Received: 17 October 2023 | Revised: 28 November 2023 | Accepted: 2 December 2023

DOI: 10.1002/agt2.478

Aggregate WILEY Open Access

REVIEW

Organoids in concert: engineering in vitro models toward enhanced fidelity

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Funding information
Health and Medical Research Fund Scheme, Grant/Award Numbers: 01150087, 16172691; Research Grants Council of Hong Kong ECS, Grant/Award Number: PolyU 251008/18 M; GRF, Grant/Award Numbers: PolyU 151061/20 M, PolyU15100821M; NFSC/RGC schemes, Grant/Award Number: N_PolyU 520/20; ITF MHKJFS, Grant/Award Numbers: MHP/011/20, MHP/037/23, 2023YFE0210500; Hong Kong Polytechnic University Project of Strategic Importance, Grant/Award Number: ZE2C; Brigham Research Institute

Abstract

Organoids have emerged as a powerful platform for studying complex biological processes and diseases in vitro. However, most studies have focused on individual organoids, overlooking the inter-organ interactions in vivo and limiting the physiological relevance of the models. To address this limitation, the development of a multi-organoid system has gained considerable attention. This system aims to recapitulate inter-organ communication and enable the study of complex physiological processes. This review provides a comprehensive overview of the recent advancements in organoid engineering and the emerging strategies for constructing a multi-organoid system. First, we highlight the critical mechanical, structural, and biochemical factors involved in designing suitable materials for the growth of different organoids. Additionally, we discuss the incorporation of dynamic culture environments to enhance organoid culture and enable inter-organoid communication. Furthermore, we explore techniques for manipulating organoid morphogenesis and spatial positioning of organoids to establish effective inter-organoid communication networks. We summarize the achievements in utilizing organoids to recapitulate inter-organ communication in vitro, including assembloids and microfluidic multi-organoid platforms. Lastly, we discuss the existing challenges and opportunities in developing a multi-organoid system from its technical bottlenecks in scalability to its applications toward complex human diseases.

KEYWORDS
assembloids, biomaterials, in vitro models, microfluidics, organoids

1 | INTRODUCTION

Animal models are conventionally and commonly used to study the development of human diseases and evaluate the efficacy of treatments. However, animal models have faced persistent criticism from two aspects. On one hand, the reliability of animal models, especially in some complex human diseases, is still questionable.[1] As humans and animals are genetically different, animal models cannot fully recapitulate the pathophysiological conditions of human in a number of scenarios. Consequently, it is possible to obtain inaccurate readouts from animals. Besides, animal testing is also

associated with ethical concerns. The growing demand for the "3R" principle—replace, reduce, and refine the use of animals—reflects an escalating desire from the public for a more ethically conscious research environment.[2] Therefore, there is always a need to develop the alternative for animal models.

Organoids are an emerging in vitro model that revolutionizes our understanding of organogenesis and disease development. By definition, organoids are three-dimensional (3D) structures formed by differentiated stem cells, which resemble the key biological, structural, and functional features of human organs.[3] In the literature, the terms "organoids" and "spheroids" are often used interchangeably. In our delineation, spheroids refer to simpler 3D aggregation of cells (usually without differentiation) that recapitulate the complexity of tissues, whereas organoids are miniaturized organs mimicking a higher level of organ-like complexity.[4] Compared to the two-dimensional (2D) monoculture of cells, organoids containing various organ-specific cells recapitulate both cell–cell and cell–matrix interaction present inside the human body.[5] Compared to animal models, organoids are more genetically close to human organs. As a "mini organ" in vitro, organoids are readily accessible and easier to manipulate compared to animal models.[3b] To date, various organoids mimicking human organs have been developed including the intestine,[6] eye,[7] brain,[8] kidney,[9] lung,[10] heart,[11] skin,[12] and others. Several recent articles provide comprehensive reviews of the state-of-art achievements in growing organoids to recapitulate different organs in vitro.[3a,3b,13] The successful establishment of an organoid-based model for human organs allows modeling complex physiological processes such as organ development,[14] genetic disease development,[15] and cancer development,[16] which are difficult to achieve in the conventional in vitro model. Moreover, patient-specific organoids can be developed using patient-derived cells, allowing precision medicine in drug screening and toxicity testing.[17]

Over the past decade of organoid research, the protocol for developing different types of organoids has been continuously optimized. Two major sources of stem cells have been identified for the growth of organoids: (i) pluripotent stem cells,[8–10,18] including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and (ii) adult tissue-derived stem cells.[19] The selection of cell sources depends on the downstream application of the cultured organoids.[20] Pluripotent stem cells recapitulate the fetal stage of development but do not maturate in adult-like tissues. Therefore, these cells could effectively model congenital diseases resulting from anomalies in fetal developmental stages as outlined in the guideline from the International Society for Stem Cell Research.[21] Due to their amenability, pluripotent stem cells could recapitulate the continuum of multi-organ development. A single organoid derived from pluripotent stem cells can generate multiple types of organ buds such as the liver, pancreas, and intestine.[22] However, the use of pluripotent stem cells for organoid growth is often constrained by their limited accessibility, low efficiency, and reproducibility in forming organoids. Alternatively, adult tissue-derived stem cells are robust in the expansion and generation of organoids in a reproducible manner.[23] These cells are maturated and potentially useful for modeling aging-related diseases.[24] In contrast, it should be noted that adult stem cells can be differentiated into limited cell types, which are often restricted in epithelial cells.

The majority of existing studies on organoids focus on one single type of organoids at a time, lacking a comprehensive approach to recapitulate the complex inter-organ communications observed in vivo. In the human body, there exists a system for inter-organ communications, where physically separated organs communicate through the circulation of signal molecules in the blood or lymphatic vessels.[25] This coordination between organs involves various metabolic activities. Taking drug metabolism as an example, when a drug is administered, it undergoes initial metabolism in the liver before entering the circulatory system and reaching the target organ.[26] Hence, hepatic drug metabolism becomes the primary origin of drug-induced toxicity and the most frequent reason for failure or withdrawal of the drug.[27] However, in many drug screening studies using organoids,[28] the active pharmaceutical ingredients are directly applied to the organoids, which does not reflect the physiological process of drug metabolism. Such single-organoid models overlook the potential drug-induced toxicity on the liver and other non-target organs that can arise during metabolism. Apart from drug metabolism, the development of pathological conditions such as obesity, diabetes, and atherosclerosis is closely associated with the dysregulation of inter-organ communications.[29] For example, the development of type-2 diabetes is not solely attributed to the dysfunction of the pancreas but also influenced by the dysregulation in other organs including gut skeletal muscle, liver, brain, and immune system.[30] Investigating the intricate communication among these organs will provide valuable insights into the underlying mechanisms and potential risks associated with diabetes development. Furthermore, understanding the interplay between these organs would open up possibilities for developing novel therapeutic interventions for diabetes by targeting not only the pancreas but also other contributing organs.[31]

