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Review

Human Brain Organoids-on-Chip: Advances, challenges, and perspectives for pre-clinical applications

Héloïse Castiglione [1,2], Pierre-Antoine Vigneron [2,3], Camille Baquerre [1], Frank Yates [2,3], Jessica Rontard [1] and Thibault Honegger [1,*]

[1] NETRI, 69007 Lyon (France)
[2] Sup'Biotech/CEA-IBFJ-SEPIA, Bâtiment 60, 18 route du Panorama, 94260 Fontenay-aux-Roses (France)
[3] Sup'Biotech, Ecole d'ingénieurs, 66 rue Guy Môquet, 94800 Villejuif (France)
* Correspondence: thibault.honegger@netri.com

Abstract: There is an urgent need for predictive *in vitro* models to improve disease modeling and drug target identification and validation, especially for neurological disorders. Brain organoids, as alternative methods to *in vivo* studies, appear now as powerful tools to decipher complex biological processes thanks to their ability to recapitulate many features of the human brain. Combining these innovative models with microfluidic technologies, referred to as Brain Organoids-on-Chips, allows to model the microenvironment of several neuronal cell types in 3D. Thus, this platform opens new avenues to create a relevant *in vitro* approach for pre-clinical applications in neuroscience.
The transfer to the pharmaceutical industry in drug discovery stages and the adoption of this approach by the scientific community requires to propose innovative microphysiological systems allowing to generate reproducible brain organoids with high quality in terms of structural and functional maturation, and compatible with automation processes and high-throughput screening.
In this review, we will focus on the promising advantages of cerebral organoids for disease modeling and how their combination with microfluidic systems can enhance the reproducibility and quality of these *in vitro* models. Then, we will finish by explaining why Brain Organoids-on-Chips could be considered as promising platforms for pharmacological applications.

Keywords: brain organoid-on-chip, predictive human based *in vitro* models, standardization, reproducibility, neurotoxicity

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1. Introduction

Neurological disorders including Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, stroke, and brain injuries represent a significant burden for society, and affect up to one billion people worldwide, globally irrespective of sex, age, and education. They are currently a leading cause of disability, and the second largest cause of mortality in the world, with 9 million of deaths per year [1]. Yet, no effective treatment exists for many of these disorders. In addition, neurology is one of the most failure-prone areas in the drug development pipeline, despite considerable investment [2]. These high drug attrition rates suggest the limitations of current experimental tools leading to clinical applications. Indeed, *in vivo* models have failed to translate into any noteworthy advances that could help to discover treatments for neurological disorders. One of the potential reasons for this failure is the gap between rodent models and humans [3].

Modeling the brain remains an elusive challenge because of its inherent complexity, which participates in this hampered translational success. This complexity starts at the cellular level: the human brain is composed of a cellular heterogeneity with approximately 170 billion cells, organized in an intricated network with 86 billion neurons, and 85 billion non-neuronal cells [4], [5] that comprise glial cells – among which microglial cells, resident immune cells of the central nervous system - and endothelial cells [6]. In

addition, neurons are organized in a complex neuronal network, estimated to be composed of nearly 10^{14} synapses, with approximately 7,000 synapses per neuron in the neocortex [7], ensuring neuronal communication through chemical and electrophysiological signaling. Another parameter that worsens this incomplete understanding is the brain plasticity, which role remains elusive, but can result in neuronal networks reorganization, in strengthened or depressed synapses, or even in changing the functions of a given neuronal population. However, it is still not well explained how this plasticity occurs [8]. Remarkably, the brain is considered to be an immune-privileged tissue in which, which means that the immune responses are tightly controlled and regulated [9]. Such a property is beneficial to protect the brain cells from immune response-mediated damages but also complicates the predictability of brain responses to drugs. Another level of complexity linked to this immune-privileged tissue arises with the blood-brain barrier (BBB), formed by the vasculature enwrapped in astrocytic endfeet. The unique ability of the BBB to selectively filter the blood poses challenges for drug delivery into the brain, drug candidates' distribution, and neurotoxicity evaluation. In the context of drug discovery and drug screening it therefore appears necessary to be able to model *in vitro* the BBB functions [10], [11]. Overall, this complexity of the human central nervous system complicates the development of relevant and predictive *in vitro* pre-clinical models of the human pathophysiology.

Each model has its own advantages and limitations: conventional two-dimensional (2D) cell cultures are simplistic and cost-effective, but the information could be far from human physiology as they lack three-dimensionality (3D); 3D cell culture systems are more appropriate to model complex functions of the brain *in vitro,* but they lack reproducibility; finally, microfluidic devices can recapitulate physiological environments under controlled flows, thus enabling predictive *in vitro* models [10].

In the last decade, different research groups in academia have developed pluripotent stem cells (PSCs), mainly human induced PSC (hiPSCs)-based protocols to generate 3D, multicellular, cerebral organoids (for a review of existing protocols, see [12]). Their use to model brain biology, early neural development, and human acquired and genetic diseases has provided new insights into neurological disorders, including microcephaly, autism, Parkinson's disease, and Alzheimer's disease [13]. However, the adoption of organoid technology for large-scale drug screening in the industry has been hampered by challenges associated with reproducibility, scalability, and translatability to human diseases. Organ-on-Chip (OoC) technology has become more widely used in recent years due to its ability to mimic physiological conditions in an *in vitro* setting [14]. The combination of brain organoids and microfluidic systems, named Brain Organoids-on-Chips, could accelerate pharmaceutical testing compared to animal models and 2D cultures [14]. The combination of brain organoids and microfluidic systems, named Brain Organoids-on-Chips, could accelerate pharmaceutical testing compared to animal models and 2D cultures by meeting the expectations of both the pharmaceutical and biotechnological industries.

