Thinking in 3 dimensions: philosophies of the microenvironment in organoids and organs-on-chip

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Abstract

Organoids and organs-on-a-chip are currently the two major families of 3D advanced organotypic in vitro culture systems, aimed at reconstituting miniaturized models of physiological and pathological states of human organs. Both share the tenets of the so-called "three-dimensional thinking", a Systems Physiology approach focused on recapitulating the dynamic interactions between cells and their microenvironment. We first review the arguments underlying the "paradigm shift" toward three-dimensional thinking in the in vitro culture community. Then, through a historically informed account of the technical affordances and the epistemic commitments of these two approaches, we highlight how they embody two distinct experimental cultures. We finally argue that the current systematic effort for their integration requires not only innovative "synergistic" engineering solutions, but also conceptual integration between different perspectives on biological causality.

1.Introduction

"Thinking in three dimensions", the watchword launched by Mina Bissell (Bissell, 2016; Simian & Bissell 2017), is borne out of extrapolation from in vitro culture typologies. It conveys the claim that the shift from 2D to more physiologically relevant organotypic 3D culture requires more than a plethora of powerful enabling technologies: it demands a new style of scientific thinking, a veritable "new paradigm" (Nelson & Bissell, 2005). This exquisitely epistemic claim is shared by many in the increasingly wider interdisciplinary biomedical and bioengineering community currently working at the forefront of 3D advanced in vitro organotypic systems (Chicurel, Chen & Ingber, 1998; Huang and Wikswo, 2006; Sasai, 2013a).

This "reconstructionist" or "post-reductionist" program (Wikswo et al., 2006; Wikswo, 2015) challenges the one-sided focus on the regulatory genome typical of the "network paradigm" which dominates the mainstream conceptual framework of Systems Biology (Mesarovic & Sreenath, 2005; Huang & Wikswo, 2006). Systems Biology has developed a "horizontal" understanding of biological complexity (Huang & Wikswo, 2006), in shifting the emphasis on relations rather than 'things' (Bertolaso, 2017) and focusing on "system-wide behavioral patterns" rather than on the specific gene "identities", which were the lynchpin of molecular biology

(Thacker, 2004, p. 155-157; Huang & Wikswo 2006; Green & Batterman, 2017; cfr. Griffith & Stotz, 2013, p. 40; Caianiello, 2019). Despite that, this approach is charged of missing the dynamic interactions between cells and their microenvironment and their explanatory relevance in development, regeneration and patho-physiological processes in multicellular organisms.

It has been mostly biomedical research, driven by the stringent requirements for "wet" functional tests[1] and by the need to bridge the extant gap between preclinical models and clinical outcomes (Hachey & Hughes 2018; Ingber, 2020a), to take up this integrative challenge (Stéphanou et al., 2018), by developing more physiologically realistic 3D organotypic in vitro culture technologies.

2D in vitro cell culture is the mainstream biotechnology in basic and translational biomedical research as well as in biopharmaceutical manufacturing because of its huge advantages in quantitation, standardization, replicate sampling, and high-throughput screening. In the standard 2D cell culture (**Figure** 1), cells grown on a rigid surface and bathed in cell-type specific media form monolayer at the solid-liquid interface, which is subsequently harvested and passaged in fresh culture dishes (thus breaking cell-substrate and cell-cell adhesion). However, the very environmental conditions imposed on cells to support growth and viability are quite different from the physiological tissue environment[2]. As cell spreading is limited to two dimensions, cells only establish lateral attachment, whereby apical-basal polarity is forced; surface stiffness and topography impact on adhesion and differentiation; the liquid/cell ratio, as well as oxygen concentration, are much higher than in vivo; gradients, as well as the physical and mechanical cues which only emerge in 3D setting, are not recapitulated in the in vitro condition. Even if in 2D in vitro culture cells do not retain the native cell- and cell-matrix interactions, this setting is well tuned to the investigation of regulatory processes at the cellular level, which is the mainstay of fundamental molecular biology. However, this becomes a serious shortcoming when investigating the specific cell interactions (and the related signaling dynamics) that take place at the tissue level, that is in a

[1] See Carusi et al., 2012; Weigelt, Ghajar & Bissell, 2014; Bertolaso 2016. Such a basic requirement is hardly to satisfy by mere in silico "simulation-based screening of therapeutic agents" as initially purported by Kitano himself, 2002, and soberly revised in Kitano, 2010 under the banner of "Systems Physiology".

[2] As humorously synthesized by Watson et al 2017, "cell biology using immortalized cells cultured as monolayers on stiff plastic in high glucose media is in fact studying cancerous, inbred, fat, lazy, and diabetic cells that gorge themselves on sugar once a day, don't exercise, don't sleep, and do not experience fluctuations in thyroid, stress, sex, or other hormones. They talk only to cells of like mind, live in the dark and in their own excrement, and don't bury their dead".

homogeneous cell population, or at the organ level, that is among heterogeneous populations of cells (Freshney, 2010, 10; cfr. Pamies & Hartung, 2016).

The aim of 3D organotypic systems is to overcome these shortcomings by recapitulating in vitro the third dimension (architecture, biochemical and biomechanical gradients), which has proved essential for the establishment of cell-cell and cell-matrix interactions, cellular signaling networks, unaltered gene expression patterns, cell growth, polarization, morphology, and motility, as well as for yielding reliable pharmacokinetic measures (Charwat & Egger, 2018; Skardal, Shupe & Atala, 2016; Baker & Chen, 2012; Pampaloni et al., 2007; Griffith & Swartz, 2006).

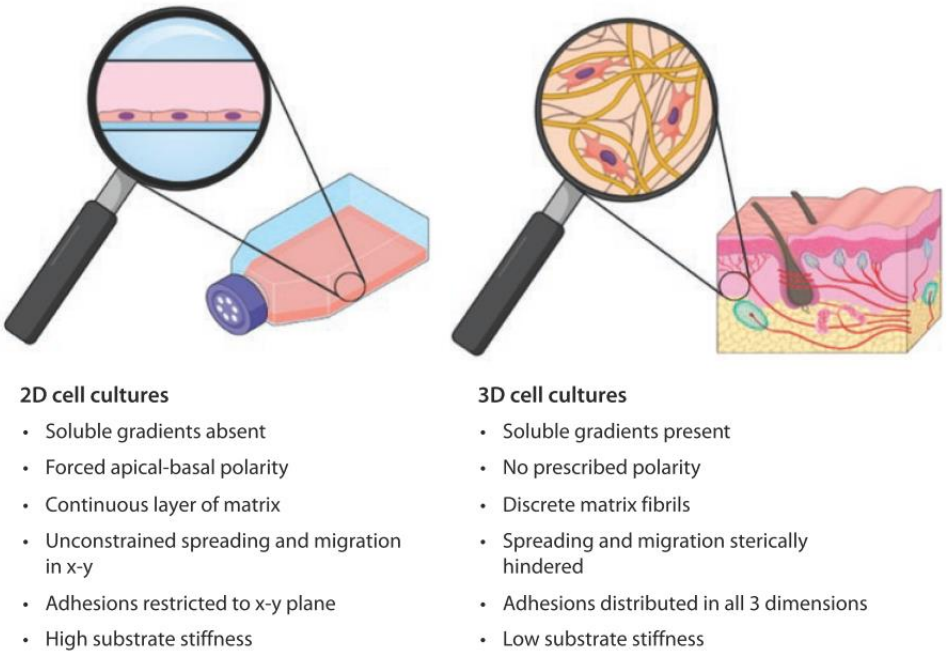

Figure 1. Schematic representation of the differences between 2D and 3D cell cultures, from Charwat and Egger, 2018.

Within the wide variety of techniques that have been recently developed in the burgeoning field of 3D organotypic culture, two major families have become established since the 2010s: organoids and organs-on-a-chip (hereinafter OoCs). However, they are so different in their approaches that it is appropriate to speak of two different "experimental cultures" (Rheinberger, 2007), which can be traced back respectively to *stem cell research* and to *tissue microengineering*. The analysis

of the major differences between these approaches allows appraising two different *philosophies of the microenvironment* at work in practice, and the respective limits that their development in the last decade has brought to the fore (see section 5).

We have chosen, as case studies, organoids and OoCs for two chief reasons. First, they are paradigmatic examples of how experimental practices are epistemically affected, in this case by different perspectives of microenvironment. Secondly, they currently represent cutting-edge technologies for biomedical engineering research in the context of regenerative and personalized medicine.

Thus, this paper seeks to address three main issues: first, how the concept of microenvironment is operationalized in organotypic technologies (i.e., organoids and OoCs); secondly, how this operationalization reveals different assumptions on biological causation; thirdly, we make the point that the current systematic effort for the integration of these two families of technologies requires not only innovative "synergistic" engineering solutions (Takebe, Zhang & Radisic Zhang, 2017), but also conceptual negotiations between different takes on biological causality. Indeed, the different views of the microenvironment that underly organoid and OoCs technologies affect not only the way they are built and their affordances but also how we model and explain the relationship between cells, tissues, and organs in patho-physiological processes.

