# Engineering multiscale structural orders for high-fidelity embryoids and organoids

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## ABSTRACT

Embryoids and organoids hold great promise for human biology and medicine. Herein, we discuss conceptual and technological frameworks useful for developing high-fidelity embryoids and organoids that display tissue- and organ-level phenotypes and functions, which are critically needed for decoding developmental programs and improving translational applications. Through dissecting the layers of inputs controlling mammalian embryogenesis, we review recent progress in reconstructing multiscale structural orders in embryoids and organoids. Bioengineering tools useful for multiscale, multimodal structural engineering of tissue- and organ-level cellular organization and microenvironment are also discussed to present integrative, bioengineering-directed approaches to achieve next-generation, high-fidelity embryoids and organoids.

# MAIN TEXT

## Introduction

Life is based on hierarchical structures spanning from cellular to organismal levels. Such a hierarchy of biological structures is characterized by multiscale orders that delineate how distinct structural units form, organize, and communicate at different length scales. Embryonic development is an essential process shaping the multiscale orders of life. However, how such orders are formed in mammals, especially in humans, remains mysterious. Filling this knowledge gap is essential for elucidating the fundamental relationship between growth, form, and function in humans, as well as for developing treatments of disorders that cause diseases, degeneration, and aging.

From a reductionist point of view, reconstructing mammalian embryogenesis *in vitro* provides an exciting approach to unveil the mechanisms for shaping the multiscale orders of life. In the past decade, stem cell-derived embryoids and organoids have been developed to recapitulate different cell lineage diversification and tissue morphogenesis events during embryogenesis (Fu et al., 2021; Kim et al., 2020c; Metzger et al., 2018; Rossant and Tam, 2017; Shahbazi et al., 2019; Shao and Fu, 2020; Wu and Belmonte, 2016). However, these embryo- and organ-like entities only recapitulate certain aspects of the multiscale orders manifested during embryogenesis. Their limited biological fidelity, with restricted developmental potential or tissue- or organ-level phenotypes and functions, not only hinders mechanistic understanding of natural developmental processes, but also hampers translational applications. Recently, through integrating bioengineering technologies, there is an emerging trend in the development of embryoids and organoids to reconstruct higher-order developmental events, including long-range tissue patterning and morphodynamics, tissue-tissue interactions, as well as organism-level organizations and functions. Embryoids and organoids with multiscale orders could also help reveal some previously unappreciated aspects of developmental programming, such as those *via* mechano-biological orchestrations.

In this review, we aim to put together a conceptual and technological framework useful for developing high-fidelity embryoids and organoids that display hierarchies in multiscale orders. From a structural perspective, we propose the concept of multiscale orders in mammalian embryogenesis based on distinct tissue structural units as well as their organization, communication, and progression through increasing spatial and temporal scales. Under this conceptual framework, we discuss recent trends in the development of embryoids and organoids that acquire higher-level orders through diverse bioengineering approaches. Based on key engineering principles emerging from recent progress, we further discuss different bioengineering tools and present a roadmap for building high-fidelity embryoids and organoids through multiscale, multimodal structural engineering of tissue- and organ-level cellular organizations and microenvironments.

## Multiscale orders in mammalian embryogenesis

Mammalian embryogenesis has long been viewed as a hierarchical process that unfolds through broad spatial and temporal scales. However, the traditional concept of the molecule-cell-tissue-organ hierarchy in development only reflects limited levels of structural orders, insufficient for resolving the multiscale orders instructing cells to assemble into complex tissues, organs, and organisms. This limited perspective could obscure our efforts in understanding and reconstructing the multiscale structural orders of mammalian embryogenesis.

Herein, we propose, from the viewpoint of structural engineering, the concept of multiscale orders in mammalian embryogenesis based on distinct tissue structural units as well as their organization, communication, and progression through increasing spatial and temporal scales. Specifically, we consider microscale orders as the ways that cells and local extracellular matrix (ECM) organize within a basic structural unit of a tissue. Classical developmental biology concepts such as cell fate specification, lumenogenesis, epithelial-mesenchymal transition (EMT), mesenchymal-epithelial transition (MET),

apical constriction, cell intercalation, and cell alignment, *etc*., are common examples of microscale orders involved in mammalian embryogenesis (**Figure 1A**). Towards a higher level, mesoscale orders are herein defined as the ways that multiple structural units (*i.e.*, microscale orders) organize and / or interact relative to each other, either within the same tissue or between different but interconnected tissues. Spatiotemporal changes of such organizations and interactions are also considered as mesoscale orders. For instance, long-range or periodic tissue patterning / morphogenesis (*e.g.*, body axis elongation, segmentation, self-similar branching, *etc.*), directional cell migration, tissue-tissue coupling (*e.g.*, vascularization, innervation, biomechanical or bioelectrical interactions), are prevalent examples of mesoscale orders in mammalian development (**Figure 1B**). Further up the scale, macroscale orders are herein defined as the ways that multiple mesoscale orders (*e.g.*, assemblies of tissue structural units) further organize and interact in relation to each other along additional dimensions in space and time, approaching the physiological size and architecture of tissues, as well as their communications, at the organ scale and beyond. Mammalian body plan, fetal-maternal interactions, circadian clock entrainment, postnatal development, and host-microbe interactions, are representatives of macroscale orders in mammalian development (**Figure 1C**). Altogether, this conceptual framework helps not only illustrate the structural orders and complexities of mammalian embryogenesis, but also integrate classical developmental biology concepts into a coherent, multiscale map to guide rationally designed structural engineering of embryoids and organoids with high-fidelity *in vitro*.

## Engineered high-order, high-fidelity embryoids and organoids

Existing embryoids and organoids recapitulate mostly microscale orders but little mesoscale or macroscale orders in mammalian development. Recent efforts have integrated bioengineering tools to achieve structurally guided organization of stem cells and their niche to form embryoids and organoids displaying mesoscale and macroscale structural orders. Here we review these progresses.

## Models of peri- and post-implantation development

Mouse embryonic stem cells (mESCs) and human pluripotent stem cells (hPSCs) can self-organize in 3D ECM matrix to form an "epiblastoid", a rudimentary model recapitulating peri-implantation proamniotic cavity formation within the epiblast (EPI) (Bedzhov and Zernicka-Goetz, 2014; Taniguchi et al., 2015). To engineer epiblastoids with mesoscale orders, ECM patterns and geometric confinement have been applied to shape epiblastoids into arbitrary morphologies (*e.g.*, tubular or branching) (Taniguchi et al., 2015) (**Figure 2Ai**). Soft matrix, micropatterns, and microfluidic devices have also been employed to structurally engineer differentiation and morphological reconfiguration of epiblastoids, mimicking mesoscale orders such as amniotic ectoderm (AE) morphodynamics and AE-EPI patterning (**Figure 2Aii-iii**) (Nasr Esfahani et al., 2019; Shao et al., 2017a; Shao et al., 2017b; Zheng et al., 2019b; Zheng et al., 2021). By assembling mouse epiblastoids with mouse trophoblast stem cells (TSCs) and extraembryonic endoderm stem cells (XENs), mouse embryoids with mesoscale orders have also been generated to recapitulate post-implantation organization and progressive development of embryonic and extraembryonic tissues, as well as anterior-posterior (A-P) patterning in early gastrulation (**Figure 2Aiv**) (Harrison et al., 2017; Sozen et al., 2018; Zhang et al., 2019).

Epiblastoids have provided mechanistic insights into microscale orders such as proamniotic lumenogenesis mediated by cell contractility and paracellular water flux (Kim et al., 2021; Taniguchi et al., 2017). Epiblastoids with mesoscale orders have further revealed autonomous, potentially mechanosensitive, mechanisms underlying AE development, as well as a critical role of AE-EPI crosstalk in mesoderm specification (Shao et al., 2017b; Zheng et al., 2019b), consistent with findings from primate embryos (Sasaki et al., 2016; Yang et al., 2021). Micropatterns and microfluidic technologies have also improved the standardization of epiblastoid-derived models such as the amnioid and post-implantation amniotic sac embryoid (PASE), important for mechanistic studies or high-

throughput screens (Nasr Esfahani et al., 2019). The morphological malleability of epiblastoids also provides tissue primordia with desirable morphological fidelity for generating different embryoids and organoids (Karzbrun et al., 2021).

Unlike in mouse embryoids, some critical mesoscale orders, *e.g.* a stably-formed A-P body axis, are still absent in aforementioned human epiblastoids (Simunovic et al., 2019), probably due to the absence of primitive endoderm-like tissues and thus proper embryonic-extraembryonic interactions (Sasaki et al., 2016; Stuckey et al., 2011). Incorporation of extraembryonic tissues in human epiblastoids to establish additional mesoscale orders is an important area awaiting future studies.

**Models of gastrulation and body axis development**

Formation of the primitive streak (PS) and the trilaminar germ disc containing the three definitive germ layers are prominent mesoscale orders manifested during the gastrulation. Conventional embryoid bodies could model some primitive features of the gastrulation with microscale orders, such as cell lineage specification and EMT. Recently, "2D gastruloid" recapitulating a PS-like thickened structure as well as concentrically arranged neuroectoderm, mesoderm, and endoderm domains has been generated with hPSCs and mESCs, respectively, on ECM micropatterns (Martyn et al., 2018; Martyn et al., 2019; Morgani et al., 2018; Warmflash et al., 2014) (**Figure 2Bi**). Interestingly, 2D gastruloids cultured on soft micropatterns form gastrulation-like foci rather than concentric ring-like domains, resembling the local initiation of PS development in early gastrulation (Muncie et al., 2020) (**Figure 2Bii**). Introduction of microfluidic morphogen gradients to 2D gastruloids induces axial, but not concentric, arrangement of the definitive germ layer domains, mimicking the linear apposition of the three germ layers *in vivo* (Manfrin et al., 2019) (**Figure 2Biii**).