In this review, we will examine the advantages offered by brain organoids as complex 3D *in vitro* models and discuss how microfluidic systems can address some of their associated challenges. Subsequently, we will provide an overview of what has already been done in the recent field of Brain Organoids-on-Chips, discussing their benefits and limitations regarding pharmacological applications. We will then address the current challenges associated with Brain Organoids-on-Chip technologies, in perspectives of further transfer as relevant pre-clinical *in vitro* models.

2. Brain organoids: advantages, limitations, and microfluidic technology as solution

Independently from the cell types used, the two-dimensionality of conventional cell culture limits the relevance of such models. Indeed, cell cultures grown in 2D cannot recapitulate the *in vivo* spatial organization of tissues, with 3D architectures enabling cell-cell and cell-matrix interactions which play a central role in cell morphology, polarity, and gene expression [15]. In addition, cells cultured in 2D conditions are directly in contact

with plastic substrates or adherence coating substrates, which biases their interactions with their microenvironment, as they do not reproduce the extracellular matrix (ECM) characteristics [16]. Moreover, in conventional 2D cultures, the friction of liquid against cell membranes, emphasized by medium renewal and handling of culture plates, induces non-physiological mechanical forces such as shear stress which can impact cell division, morphology, and can ultimately lead to cell death [17].

2.1. Brain organoids: promising in vitro *3D models of the human brain*

Brain organoids (also called cerebral organoids) are self-assembled and self-organized cellular aggregates in 3D, obtained *in vitro* by neural differentiation of PSCs. They comprise different cell types observed in the developing human brain, including neural progenitors, neurons, and glial cells. Contrary to traditional 2D cell cultures and neurospheroids, brain organoids assemble into cellularly complex 3D architectures like certain regions present in the human brain. These cerebral regions comprise forebrain, hindbrain, cortex, hippocampus and choroid plexus [18]. Brain organoids recapitulate the developing human brain not only at the cellular level, but also regarding organizational levels, as well as global developmental trajectories. The cell-cell interaction enables to recapitulate the cellular microenvironment more realistically, by promoting exchanges of information between cells and with the ECM, as well as improving cell differentiation [19].

Several methods for generating brain organoids generation exist, resulting in different types of cerebral organoids. These methods can be classified in two main differentiation protocols: unguided and guided. Both methods rely on the aggregation of human PSCs into embryoid bodies (EBs), that are harvested and then induced thanks to specific culture media. The unguided methods enable the generation of "whole-brain" organoids. They rely entirely on spontaneous morphogenesis and intrinsic differentiation capacities within the organoids towards a global cerebral identity, without orienting the differentiation into a specific cerebral region. The first unguided protocol was established by Lancaster and Knoblich in 2013, following the publication of their article the year before about microcephaly modeling using brain organoids [18], [20]. The guided methodology (also called region-specific) is an alternative method, in which external patterning factors are added during the differentiation process to drive the differentiation of stem cells towards specific cerebral regions [21]. Guided protocols have been established for cerebral cortex [22]–[25], midbrain [26] , cerebellum [27], hippocampus [28], thalamus [25] and hypothalamus [29]. Unguided methodologies enable to model a global cerebral development, which in return has the disadvantage of generating unique organoids with a high heterogeneity. On the contrary, guided methods are considered to generate more reproducible organoids, but are limited to a particular cerebral region. Overall, the choice between unguided and guided approaches depends on the applications and is often seen as a compromise between diversity and consistency of the model [12].

2.2. Brain organoids: examples of applications in disease modeling

Brain organoids have emerged as a revolutionary *in vitro* cellular model of the human brain development and functions, in both physiological and pathological conditions [12]. In addition, they are considered as a relevant approach to help reducing animal research and to accelerate the process of drug screening [30]. Furthermore, especially when combined with recent genetic engineering approaches, as well as when derived from patient-specific cells, they enable to model and study diverse neurological disorders: diseases occurring during neurodevelopment (like microcephaly [18], trisomy 21 [31], [32], or prenatal exposures to toxic compounds [33], [34]), cancers (glioma) [35], and also can give clue about the pathogenesis of neurodegenerative diseases (like Alzheimer's Disease [36], [37], Creutzfeldt-Jakob disease [38], or Trisomy 21 [39]) (for a detailed review: [13]).

In addition, brain organoids open up the way to personalized medicine with the utilization of patient-specific cells [12].

2.3. Challenges for the adoption of brain organoids in pre-clinical applications

Despite the major advances brought by brain organoids, there are still some limitations to these models which hamper their transfer and adoption by the pharmaceutical industry for neurological disease modeling and drug testing.

A major challenge is the high heterogeneity observed between brain organoids even when derived from the same PSCs and cultured in the same conditions. These discrepancies include differences in size and morphology between the organoids, as well as structural and functional variations when further analyzed. Such heterogeneity is mainly due to the stochastic nature of the PSCs differentiation and the organoids spontaneous self-organization, that inherently lead to differences in cell type proportions and structural organizations. Moreover, some intra-organoids heterogeneity is also observed, with differences of cellular densities and structures within the same organoid [12]. Some non-cerebral structures can sometimes be found within the organoids: the presence of germ layers other than neuroectoderm, such as mesoderm [12], [40] and the suboptimal presence of cystic cavities [41]. In addition to this inherent heterogeneity, another level of discrepancy is caused by the lack of standard criteria for the generation and culture of brain organoids, as well as the differences between protocols implemented by the laboratories. Moreover, the distinct differentiation methods (unguided and guided protocols) also exacerbate the diversity of brain organoids. Overall, this heterogeneity induces a lack of robustness, reproducibility and predictability of the model, which raises issues for transfer to an industrial scale, high-throughput screening (HTS) and testing of potential drug candidates in pre-clinical phases [42].

