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Bioengineering for intestinal organoid cultures

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Abstract

Recent advances allow access to human cell-based intestinal organoids that recreate human physiology to levels not possible with conventional 2D cell cultures. Despite their huge potential, there are many challenges that remain. This review will cover recent bioengineering approaches to improve organoid maturation, scale up, reproducibility and analysis. The first section covers the advances in engineering the culture environment, followed by the section on tools for micro-manipulation and analysis of organoids. The last section reviews the computational models developed to guide the use of engineered materials and tools, and to interpret observed results as well. The ability to use organoids for discovery research, and the need to both exert exquisite experimental control and obtain quantitative measurements from organoid models means that the field is ripe for collaborative efforts between biologists, engineers, clinicians and industry.

Graphical Abstract

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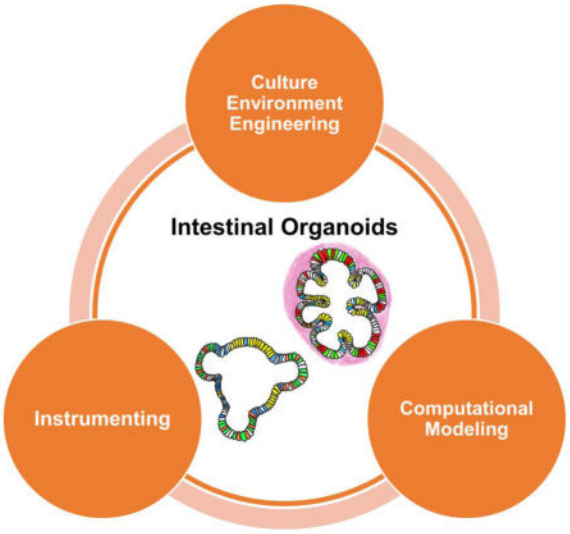

Introduction

Because of the importance of the gastrointestinal (GI) tract to health and disease, many *in vitro* and *in vivo* animal models and human tissue models of the GI tract have been developed.[1–3] Conventional *in vitro* models that rely heavily on 2D cell cultures, however, do not recapitulate the complex *in vivo* cell and tissue organization and are often representative of cancerous tissue. Animal models also fail to recapitulate much of human physiology and disease.[1] A breakthrough emerged in 2009 when Sato *et al.*[4] successfully cultured murine epithelial organoid from single $Lgr5^+$ intestinal stem cell (ISC) *in vitro*. Subsequently, human pluripotent stem cell(hPSC)-derived intestinal organoid (HIO)[5] and organoids derived from human normal and diseased biopsy samples [6,7] were developed. These 3-D, self-organized, physiologically-relevant cellular structures can be grown long-term and remain genomically stable. These systems have already proven useful in studies of GI tract development, homeostasis, human-microbiome symbiosis and pathogenesis.[1,3,8]

The speed at which organoid technologies have been developed and implemented in biomedical research is also accompanied by technical challenges and new opportunities. This short article highlights bioengineering solutions to solve unmet needs in intestinal organoid research, such as improving scalability and reproducibility, producing organoids with more mature or adult phenotypes, developing versatile functional readouts, and providing mathematical frameworks for analyses.

Engineering the culture environment of intestinal organoids

The Extracellular matrix (ECM)

The extracellular matrix (ECM) interacts with cells and tissue, providing both mechanical support and biochemical cues. Naturally-derived ECMs are readily available and widely used for intestinal organoid production and growth, with Matrigel (or similar products) being the most common commercially available products used.[4,5,9] However, there are several

limitation with these ECM products; they are often derived from tumor cell lines and have ill-defined properties, batch-to-batch variability, and non-intestine specific origin, better-defined Matrigel-alternative materials are actively sought after.[10,11]