The paper is organized as follows. Section 2 presents the historical roots and the philosophical assumptions underlying the "paradigm shift" toward three-dimensional thinking in the in vitro culture community. In Sections 3 and 4, we will distinguish two different strategies of *recapitulation*[3] of the microenvironment that inspire the two major families of organotypic cultures, organoids and OoCs. Section 5 analyzes the current efforts toward the integration of these technologies under the banner of "synergistic engineering". Finally, section 6 offers some concluding remarks concerning the philosophical questions at stake in the "synergistic" dialogue between organoids and OoCs.

2. 3D in vitro cultures: historical roots and philosophical assumptions

[3] This term is ubiquitous in the current literature of both fields and appears in its "mature", substantivized form at the dawn of the organoid literature (Sasai, 2013a) as well as of the organ chip one (Huh et al., 2010). It doesn't appear to be native to tissue engineering, as it is absent in the earlier papers of this field (Langer & Vacanti, 1993). This unreflected notion seems to cover, with an interesting ambiguity, both aspects of the older evolutionary-developmental meaning, insofar as it designates the reproduction in vitro of a physiological (or pathological) process in time, and a new one, of reconstituting the contextual conditions for that very process to occur (cfr. Griffiths & Swartz, 2006). Recapitulation seems thus to convey a basic commitment to a relational view of cell-environment dynamics.

At the beginning of the 20th century, it was soon observed that cells lose their phenotypic and functional properties in 2D environments due to the lack of relevant environmental cues, a process named "dedifferentiation" (Champy 1913). This intuition was soon after confirmed by David Thompson's experiments on in vitro chick embryos, which assessed that functional histological differentiation is under the "somatic control" of the basement membrane[4] (Thompson 1914). In 1925, the Russian-born American biologist Alexander Maximow named the reconstitution of "controlled growth" conditions "organotypic" or "organoid" culture (Maximow, 1925), thereafter shortened to "organ culture" (Fell, 1976).

The early split in tissue culture between 2D and 3D systems gave way respectively to *cell* and *organ* cultures (Caianiello 2021). The former, mostly driven by the upcoming field of virology and later by molecular biology, aimed at yielding the proliferation of large homogeneous populations of cells, marking "the transition from an organismic and cellular to a subcellular, and finally to a molecular biological knowledge regime" (Rheinberger, 2011; cfr. Freshney 2010, p. 10). The latter aimed instead at reproducing the environmental conditions fostering differentiation and focused on "the responses of organized, functional cells to environmental factors" (Fell 1972, 1976). Organ culture experiments revealed that, when dissociated cells were grown in a 3D culture setting, they did *re-differentiate*, i.e., reacquired their original histogenic capacity (Moscona and Moscona 1952).

In tissue culture, the survival of cells outside the body was dependent upon an artificial reconstitution of the natural local conditions that foster normal cell behavior in vivo, the establishment of "a new type of body in which to grow a cell" (Landecker, 2004; Caianiello, 2021). Much tissue culture research engaged in a steady refinement of the chemical knowledge of culture media, from the tinkering with natural substances to the progressive biochemical identification of their components, to the standardization of completely synthetic and chemically controlled media (Yao & Asayama, 2017). In cell culture, the dedifferentiation problem was largely overcome in the 1950s with the introduction of serum-free selective media, which, supplemented with appropriate inducers, allow to obtain differentiated phenotypes also in cell culture (Freshney, 2010, p. 8-9, 17). However, organ culture research went from the very start beyond the mere biochemistry of culture media, developing tools, specialized devices and techniques to analyze, reconstitute, and experimentally manipulate the tissue- and organ-specific 3D

[4] Basement membrane is a type of thin extracellular matrix located between epithelial tissues and the underlying connective tissue.

microenvironment for supporting stable differentiation and function, as well as to investigate "the reciprocal effect of the tissue on its environment" (Fell, 1972, p. 10).

Thus, it was mostly *organ* culture that brought about since the 1950s the revision of the Bernardian concept of *milieu interieur* that was epitomized by the new notion of *microenvironment*, or more precisely, by a multiplicity of "innumerable localized cellular or *micro-environments* (…) which are enjoyed by the various cells and tissues in the body" (Willmer, 1965, p. 11; our emphasis). Moreover, organ culture provided the experimental setting for pushing forward the knowledge about both the biochemical and biomechanical properties of the organ-specific microenvironment. It accumulated evidence of the instructive role of the extracellular matrix (hereafter ECM) on cell behavior and fate (Grobstein, 1963; Moscona, 1964; Weiss, 1968), raising early doubts about the irreversibility of the cellular specification[5] (Harrison, 1933), and fostered the development of ECM biology (Hay, 1981). Last but not least, organ culture outlined the program of an "ecological" view of the cell, supporting the notion of a dynamic interaction between cell population and microenvironment in morphogenesis (Weiss & Moscona, 1958; Gross, 1956; Drack & Wolkenhauer, 2011; see Nurse & Hayles, 2011).

This largely premolecular conceptual legacy was sidelined as the molecular explanatory level became dominant (Ingber et al., 2006), but also because of the formidable technical hindrances to the in vitro reconstitution of a physiologically realistic microenvironment (Iskratsch, Wolfenson & Sheetz, 2014; Charwat & Egger, 2018; Brinkmann et al., 2018).

Only in the last decade, the merging of cell biology with tissue microengineering, microfluidics, the nanotechnological boost in the development of sophisticated biomaterials, together with the advancement in stem cell research and cell reprogramming, are giving hope to finally overcome the shortcomings of 2D cell culture systems (van der Meer & van der Berg, 2012). A wide array of new 3D advanced organotypic cultures are currently being developed and can be implemented with human and patient-derived cells. They promise to fulfill the 3Rs[6] ethical commitment to minimize the use of animal models (Tannenbaum & Taylor

[5] This insight was set on solid experimental ground only with the advent of reprogramming research, and the findings which earned the Nobel prize to Gurdon and Yamanaka (see Laplane, 2015).

[6] 3Rs is the abbreviation for Replacing, Reducing, and Refining the use of animals in research (Porter et al. 2020).

Bennett, 2015; Ingber, 2020) and host a great potential for personalized and regenerative medicine.

Within the wide variety of techniques that have recently been developed in this burgeoning field, two major families of 3D organotypic systems have become established since the 2010s: *organoids* and *OoCs*. They share the same goal: to build *miniaturized* but *physiologically realistic models* of *organ physiology* or *disease* for basic research, drug discovery, toxicological assays and regenerative medicine[7]. Both approaches aim at exerting an epigenetic "spatiotemporal control of dynamic cellular interactions" (Sasai, 2013a). Thus, both systems strive to recapitulate organ function not only in space (3D) but also in time (4D; see Weigelt, Ghajar & Bissell, 2014; Tibbitt & Anseth, 2012).

However, they are so different in their approaches that it is appropriate to speak of two different "experimental cultures" (Rheinberger, 2007), stemming from stem cell research (organoids) and from tissue microengineering (OoCs). In our view, their major difference is in the *engineering strategy* and notably in the choice of *which microenvironmental cues* need to be experimentally controlled so as to recapitulate the complexity of organ physiology and pathophysiology (Ulieru & Doursat, 2011; see Thacker, 2004). This choice however entails different perspectives on the microenvironment, and therefore two different narratives and epistemological approaches about the cell-microenvironment interactions.

Organoid technology harnesses the capacity of stem cells (embryonic, induced pluripotent or tissue-resident progenitor cells) to self-organize into organ-like multicellular constructs, producing their own 3D niche. This outcome is obtained by means of a "multicellular engineering" process, consisting in the timed exposition of cells to the signaling and structural molecules (e.g., growth and developmental factors) that control in vivo the survival and differentiation of stem cells. This protocol allows to obtain "spatiotemporal control of dynamic cellular interactions" from bottom up, that is by providing guidance cues for steering multicellular self-organization toward the desired form and function, a strategy that has been dubbed "emergent engineering" (Kamm et al., 2018). At the operational level, the microenvironment is thus epitomized as the time-dependent set of signals that the cells receive from their niche, which is faithfully represented

[7] Both approaches belong to the framework of "Tissue Engineering 2.0" (Woodford & Zandstra, 2012), the lynchpin of regenerative medicine (Chen, 2014; Oliveira, Reis & Mano, 2015).

in terms of composition but not with respect to their specific spatial location[8]. This compositional view of the microenvironment entails an emphasis on the causal relevance of the biological signals exchanged by cells. It is their combination in "core control modules" that is held sufficient to drive morphogenesis, while the emerging properties of the microenvironment (in terms of 3D architecture, biomechanical forces and biochemical gradients) are but derivative outcomes of cells' self-organization which need not be controlled independently[9].

Organs-on-a-chip are multilayered microfluidic platforms that mimic specific organ functionalities and pathological processes by precisely manipulating the cells and their microenvironment by means of bioactive materials and microfluidic control. The strategy of control relies on engineering the precise configuration of all relevant instructive physical, mechanical, and biochemical cues in the same spatiotemporal order they encounter in the in vivo setting, in order to elicit the targeted cell behaviors. Thus, the notion of microenvironment is operationalized as the ordered, emergent configuration of *all* the relevant instructive signals that impinge upon cells in the native organ at a given time[10]. In this view, the emphasis is on the microenvironment as an emergent outcome of cellular topological organization, which is recognized and exploited as a relatively independent causal factor that affects cell behaviors.