Another central limitation is the progressive appearance of a necrotic core at the center of brain organoids as they grow, due to the lack of vasculature for proper oxygen and nutrients supply [12], [43]. Indeed, there is a diffusion limit for oxygen and nutrients/wastes exchanges from the culture medium around 400 µm from the surface of the organoid [44]. Therefore, since the progenitor cells at the core cannot be properly supplied in oxygen and nutrients, they progressively undergo necrosis. This necrotic core prevents further growth, limiting the size of organoid up to 4-5 mm in diameter, and impedes their maturation, thus preventing the organoids to recapitulate later stages of human brain development [12].

Similarly to the absence of vascular cells, microglial cells are also often absent from this model due to their mesodermal lineage [42]. This lack of brain immune responses modeling in brain organoids could raise some issues in pre-clinical studies, for drug screening, drug delivery and neurotoxicity evaluations.

Finally, even if brain organoids recapitulate many key features of early human brain development, not all aspects of neurodevelopment are fully recapitulated, including the formation of distinct cortical neuronal layers, the gyrification, and the establishment of complex neuronal circuitry [12]. In addition, current brain organoid models fail to recapitulate most of the late brain development events, such as gliagenesis and myelination, mainly due to the longer time needed for maturation. Moreover, this is worsened by the absence of microglial cells, which play a significant role in the brain maturation by inducing the formation of mature dendritic spine and synapses.

Facing these limitations, microfluidic devices are considered as promising alternative culture systems to improve overall culture conditions and reduce the heterogeneity of the generated organoids in the context of neuroscience research [10], [45].

2.4. Microfluidic systems: promising technologies to tackle brain organoids limitations

Microfluidic cell cultures rely on engineering sciences and take advantages of technologies to adapt to biological questions. Thanks to tightly controlled fluid flows, OoCs are considered to improve the culture conditions, especially by reducing the shear stress experienced by cells, improving oxygen supply and distribution, enhancing nutrients/waste exchanges, and by facilitating the implementation of chemical gradients. It has been shown that the ability to modulate these flows have an impact on cell morphology, migration [46], and differentiation particularly for stem cells [47]. In addition, the controlled flows and microenvironment are also considered to improve the reproducibility of the cell cultures, by reducing heterogeneity between batches. Regarding organoids, controlling the flows could allow an enhanced penetration of the nutrients in the center of the organoids. Indeed, Lancaster et al. recently proposed a way to overcome the lack of vascularization in the cerebral organoids, by culturing them in microfluidic devices to facilitate nutrients and oxygen uptakes within the cerebral organoids [30].

Another advantage of microfluidic systems arises from the flexibility of possible designs [48]. For instance, simple design with minimalistic human neural circuits composed of a single chamber in which one cell type is cultured [49], or more complex neural networks allowing co-cultures [50]. In more sophisticated platforms, several chambers on the device can comprise different cell types and can be separated or connected thanks to channels or porous membranes, following a logic of compartmentalization [51], [52]. In even more complex designs, several devices can be coupled, to enable connection of distinct organs forming multi-Organ-on-Chips [42].

Other benefits include the integration of multi-parametric analyses: compatibility with imaging techniques (most of microfluidic devices are optically clear, enabling fluorescence assay), and electrophysiological measurements (to monitor cells in a non-invasive approach) (Coluccio et al., 2019; Holloway et al., 2021; Nikolakopoulou et al., 2021; Miny et al., 2022).

Nowadays, microfluidic devices are perceived as alternative novel platforms to current *in vitro* models based on 2D cell cultures and *in vivo* models using animals [10], [11], [53]. Brain Organoids-on-Chips could represent good compromises between physiological relevance and reproducibility (Figure 1).

