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Rethinking embryology *in vitro*: A synergy between engineering, data science and theory

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ABSTRACT

Pluripotent stem cells, in the recent years, have been demonstrated to mimic different aspects of metazoan embryonic development *in vitro*. This has led to the establishment of *synthetic embryology*: a field that makes use of *in vitro* stem cell models to investigate developmental processes that would be otherwise inaccessible *in vivo*. Currently, a plethora of engineering-inspired techniques, including microfluidic devices and bioreactors, exist to generate and culture organoids at high throughput. Similarly, data analysis and deep learning-based techniques, that were established in *in vivo* models, are now being used to extract quantitative information from synthetic systems. Finally, theory and data-driven *in silico* modeling are starting to provide a system-level understanding of organoids and make predictions to be tested with further experiments. Here, we discuss our vision of how engineering, data science and theoretical modeling will synergize to offer an unprecedented view of embryonic development. For every one of these three scientific domains, we discuss examples from *in vivo* and *in vitro* systems that we think will pave the way to future developments of *synthetic embryology*.

1. Introduction

Reconstitution approaches try to build complex structures *in vitro* from the bottom-up by using a minimal set of ingredients (Good and Trepat, 2018; Karsenti, 2008; Liu and Fletcher, 2009; Way, 2017). While the earliest attempts to culture cells or supracellular structures *in vitro* dates back to mid twentieth century (Garber and Moscona, 1972; Moscona, 1957), serious attempts in co-culturing multiple cell types to reconstitute a structure that can mimic *in vivo* physiology can be traced back to the end of the last century (Barcellos-Hoff et al., 1989; Michalopoulos and Pitot, 1975; Rheinwatd and Green, 1975). Particularly in the last decade, remarkably complex biological tissues and their morphogenesis began to be reconstituted *in vitro* (Eiraku et al., 2008, 2011; Lancaster et al., 2013; Sato et al., 2009; Takahashi et al., 2007; Takebe et al., 2013; Wilson et al., 2009), owing to the progress in our understanding of cell and molecular biology and genetics, as well as the development of robust protocols for culture and differentiation of pluripotent stem cells. Since then, there has been growing interest in using similar bottom-up approaches to engineer multicellular systems at the tissue scale (Haase and Freedman, 2020; Kamm et al., 2018; Kicheva and Rivron, 2017) (see Table 1). Pluripotent stem cells (PSC) or induced pluripotent stem cells (iPSC), when subjected to a temporally defined series of growth factors and small molecules, have been shown to form structures at the micrometer-to-milimeter scale which are remarkably similar to organs (Rossi et al., 2018) and are collectively termed as organoids (Huch et al., 2013a; Huch et al., 2013b; Lancaster and Huch, 2019; Lancaster and Knoblich, 2014; Sato et al., 2009; Simian and Bissell, 2017; Takasato et al., 2015; van den BrinkAnna et al., 2020). Recently, the stem cell and developmental biology communities have taken advantage of the organoid technology to surpass the limitations on the accessibility of mammalian embryos, recapitulating early stages of embryonic development to an astonishing detail at both transcriptional and morphogenetic levels (Baillie-Benson et al., 2020; Beccari et al., 2018; Moris et al., 2020; Rivron, 2018; Rivron et al., 2018; Shahbazi et al., 2019; Sozen et al., 2018; ten Berge et al., 2008; Turner et al., 2017; Van den Brink et al., 2014; Warmflash et al., 2014; Zheng et al., 2019) (see Table 1). Two main broad categories of synthetic systems can be distinguished: systems that faithfully recapitulate embryo geometry and morphogenesis at very early embryonic stages (ETS/ETX embryos or blastoids (Harrison et al., 2017; Rivron, 2018; Rivron et al., 2018; Sozen et al., 2018)) and systems that show limited morphogenesis yet they are capable to reach more developed stages when assessed at transcriptional level (gastruloids, embryonic explants from *Xenopus* and Zebrafish (Beccari et al., 2018; Bérenger-Currias et al., 2020; Fulton et al., 2020;

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Table 1
Some examples of *in vitro* systems. ASCs: adult stem cells, PSCs: pluripotent stem cells, ESCs: embryonic stem cells, XEN: Extraembryonic endoderm, TSCs: trophoblast stem cells. For a detailed list of *in vitro* systems, see Ref. (Rossi et al., 2018).

Embryonic *in vitro* systems			
Organoid type	*Cell type*	*Culturing system*	*References*
Embryoid bodies	ESCs	Suspension/ Methylcellulose/ Hanging drop	(Desbaillets et al., 2000; Doetschman et al., 1985; Kurosawa, 2007)
Gastruloids	ESCs	Suspension/ Micropatterns	(Beccari et al., 2018; Marikawa et al., 2020; Moris et al., 2020; Turner et al., 2017; Van den Brink et al., 2014; Warmflash et al., 2014)
Neural tube	ESCs	3D PEG hydrogel/ Matrigel scaffold	(Ishihara et al., 2017; Meinhardt et al., 2014)
ETS embryos	ESCs + TSCs	3D matrigel scaffold	Harrison et al. (2017)
ETX embryos	ESCs + TSCs + XEN cells	AggreWell plate system	Sozen et al. (2018)
Blastoids	ESCs + TSCs	Suspension	(Rivron, 2018; Rivron et al., 2018)
Amniotic-sac-like embryoids	ESCs	Microfluidic device	Zheng et al. (2019)
Explants	*Xenopus* or Zebrafish blastula	Suspension	(Fulton et al., 2020; Green et al., 2004; Schauer et al., 2020; Williams and Solnica-Krezel, 2020)
Neuroloids	ESCs + XEN cells	Suspension	Bérenger-Currias et al. (2020)
Trophoblast	Placenta derived cells	3D matrigel scaffold	Turco et al. (2018)
Adult *in vitro* systems			
Organoid type	*Cell type*	*Culturing system*	*References*
Intestinal	ASCs	3D matrigel scaffold	Sato et al. (2009)
Optic cup	ESCs	3D matrigel scaffold	Eiraku et al. (2011)
Cerebral	PSCs	3D matrigel scaffold and Bioreactor	Lancaster et al. (2013)
Liver	ASCs	3D matrigel scaffold	Huch et al. (2013b)
Pancreas	ASCs	3D matrigel scaffold	Huch et al. (2013a)
Kidney	PSCs	Transwell filter/3D matrigel scaffold	(Freedman et al., 2015; Takasato et al., 2015)
Fallopian tubes	ASCs	3D matrigel scaffold	(Kessler et al., 2015)

Green et al., 2004; Marikawa et al., 2020; Moris et al., 2020; Schauer et al., 2020; Turner et al., 2017; Van den Brink et al., 2014; Warmflash et al., 2014; Williams and Solnica-Krezel, 2020)). This has led to the establishment of the field of *synthetic embryology*.