Two additional examples of naturally-derived ECMs include Collagen I and decellularized intestinal ECM, which is the compositionally complex but intestine-specific. Collagen I has been used to culture both human and murine reconstituted intestinal organoids.[12,13] In a comparative analysis, gene expression in human epithelial organoids after 1 week of co-culture with intestinal subepithelial myofibroblasts embedded in Collagen I was relatively similar to Matrigel control[13] but cell monolayers were also formed in Collagen I, suggesting that the growth properties in different matrices are altered. Other differences in collagen I cultures include formation of a smoother epithelium and decreased budding.[14] In this context, it is interesting to note that matrix stiffness has recently been linked with maintenance of the stem cell identity, with softer matrices being required for cellular differentiation.[10] Recent work placed both murine colonic crypt and human rectal crypts on top of neutralized Collagen I rather than embedding in the gel, which formed 2D monolayers containing both proliferative and differentiated cells. These monolayers were capable of converting to 3D organoids when embedded in Matrigel and the opposite was also possible, up to five passages in murine colonic monolayers in particular.[15]

One perceived advantage of using decellularized ECM is that the 3D microstructure of the intestine and intestine-specific ECM molecules are preserved. [16,17] Interestingly, reseeding the decellularized human or porcine ECM with HIOs was shown to be possible; however, reseeded matrices did not assume the normal crypt-villus structure of the intestine, and, reseeded scaffolds did not persist when transplanted into immunocompromised mice. [16] In contrast, simply transplanting hPSC-derived intestinal organoids into mice led to remarkable engraftment and growth into crypt-villus structures.[18]

Synthetic ECMs have been designed to customize mechanical and biochemical properties, and create controlled environments. DiMarco *et al.*[19] developed an elastin-based engineered-ECM (eECM), where stiffness and adhesion site density could be independently controlled. Mouse organoid formation efficiency was similar to collagen I with the best efficiency observed with an eECM of 180 Pa mechanical stiffness and 3.2 mM of Arg-Gly-Asp (RGD) cell-binding sites. (Figure 1A) A minimal yet cell-supportive, defined ECM was engineered by the Lutolf lab using a chemically inert polyethylene glycol (PEG) polymer with adjustable stiffness as the base material and varying densities of key cell-adhesive components including RGD peptide and laminin-111.[10] This study showed that different matrix parameters are preferred at different stages of organoid formation; stiffer matrix was required during initial stage of ISC expansion then subsequent softening of the matrix was essential for continued expansion and organoid formation, and only laminin-111 supported budding of organoids whereas laminin-111-derived peptides did not.(Figure 1B) Interestingly, the reported optimal ECM mechanical stiffness for organoid formation is similar for both the elastin-based eECM[19] and the PEG-based synthetic ECM[10]. The use of artificial matrices with minimal components allows the deconvolution of relevant environmental cues, otherwise unachievable using naturally-derived matrices. While tubular scaffold of polyglycolic/poly-L-lactic acid (PGA/PLLA) demonstrated the potential to guide

expansion of HIOs mimicking human adult small intestinal architecture when implanted to mice[16], synthetic ECMs when used *in vitro* have yet to match Matrigel for their ability to support long-term growth and differentiation.

Microfabrication-assisted 3D culture

The intestine has villi and crypts that not only increase surface area but also form niches for stem cell self-renewal and differentiation. This has inspired man-made, intestinal villus and crypt-like, 3D microstructured culture substrates, which have also been used, either alone or in combination with ECM material to culture intestine cells. Initial demonstrations used immortalized intestinal cell lines[20–22] but the strategy also translates to culture of primary cells. These crypt-like domains in colonic organoids derived from both mouse and human; however, do not have discrete proliferative and non-proliferative regions, as would be the case *in vivo*.[7,23] Wang *et al.*[24] attributed this lack of spatial organization partially to the current colonic organoid expansion protocol not precisely recapitulating *in vivo* microenvironment, particularly the microstructure. An effort to combine ECM guidance cues together with topography led to the development of polydimethylsiloxane (PDMS) microwell arrays filled with Matrigel. Isolated individual crypts cultured in these hybrid microstructures expanded while maintaining a stem cell population within the Matrigel-filled microwells, while the exposed top surface between adjacent crypts became confluent with a differentiated epithelium (Figure 1C).

