A Novel Method for Generating Single, Intestinal Organoids for High Throughput Screening

Application Note

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Introduction

For successful drug development there is a constant need to recapitulate the *in vivo* environment as much as possible. However, currently many high throughput screening (HTS) applications utilize two dimensional (2D) monocultures that may not accurately reflect *in vivo* conditions. The *in vitro* organoid model allows for the study of *in vivo* developmental and biological processes, including tissue renewal, stem cell functionality, and drug responsiveness[1]. As organoids are self-organizing and more accurately resemble the morphological and molecular biology of the tissue they are derived from, they have great potential for serving as better model systems for these applications[2]. Most currently established methods call for generating organoids in microplates or multiwell plates which results in the formation of multiple organoids per well. Thus, there is a need for methods to form reproducible, single organoids per well for HTS applications. Here we demonstrate the formation of gastrointestinal organoids derived from human induced pluripotent stem cells (hiPSC) in Corning® 96-well spheroid microplates in combination with Corning Matrigel® matrix, resulting in a single intestinal organoid in each well. Differentiation into definitive endoderm and intestinal lineage was confirmed by flow cytometry and immunostaining methods.

Materials and Methods

Normal human iPS cells (iXCells Biotechnologies Cat. No. 30HU-002) were cultured on Corning Matrigel hESC-qualified matrix (Corning Cat. No. 354277) coated 6-well cell culture plates (Corning Cat. No. 3506) using mouse embryonic feeder (MEF) conditioned medium (iXCells Biotechnologies Cat. No. MD-0015). Pluripotency was routinely confirmed by flow cytometry analysis via markers TRA-1-60 (Miltenyi Cat. No. 130-100-350), SOX2 (Miltenyi Cat. No. 130-104-993), Nanog (Miltenyi Cat. No. 130-105-080), Oct3/4 (Miltenyi Cat. No. 130-109-763) and isotype controls (Miltenyi Cat. Nos. 130-104-611 and 130-104-613), utilizing a Miltenyi Biotec MacsQuant®.

At harvest, iPSC were released as single cells with Accutase® cell detachment solution (Corning Cat. No. 25-058-CI) and seeded into 96-well spheroid microplates (Corning Cat. No. 4515) at 2,000 cells per well in 100 µL MEF conditioned medium containing 10 µM Y-27632 dihydrochloride (Sigma Cat. No. Y0503). After overnight incubation, uniform, single spheroids formed in each well. Medium was exchanged daily for 5 additional days using a modified version of the McCracken, et al. protocol[3]. Media formulations are provided in Table 1. On day 6 post-seeding, spheroids were either dissociated using TrypLE™ Select enzyme (10X) (Thermo Fisher Cat. No. A1217701) and analyzed via flow cytometry for marker expression, fixed as whole spheroids for confocal imaging to confirm definitive endoderm with SOX17 and CXCR4 expression (Table 2), or continuously cultured to initiate mid/hindgut differentiation.

For mid/hindgut differentiation, previously established protocols were modified[4,5]. Briefly, medium was exchanged daily for 4 additional days to contain RPMI with Corning glutagro™ (Corning Cat. No. 10-104-CV) supplemented with 100 ng/mL of recombinant human FGF-4 (R&D Systems Cat. No. 235-F4), 6 µM CHIR 99021 (Tocris Cat. No. 4423) and 2% defined FBS (HyClone Cat. No. SH30080.02). On day 10 post-seeding, spheroids were either dissociated using TrypLE Select enzyme 10X and analyzed via flow cytometry for CDX2 marker expression, fixed as whole spheroids for confocal imaging to confirm mid/hindgut differentiation via CDX2 expression (Table 2), or cultured further to develop into intestinal organoids.