Given the complexity of the microenvironment, the dynamics of which involves a complex orchestration of biochemical, biophysical, and mechanical cues originating at different spatial and organizational scales (Tabata & Lutolf 2017, Laplane et al. 2019), selectivity about the level of control is largely a pragmatic necessity (O'Malley et al. 2014; Sasai 2013a; Wikswo et al. 2006). Partly, the choice of the level of control between the "bottom up" strategy of organoids (i.e., from biochemical signals to tissues and organs) and the "top down" strategy[11] of OoCs (i.e., from diverse microenvironmental cues to cells) matches a difference in the

[8] This operationalization diverges significantly from the original definition of the stem cell niche by Schofield 1983, as a system that determines the stemness property of cells according to their position. This view, as highlighted by Laplane, 2015, is committed to a "strong" interpretation of the role of the microenvironment.

[9] Cfr. Sasai 2013a, who emphasizes the similarity with the logic of reprogramming research: "From recent iPS cell research, we know that a core control module that consists of only a few regulatory factors is sufficient to convert fibroblasts into pluripotent stem cells or cardiomyocytes. Likewise, mechanism-based control of tissue self-organization by a small number of "acupoints" should give us a new methodology of multicellular engineering" that is sufficient to "manipulate complex multicellular behaviors such as self-organization, and perhaps even design them for a new purpose" (Sasai, 2013a; see Davies, 2018).

[10] This approach is in fact inspired by "an integrated view of cell regulation that incorporates mechanics and structure as well as chemistry" (Chicurel, Chen & Ingber, 1998).

[11] This dichotomy is widespread in the literature, but we avoid adopting it for reasons that will become clear in Section 5.

respective processes they originally targeted: aspects of organogenesis and morphogenesis for organoids (and therefore also of developmental pathologies) and key organ physiological functions (and dysfunctions) involving a barrier or interface between interacting tissues in the case of OoCs (Takebe, Zhang & Radisic, 2017; Jackson & Lu, 2016: Brassard & Lutolf, 2019).

In the three next sections, we will introduce these different styles of "multicellular engineering", their different abstractions and assumptions about which aspects of the microenvironment are screened off in the experimental reconstitution, and highlight the practical consequences of their tradeoffs between biomimicry and control with respect to basic and translational research.

3. Organoids

Under the current definition, organoids are three-dimensional in vitro structures that self-organize from stem cells or organ-specific progenitors into a near-native microanatomy, developing organ-specific cell types and tissue compartmentalization, at a miniaturized scale spanning from micrometers to millimeters, so as to recapitulate at least some organ-specific functions (Fig. 1) (Simian & Bissell, 2017; Kretzschmar & Clevers, 2016; Fatehullah, Tan & Barker, 2016). Besides the difference in scale[12], what marks them out from OoCs is the ability to model *morphogenesis* and *organogenesis*, whereby the choice of the starting stage – whether Embryonic Stem Cells (ESC) induced Pluripotent Stem cells (iPSC), progenitor or Adult Stem Cells (ASC) – depends upon the type of organ and the targeted physiological or pathological process (Rossi, Manfrin & Lutolf, 2018).

Organoids' strategy for recapitulation is to harness the self-organizational potential of cells in vitro (Brassard & Lutolf, 2019). We will focus particularly on the theoretical framework provided by one of the pioneers of organoid technology, Yoshiki Sasai, as his reflections epitomize, in our view, the early organoid philosophy, and represent, with his emphasis on self-organization, the terminus a quo of the further developments of this field[13]. Sasai defined self-organization as

[12] From micrometers to few millimeters in organoids; from nano- to micrometers in organ chips, "the same scale as that in which living cells and tissues normally reside" (Huh et al., 2013).

[13] Yoshiki Sasai worked at the RIKEN Centre for Developmental Biology in Kobe, Japan. He discovered in 2007 the ROCK Y-27632 inhibitor that enhanced the survival of human ESC and consistently raised the efficiency of stem cell culture. He became famous for his work on the differentiation of human embryonic stem cells into some brain and eye structures, but his reputation was tarnished by the unfortunate accident of his supporting the faked results obtained by his collaborator Haruko Obokata, co-authoring two papers on *Nature* that described the stimulus-triggered acquisition of pluripotency (STAP) as a method for generating pluripotent stem cells (Obokata et al., 2014). For this reason, he

"the spontaneous formation of ordered patterns and structures from a population of elements that have no or minimal pattern" (Sasai, 2013a). In Sasai's view, complex patterning events and changes at the macroscale emerge from "relatively simple local interactions" between cells and tissues (Sasai 2013b), which elicit the contextually appropriate cell-autonomous self-organizational event (Turner, Baille-Johnson & Martinez Arias, 2015). The requirement for self-organization is therefore that pattern formation be driven by "system-autonomous" mechanisms (Rossi, Manfrin & Lutolf, 2018), while the external system provides only "permissive conditions" rather than "biased information" for patterning (Sasai, 2013a). Consistently, the essence of organoid protocol is the suppression of most external signals (Turner, Baille-Johnson & Martinez Arias, 2015)[14].

According to Sasai, the three basic processes of tissue self-organization (self-assembly, self-patterning, and self-morphogenesis) are all driven by the "internal" system according to local rules and without external cues. Unlike physical systems, the rules of interaction among the components of the system evolve in time and space, and are indirectly affected by past actions, a "stigmergy" or history-dependence that is the hallmark of the biological mode of self-organization. Even in self-driven morphogenesis, tissue mechanics is seen as "intrinsic", in so far as the agency of the process is ultimately located in the "cooperative response" of cell collectives (Sasai, 2013a). Thus, a clear border is established between the "self", the locus of activity circumscribed by the cellular capacity of integrating and processing information and of decision-making, and the contextual environment that cells shape and modify in time. As the causally relevant fraction of the microenvironment comprises only cells and their biochemical signals, the control of the microenvironmental dynamics may be safely exerted at the biochemical level. Thus, it is these signals that are the "controllable observables" (Pattee, 2000) by which to drive the morphogenetic process, while the contextual configuration of spatial organization and mechanical forces which rules their presentation to cells is logically reduced to a derivative, albeit emergent, product of cell interactions.

This original program is reflected in the two seminal "reconstitution experiments" (Sasai, 2013a) that paved the way to organoid technology, based

committed suicide in the same year. His major theoretical papers (Sasai, 2013a, 2013b) are nevertheless ubiquitously quoted as the fundamental reference for the organoid approach (cfr. Ebrhamikani & Levin, 21).

[14] See Fatehullah, Tan & Barker, 2016: "the fact that primary-tissue-derived organoids lack mesenchyme/stroma provides a reductionist approach for studying the tissue type of interest without confounding influences from the local microenvironment".

respectively on ASCs and iPSCs/ESCs. Although much water has flowed under the bridge since these two pathbreaking achievements, we will refer only to these "archetypal organoid systems" (Brassard & Lutolf, 2018) in more detail, as they represent the original organoid philosophy before the onset of the "synergistic" dialogue with OoCs technology.

The first archetypal organoid system was created by Clever's group in 2009. They derived a *mini-gut* from a single mouse LGR5+[15] adult intestinal stem cell, following the discovery that these self-renewing crypt base columnar cells were the true stem cells of the intestine that are able to generate all cell types of the epithelium[16]. They embedded single LGR5+ cells in Matrigel, a laminin-rich gel acting as a substitute for the basal lamina, and cultured them in a serum-free medium supplemented with growth factors (e.g., R-spondin 1, a WNT agonist that binds to LGR5, Epithelial Growth Factor and the BMP Noggin) recapitulating the minimal endogenous stem cell niche in vivo. This 'control module' was found sufficient for preserving stemness and the ability of intestinal stem cells to expand and generate the full complement of stem, progenitor, and differentiated cell types as in vivo (Sato et al., 2009; Sato & Clevers, 2013). Thus, mini-gut organoids recapitulated the complete differentiation hierarchy from ASCs and the self-renewing crypt-villus architecture of the gut (Sato & Clevers, 2013) without a cellular mesenchymal niche and independently of positional cues from the environment (Sato et al., 2009).

The second archetypal "reconstitution experiment" was the *optic-cup* morphogenesis from mouse and human embryonic stem cells (ESCs) in 3D culture by the Sasai group (Fig. 2) (Eiraku et al., 2011; Eiraku & Sasai, 2012). Its outspoken goal was to fill the gap in the knowledge on the molecular mechanisms of cellular differentiation and the "control of complex 3D shape and pattern" (Nakano et al., 2012). It was on the basis of this remarkable example of "structural self-organization at the multicellular level" that Sasai systematized the early organoid philosophy (Sasai, 2013a, b).

As in most organoids derived from ESCs and iPSCs, the protocol required firstly the aggregation of embryoid bodies[17] from dissociated stem cells. To promote tissue formation in 3D, they employed a three-dimensional aggregation culture

[15] LGR5+ is the abbreviation for Leucine-rich repeat-containing G-protein coupled receptor 5. This protein is a member of the Wnt signaling pathway and is expressed by adult stem cells at the base of the intestinal crypt.