Micropatterned culture platforms have also been adopted to mimic the early development of embryonic stem cells [25,26]. For example, spatially confining stem cells cultured on a 2D surface into small islands leads to more homogenous self-organized germ layer differentiation.[25] Using a similar micropatterning approach, a xenogeneic-free protocol for generating HIOs was recently reported.[27] In contrast to conventional methods used to generate HIOs, which relies on chemical cues used in a stepwise manner to mimic different stages of embryonic development *in vitro* [5], the xenogeneic-free protocol utilized stochastic differentiation, which led to all of the germ layers to being incorporated into the organoids. Unique features of this method include spatially-segregated formation of more uniformly-sized HIOs and presence of mesoderm-derived smooth muscle cells and ectoderm-derived enteric neurons that collectively create peristalsis-like motility. Drawbacks to this method include the relatively inefficient generation of intestinal organoids, since many non-intestinal 3D structures are generated due the stochastic differentiation procedure used; gene expression profiles were highly varied within organoids owing partly due to a more complex cellular composition.

Instrumenting intestinal organoids

Modulating and measuring the organoid environment

Intestinal motility and luminal flow are key intestinal functions required for efficient nutrient absorption, waste secretion and sustaining stable host-microbe interactions.[28] While flow and some motility is relatively straightforward to incorporate in epithelial cell-lined microfluidic devices[29–32], it is much more difficult to perform long-term luminal perfusion of intestinal organoids while preserving organoid structure and function. Since

postnatal intestinal development and pathophysiology require host-microbiota-virus interaction, inoculating the intestinal organoids with bacteria and viruses is crucial part of many studies.[3]

Epithelium-only human organoids can be readily transferred from a 3D organoid culture onto 2D Transwell cultures where cells form a polarized monolayer.[33] With both apical and basal side of the epithelium exposed to fluid that is readily accessed by simple pipetting, co-culture with bacteria and measurement of transepithelial electrical resistance (TEER) is straight forward. (Figure 2A) [17,33,34] TEER measurements of primary intestinal cells are reported to exhibit physiological values whereas traditional cell culture lines, such as polarized Caco-2 cell monolayers typically have TEER values exceeding physiological values.[17]

Unlike epithelium-only patient-derived organoids, HIOs possess a mesenchymal layer and do not readily spread into clean epithelial monolayers on Transwells (our unpublished data). Thus, luminal side bacteria interaction requires microinjection of bacteria into the organoid. [35,36] As long as microinjection procedures are performed carefully, bacteria leakage is minimized.[35] The process is, however, labor intensive and inherently inefficient when a large number of organoids are involved.

High-throughput batch processing

Conventional methods of passaging, sorting, and analyzing intestinal organoids take time and require practiced skills. A number of recent efforts have aimed to overcome this limitation to enable higher-throughput handling. An example is the microfluidic filtering and trapping device designed for epithelium-only organoids.(Figure 2Bi)[37] In this work, Jin *et al.* performed multiplex measurements of epithelial swelling in response to external osmolality changes or secretory diarrhea-inducing viral toxins. Here, the change in external fluorescent fluid volume within a fixed field-of-view was monitored to indirectly measure the epithelial volume change held in place by PDMS pillars. This approach circumvents the potential error arising from a platform dependent on microinjection into organoids embedded within ECM. Such error can arise from optical path perturbations by the ECM thereby creating imaging errors, and from pressure build-up due to the surrounding ECM limiting expansion due to stiffness mismatches at the ECM and tissue interface. (Figure 2Bii)[37]

Another organoid arraying device was recently described to facilitate single cell-level spatio-temporal tracking of ISCs and Paneth cells (PCs).[38] In a polystyrene-coated PDMS microwells, cells in suspension are centrifuged to have a small population of cells per microwell (<7cells/well). Wells were subsequently filled with Matrigel and culture media. Cell-containing wells are tile-scan imaged over time. With this approach, a large number of replicated data were collected in a single data acquisition cycle and analyzed based on initial cellular composition, intercellular distance over time, and morphology evolution as the cells start to form organoids. For gene expression analysis, each cell/organid-containing magnetic polystyrene raft was released from the PDMS wells. These types of regularly patterned microdevices enable multiple data points from every well of the array with the assistance of

computer interface. These throughput capabilities enhance the likelihood that intestinal organoid systems can be scaled appropriately for industry applications.

