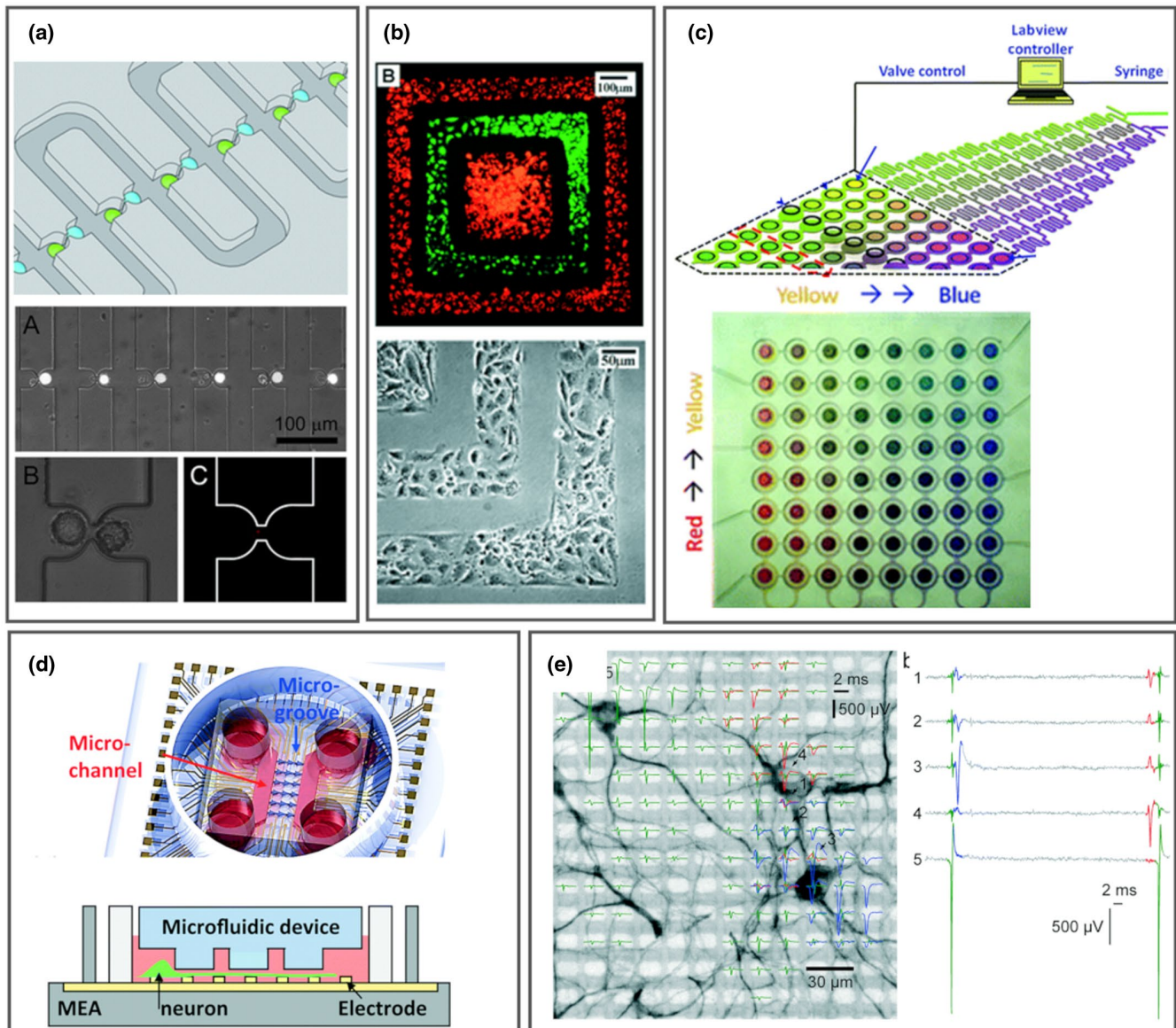


FIGURE 1 Advantages of microfluidic cell culture systems. (a). Control of fluid flows used to provide precision cell positioning (Frimat et al., 2011) (image reproduced from Frimat et al., 2011 LoC with permission from The Royal Society of Chemistry). (b). Patterning of protein disposition on surfaces using microfluidic stenciling and transfer printing to control patterning (Chiu et al., 2000) (image adapted from Chiu et al., 2000 with permission from PNAS, Copyright (2000) National Academy of Sciences, U.S.A.). (c). Microfluidic systems allow for precise control over mixing and chemical gradients for drug screening (adapted from Kim et al., 2012 with permission from The Royal Society of Chemistry). (d) Physical guidance of axons through microchannels, and integration of electrodes for cell monitoring and stimulation. (Reproduced from Jang et al., 2016 with permission from The Royal Society of Chemistry.) (e). Integration of electrodes providing recording of *in vitro* neuronal activity at subcellular, cellular, and network level (reproduced from Müller et al., 2015 Published by The Royal Society of Chemistry)

binding dynamics (Chiang & West, 2013) and nano/femtoliter chemical release has allowed for investigations using “artificial chemical synapses” (Peterman et al., 2004).

2.3 | Integration of analysis

The use of MPS provides the additional benefit of enabling integration of monitoring systems, such as transepithelial/endothelial resistance (TEER), impedance and electrophysiological systems. Such techniques often allow for real-time, long term, label-free, and

relatively non-invasive monitoring of cell culture conditions and cellular processes.

Gene expression and protein translation changes may occur on the scales of seconds to minutes, with phenotypic or morphological changes occurring on the scale of minutes to hours. Current cell assays are rarely capable of encompassing all of these timescales, gathering continuous data that can report on cell function under different conditions. Organ-on-chips and MPS have the potential to push the boundaries of on-board sensing devices that continuously monitor cell metabolism, phenotype, and growth to allow collection of multiparametric data over hours and days if not weeks (Modena et al., 2018).

TABLE 1 Commercially available microfluidic devices for *in vitro* CNS modeling

MPS	Type	Name	Company	Key features	Dimensions	Material	Refs
Neuronal circuit	Microchannel axon guidance	AXIS™ Axon Isolation Device	Merck	Available as two or three cell culture compartment formats	Axon guidance channels 5 μm H x 10 μm W available in 150, 450 or 900 μm L	Silicone (PDMS) chip- glass base	
		Ananda Neuro Device	Ananda™	Fits in any 35 mm plastic or glass bottom dishes or on coverslip.	1 mm length axon channels	Silicone (type not stated) chip - glass base	Magdesian et al. (2017)
		XonaChips®	Xona Microfluidics	Ready assembled. Made from stable hydrophilic plastic. Two or three channel chips available	150, 450, or 900 μm length axon guidance channels	Plastic cyclic olefin copolymer. Silicone also available	Nagendran et al. (2018)
		Ufluidic axon chip	Ufluidic	Microscope slide format, 3 devices per slide	10 μm H x 10 μm W x 250, 500 or 750 μm length axon channels	Silicone (PDMS) bonded to glass	
		OMEGA4 Neuronal Co-Culture Device+OMEGA ACE Triple-Chamber Nerve-on-a-Chip®	eNUVIO	Pre-bonded, 2 devices per chip, open channel format. Compatible with patch clamp	~640 μm long axon channels	Silicone (type not stated) to glass	
	3D		AxoSim	Dual-hydrogel scaffold created on the membranes of transwell inserts	5–10 mm long axon channel	Polyethylene glycol dimethacrylate	Sharma et al. (2019)
	Neurotrophic	OMEGA ^{AC} axon guidance and chemotaxis device	eNUVIO	Pre-bonded. Four chamber device (2 chemical source, 1 sink, 1 neuron channel)	Not stated	Silicone (type not stated) bonded to glass	

(Continues)

TABLE 1 (Continued)

MPS	Type	Name	Company	Key features	Dimensions	Material	Refs
Blood vessel models	Membrane-based dual chamber	Emulate Chip-S1™	Emulate Inc.	Side channels allow for pressure control of stretch. Automated platform enables control of flow and stretch in 12 chips	1,000 × 1,000 µm top channel, 1,000 × 200 µm lower channel.	PDMS	Vatine et al. (2019)
		The BI/OND CHIP	BI/OND	Two layer chip. Customisable membrane thickness and porosity. 6 chip holder for flow control	Channel heights available from 40 to 120 µm	PDMS	
	Pinned hydrogel	OrganoPlate® 2-lane + 3-lane	Mimetas	Phase guides separate a gel from a hydrostatic fed perfusion lane. 96 two lane or 40 three lane devices in a 384-well-plate format. OrganoTEER® and OrganoFlow® platforms provide flow and TEER measurement.	400 µm wide channels	thermoplastic	Bolognin et al. (2018)
		Synvivo chip	Synvivo	Radial design with large central gel chamber flanked by microchannels separated by micropillars. TEER compatibility available	Chips available in various dimensions	PDMS bonded to glass	Brown et al. (2019)
		3D Cell Culture Chip	AIM Biotech	Microscope slide format, three devices per slide. Central gel channel flanked by perfusion channels separated by micropillars	Channels 10.5 mm L × 0.25 mm H × 0.5 mm W perfusion channel 1.3 mm W gel channel	Thermoplastic with gas-permeable plastic laminate on base	Campisi et al. (2018)

Note: A non-exhaustive list of commercially available microfluidic devices that can be readily applied to CNS disease modeling of the BBB or used in the construction of neuronal circuits. References are given where examples of use of the device in CNS disease modeling has been published.

While many microfluidic chips are optically clear allowing for fluorescence assays, which are increasingly becoming automated, growing attention has focused on electrical systems that monitor properties of cells in a non-invasive manner. Pioneering work by Giaever and Keese explored how electrical impedance spectroscopy could be used to measure cell adhesion, differentiation, and even micromotion (Giaever & Keese, 1984; Lo et al., 1995). Further developments in this vein have led to devices integrated with traditional cell culture formats (multi-well plates and Transwell devices) for measuring TEER (Wegener et al., 2004). Examples of microfluidic chips with integrated sensing are becoming more widespread. There are two main types of device—those which integrate electrodes above and below cells grown on a porous membrane (analogous to a Transwell system) and those which integrate electrodes directly into the surface on which the cells are grown (Benson et al., 2013). In the case of the BBB, the Transwell device is potentially more relevant, as both the TEER of the endothelial cells, along with the ability of molecules to be transported across the barrier are interesting complementary parameters (Griep et al., 2013; Park, Mustafaoglu, et al., 2019). Neuronal firing can also be usefully interrogated using multi-electrode arrays, which pick up on action potentials at given frequencies. Maoz et al. have even demonstrated integration of both multi-electrode array and transepithelial electrical resistance onto a single device for simultaneous measurements of cellular electrical activity and barrier function (Maoz et al., 2017). Although at present such arrays cannot access intercellular electrical activity (e.g., picked up via patch clamp), monitoring neuronal firing in mono-culture or even in organoids or spheroids can be extremely valuable for drug discovery. Given the numbers of neurons present in any given network, leveraging the power of microelectrode arrays (MEAs) within a microfluidic chip is hugely beneficial (Müller et al., 2015) (Figure 1d,e) particularly when coupled with the ability to accurately pattern and position cells (Soscia et al., 2017). For a detailed review of sensor integration into MPS please see Kilic et al. (2018).

3 | MICROFLUIDIC TOOLS TO STUDY NEURONAL FUNCTION

In recent years, microfluidic technologies have increasingly been used in neuroscience research, to extend the experimental capabilities for studying axonal guidance and transport, synapse formation and function, and neuronal function across subcellular to network levels (Neto et al., 2016). Through precise control of microenvironments, MPS offer new opportunities to create *in vitro* physiologically and pathophysiologically relevant models.