[16] Many years of research on the signaling cascades involved in organogenesis were necessary (see Bjerknes & Cheng, 2006; Lancaster & Huch, 2019), in order to provide a detailed molecular description of the stem cell niche and overcome the historical difficulty to expand in vitro ASCs beyond the Hayflick limit (Hayflick & Moorhead, 1961).

[17] Embryoid bodies are three-dimensional aggregates of pluripotent stem cells.

(SFEBq culture[18]). The medium was supplemented with a minimal amount of extrinsic growth factors and low concentrations of dissolved Matrigel serving as ECM substitute[19]. After some days, the neuroepithelium gave rise to optic vesicle-like structures forming a bilayered optic-cup-like structure, the inner side differentiating into Neural Retina (NR) and the outer side in Retinal Pigment Epithelium (RPE). NR formed a fully stratified architecture as in the postnatal eye in extended culture with a culture medium promoting retinal maturation (Lancaster & Huch, 2019 ; Llonch, Carido & Ader, 2018).

Whereas in vivo eye-cup morphogenesis is driven by the reciprocal embryonic induction between the optic vesicle and the surface ectoderm, optic cup organoids do not require the action of the surface ectoderm (Furhmann, 2010). In optic cup organoids, structural self-organization is triggered by sequential changes in the mechanical properties of the differentiating domains, reducing contractility and rigidity in the NR, while RPE maintains high levels of both. At a later stage, cellular proliferation generates the pushing force that makes the more flexible NR bend inwards within the more rigid RPE (Rossi, Manfrin & Lutolf, 2018). This allowed Sasai to state that physical forces are generated *internally* by "domain- and phase-specific changes in tissue properties" (Eiraku, Adachi & Sasai, 2012).

In its current deployment, however, organoid technology is perfectly equipped also to provide the local system with non-endogenous and not self-generated signals. The core of Sasai's original philosophical assumption about structural complexity emerging from "relatively simple local interactions" holds just the same even if such interactions are not all "programmed internally" (Sasai, 2013a and b). Depending on the organ and the source of stem cells employed, current research exploits a continuum from overwhelmingly intrinsic to extrinsic factors, ranging from ESC or iPSC, to progenitor cells, ASCs and finally co-culturing protocols (Rossi, Manfrin & Lutolf, 2018). The technical requirement is that these signals, regardless of their origin in the organism, be presented at the relevant *time points* in order to *act locally*. In this sense, the emphasis on endogenous self-organization, far from being necessary, may even misrepresent the actual heuristic power of organoid technology.

One of the major affordances of this technology is the possibility of experimentally dissecting the complex cross-talk between signaling networks, pinning down the individual contribution of specific genes and key signaling

[18] SFEBq stands for serum-free culture of embryoid body-like aggregates with quick aggregation. SFEBq allows for the growth of a species-specific number of ESCs in 96-well plate.

[19] The medium was also designed to facilitate selective neural differentiation toward the default rostral forebrain fate.

pathways in development and their dysregulation in disease (Fatehullah, Tan & Barler, 2016). Genome, transcriptome, and proteome profiling[20] make a powerful platform for 'exploratory experimentation' (Burian, 2007) about causal genetic and epigenetic changes involved in disease progression and drug resistance, enabling the identification of new biomarkers (Green, Dam & Svendsen, 2021; Tiscornia, Lorenzo Vivas & Izpisúa Belmonte, 2011).

The *technical* limits of organoids are by now well documented and depend mostly upon the aspects of microenvironmental complexity that organoid philosophy chooses to screen off (Hofer & Lutolf, 2021). The lack of vascularization limits their growth, as the inside becomes necrotic when tissues exceed 100 microns. The lack of fluid flow precludes also the recapitulation of cells interactions with immune cells (Kim & Ingber, 2013; Bhatia & Ingber, 2014; Shin et al., 2020). Organoids do not allow modeling cell-stroma interaction, unless in co-culture protocols. Niche factors are diffused in a uniform culture environment (Brassard & Lutolf, 2019), not reflecting the spatial organization of signaling centers that rules their presentation to cells in vivo (Tabata and Lutolf, 2017; Rossi, Manfrin & Lutolf, 2018). Thus, although morphogens and cell-secreted soluble factors do self-organize in biochemical gradients, this uncontrolled spontaneous process is *noisy* and *highly variable*, and often fails to simulate the graded distribution essential for tissue patterning during organogenesis in vivo (Park, Georgescu & Huh, 2019; Shin et al., 2020)[21].

So, organoids have no tools to represent the multiscale "mechanochemical coupling and feedback" (Sasai, 2013a) between cells and their microenvironment. This is particularly evident in the use of ECM-substitute, limited to providing minimal enabling conditions for fostering organoid 3D self-organization[22].

[20] These technologies are enhanced by genome and even epigenome editing tools (Drost & Clevers, 2018; Smith & Tabar, 2019; Caporale & Testa, 2019) and by comparative studies of healthy and disease organoids.

[21] On the contrary, providing "biased" local mechanical microenvironmental cues, such as fluid-flow and peristalsis-like deformations, as allowed by a gut-on-a-chip setting (see §3.2), has yielded a more complete functional and morphological differentiation of villi-like structures (Kim & Ingber, 2013).

[22] The ECM is a major component of the bioactive environment and has an important regulatory role. It binds and integrates different signals, such as growth factors together with adhesion molecules. The ECM can store growth factors, covering also functions of memory storage that may prove crucial in perpetuating disease or restoring normal tissue behaviors (DuFort, Paszek & Weaver, 2011; Bhat & Bissell, 2014). Moreover, the ECM selectively presents growth factors to cells in a spatially patterned and regulated fashion, as "organized solid-phase ligands", establishing gradients of morphogenes (Hynes, 2009; Ingber, 2013). Most importantly, the ECM is a "physical information-bearing structure" (Levin, 2012) and its structural self-assembly process makes of it more than the collective product of the cells it embeds (Mouw, Ou & Weaver, 2014). The informational role of the ECM in conveying to cells a picture of their surrounding environment is continuously updated by its tightly regulated remodeling dynamics, driven by matrix metalloproteinases activation, which is "controlled at multiple levels from transcriptional to posttranslational regulation" (Lu et al., 2011).

Unsurprisingly, most organoid studies do *not* address how the ECM modulates tissue formation over time, nor the role of factors expressed by the developing organoid itself to promote its own organization (Ader & Tanaka, 2014)[23]. Furthermore, the lack of control over the mechano-structural cues of the microenvironment does not allow organoids to model and control biological interactions at higher levels of organization (Park, Georgescu & Huh, 2019). Thus, a major shortcoming of the choice to control self-organization through the timed exposure to "core control modules" is that the causal action played by the topological structure and mechanical properties of the ECM is not recapitulated.

As Lancaster aptly summarized, self-organization is both the strength and the weakness of organoid technology (Clevers et al., 2017). On the one hand, organoids do achieve a remarkable level of structural complexity, approximate the cellular heterogeneity of native organs, and represent the different functional tissues of early organogenesis. On the other, the lack of maturation and correct spatial segregation at the macroscale (Marti-Figueroa & Ashton, 2017), off-target differentiation and variability hamper experimental standardization. Thus, the stochasticity inherent to self-organizing processes takes the upper hand without the spatiotemporal control of complex multiscale biochemical and biophysical interactions that dynamically orchestrate morphogenesis in vivo (Marti-Figueroa & Ashton, 2017).

The cumulative result of these shortcomings is that organoidgenesis not always matches organogenesis. The risk that organoids may go their own way of development, no longer mirroring the in vivo one, is increasingly recognized as a major challenge. Although brain, intestinal, and even tumor organoids provide some evidence that they recapitulate the genomic, transcriptomic and epigenomic profile of the tissue of origin, this success is partly challenged by further evidence that the mutational landscape tends to diverge in time (Broutier et al., 2017) and that in vitro specimens exhibit spurious methylation patterns (Di Lullo & Kriegstein, 2017; see Caporale & Testa, 2019).

In so far, the ECM is an example of the integrative and regulatory role of "biodynamic interfaces" as defined by Arora, Giuliani & Curtin, 2020.

[23] In vivo, in fact, mechanical forces act in concert with soluble morphogens and ECM signals to coordinate organ development and maturation (Park, Georgescu & Huh, 2019).