Process engineering tools were also developed to sort hindgut spheroids based on morphological features, such as size and inner mass of the spheroids. The relationship between these features and maturation of the spheroids to intestinal organoids was used to optimize the paramaters for selecting spheroids.[39] The microscope used for the automated capillary-based sorting was instrumented with an image processing unit, a 3D stage control, and a harvesting unit. (Figure 2C) After the image processing unit determined which spheroid to harvest based on morphological features, the 3D stage control positions the tip of a glass capillary close to the spheroid of interest and pulls it up. The speed of the whole procedure is reported to be one spheroid per minute. Using this sorting method, the portion of spheroids that matured to be intestinal organoids were enriched from 13% to 20%, by size exclusion of spheroid diameter smaller than 75 μm, between day 5 and day 10. This approach may be adopted to culture more consistent intestinal organoid using various morphological features.

Computational modeling of intestinal organoid systems

Modeling morphogenesis

Several computational models of the morphological development of the gut have been developed. Buske *et al.*[40] modeled how proliferation-induced local shape and mechanical property fluctuation in epithelial intestinal organoids induce crypt-like domains where stem cells are localized. The model provides an explanation for how geometric and mechanical features of the culture environment is critical and why flat hard plastic dish culture may be unable to support organoid formation. Shyer *et al.*[41] simulated how villus morphogenesis is mechanically-coupled with muscle layer development during embryonic chick gut development. Circumferential compression and successive longitudinal compression applied to the epithelium by surrounding muscle layers result in the formation of longitudinal ridges and the formation of zigzag patterns and villi, respectively, within 2 weeks of fertilization. (Figure 3A) On the other hand, due to a thicker epithelium, it has been put forward that pattern formation in the mouse gut requires localized biochemical signals in addition to mechanical forces.[42–44]

After crypts have formed, crypt fission becomes a critical process for tissue growth and maintenance.[45,46] Computer-aided simulations of these processes have been reported.[47–49] In a recent study, Langlands *et al.*[49] studied the mechanisms of crypt fission from a cell population and biomechanical perspective using mouse the small intestine and organoids as the experimental test-bed. *In situ* imaging of mouse small intestinal crypt fission show Lgr5+ stem cells at the bifurcation sites with Paneth cells just adjacent to them. Weaker attachment to the basement membrane of Lgr5+ cells compared to that of Paneth cells, led to preferential buckling at stem cell clusters. Mouse epithelium-only organoid cultures allowed precise modulation of the number of Paneth cells relative to the number of stem cells. Experimentally, the authors found that having more Paneth cells created organoids with fewer crypt-like domains and changed the crypt shapes to be more spherical. In their computational model, however, where only the mechanical stiffness difference between the

two cell types were assumed, increased relative population of Paneth cells led to increased budding of crypt-like domains. (Figure 3B) This disagreement between experiment and model may be explained by formation of more Lgr5+ cell clusters via mitosis in the experiments; something not included in the model.[49] Temporal patterns of crypt budding has also been analyzed and modeled using epithelium-only organoids. Crypt budding in organoids were observed to occur every 12 hours. Although the physiological relevance of this finding is still controversial, computational models suggest that the circadian rhythm synchronizes this rhythmic cell cycle entry.[50]

Currently, modeling efforts related to organoid morphogenesis have focused more on mechanical mechanisms. As biomechanical interactions and biochemical signals are intimately coupled, morphogenesis models that integrate both aspects may provide further insights.