Considering the physiological and pathological importance of inter-organ communications, it becomes imperative to integrate this aspect into in vitro organ models. The advancement of multi-organ in vitro models holds the potential to enhance the fidelity of the in vitro organ systems and enable the study of complex pathological conditions. By incorporating multiple organoids and establishing communication among them, it offers a more comprehensive understanding of organ functions and disease progression, bridging the gap between traditional in vitro models and the complexity of the human body. In this review, we provide a summary of recent developments in engineering organoids, focusing on various aspects including organoid culture matrix, organoid culture environment, and strategies for manipulation of organoid morphogenesis. While our primary focus is on organoids, we have included some spheroid examples to demonstrate the potential pathways for organoid research. We highlight the significance of these advancements in paving the way for the development of multi-organoid systems. Additionally, we discuss the current progress in multi-organoid systems, exploring the integration of different organoids and their potential applications in studying inter-organ communications and complex physiological processes. Lastly, we discuss the existing challenges and opportunities in this emerging field, shedding light on future directions and the potential

TABLE 1 Comparison of the structural, mechanical, and biochemical properties of the representative biomaterials for organoid culture.

	Structural Properties		Mechanical Properties		Biochemical Properties
Matrix Materials	**Controlled macroscopic structure**	**Presence fibrous structure**	**Controlled elastic properties**	**Controllable viscoelasticity**	**Presence of essential biomolecules**
Naturally derived materials					
Matrigel	🟢	🔴	🔴	🔴	🟢
Collagen	🟢	🟢	🟡	🟡	🟡
Alginate	🟢	🔴	🟡	🟢	🔴
Decellularized ECM	🟢	🟢	🔴	🔴	🟢
Synthetic materials					
Polyethylene glycol (PEG)	🟢	🔴	🟢	🔴	🔴
Polyisocyanide (PIC)	🟢	🟢	🟢	🟢	🔴

🟢 Excellent 🟡 Neural 🔴 Poor

impact of multi-organoid systems in advancing our understanding of organ development, disease modeling, and drug discovery.

2 | BIOMIMETIC MICROENVIRONMENT: FROM PHYSICAL TO CHEMICAL PROPERTIES

The selection of matrix materials is critical to successfully growing organoids as they provide not only mechanical support but also the necessary chemical stimulation. Currently, Matrigel stands as the most commonly used matrix material for the organoid culture.[32] It is a mixture of basement membrane proteins secreted from Engelbreth–Holm–Swarm mouse sarcoma cells.[33] Despite its widespread use, the chemical composition of Matrigel is poorly defined. In a proteomic analysis of Matrigel, it was found that, apart from the known constituents such as collagen, laminin, and entactin, there were 1,800 more proteins as well as a variety of growth factors presented in Matrigel.[34] The undefined composition and its animal-based origin raise concerns about its batch-to-batch variation and the presence of xenogenic contaminants. Additionally, it offers limited flexibility in manipulating the materials' properties.

Therefore, there is a continuous need to develop chemically defined and tunable materials to replace Matrigel. From naturally derived materials such as collagen and decellularized ECM to synthetic materials such as polyethylene glycol (PEG), different types of hydrogels have been developed to serve as an alternative to Matrigel for the growth of organoids. Several reviews have comprehensively summarized the recent development of chemically defined hydrogels for the organoid culture.[35] Instead of repetitively going through the compositions of these novel Matrigel alternatives, this section focuses on three pivotal factors in designing an ideal material system to foster organoid growth: structural, mechanical, and biochemical properties (Figure 1). To contextualize three factors into content, the strengths and weaknesses of existing representative biomaterials for the organoid culture were analyzed accordingly in Table 1.

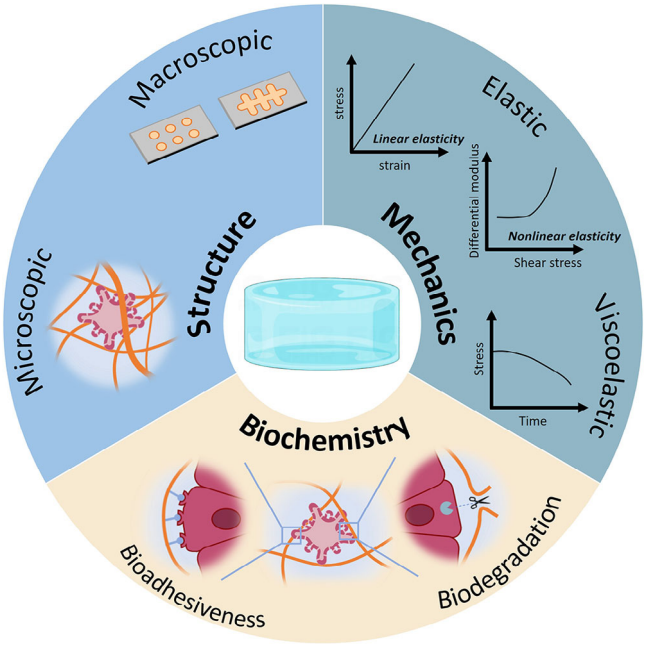

FIGURE 1 Design principles of optimal hydrogel system for organoid growth.

2.1 | Structural properties

The structural aspect of the organoid growth matrix is twofold, macroscopic structure and microscopic structure. Here, the macroscopic structure refers to the shape and geometry of the matrix, which is important to reduce the variation in the size and shape of the organoids. During the development of organoids, despite uniform encapsulation of stem cells in a homogeneous hydrogel scaffold, the developed organoids often exhibit significant variations in size and shape.[36] The

variation in organoid morphology leads to low reproducibility in the experiments. This limitation can be addressed by applying external structural confinement during organoid development via engineering the macroscopic structure of the matrix. Taking intestinal organoids as an example, the conventional 3D culture approach barely controlled the number, size, and location of the crypt-like domain and the villus-like domain in the organoids.[37] To achieve structural control of the intestinal organoids, Lutolf's group micropatterned the hydrogel scaffold with a biomimetic structure of crypts in the mouse small intestine.[38] Mouse intestinal stem cells were then perfused into the hydrogel scaffold. After 5 days, the stem cells grew and self-organized into a patterned intestinal organoid following the macroscopic structure of crypt-like and villus-like domains created in the hydrogel scaffold. Using such a system, the authors were able to investigate further the influence of the macroscopic structure in organ morphogenesis.[39]

