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SPOTLIGHT

Exploring landscapes of brain morphogenesis with organoids

Denis Jabaudon[1,2,]* and Madeline Lancaster[3,]*

ABSTRACT

The field of developmental neuroscience is benefitting from recent technological advances that allow access to organogenesis *in vitro* via organoid preparations. These methods have been applied to better understanding neural identity, and have opened up a window into the early events that occur during development of the human brain. However, current approaches are not without their limitations, and although brain organoids and other *in vitro* paradigms recapitulate many processes with remarkable fidelity, there are clear differences between brain organoid development *in vitro* and brain development *in vivo*. These topics were discussed extensively at a recent workshop organized by The Company of Biologists entitled 'Thinking beyond the dish: taking *in vitro* neural differentiation to the next level'. Here, we summarize the common themes that emerged from the workshop and highlight some of the limitations and the potential of this emerging technology. In particular, we discuss how organoids can help us understand not only healthy and diseased brain, but also explore new arrays of cellular behaviors.

KEY WORDS: Neural development, Organoids, *In vitro*, Neural differentiation

Introduction

Developmental biology aims to understand how organisms form in space and time. Developmental processes can be studied with different granularities: at a tissue level (e.g. in terms of morphogenesis, which includes processes such as invagination, budding and gyrification), at a cellular level (e.g. with regard to cell proliferation, migration and axonogenesis), and at a molecular/genetic level (e.g. in terms of molecular pathways, transcriptional programs and DNA conformation). These processes are interwoven in both cell autonomous and non-autonomous ways, such that their unfolding is difficult to study *in vivo*. The recent advent of techniques that allow organ-like structures ('organoids') to be grown *in vitro* provides alternative, more tractable systems in which to study these complex processes (Kretzschmar and Clevers, 2016; Lancaster and Knoblich, 2014). Despite the promises offered by organoids in the study of normal and abnormal development, their similarities to and differences between *in vivo* developing organs remain to be fully determined. This was the topic of a recent workshop organized by The Company of Biologists entitled 'Thinking beyond the dish: taking *in vitro* neural differentiation to the next level', in which developmental biologists, engineers, chemists and cellular biologists met to discuss and think about the next steps in organoid technologies, with a particular focus on the brain. Here, we summarize the main discussions and conclusions of this meeting, and raise some questions that, to our knowledge, have not been considered in this context. In addition, although similarities between organoids and actual organs are astounding (Kadoshima et al., 2013; Lancaster et al., 2013), including when examined at the single-cell level (Camp et al., 2015; Quadrato et al., 2017), we consider whether differences between organoid development *in vitro* and organ development *in vivo* can in fact teach us something about what cells are actually capable of, by unleashing the realm of possible cellular behaviors.

Brain development in a dish

Assembly of cells into an organ involves both cell autonomous processes (i.e. events that happen within the cell with little or no influence from the environment) and non-cell autonomous processes (i.e. events that directly result from interactions of cells with their environment). Environmental conditions are sensed by surface receptors and transduced via molecular cascades into cellular behaviors. Such signaling pathways include, for example, the Bmp, Wnt and Fgf pathways, and can be triggered by direct contact with other cells or extracellular components, such as particular nutrients or factors in the extracellular matrix (Simian et al., 2001; Warmflash et al., 2014). Studying and manipulating these processes *in vivo* is difficult for several reasons, including their inaccessibility, thus *in vivo* approaches to development have often been limited to small or transparent organisms such as *Xenopus* or zebrafish, or to the initial stages of differentiation. To circumvent these limitations, developmental biologists have gone *in vitro*, i.e. they have extracted cells from their original ecosystem and have looked at their behavior in a dish (Temple, 1989). This is usually done in two dimensions (2D), which provides an additional level of simplification when examining cells through a microscope and when trying to describe events such as cell division or migration. These 2D systems have proven invaluable in progressing our understanding of cellular and developmental biology: cell-cell interactions can be studied in exquisite detail and can be manipulated by bath-application of compounds; processes such as axonal extension can be directed; and events such as cell proliferation, differentiation and fate specification can be regulated genetically or pharmacologically (Chambers et al., 2009; Eiraku et al., 2008; Gaspard et al., 2008; Ming et al., 2002). Despite being so valuable in bringing forward our understanding of the cellular and molecular underpinnings of developmental physiology, 2D systems have not been able to help us much with aspects relating to morphogenesis, i.e. to the assembly of specific structures in space, and to the consequences of specific spatial configurations on cell fate and differentiation.