Analysis of the microenvironment

Microscale culture platforms limit the use of conventional macroscopic physical sensors for static and dynamic environment measures near tissue constructs. The *in vivo* intestinal oxygen (O_2) environment is very characteristic; however, little work has been done to understand the effect of O_2 microenvironments on *in vitro* intestinal organoid cultures. A recent study compared the O_2 gradient surrounding tissue-derived organoids in submerged or air-liquid interface (ALI) using a numerical simulation tool.[51] Combining measurement of O_2 concentrations right beneath the organoids, and Michaelis-Menten kinetics, oxygenation was visualized in different culture platforms. Organoids in ALI platform generally experience higher O_2 tension and smaller O_2 gradient than those in submerged cultures. Within 2 weeks of culture the organoids grew up to 1 mm and exhibit more circular morphology in ALI culture. (Figure 3C) It is still unclear whether such environment creates more physiologically relevant organoids. For organoid cultures with a fluidic component, flow patterns surrounding the tissue construct can be simulated.[17,37] Such an approach allowed the visualization of flow pattern change in response to tissue entrapment (Figure 2Biii,iv)[37], and shear stress calculations made to match experiments to the *in vivo* fluid flow in the luminal environment[17]. While these advances are encouraging, much work remains for microenvironment simulation technologies to be fully exploited for in-depth physiologic interpretations.

Conclusion

This short review highlights recent advances in bioengineering tools to create well-defined intestinal organoid cultures. Compared to the tremendous pace of scientific advances in the field of intestinal organoid biology, advances in bioengineering tools and methods for organoid manufacture, maturation, quality control, and analysis are less developed. Further integration of experiments with computational models should synergistically enhance intestinal organoid cultures to provide more biological insights as well as significantly reducing cost and time. Many opportunities exist for bioengineers to contribute, and the key will be the close communication and collaboration between engineers, biologists, and end-users of the intestinal organoids such as pharmaceutical companies.

Acknowledgments

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Highlights

- Synthetic extracellular matrix with appropriate cues can form intestinal organoids
- Microtechnology allows growing intestinal organoids in physiologic microenvironment
- High-throughput batch processing tools enable efficient organoid handling
- Simulation of morphogenesis and culture environment can complement experimental data