The microscopic structure describes the microscale structural properties such as pore size, fiber density, and fiber diameter. In particular, the fibrous structure of the matrix is gaining increasing attention for organoid culture. The native extracellular matrix (ECM) is a fibrous network.[40] Recapitulating the fibrous network in the organoid growth matrix would provide a biomimetic organ environment. Furthermore, the presence of fibrous structures in the organoid growth matrix could guide the development of organoids and, potentially, the communication between organoids. For example, the migration of cells from organoids is affected by the fibrous network in the matrix. It has been reported that an improved metastatic property of tumor organoids was observed when culturing in a fibrous matrix.[41] The fiber in the organoid growth matrix facilitates cell migration and leads the cells of the organoids to be more invasive and to migrate out from the substrate. Besides, the fibers in the ECM have been found to play an important role in mechanotransduction and long-distance cell–cell interactions.[42] With the presence of fiber, the mechanical signals can be transmitted over distances exceeding 1,000 μm within the matrix.[43] This enables enhanced intercellular communication over significantly longer ranges compared to non-fibrous hydrogels. Given the discovered role of fiber in signal transmission between individual cells, it is plausible that the presence of fibers would facilitate inter-organoid communications. However, no evident fibrous network was observed in the Matrigel,[33,44] which is hence considered to be a non-fibrous hydrogel.[45] The absence of the fibrous network in this material diminishes its capability of facilitating inter-organoid communications.

2.2 | Mechanical properties

The mechanical properties of the matrix include both elastic (linear and nonlinear) and viscoelastic properties. As a 3D complex structure derived from the self-assembly and differentiation of stem cells, the stiffness of the matrix has a direct impact on the development of organoids from both cell proliferation and differentiation. Generally, organoids prefer a soft environment with a stiffness of less than 2 kPa.[46] Increased matrix stiffness beyond this range has been observed to inhibit cell proliferation and impede the development of different types of organoids.[47] For example, Broguiere et al. studied the effects of hydrogel stiffness in the growth of intestinal organoids in a fibrin-based hydrogel by changing the concentration of fibrin.[47b] It was found in this study that increasing hydrogel storage modulus by raising the fibrin concentration resulted in a decrease in the percentage of colony formation and cystic organoids. As it has been known that the matrix linear elasticity regulates the differentiation of individual stem cells,[48] the matrix elasticity also affects the development of organoids in an organ-specific manner. For example, brain organoids exhibit a more mature morphology under stiffer environments, whereas intestinal organoids differentiate more in a soft matrix.[39,49]

With a developing understanding of the mechanical properties of ECM, more unique features of the native ECM mechanics have been identified, including nonlinear elasticity and viscoelasticity. These properties have gained attention in recent studies to examine their potential influence on the development of organoids. Nonlinear elasticity describes the stiffening of the matrix above the critical stress, which protects the organ against extreme deformation.[50] In a liver tumor spheroid model, it was found that the hydrogel with a higher value of critical stress stimulated the stress fiber formation in the cells and activated the mechanotransduction.[51] Besides, the stiffened environment above the critical stress elevated the proliferation and migration of the cells,[51] resulting in the increased invasion of cells from cancer organoids.[52] While the impact of nonlinear elasticity on organoid development is still in its early stages of exploration, the influence of viscoelasticity on organoid development is more investigated. The viscoelasticity refers to the relaxation of stress in the matrix under a constant strain, serving as a protective mechanism for cells from prolonged exposure to external mechanical loading in the ECM.[53] Several recent studies showed that viscoelasticity affects the morphogenesis of organoid development.[54] The intestinal organoids remained a spherical shape in the pure elastic hydrogels, whereas they broke their symmetry with enhanced branching when in the viscoelastic hydrogel.[54a] Based on that, a hydrogel with locally patterned viscoelasticity was able to control the morphogenesis of intestinal organoids in the crypt-like domain.[54b]

2.3 | Biochemical properties

Regarding the biochemical aspect of the matrix, it is the conjugation of biomolecules to the matrix to facilitate organoid culture. While each type of organoid requires organ-specific biochemical stimuli, two categories of biomolecules are commonly needed for the growth of various organoids: cell-adhesive ligands and biodegradable motifs. Supporting the adhesion of cells onto the matrix is the first step in organoid culture. Synthetic polymers such as PEG and polyisocyanide (PIC) do not contain the cell-adhesive ligand, and therefore cell-adhesive ligands, for example, arginylglycylaspartic acid (RGD) sequence, are conjugated to the polymers to support organoid growth.[55] Notably, it was found that only the RGD sequence conjugated to the hydrogel, that is, attached to the matrix structure, supports the growth of organoids, whereas the free RGD dissolved in the medium inhibited the growth of organoids.[47b] Along with the growth of

