

DIRECTED DIFFERENTIATION OF GASTROINTESTINAL EPITHELIAL ORGANOIDS USING ATCC® CELLMATRIX BASEMENT MEMBRANE FROM MULTIPLE HUMAN ATCC iPSC LINES

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ABSTRACT

Three human iPSC lines were tested for their ability to generate gastrointestinal organoids using defined culture media in a 2D/3D culture system using ATCC CellMatrix Basement Membrane. Starting from monolayer cultures, iPSCs were driven towards a fore- or antral-gut fate using various niche factors. After 4 weeks in culture organoids expressed tissue-relevant markers indicating the presence of multiple differentiated cell types including secretory goblet cells, absorptive enterocytes, and paneth cells (Figure 1).

INTRODUCTION

Organoids are complex, 3D organ-like microtissues comprised of multiple differentiated cell types that are structurally organized similar to that found *in vivo*, with central lumens and other *in vivo*-like architectural features. Organoids generated from induced pluripotent stem cells (iPSCs) have been described for various tissues including gastric¹, intestine², cerebrum³, optic cup⁴, lung⁵, pituitary⁶, kidney⁷, and liver⁸.

Organoids are an attractive alternative to other *in vitro* models; better recapitulating the *in vivo* tissue phenotype than 2D cell culture or simple 3D spheroid aggregates, but without the challenges associated with organ explants or tissue slices such as a limited lifespan in culture. In fact, organoids retain many favorable features typical of cell culture, such as the ability to maintain stable cultures for months, as well as a capacity to cryopreserve and recover cultures. Organoids are also amenable to many standard laboratory techniques including genetic modification with CRISPR/cas9, immunofluorescent staining, RNA expression quantification, western blot, and toxicity assays.

Organoid cultures have been utilized for both basic and translational research. For example, cerebral organoids have served as models for microcephaly³ and Zika virus infection⁹. Intestinal organoids have been demonstrated to engraft *in vivo*, utilized as a model for cystic fibrosis¹⁰ using patient derived lung-iPSCs, and to explore host-bacterial interactions with Salmonella¹¹. Kidney organoids have been used to study nephrotoxicity⁷ and gastric organoids were shown to be a suitable model for *H. pylori* infection¹.

Here we report a protocol for the generation of gastrointestinal (GI) organoids from three ATCC human iPSC lines (Table 1) utilizing a combination of 2D and 3D culture methods (Figure 2) in a defined media.

METHODS

iPSC culture

The iPSC lines used are listed in Table 1. All iPSCs were routinely cultured according to the ATCC human iPSC culture guidelines¹³. Briefly, iPSCs were maintained on CellMatrix Basement Membrane Gel (CellMatrix; ATCC® ACS-3035™)-coated dishes in feeder-free and serum-free conditions using Pluripotent Stem Cell SFM XF/FF (ATCC® ACS-3002™) medium. The medium was changed daily. During routine iPSC culture, the cells were passaged every 3-5 days using Stem Cell Dissociation Reagent (ATCC® ACS-3010™) and seeded as small aggregates. For organoid generation, low passage iPSCs were used that showed no signs of spontaneous differentiation.

ATCC® No.	Initial Cell Type	Description	Reprogramming Method
ACS-1019™	Foreskin fibroblast	Human Male	Sendai virus
ACS-1024™	CD34+ Bone Marrow Cell	Human African American Male	Sendai virus
ACS-1031™	CD34+ Bone Marrow Cell	Human Asian Female	Sendai virus

Table 1. Summary of iPSC lines used to generate organoids.

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Notes

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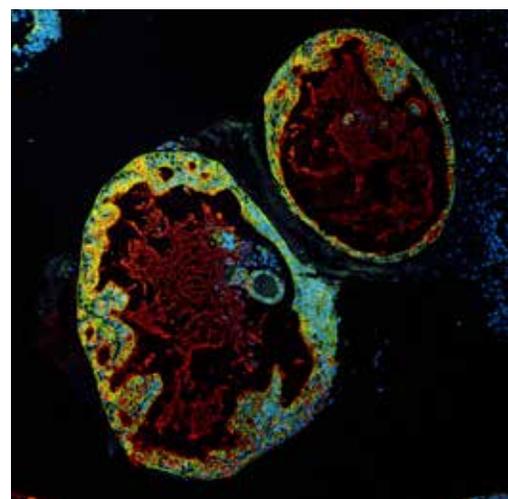


Figure 1. iPSC-derived organoids.