3.1 | Directing neurons: Guiding and compartmentalization

In 1977, Campenot et al. developed a compartmented culture system to separate the neuronal axon from the soma. Consisting of a Teflon separator attached to a glass substrate, this device provided

new possibilities to investigate various neuronal functions such as axonal transport and migration. However, this system had a number of drawbacks including: the technical difficulty of assembly and use, limited imaging possibilities, and leakage of media due to poor adhesion of the grease seal. The crucial limitation, however, was that this system relied on neurotrophin gradients to direct axon growth between compartments, meaning that neurotrophin independent CNS neurons were not compatible, and the system was limited to peripheral nervous system (PNS) neurons. The advent of microfluidic devices has enabled vast improvements in spatially separating neuronal axons and soma, allowing culture of CNS neurons through physical guidance in subcellular microchannels, connection of multiple compartments, and devices providing directional neurite outgrowth.

Based on the microfluidic design proposed in a landmark paper from 2003 (Taylor et al., 2003), several examples of how neuronal cell cultures can be grown, manipulated, and studied in microfluidic devices have been provided in the last 15 years, with a number of device options commercially available for neuroscientists to study axonal growth and connectivity, such as Merck's AXIS™ Axon Isolation Devices, Ananda's™ Neuro Devices, Ufluidic's Axon chips, eNUVIO OMEGA4 Neuronal Co-Culture chips and Xona Microfluidics® XonaChips (summarized in Table 1). Various modifications of the device layout and the development of new methodologies based on the device topology have since been reported, expanding the breath of applications that miniaturized technologies can provide in neuroscience research, such as axonal response to injury (Taylor et al., 2005), myelination (Park et al., 2009), synaptic formation and function (Shi et al., 2013), probing the direct and indirect response of neuronal cultures to chemical stimuli (Robertson et al., 2014), neurite growth (Frimat et al., 2010) (Nagendran et al., 2018), proof-of-concept pharmacology (MacKerron et al., 2017), and drug screening (Fantuzzo et al., 2020). Microfluidic topologies have also shown the capability to promote the asymmetric spatial organization of dendritic and axonal subcellular components, leading to the formation of unidirectional connections where axons from one culture are free to grow in a permissive direction, but extension from a neighboring culture is impeded, thus forming asymmetric connections (Holloway et al., 2019). Directional neuronal connectivity is a key feature of the functional anatomy of the brain and *in vitro* models enabling asymmetric neuronal patterning opens up new possibilities to mimic key regions of the nervous system. Microfluidic designs based on directional bias and axonal edge guidance have been successfully demonstrated mostly using rodent cultures (Holloway et al., 2019; Peyrin et al., 2011; Renault et al., 2016) (Figure 2a,b), and also more recently with induced pluripotent stem cell (iPSC)-derived neuronal cultures (Gribaudo et al., 2019). Other methods to direct neuronal outgrowth include direct micro-manipulation (Magdesian et al., 2017) as well as use of gradients of neurotrophic factors using spatio-temporal fluid control (Millet et al., 2010; Taylor et al., 2015) (Figure 2c). Micro-contact printing may also be used to pattern surface proteins promoting neuronal outgrowth in defined circuits (Offenhäusser et al., 2007), allowing for open top platforms, easy access, and integration of electrophysiological measures. Fidelity and longevity of the surface patterning along with conformity of cell growth to the exact pattern remain a drawback of this technique.

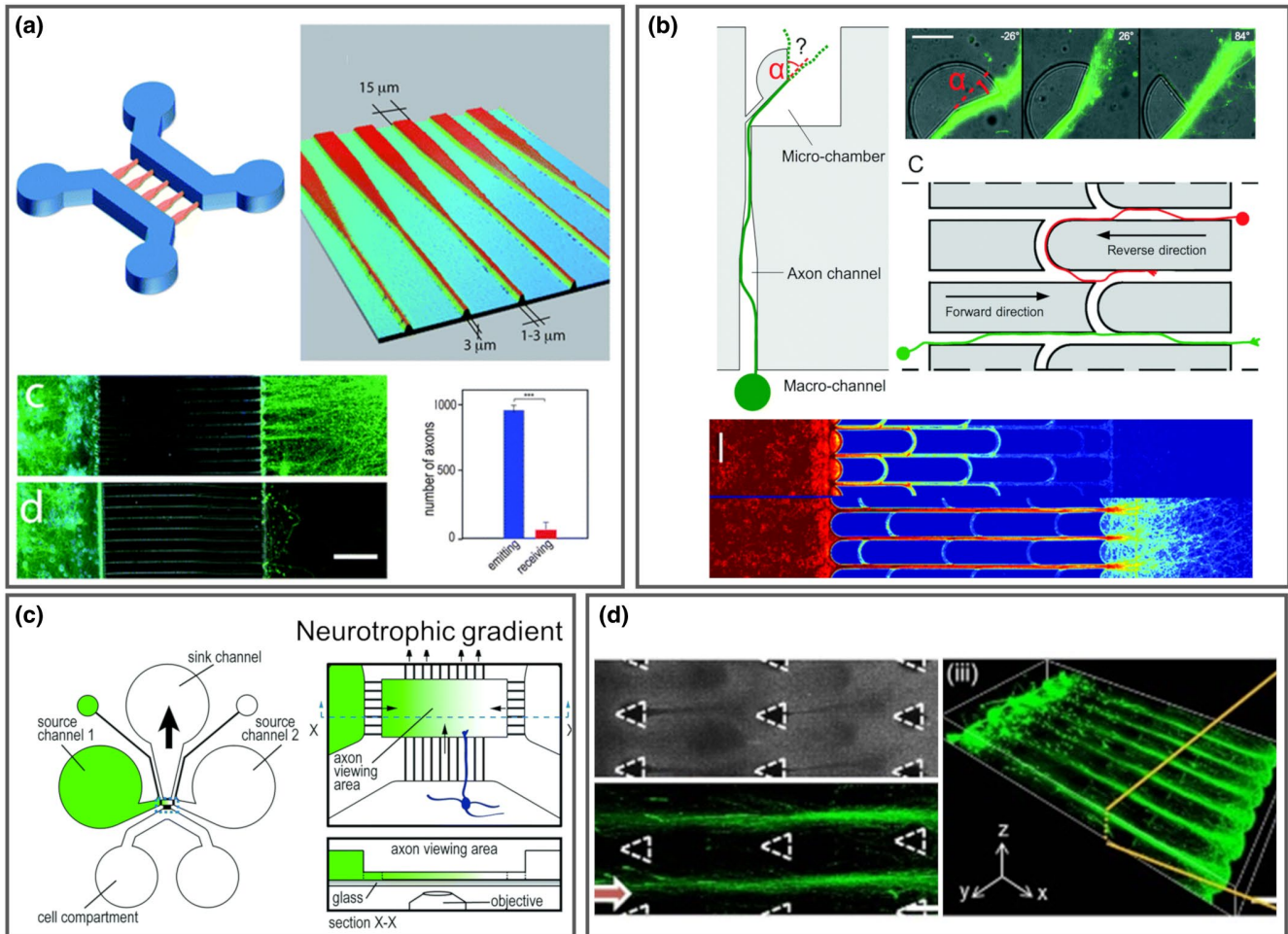


FIGURE 2 Methods for directing neurons and simplistic cell circuits. (a) Peyrin et al. use funnel shaped neurite outgrowth channels to preferentially guide axons from one chamber to the other (reproduced from Peyrin et al., 2011 with permission from The Royal Society of Chemistry). (b) Renault et al. exploit a prohibitive critical angle of growth to create return loops within the outgrowth channels (reproduced from Renault et al., 2016 with permission from The Royal Society of Chemistry). In the permissive direction the angle is too great and the axons predominantly continue, in the non-permissive direction axons follow the edge of the channel and are looped back. (c). Gradients of neurotrophic factors can be used to guide axon growth (reproduced from Taylor et al., 2015 with permission from The Royal Society of Chemistry). (d). Alignment of ECM fibers during gelation using flow, can be used to guide axon growth in 3D aligned bundles (reproduced from Bang et al., 2016 with permission from Advanced Healthcare Materials, John Wiley and Sons)

Neural circuits have been formed in 3D hydrogels using fluid flows to align ECM fibers during gelation to guide axon growth. Bang et al. used this technique to create neuronal bundles and fasciculated axons (Bang et al., 2016) while Kim et al. exploited the fibrillogenic properties of collagen, to create an anisotropically organized CA3-CA1 neural circuit (Kim et al., 2017). However, with the greater physiological relevance provided by directing 3D neuronal circuits, this also provides an added layer of complexity in probing neuronal function and analysis workflows.

3.2 | Electrophysiological probing neuronal function

The above outlined methods for creating neuronal circuits *in vitro* are now increasingly being integrated with MEAs for

electrophysiological interrogation, combining the advantageous spatial cell patterning capabilities and precise control of chemical cues provided by microfluidics with electrical stimulation and recording of neuronal networks. Throughout the years, several methodologies have been developed, from manual whole cell patch clamping in standard two-chamber microfluidic devices (Jokinen et al., 2013), to tubeless devices based on capillary forces for drug delivery (Resto et al., 2017), to microfluidic planar patch clamp systems (Xu et al., 2014) with automated drug delivery (Yuan et al., 2016). However, it is the integration of microfluidics with MEA substrates, possibly due to the planarity of both technologies, that has produced higher-throughput and innovative computational solutions. Earlier studies combined commercially available MEA substrates with standard two-chamber devices (Kanagasabapathi et al., 2011) and lately with more

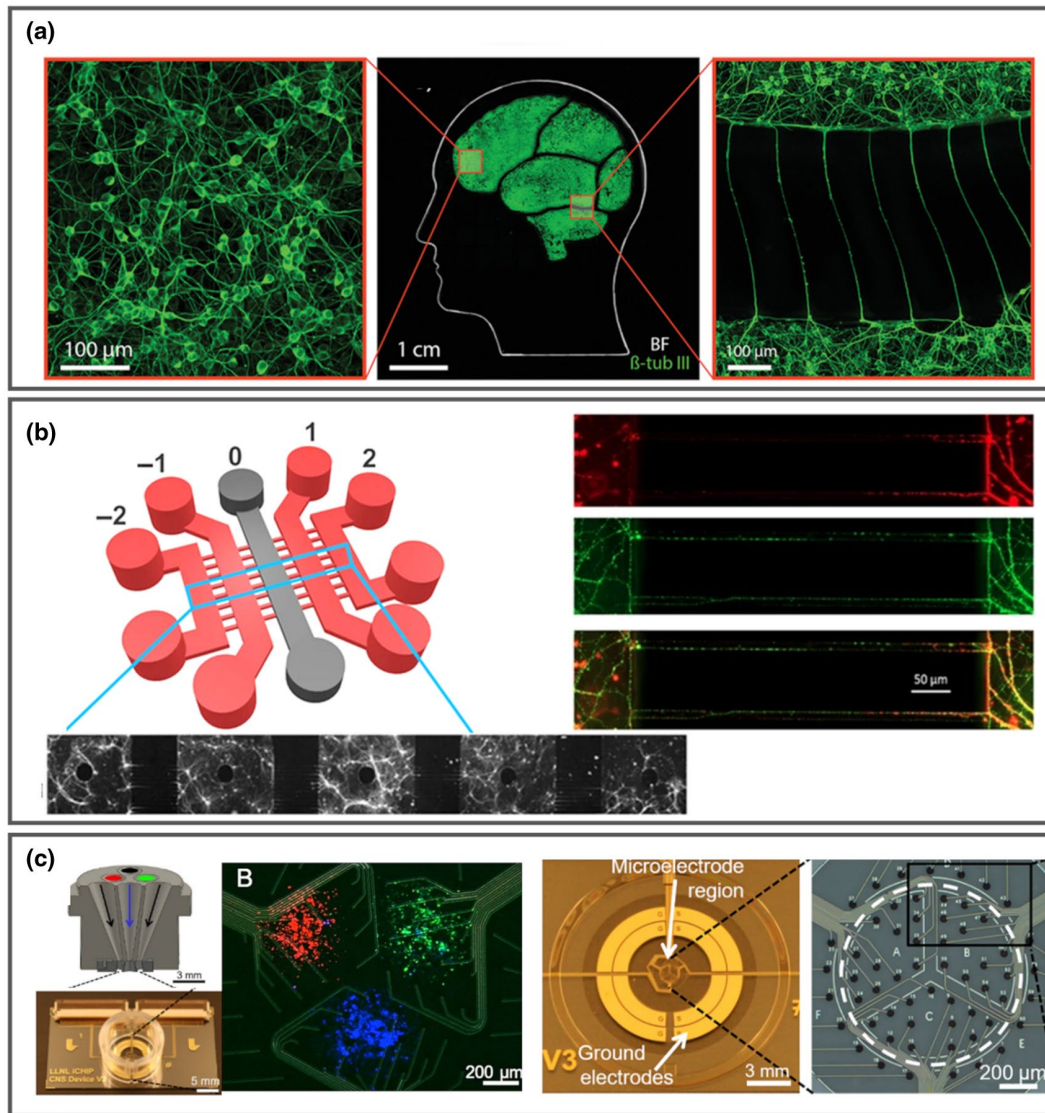


FIGURE 3 *In vitro* neural networks mimicking connectivity between brain regions. (a) Kajtez et al. combine 3D printing with microfabrication techniques to create macro- and microscale architecture in a simplified nigrostriatal pathway using human stem cell-derived neurons (figure reproduced from Kajtez et al., 2020 under the terms of the Creative Commons Attribution License). (b) Samson et al., use a five-chamber neural guidance device to connect primary hippocampal neurons, revealing network level activity buffers against excitotoxicity. (reproduced from Samson et al., 2016 under the Creative Commons Attribution 4.0). (c). Soscia et al. use a removable insert to pattern primary rodent hippocampal and cortical neurons onto an MEA which showed characteristic bursting and communication between neuronal compartments (modified from Soscia et al., 2017 under the Creative Commons Attribution License)