In vitro approaches can be used to develop methods to build structures mimicking their *in vivo* counterparts (Sozen et al., 2018). Intriguingly, they can also be seen as platforms to explore many possible conditions revealing what is necessary and what is sufficient to build a certain multicellular structure (Van den Brink et al., 2014; Warmflash et al., 2014). The quote *"What I cannot create, I do not understand"* from Richard Feynman has been used to exemplify the bottom-up approach usually taken by engineers and physicists towards understanding biological systems (Kicheva and Rivron, 2017; Way, 2017). However, the opposite (*"What I can create, I do understand"*) is not necessarily true, especially in complex biological systems where most of the phenomena observed is emergent (Good and Trepat, 2018). Currently, we know how to form millimeter-scale structures mimicking brains, kidneys or guts, despite not fully understanding how they self-organize. Here the term "self-organization" is referred to as the spontaneous emergence of increasing order in a multicellular structure in the presence of spatially homogeneous signaling cues (Haken, 2008). We argue that *in vitro* approaches not only allow guiding the self-organization of multicellular structures, but also provide a platform to understand how self-organization occurs *per se*. This is a consequence of the high level of control (chemical and mechanical) in manipulating the *in vitro* system as opposed to the developing embryo.

Here, we review the state-of-the-art of synthetic embryology and propose an interdisciplinary approach to understand self-organization in such *in vitro* systems by combining engineering, data science and mathematical modeling. We discuss engineering tools (Section 2) ranging from those used for controlling the environment (bioreactors, microfluidic systems), cell arrangement (micropatterning (Warmflash et al., 2014) and microwell arrays (Kim et al., 2013)) and high-throughput screening for drug testing (Vrij et al., 2016) to those used for quantitative high-content imaging together with deep learning techniques (Jones et al., 2009). This is followed by recent developments in data analysis tools (Section 3) that allow large scale image analyses as well as interpretation of high-content -omics data. Towards the end we postulate how theoretical and computational approaches, that span both agent-based and continuum models (Section 4), can capture the underlying biophysics of synthetic systems. Until recently, a major limitation in the application of such techniques in the field of synthetic embryology has been the separation of expertise between embryologists/developmental biologists, bioengineers and theoreticians. However, interdisciplinary approaches have started to prosper, in particular efforts between experimentalists and bioinformaticians have helped elucidate the transcriptional state of cells in most *in vitro* systems known to date. Recently an image-based screen to assay an annotated library of compounds, combined with RNA sequencing was used to understand the initiation of transcriptional programs that guide the cell-fate transitions within intestinal organoids (Lukonin et al., 2020). Therefore, thanks to the great variety of tools available, we envision *in vitro* approaches in multicellular systems as a platform to test hypotheses and explore concepts in developmental biology which otherwise would be impossible to test in a real embryo.

2. Engineering devices to generate and image organoids

2.1. Environmental control and microfluidic tools

Adult and embryonic stem cells have the potential to self-organize into complex structures resembling organs and embryos when cultured under favourable conditions - appropriate nutrient-rich media and optimal environmental conditions - that can promote the cellular activities necessary for tissue patterning. While in some cases this is achieved by replacing the culture media daily to ensure waste removal and nutrient replenishment, it is often important to utilize automated devices capable of monitoring and maintaining the environmental conditions. For example, using microfluidic devices and relatively simple static systems, the pH, CO_2, nutrient and oxygen levels, and waste removal can be efficiently controlled continuously (Bhatia and Ingber, 2014; Huh et al., 2010; Yu et al., 2017). Moreover, microfabrication allows to generate environments with different geometries and sizes, thus favouring stem cell self-organization (for a review, see (Brassard and Lutolf, 2019)). For instance, a microfabricated approach has been used recently to generate and grow mouse blastocyst-like structures (blastoids) (Rivron et al., 2018).

On the other hand, when trying to reproduce the *in vivo* physiology as close as possible *in vitro*, the environmental conditions need to be modified in a tightly controlled spatio-temporal fashion. The effect of temporally modulated signals has been demonstrated on 2D myoblast cell cultures combined with a microfluidic platform which showed that cell differentiation is largely impaired when TGF-β pulses are applied, but less so under continuous stimulation (Sorre et al., 2014). More recent studies on gastruloids generated from epiblast-like cells demonstrated that, upon activation or inhibition of the Wnt pathway starting at day 3, they were able to recapitulate endoderm and ectoderm patterning, respectively (Girgin and Lutolf, 2020; Hashmi et al., 2020). However,

when gastruloids are formed from more pluripotent-like cells and exposed to Wnt activators at day 2, they generate mesodermal structures (Beccari et al., 2018). Similarly, spatial and geometrical configurations play a crucial role in the patterning of *in vitro* systems (Vianello and Lutolf, 2019), as exemplified by the fact that the primitive streak structure generated in embryoid bodies was positioned at the point of contact with the agarose microwells in which the cells were seeded (Sagy et al., 2019). These studies demonstrate how external geometry and spatio-temporal regulation of signals are key ingredients for embryonic patterning and need to be precisely controlled in *in vitro* systems. For instance, in some cases it is important to transiently apply pulses of signaling molecules or locally activate a group of cells (Sonnen et al., 2018). Such level of control can only be achieved with the help of advanced engineering devices that are capable of modifying the environmental conditions with high precision (for reviews, see (Samal et al., 2019; Yin et al., 2016; Yu et al., 2019)). The first attempt in this direction used a "Y"-channel device made of the biocompatible material polydimethylsiloxane (PDMS). The authors used this device to culture mouse embryonic bodies and induce neural differentiation by applying a flow of retinoic acid (Fung et al., 2009). More recently, Manfrin et al. utilized a multi-layered microfluidic device and applied transient and opposing gradients of BMP4 and NOGGIN to guide the appearance of differentiation domains resembling early gastrulation in human embryonic stem cells (hESC) confined in a 2D circular geometry (Manfrin et al., 2019). In another example, Zheng et al. used a liquid flow system to seed and aggregate hESCs into gel pockets and, by applying BMP4 to only one half of the aggregate, the authors recapitulated the dorsal-ventral patterning of the human epiblast and amniotic ectoderm development (Zheng et al., 2019) (see Fig. 1a). While other strategies to generate similar microfluidic devices exist (O'Grady et al., 2019), we have only started to explore the potential of microfluidics to the study of *in vitro* embryonic systems.

More complex devices that allow long term maintenance, improved growth and high-throughput culture of organoids exist. These devices, termed bioreactors, include multi-layered microfluidics, stirred tanks, rotating wall vessels, clinostats and any modular combination of those. These devices allow the generation and control of complex environments with high precision (for a review, see (Phelan et al., 2018)). Therefore, it is in principle possible to optimize growth conditions for the generation and development of organoids. Bioreactors have been designed recently to improve the culturing conditions of a variety of *in vitro* cellular systems (see Fig. 1b), such as human lung adenocarcinoma spheroids (Phelan et al., 2019), brain organoids (Qian et al., 2016, 2018; Romero-Morales et al., 2019), hematopoietic progenitor cells (Wuchter et al., 2016), retinal organoids (Ovando-Roche et al., 2018) and kidney organoids (Przepiorski et al., 2018). Despite the complexity of these devices makes them technically challenging to fabricate, we expect that bioreactors and more complex microfluidic devices will be increasingly adopted to study *in vitro* embryonic systems trying to mimic as close as possible their *in vivo* counterparts.