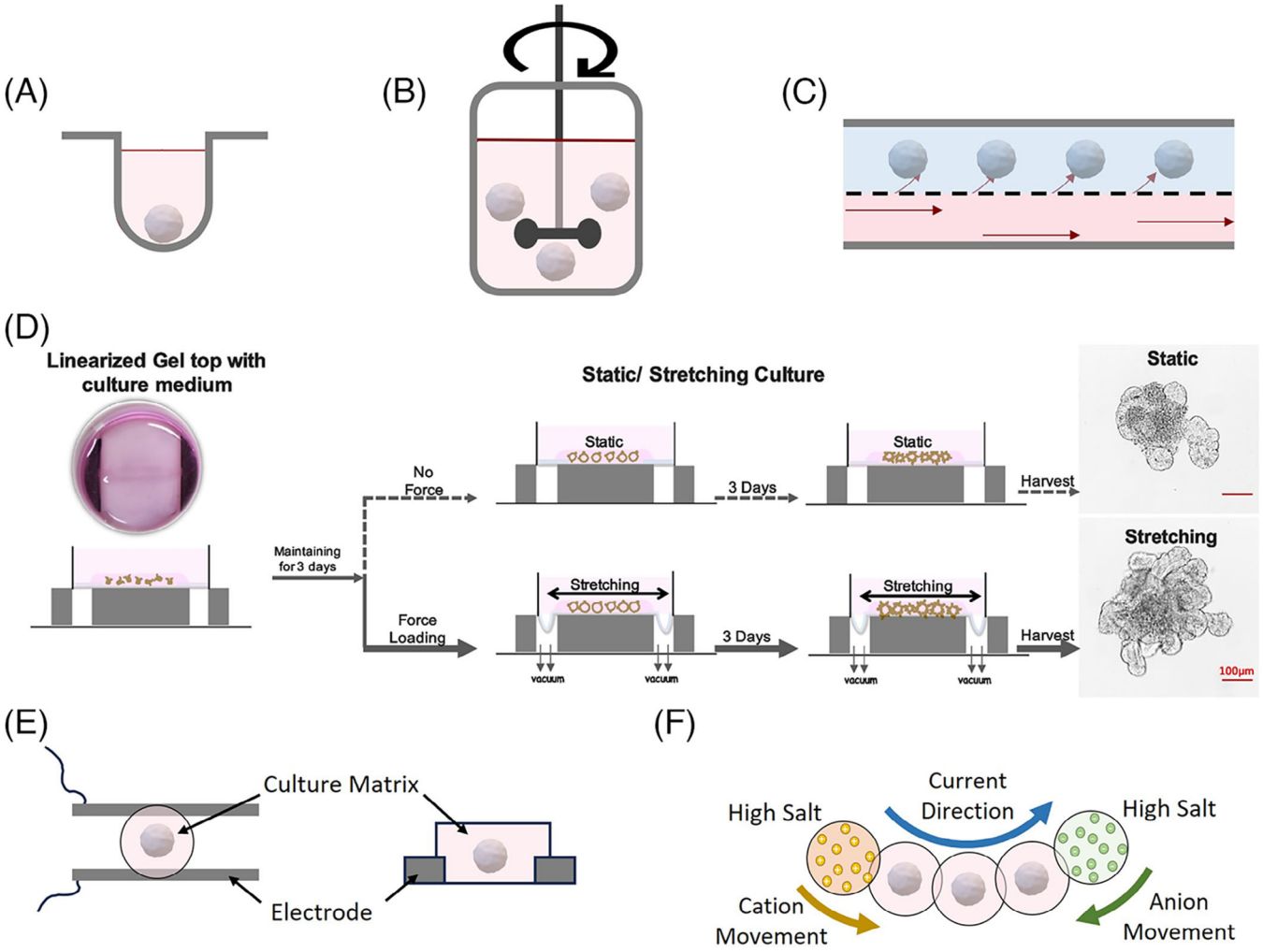

FIGURE 2 Schematics of organoid culture systems. (A) A static culture system. (B) A spinning organoid bioreactor. (C) A microfluidic organoid culture device. (D) A mechanical stretching organoid culture system (Reproduced with permission: Copyright 2022, SpringerOpen. Ref.[65]). (E) A rigid electrode-based organoid electrical stimulation culture system. (F) A microscale soft ionic power system for the organoid electrical stimulation culture system

organoids, it is ideal to have a matrix that is biodegradable and can be remodeled by the organoids. Similarly, while most naturally derived polymers are biodegradable, proteinase-sensitive motif needs to be conjugated to the hydrogels formed from synthetic polymers.[35b] Matrix metalloproteinase (MMP)-sensitive motif is one of the common candidates to introduce biodegradability to the synthetic hydrogel. It was found that when cultured in a biodegradable matrix, an increased polarization of the organoids was observed compared to the non-degradable group.[56] In addition to the two universally beneficial biomolecules, other biomolecules such as ECM-mimic proteins can be incorporated into the matrix materials to facilitate the development of organoids. The selection of such organoid development ligands is usually organ-specific. For example, laminin is needed for the growth of the intestinal organoids.[47b]

3 | PHYSIOLOGICAL ENVIRONMENT: FROM STATIC TO DYNAMIC CULTURE

Conventionally, organoids are cultured in a static environment, where the stem cells are encapsulated in the matrix and submerged in the culture medium (Figure 2A). Although being simple and widely used, the static culture does not account for the effects of fluid flow, external stimuli, oxygen gradients, nutrient transport, and waste removal on organoid development and function. Consequently, several limitations arise, including low cell viability of organoids in the long term and limited differentiation capacity.

3.1 | Physiological flow

In the static culture environment, the transport of nutrients and exchange of waste across the organoids relies only on the diffusion of the molecules. Without the presence of vasculature, the diffusion of nutrients and oxygen into the organoids is generally limited to a distance of 200 μm.[57] Consequently, it becomes challenging to maintain the viability of organoids with a diameter beyond 400 μm. To overcome that, dynamic flow is created in the organoid culture chamber for enhanced diffusion. Broadly, the dynamic flow can be created in an organoid culture system via spinning bioreactors,[58] and microfluidic devices.[59] In the context of the organoid culture, a bioreactor is a culture chamber where the organoids are suspended in the culture medium that is continuously agitated to create a dynamic flow (Figure 2B). With the enhanced mass transport from agitating/spinning bioreactor, both the size of viable organoids and the span of culture time are

significantly increased.[60] In a multi-well spinning bioreactor, Qiao et al. were able to expand the size of brain organoids to a millimeter scale and maintain the organoids viable for over 400 days.[58a] Alternatively, a dynamic culture system can be created in a microfluidic device. Typically, a microfluidic device for the dynamic culture of organoids contains a culture chamber of organoids which is connected to a supply channel with a continuous flow of the culture medium (Figure 2C). The presence of dynamic flow in the microfluidic device not only enhanced the diffusion of the molecules but also introduced the convection of the molecules.[61] Moreover, the fluid flow created in the microfluidic device is found to be beneficial in the differentiation of different organoids such as kidney organoids,[62] liver organoids,[63] and brain organoids.[64] This is because the shear stress exerted from the dynamic flow serves as a mechanical stimulus to cell differentiation. For example, in comparison to the organoids statically cultured in the U-shape microwell, an enhanced vascularization and maturation of kidney organoids was observed when subjected to continuous directional flow for 10 days.[62]

3.2 | Mechanical forces

In addition to introducing dynamic flow, other approaches have also been developed to simulate physiologically relevant dynamic environments such as stretch and mechanical loading to meet organ-specific requirements in the organoid culture. For example, mechanical forces play a critical role in maintaining intestinal functions such as intestinal peristalsis, where the epithelial cells experience cyclic contraction and relaxation. To incorporate such a dynamic environment into the organoid culture, intestinal organoids were exposed to cyclic stretch after a 3-day static culture (Figure 2D).[65] It was found that the organoids exhibited a larger size and a higher crypt number under cyclic stretching. Moreover, the presence of mechanical stretching improved the stemness of the intestinal stem cells and facilitated organoid growth. Similarly, the cyclic stretch was applied to the lung organoids in a customized organoid stretching module to recapitulate the continuous breathing movement of a human lung.[66] The organoids exposed to cyclic stretch exhibited an increased expression of several mesenchymal genes that are associated with postnatal lung development. Apart from stretching, compression can also be incorporated into an organoid culture system to mimic the mechanical loading on the bone. To achieve that, a stem cell-laden scaffold was mounted to a mechanical tester and cultured under loading for 45 days.[67] Such a compression bioreactor can be potentially applied to bone organoids to investigate the role of mechanical loading in organoid development.