bespoke layouts such as van de Wijdeven et al.'s device containing three nodes connected in series, with integrated MEA. This system allows for measurement of extracellular electrophysiological activity in response to axotomy, with separate synaptic channels enabling mechanical shearing of axons via introduction of an air bubble through pipetting (van de Wijdeven et al., 2019).

3.3 | Toward networks

Neural circuits are anatomical connections within the brain with far-reaching implications for many neural functions. As an

example, the hippocampus, known to be involved in spatial navigation, consists of multiple sub-regions which are interconnected in a predefined, mostly unidirectional, way (e.g., mossy fibers) (Strange et al., 2014). Similarly, the midbrain is another example of a source of projection neurons (Caggiano et al., 2018; Tovote et al., 2016). Dopaminergic neurons in this area project to areas across the brain, including the striatum and multiple cortical areas. The majority of studies carried out on such specific brain regions have been performed *ex vivo* using brain slices. However, this approach has obvious limitations due to undesired axotomy, uncertainty on cell topological connections and short experimentation times due to cell death. Therefore, *in vitro* models where different

cell populations can be grown and connected “ad hoc” present simplified but valuable tools to investigate network function as well as neuropathological conditions. Microfluidic technologies have been employed to create human brain-mimicking neuronal circuits for the study of cortico-striatal networks using calcium imaging (Lassus et al., 2018), to model neuroprotective mechanisms (Samson et al., 2016), to study structure–function relationship by precise neurite guidance achieved by electric fields [77], to highlight brain region-specific cell identities (Kamudzandu et al., 2019), physiology and function (Dauth et al., 2017), to create 3D structured circuits (van de Wijdeven et al., 2018) and for drug screening (S. R. Lee et al., 2019) (Figure 3). Kajtez et al., have also recently used a hybrid fabrication technique by integrating 3D printing with soft lithography to provide rapid prototyping at both micro- and macroscale, enabling open-well compartmentalized devices with greater freedom of device design (Figure 3a) (Kajtez et al., 2020). Using this system, they demonstrate a proof-of-principle human *in vitro* model of the nigrostriatal pathway using human stem cell-derived neurons (Kajtez et al., 2020). Such advancements in device fabrication techniques could in future be utilized to create more physiologically relevant neural network mimetics of key pathways to interrogate network level connectivity in pathophysiological contexts.

4 | HETEROTYPIC CELL CULTURE SYSTEMS: TOWARD ORGAN-ON-CHIP MODELS

Historically, the neuron has provided the central focus for studies of neurological diseases. Recent years have, however, seen a conceptual shift from this neuro-centric view to one that emphasizes the importance of multidirectional interactions between all brain cell types, including neurons, glia, vascular, and perivascular cells. This concept of the NVU, (formalized at the 2001 NINDS Stroke Progress Review Group meeting) has emerged as a new paradigm from which to investigate brain physiology and pathobiology and has revealed commonalities of pathogenic processes across many neurological diseases, such as glial activation and BBB compromise. As such, numerous efforts have been made to replicate the heterotypic cellular interactions and physio-chemical environment that underpin the BBB and NVU *in vitro*, to enable a greater insight into neurological diseases.

4.1 | Blood–brain barrier models

The BBB is a dynamic and highly selective conduit that maintains the unique chemical environment of the brain (Langen et al., 2019). This barrier provides a considerable obstacle in delivering neuropharmaceuticals to the brain, while its disruption has been implicated in the pathophysiology of a number of diseases, including Alzheimer's, multiple sclerosis, epilepsy, stroke, and Parkinson's

disease. Early attempts to replicate this structure *in vitro* involved the culture of BECs on Transwells™ under static conditions to allow the study of transcellular and paracellular permeability. Such systems have since allowed for a dissection of the components that make up the BBB providing an insight into the roles of astrocytes, pericytes, and the ECM in BBB maintenance. While the mainstay for such investigations has been traditional Transwell assays, microfluidic technologies are now emerging that provide a greater level of control over microscale architecture and physiochemical cues, such as flow, that regulate BBB function.

The significance of the *in vivo* microenvironment in maintaining BEC properties was highlighted by observations that proliferating rat cerebral capillary fragments gave rise to confluent monolayers *ex vivo* but their endothelial characteristics were lost over time in culture (Panula et al., 1978). Early blood–brain interphases were generated by co-culturing primary BEC and brain parenchymal cells, often from different species, in different configurations on Transwells, with endothelial cells (ECs) suspended on membranes that represent an artificial barrier, typically thick (10–50 μm) rigid substrates with pores (0.4–3 μm diameter) (Figure 4a). These models helped to show that ECM composition, pericytes, astrocytes, or their conditioned media upregulated tight junction protein (TJP), polarized apico-basal transporter expression, and increased TEER (Abbott et al., 2006; Nielsen et al., 2017). Such transwell models have enabled high-throughput pharmacokinetic, pharmacological, and drug toxicity applications, but fail to recapitulate 3D BBB architecture, wall shear stress (WSS) and have a high media to cell ratio which dilutes paracrine signaling, thus limiting their translation to human BBB studies.

MPS have recently enabled dynamic neurovascular microenvironments to be precisely defined, sampled, and manipulated, while maintaining paracrine and juxtacrine signaling at their interphases (Lee & Leong, 2020). A number of approaches have been made to recapitulate the BBB (Figure 4b–e), each with their own benefits and limitations which should be considered with respect to the intended study (for a detailed discussion of the parameters which can be used to benchmark *in vitro* BBB models see DeStefano et al., 2018). Multi-layered microchambers separated by porous membranes (Figure 4b) such as the bi-layered Emulate chip (Table 1) enable transwell-like connectivity with improved compatibility with microscopy techniques, while also allowing for perfusion to recapitulate vascular WSS (Griep et al., 2013; van der Meer et al., 2010). Compartmentalized membrane-based devices allow for easy sampling of vascular and parenchymal compartments while also enabling integration of electrodes to monitor TEER. Griep et al. demonstrated the dynamic and responsive nature of this barrier with the introduction of flow and WSS increasing TJP expression and elevating TEER by a factor of 3, while addition of inflammatory mediators disrupted barrier function, reducing TEER by a factor of 10 (Griep et al., 2013). Ahn et al. incorporate a 3D culture in the lower pericyte/astrocyte chamber, with flow maintained over the apical EC monolayer, showing co-culture to upregulate endothelial expression of TJP and membrane transporters and receptors (Ahn et al., 2020). Using this model Ahn et al.

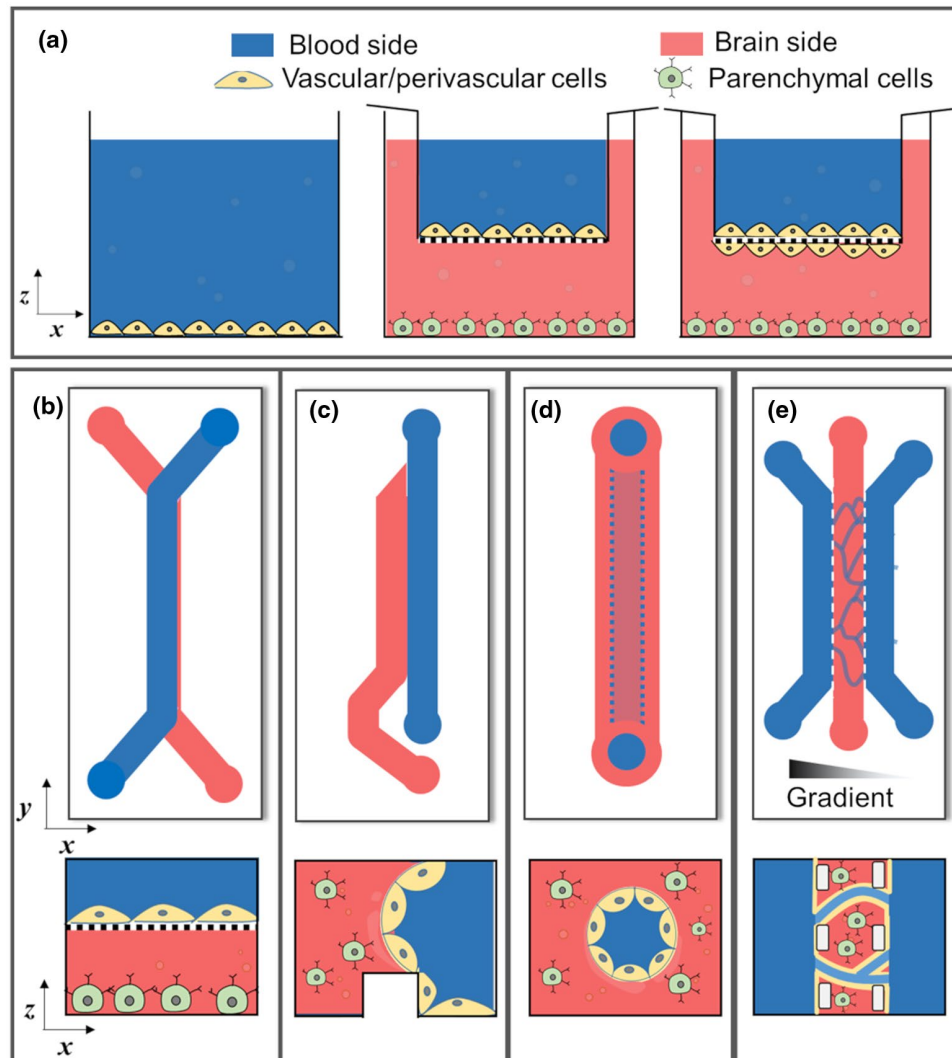


FIGURE 4 Approaches to NVU and BBB models. (a) Macroscale approaches to developing BBB models typically consist of cell monolayers (left) and Transwell systems (center and right), the relatively low surface area to volume ratio (typically $\sim 10 \text{ cm}^{-1}$) can result in poor gas exchange and distance between heterotypic cells can result in a large dilution of paracrine signaling factors. Microfluidic systems typically have surface area to volume ratios of $\sim 800 \text{ cm}^{-1}$ and show rapid gas exchange, however, rapid accumulation of waste products and consumption of nutrients necessitates flow. Multiple approaches to dynamic microfluidic BBB models have been made. (b) Two layer systems, utilize a membrane to separate blood and brain compartments, such as in the Emulate chip, similar to a transwell system but incorporating flow and with more physiological cell-extracellular fluid ratios. (c). Devices using pillars or phase guides on one edge (as in the Mimetas OrganoPlate[®], the SyM-BBB (Prabhakarandian et al., 2013) and SynVivo's SynBBB) pin a cell laden gel allow for a lumen with one region in direct contact with parenchymal cells in 3D. (d). Fully 3D defined lumens can be created in a gel using sacrificial (Golden & Tien, 2007) or removable moulds (Bouhira et al., 2020) or by taking advantage of Saffman-Taylor instability such as (Herland et al., 2016). These create relatively large (100–300 μm) lumens in straight channels allowing for direct cell-cell contact. (e) Pinning cell laden gels to a central channel and creating a gradient of pressure or growth factors can be used to stimulate vasculogenesis and create perusable capillary like networks of physiologically relevant sizes with direct contact between parenchymal and vascular cells as in AIM Biotech chips

were able to study receptor-dependent transcytosis of nano-particle coupled drug delivery (Ahn et al., 2020).