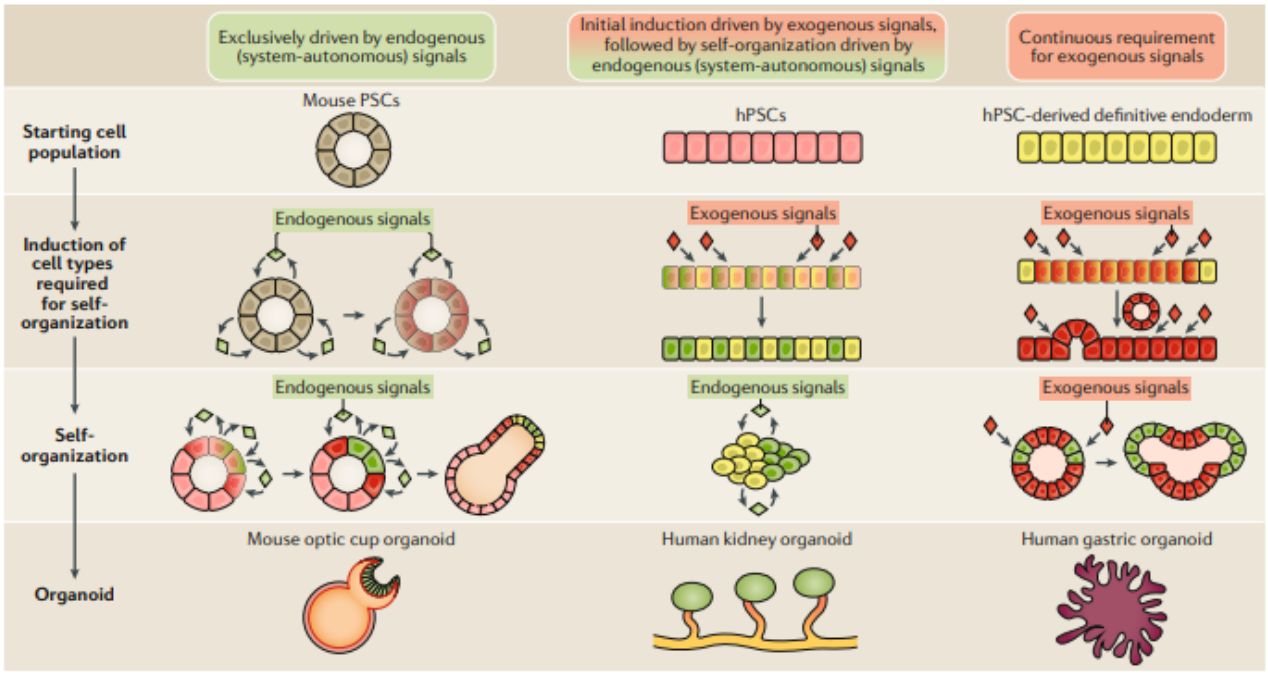

Figure 2 The development of organoids through the self-organization process of stem cells induced by endogenous or exogenous signals (Rossi et al., 2018).

4.Organs-on-a-chip

The breakthrough of microfluidic OoCs was almost contemporary to the first 'modern' organoids (Huh et al., 2010), although its concept traced back to the late 1990s. Like organoids, OoCs aim at recapitulating "complex structures and function of living human organs" in 3D (Ingber, 2013), but the strategy to attain this goal is almost the reverse than the one pursued by organoid research. Whereas in organoids the epigenetic control on morphogenesis is exerted by a chemical cell niche (i.e., biological signals) engineering, the concept underlying OoCs fabrication is to gain and exert active control on cellular and intracellular processes by imposing *external constraints* on cells. As Wikswo et al. (2006) emphasize, the core engineering concept is to open the internal cellular feedback loop and replace it with external control methods inspired by control theory. Thus, rather than providing cells with signals that steer their intrinsic self-organizational potential, the OoCs strategy is to dynamically modulate all instructive physical, mechanical, and biochemical cues that are needed to elicit the targeted cellular response and behavior (Ingber, 2016; Huh et al., 2013).

OoCs technology stems from the merging of tissue engineering with microelectronics, microfabrication, and microfluidics (Perestrelo et al., 2015). These small devices, reaching the size of a computer memory stick, couple biological processes to in silico models. They represent an evolution of BioMEMS[24] and BioNEMS[25], as the microfabrication techniques developed for silicon chips are adapted to build microfluidic microbioreactors with flexible and optically clear elastomeric biomaterials such as PDMS (poly-dimethylsiloxane). Microfabricated microchannels of less than 1 mm allow BioMEMS to direct fluids "as microchip[s] move electrons" (Eisenstein, 2015; cf. Perestrelo et al., 2015; Thorsen et al., 2002), exploiting the peculiar properties of laminar flow at the microscale (De Ninno et al., 2010).

The idea of an "animal on a chip" was implemented with a microscale "cell culture analog, a direct physical replica (…) of a mathematical model that divides the body in compartments usually corresponding to organs" (Shuler et al., 1996). This pioneering work by Shuler and coworkers first demonstrated the possibilities of integrating multiple microscale cultures with microfluidic systems, thereby controlling transport rates and quantities between organ compartments, which could be made more realistic by including organ-specific microenvironmental cues (Moraes et al. 2013).

OoCs are "synthetic culture systems" (Bhatia & Ingber, 2014) based on "reverse-engineering human pathophysiology" (Ingber, 2016), a reductionist analysis that abstracts from the "exceedingly complex in vivo counterparts" (Hayward, Kouthouridis & Zhang, 2021). The basic requirement for this task is to gain active and dynamic control of all the instructive microenvironmental cues involved in function, by recapitulating the interface between a parenchymal and a vascular tissue, cell-cell, and cell-matrix interactions, and precisely tailoring the organ-specific physical, biochemical and mechanical microenvironment.

Tissue-tissue interfaces are usually reconstituted by lining respectively vascularized endothelial and epithelial or other functionally relevant cells[26] on two

[24] BioMEMS is the abbreviation for biological microelectromechanical systems (see Voldman, 2003; Wikswo et al., 2006). They were first applied in the 1990s to the construction of miniaturized analysis systems. These Labs-on-a-chip (LOC), or Micro Total Analysis Systems (μTAS), thanks to the integration of in-line electrical, chemical, mechanical and optical microsensors as well as micromechanical actuators, are able to perform several high-throughput analytical assays at the biochemical, genetic and metabolic level in parallel and with minimal consumption of reagents (van den Berg & Lammerink, 1997; Reyes et al., 2002; Mark et al., 2010).

[25] BioNEMS stands for biological nano-electrical-mechanical systems.

[26] Usually established cell lines or primary cells, although much work is in progress on stem cells, which exploits the physical and mechanical control of the stem cell niche to drive cell fate and maturation. In the case of interfaces such as the Blood-Brain-Barrier, the relevant cells are usually astrocytes. As an example of alternative technical approaches to tissue-tissue interface recapitulation, see Prabhakarpandian et al., 2013.

microfabricated channels within a single multilayered microchip[27] (Fig. 3). Nano- and microfabrication technologies tailor channel topography at the same scale of cells and tissues, thereby controlling the spatial-temporal positioning of cells as well as recapitulating complex surface patterns that may induce cells to mimic tissue-specific microarchitecture (Huh et al., 2013; Huh, Hamilton & Ingber, 2011; De Ninno et al., 2010; Nawroth et al., 2018).

Both the biochemical and biomechanical properties of microenvironment can be tightly controlled in space and in time by leveraging on the properties of laminar flow at the scale of microchannels. These devices can precisely recapitulate the *spatio-temporal* structure of the biochemical environment by generating complex concentration gradients of chemicals and molecules (cell metabolites, small molecules, growth factors, chemokines, nutrients, oxygen). All these parameters can be independently varied to mimic both physiological and pathophysiological processes of tissues and organs. These steps of accurate microenvironmental sculpting, together with tightly controlled fluid flow, recreate in vitro the physiological conditions for extended cell viability and stable functional differentiation (Markov et al., 2012).

However, the major affordance of OoCs is that they are "*dynamic* microphysiological systems" (Ingber, 2020) that actively engineer organ-level biomechanical stimuli: fluid shear stress through the alteration of fluid flow rates; organ-relevant motions such as breathing and peristalsis through the application of cyclic suction to lateral hollow channels affixed at each side of the culture channels; organ compression through the increase in pressure in the air chambers. Further methods also include the local imposition of mechanical forces (Leduc, Messner & Wikswo, 2011) and electrical fields (Batia & Ingber, 2014; Ting & Sniadecki, 2011; Pavesi et al., 2016). It is this capacity that allows them to represent the third dimension. All such cues and culture parameters (e.g., biochemical gradients, mechanical forces, delivery of drugs or toxins and even of cell types) can be actively modified independently as well as in combination through, for example, the simultaneous exposure of cells to different mechanical forces such as shear stress, cyclic strain, electric and mechanical stimuli (see Sidorov et al., 2017). As such, OoCs play an important role in drug screening and toxicological assays, insofar as they may deliver drugs, toxins, and soluble cues independently in each

[27] Such a microchip consists of elastomeric, optically transparent biomaterial such as PDMS, which is compatible with live-cell microscopy and high-throughput screening.

channel with high spatio-temporal precision and assess their effect on cell behavior (Jackson & Lu, 2016; Huh et al., 2013[28]).

The first application of OoCs technology was the *lung-on-a-chip* (Huh et al. 2010) that recapitulates the alveolar-capillary interface by means of two microfluidic channels separated by a flexible ECM coated porous membrane (Huh et al., 2010) (Fig. 3). From one side of the membrane, alveolar epithelial cells are cultured at an air-liquid interface, while on the other side, capillary endothelial cells are perfused by a culture medium mimicking the vasculature hemodynamics. The organ-level function of the lung – breathing – was reconstituted by applying cyclic suction to the affixed hollow side channels, deforming rhythmically the central membrane. Since 2010, several other OoC models (e.g., eye, skin, heart) have been developed to evaluate the efficacy and safety of some drugs in different organs (Sontheimer-Phelps, Hassell & Ingber, 2019; Ingber, 2020; Zhang et al., 2018).