2.2. High throughput screening

Engineering-inspired and automated approaches to generate and maintain organoids have been recently used in the fields of personalized medicine and drug discovery (for a review, see (Takahashi, 2019; Takebe and Wells, 2019)). For instance, drug screening, in combination with genetic and molecular analyses, has been applied to cancer organoids derived from patient tumors (Francies et al., 2016). In addition to molecular testing, multi-electrode arrays and large liquid handling systems

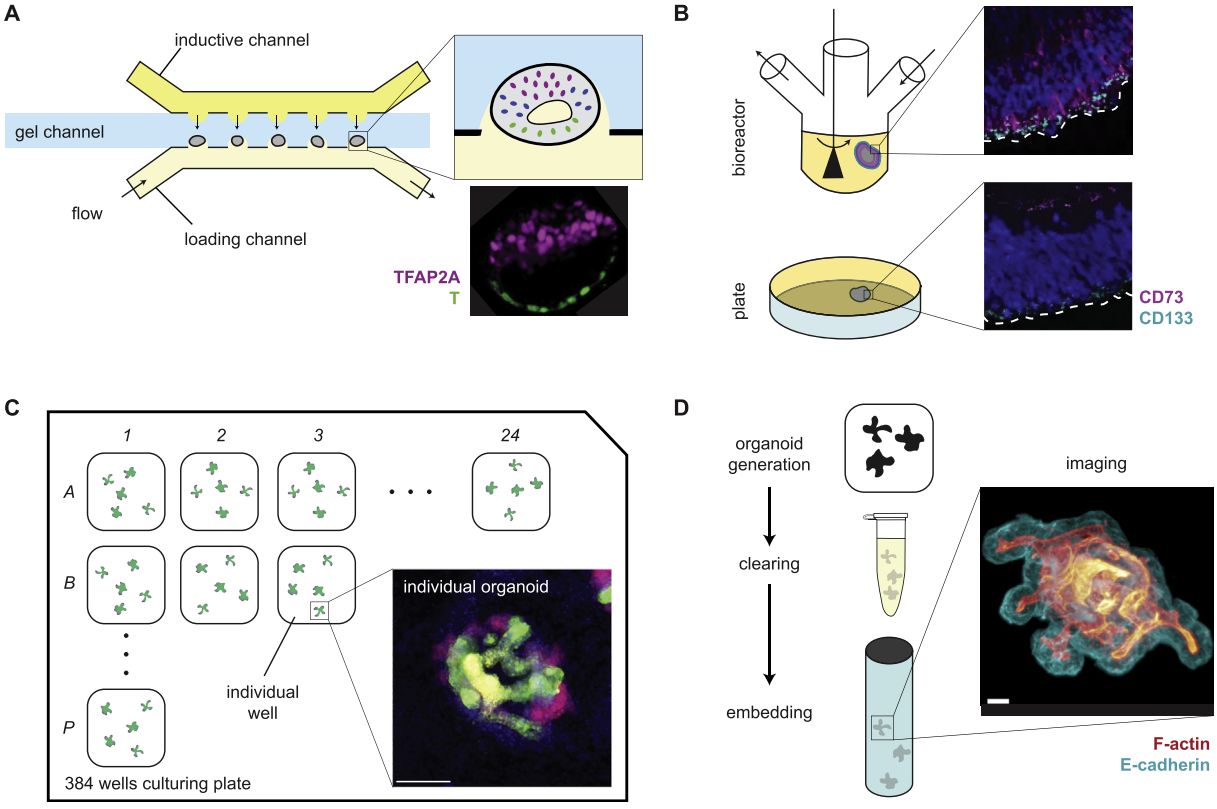

Fig. 1. Engineering approaches to culture and image organoids. a) Microfluidics device used to generate posteriorized embryonic-like sac, imaged at 36 h with confocal microscopy. Image adapted from Ref. (Zheng et al., 2019). b) Retinal organoid imaged after 16 weeks of culturing in a bioreactor show photoreceptors cells compared to control organoid. Image adapted from Ref. (Ovando-Roche et al., 2018). c) Confocal image of one of the thousands kidney organoids generated in an automated manner in a 384-wells plate. Image adapted from Ref. (Czerniecki et al., 2018). d) Human colonic organoids imaged with light sheet microscopy after immunolabeling and clearing. Image adapted from Ref. (Dekkers et al., 2019).

have been used to perform morphological screening and phenotyping of brain (Durens et al., 2020) and kidney organoids (Czerniecki et al., 2018), respectively (see Fig. 1c). Moreover, Mead et al. established a high-throughput screening assay on a miniaturized system and proved its power in identifying clinically-relevant small molecules which can be translated *in vivo* (Mead et al., 2020). Recently, a microengineered device, combined with automated handling of organoids, has been used to generate, phenotype and drug-test thousands of gastrointestinal and colorectal organoids (Brandenberg et al., 2020).

While the previous studies exemplify the power of engineering devices in *in vitro* systems, only a handful of examples exist in which they have been used for high-throughput studies in the context of synthetic embryology (Ranga et al., 2014; Vrij et al., 2016). We expect that, in the future, automated and high-throughput approaches will become an essential ingredient in the field of synthetic embryology for three main reasons. First, they will allow us to answer fundamental biological questions that have been so far challenging to investigate, for instance to elucidate the role of different biochemical and mechanical clues on biological patterning. This can only be achieved by growing synthetic embryos under a large variety of different environmental conditions, and using highly scalable high-throughput approaches will greatly aid this effort. Second, reproducibility is a challenge in organoid derivation protocols (Rossi et al., 2018), and synthetic embryology is no exception. The use of automated devices will help us improve the success rate of organoid generation and reduce the sample-to-sample variability. Third, when phenotypic variability cannot be suppressed by using automated devices, we will be able to embrace it and leverage statistical power by generating and analyzing hundreds or thousands of organoids. This will prove crucial to study the sources of noise that lead to the observed phenotypic variability.

2.3. Imaging of organoids

Phenotypic variations of *in vitro* systems manifest themselves in a wide range of scales, ranging from the transcriptional state and electrical stimuli response of single cells, to the morphology and timescale of developmental progression of the whole organoid. Therefore, in order to investigate these widely different phenotypes, one needs to probe and visualize features of the whole-organoids as well as subcellular features. A variety of platforms tailored to the visualization and functional probing of organoids have been developed, ranging from imaging-based methods (for a perspective, see (Rios and Clevers, 2018)) to neuronal recording and laser ablation experiments. In particular, imaging techniques that allow non-invasive optical sectioning provide an ideal way to fully appreciate the 3D complexity of organoids structures. Confocal 3D imaging, for example, has allowed to elucidate the differentiation state of breast organoids at cellular resolution (Jamieson et al., 2017). In the last decade, light sheet fluorescence microscopy (LSFM) (Huisken et al., 2004) has established itself as the technique of choice to perform live imaging at high speed and limited phototoxicity. While it has been originally used to study embryonic development (Keller et al., 2008; McDole et al., 2018; Shah et al., 2019), recently light sheet microscopes have been used to study a variety of *in vitro* systems. This has enabled researchers to quantitatively determine cell lineages and cell movements in kidney organoids over 24 h of development (Held et al., 2018). Light sheet microscopy has also been used to perform label-free imaging of colorectal cancer organoids to quantify treatment response (Favreau et al., 2020). Importantly, light sheet microscopy can be combined with other advanced imaging techniques such as two-photon microscopy to improve optical penetration in large samples. This technique has been used to perform large field-of-view and fast volumetric imaging of mouse embryoid bodies and brain organoids (Rakotoson et al., 2019). Not only does the field of *in vitro* synthetic development benefit from the advantages of LSFM (particularly the low levels of phototoxicity that allows long-term imaging), but the unconventional constraints on sample mounting that these *in vitro* models present also prompt novel configurations of the microscope itself. For instance an inverted light sheet configuration customized for multi-sample imaging was recently used for imaging morphogenesis in intestinal (Serra et al., 2019) and brain (He et al., 2020) organoids for several days. Additionally, optical imaging techniques can be combined with other probing methods. For instance, clearing methods allow to obtain single snapshots of immunostained organoids at very high spatial resolution and depth (Dekkers et al., 2019) (see Fig. 1d). Other examples of complementary techniques include electrophysiology studies of neuronal *in vitro* systems with the use of patch clamping (Paşca et al., 2015) or micro-electrode arrays (Jo et al., 2016), and the use of optogenetics to spatio-temporally control gene expression and cell behavior (Hartmann et al., 2020; Mumford et al., 2020; Repina et al., 2019; Shiri et al., 2019). We expect that similar approaches will be soon adopted in the field of synthetic embryology and, by providing us with a unique view of *in vitro* embryonic systems, they will be crucial to advance the field.