Compartmentalized models with BEC monolayers are disadvantaged by artificial barrier interphases that limit direct neurovascular coupling. These systems often consist of cell monolayers that do not recapitulate 3D ECM and heterotypic cell interactions, also using rectangular channel cross sections that result in poor cell coverage and flow dead zones at the corners. Other microfluidic designs have

focused on recapitulating the 3D NVU architecture to preserve cell associations by using cells embedded in gels. One approach to compartmentalize a 3D matrix has been through the use of surface tension to pin a gel at a change in channel geometry such as with phase guides or micropillars (Figure 3c), as incorporated in the SynBBB, B³C BBB models, or the commercially available MIMETAS OrganoPlate[®] (Table 1). These allow for the controlled dimensions of the vascular channel to be maintained while allowing 3D culture

and direct contact with perivascular cells and astrocyte endfeet. BBB permeability and transcellular flux can be assessed by live imaging of fluorescent molecules of different sizes and properties. Using the B³C BBB chip (Figure 4c) Deosarkar et al., show that such

an approach results in significantly improved barrier characteristics when directly compared to transwell models using the same cells, to values that approach those found *in vivo* (Deosarkar et al., 2015) (Figure 5c).

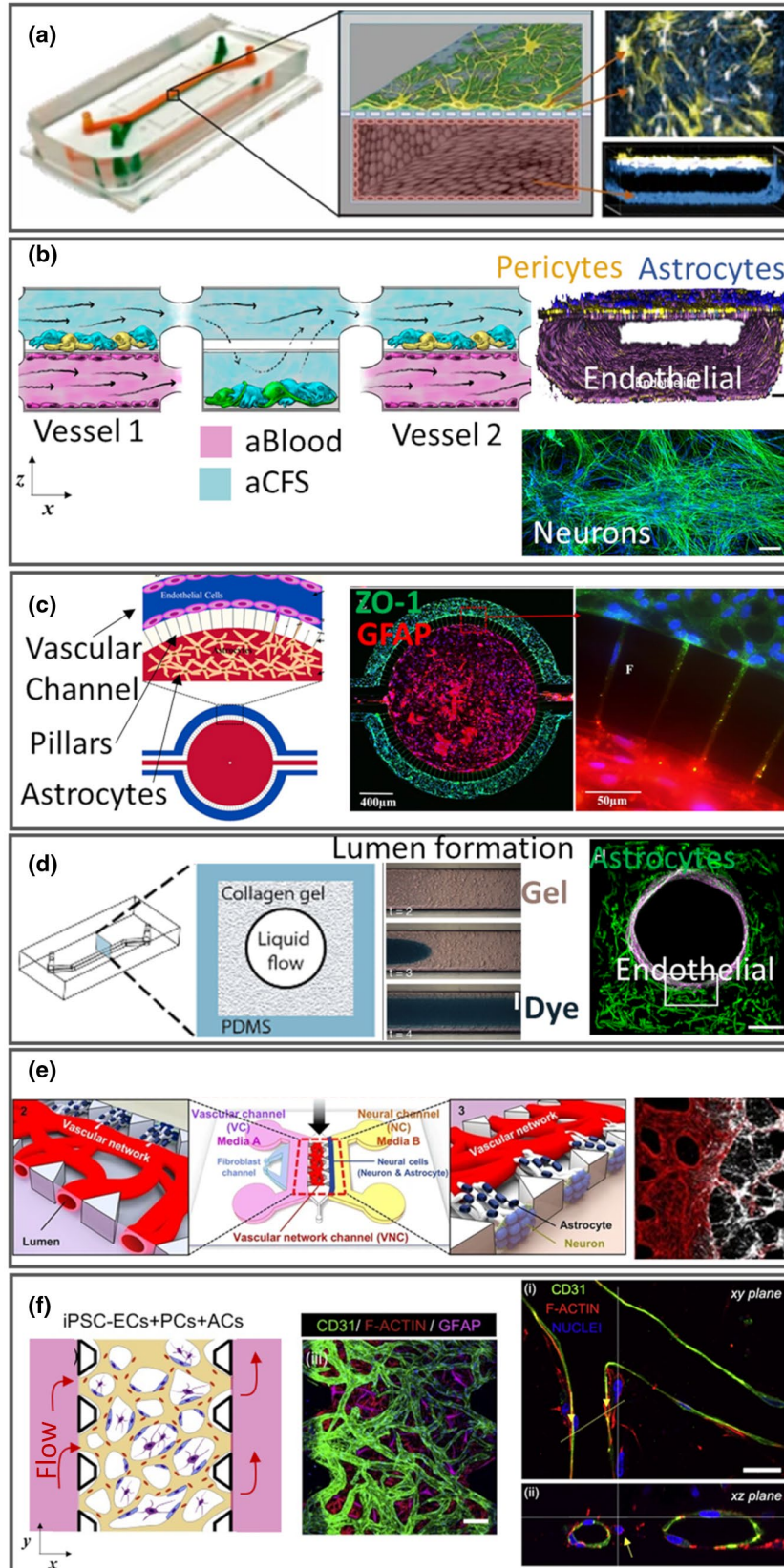


FIGURE 5 Microfluidic BBB and NVU models. (a) Park et al. use an Emulate two layer chip to co-culture iPSC derived human brain endothelial cells with primary human brain astrocytes and pericytes suspended on a membrane (modified from Park, Mustafaoglu, et al., 2019 under the creative commons license 4.0). (b) By utilizing multiple connected chips as a full NVU model, incorporating endothelial lined channels separated by a membrane from an astrocytes/pericyte co-culture that links to a neuron/astrocyte culture, Maoz et al. (2018) were able to study metabolic fluxes and interactions across the NVU. (c) Deosarkar et al.'s B³C BBB chip (now commercially available as Synvivo's SynBBB) uses micropillars to pin a gel to a central region allowing 3D culture of astrocytes with endothelial cells in the surrounding channel (adapted from Deosarkar et al. (2015) 2015 PLOS ONE under the Creative Commons Attribution License). (d) Herland et al., take advantage of Saffman–Taylor instability by using fluid flow to displace collagen during gelation, creating a circular lumen for use as a 3D BBB model (Herland et al., 2016). (e) Bang et al. use vasculogenesis through a central gel pinned by surface tension to develop an NVU model with neurons astrocytes and endothelial cells, supported by fibroblast derive factors released from a separate fibroblast compartment (reproduced from Bang et al., 2017 under the Creative Commons Attribution 4.0 International License). (f) Campisi et al. use a similar technique to provide a human BBB model with endothelial cells, pericytes and astrocytes (reproduced from Campisi et al., 2018)

Using the above approach only one side of the vascular channel is in contact with the parenchyma. To address this, researchers have utilized the phenomenon of viscous fingering by which a flow of a lower viscosity fluid can replace a denser one, in this case a hydrogel, to create a fully circular cross-section lumen (Figure 4d). Herland et al., utilized this approach to uncover distinct contributions of astrocytes and pericytes to BBB inflammatory responses (Herland et al., 2016) (Figure 5d). De Graaf et al. have since refined this technique, demonstrating scalability for production of blood vessel models (de Graaf et al., 2019). Other approaches to forming circular lumens, have been through sacrificial moulds or removable templates, providing more flexibility in vessel geometries, allowing investigation of disturbed flows at vessel bifurcations on BBB integrity (Bouhria et al., 2020). More recently, this approach has also been used to demonstrate that severe acute respiratory syndrome coronavirus 2 spike protein alters barrier function (Buzhdygan et al., 2020). These approaches are limited in that they produce relatively large diameter lumens compared with the brain capillaries of the BBB which does not allow the complete ensheathment of the vasculature with pericytes or astrocyte endfeet as occurs *in vivo*.

Cellular self-organization has also been utilized for the production of 3D networks (Figure 4e). By confining a hydrogel to a region flanked by perfusion channels, gradients of growth factors or fluid flow can be used to stimulate vasculogenesis or angiogenesis through the gel (Figure 5e,f). Such models produce vessels highly similar to *in vivo* vascular beds, although they often require support from fibroblasts to allow lumen formation and vessel stabilization. Campisi et al. have, however, recently been able to successfully produce luminized vessels by co-culturing hiPSC-BEC with human primary brain pericytes and astrocytes (Figure 5f) allowing pericytes and astrocytic end feet to directly contact the perfused vascular network (Campisi et al., 2018). This model recapitulated physiological permeability coefficients and preserved intra-luminal mechanotransduction. Such platforms are well suited to studies of vasculogenesis, vascular remodeling, and could be used in conjunction with iPSC technologies to study the effect of genetics on vessel formation, such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Homogenous WSS or TEER measurements are, however, difficult to achieve due to the unpredictable geometry of vessels formed.

4.2 | Neurovascular unit models

The NVU represents a functional unit of the brain. By extending BBB models to include neurons, microfluidic *in vitro* systems have been able to better model the NVU, demonstrating physiologically relevant BBB integrity, cell–cell interaction and paracellular signaling and metabolic coupling.

Maoz et al. demonstrate the power of utilizing BBB chips with neuronal culture by connecting a BBB chip to an hES-derived neuronal culture upstream of a second BBB chip. This enabled influx of perfused metabolites to be traced across the BBB, through neuronal compartment and efflux out of the second BBB chip, to show that metabolites produced by the endothelium and cells of the perivascular niche directly influence GABA and glutamatergic neurotransmitter synthesis within the brain compartment, thus identifying a previously unknown metabolic coupling between BBB and neurons (Maoz et al., 2018) (Figure 5b).

Bang et al. take a vasculogenesis approach, culturing HUVECs in a central fibrin gel channel flanked by a vascular channel and neuronal/astrocyte co-culture channel. This allows the formation of capillaries in direct contact with astrocytes and neurons, while separate channels enabled media to be optimized for each culture (Bang et al., 2017) (Figure 5e). This approach, however, required an additional culture channel containing lung fibroblasts which continually condition the media to support HUVEC vessel formation. The use of brain resident vessel support cells, such as pericytes, could be used in future to negate the need for non-brain resident cells.

Adriani et al. employed micropillars to pattern two distinct cell laden hydrogels of neuronal and astrocyte cultures with a third channel being lined with human BECs to form the BBB interphase (Adriani et al., 2017). This model displayed size-selective barrier permeabilities and an active neuronal network. While the permeability coefficients were supraphysiological, likely due to combining cells from different species and the absence of pericytes, it provided functionally coupled 3D NVUs for assessing the effects of perfused drugs on neuronal activity. A subsequent brain-on-chip model integrated human ES-derived motor neuron spheroids with iPSC-BECs in a common collagen gel, revealing BEC to promote neuronal connectivity and increase synchronized activity through paracrine and contact-dependent signaling (Osaki et al., 2018).