Definitive endoderm differentiation

All niche factors were reconstituted according to the suppliers' instructions and stored as single use aliquots at -80°C.

iPSCs were seeded into CellMatrix-coated 24-well multiwell plates and grown to 30-50% confluence. The media was then replaced with definitive endoderm (DE) differentiation media consisting of RPMI-1640 (ATCC® 30-2001), B27 supplement (Gibco), and 100 ng/mL activin A (R&D Systems) for three days. On the first day the DE medium also contained 2 µM CHIR99021 (R&D Systems). The culture medium was made fresh and changed daily for three days.

Hindgut spheroid generation

Following DE induction mid/hindgut spheroids were generated by four days of culture in RPMI-1640, B27, 100 ng/mL activin A, 3 µM CHIR99021, and 500 ng/mL fibroblast growth factor 4 (FGF4; R&D Systems). The media was made fresh and changed daily.

Posterior foregut spheroid generation

Following DE induction posterior foregut spheroids were generated by three days of culture in RPMI-1640, B27, 2 µM CHIR99021, 500 ng/mL FGF4 and 200 ng/mL noggin (R&D Systems). On the first day the media also contained 2 µM retinoic acid (Sigma). The media was changed daily. Media was made fresh every day.

Intestinal organoid generation

Mid/hindgut spheroids were collected and re-suspended in ice cold, undiluted CellMatrix. Approximately 10-20 spheroids were plated per well of a 24-well plate in 50 µL drops. After plating the plate was kept at 37°C for 15 minutes to polymerize the gel, forming a 3D dome within the center of the well. The solidified gel was overlaid with pre-warmed complete intestinal organoid media consisting of Advanced DMEM:F12, N2 supplement (Gibco), B27 supplement, L-Glutamine (ATCC® 30-2214™), 10 mM HEPES (Sigma), 100 ng/mL epidermal growth factor (EGF; R&D Systems), 100 ng/mL noggin (R&D Systems) and 500 ng/mL R-spondin-1 (R&D Systems). Media was changed every 3-4 days and organoids were passaged by mechanical dissociation every 7-14 days into fresh CellMatrix. Complete media was stored for no more than three days at 4°C.

Gastric organoid generation

Posterior foregut spheroids were collected and re-suspended in undiluted CellMatrix. Approximately 10-20 spheroids were plated per well of a 24-well plate in 50 µL drops. After seeding, the plate was kept at 37°C for 15 minutes to polymerize the gel, forming a 3D dome within the center of the well. The solidified gel was overlaid with pre-warmed complete gastric organoid media consisting of Advanced DMEM:F12, N2 supplement, B27 supplement, L-Glutamine, 10 mM HEPES, 100 ng/mL EGF, 200 ng/mL noggin, and 2 µM retinoic acid. Media was changed every 3-4 days and organoids were embedded in fresh CellMatrix every 7-10 days. Complete media was stored for no more than three days at 4°C.

Immunofluorescence and immunohistochemistry

Cell monolayers, spheroids, or tissues were washed twice in Dulbecco's Phosphate Buffered Saline (PBS; ATCC® 30-2200™) and fixed in 4% paraformaldehyde for 15 to 30 minutes at room temperature. Fixed spheroids/organoids were embedded in paraffin, sectioned at 5-10 µm, and mounted on slides for subsequent staining. For immunofluorescent staining, cell monolayers or tissue sections were blocked and permeabilized in PBS containing 5% donkey serum, 0.5% Triton-X, 0.2% Tween-20, and 1% bovine serum albumin for 1 hour at room temperature. Primary antibodies were diluted in blocking buffer and incubated with cells overnight at 4°C. Fluorescent secondary antibodies were diluted in blocking buffer and incubated with cells for 1 hour in the dark at room temperature. Histological staining with hematoxylin and eosin (H&E) and PAS-Alcian Blue was performed using standard protocols.

RESULTS AND DISCUSSION

In vivo the DE gives rise to the digestive tract and is a critical first step of the GI organoid developmental process. Immunostaining revealed greater than 80% expression of the characteristic DE markers FOXA2 and SOX17, and minimal expression of the mesoderm marker brachyury (data not shown) following three days in DE differentiation media (Figure 3).