3.3 | Electrical stimulation

Electrical signals play a vital role in regulating the development, communication, and functioning of certain organs such as the brain, heart, and muscle.[68] At the cellular level, electrical stimulation has been found to affect the proliferation, migration, differentiation, contraction, and orientation of the electrically excitable cells from these organs.[69] Therefore, electrical stimulation at a physiological level is another critical aspect to be included in a dynamic organoid culture environment. Accumulating evidence has demonstrated the role of electrical stimulation in directing the growth, differentiation, and functions of various organoids including cardiac organoids, brain organoids, and muscular organoids.[70] For example, Chiu et al. investigated how the pattern of electrical stimulation affects the structure and functionality of the cardiac organoids.[71] Compared to monophasic stimulation, biphasic electrical field stimulation was found to improve the functionality of the cardiac organoid reflected by the raised cell density of the organoids, and stronger beating at a lower threshold of excitation voltage. Another example demonstrating the impact of electrical signals in the organoid culture is the enhanced vascularization in a myovascular organoid under chronic electrical stimulation.[72] To implement electrical stimulation in an organoid culture system, a typical setup is to embed the electrode into the culture matrix (Figure 2E). While this type of setup is simple to construct, the use of rigid electrodes often leads to limited adhesion between the electrodes and the organoids. The development of soft electronics enables the incorporation of flexible electrical stimulators into the organoid culture system. For example, a conductive hydrogel containing electrode arrays was developed to apply electrical stimulation through the direct contact.[73] Furthermore, Zhang et al. took the advantage of the directional ion movement along the concentration gradient to develop a microscale soft ionic power source that provides electrical stimulation during the growth of neural progenitor cells embedded in Matrigel droplets (Figure 2F).[74] This device provides a biocompatible and simple strategy to introduce electrical stimulation through ionic current in the organoid culture.

4 | MORPHOGENESIS: FROM SELF-ASSEMBLY TO INSTRUCTED SHAPE-FORMATION

Conventionally, organoids are formed almost purely from self-assembly of the stem cells for the microscale tissue to morph, through differentiation-directed spatial patterning of necessary resulting cell types, into their target organ-specific organoids. Such a method is convenient, does not require additional tools, and is relatively mature. This meets the need to study organogenesis and disease development in a relatively simple setting. However, the lack of control over the morphology and the spatial distribution of the organoids makes it challenging to establish reliable inter-organ communications in an in vitro multi-organoid system. Taking the concept of organoid development a step further, constructing a complex organoid system containing two or more organoids of hetero-types termed assembloids can improve the fidelity and complexity of organoids as an in vitro model.[75] Through fusion and functional integration, organoids can be integrated together and establish inter-organoid connections, mimicking the inter-organ communication in the human body, which is missing in a single and homogeneous organoid system. Despite the potential assembloids, two major limitations remain to be addressed, geometrical control of the morphology of individual organoids and the precise positioning of the organoids.

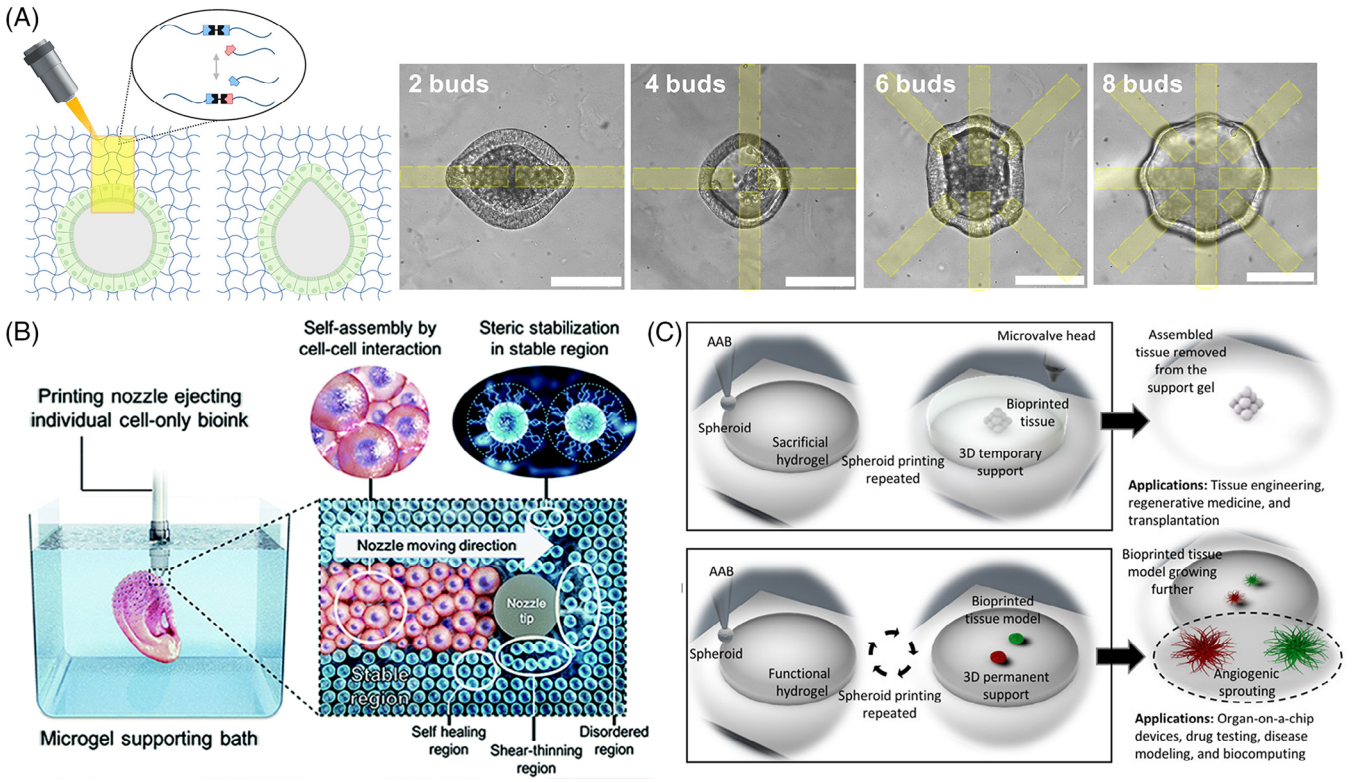

FIGURE 3 Strategies in manipulating the morphogenesis of organoids. (A) Controlling the morphogenesis of organoids by locally patterning the mechanical properties of the organoids-growth scaffold (Reproduced with permission: Copyright 2023, American Association for the Advancement of Science. Ref. [54b]). (B) Individual cell-only bioprinting to control the spatial distribution of the organoid-forming stem cell (Reproduced with permission: Copyright 2019, Royal Society of Chemistry. Ref.[78]). (C) Individual organoid-only bioprinting to control the spatial distribution of organoids (Reproduced with permission: Copyright 2020, American Association for the Advancement of Science. Ref.[80b])