More recently, iPSC technologies have been used in the Emulate dual compartment chip to create isogenically derived human iPSC-NVUs to provide a platform for studying personalized medicine and patient-specific disease (Vatine et al., 2019). This model demonstrated physiologically relevant TEER ($>1,000 \Omega/\text{cm}^2$) and could

appropriately distinguish between substances of known *in vivo* BBB permeabilities, while also protecting neural cells from plasma-induced toxicity upon whole blood perfusion. Two NVU disease models were generated using hiPSC-derived from Huntington's disease patients, or by using Crispr/Cas9 to modulate the monocarboxylate

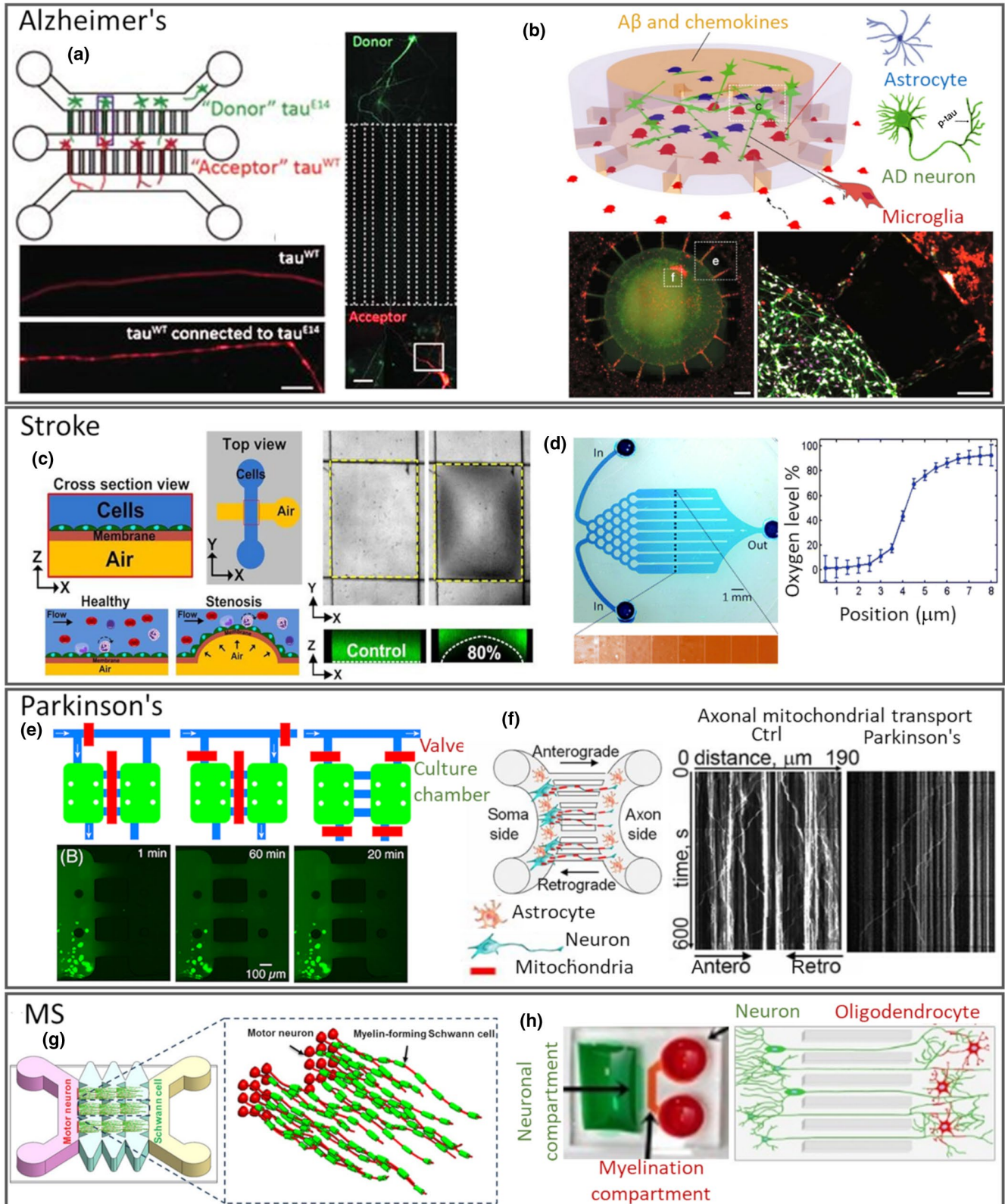


FIGURE 6 Microfluidic models amenable to neurological disease modeling. (a). Use of a three chamber axon guidance microfluidic device to study TauE14 (green) axonal transport, prion seeding and native Tau (red) aggregation (modified from Hallinan, Vargas-Caballero, et al., 2019 used under CC-BY). (b). A 3D human tri-culture model of Alzheimer's disease demonstrating A β aggregation, phosphorylated tau accumulation and microglial recruitment (adapted by permission from Springer Nature: Park et al., 2018). (c) Microfluidic methods such as those by Menon et al., might be used for stroke studies. Here air pressure under a flexible membrane is used to induce controllable vessel stenosis to study leukocyte-endothelial interactions in atherosclerosis (adapted from Menon et al., 2017 (Venugopal Menon et al., 2018) under the CC BY license). (d) Microfluidic methods such as demonstrated by Lo et al., (Chen et al., 2011) can create spatially defined oxygen concentrations which might be used to study pathogenic events in the penumbra in stroke (adapted from Lo et al., 2010 (Chen et al., 2011) with permission from the Royal Society of Chemistry). (e) Parkinson modeling has focused on studying the pathogenic potential of α -syn. By utilizing pneumatic valving, Fernandes et al., created individually addressable chambers which can be linked to allow diffusion of α -syn between chambers to study neuronal transmission and microglial responses (adapted from Fernandes et al., 2016 under the terms of the CC BY). (f). Prots et al. developed a model using iPSC derived neurons and astrocytes from Parkinson's patients, using an axon guidance device they demonstrate disrupted axonal transport as shown by mitochondrial kymographs (adapted from Prots et al., 2018 under the terms of the CC BY). (g,h) While modeling of MS using *in vitro* microfluidic modeling is in its infancy, various models have demonstrated *in vitro* myelination. Hyung et al. provide a 3D myelination model neurons guided using an aligned cross-linked hydrogel and optogenetic stimulation of myelination (adapted from Hyung et al., 2019 Biotechnology and Bioengineering with permission from John Wiley and Sons). (h) Lee et al. use subcellular electrical stimulation to enhance *in vitro* myelination of axons by oligodendrocytes in an axon guidance microfluidic device (adapted from Lee et al., 2017 under the terms of CC BY 4.0)

transporter 8 (MCT8) transporter as a model for MCT8 deficiency, a severe form of psychomotor disability, both showing appropriate disruption of barrier integrity. Paving the way for full NVU models of human disease.

5 | MICROFLUIDIC MODELS OF NEUROLOGICAL DISEASES

5.1 | Alzheimer's disease

The past three decades, have shown great advances in the understandings of the cellular and molecular events underlying Alzheimer's disease such as the aberrant accumulation of amyloid- β (A β) and the hyperphosphorylated microtubule-associated protein Tau. Exact pathological mechanisms of initiation and progression remain unclear and have hampered disease modeling and development of effective treatments. Of more than 400 trials in humans, no disease modifying drugs have yet made it to the market.

In recent years microfluidic devices have enabled new insights into the trafficking and accumulation of pathological A β and tau in human cells.

Using microfluidic devices, several studies have analyzed the different species that can propagate from cell to cell. Tau dimer, trimer, or oligomer aggregates as well as short fibrils, but not monomers or long fibrils, can be taken up by neurons and transported both anterogradely and retrogradely (Usenovic et al., 2015; Wu et al., 2013). Tau oligomers, but not monomers, also induce an increase in aggregated and phosphorylated Tau, alongside neurite retraction, loss of synapses, aberrant calcium homeostasis, and imbalanced neurotransmitter release (Usenovic et al., 2015). Another study shows that phosphorylated high-molecular-weight Tau, although very low in abundance, is taken up, transported in axons, and transmitted through synapses to connected neurons (Takeda et al., 2015). Tau pathology is propagated via synaptic activity (Wu et al., 2016), possibly through exosomes (Wang et al., 2017), as well as non-synaptic mechanisms (Calafate et al., 2015). Studies using MPS have

contributed to elucidate the prion-like propagation of Tau, and to clarify tau species involved and effects on recipient cells (reviewed in (Hallinan, Pitera, et al., 2019)) and have more recently been used to screen Tau targeted prospective therapeutics. Using a three chamber MPS Nobuhara et al. showed that some Tau antibodies, targeting the protein mid-domain, efficiently blocked the Tau uptake, aggregation, and spreading (Nobuhara et al., 2017).

There is also accumulating evidence of trans-synaptic transmission of A β . Song et al. showed A β absorption by axonal processes and retrograde transport (Song et al., 2014). Using a microfluidic-based reconstructed neuronal network, Deleglise et al. showed A β induces a dying-back process and synaptic alterations in presynaptic neurons, as well as hyperphosphorylated Tau in postsynaptic neurons (Deleglise et al., 2014). By creating an MPS generating spatial gradients of diffusible oligomeric assemblies, Choi et al. found A β exposure produced an atrophy effect on neurons cultured under continuous flow (Choi et al., 2013).

Use of 3D cultures, closer to the *in vivo* physiology, allows more authentic recapitulation of molecular and cellular events underlying Alzheimer's disease. The first 3D culture to model formation of both A β plaques and hyperphosphorylated tau tangles was published in 2014 (S. H. Choi et al., 2014). Since then, other examples of 3D cultures analyzed the role of A β secretion and accumulation (Papadimitriou et al., 2018; Pavoni et al., 2018). Advantages and limitations of these 3D models are reviewed elsewhere (Siney et al., 2018). Nonetheless, combining these powerful 3D models with microfluidic platforms allows new opportunities to model aspects of the disease beyond a neurocentric viewpoint. The role of microglia in Alzheimer's disease has been widely documented for several years. MPS have allowed exploration of microglial responses to A β gradients (Cho et al., 2013), as well as microglia recruitment or neuron-astrocytes interactions in a ground breaking 3D human Alzheimer's disease tri-culture model (Park et al., 2018) (Figure 6). BBB dysfunction is a well-known aspect of the disease. Shin et al. recently used a microfluidic model of the BBB to study the influence of A β on BBB phenotype. By incorporating 3D culture of human neural progenitors with mutations in the amyloid precursor protein gene,

the authors were able to replicate robust extracellular deposition of amyloid plaques which lead to neurofibrillary tangle formation and subsequent tauopathy. This system also showed increased BBB permeability, decreased expression of TJPs, and increased reactive oxygen species, thus recapitulating several key aspects of BBB dysfunction observed in Alzheimer's disease patients (Shin et al., 2019). Others have used MPS to demonstrate that Alzheimer's patients' serum can result in vascular dysfunction (Bersini et al., 2020) and to gain insights into cerebral amyloid angiopathy and vascular A β transport (Robert et al., 2017).

Such MPS, while still in development, are already being used to understand Alzheimer's disease pathophysiology at the molecular, cellular, and network level that could previously only be examined in large-scale animal studies.

5.2 | Stroke

Stroke, caused by a disruption of blood flow to the brain, is the largest cause of adult disability. However, despite significant research efforts, with over 1,000 neuroprotective therapies tested pre-clinically, no neuroprotective treatment has yet been approved (O'Collins et al., 2006). Despite significant differences between human and rodent metabolic rates, inflammatory responses and neurobiology, methods to test stroke therapeutics using human cells are extremely limited; a 2012 literature search by Antonic et al. found only 30 of 30,000 stroke studies identified, used human *in vitro* models (Antonic et al., 2012). Current *in vitro* models of stroke largely consist of oxygen-glucose deprived media applied homogeneously over a cell culture. Such models fail to replicate the focal injury experienced in the majority of ischemic strokes, and are often performed on a single cell type, thus omitting the heterotypic interactions that underlie many of the pathological mechanisms in stroke.