The lung-on-a-chip system has been employed for testing the organ-level response of human lungs to toxic nanoparticles, as well as to bacteria and cytokines. It demonstrated for the first time that nanoparticles introduced in the air channel penetrate the epithelial-endothelial barrier only when mechanical stress is applied, but not when the culture is kept under static conditions (Huh et al., 2010; van der Meer and van den Berg, 2012), unveiling unknown mechanosensitive responses to nanoparticulates[29]. Lung-on-a-chip could visualize in real time with high-resolution microscopic imaging the immune response to Escherichia coli infection, showing how quiescent circulating neutrophils activate in response to bacterial presence and transmigrate into the alveolar channel. Furthermore, the lung-on-a-chip has been employed to get insight into tumor cell migration and invasion by introducing a low number of NSLCL (non-small-cell lung cancer) cells in the epithelial-lined channel. The model shows how breathing motions suppress cancer cell response to tyrosine kinase inhibitor therapy in the aerated parts of the lung, providing a rationale for the difficulty of completely eradicating the disease after effective regression of larger tumors (Hassell et al., 2017).

In the same vein, the development of *bodies-on-chips* has enriched the understanding of the interactions among multiple organs through fluidic channels mimicking circulating blood flow into a series of single OoCs. Many bioengineered "homunculi" are being developed as testing platforms for drug pharmacokinetics

[28] Cfr. De Ninno et al., 2013: "we can, in principle, talk even with single cells".

[29] See also the "smoking-lung-on-a-chip" by Benam et al., 2016.

and have already proved their capacity to detect unsuspected systemic effects of drugs and chemicals (Perestrelo et al., 2015; Skardal, Shupe & Atala, 2016; Zhang et al., 2018). Bodies-on-chips have also been employed as heuristic models for human pathophysiology, as in the recent multiorgan tumor model for the study of metastasis (Sung, Wang & Shuler, 2019; Sontheimer-Phelps, Hassell & Ingber, 2019) and for the modeling of host-microbiome interaction (Hawkins et al., 2020; see Kim et al, 2017).

The limitations of the "modular abstraction" (Hayward, Kouthouridis & Zhang, 2021) underlying OoCs are manifold: poor cellular heterogeneity, low cell number, the insufficiency of size for realistically reproducing 3D tissue organization and for studying the effects of large-scale forces on large organs, among others[30]. This class of shortcomings is partly compensated by the extraordinary manipulability that characterizes these systems, which makes it extremely easy to selectively add cell types or soluble factors[31] until one identifies "the correct combination of cells necessary to achieve the functionality of interest" (Huh et al., 2013).

However, the major hindrance to the recapitulation of macroscopic organs patho-physiology in microscale constructs on chip is the problem of scaling, both with respect to physiological parameters such as metabolic rate and in the respective size of cells, tissues, and organs. This problem is exacerbated in multiple OoC systems, in which allometric scaling of organ size does not allow to scale function efficiently (Kamm et al., 2018; Wikswo et al., 2013; Zhang & Radisic, 2018). Thus, there is currently no hope for OoCs to reach the biomimetic realism of organoids.

Nonetheless, OoCs offer several advantages, which are all related to the choice to exploit the microenvironment and its emergent behavior to induce cell function. They can dynamically elicit and precisely measure the cellular response to diverse stimuli stemming from different scales – from the single cell to the tissue, from organ to systemic cues and, inversely, to disentangle the specific contribution of cellular, molecular, chemical and physical cues to tissue and organ patho-physiology (Bhatia & Ingber, 2014). They can recapitulate functionally relevant biological interactions at higher levels of organization and measure their effects at

[30] Other problems are related to the biocompatibility of the materials employed, currently addressed by innovative biomaterial research, to the difficulty to collect cell samples from chips (Regmi et al., 2022), as well as to the technical difficulty to connect these tiny devices with large scale external devices. A lesser problem concerns the high technical complexity of these devices, as the number of ready-made commercialized chips, endowed with user-friendly automated instruments for culture and fluidic control, is steadily increasing (Ingber, 2020; Sontheimer-Phelps, Hassell & Ingber, 2019; Takebe, Zhang & Radisic, 2017; Zhang & Radisic, 2017; Sbrana & Ahluwalia, 2012).

[31] See Ingber, 2020a: "Circulating or resident immune cells, connective tissue cells, nerve cells, and other cell types can be integrated into the Organ Chips as needed to recapitulate increasing levels of complexity".

the molecular scale, thereby capturing the context dependency of molecular processes ("molecular semantics", Laubichler & Wagner, 2001). Their ability to assess the response to drugs at different scales might help overcome the lingering reductionist bias in pharmacological research (Wikswo, 2014; Norris et al., 2017; Geerts et al., 2020).

In brief, we think that what makes of OoCs a "novel form of mechanistic human experimentation in vitro" (Ingber, 2020) is their affordance to work as *multiscale models* and as powerful tools to integrate "finer scale processes with higher levels of organization at which emergent processes occur" (Mao & Green, 2017).

It is telling that several researchers in the OoCs community support the view that a precise set of rules governing cell-microenvironment communication can be identified. These rules would make up a veritable "morphogenetic language or code" that is so accurate that its decryption allows researchers to draw "maps between environmental conditions and the cellular phenotype" (Warrick, Murphy & Beebe, 2008; Rozario and De Simone, 2010; De Ninno et al., 2010). Such a view sets a larger share of cells' "decision-making" as dependent on the highly ordered nature of the instructive cellular and noncellular microenvironment. It lightens the computational burden of the genomic networks of the cell placing more emphasis on the emerging informational content of its niche**,** in line with the claim that there is no complete algorithmic logic of a program in the DNA sequence (Noble, 2015; see Huang & Wikswo 2006). In fact, such a "code" would greatly simplify cells' work of integrating and interpreting correctly the multiple chemical, adhesive and mechanical inputs simultaneously and make the appropriate developmental decisions.

Such higher-level code can be seen as an instantiation of the "language of form" (Nelson & Bissell, 2006), in so far as it brings to the fore the informational relevance of the niche mesoscopic organization. This "macrostate" (Levin, 2020) becomes "cause in the matter" (Strohman, 2000): cells would sense the specific and time-dependent constellation of all the diverse signals in their precise spatial order and architectural organization as "a whole" (Warwick, Murphy & Beebe, 2008), while their response would be limited to switching "on one of a limited number of specific and reproducible phenotypic responses (e.g., growth or differentiation or apoptosis)" (Ingber, 2006; Ingber & Levin, 2007).

The notion of morphogenetic code, whatever the relevant description of the informational macrostate impacting on cell decisions – biophysical, as in the bio-

electric code (Levin & Martyunik, 2018), biomechanical or mechanochemical – entails the philosophical move of "expanding the understanding of causation" from the molecular level to emergent "causally effective physiological/biophysical spatially distributed states" (Levin, 2020). The consequences of this move would not be simply philosophical: their practical and therapeutic application involves devising strategies of higher-level control in the clinic (i.e., cancer treatment), in regenerative medicine and synthetic biology, which would not need meshing directly into the complexity of the cellular machinery (van der Meer & van der Berg, 2012; Pezzulo & Levin 2016).

A proof-of-principle of the possibility to exploit higher-level control in medical applications seems to be the "reprogramming" of the macrostate (i.e., bioelectric gradients) to normalize cancer cells (Chernet & Levin 2013; Chernet et al., 2014; Levin, 2021[32]). Lately, many bioengineered models of the tumor microenvironment are actively investigating the microenvironmental regulation of cancer dormancy (Pradhan et al., 2018; Rao, Kondapaneni & Narkhede, 2019), and OoC models are already providing experimental support to the role of mechanical cues in tumor growth and metastasis as well as in chemoresistance (Skardal et al. 16; Hassell et al., 17; Nashimoto et al., 2020).

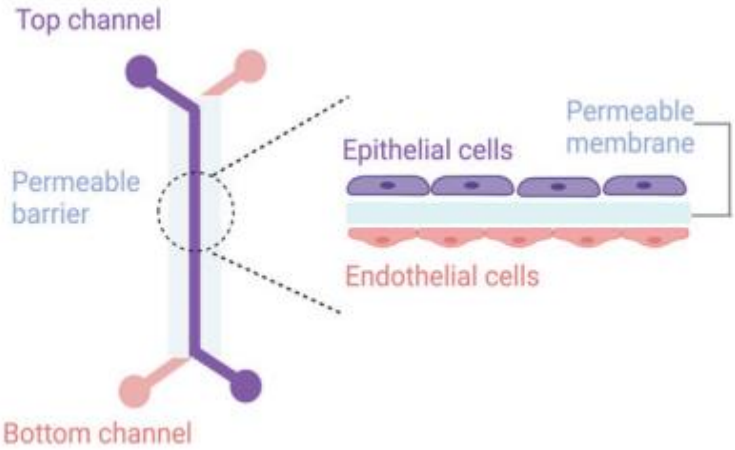

Figure 1 Schematic model of organ-on-a-chip: the chip consists of two channels. The upper level contains epithelial cells, whereas the lower one endothelial cells. The two channels are separated by a permeable membrane (Parvatam and Chavali 2022).