The development of body axes and related trunk structures features multiple mesoscale orders such as tissue elongation, axial patterning, and periodic somite segmentation, which are absent in 2D

gastruloids. Recently, 3D gastruloids have been developed from free-floating mESC and hPSC aggregates, respectively, upon temporal modulation of exogenous morphogen signals (Moris et al., 2020; van den Brink et al., 2014) (**Figure 2Biv**), recapitulating tissue elongation and A-P patterning of neuroectoderm, mesoderm, endoderm, and anterior cardiac crescent domains. Upon extended culture under mechanical shaking, mesoscale orders would emerge in 3D gastruloids, including the formation of a gut tube-like structure and a spinal cord-like neuronal architecture (Olmsted and Paluh, 2021; Vianello and Lutolf, 2021) (**Figure 2Bv**). High-fidelity 3D gastruloids featuring multi-axial tissue patterning along two or more of the A-P, dorsoventral (D-V), and mediolateral (M-L) body axes have recently been generated *via* extended culture or assembly with an engineered signaling center (Beccari et al., 2018; Xu et al., 2021). The assembled multi-axial gastruloids exhibit additional mesoscale orders such as directional cell migration, long-range tissue folding in the neuroepithelium (NE), somite-like segmentation, and tissue vascularization, thereby exhibiting macroscale orders seen in the neurula-stage mouse embryo (Xu et al., 2021) (**Figure 2Bvi**). By embedding mESC-derived gastruloids in a soft matrix, bi-lateral somitogenesis, as well as a spinal cord- and a gut-like structures, have been induced (Umemuraa et al., 2020; van den Brink et al., 2020; Veenvliet et al., 2020) (**Figure 2Bvii**). Such trunk-like mouse gastruloids also exhibit oscillation of segmentation clock genes, a critical microscale order underlying the mesoscale somitogenesis. By inducing hPSCs towards presomitic mesoderm fate, human segmentation clock models have also recently been generated (Diaz-Cuadros et al., 2020; Matsuda et al., 2020).

Gastruloids have provided insights into the regulation of mesoscale orders in the gastrulation and body axis development. For example, 2D gastruloids have been applied to elucidate how mechano-biological coupling, *e.g.*, cell density-dependent receptor positioning (Etoc et al., 2016), dynamic signaling wavefront (Chhabra et al., 2019), and tissue stiffness- or geometry-controlled cell fate

regionalization (Heemskerk et al., 2019; Muncie et al., 2020), plays a critical role in germ-layer patterning. The assembled gastruloid also supports the functional role of a morphogen signaling center as an organizer – a classical concept first established in amphibians and birds – in organizing mesoscale order development in mammalian embryogenesis (Xu et al., 2021). Combined with single-cell transcriptomics, gastruloids also provide unprecedented opportunities for comparative studies on the gastrulation and body axis development between humans and mice (Moris et al., 2020).

The reproducibility and standardization of gastruloids remain unresolved challenges. Building high-fidelity human gastruloids with meso- and macro-scale orders, such as gastrulation-like directional, collective cell migration, multi-axial body patterning and morphogenesis, as well as trunk segmentation, would also be of major interest for future works. Such models would not only be valuable for studying classical developmental theories, such as the clock-wavefront segmentation model (Hubaud and Pourquie, 2014), in shaping mammalian embryos, but also provide unique opportunities for studying developmental disorders such as situs inversus and spondylocostal dysostosis.

**Models of neurulation and ectodermal organogenesis**

Neural rosettes have long been applied to model microscale orders, *e.g.*, cell fate diversification and neuroepithelial (NE) polarity, during neurulation (Elkabetz et al., 2008). However, neural rosettes fall short of capturing mesoscale orders in the neurulation. By culturing hPSCs on ECM micropatterns, "2D neuruloids" featuring concentric tissue domains have been generated to model M-L axial patterning of the neural plate (Britton et al., 2019; Tewary et al., 2019; Xue et al., 2018). Extended culture of micropatterned neuruloids further leads to a semi-3D structure, featuring a lumenal NE tissue enveloped by neural crest (NC) and non-neuroectoderm (NNE) cells as seen *in vivo* (Haremaki et al., 2019) (**Figure 2Ci**). Micropatterned 3D culture, microfluidic morphogen gradients, low-stiffness ECM matrix, as well as mechanical stretch have also been applied to generate neuruloids that exhibit mesoscale orders

such as bi-lateral or medial folding of the neural plate and its patterning along the A-P or D-V axis, respectively (Demers et al., 2016; Fattah et al., 2021; Karzbrun et al., 2021; Libby et al., 2021; Meinhardt et al., 2014; Ogura et al., 2018; Ranga et al., 2016; Rifes et al., 2020; Takata et al., 2017; Zheng et al., 2019a) (**Figure 2Cii-iv**).

As an important ectodermal organ, brain and its development have received considerable attention. hPSC-derived cerebral organoids (hCOs) have been established for modeling the development of different brain subdivisions (Jo et al., 2016; Lancaster et al., 2013; Mansour et al., 2018; Qian et al., 2016; Valiulahi et al., 2021). Recently, spinning bioreactors (Qian et al., 2016), microfluidic perfusion chips (Berger et al., 2018), and micro-confinements (Zhu et al., 2017) have been utilized to improve the scalability and reproducibility of hCOs. Geometrical confinement is also reported to affect the development of multiple neural lineages in hCOs (Sen et al., 2021) (**Figure 3Ai**). Organization of neurons into nerve tracts has also been demonstrated in hCOs under air-liquid interface culture or geometric guidance (Giandomenico et al., 2019; Kawada et al., 2017). To enhance mesoscale orders like cortical compartmentalization and axial patterning, polymer scaffolds and genetically engineered SHH-expressing signaling centers have also been applied to hCOs (Cederquist et al., 2019; De Santis et al., 2021; Lancaster et al., 2017) (**Figure 3Aii-iii**). To generate high-fidelity hCOs with cortical convolution, either physical confinement or accelerated neural progenitor proliferation has been used to introduce residual compressive stress in the cortical layer, causing periodic tissue folding through a buckling-like process (Karzbrun et al., 2018; Li et al., 2017) (**Figure 3Aiv**).

Models of neurulation and neural organogenesis have validated the roles of classical concepts such as apical constriction and morphogen signaling centers in driving human neural tube morphogenesis and axial patterning. They also revealed a shared, yet previously underappreciated, mechanosensitive properties of neural development, for which appropriate states of ECM stiffness,

tissue size and geometry, cell contractility, and residual stress are needed to regulate micro- and meso-scale orders, *e.g.*, NE polarity, neural plate folding, neural patterning, and cortical convolution (Fattah et al., 2021; Karzbrun et al., 2018; Karzbrun et al., 2021; Knight et al., 2018; Ranga et al., 2016; Xue et al., 2018; Zheng et al., 2019a). Micropatterns and light-induced genetic manipulation also endow enhanced controllability and standardization (De Santis et al., 2021; Karzbrun et al., 2021), enabling translational applications in modeling developmental abnormalities or drug screening.

However, there is still a lack of integration of multiple mesoscale orders in existing neuruloids or hCOs. This limitation obstructs the formation of macroscale orders, such as multi-axial patterning and morphogenesis of the neural tube, or the organization and morphodynamics of consecutive brain vesicles, which are utmost important hallmarks of early neurodevelopment. Engineering advances in the development of neuruloids and hCOs still await to be applied to reconstruct high-order, high-fidelity models for other ectodermal organs. Despite some pioneering work on stem cell-based models of eye development, advanced organoids for modeling the development of the eye, inner ear, and skin appendages remain to be demonstrated (Achberger et al., 2019; Decembrini et al., 2020; Eiraku et al., 2011; Gabriel et al., 2021; Koehler et al., 2013; Koehler et al., 2017; Lee et al., 2020; Mattei et al., 2019; Nakano et al., 2012; Okuda et al., 2018). Future works on these areas should broaden our understanding of complex neurodevelopmental diseases as well as advance regenerative treatments for ectodermal organs.

**Models of mesodermal organogenesis**

Human stem cell-based organoids have been generated for various mesodermal organs and tissues, including the heart, kidney, skeletal muscle, blood, vasculature, tonsil, cartilage, bone, testis, endometrium, and fallopian tube (de Peppo et al., 2013; Foltz et al., 2021; Jiang et al., 2021; Kim et al., 2020b; Montel-Hagen et al., 2019; Motazedian et al., 2020; Pendergraft et al., 2017; Sharma et al., 2020;

Turco et al., 2017; Wimmer et al., 2019a; Wimmer et al., 2019b; Yucer et al., 2017). Through proper mechanobiological engineering of the culture niche, heart and kidney organoids have been shown to recapitulate *in vivo*-like orders at multiple levels.

By culturing gastruloids or mesendoderm cell aggregates under mechanical shaking, or embedding hPSC-derived cardiac spheroids in a soft matrix, "cardioids" with multiscale structural orders have been developed to model the coordinated emergence of heart fields and the foregut (Drakhlis et al., 2021; Rossi et al., 2021; Silva et al., 2021) (**Figure 3Bi-ii**). Micropatterns have also been applied to generate cardiac chamberization models in a high-throughput format (Hoang et al., 2021) (**Figure 3Biii**). Furthermore, groove-like substrate topography (Lind et al., 2017), elastic tissue anchorages and mechano-electrophysiological training (Nunes et al., 2013; Ronaldson-Bouchard et al., 2018) could also instruct both micro- and meso-scale orders such as cell alignments, helical muscle fiber patterns (Fleischer et al., 2017), as well as regional patterning and communications along the atrioventricular axis (Zhao et al., 2019) (**Figure 3Biv**), showing greater fidelity to native tissue architecture and functional maturation in the heart. Similar approaches have also been extended to reconstruct multiscale orders in skeletal and smooth muscle models (Al Tanoury et al., 2021; Maffioletti et al., 2018).