A number of MPS have been developed that allow for precise temporal and spatial control of oxygen (O₂) levels, even allowing for different shapes of gradient within the same cell culture chamber (Oppegard et al., 2009; Polinkovsky et al., 2009). Mauleon et al. have used such techniques to apply an O₂ gradient over brain slices (Mauleon et al., 2012). By bathing hippocampal slices in artificial cerebrospinal fluid in an open top microfluidic perfusion device, Mauleon and colleagues spatially controlled localized O₂ delivery with a resolution of 500 μ m. Using this technique hypoxic insults are specifically delivered to either the dentate gyrus or the CA1 hippocampal regions across the full 350 μ m thickness of the slice, while using fluorescent imaging to visualize real-time calcium responses. In future, such techniques could be used to delineate the cellular and molecular mechanisms that confer selective neuronal vulnerability of human hippocampal CA1 neurons observed in stroke (Bartsch et al., 2015) to potentially reveal novel therapeutic targets. Many of these microfluidic systems rely on the gas permeability of polydimethylsiloxane (PDMS) cell culture devices and complex switching of compressed gas supplies, requiring specialist setups. More recently, Sun et al., and Sleeboom et al., have demonstrated

O₂ control using O₂ consuming or producing chemical reactions, in aqueous flow channels that run in close proximity to the cell culture channel. This enables control of O₂ gradients in a significantly simplified setup (Sleeboom et al., 2018; Sun et al., 2018). Such systems have not yet been applied to model stroke.

In addition to providing controlled hypoxia, microfluidic devices have also allowed for more detailed study of specific pathological processes that underlie the delayed and progressive neuronal damage which follows cerebral ischemia. Initial energy crisis results in loss of ionic gradients and depolarization leading to excessive release of excitatory amino acids, particularly glutamate, termed excitotoxicity. This initiates a plethora of downstream events, including calcium-dependent activation of death pathways and oxidative stress, which work synergistically to drive neuronal death. To model these events *in vitro*, spatially defined and compartmentalized systems are required. Samson et al., have recently used microfluidic devices to culture environmentally isolated but synaptically connected hippocampal neurons to deliver a localized excitotoxic insult. Using a five compartment device, Samson et al., were able to replicate secondary spreading toxicity following glutamate exposure to one of the compartments and demonstrate a previously unknown innate GluN2A-dependent neuroprotective signaling that rapidly quenches excitation within a neural network (Samson et al., 2016). Similarly Hernández et al., used glutamate-induced excitotoxicity in a compartmentalized microfluidic neuronal circuit to study axonal degeneration, showing co-activation of two independent degenerative mechanisms in axon and soma, with axonal degeneration progressing via necroptotic kinases RIPK1 and RIPK3, while apoptotic events predominate in the soma (Hernandez et al., 2018). The use of microfluidic neuronal circuits consisting of multiple neuronal types, such as that presented by Kamudzandu et al. which mimics the circuitry of the basal ganglia, may further shed light on the influence of excitotoxicity on network activity (Kamudzandu et al., 2019).

Such studies have proven highly useful in dissecting neurotoxic events in the context of connected networks and may help to unveil new therapeutic approaches to stroke treatment. NVU disruption is a key feature in the pathogenesis of stroke and as microfluidic models of the NVU improve, their application to stroke research may also provide an invaluable tool in dissecting human NVU pathobiology. Vascularized microfluidic systems have already been utilized to study thrombosis, thrombolysis, endothelial inflammatory activation in embolic occlusion (Nemcovsky Amar et al., 2019), and leukocyte recruitment in arteriosclerosis under defined shear rates (Costa et al., 2017; Herbig et al., 2018; Loyau et al., 2018; Venugopal Menon et al., 2018). Application of such techniques in human NVU models could provide a powerful investigational tool in studying disruption of neurovascular coupling and the development of new therapeutic strategies for stroke.

5.3 | Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder affecting over 6 million people worldwide (Dorsey et al., 2018), characterized

by loss of dopaminergic neurons in the substantia nigra involving the accumulation of intracellular protein inclusions comprised largely of alpha-synuclein (α -syn). While the exact mechanisms of PD remain to be fully understood, microglia activation, mitochondrial dysfunction, oxidative stress, and chronic neuroinflammation have all been demonstrated to play pathological roles (Dexter & Jenner, 2013). Toxin-based models of PD replicate many of the known mechanisms of cell death, however, the progressive nature of the disease and dopaminergic selectivity is not mirrored. Drugs that have proved successful in combating toxin-induced cell death have yet to translate into neuroprotective therapies for PD. Genome-wide association studies have informed the design of genetic models of PD, however, there are as yet no animal models that fully recapitulate PD pathology (Dexter & Jenner, 2013). While a current lack of in-depth mechanistic understanding of the human disease pathobiology hinders *in vitro* modeling, microfluidic systems have been used to allow interrogation of specific pathological events that characterize the human disease.

While some studies have used MPS to explore neurotoxin models of PD, such as concentration gradients of 6-hydroxydopamine (Seidi et al., 2011), the main focus of microfluidic systems has been in building a more detailed understanding of α -syn inclusion formation and trafficking, through taking advantage of precise control over chemical environments and compartmentalized cultures. Perrino et al. have used an automated microfluidic platform to precisely regulate α -syn levels over yeast cells, revealing the dependence of inclusion formation on concentration (Perrino et al., 2019), while the use of yeast cells provides a high-throughput assay, physiological relevance is limited. Others have since used human dopaminergic neurons in compartmentalized culture to demonstrate that uptake of exogenously administered oligomeric misfolded α -syn and α -syn fibrils can occur both through the axon and soma. Transport can be either anterograde or retrograde, with retrograde transport found to be almost twice as efficient as anterograde transport (Brahic et al., 2016; Freundt et al., 2012). Gribaudo et al. have additionally studied the influence of two structurally and functionally distinct α -syn forms, fibrils, and ribbons, on human neuronal function. Both forms were transported, between neurons and elicited endogenous α -syn aggregation, disrupting synaptic integrity and mitochondria morphology, with ribbons bearing a more potent seeding activity (Gribaudo et al., 2019). Prots et al. further demonstrate the pathological role of α -syn using iPSC-derived neurons from a PD patient carrying an α -syn gene duplication. Using a commercially available microfluidic axon guidance device from Xona Microfluidics, Prots et al. show that increased α -syn oligomers disrupt axonal integrity and impair axonal mitochondrial transport (Prots et al., 2018). Other microfluidic systems have been used to investigate the interplay between different cell types under pathological conditions. Fernandes et al. developed a microfluidic platform with two cell culture chambers connected by three channels and equipped with integrated pneumatic valves for precise temporal control of cell treatment and diffusion or perfusion between compartments.

Using this system Fernandes et al. were not only able to observe the release and spread of GFP tagged α -syn between H4 neuroglioma cells, but were able to co-culture with N9 microglial cells demonstrating increased levels of reactive oxygen species in H4 cells cultured in the presence of activated N9 cells (Fernandes et al., 2016). While such systems predominantly use 2D cultures it is increasingly being recognized that 3D architectures are required to recapitulate *in vivo* like gene expression profiles (Baker & Chen, 2012) and to reproduce more physiological equilibration and transport of soluble factors (Ramanujan et al., 2002). Using iPSC-derived dopaminergic neurons expressing the most common pathogenic mutation causing autosomal dominant PD (LRRK2-G2019S) Bolognin et al. were able to show, using the Mimetas 3D cell culture OrganoPlates, that in the absence of stressors, 2D cell culture systems fail to exhibit robust endophenotypes (Bolognin et al., 2018). In 3D cultures of LRRK2-G2019S neurons, compared to isogenic wild-type lines, showed time-dependent dopaminergic degeneration, altered mitochondrial morphology, and enhanced cell death all in the absence of exogenously administered stressors (Bolognin et al., 2018). Such high-throughput 3D assays may thus allow for more effective screening of therapeutics for PD.

5.4 | Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease affecting more than 2 million people worldwide (Wallin et al., 2019). Commonly used experimental systems to study MS include neuro-inflammatory animal models such as experimental autoimmune encephalomyelitis, viral induced demyelination and inflammation, and toxin-induced demyelination such as cuprizone treatment. While neuro-inflammatory models reproduce the widespread inflammatory features of MS, toxin-based models are more suited to dissect specific mechanisms of demyelination (Hooijmans et al., 2019). The lack of separation between neurons and myelinating cells in traditional cell culture systems has hampered the detailed mechanistic study of myelination and myelin maintenance *in vitro*. Microfluidic solutions for the spatial organization of multiple cell types in culture, are now being applied to enable more effective investigation of events underpinning MS.

Groups including Ristola et al. and Kerman et al. have provided improvements over traditional primary rat dorsal root ganglion neuron-oligodendrocyte co-culture methods, by using microchannels to guide axons from one microfluidic compartment into a separately addressable oligodendrocyte compartment, resulting in the aligned deposition of myelin segments, thus enabling precise study and manipulation of myelination and demyelinating events (Kerman et al., 2015; Ristola et al., 2019). Vaquié et al. build upon this technique by perturbing the system using laser axotomy in both PNS and CNS models (using Schwann cells or oligodendrocytes, respectively) (Vaquié et al., 2018). As found *in vivo* Vaquié et al. observed that the PNS model recapitulates regeneration processes of Schwann cell de-differentiation, axonal regrowth, and remyelination over similar

time scales to those observed *in vitro*, while CNS oligodendrocytes remain inactive or die after laser axotomy, replicating the limited regenerative capacity seen *in vivo*. More recently Licht-Mayer et al. used an axonal guidance device as a model of demyelination using a co-culture of neurons with oligodendrocyte precursor cells and inducing demyelination by exposing the axonal compartment to lysolipid, (which is similarly used in animal models of demyelination). Licht-Mayer et al. used this system to help uncover a novel process in which mitochondria move from the neuronal cell body to the demyelinated axon, increasing axonal mitochondrial content, and that enhancing this process protects acutely demyelinated axons from degeneration (Licht-Mayer et al., 2020). Understanding such mechanisms of protection may have profound future implications for treatment of MS.

Although only to date performed in a Schwann cell model, Hyung et al. have extended *in vitro* myelination models into 3D. By using compartmentalized system with a hydrogel pinned to a central region by an array of micropillars separating two main cell culture channels they demonstrate that optogenetic neuronal stimulation promotes axon outgrowth and myelination (Hyung et al., 2019). Stimulation produced longer axons and increased the thickness of the myelin sheath to levels more comparable to *in vivo* estimates. Yang et al. replicate this activity dependent myelination using a microfluidic device with integrated platinum electrodes for biphasic electrical stimulation of cultures (Yang et al., 2012). Such studies highlight the importance of not only mimicking the physical structure and microenvironment to improve physiological relevance but also that relevant electrochemical stimulation may provide more *in vivo* like responses. While such devices have not yet been used to specifically model MS, the recapitulation of myelinating and demyelinating events achieved in such systems provides a great opportunity to introduce toxins traditionally used in MS modeling, or activated T cells to perturb the system to investigate potential therapies to promote demyelination and neuronal survival.

Understanding the migration characteristics of immune cells derived from cerebrospinal fluid of MS patients represents another challenge to which microfluidic *in vitro* systems are ideally suited and this is now being pursued by a number of research groups. Chemotaxis devices have allowed for the study of primary human T cell and dendritic cell migration to both soluble and matrix immobilized chemokine gradients such as CXCL12, a chemokine found to be increased in the cerebrospinal fluid of MS patients, and both CCL19 and CCL21 chemokines which are increased in the CNS of experimental autoimmune encephalomyelitis models of MS. In a device by Mehling et al. a gradient of CXCL12, was established by switching of a source-sink flow using integrated microfluidic valves. Multiple inlet and outlet channels along the path of the gradient, perpendicular to the gradient, allow for the initial positioning of T cells and their subsequent retrieval, through different channels based on how far they migrate along the gradient (Mehling et al., 2015). This system allowed for the division of T cell subsets in relation to migration speed, and subsequent analysis using microfluidic droplet digital-PCR to match cell behaviors to RNA profiles. While yet to be realized, combining

primary T cell culture and chemotactic studies with BBB models or *in vitro* myelination models, such as those previously described, could be used in future study of MS related disease mechanisms and potential therapeutics.