Recently, however, great progress has been made in developing technologies that allow specific cell types to grow *in vitro* in three dimensions (3D) and generate structures that resemble the organ from which they originate (Clevers, 2016; Huch and Koo, 2015). These mini-organs, called organoids, can arise from a variety of cells [e.g. embryonic stem cells (ESCs), adult stem cells and induced pluripotent stem cells (iPSCs)], the fate of which can be

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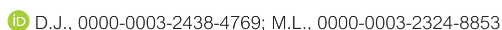
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directed to generate distinct types of tissues/organs, including the brain, gut and liver. As these organoids can be generated from healthy as well as from abnormal cells, they are raising great expectations in translational and personalized medicine. Indeed, these organoids can be generated from cells of human patients, such that the (abnormal) development of specific organs can, in principle, be studied *in vitro* to identify, and potentially one day to correct, abnormal developmental trajectories.

Current capabilities and limitations of *in vitro* models

Perhaps the main strength of current *in vitro* models of brain development is that they can be generated from human cells. By definition, human-specific features, and defects that affect those features, cannot be directly studied in animal models. Brain organoids seemingly faithfully display a number of features of the human brain, and can be used to model congenital defects that are not necessarily well modeled in mice (Bershteyn et al., 2017; Birey et al., 2017; Lancaster et al., 2013). This strength has led to a huge influx of researchers adopting these methods for the study of human neurological conditions.

Despite this promising potential, brain organoids suffer from a number of limitations, the main one being that only relatively early stages of differentiation can be replicated. In particular, it is unclear to what extent growth can be sustained *in vitro* while still reasonably reflecting the *in vivo* situation. Physical limitations likely underlie this current inability of organs to develop past a certain stage, and a major challenge appears to be the ability to provide structures with an adequate supply of oxygen and nutrients past a certain stage of development at which diffusion alone no longer suffices. Thus, the topic of vascularization was a major one at the workshop, with Abed Mansour presenting his recently published work (Mansour et al., 2018) that takes advantage of transplantation in the mouse to promote the *in vivo* vascularization of brain organoids. But there was also extensive interest in *in vitro* vascular models, such as those presented by Sharon Gerecht and Chris Hughes (Kusuma et al., 2013; Sobrino et al., 2016), which allow for the *in vitro* vascularization of organoids and the delivery of crucial nutrients through living blood vessels. These systems offer the potential to transcend the size limits that currently limit organoid development *in vitro*. Although both *in vivo* transplantation and *in vitro* vascularization have the potential to overcome current nutrient limitations, each has certain advantages over the other. Specifically, transplantation has the potential to look at brain development in the context of the whole organism, allowing the investigation of extra-CNS-derived cell types and metabolites. However, transplantation is an involved procedure that can only be performed with limited samples; by contrast, *in vitro* vascularization may be more appropriate for high-throughput experiments such as drug screening.

The brain, and more generally the nervous system, also presents a specific challenge in that many of the connections between brain regions are thought not only to rely on cell-intrinsic developmental programs of axonal guidance, but also on activity/input-dependent processes derived from target and source areas as well as from external signals from the environment. At this stage, it feels like the field is hitting a roadblock in which some early developmental processes, including morphogenetic processes, can be replicated with a reasonable level of reliability, whereas the extent to which later occurring processes, such as synaptogenesis and wiring, faithfully replicate the process *in vivo* is unknown. Therefore, as Pierre Vanderhaeghen (VIB-KU Leuven Center for Brain and Disease Research, Belgium) pointed out, there is a continual need for *in vivo* confirmation, and future studies will no doubt require careful comparative analyses including, for example, those using chimeric models of human ESC/iPSC-derived neural cells transplanted into the mouse brain (Espuny-Camacho et al., 2013). In a more distant future, additional approaches allowing environment- and/or sensory experience-like manipulations of activity, including through advanced optogenetic and chemogenetic means as well as co-culture with sensory organ-like structures or neuromuscular components, might contribute to emulate *in vivo*-like conditions and act to sculpt neuronal connectivity into more meaningful functional circuit components.

The concept of ‘fit-for-purpose’

The increasing attention on brain organoids for the study of neurological conditions has also spotlighted certain other limitations, most notably issues with reproducibility. Reproducibility is a complicated topic and encompasses variations in methodology, variability in starting materials (i.e. cell lines), lab-to-lab and individual-to-individual variability, and even technical differences as trivial as pipette tip material. Add to this the intrinsic heterogeneity of these self-organizing structures and inherent stochasticity in their tissue morphogenesis, and it’s no wonder that the outcome varies considerably.