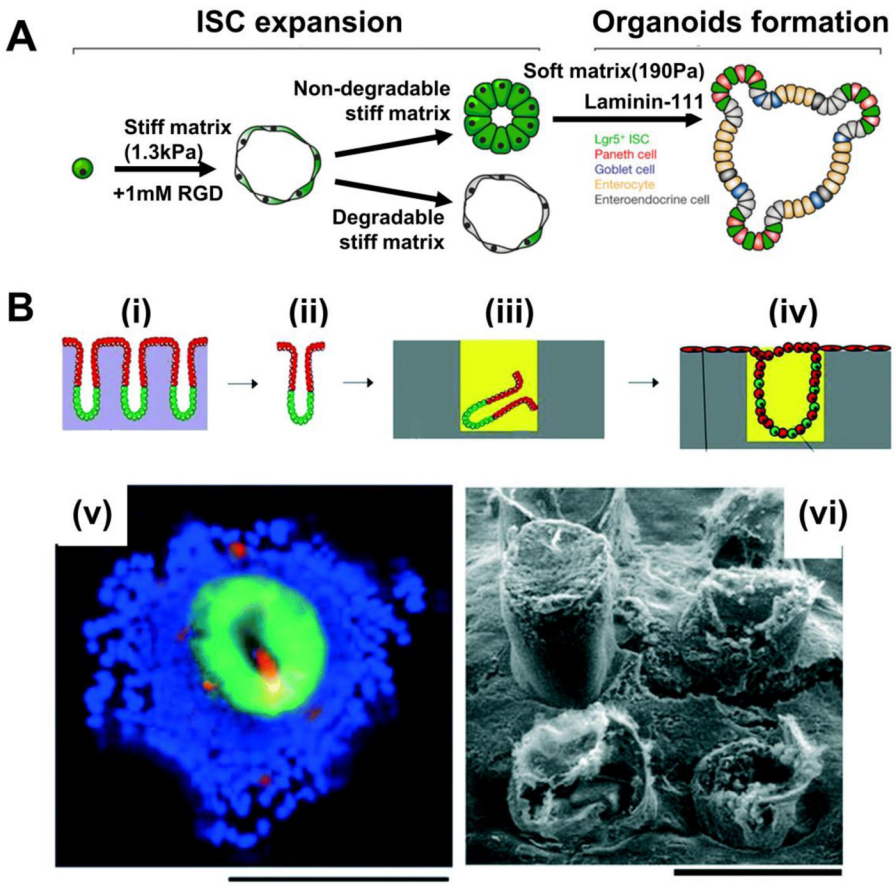

Figure 1. Engineering the culture environment of intestinal organoids

(A) Recombinantly engineered ECM with lower stiffness and high concentration of cell-binding sites increased organoid formation efficiency to levels comparable to collagen I cultures. Adapted from [19] with permission. Scale bar=50 μm.

(B) Optimal mechanical and biochemical properties of synthetic ECM should be modulated depending on the organoid formation stage (i) and confocal images of differentiated cells in the enteroid, grown in synthetic ECM (ii). Adapted from [10] with permission

(C) Schematics illustrating the experimental procedure for generating colonic epithelium using microstructured scaffolds: (i,ii) isolation of colonic crypt, (iii) embedding the crypt in Matrigel-filled microwell, and (iv) *in vitro* 2D/3D colonic epithelium with polarized proliferative and non-proliferative architecture. Immunofluorescence image (green = GFP, red = MUC2, blue = nuclei stain)(v) and scanning electron microscopy(iv) of the epithelium. Adapted from [24] with permission. Scale bars=200 μm.

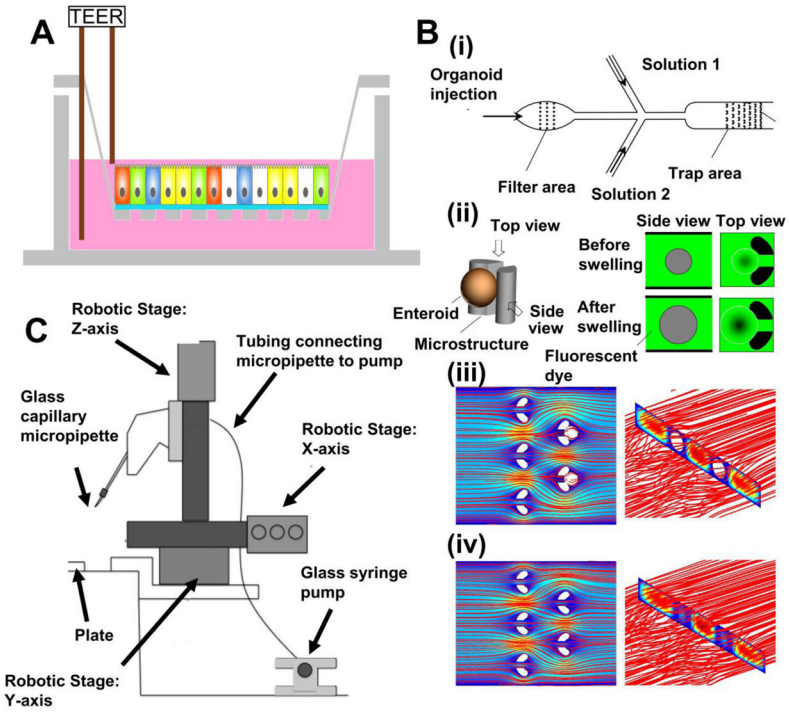

Figure 2. Instrumenting intestinal organoids

(A)TEER measurement across a monolayer created from enteroids.

(B) (i) Microfluidic device for trapping enteroids filtered based on size. (ii) Enteroid swelling indirectly measured by imaging the external fluid volume change in a fixed field-of-view. Numerical simulations of fluid flow patterns in the device with microstructure-trapped enteroids (iii) and without them (iv). Adapted from [37] with permission.