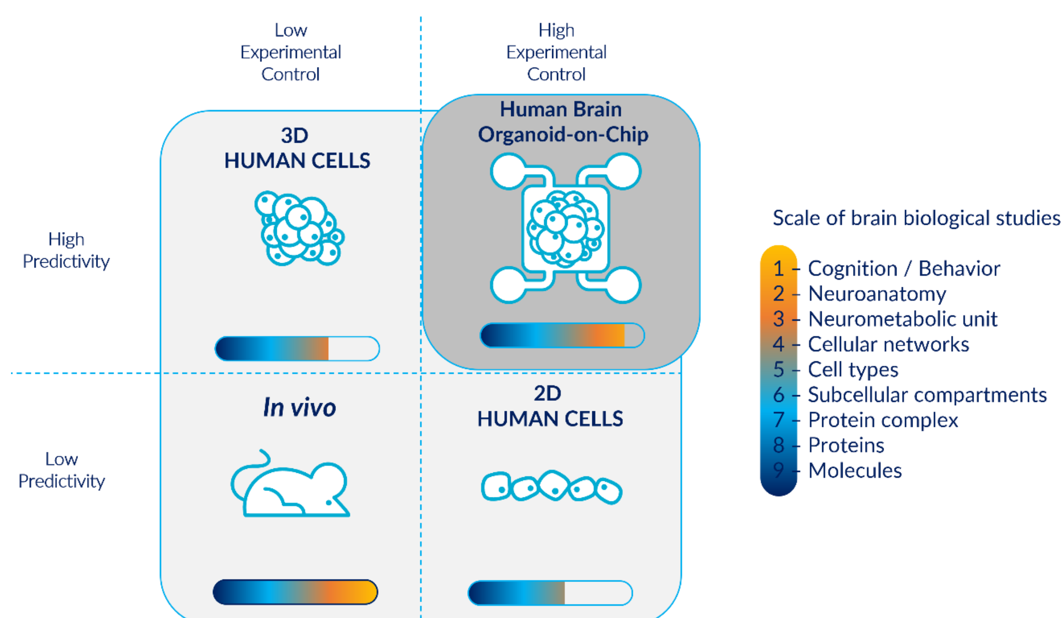

Figure 1. Human brain Organoid-on-Chips as physiologically relevant and reproducible models for the study of brain biological organization. Even if in vivo models enable to perform the highest diversity of biological neurological studies, they are associated with low predictivity and experimental control. They are also considered as a black box model: being often privileged by re-searchers despite the availability of other models. On the contrary, in vitro 2D human cells allow a high

experimental control, however they display a low predictivity and do not permit brain bio-logical studies at higher scale than the cellular level. By opposition, 3D structures such as brain organoids enable more complex studies with high predictivity but are associated with lower experimental control and high inter- and intra-batch heterogeneity. Combination of brain organoids with microfluidic technology provides a model with a high experimental controllability, particularly for the modeling of physiological microenvironments in a minimalist approach, while ensuring a high predictivity, and enabling a wide range of biological studies.

3. Brain organoids-on Chips: state-of-the art of promising models

Brain organoid-on-chip systems have emerged quite recently as a new field of research. They combine cerebral organoids culture with microfluidic devices, to improve the culture conditions, physiological relevance, reproducibility, and industrial transferability of the brain organoids. In the literature, there are currently few articles dealing with human brain organoid-on-chips. They can be classified according to their global architecture and manufacture process of the microfluidic device (Table 1):

(i) Microfluidic devices: composed of 3D cell culture areas, and channels for culture medium flows;

(ii) Microfluidic devices with micropillar arrays: comprising micropillars between which cells are cultured in 3D, from the iPSCs seeding, to the organoid generation, and further growth and expansion;

(iii) Microfluidic device with an air-liquid interface: air-liquid integrated to the culture platform.

Table 1. State-of-the-art of recent Brain Organoids-on-Chip technologies with their advantages for brain organoids cultures (W: width; L: length; D: diameter; H: height).

Type of device	Fields of study	Protocol for organoid generation	Characteristics of the microfluidic device	Advantages of the microfluidic technology for brain organoids culture	References
Microfluidic devices with 3D culture areas and channels	Neurodevelopmental toxicity: tests of prenatal nicotine exposure effects on neurodevelopment	Whole-brain (Lancaster's protocol)	Culture channels (w: 2.5 mm x L: 14 mm) - Perfusion channel in between - Medium flow channels - Continuous flow (syringe pump)	Enhancement of cellular viability and growth	[54] [33]
	Modeling of the cerebrospinal fluid flow	Whole-brain (Lancaster's protocol)	- Culture chambers (D: 8 mm) between medium chambers - Periodic flow: device placed on a bi-directional rocker - Microfluidic device with bi-directional fluid flows	- Enhancement of viability and growth - Improvement of oxygen diffusion within the organoids - Acceleration of the maturation (structural + functional) - Enhanced reproducibility	[55]
	Modeling of cerebral folding and diseased modeling of lissencephaly	Whole-brain (not Lancaster's protocol)	- Constrained culture chamber (150 μm height) - Medium perfusion through a semi-permeable membrane between the chamber and a medium reservoir above	- Appearance of surface wrinkles and folding in organoids - Lissencephalic organoids displayed reduced convolutions - Entirely *in situ* organoids culture	[56]
	Vascularized brain organoid in a microfluidic device	Whole-brain (adapted from Lancaster's protocol)	- Individual culture chamber (D: 2 mm) per device, surrounded by channels for endothelial cells/pericytes to vascularize the organoid	- Perfusion and permeability of the vascular network - Improved neuronal maturation - Could be used as a model of BBB	[57]
Microfluidic devices with micropillar arrays	Neurodevelopmental toxicity: tests of prenatal cadmium exposure effects on neurodevelopment	Whole-brain (Lancaster's protocol)	- Micropillar arrays with octagon-shaped pillars (D: 1 mm x H: 0.8 or 0.6)	- Characterizations of the organoids (neurogenesis and brain structures) - Entirely *in situ* organoids culture	[58], [59]
	Neurodevelopmental toxicity: tests of prenatal exposure effects on neurodevelopment with valproic acid and breast cancer-derived exosomes	Cortical (guided differentiation)	- Micropillar arrays (D: 1 mm x 1 mm)	- Entirely *in situ* organoids culture	[60], [61]
Microfluidic devices with air-liquid interface	Neurodevelopmental toxicity: tests of prenatal cannabis exposure effects on neurodevelopment	Whole-brain (STEMdiff cerebral organoid kit), adapted from Lancaster's protocol)	- Culture chambers with an integrated air-liquid interface (D: 2mm)	- Improved viability + reduced hypoxia + enhanced homogeneity of diameters - Entirely *in situ* organoids culture - Possibility of in situ Matrigel embedding (Lancaster's protocol)	[34]