After 3 to 4 days in the fore- or hindgut differentiation media, floating or semi-adherent spheroids as well as adherent, elongated tube-like structures formed (Figure 4). Spheroids produced by the hindgut differentiation media were positive for the intestinal marker CDX2 but negative for the hindgut marker SOX2, while spheroids produced from foregut differentiation media were CDX2-/SOX2+.

Within seven days after spheroids were embedded in CellMatrix and maintained in organoid generation media, large simple round organoids became visible, approximately 100-200 µm in diameter (Figure 5). By day 30, the organoids had greatly increased in size and complexity, with diameters of 1500-2000 µm and many visible tube and crypt/bud-like structures (Figure 6).

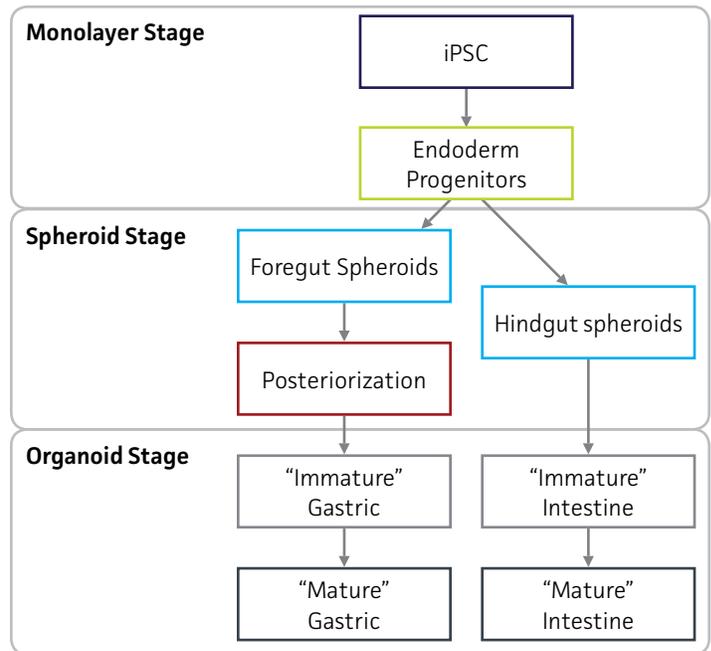


Figure 2. Summary of the workflow utilizing a 2D/3D culture system for the generation of GI organoids from human iPSCs.

Immunostaining of day 30+ organoids revealed complex folding architecture, columnar epithelium, crypt-like structures, and expression of numerous gastrointestinal markers (Figure 7) including foregut marker PDX1, hindgut marker CDX1, MUC1 and MUC5AC positive mucin secreting cells, lysozyme (LYSO)-positive paneth cells, and villin (VILL)-positive enterocytes. The cells surrounding the organoids contained a heterogeneous population of cells expressing vimentin (VIM), fibronectin, and smooth muscle alpha actin (α -SMA). Mucin expression was restricted to goblet cells and the central lumen of the organoids, which also contained dead cells and debris (Figure 8). Histological staining with H&E and PAS-Alcian Blue showed the presence of both acidic and neutral mucins, as well as a tissue-like morphology within mature organoids (Figure 9).

Organoids can more faithfully recapitulate complex physiological structures than simple 3D spheroids or traditional 2D culture models. However, organoids are not without their limitations. While iPSC-derived GI organoids exhibit many features of *in vivo* tissue, they do not fully capture the phenotype of mature tissue. For example, they lack many aspects of *in vivo* tissue such as vasculature and interactions with the nervous and immune systems. Further, while organoids display *in vivo*-like cellular organization, it is not as consistent as that found in primary GI tissue and any individual organoid may vary significantly in size, complexity, viability, and expression of specific cell types. While the differentiation media utilized to generate organoids may be fully defined, most organoid protocols rely on an undefined, mouse-derived extracellular matrix, which limits their clinical applications. Despite these challenges, organoid culture methods represent a useful tool for increasing our understanding of organ development and hold promise for personalized medicine approaches such as in drug screening or regenerative medicine.