To model kidney development, mESC-derived ureteric bud (UB) progenitors have been shown to undergo fractal-like branching at the presence of embryonic metanephric mesenchyme (MM) cells, generating high-fidelity models of the collecting duct system with mesoscale orders (Taguchi and Nishinakamura, 2017) (**Figure 3Bv**). hPSC-derived UB organoids, however, only exhibit limited branch bifurcation (Mae et al., 2020; Taguchi and Nishinakamura, 2017; Zeng et al., 2021). Kidney organoids manifesting multi-lineage renal cell diversification as well as mesoscale organizations of collecting duct, renal vesicles, proximal-distally patterned nephron tubules, and glomeruli have also been created from

both mESCs and hPSCs (Czerniecki et al., 2018; Morizane et al., 2015; Phipson et al., 2019; Taguchi et al., 2014; Takasato et al., 2015). Biophysical niche cues, such as ECM stiffness, tissue size and geometry, as well as fluid flow, have also been reported to promote the formation of mesoscale orders in kidney organoids, including renal vesicle formation (Garreta et al., 2019), kidney cystogenesis (Cruz et al., 2017), glomerular regionalization (Lawlor et al., 2021), and tissue vascularization (Homan et al., 2019) (**Figure 3Bvi-viii**).

Development of heart and kidney organoids demonstrates a common role of tissue-tissue interactions, such as those between the cardiac mesoderm and anterior endoderm, atrial and ventricle cardiac regions, as well as the UB and MM, in regulating multiscale orders in human heart and kidney development. Accompanied with single-cell multiomics, these organoids help reveal unique molecular networks underlying tissue co-development (Silva et al., 2021). They also enable disease modeling and drug screens wherein tissue- and organ-level phenotypes and functions are required (Drakhlis et al., 2021; Zhao et al., 2019). However, current human heart and kidney organoids fall short of exhibiting several important meso- and macro-scale orders, such as the folding and chiral looping of the heart tube, the four-chambered heart architecture, the proximal-distal organization of renal compartments, and the hierarchical branching of collecting ducts.

## Models of endodermal organogenesis

Endodermal organoids have been created from hPSCs to model the development of the intestine, stomach, esophagus, liver, biliary duct, pancreas, and lung (Chen et al., 2017; Dye et al., 2015; Gotoh et al., 2014; Huang et al., 2015; Huang et al., 2021; McCracken et al., 2014; Miller et al., 2019; Sampaziotis et al., 2017; Spence et al., 2011; Takebe et al., 2013; Trisno et al., 2018; Wu et al., 2019; Zhang et al., 2018b). Through sequential treatments with different soluble morphogens, region-specific human intestinal (hIOs) and gastric organoids (hGOs) have been generated, recapitulating characteristics

of different parts of the intestine and stomach, respectively (McCracken et al., 2017; Tsai et al., 2017) (**Figure 3Ci**). By modulating both static biophysical cues (*e.g.*, ECM stiffness, geometric confinement, and actomyosin cytoskeleton) and dynamic mechanical stimulations (*e.g.*, tissue stretch and fluid flow), micro- and meso-scale orders such as tissue morphogenesis, maturation, peristalsis, and endocrine secretion have also been enhanced in hIOs, hGOs, and hPSC-derived islet organoids, respectively (Cruz-Acuna et al., 2017; Hogrebe et al., 2020; Lee et al., 2018; Mamidi et al., 2018; Nair et al., 2019; Patel et al., 2021; Poling et al., 2018; Tao et al., 2019) (**Figure 3Cii-iv**).

However, several important meso- and macro-scale orders, *e.g.* axial patterning of the stomach / intestine, periodic morphogenesis of intestinal villi, tubular shape of the intestine and esophagus, asymmetrically expanded chamber of the stomach, intestinal looping, orthogonally apposed smooth muscle layers, airway branching morphogenesis, as well as the sequential assembly of multiple organs along the gastrointestinal (GI) tract, are still limited or absent in hPSC-derived endodermal organoids.

Of note, efforts in reconstructing meso- and macro-scale orders have been recently pioneered in adult stem cell (ASC)-derived GI organoids. For example, dynamic niche mechanics, as well as high-throughput screening, have been leveraged to optimize conditions for crypt morphogenesis in epithelial organoids derived from human intestinal stem cells (hISCs) (Brandenberg et al., 2020; Gjorevski et al., 2016; Hushka et al., 2020) (**Figure 3Cv-vi**). Using photo-patterned matrix mechanics, or scaffolds with protrusive and recessive structures that resemble the villus and crypt domains, intestinal organoids derived from hISCs with high fidelity and standardization on epithelial topography and cell fate patterning have also been generated (Gjorevski et al., 2022; Nikolaev et al., 2020; Wang et al., 2017) (**Figure 3Cvii**). These intestinal organoids are perfusible and can recapitulate epithelial homeostasis. Recently, bioprinted, centimeter-long linear aggregates of hISCs have been guided to form a tube-like GI organoid exhibiting proper A-P patterning and repeated crypt structures (Brassard et al., 2021)

(**Figure 3Cviii**). These GI organoids partially recapitulate macroscale orders of human GI mucosa, in a scalable and standardized format, thereby presenting attractive approaches for studies on epithelial homeostasis, host-microbe interactions, disease mechanisms, and drug screening. Given the recent progress in deriving hASC-like cells from hPSCs (Forster et al., 2014; Mithal et al., 2020; Takahashi et al., 2018), abovementioned strategies might be applicable for deriving high-order, high-fidelity endodermal organoids from hPSCs.

**Models of tissue-tissue coupling**

Tissue vascularization and innervation are needed for establishing mesoscale orders in organoid development and maturation (**Figure 3Di**). Tissue engineering strategies, including organoid-endothelial cell co-culture (Kitano et al., 2017; Takebe et al., 2015), 3D bioprinting of structured canalization in a tissue volume (Grigoryan et al., 2019; Hinton et al., 2015; Skylar-Scott et al., 2019b), and microfluidic chips featuring compartmentalized vasculogenesis (Salmon et al., 2021), have been utilized for generating tissue-vasculature coupling in various organoids. To recapitulate co-development of organ primordia and their vasculature, organoid-resident angiogenic niche and endothelial progenitor cells, as well as synthetic gene regulatory network for vasculature development, have been applied, demonstrating a critical role of biochemical and biomechanical tissue-tissue coupling in promoting organoid vascularization and maturation (Cakir et al., 2019; Homan et al., 2019; Low et al., 2019; Velazquez et al., 2020). Abovementioned strategies have also been applied to engineer tissue-nerve coupling (Giandomenico et al., 2019; Martins et al., 2020; Olmsted and Paluh, 2021; Osaki et al., 2020; Workman et al., 2017) and organized epithelium-muscle-nerve architecture (Eicher et al., 2022) in organoids.

Recently, structurally engineered tissue assembly has been utilized as a strategy to reconstruct meso- and macro-scale orders such as intra/inter-organ architecture and crosstalk, emergent interfacial

tissues, and long-range periodic morphogenesis. For example, assembled multi-tissue organoids, termed assembloids, have been developed to model tissue-tissue reciprocity and complex organ structures such as those found in the brain, bladder, kidney, and musculoskeletal organs (Andersen et al., 2020; Bagley et al., 2017; Birey et al., 2017; Kim et al., 2020a; Miura et al., 2020; Wang et al., 2021; Xiang et al., 2017; Xiang et al., 2019; Zeng et al., 2021) (**Figure 3Dii**). Assembling anterior and posterior gut organoids lead to the emergence of hepatic, biliary, pancreatic multi-organ anlages at the organoid interface (Koike et al., 2019; Marsee et al., 2021) (**Figure 3Dii**). Furthermore, tissue-scale periodic folding has been demonstrated through assembling spheroids of mesenchymal cells into prescribed positions within a thin ECM film (Hughes et al., 2018) (**Figure 3Diii**) modeling mesenchymal condensation-driven morphogenesis in epithelial organs such as the skin and the intestine (Glover et al., 2017; Shyer et al., 2013; Shyer et al., 2017). To recapitulate inter-organ communications *via* soluble signals, a variety of technological platforms, such as interconnected fluidic chips or robotic liquid handling, have been employed to control the cross-talk between different organoids (Edington et al., 2018; Novak et al., 2020) (**Figure 3Div**). There remains room for abovementioned models to improve and faithfully recapitulate meso- and macro-scale orders such as organ-scale compartmentalization, mechanobiological tissue coupling, and interfacial tissue emergence within complex organs.

## Models of mammalian embryo and organismal biology

To decipher the developmental autonomy of mammalian embryos, *in vitro* construction of stem cell-based "artificial embryos" has been an active research direction recently. Mouse blastoids resembling pre-implantation blastocysts have been generated using mESC-mTSC assembly, mouse extended pluripotent stem cell (mEPSC) aggregates, and mEPSC-mTSC assembly, respectively (Li et al., 2019b; Rivron et al., 2018; Sozen et al., 2019) (**Figure 3Ei**). Even though mouse blastoids could implant in the mouse uterus and form patterned deciduae, they show retarded or malformed phenotypes and fail to

develop beyond that equivalent to 6.5-8.5 dpc in natural decidualization, likely due to failure in assembling the Reichert's basement membrane (Sozen et al., 2019). Of note, a microfluidic chip has recently been developed to model first interactions between the mouse blastocyst and the maternal blood vessels (Govindasamy et al., 2021) (**Figure 3Eii**), suggesting opportunities to leverage *in vitro* engineered systems for studying the implantation and placentation. This effort might provide new mechanistic insights to promote the progressive development of implanted blastoids.