(C) Instrumentation for automated high-throughput sorting of intestinal organoids. Adapted from [39] with permission.

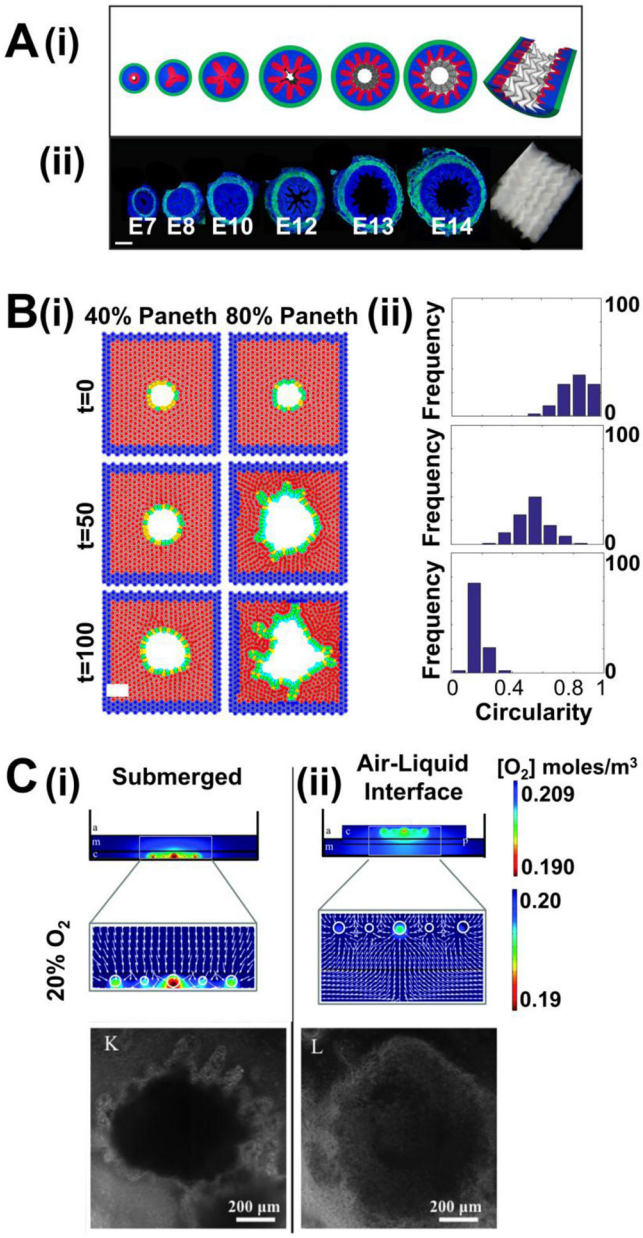

Figure 3. Computation modeling of the intestinal organoid systems

(A) Computational simulation of villification assisted by circumferential and longitudinal compression (green=muscle layer; blue=mesenchyme; red=endoderm) (i) and corresponding chick intestine dissections (blue = DAPI nuclei stain; green = αSMA smooth muscle actin stain) (ii). Adapted from [41] with permission. Scale bar=100 μm.

(B) (i) Mathematical modeling showing increased proportion of stiff Paneth cells in epithelium lead to buckling of the epithelium, hence crypt fission. (Blue=non-epithelial cells; red=Matrigel or stromal cells; yellow=Lgr5+ cells; green=Paneth cells) (ii) Histograms of final circularity index distribution with respect to Paneth cell composition. Adapted from [49] with permission.

(C) Numerical simulations for the oxygenation of tissue-derived intestinal organoids embedded in collagen (i, top) and cultured at an air-liquid interface (ALI) (ii, top) under ambient air. Brightfield images of organoids grown in submerged culture (i, bottom) and ALI culture (ii, bottom) for 2 weeks. Adapted from [51] with permission.