Table 1. Media Formulations Utilized for Definitive Endoderm Differentiation

Media Formulation	Vendor/Cat. No.
Day 1 Definitive Endoderm	
RPMI with Corning glutagro	Corning/10-104-CV
100 ng/mL of Activin A	eBioscience/34-8993-82
Day 2 Definitive Endoderm	
RPMI with Corning glutagro	Corning/10-104-CV
100 ng/mL of Activin A	eBioscience/34-8993-82
0.2% defined HyClone™ FBS	GE/SH30080.02
Days 3-5 Definitive Endoderm	
RPMI with Corning glutagro	Corning/10-104-CV
100 ng/mL of Activin A	eBioscience/34-8993-82
2% defined HyClone FBS	GE/SH30080.02

Table 2. Antibodies: Flow Cytometry and Immunofluorescence

Antibody	Purpose	Vendor	Primary	Isotype Control
SOX17	Flow	R&D Systems	IC19241G	IC002G
SOX17	IF	R&D Systems	NL1924R	NL001
CXCR4	Flow	Biolegend	306510	400220
CXCR4	IF	BD Biosciences	560936	555576
CDX2	Flow/IF	BD Biosciences	563428	554680
Lysozyme	IF	Abcam	ab2408	ab172730
Villin	IF	Abcam	ab130751	ab172730
Muc2	IF	Abcam	ab11197	ab91353
Chromogranin A	IF	Abcam	ab15160	ab172730
Vimentin	IF	Abcam	ab92547	ab172730

FL = Flow cytometry, IF = Immunofluorescence

On day 10 post-seeding, mid/hindgut spheroids were embedded in Corning® Matrigel® matrix (Corning Cat. No. 354234) per McCracken, et al. protocol. Medium was aspirated and replaced with 50 µL per well of undiluted Corning Matrigel matrix. This was accomplished by pre-chilling Corning spheroid microplates at 4°C for 10 minutes prior to placing microplate on Corning CoolBox™ XT cooling and freezing module (Corning Cat. No. 432021) to keep the microplate cold while pipetting. To ensure the spheroid would be centered in the bottom of each well, spheroid microplates were spun in a 4°C pre-chilled centrifuge at 50 x g for 7 minutes. Matrigel matrix was allowed to gel in the spheroid microplate at 37°C for 30 minutes prior to the addition of 100 µL intestinal medium, which consisted of Advanced DMEM (Thermo Fisher Cat. No. 12634-010) supplemented to 2X B27 (Thermo Fisher Cat. No. 17504044), 2 mM L-glutamine (Corning Cat. No. 25-005-CI), 15 mM HEPES (Corning Cat. No. 25-060-CI), 500 ng/mL R-spondin1 (Peprotech Cat. No. 120-38), 100 ng/mL Noggin (R&D Systems Cat. No. 6057-NG), and 100 ng/mL EGF (Corning Cat. No. 354052). Medium was exchanged every 2 to 4 days as needed for the duration of the culture. After 2 weeks in Matrigel matrix, an additional 50 µL of Matrigel matrix was added to each well, as described previously, prior to addition of fresh medium. Organoids were processed on day 38 post-seeding by fixing in 4% paraformaldehyde prior to paraffin embedding, sectioning, and staining. Histological preparations and imaging were performed by the Histology and Imaging Core at the University of New England, Biddeford, ME.

For proof-of-concept screening assay, organoids were removed from Corning Matrigel matrix by washing several times with cold hanks buffered saline solution (HBSS) (Corning Cat. No. 21-022-CM) followed by a 1-hour incubation at 4°C with cold Corning Cell Recovery solution (Corning Cat. No. 354253). Organoids were washed 1 additional time with cold HBSS before imaging and assaying. In order to achieve complete lysis, organoids were incubated on a plate shaker for 5 minutes in Sodium Dodecyl Sulfate (SDS) solution (Corning Cat. No 46-040-CI) diluted to 1% with HBSS. After 5 minutes, an equal volume of CellTiter-Glo® 3D (Promega Cat. No. G9681) was added and assayed per vendor protocol. Plates were read for luminescence with PerkinElmer EnVision™.

Results and Discussion

Traditionally, organoid formation protocols utilizing microplates or multiwell plates have relied on 2D definitive endoderm differentiation in order to form three dimensional (3D) structures, which can be further differentiated. This results in the formation of multiple, non-uniform, organoids per cell culture well which may not be ideal for HTS applications. By initiating the definitive endoderm differentiation in the Corning spheroid microplate, a single spheroid per well of a uniform size can be achieved. This technique in combination with Corning Matrigel matrix creates an organoid platform that is amendable to HTS applications.