Organoid generation critically relies on the contribution of various niche factors and the temporally restricted activation of specific tissue-appropriate signaling pathways. We found significant lot-to-lot and supplier-to-supplier variability in the activity of many of the recombinant proteins and small molecules utilized in the protocol. Optimization of the concentrations of these niche factors and testing material from multiple suppliers is vital to ensure successful organoid generation and should be the first step in troubleshooting. Additionally, organoid generation depends on a specific sequence of developmental events that is heavily dependent on the prior stages. In particular, if the initial step of DE formation from iPSCs is not 80-90% we found organoid generation to be generally unsuccessful. Finally, some differentiation markers are not evident until organoids have reached a certain stage of maturity; we recommend maintaining GI organoids in culture for at least 30 days and in some cases 60+ days may be required for the full complement of cell types to be present.

CONCLUSIONS

Human iPSC-derived organoids are an exciting new model for studying organ and tissue development, function, and disease. Here we report that ATCC provides iPSC lines and reagents that support the generation and maintenance of gastric and intestinal organoids using defined media conditions. These organoids are suitable for use with many standard experimental techniques (e.g., immunohistochemistry), can be generated from a diverse panel of iPSC types, and provide physiologically relevant complexity not captured in traditional 2D monocultures.

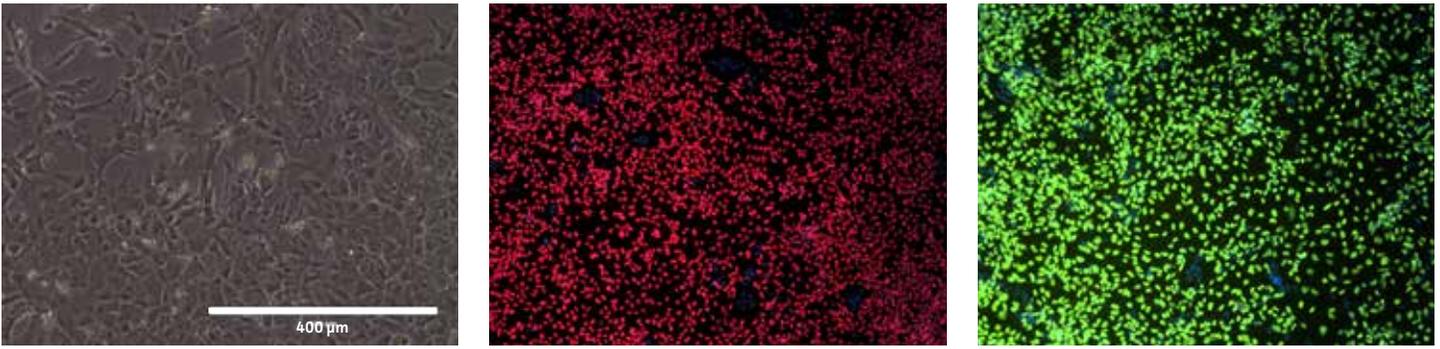


Figure 3. Definitive endoderm generation from ATCC iPSCs. Phase contrast (left) and immunofluorescent staining (middle, right) showing expression of definitive endoderm markers SOX17 (green) or FOXA2 (red) after iPSCs were cultured for 3 days in definitive endoderm media.

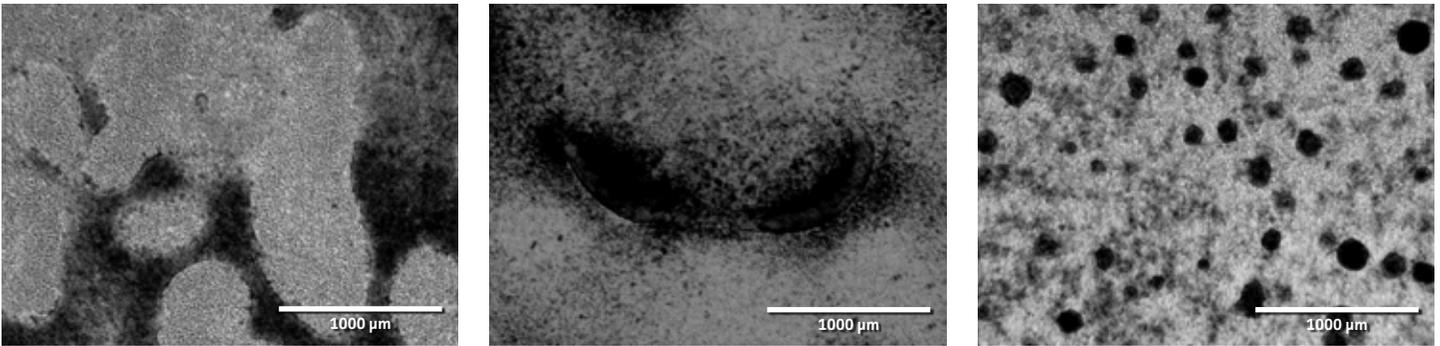


Figure 4. 2D to 3D culture. Phase contrast images following gut morphogenesis of definitive endoderm monolayer resulting in adherent tube-like structures (left, middle) that eventually form spheroids (right).