Recently, human blastoids were also generated using naïve hPSCs, hEPSCs, or reprogrammed cells, respectively (Fan et al., 2021; Liu et al., 2021; Sozen et al., 2021; Yanagida et al., 2021; Yu et al., 2021). Notably, triple inhibition of Hippo, TGF-β, and ERK pathways has been identified critical for efficient generation of blastoids with naïve hPSCs (Kagawa et al., 2021). Some human blastoids also show peri-implantation-like development *in vitro*. However, the developmental fidelity of these human blastoids remains limited (Sozen et al., 2021). There are also debates about the true identity of trophectoderm-like cells in the human blastoid generated by the reprogramming method (Zhao et al., 2021), highlighting the challenge in assigning cell fates in human blastoids using few and limited human reference datasets. High-throughput screens for fine-tuning initial cell states, initial cell aggregation sizes, and biological and mechanical properties of the developmental niche might help generate next-generation blastoids with improved fidelity and developmental potential, which will serve as valuable experimental tools to advance fundamental understanding of human blastocyst development.

Embryoids and organoids could provide new tools to investigate organismal biology of mammals, such as circadian clock development (Yagita et al., 2010), circadian entrainment for fetal organ maturation (Alvarez-Dominguez et al., 2020), cell state transition from fetal to neonatal and adult stages (Navis et al., 2019), and host-microbe interactions (Min et al., 2020; Nikolaev et al., 2020; Puschhof et

al., 2021) (**Figure 3Eiii-iv**). Although *in vitro* models for human organismal biology are still in their infancy, the time is now ripe for exploring human embryoids and organoids for such studies.

## Emerging engineering principles for building high-order, high-fidelity embryoids and organoids

From a retrospective view, we have observed several common engineering principles that have emerged from recent progresses towards high-order, high-fidelity embryoids and organoids.

Firstly, guided organization of stem cells, instead of their self-organization, could promote meso- and macro-scale orders in embryoids and organoids. Although stem cells possess innate self-organizing properties, allowing them to form tissue-like structures, unguided organization of stem cells mostly gives rise to tissue structural units (*i.e.*, microscale orders) in an uncontrolled, disorganized manner, thereby largely insufficient for modeling meso- or macro-scale orders in embryogenesis.

Secondly, structured tissue boundaries are critical for achieving meso- and macro-scale orders (**Figure 4**). Structured tissue boundaries could come in the form of gradients of tissue stiffness, geometry, forces, compositions, biological signals, gene expression activities, *etc.*, in both space and time. It should be noted, however, that microenvironments with spatial heterogeneity no larger than the scale of a cell (*e.g.*, ECM hydrogels or topographical substrates that contain uniformly distributed subcellular features) would appear to cells as an effectively "bulk" or "homogeneous" environment (**Figure 4A**). Thus, structured tissue boundaries discussed henceforth refer to those guiding stem cell organization with spatial heterogeneity greater than the cellular scale (**Figure 4B-C**).

Thirdly, mechano-biological coupling plays an important role in dictating meso- and macro-scale orders. Although mechanical cues have long been appreciated as potent regulators of microscale orders (*e.g.*, cell differentiation, migration, *etc.*), their roles in shaping meso- and macro-scale orders in mammalian development, especially in human development, are less clear. Recent progress discussed above provide undisputable evidence that spatiotemporal changes of either niche mechanics or tissue-

resident forces (tensile, compressive, or shear force) could induce mesoscale orders in diverse developmental models.

Fourthly, to develop spatiotemporally structured tissue boundaries and mechano-biological coupling, it requires engineering over multiple aspects of stem cells and / or their niche. Therefore, the capability of "orthogonal engineering" on multiple cell and niche parameters would be essential, which demands the integration of different engineering modalities (*e.g.*, micropatterns, microfluidics, light, synthetic genetic circuits, controlled tissue assembly, *etc.*) as those exemplified in recent studies.

Together, we propose multiscale, multimodal structural engineering (MUSE) of cells and niche signals as an emerging strategy for reconstructing high-order, high-fidelity embryoids and organoids.

## Bioengineering warehouse for multiscale, multimodal structural engineering of embryoids and organoids

The past two decades have witnessed the development and employment of novel bioengineering tools for manipulating biological processes spanning from single-molecule to whole-organ levels. However, it remains challenging to rationally adapt and apply these tools to reconstruct the multiscale orders of the natural development in embryoids and organoids. Recent advances and emerging applications of MUSE in embryoids and organoids have provided valuable insights about the unique applications of different engineering modalities and their combinations for reconstructing the micro-, meso-, and macro-scale orders of mammalian life, respectively (**Figure 4**).

### Engineering modalities for reconstructing microscale orders

As discussed above, self-organization of stem cells and their progenies often drives the microscale order development in embryoids and organoids. Stem cell self-organization is sensitive to the local cell microenvironment (**Figure 4A**). Besides modulating exogenous soluble factors in culture environments

to mimic *in vivo*-like biochemical signals (**Figure 4Ai**), a number of solid-state tools have also been applied to control bulk properties of stem cell culture environment to influence stem cell differentiation and self-organization *via* mechano-biological coupling (**Figure 4Aii**). For example, ECM-bound growth factors have been employed in bioengineered scaffolds to regulate stem cell fate and functions (Mitchell et al., 2016). Binding to polymeric backbones of scaffolds alleviates the low-stability of growth factors in a proteolytic-prone microenvironment. Binding of growth factors to the ECM could also alter their biochemical activities owing to mechano-biological coupling with cytoskeletal contractile forces (Stejskalova et al., 2019). The porous nature of biomaterials further adds another biomechanical signal potent for modulating mechano-biological interactions (*e.g.*, molecular tethering) between cell surface receptors and ECM proteins (Trappmann et al., 2012).

Mechanical stiffness of the ECM, which characterizes their bulk resistance against deformation under forces, has long been viewed as a potent regulator of stem cell fate and self-organization (Kratochvil et al., 2019; Shao and Fu, 2014; Shao et al., 2015). Recently, the viscoelasticity, or the stress-relaxation behavior, of ECM has also been identified as a biomechanical property affecting cell fate and functions (Chaudhuri et al., 2015; Chaudhuri et al., 2020). Of note, while ECM stiffness is a static constant property, viscoelasticity represents the ability of biomaterials to exhibit different levels of resistance to deformation depending on the rate of mechanical forces applied on it. Therefore, cell types with different contractile rates could exhibit different mechanoresponsive behaviors when sensing the same viscoelastic matrix owing to its "rate-dependent apparent mechanical stiffness" (Adebowale et al., 2021; Indana et al., 2021; Wei et al., 2020). Inspired by the adaptive nature of native ECM, stimulus-responsive biomaterials have been developed that could respond to environmental signals (such as hydrolysis, pH, nucleic acid, enzyme, redox condition, temperature, light, *etc.*) and undergo chemical or structural changes to facilitate versatile manipulation of cell behaviors (*e.g.*, differentiation, migration,

organization, *etc.*) (Badeau and DeForest, 2019). Recently, advances in assembling molecular "logic gates" lead to novel stimulus-responsive biomaterials through orthogonal molecular inputs (Badeau et al., 2018; Gawade et al., 2019; Zhang et al., 2020). To engineer ECM topography at a nano- and micro-scale, various technologies (*e.g.*, reactive ion etching, focused ion beam, colloidal assembly, electrospinning, *etc.*) have been used. The size, shape, spatial organization, and curvature of nano- and micro-scale topographical features have all been shown potent in regulating stem cell behaviors (Chen et al., 2014). Given their independent manufacturing processes, nano/micro-topography and aforementioned mechanical properties of hydrogel-based biomaterials can be combined to enable additional levels of modulation of the stem cell niche. Aside from above, other physical properties of the microenvironment, such as the electric conductivity and photothermal effect, have also been recently employed to modulate cell fate and functions (Carrow et al., 2020; Rocha et al., 2021; Xiong et al., 2021). These advances show the emergence of orthogonal, multimodal engineering of both biochemical and biomechanical properties of the stem cell niche for integrative programming of self-organized microscale orders.

So far, the design of abovementioned biomaterials relies largely on biomimicry and bioinspiration. It remains a major challenge, however, to optimize biomaterial functions or to translate current biomaterials to new applications. This bottleneck reflects our limited understanding on the role of intricate cell-ECM interactions in determining stem cell fate and functions and regulating native development, thereby restricting quantitative, rational biomaterial designs for embryoids and organoids. Recent advances in high-throughput technologies and data sciences have brought new opportunities to address these challenges through ECM library screening (**Figure 4Aiii**). In past decades, nanoliter synthesis and robotic spotting have been leveraged to create libraries of acrylate-based polymers (Anderson et al., 2004), native ECM proteins (Flaim et al., 2005), topographical features (Unadkat et al.,

2011), and PEG-based hydrogels (Gobaa et al., 2011; Ranga et al., 2014), respectively. These ECM libraries have been applied for culturing hPSCs, mESCs, as well as organoids. However, current ECM libraries still contain limited classes of biomaterials, hindering their extended applications. Expanding these ECM libraries would naturally be of interest. By integrating ECM library screening with artificial intelligence, it will be possible to achieve data-driven optimization of synthetic stem cell niche for controlling stem cell behaviors and their self-organization.

It is also possible to directly engineer stem cells to modulate their fate, function, and self-organization (Javdan and Deans, 2021; Mansouri and Fussenegger, 2021) (**Figure 4Aiv**). A synthetic Notch juxtacrine signaling system has been developed to generate artificial genetic programs that can lead to predictable self-organizing structures that are robust, reversible, and self-repairing (Toda et al., 2018). A synthetic Nodal-Lefty network has also been used to reconstruct an activator-inhibitor circuit, which could induce the formation of Turing-like patterns (Sekine et al., 2018). Given the rapid advances in gene editing and synthetic biology, engineering microscale orders in embryoids and organoids through rationally designed synthetic genetic circuits should be a promising future direction.

## Engineering modalities for reconstructing mesoscale orders

Mesoscale orders in embryoids and organoids are generated based on the organizations and interactions between multiple microscale orders (*i.e.*, tissue structural units). Spatiotemporally structured tissue boundaries often act to shape such mesoscale orders (**Figure 4B**).