Altogether, these studies demonstrate how the organoid field has constantly provided new technical challenges and has been pushing the limits of what is technically possible in the domains of sample visualization and probing. As a consequence, organoid culturing technologies and the engineering systems to handle and probe them are advancing at an impressive pace. Therefore, we are quickly approaching the day in which we will be able to visualize every single cell in an *in vitro* embryonic system under physiological conditions over several days of development. The next great challenge will then be to extract quantitative and interpretable features out of the immense amount of data we will generate.

3. Quantitative analysis of organoids

Owing to the rapid advancements in the experimental techniques available to study organoids, we will soon be able to generate more data than we can handle (Gomez-Cabrero et al., 2014; Manzoni et al., 2018). Therefore, computational tools will be needed to quantify, filter and interpret significant patterns within the datasets in an automated fashion. In the organoids field, two major categories of data can be generated: imaging and -omics. In what follows, we will review and comment on the main computational tools developed for each data type and provide examples of how they have been used to aid biological discoveries in the recent years.

3.1. Image analysis

Microscopy experiments can either generate imaging data for a large number of samples (e.g. confocal high throughput systems) or at high temporal resolution (e.g. LSFM). As such, the output of a microscopy experiment is a multidimensional dataset given by the spatial dimensions (2D or 3D), and the numbers of timepoints and fluorescence channels. Image analysis tasks are very diverse and include: registration, object detection, classification and segmentation, object tracking over consecutive timepoints and object analysis such as morphometrics, parametrization and fluorescence quantification. Therefore, computational tools will vary depending not only on the data structure but also on the analysis task.

The field of image analysis has developed at a fast pace in the last two decades (for a review, see (Peng, 2008)), leading to three main types of softwares and algorithms: proprietary, open-source and custom-written. Proprietary softwares (such as Imaris, Velocity, Amira, Harmony and ZEN, see (Li et al., 2016) for a review) are commercially available, often provided with the imaging instrument and routinely maintained and documented to ensure ease-of-use. However, the algorithms used are at best commented in a manual, and usually unavailable for the user. On the other hand, open-source softwares such as FIJI (Schindelin et al., 2012), CellProfiler (Lamprecht et al., 2007) and KNIME (Berthold et al., 2009), provide fully transparent platforms to design and share image analysis pipelines. However, even though a large community is devoted to their

maintenance and documentation, open-source softwares often assume a certain degree of programming knowledge of the user. Recently, in the organoid field, custom-written software has been increasingly employed to analyze microscopy images. In our opinion, this is due to two main reasons: first, organoid experiments are posing biological questions of ever increasing complexity, and traditionally simple parameters such as radius length, average fluorescence intensity and cell counts are not adequate to describe the full phenotypic complexity of organoids. Second, even though the full 3D complexity of organoid development cannot be underestimated, softwares devoted to high-throughput imaging and screening are traditionally limited to 2D analysis. Therefore, quantification pipelines that are tailored to the question of interest are not readily available within the proprietary and open-source analysis pipelines.

Originally, traditional image analysis techniques have been developed to perform morphometric quantification of complex heterogeneous organoids based on bright field images (Borten et al., 2018; Bulin et al., 2017). More recently, the advent of machine learning (LeCun et al., 2015) has revolutionized the field of image and data analysis (Berg et al., 2019), and it has also been employed to perform automated segmentation of organoids in their 3D culture environment (Benning et al., 2020; Kassis et al., 2019). While such efforts have allowed the development of morphology-based screening and drug testing, when applied to the study of the self-organizing properties of organoids, they are limited in two crucial ways: first, segmentation is performed in whole-organoid structures and lacks the sufficient spatial resolution to detect single cells, which are the core components responsible for the emerging organizing properties of *in vitro* systems. Second, morphometry alone can not provide meaningful insights in the expression profile of the genes responsible for the cell behavior. To this aim, a combination of fluorescence high-content imaging and morphometry analysis is necessary.

The previous image analysis techniques have been extensively used to study *in vivo* systems. For example, using advanced cell tracking algorithms, it has been possible to visualize the trajectories and divisions of all the cells in developing zebrafish (Keller et al., 2008; Shah et al., 2019) and mouse (McDole et al., 2018) embryos. However, a similar single cell dynamics quantification approach in organoids still relies at least partially on manual annotation (Hashmi et al., 2020; He et al., 2020) (see Fig. 2a), and only recently automated approaches have been developed (Kok et al., 2020). For example, Serra et al. developed an image analysis pipeline to extract single cell as well as whole-organoid level features from time-course and time lapse experiments (Serra et al., 2019) (see Fig. 2b). The authors used a computational and data-driven approach to describe the self-organizing properties of intestinal organoids generated from single cells. More recently, Hof et al. imaged hundreds of pancreas and liver organoids using LSFM and quantified their properties across different scales, ranging from micrometer (single-cell) to macroscopic (whole-culture) features (Hof et al., 2020). These studies provided a unique view of the development of *in vitro* systems, and in the future we expect an increasing amount of similar automated, high-content image analysis pipelines to be developed.

Despite being more optically accessible compared to their *in vivo* counterparts, organoids are rather heterogeneous and opaque objects that refract, scatter and absorb visible light. Therefore, as organoids grow, they become less optically accessible, and eventually the signal-to-noise ratio (SNR) in the innermost region falls below the detectable limits of the instrument. Several computational approaches exist which help restore information content and resolution of microscopy images. These include, but are not limited to, deconvolution (Preibisch et al., 2014) and denoising algorithms (Buades et al., 2005). These have been applied, for instance, to the extraction of single cell shapes within liver tissues (Morales-Navarrete et al., 2015). However, traditional computational algorithms rely on a number of user-defined parameters, thus leading to the introduction of artifacts in the final image. By using previously annotated data, artificial neural networks are nowadays capable of learning