5.5 | Brain cancer

Primary brain tumors refer to a heterogeneous group of tumors which originate within the CNS, ~75% of which are gliomas (Lapointe et al., 2018). While only accounting for ~3% of all new adult cancers, in children brain cancers are the most common form of solid tumors (Logun et al., 2018; Ostrom et al., 2014). Brain cancers lead to a high mortality rate and the unique microenvironment of the brain, particularly the restrictive nature of the BBB, make these cancers notoriously difficult to treat. MPS have already enabled some unique insights into the heterogeneous cell-cell and cell-environment interactions that influence tumorigenicity, such as investigations by Lei et al demonstrating that nerve bundles can provide biophysical support for directional cancer cell migration (Lei et al., 2016).

An important benefit of MPS in cancer research is the ability to integrate patient-derived biopsies and tumor cells to provide direct translational relevance. By integrating bio-printing with on-chip culture, Yi et al. not only mimic heterotypic cellular interactions in a relevant brain-derived ECM 3D microenvironment with a hypoxic tumor core but also were able to reproduce clinically observed patient-specific resistances to chemoradiation and temozolomide using patient-derived cells (Yi et al., 2019). Future work might additionally enable investigations of immune responses, potentially offering an advantage over current xenograft animal models, which require immune compromised animals.

The use of vascularized models has allowed the study of both anti-angiogenic and anti-cancer therapies (Sobrinho et al., 2016) as well as in brain-specific models, for instance to assess strategies to enhance drug delivery through the BBB (Bonakdar et al., 2017) and to delineate pathways of brain metastasis. Indeed most malignant brain lesions are secondary brain tumors metastasized from other organs. With metastasis occurring in up to 30% of adults who have a malignant primary tumor at another site, with the highest incidence being in lung cancer (30%–50% of patients) (Weller et al., 2015). A number of studies have made use of BBB MPS to model brain metastasis, providing insights such as the role of astrocytes in restricting cancer cell extravasation (Xu et al., 2016). Such systems might in future take advantage of establish panels of brain metastatic cell lines to investigate brain tropism and mechanisms that enable cancer cells to surmount the BBB (Valiente et al., 2020).

As is discussed in section 7.1 the ability to connect multiple MPS which mimic different tissue microenvironments opens up the opportunity to investigate organ-organ interactions and may be used to shed light on cancer metastasis. This approach has already been applied to lung-brain metastasis. Yi et al. demonstrate this through the connection of a lung on chip model incorporating flow and cyclic stretch, upstream of three separate organ specific 3D culture of

cells (Astrocytes, osteoblasts, and hepatocytes) demonstrating the importance of lung stromal cells on epithelial–mesenchymal transition and metastatic capacity (Yi et al., 2019).

For in depth reviews on how microfluidics models are not only advancing understanding of the brain tumor microenvironment but also cancer cell extravasation, diagnostics and drug efficacy screening see (Logun et al., 2018), (Cai et al., 2020), and (Coughlin & Kamm, 2020).

5.6 | Traumatic brain injury

Traumatic brain injury (TBI) is not only a leading cause of mortality and morbidity in adults but is also a risk factor for the future development of neurodegenerative diseases such as AD and PD (Gupta & Sen, 2016). The initial mechanical shear, stretching, and compression, resulting from inertial forces induced during trauma such as motor vehicle accidents, falls, and sporting injuries, not only causes immediate physical injury to brain tissue but also sets in motion a range of protracted structural and biochemical changes including; anterograde degeneration, mitochondrial dysfunction, and secondary injury from excitotoxicity and inflammation (Yap et al., 2017). Recently CNS microfluidic models have been applied to TBI research to allow a more precise and targeted control of mechanical injury than can be produced in traditional *in vitro* models. A number of these developments have been made using neuronal cultures in axon guidance devices, taking advantage of high spatial precision to study axonal injury and white matter damage. One method to induce mechanical injury which has been used both in brain slices and with primary cell cultures, is by making use of flexible device materials (such as PDMS) and a pressurized pneumatic channel to deform the axonal channels mimicking the stretching of axons caused by shear deformation during TBI (Dollé et al., 2013; Fournier et al., 2014; Yap et al., 2017). Other approaches such as vacuum aspiration (Taylor et al., 2005), chemical treatment, laser-based axotomy (Kim et al., 2009), and electro-mechanical shear in 3D cultures (LaPlaca et al., 2005) have all been explored and the benefits and limitations of each of these approaches have been reviewed elsewhere (Shrirao et al., 2018). While these systems provide reproducible and precise injury, they have yet to fully recapitulate the many pathogenic events that follow TBI, such as inflammatory responses and the corresponding swelling and tissue hypoxia. Instead the current benefit of current MPS in this case, is the ability to study a specific mechanism within precisely defined conditions.

6 | FUTURE DEVELOPMENT AND CHALLENGES

The desire in the pharmaceutical industry is to access models predictive of target efficacy and drug safety and the dose of drug needed to achieve therapeutic benefit in the clinic. A lack of efficacy and safety are the two most significant reasons why drugs in clinical

trials fail to progress (Harrison, 2016). Looking to the future, the hope is that new human CNS models in development, are better able to predict target efficacy and safety, compared to historically used models, reducing clinical attrition and late-stage failure.

In the past, targets have been identified using models as a starting point, with a strong dependence on animal models. The pharmaceutical industry is now taking advantage of advances in genetics and computational biology, to interrogate large data sets from human populations, enabling target identification by linking genetic variants to human disease (Nelson et al., 2015), for example, LRRK2 to PD (Tolosa et al., 2020). The next step is target validation, to generate experimental evidence that therapeutic modulation of the target will provide efficacy in disease. A number of MPS now provide good candidates to manipulate a target *in vitro* with enhanced physiological relevance, combined with the ability to perform multiparametric assessments of the consequences of target perturbation in longitudinal studies. Integration of other technologies including real-time monitoring and iPSC technologies shows great promise in enhancing the utility of these systems. However, a number of challenges still remain, which hold back widespread adoption of these techniques, including issues with throughput, materials, robust validation, and standardization.

6.1 | Multi-organ systems

MPS are currently providing new insights into physiological and pathophysiological phenomena in the context of a specific functional unit of an organ or tissue, yet they fall short in modeling systemic responses and multi-organ interactions which typically necessitate the use of animal models, such as in the study of the gut brain axis, brain cancer metastasis, and neuro-immune networks. There have been a number of efforts to link multiple MPS to mimic key organ–organ reciprocal actions and more ambitiously in “Body on Chip” projects, such as the \$37 million Defense Advanced Research Projects Agency (DARPA) backed “Body on chip” project at the Wyss Institute for Biologically Inspired Engineering to integrate 10 human organ-on-chips. The MINERVA (MIcrobiota-Gut-BraiN EngineeRed platform to eVALuate intestinal microflora impact on brain functionality) project, funded by the European Research Council represents a specifically brain focused attempt to connect multiple microfluidic cultures (microbiota, gut epithelial barrier, immune cells, BBB, and brain) aiming to study the impact of intestinal microflora on neurodegeneration (Raimondi et al., 2019). A discussion of such programs is beyond the scope of this review, but Sung et al. provide a detailed analysis of how such ambitious large-scale projects may in future prove useful in delineating the contribution of organ–organ cross-talk in health and disease (Sung et al., 2019). While linking multiple MPS comes with a unique set of challenges along with significantly increased complexity and expense, specific interactions between defined functional units are already being used to provide physiologically relevant insights, such as BBB-brain metabolic coupling (Maoz et al., 2018) and brain metastasis (Yi et al., 2019).

6.2 | Incorporating iPSC technologies

Obtaining relevant human cells for CNS studies is particularly challenging, not only due to the scarcity of living tissue, (such as from epilepsy surgery) but also due to the post-mitotic nature of neurons, limiting their expansion *in vitro*. The ability to reprogram terminally differentiated somatic cells into stem cells by induced expression of pluripotency factors (OCT3/4, SOX2, c-Myc, Klf4) (Aasen et al., 2008; Takahashi et al., 2007; Takahashi & Yamanaka, 2006) has increased the availability of stem cells. Developmentally inspired protocols now allow the production of human brain cells which carry the unique genetic sequence of the adult somatic cell, thus enabling investigation of disease-specific phenotypes such as in Schizophrenia (Brennand & Gage, 2011; Brennand et al., 2015; Marchetto et al., 2011; Wen et al., 2014), Parkinson's (Sánchez-Danés et al., 2012), and Alzheimer's diseases (di Domenico et al., 2019; Julia et al., 2019). The differentiation of mature functional iPSC-derived cortical neurons is, however, requires prolonged cultivation times, with maturation occurring over a period of months (Kirwan et al., 2015; Odawara et al., 2016), as neural progenitor cells differentiate into neurons and astrocyte lineage cells, that participate in spontaneous network activity (Kirwan et al., 2015; Odawara et al., 2016; Shi et al., 2012). Single-cell RNA-seq data from human fetal and adult brain confirms that iPSC-derived cortical neurons are highly similar to primary cell neurons and with extended time in culture develop a more adult phenotype (Handel et al., 2016). Protocols have also been established for derivation of specific neuronal identities including GABAergic (Yang et al., 2017), glutamatergic (Cao et al., 2017), and dopaminergic (Mahajani et al., 2019) neurons, while novel methods of rapid neuronal induction and maturation have also been developed by inducing lineage-determining transcription factors (Yang et al., 2017). Methods for differentiating other key brain types including astrocytes (Lundin et al., 2018), microglia (Hasselmann & Blurton-Jones, 2020), and pericytes (Faal et al., 2019) have also been developed and many of these protocols have already been integrated into MPS (Appelt-Menzel et al., 2017; Prots et al., 2018; Usenovic et al., 2015). While integration of iPSC technologies may have the potential to greatly improve translational relevance for these models, as outlined in reviews by A. Sharma et al. (2020) and Pasteuning-Vuhman et al. (2020), to be widely adopted, they need to be carefully characterized both phenotypically and functionally. Without careful phenotypic characterization, cell identity of differentiated cells can be mistaken. This has been recently suggested in a preliminary report that provides single cell sequencing and immune-staining evidence that a widely used brain endothelial differentiation strategy in fact produces neuroectodermal epithelial cells that form tight junctions reminiscent of those seen with BECs (Lu et al., 2019). Thus robust characterization and reliable methods are required for iPSC technologies to succeed in delivering on their potential to significantly improve CNS disease modeling.

6.3 | Advancing organoid models

Recent developments in 3D culturing of human iPSCs into neural "organoid" tissues offer a promising horizon to explore (Camp et al., 2015; Kelava & Lancaster, 2016; Pasca et al., 2015; Qian et al., 2016). Various region-specific brain organoids have already been described, including retinal, whole-brain, cortical, forebrain, and midbrain (Eiraku & Sasai, 2012; Eiraku et al., 2011; Kadoshima et al., 2013; Lancaster et al., 2013; Lindborg et al., 2016; Marton & Pasca, 2016; Pasca et al., 2015; Qian et al., 2016). Such organoid cultures show great promise to extend the possibilities of *in vitro* neurological research but are beyond the scope of this review (for reviews please see (Wang, 2018) and (Setia & Muotri, 2019)). One notable limitation of organoids is the lack of vascularization, leading to a necrotic core of cells at sizes above ~300 μm due to the limitations of oxygen and nutrient diffusion. Combining organoid cultures with microfluidic techniques to provide perfusion (Yu et al., 2019) has shown to improve viability. Exploiting microfluidic techniques that promote angiogenesis to create perfusable vessels may allow further advances in organoid models (Shi et al., 2020). The use of microfluidic technologies is already advancing organoid culture, as reviewed by Park et al. (2019).