3.1. Microfluidic devices

In 2018, Wang and colleagues have developed an innovative Brain Organoid-on-Chip technology to improve brain organoids quality, compared to conventional cell culture support [54]. The microfluidic device has been designed with a perfusion system to enhance oxygen and nutrients supply in the center of the organoids, using whole-brain organoids generated following the Lancaster's protocol [18]. Concretely, the device is composed of two culture channels into which the organoids were cultured from the EBs stage and maintained up to 33 days. The culture channels surround a central perfusion channel with a continuous flow of culture medium provided by a syringe pump system, and they are wrapped between two additional medium channels with flows of medium. The architecture of the device enables medium flows and facilitates nutrients/waste exchanges between the culture channels and the other channels. This system has proved to enhance cellular viability and organoids growth, with improved cortical development, compared to static culture conditions.

In a consecutive article from the same research team, this Brain Organoid-on-Chip technology served as a model of human brain development to study the effects of prenatal nicotine exposure on neurodevelopment [33]. They have demonstrated that brain regionalization, neuronal outgrowth and cortical development were disrupted in the nicotine-treated organoids, with premature neuronal differentiation, thus suggesting that nicotine exposure impaired neurogenesis in early fetal brain development. This study also highlights that Brain Organoids on-Chip technologies could be adequate models and powerful tools in pre-clinical *in vitro* studies.

The main advantage that offers this microfluidic device lies in the perfusion system, both enabled by continuous medium renewal with a pump, and by the presence of several channels surrounding the culture channels. The design of the device also seems to improve flow exchanges between the channels, and to enhance the perfusion through the Matrigel - an equivalent of ECM acting as a scaffold to facilitate the organoids expansion in 3D and bringing supplementary growth factors to the organoids - in the culture channels. This device also seems adapted to real-time imaging. Moreover, this platform enables to study a variety of other prenatal exposures, as well as neurodevelopment in both physiological and pathological contexts. However, this microfluidic system does not permit to culture organoids individually, which can be suboptimal for some pathological studies and regarding drug testing applications. It also requires a syringe pump for continuous medium renewal, which is not an easy-to-use format, and which represents a supplementary constraint in laboratories and in industry.

Another potential drawback that could be associated with this system is that the cerebral 3D cellular microenvironment is not entirely recapitulated. Indeed, the unidirectional medium flows established in the channels of the device do not model the complexity of fluid flows in the brain.

To face this issue, Cho *et al.* have developed a specific microfluidic device, coupled with an improved ECM for the EBs embedding [55]. More precisely, their work was based on two bioengineering strategies: (i) addition of an ECM specific to the brain (brain extracellular matrix), to bring additional brain-specific cues; and (ii) dynamic culture conditions in a microfluidic device, to model a bidirectional fluid flow. Regarding the microfluidic device, there are two large open culture chambers per device for the brain organoids culture, surrounded by three medium chambers containing culture medium. The different chambers are fluidly connected by vertical microchannels that enable medium flows from a chamber to another. Moreover, a periodic and gravity-driven flow from left-to-right is created by putting the device onto a bi-directional rocker. Concerning the organoids, they are also whole-brain organoids generated following the Lancaster's protocol [18]. In this study, they have demonstrated that this microfluidic device improves oxygen diffusion within the cerebral organoids, using oxygen-sensing nanoparticles (PtTFPP-PUAN). The

microfluidic conditions also contributed to enhance the organoids viability and growth, to reduce their heterogeneity, to increase the neurogenesis and corticogenesis, as well as accelerating maturation (mature neurons, astrocytes, synapses, and electrophysiological signals) after 60 days of culture, compared to classical culture conditions. Such results were even exacerbated when the microfluidic culture was coupled with an embedding in human brain ECM-enriched Matrigel.

The design of this microfluidic device has been adapted to the protocol, with a system of open chambers that facilitates the introduction of the organoids, covered by the addition of lids afterwards. Such design proves the flexibility of microfluidic devices, that can be adapted to experimental constraints. This design also offers other advantages. Indeed, the combination of microchannels that connect the medium and culture chambers, with a left-right flow of medium, seems to play a central role in the improvements observed regarding organoids viability and maturation. In addition, this improved medium flow, renewal, and diffusion through the organoids are permitted in a simple pump-free manner, adapted to an industrial transfer. Moreover, this platform appears to be adapted to a whole range of studies of brain development and functions, in both physiological and pathological conditions.

Nonetheless, this microfluidic device does not seem suitable to culture brain organoids from their earliest stages either. Indeed, EBs could be easily introduced inside the chambers, but the Matrigel embedding step required in the Lancaster's protocol does not seem feasible inside this format, due to presence of lateral microchannels that would be blocked by polymerized Matrigel. As for the previously described articles (Wang, Wang, Guo, *et al.*, 2018; Wang, Wang, Zhu, *et al.*, 2018), this device does not permit to culture organoids individually, which can limit drug testing applications.

The culture of cerebral organoids requires long culture times for the organoids to expand and mature. In this previous article, this point was not addressed, since they still required quite long culture times, up to 120 days to observe a distinct separation between upper and deep cortical layers (immunostained by Satb2 and Ctip2 respectively).