[32] Chernet and Levin, 2013 also argue that the cell-level view, focused on cell cycle checkpoints and TGF-β molecules, predicts that cancer and regenerative potential be coupled, while morphogenetic field models, which conceive of cancer as a failure to transmit or receive anatomical cues, suggest an inverse relationship between regeneration and cancer.

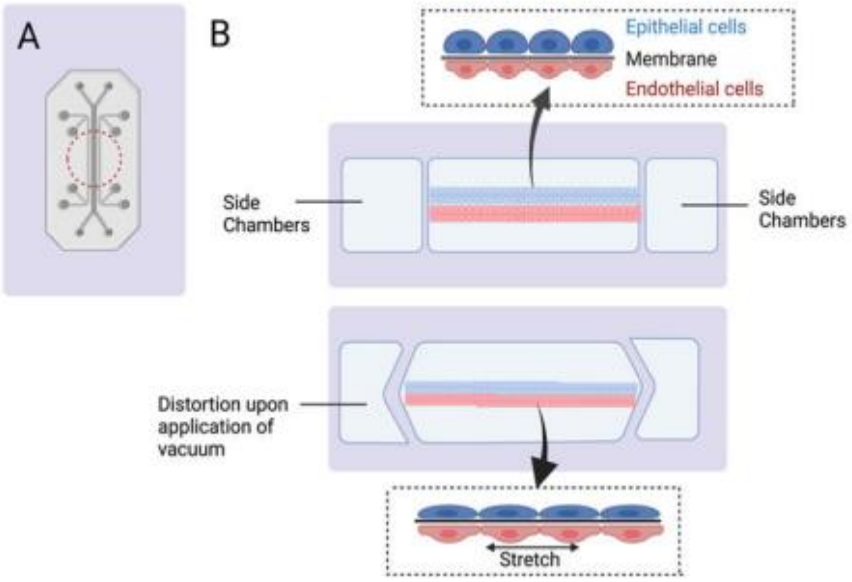

Figure 2 A lung-on-a-chip: the chamber consisting of epithelial and endothelial cells (at the center) is surrounded by two side chambers undergoing distortion after the application of vacuum. As a result, the middle chamber can stretch, mimicking physiological breathing (Parvatam and Chavali 2022).

5. Synergistic Engineering: the new frontier

In the previous two sections, we have examined the history, the experimental setups, and the different strategies of selective recapitulation of the microenvironment of organoids and OoCs. These two technologies, both harnessing the self-organizing abilities of cells for obtaining high-fidelity 3D miniaturized organs, represent a promising route for advancing basic research on organ pathophysiology, drug discovery, and regenerative medicine, including the engineering of transplantable organs.

The main strengths of organoids are the unprecedented level of structural and functional complexity they achieve, the possibility to recapitulate developmental pathologies, their affordances to experimentally dissect the complex cross-talk between signaling networks, to pin down the individual contribution of specific genes and key signaling pathways in development and their dysregulation in disease (Fatehullah, Tan & Barler, 2016), and to identify new biomarkers. They also unveil mechanisms of tissue-autonomous self-organization in homeostasis, regeneration and development (Rossi et al. 2018). The strength of OoCs is in their ability to disentangle the respective contribution on organ pathophysiology of diverse stimuli stemming from different scales by dynamically controlling in space

as well as in time all instructive physical, mechanical, and biochemical cues in the relevant microenvironment (Ingber, 2016; Huh et al., 2013). Therefore, they are better poised to address experimentally the feedback on gene expression elicited by higher-level organizational entities, processes, and forces, and to support the challenge of multiscale modeling.

The main shortcoming of organoids lies in the danger that *organoidgenesis* does not match *organogenesis* because of the stochasticity of self-organization, which makes their mutational landscape and epigenetic profiles diverge in time. Most of the acknowledged limits of organoids are due to the lack of dynamic control on their biochemical, biophysical, and biomechanical microenvironment: lack of maturation, absence of correct spatial segregation at the macroscale (Marti-Figueroa & Ashton, 2017), off-target differentiation, and variability, which hamper experimental standardization.

Likewise, OoCs have major limitations: poor cellular heterogeneity, low cell number, the insufficiency of size for reproducing 3D tissue organization and for studying the effects of large-scale forces on large organs. Notwithstanding the unprecedented level of microenvironmental tailoring and control that they enable, they are extremely simplified models of the in vivo counterpart (Hayward et al. 2021). Finally, issues of size scaling hinder their possibility to attain a level of biomimicry comparable to organoids, and even their affordances in terms of control and monitoring of cellular behavior are not easily scalable at higher sizes.

	ORGANOIDS	**ORGANS-ON-A-CHIP**
CONSTITUTIVE MATERIAL	Stem cells	Microfluidic systems
CONTROL OF SELF-ORGANIZATION (controllable observables)	*Inside-out:* timed exposure to growth factors Targeted dimension: TIMING	*Outside-in:* biochemical gradients; biodynamic interfaces; Tissue-microarchitecture and topologies Targeted dimension: TOPOLOGY
BIOLOGICAL PROCESSES TO BE MIMICKED	Organogenesis Organ patho-physiology and its development	Organ Patho-physiology
MAIN ANALYTICAL STRENGHTS	Dissecting the cross-talk between signaling pathways; identification of new biomarkers	Disentangling the respective contribution of different kinds of stimuli to organ patho-physiology
MAIN SHORTCOMINGS	Stochasticity of self-organization; lack of control on microenvironment	Poor cellular heterogeneity; low cell number; excessive simplifications; size scaling issues

Table 1. *Comparative table of the main features of organoids and organs-on-a-chip*

Both Organoids and Organ on Chip leverage on self-organizational principles in their experimental set-up to steer multicellular processes. More than in terms of bottom-up vs top-down, the difference in their strategy is in our view better framed as inside-out (organoids) vs outside-in (OoCs). While organoids are focused on providing cells with (more or less intrinsic) signals that enable the targeted behavior within their self-organizational potential, OoCs are focused on gaining external control of the internal cellular feedback loop, i.e. externalizing control (see **Table 1**).

The practical limits of both technologies that – to some extent – embody their respective philosophies of the microenvironment are increasingly perceived and have recently elicited a massive effort for their integration (Takebe, Zhang & Radisic, 2017).

To overcome the respective shortcomings and merge the strengths of these two technologies, research has moved in fact toward "synergistic engineering", which aims at the strategic integration of the two approaches (Takebe & Wells, 2019; see Hayward, Kouthouridis & Zhang, 2021; Zhang, Wan & Kamm, 2021; Brassard & Lutolf, 2019; Park, Georgescu & Huh, 2019; Mertz, Ahmed & Takayama, 2018; Nakamura & Sato, 2018*;* Clevers et al., 2017; Picollet-D'hahan et al., 2017; Marti-Figueroa & Ashton, 2017; Skardal, Shupe & Atala, 2016; and the more skeptical views by Ingber, 2020 and Hofer & Lutolf, 2021). Synergistic engineering would combine the advantages of "cellular self-emergence to generate complex tissues" with the adoption of engineering "tools to better define the cellular microenvironment in order to reproducibly guide and direct cell behavior" (Jackson & Lu, 2016).

An early attempt in this direction, stemming from the organoid research community, is the enCor, an engineered cerebral organoid. In this case, the synergy has been pursued by engineering the embryoid body[33] from the inside out by means of a microscale internal scaffold. Microfilaments that do not constrain, as a patterned scaffold would, the self-organizing properties of cells, were sufficient to achieve a more controlled and faithful cellular heterogeneity, and the recapitulation of the correct radial organization of the cortical plate (Lancaster et al., 2017). On the OoCs side, the synergistic thrust has spurred intense research about microenvironmental control on stem cell differentiation, organogenesis, and embryogenesis (Hayward, Kouthouridis & Zhang, 2021; Rico-Varela, Ho & Wan,

[33] Embryoid bodies are three-dimensional aggregates formed in suspension of pluripotent stem cells, including embryonic and induced pluripotent stem cells (Lin and Chen 2014).

2018; Tabata & Lutolf, 2017; Caiazzo et al., 2016; Gjorevski et al., 2014; Kobel & Lutolf, 2011).

The latest cutting-edge research trend is currently focused on the development of a new generation of "organoids-on-a-chip", which is the culturing of organoids within properly adapted microfluidic culture systems (Park et al., 2019; Shirure, Hughes & George 2021; Lee et al., 2018). This further step in "engineering self-organization" makes it possible to increase organoid fidelity, size, lifespan, and maturation, although at the cost of "externally imposing biochemical and biophysical boundaries to the system" (Garreta et al., 2021). Besides transferring to these new platforms the bioengineering achievements deployed in OoCs technology, much work is devoted to developing strategies to engineer *vascularized organoids*, and significant progress has been made also in the alternative "biology-directed approach" of self-assembling microvascular networks in vitro (Shirure, Hughes & George, 2021). Even body-on-a-chip research is currently focused on multi-organoid systems on-a-chip (Skardal et al., 2020; Jin et al., 2018) or "organismoids" (Marx et al., 2021).