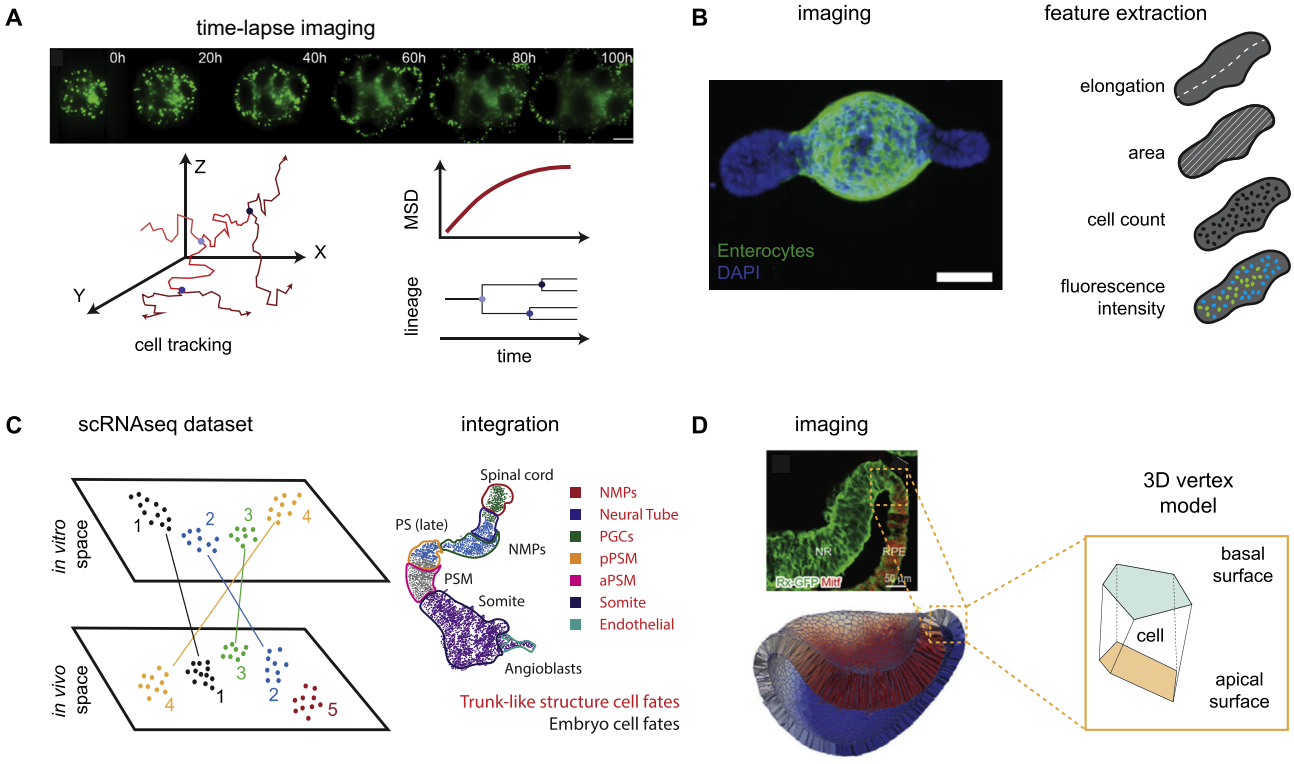

Fig. 2. Data analysis and modeling techniques to study organoids. a) 4D light sheet datasets of cerebral organoids (top) and single cell behavior analysis. MSD: mean square displacement. Image adapted from Ref (He et al., 2020). b) Single plane image of intestinal organoids used for whole-organoid and single cells feature extraction. Image adapted from Ref. (Serra et al., 2019). c) Comparison between scRNAseq datasets *in vitro* and *in vivo*. Transcriptome analysis of trunk-like structure and post-occipital E8.5 mouse embryo (right). Image adapted from Ref. (Veenvliet et al., 2020). d) Confocal image and 3D vertex model reconstruction of single cells in an optic cup organoid. Image adapted from Ref. (Okuda et al., 2018a).

complex and highly nonlinear relationships between the input image and the target data. Deep learning has been used in biology for several purposes, including neuronal segmentation in electron microscopy, image resolution enhancement in histology and classification of organoids differentiation and viability (Beier et al., 2017; Benning et al., 2020; Christiansen et al., 2018; Kegeles et al., 2020; Rivenson et al., 2017). Recently, deep learning was proposed as a tool to perform artifact-free image restoration (Weigert et al., 2018). The authors provide several strategies to generate ground truth images with high resolution and use them to train a convolutional neural network (Ronneberger et al., 2015) to enhance the SNR of fluorescence microscopy images at different spatial scales. A similar approach has been used to segment vasculatures in the mouse brain (Todorov et al., 2020). In our opinion, deep learning-based image restoration will become an essential tool in the near future to generate microscopy images of organoids with high SNR, leading to better quantification of the behavior of single cells during the development of organoids.

It is important to notice that, while embryos are less optically accessible and therefore more difficult to image and analyze compared to their *in vitro* counterparts, the visualization and quantification of single-cell behavior in their native environment has resulted in a comprehensive understanding of the dynamics that lead to the formation of different tissues within embryos. Because similar studies have not been performed yet in *in vitro* systems, we argue that the knowledge we currently have regarding the behavior of single cells within them is still limited. Therefore, we expect that studies similar to what has been done in embryos in the last decade will become increasingly popular. Recently, an image-based multivariate feature approach has been used to quantify the phenotypic landscape of intestinal organoids, as well as to infer genetic network interactions in the context of regeneration and homeostasis (Lukonin et al., 2020). Similar approaches will be important not only to provide a comprehensive mechanistic understanding of the self-organizing properties of synthetic embryos, but will also be crucial to better understand the behavior of single cells in their *in vivo* counterparts.

3.2. Transcriptomics data analysis

While optical microscopy allows the visualization of organoid development over time, only a few fluorescence proteins can be spectrally distinguished, thus limiting the number of genes for which the expression level can be visualized at a time. Recently, with the advent of single cell RNA sequencing (scRNAseq) (Tang et al., 2009), it has been possible to extract and quantify the RNA content of single cells, thus providing the full transcriptional state of every cell in a tissue. scRNAseq techniques rely on the dissociation of the tissue in single cells, which is achieved through micromanipulation (Brehm-Stecher and Johnson, 2004), flow-activated cell sorting (Julius et al., 1972), microdissection (Nichterwitz et al., 2016), microfluidics (Marcus et al., 2006) or microdroplets (Thorsen et al., 2001). This is followed by cell lysis, reverse transcription of RNA into cDNA, synthesis of complementary second strand and amplification via polymerase chain reaction (PCR) (for a review, see (Hwang et al., 2018)). Regardless of the specific technique and biological sample, scRNAseq datasets consist of a 2D gene expression matrix representing the counts of RNA per gene and per isolated cell. Similarly to the tools developed for image analysis, scRNAseq analysis softwares are either proprietary (such as 10× Genomics or Fluidigm), or open-source (for example Seurat (Stuart et al., 2019) and Scanpy (Wolf et al., 2018)). Nevertheless, every analysis pipeline includes, without exceptions, quality controls measures, data normalization and clustering in different cell types. The final results can then be used to perform more advanced analysis, including but not limited to gene regulatory network inference, lineage tracing, spatial reconstruction and data integration.