One experimental strategy that could overcome this issue is the culture of brain organoids in a device with a constrained environment to model the cerebral wrinkling and folding [56]. For this purpose, they designed a device inside which the organoid is compressed in a small culture chamber of only 150 µm in height (compared to 8 mm in Cho *et al.*). The chamber was covered with a semi-permeable membrane and a medium reservoir to facilitate the diffusion of nutrients. The device was composed of different parts which were progressively assembled to facilitate the different steps of brain organoids culture (for instance, EBs were positioned in an open chamber, which was sealed to the rest of the device afterwards). Moreover, a bottom coverslip positioned under the device facilitates *in situ* imaging during the culture. Interestingly, this platform is also adapted to *in situ* immunostaining of the organoids. The generated organoids were whole-brain organoids, but according to a different protocol and timescale than the Lancaster's method, and with smaller EBs at the initial stages. This study demonstrated the appearance of surface wrinkles in brain organoids when grown in a constrained space and enabled to study a model of lissencephaly. Thus, this device provided for the first time a platform to study mechanisms underlying brain folding, as well as associated pathologies. In addition, the constrained environment and the resulting surface wrinkles could also imply an accelerated maturation of the organoids, compared to non-spatially restrained cultures.

However, the step-by-step assembly of this device does not seem compatible with scaled-up fabrication processes and industrial transfer. Nevertheless, approaches to accelerate the maturation of brain organoids are of great interest, especially for some neurological diseases modeling and pharmacological studies, and solutions adapted to an industrial transfer should be further investigated.

The inherent absence of vascular cells in brain organoids raises another challenge regarding their viability. To answer this issue, Salmon and co-workers developed vascularized brain organoids [57] by co-culturing a brain organoid with a vasculature composed of human PSCs-derived endothelial cells and pericytes, inside a microfluidic device. As for the brain organoids, the 3D vascular network was obtained by differentiation of human PSCs in a 3D culture. The brain organoids were whole-brain organoids, following the Lancaster protocol [18]. The aim of this study was to recapitulate the temporal synchronization and spatial orientation of both cultures in an *in vivo*-like manner. Regarding the structure of the microfluidic device, it is composed of an individual culture chamber for the brain organoid culture, surrounded by lateral channels for endothelial cells and pericytes to vascularize the central organoid. In this study, the authors have demonstrated the feasibility of co-culturing brain organoids with a 3D vasculature. They also enhanced both perfusion and permeability of the generated vascular network and achieved accelerated maturation of the organoids after 15 and 30 days of culture, compared to control non-vascularized brain organoids. This device could also serve as a base to model the BBB, by addition of human astrocytes. In the end, it appears to be quite transferable to an industrial scale, considering the fabrication process, the standardizable format of the device and its suitability for drug testing applications. This device seems also adapted to vascularize other types of organoids. However, the most optimal flow regime, as well as its implications on organoids growth, remain to be determined.

All the microfluidic systems described in this part have been fabricated in-house by the experimenters themselves. Globally, a major advantage of this "home-made" strategy is that the devices are specifically designed to answer the needs of the experiments. However, the corresponding drawback is that they are not all suitable for higher production scales, due to the in-house designs and fabrication methods.

3.2. Microfluidic devices with micropillar arrays

Such devices comprise multiple micropillar structures between which cells are seeded, self-aggregate to form EBs, and further expand into organoids. Therefore, a main advantage with these micropillar devices is the entirely *in situ* generation of the brain organoids, which reduces manual transfers of the EBs for the harvesting step.

In a first article from Zhu and colleagues, they present a device composed of micropillar array with octagon-shaped micropillars [58]. The protocol followed to generate the brain organoids was the Lancaster's methodology [18], whose all the steps were performed *in situ*. The objective of this article was to design and optimize the micropillar arrays to generate brain organoids, especially the dimensions of the pillars and the distances between them. The organoids obtained were then characterized to assess correct neurogenesis, and to identify different brain structures. Later, the same research team applied this micropillar device to investigate the neural impairments induced by cadmium - known to be a neurotoxic compound with a long biological half-life - during early brain development [59]. For the treated organoids, they observed an increased cell apoptosis, impaired neural differentiation, and maturation, compared to the control organoids, suggesting that cadmium exposure impaired neurodevelopment. Therefore, this microfluidic system seems to be a relevant approach for drug testing and neurotoxicity studies.

Another team developed its own Brain Organoid-on-Chip platform with micropillar arrays and coupled with cortical organoids (guided differentiation), to study the effects on neurodevelopment of prenatal exposures with valproic acid (VPA) [60], and exosomes derived from breast cancer [61]. For the study with VPA, the treated cortical organoids exhibited impaired neurodevelopment with increased neuronal progenitors, but inhibited neuronal differentiation, and altered forebrain regionalization. Interestingly, similarities with autism patient-derived organoids were observed, highlighting the risk of autism

onset associated with prenatal VPA exposure. Regarding the study with breast cancer-derived exosomes, treated organoids displayed not only impaired neurogenesis, but also carcinogenesis with activated signaling pathways associated with breast cancer and medulloblastoma.

Overall, these devices with micropillar arrays could become interesting platforms for HTS and drug testing, especially with applications in neurotoxicity studies. However, the absence of compartmentalization between the organoids could be problematic for some studies in which individual responses of the organoids could be required (e.g.: measurement of metabolites). Moreover, flows of medium do not seem well controlled yet within the micropillar arrays, indicating a need for further microfluidic studies.