Notably, micro/milli-fluidic systems recently emerged as powerful tools for programming mesoscale orders in embryoids and organoids given their ability to structurally engineer gradients of biochemical or biophysical cues using fluid-driven mechanisms (**Figure 4Bi**). For example, based on passive diffusion or multi-parallel laminar flows, gradients of soluble factors are generated in either linear (1D) or planar (2D) patterns (Atencia et al., 2009; Berthier and Beebe, 2014; Uzel et al., 2016).

Through proper design of flow inlets or internal compartmentalization, biochemical gradients with arbitrary profiles have been demonstrated in laminar flow-based devices (Allazetta et al., 2011; Dertinger et al., 2001; Irimia et al., 2006; Jeon et al., 2000; Kim et al., 2010). These micro/milli-fluidic systems could also be applied to generate gradients of molecules immobilized on culture surfaces or within a hydrogel (Allazetta et al., 2011; Jiang et al., 2005). Aside from biochemical gradients, micro/milli-fluidic systems have also been utilized to define spatiotemporally patterned biophysical cues such as shear stress or mechanical stretch (**Figure 4Bi**). On-demand modulation of the frequency, magnitude, directionality, spatial distribution, and throughput of shear flow and mechanical stretch, respectively, have been demonstrated (Mandrycky et al., 2020; Shemesh et al., 2015; Wasson et al., 2021; Xu et al., 2018; Xue et al., 2018; Yu et al., 2014; Zhang et al., 2014). During embryogenesis, shear stress and tissue stretch act as "mechanical morphogens" to regulate tissue- and organ-level developmental events, such as convergent tissue movement (Boselli et al., 2017), epithelial tube elongation (Conrad et al., 2021), smooth muscle-blood vessel coupling (Padget et al., 2019), intestinal muscle anisotropy (Huycke et al., 2019), and tendon-bone interface (Fang et al., 2020). However, the application of micro/milli-fluidic systems to reconstruct biomechanical gradients and guide stem cell organization to model such mesoscale orders *in vitro* remain a relatively underexplored area.

Structured physical boundaries could also be generated by non-fluidic systems to guide mesoscale order formation *via* spatiotemporally heterogeneous mechano-biological coupling (**Figure 4Bii**). For example, adhesive micropatterns to guide stem cell organization and emergent tissue patterning could be generated by either stamping, photolithography, or stencil technology with pre-defined geometry, size, and molecular composition (Knight et al., 2015; Théry, 2010; Wright et al., 2007). While micropatterns are often applied to 2D monolayer culture, they could also be used in 3D embryoids and organoids development (Hoang et al., 2021; Seo et al., 2021). Compared with 2D

micropatterns, microwells, often generated by soft lithography or photolithography (Romita et al., 2020; van der Putten et al., 2021; Whitesides et al., 2001), could provide a more 3D-like, or sometimes termed 2.5D, structural boundary to guide cellular organization. In addition, free-standing scaffolds with distinct internal structures are versatile tools to guide the shape and anisotropy of cell organization in 3D *via* contact guidance or tissue anchorage (Asmani et al., 2018; Fleischer et al., 2017; Legant et al., 2009). Furthermore, mechanical forces, induced by triggerable actuations or residual stress due to structural restraints, add another level of control over the spatiotemporal tissue boundary. To this end, contact-free mechanisms of mechanical actuation, *e.g.*, acoustic radiation (Fan et al., 2018; Topal et al., 2018), magnetic field (Kim et al., 2013; Mongera et al., 2018; Sniadecki et al., 2007; Uslu et al., 2021; Zhao et al., 2013), and photothermal effect (Sutton et al., 2017), have provided novel approaches given their manipulability in both space and time and compatibility with mammalian tissue cultures. It should be noted that above engineering tools can all be integrated with engineering modalities that can promote microscale orders in embryoids and organoids (**Figure 4A**). Together, they provide opportunities for orthogonal, multimodal engineering of both micro- and meso-scale orders in embryoids and organoids.

Directed assembly of pre-made embryoids, organoids, or other types of cell aggregates has also recently been demonstrated as an engineering modality for reconstructing mesoscale orders in mammalian embryogenesis (**Figure 4Biii**). Previous studies have mostly relied on manual assembly of cell aggregates *via* sequential sedimentation into microwells (Marton and Pasca, 2020). This strategy is simple to implement; however, it suffers from the difficulty in building tissue assemblies with elaborate morphologies. Assembly methods with pre-defined contact conditions, such as those using geometric templates or aspiration-based robotic positioning (Ayan et al., 2020; Zhao et al., 2019), have provided potential solutions to this challenge. In addition, programmable, non-contact tissue assemblies *via* magnetic manipulation (Jafari et al., 2019; Kim et al., 2013; Souza et al., 2010) and acoustofluidic

positioning (Ao et al., 2021; Ozcelik et al., 2018) have also recently been demonstrated for assembling cell aggregates or organoids. By decorating cell or tissue surfaces with biomolecules (*e.g.*, oligonucleotides) for complementary interactions, it could also enable structured tissue assembly with programmable spatial arrangement (Gartner and Bertozzi, 2009; Todhunter et al., 2015). Dielectrophoresis, a phenomenon depicting directional movement of dielectric particles in electric field gradients, has also been applied to assemble mESCs into pre-specified arrays of embryoid bodies (Ahadian et al., 2014). However, the potential of dielectrophoresis for assembling organoids into complex tissue assemblies remains to be examined.

In combination with synthetic biology and chemistry, physical fields (*e.g.*, optical, magnetic, thermal, and electric) have recently been leveraged to achieve spatiotemporal reconfiguration of cells and their niche in a triggerable, reversible, and non-invasive manner, guiding mesoscale order formation (**Figure 4Biv**). For example, optogenetic tools have been applied to engineer cell signaling, fate, and functions at designated space and time, giving rise to mesoscale orders such as long-range tissue patterning (De Santis et al., 2021; Repina et al., 2020), tissue bending (Guglielmi et al., 2015; Izquierdo et al., 2018), and directed cell migration (de Beco et al., 2018). Using engineered cells with heat-responsive genetic circuit, gradient temperature fields could also be applied to define 3D spatiotemporal patterning of gene expression in engineered tissue models (Corbett et al., 2020). By integrating with membrane depolarization-based transcriptional control, electrogenetic tools have also been developed for regional, dynamic, reversible modulations of cell functions (*e.g.*, insulin secretion) through bioelectric stimulations (Krawczyk et al., 2020). Recently, spatiotemporal reconfiguration of the mechanical and biochemical niche *in situ* have also been demonstrated *via* integrating structured illumination with synthetic photo-sensitive hydrogels (Gjorevski et al., 2022) or photothermal delivery systems (Yao et al., 2021). Altogether, these advances suggest powerful, yet underexplored, physics-

driven strategies that are potentially able to reconfigure cells and their niche in both space and time and promote mesoscale order formation. Integrated with recent breakthroughs in biophotonics and bioelectronics technologies (Floch et al., 2022; Jiang et al., 2019; Li et al., 2019a; Park et al., 2021), future investigations on how such physics-driven strategies could help drive mesoscale order formation in embryoids and organoids are warranted.

## Engineering modalities for reconstructing macroscale orders

To reconstruct macroscale orders, it requires engineering modalities to integrate multiple mesoscale orders, as well as microscale orders associated with them, in both space and time. Bioprinting represents a suitable strategy for this purpose, *via* selectively distributing "bioink" (*e.g.*, cells, biomaterials, growth factors, or combinations thereof) to: (1) define the niche for microscale order self-organization, (2) arrange microscale orders in programmable spatial or temporal sequences to shape mesoscale orders, and (3) further organize mesoscale orders to form macroscale orders (Gungor-Ozkerim et al., 2018; Mota et al., 2020; Murphy and Atala, 2014) (**Figure 4C**).

As a commonly used bioprinting method, direct ink writing (DIW) *via* mechanical extrusion (**Figure 4Ci**) is compatible with a wide range of bioinks to generate compartmentalized structures in a layer-by-layer scheme (Askari et al., 2021; Zhang et al., 2021). While it is straightforward to build tissue-level architectures by DIW, controlling cell-level interactions and organizations and developing microscale orders using DIW remain a challenge. To overcome such limitations, local parameters in DIW such as cell density and degradable ECM have recently been shown critical for stem cell self-organization and emergence of microscale orders in bioprinted GI organoids, exhibiting guided fusion into centimeter-long tubes defined by pre-specified tissue boundaries (Brassard et al., 2021) (**Figure 4Ci**). Thus, integrating the top-down manufacturing process of DIW and bottom-up self-organization of

stem cells to define structures and interactions at macro/meso- and micro-scales, respectively, should be a promising strategy to build high-fidelity embryoids and organoids in the future.

Although DIW can be extended to multi-material bioprinting, by using either mixing and switching nozzles (Hardin et al., 2015; Kokkinis et al., 2018; Liu et al., 2017; Ober et al., 2015) or a multi-nozzle array (Hansen et al., 2013), most existing DIW technologies still fall short of high-speed, voxelated multi-material bioprinting, which are critical for building tissue / organ models with high spatial heterogeneity within a short printability window. Using a pressure-driven fast-switching mechanism, single-nozzle and multi-nozzle printheads have recently been developed to address such a challenge (Skylar-Scott et al., 2019a), enabling continuous, multi-material, voxelated 3D printing of various soft materials (**Figure 4Cii**). Nonetheless, applications of this technology in constructing stem cell-based embryoids and organoids still await future investigations.