scRNAseq has been extensively used for the study of *in vitro* systems (Brazovskaja et al., 2019) such as brain organoids (Fleck et al., 2020; He et al., 2020), intestinal organoids (Serra et al., 2019) and kidney organoids (Wu et al., 2018). Importantly, scRNAseq datasets from *in vitro* and *in vivo* systems can be integrated and compared to determine how similar the two are in terms of their transcriptional state (Beccari et al., 2018). However, while these studies have elucidated many molecular mechanisms responsible for the heterogeneity of cell fates, scRNAseq *per se* does not provide spatial or temporal information of the biological process. Gene expression profiling techniques exist which allow, either experimentally or computationally, to retrieve spatial (Cang and Nie, 2020; Kruse et al., 2016; Levesque and Raj, 2013; Nitzan et al., 2019) and temporal information from static scRNAseq datasets (Farrell et al., 2018; Furchtgott et al., 2017; La Manno et al., 2018; Setty et al., 2016; Wagner et al., 2018) (reviewed in Ref (Kester and van Oudenaarden, 2018)), and are starting to be applied to embryonic organoids. For instance, TomoSeq (Kruse et al., 2016) has recently allowed to determine 1023 genes showing reproducible expression patterns in gastruloids derived from human embryonic stem cells (Moris et al., 2020). Similarly, using a technique to spatially resolve RNAseq data (Peng et al., 2016), it was possible to assign opposite halves of ETX organoids (see Table 1) to spatial domains in mouse embryos at different stages (Sozen et al., 2018). Recently, RNAvelocity has been developed to extract temporal information from static scRNAseq datasets using the ratio between spliced and unspliced RNA levels observed (La Manno et al., 2018). This technique, together with direct comparative approaches, was used on mouse trunk-like structures to demonstrate that they are most similar to the stage E8.5 mouse embryo (Veenvliet et al., 2020) (see Fig. 2c). We expect that similar techniques capable of mapping scRNAseq datasets from different experiments into their spatio-temporal context will be crucial to the study of synthetic embryology in two crucial ways. First, by using the wide range of publicly available embryonic scRNAseq data, they will help elucidate the similarities between *in vitro* systems and their *in vivo* counterparts and dissect the specific components needed to achieve a phenotype. This demands a concerted community effort to standardize data collection and annotation as well as accessible computational tools for reliable comparisons *in silico*. Second, they will be used to determine interactions between cell types that would otherwise be challenging to observe *in vivo*. For example, it has been recently shown that the embryonic stem cells and trophectoderm cells are both necessary to ensure proper cell differentiation (Rivron et al., 2018). scRNAseq in organogenesis has so far mostly been used to validate the *in vitro* model. This has allowed, for instance, to identify the best culturing conditions to generate neuronal cell types that are most similar to those found *in vivo*, thus establishing a tractable model of brain diseases (Tekin et al., 2018). However, the application of scRNAseq to synthetic embryology offers an alternative view: the identification of new, previously unseen, cell types will help elucidate the role of maternal, mechanical and chemical cues in early embryonic development as well as how different cell types can have a mutual influence on differentiation (Rivron et al., 2018). Therefore, we argue that the use of scRNAseq in embryonic organoids will revolutionize our understanding of early embryology (Marioni and Arendt, 2017).

In conclusion, we expect that the field of synthetic embryology will quickly adopt the wide variety of computational tools available for both image and scRNAseq data analysis. While most of the tools can in principle be applied directly with minor modifications, important aspects of embryonic *in vitro* systems need to be taken into account when considering specific analysis tools. For instance, automated algorithms to track single cells in a developing embryo rely on the assumption that cells move in a coordinated manner. This information is then used to correct cell tracking errors (McDole et al., 2018). It is not clear whether a similar approach could be used in amorphous and highly dynamical aggregates such as gastruloids. As another example, computational spatial reconstruction of scRNAseq data assumes that reproducible, spatially distinct domains of gene expression exist in the tissue (Nitzan et al., 2019). This assumption might not hold for organoids, which are difficult to generate in a reproducible manner. Therefore, to gain a comprehensive understanding of single cell dynamics in embryonic organoids, we will need an effort from the whole community to establish and share computational tools that are specific to the study of embryonic *in vitro* systems.

4. Mathematical modeling of organoids

To gain mechanistic insight into how *in vitro* systems self-organize, high-quality quantitative experimental data alone is not enough unless accompanied by theory and modeling to test hypothesis and make predictions (Gunawardena, 2014; Phillips, 2015; Phillips et al., 2019; Sharpe, 2017). The concept of theory in developmental biology has evolved from the work on theoretical biology in the early twentieth century (Thompson, 1917; von Bertalanffy, 1933; Waddington, 1968) to more modern views introducing concepts such as dynamical systems theory (Minelli and Pradeu, 2014). The words *theory* and *mathematical/computational modeling* are becoming increasingly popular in the developmental biology field both *in vivo* and *in vitro* (Dahl-Jensen and Grapin-Botton, 2017; Sharpe, 2017; Zheng and Sriram, 2010). Although these two words are often used interchangeably they have different meanings. A theory aims at making generalized statements to explain a variety of similar phenomena while a model usually aims at explaining a specific phenomenon (Suppe, 2000). An example drawn from physics is pattern formation, which has been extensively studied within the theoretical framework of nonlinear dynamical systems and accounts for a variety of phenomena both in inert as well as in living matter (Cross and Greenside, 2009). Yet a model explains a particular phenomenon; for instance Turing models describe developmental patterning (Gierer and Meinhardt, 1972; Turing, 1990) or Cahn-Hilliard models describe phase separation (Cahn and Hilliard, 1958; Hyman et al., 2014).

In multicellular systems, changes in form and shape of tissues are tightly coupled to the patterned expression of genes. Hence, a theoretical framework of morphogenesis must capture the interplay between pattern formation and the mechanics of tissues. There is now increasing evidence of the role of mechanics and biochemical signals in development (Aoki et al., 2017; Cho et al., 2017; Li et al., 2018; Shyer et al., 2015; Walko et al., 2017) (reviewed in Ref. (Hannezo and Heisenberg, 2019)). Mechanochemical feedback can occur at the cellular or supracellular levels. For example, in the *C. elegans* zygote, the coupling between advective transport and reaction-diffusion can determine cell polarity (Goehring et al., 2011). Another example is mechanosensation: external forces acting on a cell can deform the cell nucleus through the actomyosin cytoskeleton which in turns affects gene transcription and cell fate (Cho et al., 2017; Hannezo and Heisenberg, 2019; Li et al., 2018; Lomakin et al., 2020; Venturini et al., 2020). At the tissue level, the activity of signaling pathways can regulate cell density and actomyosin contraction, ultimately orienting collective cell migration (Aoki et al., 2017). All the previous studies have prompted theoreticians to explore the complex interplay between mechanics and patterning with the help of mathematical formulations (Glen et al., 2019; Goehring and Grill, 2013; Howard et al., 2011; Mietke et al., 2019; Recho et al., 2019). As a consequence of the rapid advances in tissue engineering, the theoretical approaches and computational tools first developed to understand developmental processes *in vivo* have now found a niche in studying simplified *in vitro* systems such as organoids and synthetic embryos (Montes-Olivas et al., 2019), where there is the possibility to better control and manipulate the experimental systems. For example, mathematical models accounting for the patterning of the germ layers during gastrulation have been studied in the context of 2D human gastruloids (Chhabra et al., 2019; Etoc et al., 2016; Martyn et al., 2019). Regarding organoid mechanics, recent examples include modeling of closed lumen-filled epithelial tissues (Mercker et al., 2016; Okuda et al., 2018b; Rozman et al., 2019), which are applicable to both optic cup (Okuda et al., 2018a) and intestinal organoids (Okuda et al., 2018b; Yang et al., 2020). Such computational studies have provided great insight on how apical, basal and lateral tensions as well as osmotic forces impact on crypt morphogenesis (Yang et al., 2020) and invagination (Okuda et al., 2018b). Expansion of such theoretical frameworks to other lumenous systems such as ETX embryos (Sozen et al., 2018) as well as non-lumenous organoids such as gastruloids (Beccari et al., 2018; Turner et al., 2017; Van den Brink et al., 2014) or brain organoids (Lancaster et al., 2013) is still missing, and we expect such descriptions to be developed in the near future.