3.3. Microfluidic devices with an air-liquid interface

Ao and colleagues have developed an in-house device made of individual chambers for brain organoids culture, with a generation protocol entirely *in situ* [34]. The chambers contain an air-liquid interface, supposed to promote the oxygenation within the medium, to minimize the hypoxic core formation within the organoids. The microfluidic device also induces a physical restriction for the growing organoids to control their size to be under 2 mm, to enhance their reproducibility in terms of dimensions. The authors demonstrated that the generated organoids recapitulated structural and electrophysiological characteristics of the early human brain, while exhibiting reduced hypoxia and being more homogenous in terms of sizes, compared to conventional cultures.

This platform was also used to study neurotoxic effects on the cerebral development during cannabis prenatal exposure. The treated organoids especially displayed reduced neuronal maturation, impaired neurite outgrowth, and reduced spontaneous firing rate

Since this device is compatible with commercially available standard 6-well or 24-well plates formats, it seems well adapted to a transfer at a larger scale. Moreover, it enables to simultaneously culture a high number of brain organoids (up to 169 in a 6-well plate format), while preventing fusion and merging between the organoids, contrary to conventional culture conditions. Overall, the main advantages of this system are the reduced hypoxic core formation, and the enhanced homogeneity of sizes between the generated organoids. Ultimately, this approach seems to provide an experimental strategy to control the size of organoids. However, regarding the size homogeneity observed between the organoids, the measurements rely on the diameters of the organoids, which provides interesting information, but is generally considered as less representative than measurements of volumes, since organoids expand in 3D.

Interestingly, Giandomenico and Lancaster have recently described an innovative brain organoid culture, based on the combination of organotypic slices cultured with an air-liquid interface, for improved oxygen and nutrients supply, in order to accelerate organoids maturation [62]. This system enabled increased neuronal survival and maturation, axon outgrowth, and circuit formation, leading to an active neuronal network. However, in this protocol based on organoid slices, the organoids are not maintained intact during the culture, contrary to the protocol of Ao and colleagues where whole organoids have been cultured at the air-liquid interface thanks to a microfluidic technology.

4. Discussion: Current insight towards industrialized Brain Organoids-on-Chips for pharmacological applications

Proposing human Brain-Organoids-on-Chips as predictive platforms to study drug responses for neurological disorders requires taking into account different aspects such as (i) conceiving methods to obtain brain organoids closely mimicking the physiology of the human brain, with a high reproducibility and an increased maturation rate in order to limit the time spent in culture, (ii) having access to standardized and reproducible

manufacturing processes, while (iii) increasing the performance for high throughput analysis via non-invasive monitoring techniques such as electrophysiology, and (iv) having the capability to complexify the model by integrating several cell types or forming multi-organ/multi-organoids-on-chip (for an overview of current Brain Organoids-on-Chips advantages and limitations towards pharmacological applications, see Table 2). Since the cerebral organoid generation protocol requires expertise in stem cells and neurodevelopment, the platform must remain affordable and easily transferable to user laboratories, while being standardized and adapted to HTS and automation process for industrial transfer.

Table 2. Advantages and current limitations of Brain Organoids-on-Chip technologies regarding pharmaceutical applications

References	Scalability	Reproducibility	Maturity*	Functionality**	Drug permeability (BBB modeling)
[33], [54]	Possible	Yes	33 days	No	No
[55]	Possible	Yes	120 days	+/- (Ca^{2+}, patch-clamp)	No
[56]	Does not seem possible	Yes	20 days	No	No
[57]	Possible	Yes	30 days	No	+/-
[58], [59]	Possible	Yes	40 days	No	No
[60], [61]	Possible	Yes	70 days	No	No
[34]	Yes	Yes	90 days	+/- (2D MEA)	+/-

***Maximal timepoint**

****Electrophysiological recording assays**

4.1. Standardization methods for reproducible brain organoids generation

The stochastic nature of PSCs self-organization and differentiation inherently leads to heterogeneity and variability between cell types and structures obtained, within and between the individual brain organoids. In particular, unguided differentiation results in higher heterogeneity, compared to guided methods [12]. Yet, only one research team to date used a guided protocol to generate cortical organoids (compared to eight using unguided protocol) in a micropillar arrays-based platform [60], [61] (Table 1). However, using guided protocols seems more suitable for larger scale pharmaceutical applications, due to their inherent reduced heterogeneity.

For pharmacological studies, others key variable parameters of the organoid generation protocols should be considered: (i) the source of PSCs, ranging from commercially available PSCs lines to patients-derived cells, as well as the quality and initial state of the PSCs used to generate the organoids, (ii) experimental parameters of cell culture, and (iii) the presence of undefined components.

Culturing PSCs in feeder-dependent conditions is considered to increase variability and inconsistency of the cells, in addition to be technically more difficult [12]. Therefore, feeder-free cultures appear more appropriate for pre-clinical studies in terms of reproducibility.

Regarding other experimental parameters, including the number of passages of the PSCs used, their state of confluency before their self-aggregation in EBs, or the initial morphology of the EBs, it seems important to define quality controls and selection criteria for improved standardization and reproducibility. Another important aspect to consider is the limitation of undefined ingredients in the protocols. Notably concerning the Matrigel, commonly used either to embed the EBs [18], or diluted in the culture medium [24]. Its animal-origin, undefined composition, batch-to-batch variability, relatively high-cost, as well as the recent global shortage, provide reasons to reflect on the necessity to replace animal-derived ECMs in pre-clinical studies, and on the need to multiply industrial suppliers. Alternatives to Matrigel could include defined non-animal hydrogels. However, these latter must be carefully selected in terms of mechanical properties since they are known to influence brain organoids development [48]. Another solution could lie in the use of brain organoids protocols that do not require an ECM, as already existing [22], [23]. Interestingly, in the study of Cho and colleagues, they used a human brain ECM-enriched Matrigel for the EBs embedding [55]. They demonstrated that the presence of ECM extracted from human cortex samples led to improvements of viability and growth of the organoids, as well as an accelerated maturation at the structural and functional levels. Thinking about using human brain ECM is certainly more physiologically relevant, however, the lack of supply and costs would probably hamper its use in pre-clinical research. However, synthetic hydrogels that model the human brain ECM physical structure and proteome could be a promising alternative adapted to brain organoids culture.