To reconstruct high-fidelity organ and tissue models with complex tubular topologies, such as the lung, vasculature, kidney, and mammary gland, it is critical to enable 3D interconnected and interwoven canalization in bioprinted constructs (**Figure 4Ciii**). Establishing a functional vasculature network in organoids is also necessary for promoting their maturation to recapitulate organ-level functions and phenotypes. For such purposes, sacrificial inks (or fugitive inks), which could be extrusion-bioprinted into a supporting matrix and then removed after the matrix is cured, has recent been developed (Bhattacharjee et al., 2015; Grosskopf et al., 2018; Hinton et al., 2015; Wu et al., 2011). Besides for creating vasculatures in acellular or sparsely cellular constructs (Kolesky et al., 2016; Miller et al., 2012; Wehner et al., 2016), 3D bioprinting with embedded sacrificial ink has recently been integrated with tissue matrix assembled from embryoid bodies to generated densely cellular constructs with perfusable, interconnected vasculature (Skylar-Scott et al., 2019b). In contrast to extrusion bioprinting that generates structures through serial deposition, highly parallelized photo-crosslinking has

been leveraged to enable rapid fabrication of complex vascular topologies in 3D hydrogel constructs using stereolithographic processes (Grigoryan et al., 2019; Heintz et al., 2016; Kumar and Kim, 2020; Ma et al., 2016). To minimize phototoxicity resulted from photoabsorbers, food dye additives have recently been identified as a promising class of candidates whose absorbance spectra encompass visible light wavelengths (Grigoryan et al., 2019). Using a manufacturing pipeline with stepwise dipping-photocrosslinking-rinsing cycles, stereolithography has also recently been extended to multi-material bioprinting (Grigoryan et al., 2021). Notably, although the abovementioned platforms could generate macroscopic, vascularized models of organs and tissues that are densely populated with cells (such as the heart and the liver), it remains a challenge to ensure physiological-like organizations and communications between each cellular components within the bioprinted volume, which are essential for correctly shaping the micro/meso-scale orders desired for high-fidelity organoids.

The above bioprinting modalities are compatible with organ-on-a-chip technologies (Lin et al., 2019; Yu and Choudhury, 2019; Zhang et al., 2018a). However, it is difficult to achieve on-chip dynamic modulation of stem cell niche using DIW-based bioprinting due to enclosed chip environments. In contrast, stereolithography might still be applicable for dynamic on-chip engineering of cell culture niches. In addition, subtractive manufacturing such as laser ablation might be useful for 3D sculpting of biomaterial-based stem cell niche *in situ* to help guide the formation of multiscale orders in organoid-on-a-chip models (Nikolaev et al., 2020) (**Figure 4Civ**). Integrating embryoids and organoids with microchip technologies should be a promising direction for future innovations towards translationally applicable *in vitro* models of human development and diseases.

## Challenges and opportunities

Despite recent progresses, current embryoids and organoids mostly recapitulate only part of the meso/macro-scale orders that make up a high-level biological process or phenotype of significance in

development, disease, or regeneration. It remains a challenge for current embryoids and organoids to simultaneously recapitulate both spatiotemporally registered cell fate specifications and tissue morphodynamics as seen *in vivo*. The large and expanding bioengineering warehouse now provides a technological framework to guide rational adaptation and integration of different orthogonal engineering modalities to reconstruct such multiscale orders in next-generation embryoids and organoids. These models, together with recent advances in single-cell multi-omics and spatiotemporal cell atlas regarding mammalian development, might constitute a new foundation for advancing embryo and organ engineering for basic research and translational applications.

Reproducibility and standardization remain challenging issues for embryoid and organoid research. These issues rise largely due to variations in initial states of the stem cell lines used in embryoid and organoid research (Phipson et al., 2019), as well as some stochasticity in gene expression that might cause inconsistent cell fate decisions during stem cell self-organization (Wennekamp et al., 2013). Therefore, by introducing structured tissue boundaries with conductive biochemical and biomechanical boundary conditions to provide quantitatively defined instructions to guide stem cell organization, it could help overcome the effects of such intrinsic variations and fluctuations, improving the reproducibility and standardization of embryoids and organoids (Brandenberg et al., 2020; Lawlor et al., 2021).

Cell lineage annotation is another challenge for embryoid and organoid research. Given recent controversies on the trophoblast differentiation potential of hEPSCs (Guo et al., 2021; Io et al., 2021; Posfai et al., 2021; Tan et al., 2021) and the purported trophoblast lineage in the iBlastoids (Zhao et al., 2021), it requires caution when performing lineage annotations based on limited data sets from early human embryo specimens. In addition, functional validation of the developmental potential of hPSC-based embryoids has been challenging. This is due to both ethical and technological issues restricting

current embryo culture systems to support long-term, high-fidelity development of human embryos / embryoids *in vitro*, as well as the prohibition of transplantation of human embryoids *in vivo*, which is otherwise a gold-standard assay used for stem cell-based mouse embryoids (Li et al., 2019b; Sozen et al., 2019). Embryo models derived from stem cells of non-human primates (Chen et al., 2021), which share close relationship to humans, might provide an alternate path for generating primate embryoids, whose developmental potential and biological fidelity could be thoroughly examined with both long-term *in vitro* culture and *in vivo* transplantation assays

Since development extends far beyond the moment of birth, organoid technology also provides opportunities to investigate the biology and medicine throughout the entire life cycle of humans. However, stem cell-based models for age-related diseases and conditions, especially those in children or elderlies, are still largely missing, due to the difficulty in faithfully recapitulating age-related phenotypes *in vitro*. Based on recent progresses, building a panoramic library of stem cell-based models for all stages of human life might help establish new platforms to improve our understanding and treatment of previously underexplored human health problems such as pediatric diseases and conditions, developmental origins of late-onset diseases, and aging.

## Conclusions

Embryoids and organoids have been held with high expectations to advance our understanding of human development and diseases, to revolutionize experimental tools for disease modeling and drug discovery, and to supply functional replacements for tissue and organ regeneration. All these expectations require embryoids and organoids to capture tissue- and organ-level phenotypes and functions beyond those exhibited in conventional tissue culture models. Following the conceptual framework of multiscale orders in mammalian embryogenesis, recent progresses have indeed achieved to reconstruct high-fidelity embryoids and organoids through engineering meso- and macro-scale orders. Recent advances have

further suggested the MUSE – multiscale, multimodal structural engineering – of cells and their niche as a promising strategy to construct high-order, high-fidelity embryoids and organoids *via* spatiotemporally structured tissue boundaries. Lessons from recent works have further inspired a technological framework to execute the MUSE strategy and guide rational, selective applications of different engineering modalities to reconstruct micro-, meso-, and macro-scale orders, respectively. Such a technological framework can be further expanded through integration with advances in related fields such as multiphysics and multifunctional biomaterials, automation and data sciences, as well as human-machine interface (or more specifically, organoid-machine interface). With the continuous evolution of stem cell-based embryoids and organoids, fueled by an expanding bioengineering warehouse, we envision a bright future for this field with fruitful applications in both fundamental and translational researches.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## FIGURE CAPTIONS

**Figure 1. Multiscale Orders in Mammalian Embryogenesis.**

From a structural perspective, a conceptual framework of multiscale orders in mammalian embryogenesis is proposed. It is based on (**A**) the formation of distinct tissue structural units and (**B&C**) their organization, communication, and progression through increasing spatial and temporal scales. This conceptual framework also integrates classical developmental biology concepts (as representatively shown herein) into a coherent, multiscale map to comprehensively illustrate the structural orders and complexities of mammalian embryogenesis. This conceptual map also serves to guide rationally designed structural engineering of high-fidelity embryoids and organoids *in vitro*. (Abbreviations: EPI, epiblast; ICM, inner cell mass; TE, trophectoderm; PrE, primitive endoderm; AE, amniotic ectoderm; MHP, medial hinge point; DLHP, dorsolateral hinge point; IVC, inferior vena cava)

**Figure 2. Engineered Evolution of Stem Cell Models of Early Embryogenesis.**

By leveraging diverse bioengineering approaches, high-order, high-fidelity stem cell models of early embryogenic milestones, including peri- and post-implantation development (**A**), gastrulation and body axis development (**B**), and neurulation (**C**) have been developed recently. In contrast to conventional models based on self-organization, recent evolution of embryoids, gastruloids, and neuruloids acquires meso- and macro-scale orders through guided stem cell organization, which is dictated by structurally engineered tissue boundaries. These models provide important platforms for understanding human embryonic programming, recapitulating developmental abnormalities, as well as embryo toxicology screening.

**Figure 3. Engineered Evolution of Stem Cell Models of Organogenesis and Organismal Biology.**

Self-organized, stem cell-based rudimentary models have been long used to recapitulate certain aspects of organ development. But they are mostly limited by their biological fidelity, reproducibility, and standardization. Recently, we witnessed an engineered evolution towards high-order, high-fidelity organoids, which is driven by different types of bioengineering technologies. Such evolution takes the advantage of guided organizations of stem cells dictated by spatiotemporally structured developmental boundary conditions. These models have shown meso- and macro-scale orders with greater similarities to the multiscale organizations and functionalities in ectodermal (**A**), mesodermal (**B**), and endodermal (**C**) organs. Meso- and macro-scale orders manifested in tissue-tissue coupling (**D**) and organismal biology (**E**) have also been recapitulated. Recent works have also highlighted the critical role of mechano-biological coupling in the development of high-fidelity organoids. These advances provide important foundations to attack basic questions in developmental biology and to facilitate translational applications in disease modeling, drug screening, and regenerative therapeutics.

**Figure 4. Bioengineering Warehouse for Reconstructing Multiscale Orders of Life**

Multiscale, multimodal structural engineering (MUSE) of the cells and their niche has recently emerged as a promising strategy for building micro-, meso-, and macro-scale orders in the development of high-fidelity embryoids and organoids. Guided by this strategy, the large and still expanding bioengineering warehouse has been categorized so as to highlight unique applications of different engineering modalities and their combinations for reconstructing micro- (**A**), meso- (**B**), and macro-scale orders (**C**), respectively. This bioengineering warehouse provides a technological framework to guide rational selection and orthogonal integration of engineering modalities to reconstruct multiscale orders of mammalian life, and therefore to advance high-fidelity embryoids and organoids.