Two main classes of models have been used to study organoids: agent-based models and continuum models (see (Dahl-Jensen and Grapin-Botton, 2017; Montes-Olivas et al., 2019; Van Liedekerke et al., 2015)). In turn, agent-based models can be classified into particle-based models (Buske et al., 2012; Karolak et al., 2019; Thalheim et al., 2018) or vertex models (Okuda et al., 2015, 2018a, 2018b) (see Fig. 2d). Agent-based models rely on a defined set of interactions between individual cells which lead to emergent phenomena at the macroscopic level. A variety of particle-based softwares exist, such as CGAL (Fabri et al., 2000), CHASTE (Mirams et al., 2013; Pitt-Francis et al., 2009), EmbryoMaker (Marin-Riera et al., 2016) or ya||a (Germann et al., 2019) among others (see list in Ref (Montes-Olivas et al., 2019)), whereas vertex models are mostly custom made (Alt et al., 2017; Collinet et al., 2015; Farhadifar et al., 2007; Okuda et al., 2018a). The main advantages of these approaches is that they model interactions at the cellular level (e.g. cell-cell adhesion, proliferation), can include heterogeneities in the cell types and can describe complex 3D morphologies. The downside of these descriptions is that they are not general and each model relies on different microscopic rules depending on the problem of choice. For example, in the case of epithelial tissues, a vertex model explicitly describes the shape of the cell and can account for mechanical differences between apical and basal sides. In contrast, particle-based models miss the cell shape information, although in some cases polarity can be associated to cells to distinguish apical and basal sides (Germann et al., 2019). This is in contrast to hydrodynamic descriptions that are generic, i.e. multiple microscopic models lead to the same continuum representations (Alt et al., 2017; Marchetti et al., 2013). These models coarse-grain the tissue over lengths larger than the typical cell size and capture a great variety of phenomena in terms of few effective parameters. This is achieved by using the formalism of non-equilibrium thermodynamics and Onsager symmetry relations in the vicinity of an equilibrium state (Jülicher et al., 2018; Marchetti et al., 2013; Torres-Sánchez et al., 2019). The main downside of the continuum approaches is the fact that the connection between the cell-level details and the macroscopic effective parameters is unknown *a priori*. Some attempts have been made to connect both scales, for example by coarse-graining vertex models and deriving continuum theories in the case of epithelial tissues (Alt et al., 2017; Hannezo et al., 2014; Murisic et al., 2015). In this case, the bulk, shear and bending moduli of epithelia have been related to active tensions along the apical, basal or lateral interfaces of the cells (Murisic et al., 2015). Although a systematic coarse-graining of detailed microscopic models might be difficult to achieve, simple microscopic descriptions can be coarse-grained and mapped to the same general continuum representation. This already provides deep analytical insights on the relationship between micro and macroscales (Marchetti et al., 2013).

We would like to emphasize that the real value of these models is to make predictions to be tested *in vitro* and not to merely obtain a mathematical description of the process. *In vitro* systems offer the possibility to test some conditions which might not be possible *in vivo*. For example, it is possible now to have great control over the geometry and the external forces applied to the system (Vianello and Lutolf, 2019). Bioprinting techniques enable control over the morphology of tissues (Mironov et al., 2009), with the possibility to generate lumen, tubular or branching structures (Brassard et al., 2021). Geometrical constraints can be easily manipulated using micropatterns in 2D (Muncie et al., 2020; Warmflash et al., 2014) or embedding organoids in 3D matrigel or PEG hydrogels (Blondel and Lutolf, 2019). The latter physical constraints are known to generate chemical and physical cues that instruct tissue self-organization (Magno et al., 2020; Muncie et al., 2020; Sagy et al., 2019). Finally, manipulating the number of cells within the *in vitro* systems allows us to understand the influence of size on the timing and patterning of biological systems when compared to their *in vitro* counterparts (Orietti et al., 2020). The previous engineering possibilities offer a myriad of

conditions that can be tested against theoretical models (Yang et al., 2020), which can be further refined to account for the experimental observations. For this reason, we propose a data-driven modeling approach, a paradigm in physics and engineering (Montáns et al., 2019) which is becoming widespread in interdisciplinary areas such as biophysics, mechanobiology or systems biology. Such approach is based on using experimental measurements and observations which motivate the development of predictive mathematical models that challenge our understanding of the system (see Fig. 3). A clear example of this approach can be found in Ref. (Okuda et al., 2018a), where quantitative measurements in optic cup organoids were used to develop a vertex model which generated *in silico* predictions that were further tested experimentally by means of inhibition assays. Understanding organoids will only be possible by complementing experimental and theoretical approaches, thus leading to a system-level understanding which will enable us to engineer multicellular systems.

5. Discussion

Despite being a very young field, synthetic embryology is advancing rapidly. A large variety of protocols allow us to mimic different aspects of early embryogenesis *in vitro*. Complex engineering devices enable the culturing of hundreds of *in vitro* embryonic systems over several days of development. New designs in optical microscopy allow us to image organoids at high throughput and across spatio-temporal scales, from the whole organoid level down to subcellular structures. scRNAseq techniques allow us to detect few transcripts in single cells with high fidelity and depth. Hence, the next step and challenge is to be able to analyze, integrate and interpret the large amount of data that is generated under a theoretical framework that can generate falsifiable hypotheses. Importantly, rather than applying these techniques in isolation, combining them complementarily holds the key to a comprehensive understanding of *in vitro* embryogenesis. For instance, in the case of intestinal organoids, it was shown that the combination of high throughput culturing systems, state-of-the-art imaging, single cell transcriptomics and theoretical modeling was crucial to dissect the underlying mechanochemical interaction responsible for the first symmetry breaking event and subsequent morphogenesis (Lukonin et al., 2020; Serra et al., 2019; Yang et al., 2020). Therefore it is likely that once these approaches are integrated and applied routinely to study *in vitro* stem cell systems, in general, we will gain a holistic understanding of the mechanisms underlying embryogenesis. Moreover, we will gain meaningful insights in disease modeling that would otherwise be impossible to obtain by studying *in vivo* systems alone. Similar approaches have already been used to characterize *in vitro* the human segmentation clock, determine pathological alterations and decipher the molecular mechanisms underlying skeletogenesis diseases in patient iPSCs (Diaz-Cuadros et al., 2020; Matsuda et al., 2020). Similarly, synthetic embryology will take advantage of the modularity of development to reconstitute and elucidate parts of the mechanisms underlying the highly complex process of embryogenesis as well as abnormalities associated with development (Shahbazi et al., 2020).