Although the issue of reproducibility has been a recurrent topic at other organoid-focused conferences, the discussion sessions at this workshop delved into this topic in more depth. Of note, participants attempted to collectively understand what the issues are, which features in particular are more or less reproducible, and what we can do to address these issues as we move forward as a field. Because more and more researchers are adopting these methods, there is inherent divergence in the protocols being used, and the evolution of methods over time has led to a variety of (apparently) minor tweaks, and sometimes major modifications (Lancaster et al., 2017; Paşca et al., 2015; Qian et al., 2016; Sakaguchi et al., 2015). Indeed, it became clear during the discussions that the wide range of methods can be somewhat frustrating to newcomers in the field, and some degree of standardization across laboratories would be valuable.

However, it was pointed out that the diversity of methods can also be seen as a strength, as it provides many options for answering a given question. Thus, rather than attempting to reach an overarching template of what organoids should be able to fulfill, one of the concepts that has emerged in the field of tissue engineering, as put forward by Chris Hughes (University of California, Irvine, USA) in our discussions, is the idea of a ‘fit-for-purpose’ approach. Given organoid preparations need not be able to address all developmental questions, pathology-related questions or basic biophysical questions at the same time. Thus, some preparations might be best suited to study cell fate determination, whereas others would be more adequate for neuronal connectivity, for example. The important point when working with *in vitro* models then is to choose the method that is right for the question at hand. Following on from this, having more methods to choose from can therefore be an advantage rather than a limitation of the field, and we should not necessarily seek to standardize across laboratories to the point of only using a single method.

Nonetheless, it was agreed that the ability to systematically reproduce (within and across laboratories) a given experimental preparation, or to identify which processes are intrinsically stochastic, is essential to allow organizing principles to be identified. This is particularly important for models of neurological conditions, in which the need for reproducible *in vitro* human models is perhaps highest. The ability to detect a phenotype in any model depends on the degree of variability for the biological feature of interest. If this

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range is too large in the control condition, it is unlikely that a phenotype will be visible above the noise. Although biological heterogeneity creates a certain range of variability that is intrinsic, technical variability introduces a different kind of noise. Thus, experimental reproducibility could be improved by standardizing methodologies, perhaps through courses aimed at training current and future users in the more widely accepted methods, and by trying together to establish 'good practice' guidelines in the field, as has been the case for classical animal models.

What can organoids do: the concept of the 'morphospace'

The discussion on variability kicked off an interesting conversation on intrinsic biological heterogeneity. The overall conclusion was that, although more technical standardization across laboratories would be valuable, variability is an intrinsic feature of developing organs that might actually be desirable and/or required when studying biological processes. As Matthias Lutolf (Ecole polytechnique fédérale de Lausanne) put it, development is essentially robust self-organization. Thus, using organoids to understand these self-organizing principles may be informative of development *in vivo*. Indeed, it was agreed that the issue of faithfully emulating *in vivo* processes and in a reliable manner ('fidelity') is particularly crucial if one seeks to model diseases (i.e. are the abnormal processes observed *in vitro* truly indicative of a corresponding *in vivo* abnormality?) and understand physiological developmental processes. In contrast, discrepancies in development (i.e. the emergence of developmental trajectories that would not normally occur *in vivo*) can be informative for understanding the realm of possibilities a given group of cells is able to explore, i.e. the 'morphospace' that is accessible to developing organisms (Mitteroecker and Huttegger, 2015), a concept put forward during the meeting by James Briscoe (The Francis Crick Institute, London, UK). The idea of morphospace is well established in the fields of evolutionary biology and paleontology, in which it is used as a way to represent the possible forms and shapes of an organism. It illustrates the subset of realized forms of a structure relative to all possible forms, providing insight into the constraints that operate to restrict the evolution of a species and/or clade. In the same way, organoids allow the exploration of the space of possible forms, of which the one realized in an embryo is what the embryonic constraints determine. Anomalies *in vitro* may therefore inform us on the plasticity and developmental potential of cellular assemblies by untethering developing tissues from the systemic *in vivo* constraints imposed by millions of years of evolution. Thus, although increasing fidelity is important for modeling disease, these discrepancies may actually be highly informative from a developmental biology perspective (Fig. 1).

Where do we stand?