# FIGURES

## Figure 1

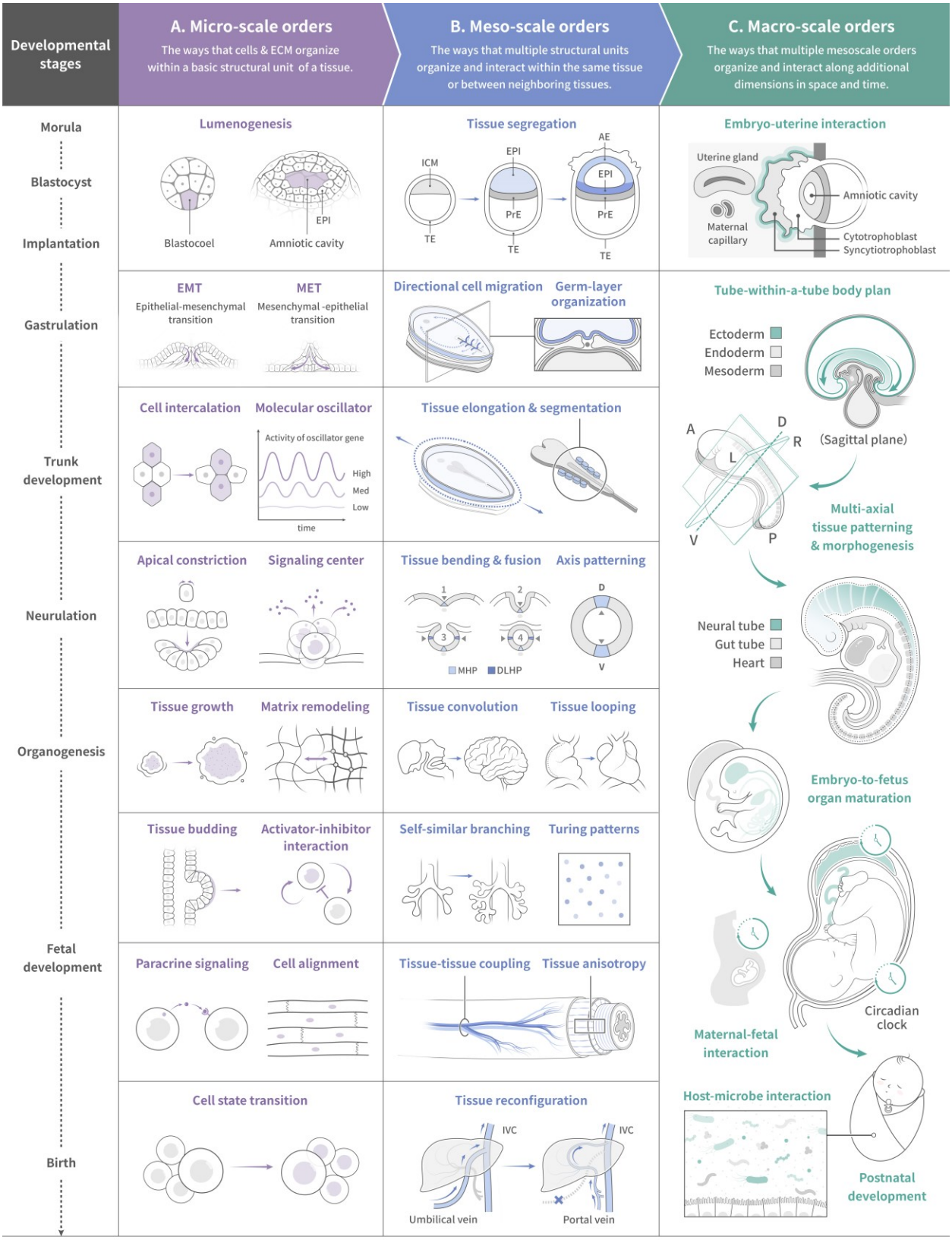


**Figure 2**

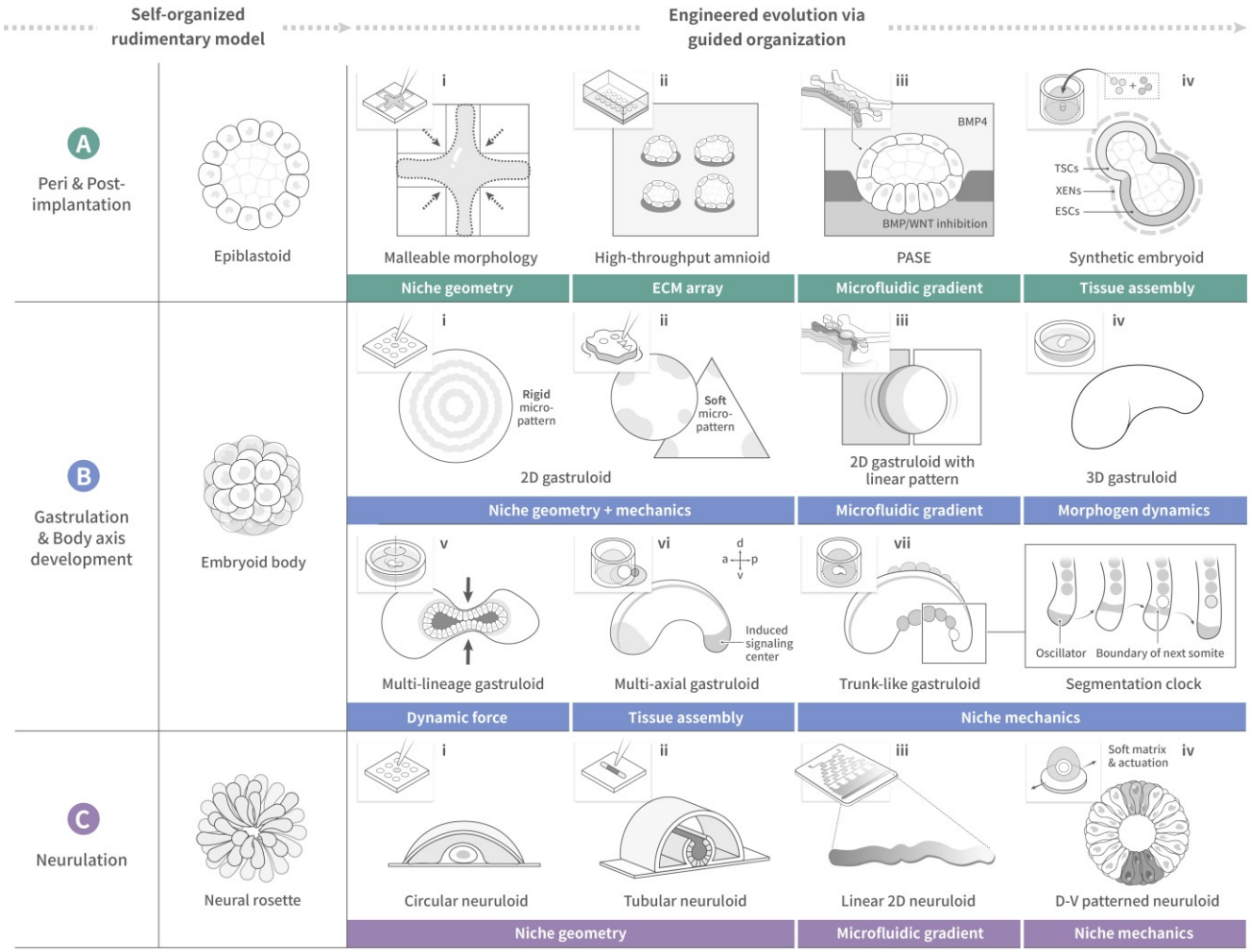

Self-organized rudimentary model
Engineered evolution via guided organization
A
Peri & Post-implantation
Epiblastoid
i
Malleable morphology
ii
High-throughput amnioid
iii
BMP4
BMP/WNT inhibition
PASE
iv
TSCs
XENs
ESCs
Synthetic embryoid
Niche geometry
ECM array
Microfluidic gradient
Tissue assembly
B
Gastrulation & Body axis development
Embryoid body
Rigid micro-pattern
Soft micro-pattern
2D gastruloid
2D gastruloid with linear pattern
3D gastruloid
Niche geometry + mechanics
Microfluidic gradient
Morphogen dynamics
v
Multi-lineage gastruloid
vi
d
a
p
v
Induced signaling center
Multi-axial gastruloid
vii
Trunk-like gastruloid
Oscillator
Boundary of next somite
Segmentation clock
Dynamic force
Tissue assembly
Niche mechanics
C
Neurulation
Neural rosette
Circular neuruloid
Tubular neuruloid
Linear 2D neuruloid
Soft matrix & actuation
D-V patterned neuruloid
Niche geometry
Microfluidic gradient
Niche mechanics