Overall, harmonization and standardization of the brain organoid protocols should be considered to facilitate adoption of these models by the pharmaceutical industry and regulatory bodies. Moreover, commercial brain organoids could also constitute a potential alternative for pharmacological studies, by facilitating standardized generation and culture protocols [13].

4.2. Standardization microfluidic fabrication process for reproducible devices

As highlighted in Table 1, microfluidic systems tend to enhance the reproducibility and quality of 3D cell culture. However, microfluidic devices implementation requires quite complex and time-consuming prototyping and fabrication processes, with specific equipment. Since the generation of brain organoids is challenging and can lead to a great variability between batches, the manufacturing processes must therefore ensure robust and reproducible microfluidic architectures. In addition, the use of microfluidic systems needs to be easy-to-use, and suppliers should give all the details and protocols to be adopted by the whole scientific community [14]. Quality criteria and device standardization are thus necessary to allow comparison and experimental reproducibility between laboratories, and to facilitate a large-scale transfer for pharmacological studies. Overall, this necessary standardization could be achieved using commercial microfluidic systems [48]. Similarly, the materials used to fabricate the devices are another important parameter to consider [63]. Indeed, the absorption properties of the material must be considered, particularly concerning some small molecules [64].

4.3. Compatibility of microfluidic technologies with HTS pharmacological assays: electrophysiology as relevant non-invasive read-out

Emergent Micro-Electrode Array (MEA) technologies enable non-invasive and long-term recording of electrophysiological signals in brain organoids. Mature brain organoids have the ability to recapitulate complex neuronal networks with electrophysiological communications (for detailed reviews, see [65], [66]).

In 2D MEA, brain organoids are positioned on the MEA support, and action potentials can be detected and recorded from neurons located on the surface of the organoid in contact with the MEA. However, this means that the recording is limited to a certain region at the surface of the organoid. To overcome this limitation, other MEA technologies

are currently being developed, including 3D MEA, to improve electrical detection in the center of brain organoids.

In perspectives, combination of Brain Organoids-on-Chip platforms with integrated MEA technologies could provide a relevant non-invasive readout appropriate for high-throughput analyses and automatization processes, and suitable for HTS compounds screening, drug discovery and toxicological evaluations.

4.4. Enhancing capabilities by coupling 3D cell culture with the Blood-Brain Barrier

One of the major obstacles in the development of efficient therapeutic drugs for many neurological diseases is the high selectivity of the BBB. Developing high-fidelity *in vitro* models of the BBB is necessary, in order to test drug permeability and distribution. Conventional *in vitro* models are based on transwell culture systems with co-cultures of several neurovascular cells – generally endothelial cells and astrocytes. However, these minimalistic models are limited in terms of physiological relevance. To address this issue, microfluidic devices have been proved to recapitulate in physiological microenvironment, the BBB complexity, thanks to the 3D geometry, capability of compartmentalization, and improved fluid flows [11]. Typically, a microfluidic-based BBB device commonly consists of a porous membrane separating two channels thus forming two distinct compartments modeling vascular and neural sides, separated by an endothelial cell monolayer. These *in vitro* models allow to investigate the ability of different compounds to interact with endothelial cells, pericytes, and astrocytes, and to transit across the BBB [67]. An interesting future perspective could be the combination of brain organoids with such BBB models, thanks to complex microfluidic platforms, notably for *in vitro* neurotoxic assessment.

Similarly, another perspective of Brain Organoids-on-Chips complexification lies in the emerging field of multi-Organs/Organoids-on-Chips, with complex co-cultures of several cell types or organoids, and the addition of porous membranes to model physiological barriers.

5. Conclusions

The synergy between brain organoid culture and microphysiological chips, termed “Brain Organoid-on-Chips", can recapitulate the complexity of the human brain while leveraging the advantages of the technology by recreating a physiological and controlled microenvironment. 3D microfluidic *in vitro* models based on brain organoids will open the field to study toxicology (ADME-tox), as well as the delivery and screening of molecules that target the brain under more physiologically relevant conditions. However, a routine use in the early phases of drug development requires a reduced variability and an increased quality in terms of culture duration and expression of maturation markers to achieve the possibility of high throughput analysis. Thus, adoption in routine of Brain Organoids-on-Chips requires:

- The establishment of standardized cell culture conditions with defined and reproducible validation and characterization criteria,
- Proof of concept that the technology allows to contribute efficiently and reliably to clinical success for novel therapeutics and improve translational research with providing evidence of successfully predicted human responses,
- Evidence that the model will reduce the need for animal testing while maintaining consistency with the scientific aims of the study.

Once validated, two axes of complexification with very strong benefits could be proposed, namely opening to personalized medicine with the use of primary human cells obtained from patients and generating Multi-Organoids-on-Chips platforms.

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