While we foresee an increasing interest in synthetic embryology, one of the biggest bottlenecks in the field to date is reproducibility (Huch et al., 2017). It is natural to expect that, under controlled experimental conditions, all samples would generate phenotypic traits in a robust manner. Importantly, in an *in vivo* context, this assumption is often met, and it is by studying the deviations from this robust patterning that we

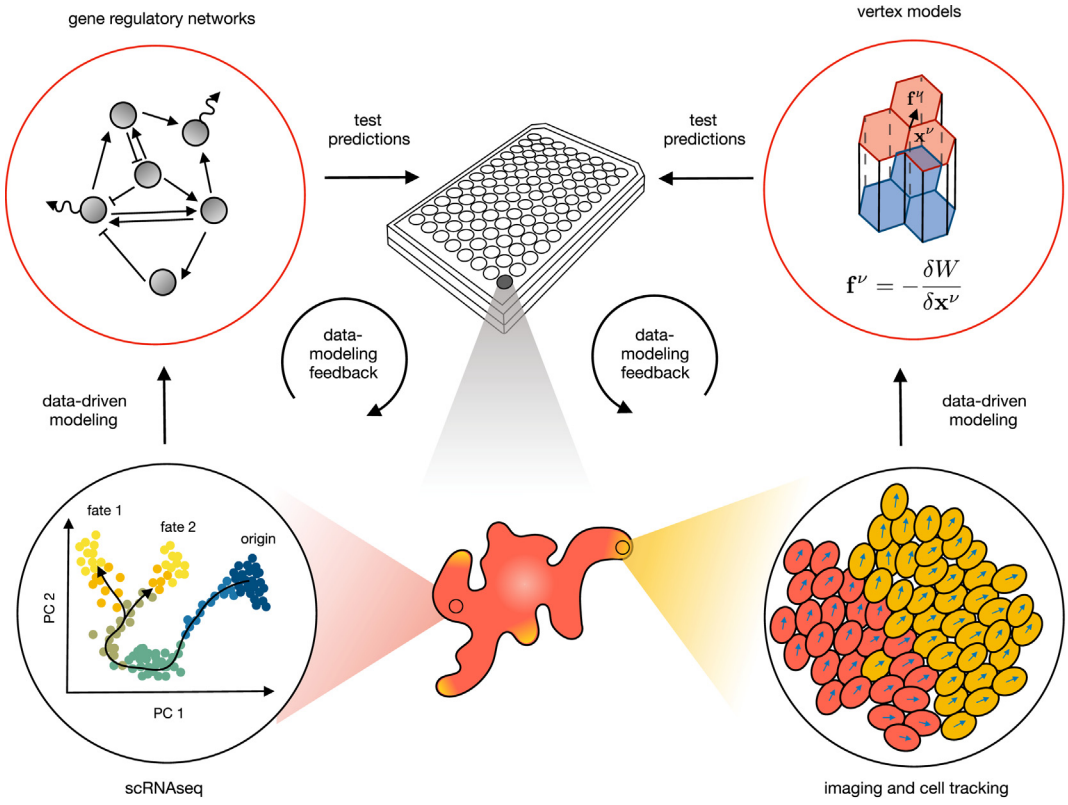

Fig. 3. A system-level understanding of organoids and synthetic embryo systems requires a data-driven modeling approach. Organoids can be cultured in large numbers by means of multiwell array systems. High-content imaging and single-cell transcriptomic technologies enable us to obtain large datasets which need to be analysed by means of image analysis pipelines or computer algorithms, respectively. Once the data is processed, computational models can be used to explain the underlying phenomena observed. Such models will generate predictions which can be further tested experimentally. Such data-modeling feedback is key to obtain a system-level understanding of organoids and synthetic embryo systems. Multiwell image adapted from MatTeK Lifesciences and the vertex model image adapted from Ref. (Alt et al., 2017).

have been able to elucidate many mechanisms underlying development, disease and regeneration. However, *in vitro* systems have been shown to exhibit batch-to-batch and sample-dependent variabilities. Therefore, it is essential to elucidate why such variability exists and how it emerges despite the controlled conditions under which organoids are generated. Stochastic variability is omnipresent in biological systems, ranging from gene expression noise at the single cell level to developmental variability of multicellular organisms (Johnston and Desplan, 2010). Collectively, stochasticity in biology is referred to as biological noise, and how biological systems cope with it is still an open question (Johnston and Desplan, 2008; Raj et al., 2010; Roorda and Williams, 1999; Vassar et al., 1993). In this context, embryonic *in vitro* systems are an ideal model to study the role of biological noise in early development and disease, and they provide a suitable framework to understand how embryonic stem cells collectively cope with noisy inputs to generate the observed emergent behaviors. In order to study biological noise, a large number of samples need to be observed to generate sufficient statistics. While, for both practical and ethical reasons, *in vivo* models are difficult to study with the necessary throughput, *in vitro* protocols are highly scalable to generate hundreds of organoids at the same time. When combined with imaging and -omics techniques and a thorough data-driven analysis, this statistical power has been instrumental in elucidating the key factors orchestrating intestinal organoid formation and regeneration (Lukonin et al., 2020). This meticulous investigation will be crucial to distinguish artificial and systematic sources of variability from biologically meaningful heterogeneity. While the former can, in principle, be limited by employing more reproducible automated and robotics systems, the latter is the object of further investigation. For instance, a set of genes were identified that showed high variability among iPSCs-derived kidney organoids (Phipson et al., 2019). Moreover, different genetic backgrounds can influence the differentiation states of PSC *in vitro*, even after resetting their epigenetic state (Ortmann et al., 2020). Understanding and isolating batch-to-batch from sample-dependent variabilities is crucial not only to answer open questions in metazoan development, but also for disease-modeling, personalized medicine and functional genomics.

There is emerging evidence that organoid and synthetic embryo systems might also offer a platform to study ecology, symbiosis or even social systems. For instance, in the embryonic context, models that combine embryonic and maternal components have been recently developed. These include models that recapitulate maternal-fetal interactions (Turco et al., 2018) or the environment of the female reproductive tract (Alzamil et al., 2020; Heidari-Khoei et al., 2020; Kessler et al., 2015). Moreover, synthetic embryonic models have shown that maternal cues might be dispensable in anterior-posterior axis elongation (Bedzhov et al., 2015) or in engineered implantation-like environments (Shao et al., 2017). In a more general context, microbe-organoid systems have been developed, for example to study Salmonella infection (Yin and Zhou, 2018), gastrointestinal microbiota (Min et al., 2020; Williamson et al., 2018), epithelial-commensal bacterial interactions (Son et al., 2020), or the anaerobic microbiome in the gut (Sasaki et al., 2020). All these studies can be used to test mathematical models (Gould et al., 2018; Labarthe et al., 2019; Takayasu et al., 2017). In the future, we envision synthetic multicellular systems as versatile tools that will help us to tackle complex problems in biomedical and ecological sciences.

The living organisms are the ultimate and uncompromising judges of our work. As Peter Dirk Nieuwkoop suggested, our understanding of the building blocks of development in isolation "*form more a camouflage for our real lack of understanding than represent a proper insight in the highly complex process of organogenesis*" (Nieuwkoop, 1969). Nevertheless, by adopting a quantitative, unbiased and reproducible *modus operandi* and by interpreting and integrating individual results, we hope that synthetic models will help us gain a system-level understanding of embryogenesis.

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Declaration of competing interest

The authors have no competing interests.

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