**Figure 3**

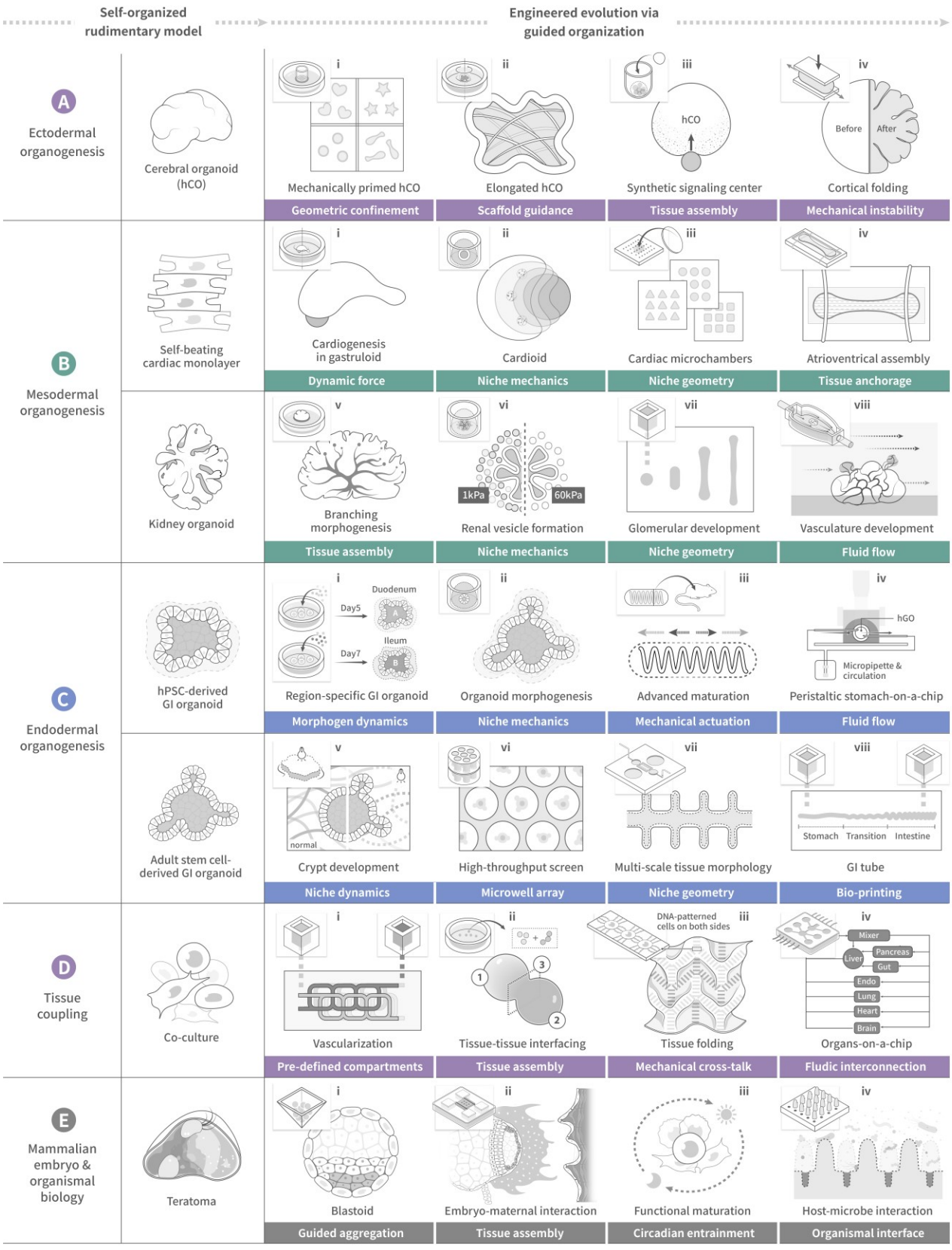
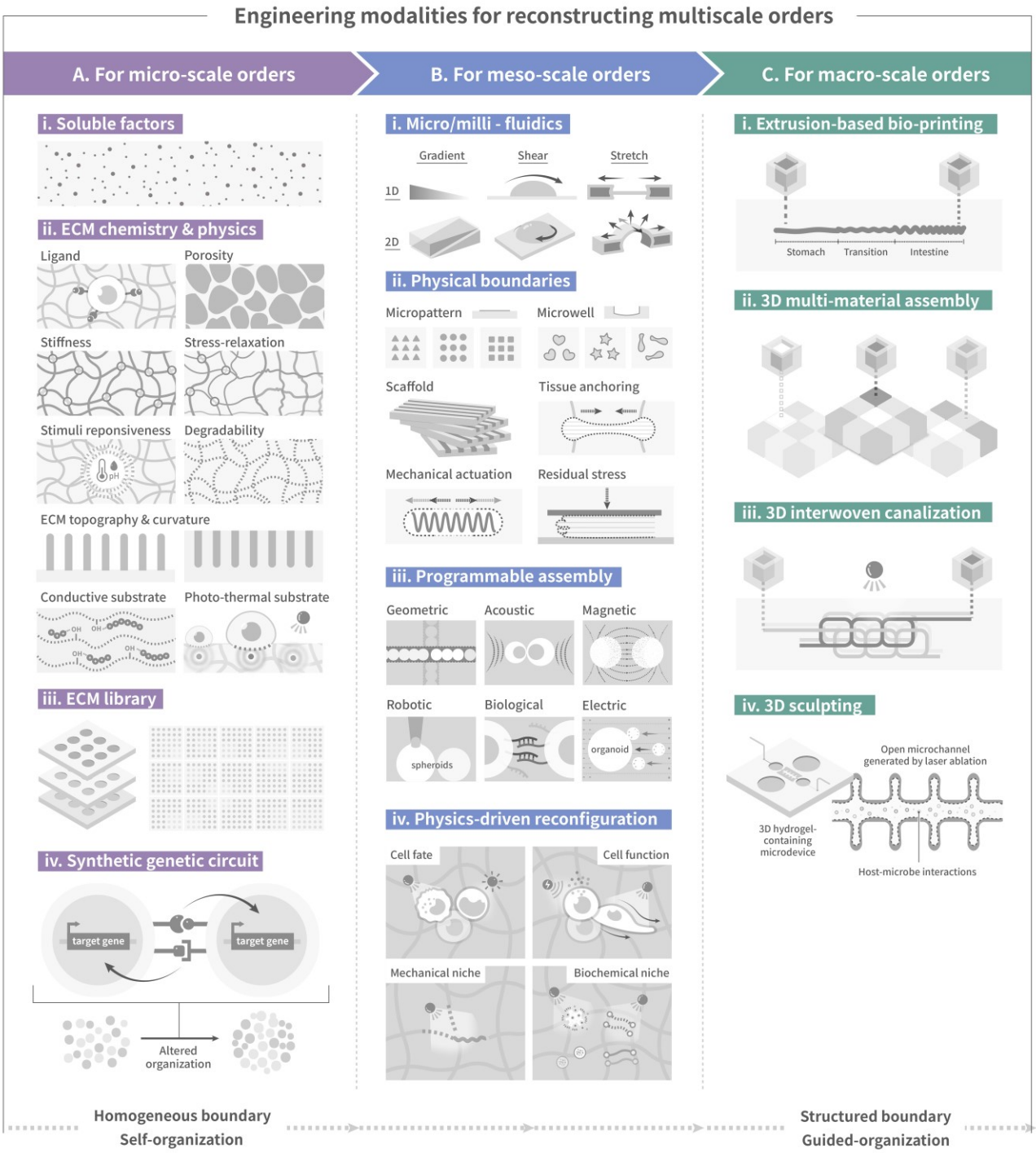

Self-organized rudimentary model
Engineered evolution via guided organization
A
Ectodermal organogenesis
Cerebral organoid (hCO)
i
Mechanically primed hCO
Geometric confinement
ii
Elongated hCO
Scaffold guidance
iii
hCO
Synthetic signaling center
Tissue assembly
iv
Before
After
Cortical folding
Mechanical instability
B
Mesodermal organogenesis
Self-beating cardiac monolayer
Cardiogenesis in gastruloid
Dynamic force
Cardioid
Niche mechanics
Cardiac microchambers
Niche geometry
Atrioventrical assembly
Tissue anchorage
Kidney organoid
v
Branching morphogenesis
Tissue assembly
vi
1kPa
60kPa
Renal vesicle formation
Niche mechanics
vii
Glomerular development
Niche geometry
viii
Vasculature development
Fluid flow
C
Endodermal organogenesis
hPSC-derived GI organoid
Duodenum
Day5
Ileum
Day7
Region-specific GI organoid
Morphogen dynamics
Organoid morphogenesis
Niche mechanics
Advanced maturation
Mechanical actuation
hGO
Micropipette & circulation
Peristaltic stomach-on-a-chip
Fluid flow
Adult stem cell-derived GI organoid
normal
Crypt development
Niche dynamics
High-throughput screen
Microwell array
Multi-scale tissue morphology
Niche geometry
Stomach
Transition
Intestine
GI tube
Bio-printing
D
Tissue coupling
Co-culture
Vascularization
Pre-defined compartments
Tissue-tissue interfacing
Tissue assembly
DNA-patterned cells on both sides
Tissue folding
Mechanical cross-talk
Mixer
Pancreas
Liver
Gut
Endo
Lung
Heart
Brain
Organs-on-a-chip
Fluidic interconnection
E
Mammalian embryo & organismal biology
Teratoma
Blastoid
Guided aggregation
Embryo-maternal interaction
Tissue assembly
Functional maturation
Circadian entrainment
Host-microbe interaction
Organismal interface

**Figure 4**


Engineering modalities for reconstructing multiscale orders
A. For micro-scale orders
B. For meso-scale orders
C. For macro-scale orders
i. Soluble factors
ii. ECM chemistry & physics
Ligand
Porosity
Stiffness
Stress-relaxation
Stimuli reponsiveness
Degradability
pH
ECM topography & curvature
Conductive substrate
Photo-thermal substrate
iii. ECM library
iv. Synthetic genetic circuit
target gene
target gene
Altered organization
i. Micro/milli - fluidics
Gradient
Shear
Stretch
1D
2D
ii. Physical boundaries
Micropattern
Microwell
Scaffold
Tissue anchoring
Mechanical actuation
Residual stress
iii. Programmable assembly
Geometric
Acoustic
Magnetic
Robotic
Biological
Electric
spheroids
organoid
iv. Physics-driven reconfiguration
Cell fate
Cell function
Mechanical niche
Biochemical niche
i. Extrusion-based bio-printing
Stomach
Transition
Intestine
ii. 3D multi-material assembly
iii. 3D interwoven canalization
iv. 3D sculpting
Open microchannel generated by laser ablation
3D hydrogel-containing microdevice
Host-microbe interactions
Homogeneous boundary
Self-organization
Structured boundary
Guided-organization