With the continued innovation of biofabrication technologies, our ability to pattern cells across multiple scales has significantly improved.[76] To this end, some recent demonstrations reveal the possibility of driving deterministic organoid morphogenesis using precisely patterned geometries, deviating from their conventional, spherical form. There exist two ways: engineering the matrix properties and direct positioning of the organoids. As discussed in the previous section, engineering the structural, mechanical, or biochemical properties has a direct impact on the morphology of the organoids developed. For example, when a photoinduced hydrogel crosslinking exchange reaction was adopted to spatiotemporally alter local curvature, crypt morphogenesis of epithelial cells could be instructed (Figure 3A).[54b] Hydrogel structures that enabled defined shape, size, and cell distributions could lead to the formation of organoids that are more native-like, predictable, and reproducible.[39]

The other strategy, precise positioning of organoids, can be achieved through 3D bioprinting at both the single-cell and organoid levels.[77] At the single-cell level, the organoid-forming stem cells are positioned to a desired 3D structure by bioprinting either the cells directly or a cell-laden bioink. Using a photocurable, self-healing, and shear-thinning microgel supporting bath, human mesenchymal stem cells were directly extruded from the nozzle as the only component of the bioink (Figure 3B).[78] Although the application of this cell-only bioprinting technique to organoids has not been fully demonstrated, the technology holds the potential to directly position different stem cells in a matrix to form a complex assembloid system. A more common approach to control the morphogenesis of organoids is printing cell-laden bioink, which is prepared by mixing the organoids-forming stem cells with a hydrogel precursor. The robotic movement of the printhead in bioprinting may be used to pattern the organoids into various predefined geometries. This is a process shown to be effective in directing their patterning into organ-like morphologies such as hollow tubes, or in promoting the functions of the obtained organoids.[79] For example, bioinks containing human intestinal stem cells were extruded into a line, which later self-organized into a connected and polarized epithelial tube.[79a] By changing the cells in the bioink, the authors were able to bioprint stomach and intestine organoids in a single line or an intestinal tube alongside intestinal connective tissues. This work demonstrated the promising potential of 3D bioprinting in creating highly customized assembloids to study inter-organ communications.

At the organoid level, bioprinting allows printing directly the organoids or organoid-laden bioink. In this case, the formation of organoids is completed before the printing. Due to the larger size of organoids, aspiration-based 3D bioprinting is more suitable for direct deposition of individual organoids and has shown a promising capability in positioning organoids to study the inter-organoid communications.[80] By controlling the spacing between two human umbilical vein endothelial cell (HUVEC) spheroids positioned, Ayan et al. examined the potential factors affecting angiogenesis (Figure 3C).[80b] Likewise, by positioning a scarred cardiac spheroid in a ring of healthy cardiac spheroids, Daly et al. established a multi-spheroid model to study cardiac fibrosis caused by myocardial infarction.[80c] Alternatively, organoid-laden bioink can be utilized in extrusion-based bioprinting.

Pre-formed organoids are first encapsulated in the bioink and extruded through the nozzle to construct a millimeter-scale 3D structure.[81] This offers higher throughput and the capability to construct a complex 3D architecture compared to aspiration-based 3D bioprinting, where only one spheroid can be bioprinted at a time. While this approach provides improved efficiency, it comes at the cost of reduced resolution in positioning organoids at a single-organoid level, which may limit its utility for establishing precise organoid–organoid interactions.

5 | INTER-ORGAN COMMUNICATION: FROM SINGLE TO MULTI-ORGANOID SYSTEMS

As aforementioned, inter-organ communication plays a critical role in regulating the proper functions of the human body and is involved in the disease development. Recapitulating such communication in vitro will allow a deeper understanding of organogenesis and disease development. However, this is often overlooked in current in vitro models. Hence, the development of multi-organoid systems has garnered considerable attention within the realm of organoid research, as it presents an opportunity to examine the interplay between various organoids and simulate intricate physiological processes. Two primary approaches exist for the construction of multi-organoid systems: the assembly of organoids into assembloids and the integration of organoids within a microfluidic platform.

5.1 | Vascularized organoids: a rudiment of assembloids

The assembly of assembloids can be achieved by mixing different cells at the early stage of the organoid culture, incorporating stem cells with developed organoids, or fusing different types of developed organoids. Vasculature plays a key role in co-opting the development of multiple organs in the body as the vascular network provides structural support as well as nutrition via both perfusions of media and angiocrines.[82] Hence, incorporating vasculature into organoids is a rudiment of assembloids and is expected to facilitate the development of multiple tissues/organs systems. A straightforward approach to vascularize an organoid is to mix stem cells that have been engineered toward different lineages. For example, a vascularized cortical organoid was developed by mixing lineages of human ESCs with one differentiated into a vascular network and the other forming the cortical organoid.[83] In this work, the authors ectopically expressed ETV2 in the human ESCs, a master regulator of endothelial lineage, and induced them to endothelial cells in cortical organoids. As a result, these ETV2+ endothelial cells formed a vascularized network in the cortical organoids. Moreover, the vasculature inside the cortical organoids acquired the characteristics of the blood–brain barrier, a unique function of vessels in the central nervous system, with increased expression of tight junction and nutrient transporters and elevated trans-endothelial electrical resistance. Although this work presents a simple approach to creating hybrid organoids containing vasculature, the success of this approach relies on the ectopic expression of transgene. Therefore, its application is constrained by genetic modification of human pluripotent stem cells. Alternatively, Rafii and colleagues developed a protocol to reset adult endothelial cells which were later mixed with cortical organoids and form a vascular network.[84] Besides, xenografting human organoids into highly vascularized animal organs such as the brain demonstrated the progressive formation of vasculature into the organoids.[85] Peninger and colleagues generated blood vessel organoids from human PSCs and transplanted them into a mouse to model diabetic vasculopathy.[86] The transplanted blood vessel organoids formed a stable and perfused vascular network in the mouse and developed diabetic-induced impaired vessel functions. These reduced vessel functions included a reduced barrier function and thickening of the vascular basement membrane. Both examples demonstrated the potential of creating vascularized assembloids with immunologically matching donor (vascular) and recipient (gut, lung, brain, etc.) organoids.