Although the cooperation between these two "experimental cultures" is steadily rising, a lingering divide can still be perceived in what the respective communities expect from such synergistic effort. For instance, the organoid community appears mostly concerned about limiting the engineering interventions to the initial conditions as far as possible, and considers microenvironmental engineering as a mere tool for "improving" self-organization (Brassard & Lutolf, 2019; see Lancaster et al., 2017). While this allows to exploit the self-organizational potential of cells to recapitulate more complex structures, this stance also reasserts what we have called the *inside-out* control strategy. From the OoCs perspective, the emphasis lies on enhancing the *outside-in* approach, i.e., engineering constraints and dynamic control of self-organizational processes (Hofer & Lutolf, 2021). Some researchers from this field are skeptical about the synergistic program, arguing that organoids will not be able to fully recapitulate organ-level responses until they cease to be impenetrable "closed structures" and become fully engineered (Ingber 2020).

According to Takebe and Wells, synergistic engineering ought to be "narrative" and aimed at controlling the dynamics of the self-organizing system by means of the "timed manipulation" of both biological and synthetic environmental instructive cues (2019). However, the in vitro mimicking of the dynamic "morphogenetic feedback" between physical tissue morphogenesis and genetic

patterning (Gilmour, Rembold & Leptin, 2017; see sect. 4) requires the establishment of *multiple layers of control* (Marti-Figueroa & Ashton, 2017; see Naganathan & Oates, 2017). This would inevitably erode the idea, characteristic of the early organoid philosophy, that multicellular self-organization of cells is an autonomous process that can be controlled inside-out. Thus, the merging of the two approaches, as it brings to the fore the oxymoronic nature of the task of *controlling self-organization*, does seem to require, as claimed by Hofer & Lutolf, 2021, a deeper "reconceptualization of the design principles of organoids".

These lingering tensions show, in our view, that the upcoming "synergistic" enterprise must yet confront the *conceptual* divide in the two operational notions of *microenvironment* which historically demarcate the two approaches.

The specificities of the two philosophies of the microenvironment may be exemplified by the differing commitments driving cancer research respectively in tumoroids and tumor-on-a-chip literature. *Tumoroid* research focuses overwhelmingly on mutational processes underlying tumorigenesis, the role of genetic mutations, and the identification of genetic biomarkers (Green, Dam & Svendsen, 2021; Bian et al., 2018; Bartfeld & Clevers, 2017; Matano et al., 2015; Nadauld et al., 2014; Fumagalli et al., 2017; Tiscornia et al., 2011). Furthermore, it stresses the role of Cancer Stem Cells following the experimental framework presented above in Section 2 (Hubert 2016; Wang 2018). In other words, tumoroid research keeps focused on the cellular or genetic level, in accord with the Somatic Mutation Theory[34] tenet that "cancer is caused by the gradual accumulation of mutations in disease-driving genes" (Drost & Clevers, 2018) and by cellular driven processes (Bertolaso 2016).

Instead, most *tumor-on-a-chip* research moves rather from an ecological view of cancer (Strand et al., 2010) focused on the multilevel relational dynamics between tumor cells and tumor microenvironment (Hachey & Hughes, 2018; Sleeboom et al., 2018; Ahn et al., 2017). While tumoroids downplay the importance of the tumor microenvironment, tumors-on-a-chip aim at engineering its specific *cellular* and *non-cellular* components (Pereira et al., 2017), at replicating complex interactions between multiple cancer associated cell types and ECM molecules, and at modeling cancer behavior at the tissue and organ level by varying the physical properties of the cancer microenvironment (Sontheimer-Phelps, Hassell & Ingber, 2109). The

[34] The Somatic Mutation Theory explains carcinogenesis as the result of the accumulation of genetic mutations in somatic cells.

most explicit genealogical reference of this approach points unsurprisingly to Paget's *Seed and Soil Hypothesis*, which emphasized the causal role of the microenvironment for the metastatic spreading to distant sites (Sung et al., 2011). Furthermore, the ability of tumors-on-a-chip to provide accurate time-series of biological data functionally contextualized at different spatiotemporal scales (Wikswo, 2014; Bardini et al., 2017), makes them an ideal "wet" platform for feeding intrinsically "sloppy" multiscale computational models (Gutekunst et al., 2007; Transtrum et al., 2015) and for validating multiscale simulations (Kam, Rejnak & Anderson, 2011).

6. Concluding remarks

From the previous sections, we can now state that cells and their microenvironments make up a complex dynamic system, whose relevant interactions can be studied both from the perspectives of elements (by manipulating their morphogenetic potential as in organoids) and of the context (by manipulating its properties as in OoCs). Although methodologically independent if not opposed, we think that these perspectives are epistemologically interdependent.

The extant conceptual difference between these two different approaches to biological causality suggests that organoids and organs-on-chips focus on two distinct, and *not convergent but epistemically complementary*, aspects of the microenvironment: on the one hand, organoids stress the causal role of the *cell activity* in shaping and controlling the microenvironment; on the other, organs-on-chips underline the role of the *context* (i.e., the emergent microenvironmental organization) in driving cellular behavior and patho-physiology. Therefore, a potential convergence of these two approaches requires a philosophical approach that takes stock of the dual dimension inherent to embodied systems (as cells-microenvironment system is). There are, in fact, no intrinsic properties to the cellular or contextual elements. Biological properties should be always understood as relational (Bertolaso 2013, Bertolaso and Ratti 2018) as they entail a reciprocal although not symmetrical epistemological relationship. Such epistemic relationship allows us to build experimental setups that can differently constrain the cellular or

the contextual dynamic features depending on the pragmatic interest (morphogenesis vs multi-scale control of cellular feedback activities).

From an epistemic point of view, these two technologies epitomize *two constitutive and coexisting dimensions* of the microenvironment: cell activity and its emergent context. Figuratively speaking, we can consider them as the concave and convex sides of a curved surface: there is only one phenomenon – organ functionality/dysfunctionality (the curved surface) – that is explained, and mimicked, by looking either at the cell side or at the microenvironment side (see Bertolaso 2016, Bertolaso & Ratti 2018, Militello & Bertolaso 2022). Both aspects constitute, complementing one another, the organ patho-physiology, so that to account for organ patho-physiological process a relational view of cellular organization is required.

To summarize, organoids and organs-on-chips exhibit some important limitations in mimicking organogenesis and organ pathophysiology. Although these limitations can be technically smoothened by synergistic engineering, we think that technological solutionism might not seamlessly overcome the conceptual differences in their respective understanding of cell-microenvironment interactions.

An epistemic synthesis of these different philosophies requires not a mere methodological integration of models, but a different understanding of the original intuition of 3D thinking that takes stock of their complementarity (Park, Georgescu & Hu, 2019).

The complementarity perspective may in fact overcome the limits of both philosophies so that they enrich one another. It opens up the possibility of capturing the dynamics of inter-level regulation in self-evolving systems by combining two different approaches that look at the system from the point of view of cell activity (thus constraining the contextual factors) or at the causal role of the constraining elements on cells' behavior.

However, we have highlighted how this constructive and positive synergistic enterprise promoted by the latest research on organoids-on-a-chip requires a revision of the original emphasis on the very notion of self-organization and on the cell-autonomous view that upheld the implementation of the initial 'philosophy' of organoids. Stem cell biologists of the organoid community and OoCs bioengineers might be now in a good position to confirm and deepen what Waddington said decades ago about self-differentiation processes: "Since (…) it appears to be necessary to specify the conditions under which a 'self-differentiation' takes place,

the idea of self-differentiation must be discarded as an exact concept for theoretical discussion" (Waddington, 1932, p. 223).

History teaches us that the process of 'discarding' in scientific practice quite often takes the form of a smooth conceptual transition that is mostly driven by the practices, and this appears to be also the case of organoid and OoCs technologies. What is, in fact, at stake is not the methodological possibility to describe context-dependent biological phenomena in terms of cell-autonomous processes, but its explanatory power (epistemic level) when facing and addressing inter-level regulatory dynamics.

The difficulty in disentangling these aspects often arises from the also well-known phenomenon in the scientific practice of transforming a methodology into an ontology, thus shifting the debate to conceptual levels where a synthesis of different viewpoints is obviously more complex. We have explored elsewhere similar tensions in cancer research (Bertolaso, 2016; Strauss et al., 2021) that witness how 'ideological' factors might play a relevant role in the debates. In this case, however, we have to acknowledge that the time seems to be ripe for scientific advancement both in conceptual and technological terms, which will give a new and positive example of how science works and why it works, precisely through the possibility of scientists to revise their conceptual assumptions and to combine different technological approaches. The commonly acknowledged role of the microenvironment in organoids and OoCs practices has the 'power' to further drive the process of innovation towards an integration of approaches that goes beyond mere juxtaposition both in terms of practices and theoretical tools and narratives.

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