Figure 5. Immature organoids. Bright field images of various day 7 organoids embedded in ATCC CellMatrix from the same preparation displaying varying morphology.



Figure 6. Mature organoid. Bright field image of day 30+ organoid embedded in ATCC CellMatrix exhibiting many tube-like structures.

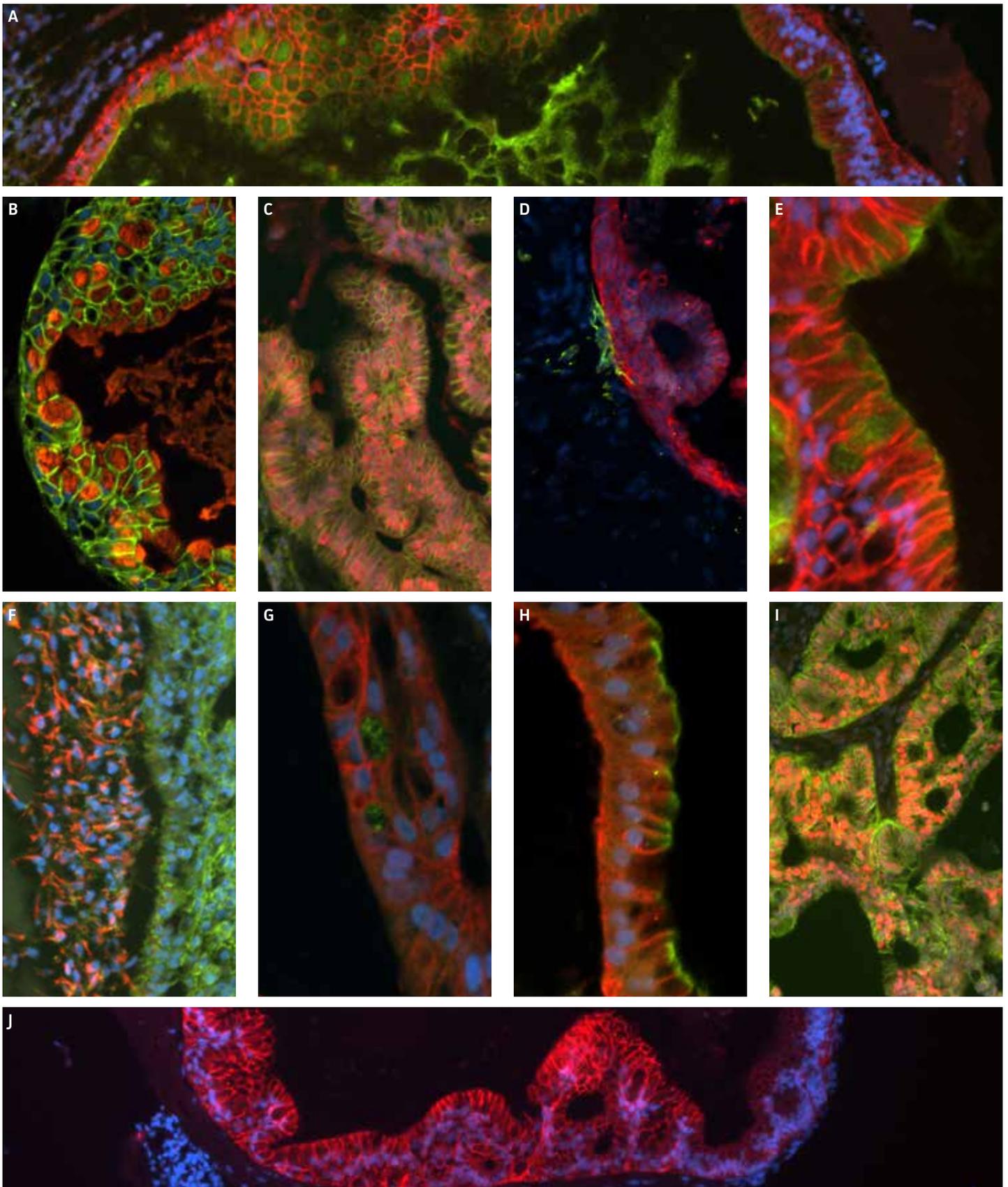


Figure 7. Day 30+ organoids exhibit complex morphology, functional secretory cells, and various gastric and intestinal tissue relevant marker expression. A. Secretory and non-secretory cells line the lumen. MUC1, green; Hoechst, blue; E-cadherin, red. B. MUC5A, red; Hoechst, blue; β -catenin, green. C. PDX1, red; Hoechst, blue; β -catenin, green. D. Smooth muscle- α actin, green; β -catenin, red; Hoechst, blue. E. MUC1, green; Hoechst, blue; E-cadherin, red. F. E-cadherin, green; vimentin, red; Hoechst, blue. G. Lysozyme, green; Hoechst, blue; E-cadherin, red. H. E-cadherin, red; villin, green; Hoechst, blue. I. E-cadherin, green; CDX2, red; Hoechst, blue. J. Complex, folded columnar epithelial architecture is present in mature organoids. E-cadherin, red; Hoechst, blue.

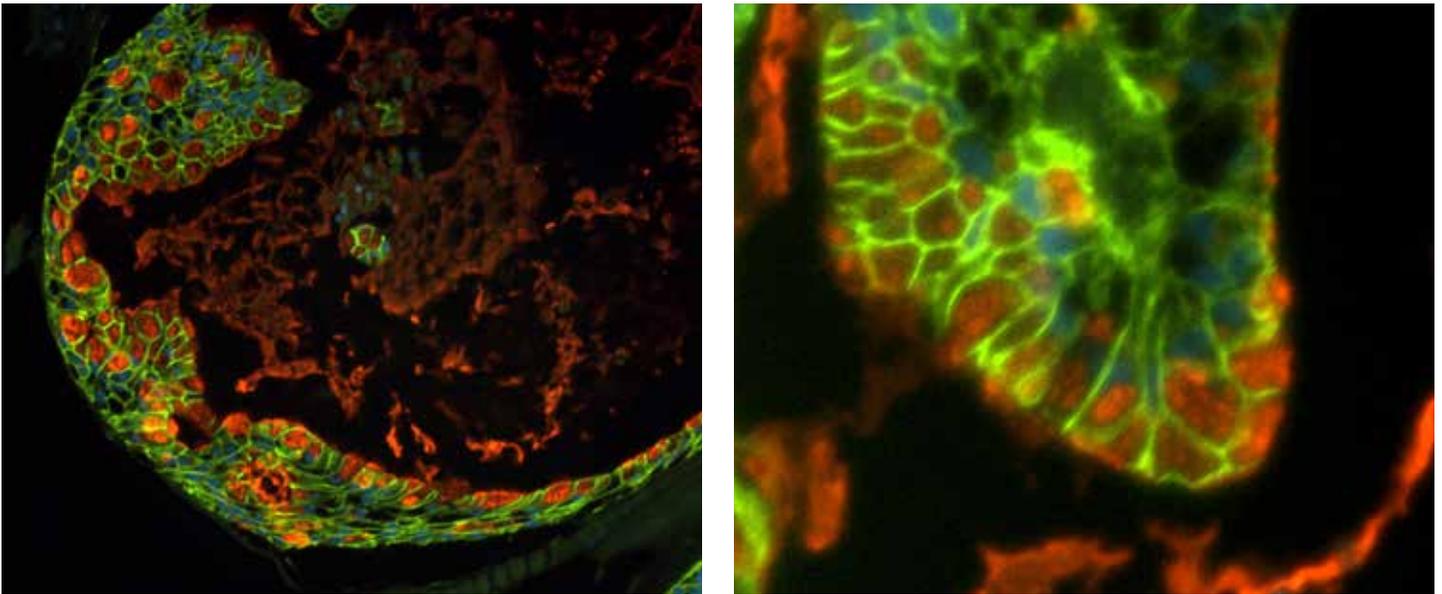


Figure 8. Functional goblet cells. Immunofluorescent staining showing expression of MUC5AC (red), Hoechst (blue) and E-cadherin (green). Secreted mucin is present within the lumen (left). Granulated appearance of staining is typical of goblet cells containing mucin within secretory vesicles (right).