5.2 | Complex assembloids: inter-connected multi-organoid systems

Further along this step, assembloids with more complex structures can be prepared through the assembly of various types of organoids. For example, to model the complexity of the brain structure, Park and colleagues developed an assembloid of forebrain and hindbrain organoids.[87] Two groups of human PSCs first underwent cortical and ventral differentiation separately to form cortical organoids and medial ganglionic eminence (MGE) organoids, respectively. The functional maturation of the cortical organoids and MGE organoids was verified by abundant synaptogenesis and the synchronized pattern of the neuronal activity, respectively. Subsequently, these two organoids were fused to form an assembloid with the regional features of the human brain. With this assembloid, the signal interactions of both forebrain and hindbrain during the brain development can be modeled in vitro. This demonstrates that assembloids offer the opportunity to model the development of complex organs like the brain. The interaction between organs that are spatially distant from each other in vivo can also be studied in an assembloid model. To recapitulate the human cortical–spinal–muscle pathway, Pasca and colleagues generated cortico–motor assembloids by fusing cortical spheroids, spinal spheroids, and skeletal muscle spheroids (Figure 4A).[88] With the three spheroids synoptically connected in the sequence of the cortical–spinal–muscle pathway, the authors were able to monitor the long-term functionality, that is, contraction of the muscle (skeletal muscle spheroids) over 8 weeks in response to the stimulation from the cortical neuron (cortical spheroids). Furthermore, Takebe and colleagues leveraged the self-organization capacity of gut organoids to generate a multi-bud assembloid containing the pancreas-, liver-, and intestine-like organoids.[89] An anterior gut spheroid and a posterior gut spheroid were first fused to create a boundary organoid. The hepatobiliary–pancreatic progenitor cells developed from this fused boundary organoids and underwent 3D differentiation to liver, pancreatic, and biliary buds in this fused organoid.

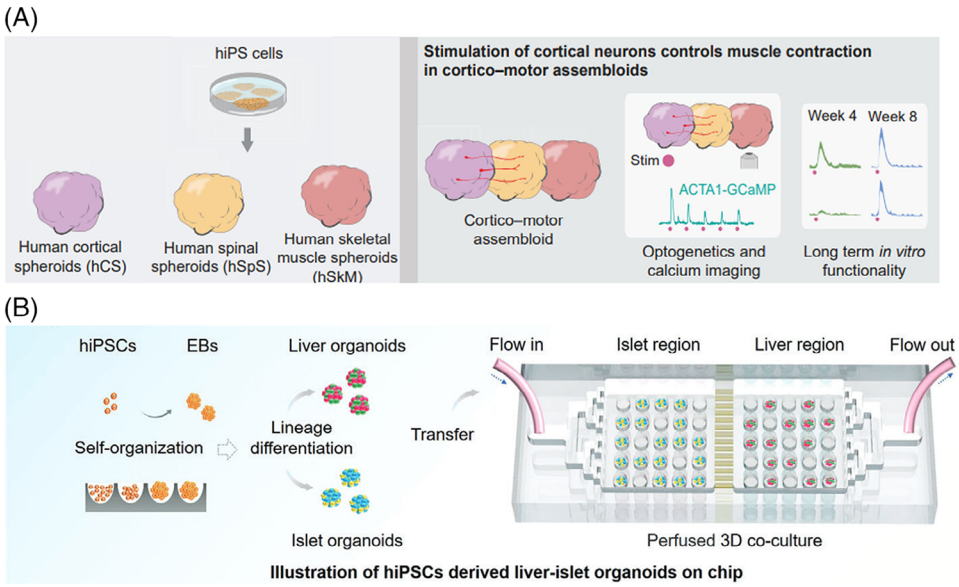

FIGURE 4 Strategies to fabricate a multi-organoid system. (A) A cortico–motor assembloid by assembly and fusion of a human cortical spheroid, a human spinal spheroid, and a human skeletal muscle spheroid (Reproduced with permission: Copyright 2020, Elsevier. Ref.[88]). (B) A microfluidic liver-islet organoid-on-chip platform by connecting organoids in two regions through a microchannel network (Reproduced with permission: Copyright 2021, Royal Society of Chemistry. Ref.[93])

Moreover, a functional link was established between the pancreatic bud and the biliary bud as increased amylase secretion was observed under the cholecystokinin treatment. The multi-bud organoids recapitulated complex human endoderm organogenesis and were utilized to study the organ-segregation error.

5.3 | Multi-organoid-on-a-chip: orchestrating distanced multi-organoid systems

It is probable to simulate more complex physiological procedures when the organs are not in direct contact and communicate through systematic responses. Inside the human body, organs are usually connected through blood vessels and/or lymphatic vessels, where signaling molecules such as angiocrines are transmitted and facilitate both the development and the function of tissues.[90] To recapitulate such inter-organ communications in vitro, a multi-chamber organs-on-chip platform was developed to connect tumor tissue and lymph nodes.[91] In this work, tumor tissue and lymph node tissue were cultured in two separate culture chambers and connected by tubing and microchannels. An on-chip circulating system was created by a peristaltic pump. By connecting the lymph node tissue with tumor or healthy tissue, this platform was able to study tumor–immune interactions. Another application of the multi-organoid-on-a-chip platform is cancer metastasis. A metastasis-on-a-chip device was developed by connecting colorectal cancer organoids in the primary tumor chamber with three downstream chambers containing liver, lung, and endothelium organoids.[92] Under the circulating flow, the migration of the fluorescently labeled tumor cell from the primary tumor site and its invasion to the secondary site was monitored continuously. By customizing the type of organoids placed in the tumor chamber and downstream chambers, this device can be applied to study the metastasis of various tumors, which is challenging in conventional animal models. As organoids are cultured in an individual chamber, it also allows the investigation of the functionality of each organoid.