6.4 | Validation

One of the most important factors in the successful uptake of new *in vitro* models is validation and robust data showing improvements against existing models with greater relevance to the clinical disease. Emerging models are often described as "validated" by using existing drugs. This is valid for benchmarking new drugs against established drugs for safety and toxicology studies. In the search for novel drugs to address a large unmet need in neurological diseases, novel mechanisms for which there is no benchmark drug are being explored. In this case it is important to understand the limitations of the model in terms of what aspects of healthy and disease tissue the model is able to recapitulate. For this, analysis of molecular markers and cellular morphology which can be compared to the clinical presentation of disease, would allow for direct comparison. This presents a challenge as many neurological disease markers remain to be standardized, often due to gaps in understanding the exact cellular and molecular underpinnings of human pathophysiology. Furthermore, while a great deal of insight has been gained from animal models of neurological disease, in many cases these fail to fully replicate the human disease. A recent paper by Donald Ingber raises the important question of; given the mismatch between clinical data and *in vivo* models, whether a requirement of animal testing to confirm *in vitro* results for publication or grant funding still makes scientific or ethical sense (Ingber, 2020). The gold standard for comparison of disease models should always be the clinical disease itself, yet given the limited depth in which human pathophysiology can be studied, validation must be appropriately designed to match available data

and experimental possibilities. Where specific readouts in animal models are known to reflect human pathophysiological responses, intelligent and target comparisons can be made for validation of new models, not only comparing new models to the clinical data but also to established animal models. To ensure that experimental results are not artifactual manifestations of the cell culture environment, it would also be possible to create equivalent systems using animal cells to allow direct comparison with extensively characterized *in vivo* models, as well as to the clinical and human tissue sample data. The results from initiatives such as the U.S. Food and Drug Administration's (FDA) Biomarker Qualification Program will likely improve the ease in which such comparisons between models and the real world disease can be made. The NIH Tissue Chips Consortium has brought together the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) the National Center for Advancing Translational Sciences (NCATS) at the National Institutes of Health (NIH), DARPA and the FDA, and enabled the establishment of Tissue Chip Testing Centers in 2016 and 2018 which allow for independent testing and validation of platforms as part of a wider Tissue Chip Validation Framework.

6.5 | Materials

Plastic has been the material of choice for biologists for almost 50 years and as such biologists are familiar with the capabilities. Some microfluidic devices manufacturers have opted for the use of thermoplastics, while in other cases limitations in machining and properties have necessitated the use of other less familiar materials. The vast majority of microfluidic laboratories experimenting with new possibilities through rapid prototyping use PDMS due to its ease of fabrication, low cost production, optical clarity, gas permeability, and relative biocompatibility. PDMS is also used for its high elasticity, as in the Emulate Chip which enables stretch to be used to mimic breathing cycles (Huh, 2015) and vascular stretch (Sato et al., 2019). PDMS is, however, highly hydrophobic with a low surface energy that, unless treated, resists biological coatings and may also absorb small molecules such as drugs, which can have significant impacts on detection of analytes and drug bioavailability (van Meer et al., 2017). Many commercial MPS are returning to the use of thermoplastics, where flexibility is not a requirement, while others are investigating novel materials with potentially superior characteristics, such as thiol-based chemistries (Ostemers) (Zhou et al., 2017). For an in-depth review of materials for microfluidic chip fabrication, see Ren et al. (2013).

6.6 | Increasing data through integration of real-time monitoring

Multi-parametric sensing is highly desirable, to generate high-content data from cell systems. In this context, the extensive developments on electrochemical enzymatic sensing of glucose and

lactate, primarily in the field of blood sugar monitoring for diabetics, are now being applied to MPS and would be a useful addition to CNS models. Indeed glucose and lactate production, along with CO₂ production and O₂ consumption yield a detailed picture of cell metabolism, and have been incorporated into MPS to monitor cell growth (Prill et al., 2014). Oxygen and glucose consumption has been usefully linked to neuronal firing and can even predict burst activity such as that seen in epileptic seizures (Ivanov et al., 2015). pH sensing may also be useful, which in many cases is a substitute for monitoring lactic acid production. Functionalization of electrodes with redox enzymes or other suitable biorecognition elements, can in principal allow sensing of a wide variety of analytes, although requiring careful design of the functionalization strategy and materials/reagents.

A major challenge in integration of sensing technology comes when the cell systems become more 3D to provide a more natural environment for the cells. In terms of electrodes, currently, the majority of systems rely on flat, rigid electrodes. New technologies are nevertheless enabling flexible polymeric devices which can be tailored to adapt to tissues in a more seamless manner (Kalmykov et al., 2019; Pitsalidis et al., 2018). Much of this development is being pushed by the neural interfacing community, faced with the sizeable challenge of implanting electrodes into brain tissue for durable and non-invasive neuronal recording. Lessons learnt from this community will inevitably translate into better electrical recording devices *in vitro*, adapted to complex biology. Transparency of the next-generation electronic materials translates into an added advantage *in vitro*, in terms of imaging cells in devices (Curto et al., 2017). Overall, the advantages obtained by electrophysiological and real-time monitoring systems are compensated by higher costs and by the increased complexity of fluidically sealing the microfluidic device.

6.7 | Increasing throughput and integrating automation

The throughput of models for the lead discovery can range from 1000s for antibody screening to 100,000, even 1,000,000s, for a small molecule screen. Small molecules are the most likely modality for CNS diseases, in which an important hurdle to overcome is drug delivery across the BBB. Novel BBB models, if appropriately scaled, could aid in assessing the bioavailability of a drug in the lead optimization phase, where typically the numbers of drugs profiled through a cell-based model is in the range of hundreds. This is within the range that could be accommodated using multi-device platforms such as the OrganoPlate[®] from Mimetas (96 two channel or 40 three channel devices in a 384-well plate format) (Wevers et al., 2016), the IMPACT Platform from Noo Li Jeon's laboratory (S.-R. Lee et al., 2019; S. Lee et al., 2019) (12 devices in a microscope slide footprint or 96 devices on a 96-well plate format) or the platform presented by Phan et al. (2017) (12 devices in a 96-well plate format), all of which provide patterning of hydrogels for culture of multiple cell types with flanking perfusion channels supplied with flow by passive hydrostatic head. Fantuzzo et al. have also taken the

concept of axon guidance first presented by Taylor et al. and developed a high-throughput arrayed neural circuitry platform with 96 devices per plate (Fantuzzo et al., 2020), while Parrish et al., 2018, have taken a membrane-based dual perfusion chamber approach to provide a 96-well microplate platform amenable for BBB studies (Parrish et al., 2018). This last example and many other MPS models require complex bespoke perfusion systems and holders to operate the devices, which may limit uptake or restrict the user to a single type of MPS, thus raising the issue of standardization within the field.

6.8 | Standardization

Standardization of microfluidic neurological models, and microfluidics in general presents a huge challenge, due to the inherently interdisciplinary nature of the field and the fast moving pace of innovation. Failure to standardize may have massive implications to the growth of the field and widespread uptake of emerging technologies, however, poorly devised standards could stifle innovation and technological optimization (Blind, 2016). Within other technology fields standards have previously arisen due to dominant commercial entities or from collectives and regulatory bodies (such as the International Organization for Standardization and the Institute of Electrical and Electronics Engineers). The microfluidics and Organ-on-chip fields are currently dominated by small biotech start-ups and academic groups. It would seem that standards are most likely emerge from discussions between Organ-on-chip communities and cross-disciplinary consortia such as ORCHID (Organ-on-Chip Development DMT), European Organ-on-Chip Society (EUROoCS), the Organ-on-a-Chip Technologies Network in the UK and the NIH Tissue Chips Consortium in the United States. Such networks provide platforms for discussion between academic and industry developers, pharmaceutical and research institute end users, and funding agencies and regulatory bodies to develop standardization strategy. Within the pharmaceutical industry, significant interest in MPS has led to the formation of the IQ Microphysiological Systems Affiliate, a collaboration of over 20 pharmaceutical and biotechnologies companies including AbbVie, AstraZeneca, BMS, GSK, Eli Lilly, Merck, Novartis, and Pfizer. This cross-pharma collaboration, created to facilitate data sharing and expedite uptake and impact of MPS, will also likely have an impact on how MPS and Organ-on-Chip technologies are standardized.

Standards that emerge will likely exist on multiple levels, reflecting the multidisciplinary nature of the field, and may include: fluidic and electrical interconnects, dimensions, materials, media, flow rates and cells, and culture protocols. Any standards that emerge should, and often do, follow currently accepted bioscience and pharmaceutical industry standards such as glass slide standard dimensions (Emulate chips and IBIDI channel slides), ANSI/SLAS multi-well plate footprint (The OrganoPlate® from Mimentas and CellASIC® ONIX Microfluidic Plates from Merck), and the Luer Lock interface (IBIDI channel slides). Increasing standardization from the Organ-on-chip,

microfluidic community must not stifle creativity and innovation but improve compatibility to facilitate and simplify collaboration and integration, accelerating innovation, and widespread adoption.

7 | CONCLUSIONS AND CONSIDERATIONS FOR EARLY ADOPTERS

As we have detailed in this review, MPS have provided an array of new opportunities to study neurological disease and new technologies are rapidly developing. The initial foray into microfluidic technologies for disease modeling has been spearheaded through close collaborations between engineers, chemists, and biologists, and while many of these pioneers are now offering expertise in their platform as outsourced research and disease modeling services (such as Aracari Biosciences Inc. and Hesperos Inc.), a number of devices have already entered the market targeting biologists as end users (Table 1) and some even offer custom device fabrication services (e.g., TissUse GmbH and uFluidic). While commercially available systems often come with SOPs and troubleshooting guides specific to the platform, early adopters should be aware of a number of unique challenges with using microfluidic devices.

With microfluidic devices containing 10s to thousands of cells rather than the millions of cells used in macroscale cultures, particular attention should be made to seeding densities and even distribution of cells. Cell clumps can easily block microfluidic devices or result in abnormal distribution and growth of cells. At the small scales of MPS, evaporation can have a large effect on volumes of cell culture, and particular care should be made to maintain high humidity. Scaling also influences surface area to volume ratio. Traditional macroscale cultures typically have a very low surface area to volume ratio when compared to MPS. For instance in the example by Walker and Beebe, a 35 -mm-tissue culture dish may have surface area to volume ratio of 11 cm^{-1} while a typical microchannel may have a surface area to volume ratio of 800 cm^{-1} . MPS show rapid exchange of gases and enable study of autocrine and paracrine signaling without the significant dilutions that occur in macroscale cultures. On the other hand, nutrient depletion and waste build-up is rapid in the absence of flow (as is the case *in vivo*). As such, the concepts of effective culture volume and a critical perfusion rate introduced by Walker and Beebe (Walker et al., 2004; Young & Beebe, 2010) should be well understood, along with an appreciation of the effects of the resulting shear and the implications that the high surface area to volume ratio and material properties have on the microenvironment. For instance, in case of PDMS devices, surface adsorption of hydrophobic dyes and drug molecules or bulk absorption of nutrients from media can confound results and while high gas permeability allows rapid O_2 exchange, water vapor permeability can result in changes in concentration of media constituents unless high humidity is maintained. Thus appropriate coatings or pre-incubation with media or serum to saturate absorption prior to cell culture, should be considered.

Shear stress can be used to enhance barrier functions of BBB models and increase phenotypic relevance of endothelial cells. However it should be noted that shear can easily be introduced where it is not intended, such as neuronal cultures, which can be highly sensitive to shear. As an example, a hydrostatic head of 10 mm feeding a channel of 100 μm high 500 μm wide and 10 mm long can introduce brief shear stress of over 2 dyne/cm^2 , while *in vivo* shear of cells in contact with cerebrospinal fluid is approximately 0.01 dyne/cm^2 with small increases having previously been shown to influence cell behavior (Park et al., 2017). A background knowledge and appreciation of fluid dynamics would benefit those adopting such studies. Furthermore, new users of MPS should carefully consider the limitations and challenges specific to the platform they intend to use when designing experiments. Examples of these considerations include; compatibility with desired readouts, such as fluorescent imaging, whether enough biological material can be harvested for the analysis of choice and compatibility of existing cell culture protocols, for example, surface coating, along with the potential increase in costs and extra instrumentation that may be required.

Given that many biological laboratories are already taking advantage of the unique experimental possibilities provided by MPS, these systems, especially when integrated with advances in stem cell technologies and real-time monitoring, show great potential in enhancing the predictive power of pre-clinical studies through integration as part of the drug discovery pipeline.

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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CONFLICT OF INTEREST

Anup D. Sharma is an employee of AxoSim Inc. No other conflict exists.

AUTHOR CONTRIBUTIONS

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