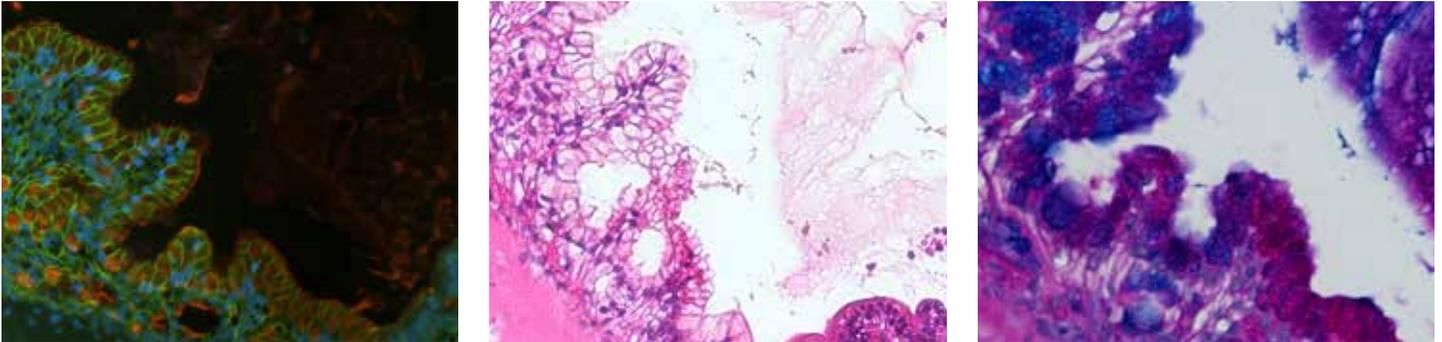
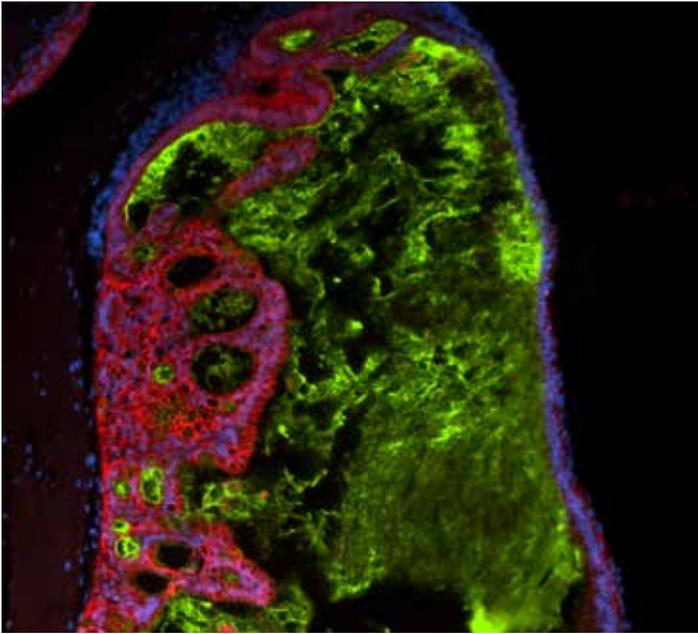


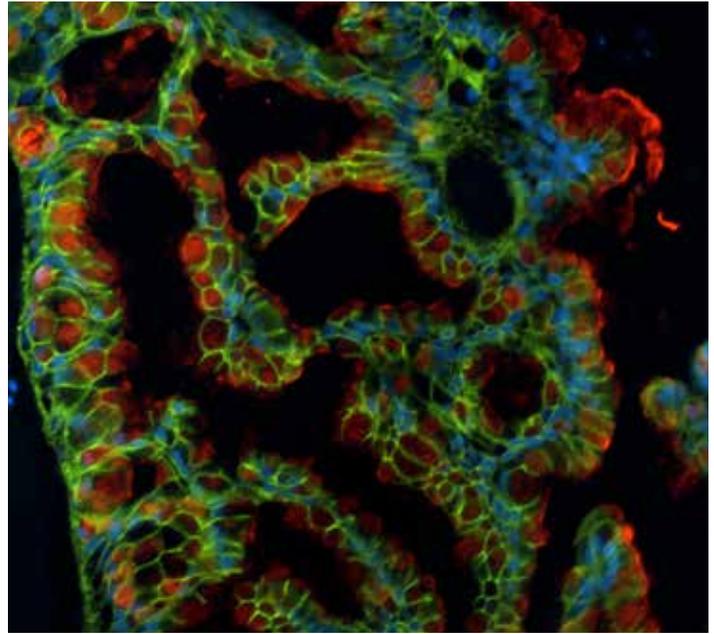
Figure 9. Mature organoids resemble tissue. Adjacent sections of a day 30+ organoid stained for MUC5A (red; left), H&E (middle) and PAS-Alcian Blue (right).