Beyond cancer research, multi-organoid-on-a-chip connecting various organoids has the potential for drug discovery and toxicity assessment in other inter-organ systems. Considering the liver's fundamental role in processing pro-drugs and nutrition, liver-on-a-chip can be connected to various types of organs-on-chips. For example, human iPSC-derived cardiac organoids connected with liver organoids assessed the cardiac toxicity of liver-metabolized Clomipramine (Figure 4B).[93] A two-layer microfluidic platform was fabricated to co-culture liver organoids on the top layer and cardiac organoids on the bottom layer. The layers were separated by a porous membrane that serves as a physical barrier between the organoids while allowing for the diffusion of molecules across the layer. The functionality of each organoid was independently monitored by analyzing urea synthesis from liver organoids and measuring the beating rate of cardiac organoids. The cardiac toxicity of a liver-metabolized drug, Clomipramine, was evaluated by monitoring the functionality of cardiac organoids underneath after supplying the drug to liver organoids. Vice versa, one could address how hormones from other organs affect the liver. For instance, human iPSC-derived liver and islet organoids cultured in two separated regions were connected through a network of microchannels to mimic the human liver–pancreatic islet axis.[94] The insulin secreted from the islet organoids flew across the microchannel and regulated the glucose utilization of liver organoids downstream. Further, more organoids can be incorporated into an organoids-on-a-chip platform. A seven-organoid platform containing liver, cardiac, lung, vascular, testis, colon, and brain organoids was developed to conducted a comprehensive evaluation of liver-metabolized pro-cancer drug.[95]

6 | CHALLENGES AND OPPORTUNITIES

The emergence of assembloids and microfluidic multi-organoid systems has revealed the potential of organoids in studying complex inter-organ interactions. These advancements offer opportunities to investigate physiological processes and disease mechanisms. However, it is important to acknowledge that the field is still in its nascent stage and faces several challenges.

From a biofabrication standpoint, one of the biggest bottlenecks is the precision and scalability of organoid positioning. Although the incorporation of 3D bioprinting allows the automation of processes, the current strategy to 3D bioprint organoids is still far from large-scale fabrication. The aspiration-assisted bioprinting of organoids provides, so far, the highest precision in positioning organoids at a single-organoid level. It allows for the meticulous manipulation and placement of individual organoids to a desired location, achieving a high level of accuracy. However, it should be noted that this approach comes with a delicate experimental setup and involves time-consuming procedures. Moreover, the manipulation of only one organoid at a time limits the efficiency and throughput necessary for constructing complex multi-organoid systems at a large scale. Alternatively, extrusion-based printing of organoids-laden bioinks is more efficient in constructing complex 3D structures with a cost in the resolution. Recently, the development of volumetric printing provides another solution to construct sophisticated 3D structures in a timely manner.[96] By projecting reconstructed lights from different angles, all directions of the object are bioprinted simultaneously. A recent pilot study on applying volumetric bioprinting to build a hepatic sinusoidal network from hepatic organoids demonstrates the potential of using this technique for high-throughput organoid bioprinting.[97] The application of volumetric bioprinting in constructing multi-organoid systems would require further development in both materials design and light manipulation to enable precise and accurate manipulation of each individual component organoid.

From a biological perspective, numerous opportunities exist for multi-organoid systems to enhance the complexity and fidelity of existing in vitro models. Despite progress in multi-organoid systems, many remain simplified, lacking the capacity to replicate sophisticated pathophysiological conditions. One of the critical components missing in most of the existing in vitro models including the multi-organoid system is the immune system. Immune cells regulate homeostasis in various organs, such as the liver, lung, and gut, and pathogenesis, such as cancer metastasis.[98] The human immune system is rather complex and contains various types of immune cells as well as a unique microenvironment. It is reported that immune organoids such as the thymus and tonsils dissected immune cell development and were robust in differentiating functional immune cells.[99] The development of immune organoids will contribute to the successful integration of the immune system into multi-organoid systems, which holds promise for a wide range of applications such as immunotherapy and vaccine development. Ultimately, multi-organoid-on-chips will leverage the benefits of both assembloids (complexity of tissues, immunization, and vascularization), and organs-on-chips (inter-organ connections, precise control of medium input, and application of mechanical forces). Equipping these tissue-resident immune cells in multi-organoid on chips will enhance our understanding of immunological response in an array of pathological conditions.

Another potential application of a multi-organoid system is to model infectious diseases including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This is because the severe phenotype of infectious diseases often attacks multiple organs and exacerbates progression with other chronic diseases. Organoid-based in vitro models have been employed for research in infectious diseases. For example, brain organoids from human iPSCs modeled Zika virus infection and their damage to brain development.[100] When human iPSC-derived pancreatic islet organoids were exposed to SARS-CoV-2, the striking induction of cytokines indicated that the virus infected and damaged the tissue.[101] However, it should be noted that these two studies used conventional organoids whose cellular components are still limited. Modeling the whole-body response during the progression of infectious disease requires a more complex in vitro system. Therefore, endowing both the vasculature and immune components together with brain, lung, pancreas, and kidney organoids will further recapitulate infectious disease and open the venue for drug discovery and vaccine development in infectious diseases.

7 | CONCLUSION

In conclusion, the multi-organoid system holds immense promise as a cutting-edge model for advancing our understanding of inter-organ interactions and complex human diseases. Although some current technical challenges remain as bottlenecks for achieving broader downstream applications of this platform, it is a blooming field anticipating significant progress in surmounting these challenges. By integrating organoids with other advancements in bioengineering, the multi-organoid system will revolutionize biomedicine and healthcare from fundamental studies on the disease development to applied research on drug discovery and disease therapy.

ACKNOWLEDGMENTS

This work was supported by Health and Medical Research Fund Scheme (01150087#, 16172691#), Research Grants Council of Hong Kong ECS (PolyU 251008/18 M), GRF (PolyU 151061/20 M, PolyU15100821M), NFSC/RGC schemes (N_PolyU 520/20), ITF MHKJFS (MHP/011/20, MHP/037/23, 2023YFE0210500) and the Hong Kong Polytechnic University Project of Strategic Importance (ZE2C). ZC and YSZ were not supported by any of these funds. YSZ acknowledged the support by Brigham Research Institute.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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How to cite this article: Z. Chen, R. Sugimura, Y. S. Zhang, C. Ruan, C. Wen, *Aggregate* **2024**, *5,* e478. https://doi.org/10.1002/agt2.478

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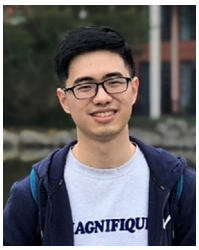

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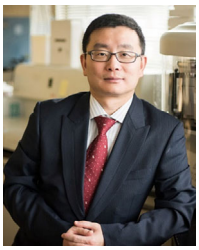

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