Overall, it became evident throughout the meeting that identifying the molecular and mechanical parameters that influence 3D tissue growth will be a prerequisite to allow for replicability. Indeed, presentations from Linda Griffith, Fan Yang and Cole DeForest focused on extracellular matrix components and standardization of the 3D microenvironment (Cambria et al., 2015; DeForest and Tirrell, 2015; Wang et al., 2014), and work presented by Guo-li Ming demonstrated the ability to produce largely identical organoids on a bigger scale (Qian et al., 2016). Patterning was also an area of focus, with Randolph Ashton, Matthias Lutolf, Madeline Lancaster and David Schaffer presenting work on intrinsic and extrinsic cues that control cell fate determination (Knight et al., 2018; Muckom et al., 2018; Ranga et al., 2016).

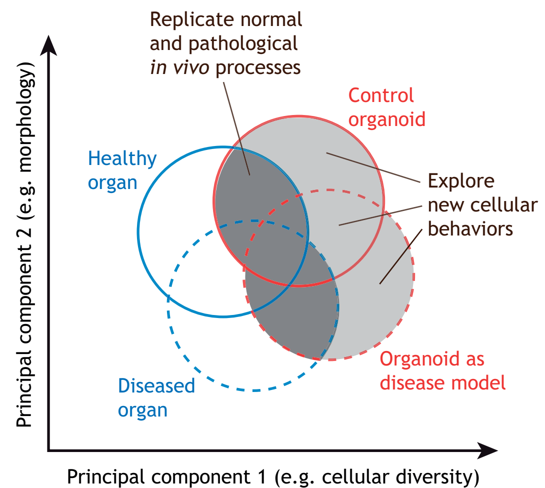

Fig. 1. Exploring possible behaviors of cellular assemblies with organoids. A number of parameters define how individual cells relate to the organ they form. Two such parameters (cellular diversity and organ morphology) are illustrated here in a principal component analysis display. These parameters only partially overlap between organs and their organoid counterparts (whether healthy or diseased), allowing the investigation of normal and abnormal development, but also the exploration of new cellular behaviors.

It is clear that more research is needed into what organoids are actually capable of and, therefore, which disorders can accurately be modeled. Several studies have already demonstrated the utility of brain organoids and other *in vitro* neural models to identify disease mechanisms, such as in the case of genetic and acquired microcephaly. Along these lines, work presented by Silvia Cappello, Marisa Karow, Femke De Vrij and Andras Lakatos (de Vrij et al., 2018; Tyzack et al., 2017) focused on human conditions for which *in vitro* neural models have enormous potential.

Although the reliability of organoid development with regard to *in vivo* processes is not essential in all cases, and may, in fact, inform us on the degrees of freedom of cell behaviors (as discussed above), it will be important to tie this back to *in vivo* findings in order to discern which features are anomalies and which may actually be reflecting processes not observed before *in vivo*. In order to accomplish this, careful *in vivo* analyses that describe cellular diversity and neural cell behaviors, such as the work described by Laurent Nguyen, James Briscoe, Denis Jabaudon and Debra Silver, are vital (Pilaz et al., 2016; Silva et al., 2018; Zagorski et al., 2017). These and many other previous studies illustrate that animal models remain essential for meaningful *in vivo* experimentation, for which *in vitro* approaches including organoids will never fully substitute. *In vivo* and organoid approaches should thus be considered complementary rather than in opposition. Once we have a grasp of the cellular processes occurring within organoids and *in vivo*, the two can be compared in order to tease apart similarities and differences. This type of comparative analysis was demonstrated at the transcriptome level by Barbara Treutlein and Gray Camp, who pointed out that the similarities in expression and cellular diversity in organoids and in brain samples are striking (Camp et al., 2015). However, differences are also observed and can be highly informative. Indeed, differences between human and non-human neural development can be explored using pluripotent stem cell-based models to provide insights into brain evolution. This focus on evolutionary differences was a recurring theme throughout the workshop, and was further exemplified by the work presented by

Pierre Vanderhaeghen, who discussed the impact of human-specific genes on cortical neurogenesis (Suzuki et al., 2018).

Finally, one important issue that will need to be addressed is what to do when organoids do not conform to the cellular and tissue level behaviors seen *in vivo*. Does this mean that the organoid is not useful, or can we learn something from it? Could it be that the organoid allows for a greater sampling of the realm of possible cellular behaviors than the more controlled environment of development? Indeed, James Briscoe raised the intriguing question: 'Is there such a thing as an *in vitro* artifact?'

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Competing interests
M.L. is an inventor on patents describing brain organoid methodologies.

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