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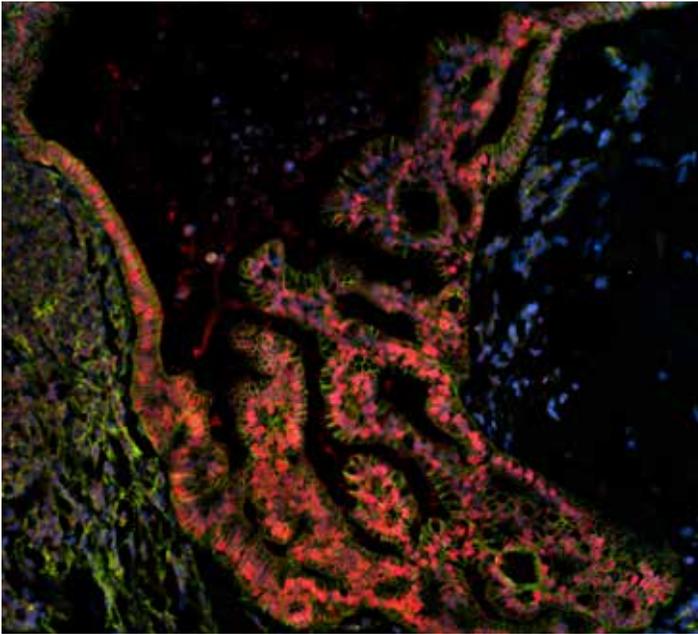
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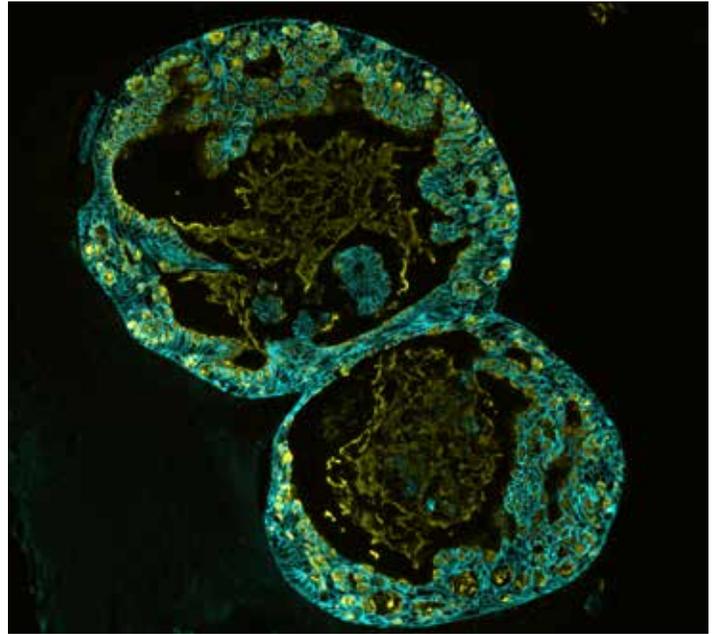
Human iPSC-derived gastrointestinal organoid sectioned and stained for cadherin-1 (red) and mucin 1 (green) and cell nuclei (blue).



Human iPSC-derived gastrointestinal organoid sectioned and stained for β -catenin (green) and mucin 5AC (red) and cell nuclei (blue).



Human iPSC-derived gastrointestinal organoid sectioned and stained for β -catenin (green) and pancreatic and duodenal homeobox 1 (red) and cell nuclei (blue).



Human iPSC-derived gastrointestinal organoid sectioned and stained for cadherin-1 (teal) and mucin 5AC (yellow).

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