Twenty-four hours after seeding iPSCs into the wells of a Corning 96-well spheroid microplate, a single uniform embryoid body forms in each well (data not shown). After 5 days following the definitive endoderm differentiation protocol described previously, a highly SOX17- and CXCR4-positive spheroid formed in each well which was confirmed via immunostaining of fixed spheroids (Figure 1) and flow cytometry marker expression of dissociated spheroids (Figure 2).

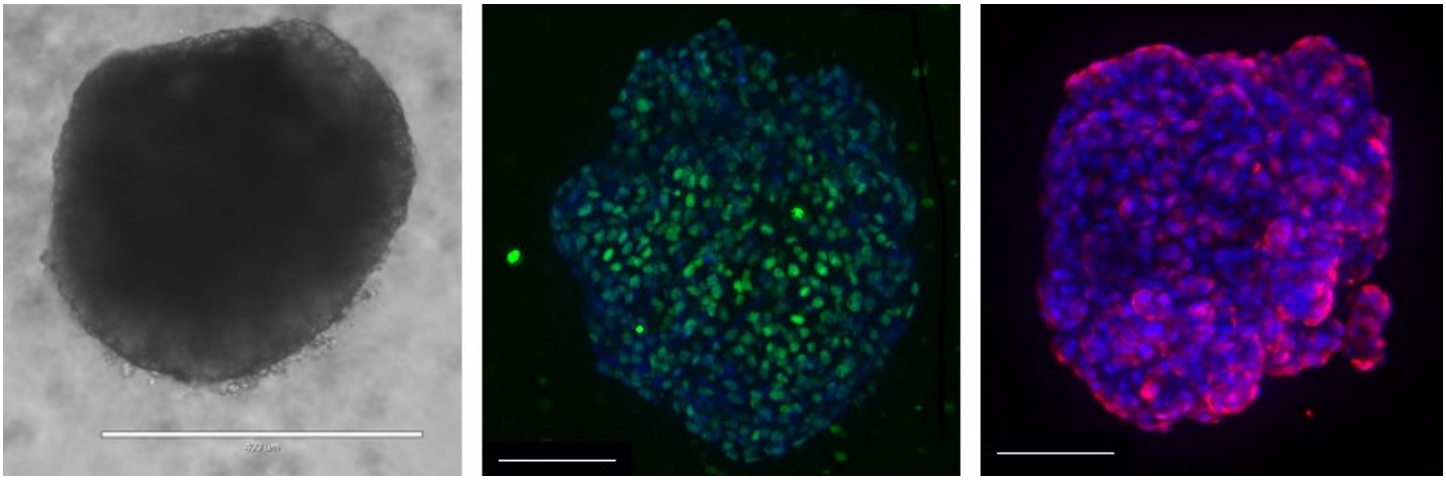


Figure 1. Representative photomicrographs of definitive endoderm spheroids. Bright field image taken with 10X objective (left) and composite of 15 confocal Z-stacked images with 25 µm slices of SOX17 (middle) and CXCR4 (right) expression with Hoechst nuclei counterstain. Confocal images obtained with Thermo Fisher CellInsight CX7. Scale bar is 400 µm for bright field and 100 µm for confocal images.

Further differentiation of definitive endoderm spheroids into mid/hindgut lineage, was similarly confirmed by assessing CDX2 expression via immunostaining of fixed spheroids (Figure 3) and flow cytometry marker expression of dissociated spheroids (Figure 4). As shown in Figure 3, the spheroids take on a more intestinal-like morphology following mid/hindgut differentiation.

CDX2 positive spheroids were then embedded in Corning® Matrigel® matrix for 4 weeks, allowing for further differentiation into intestinal organoids. Hematoxylin & eosin, as well as periodic acid-Schiff/Alcian blue staining of embedded organoids demonstrate highly organized structures with lumen and positive staining for mucin secretion (Figure 5). In order to further validate the formation of intestinal organoids, 4 week old organoids were

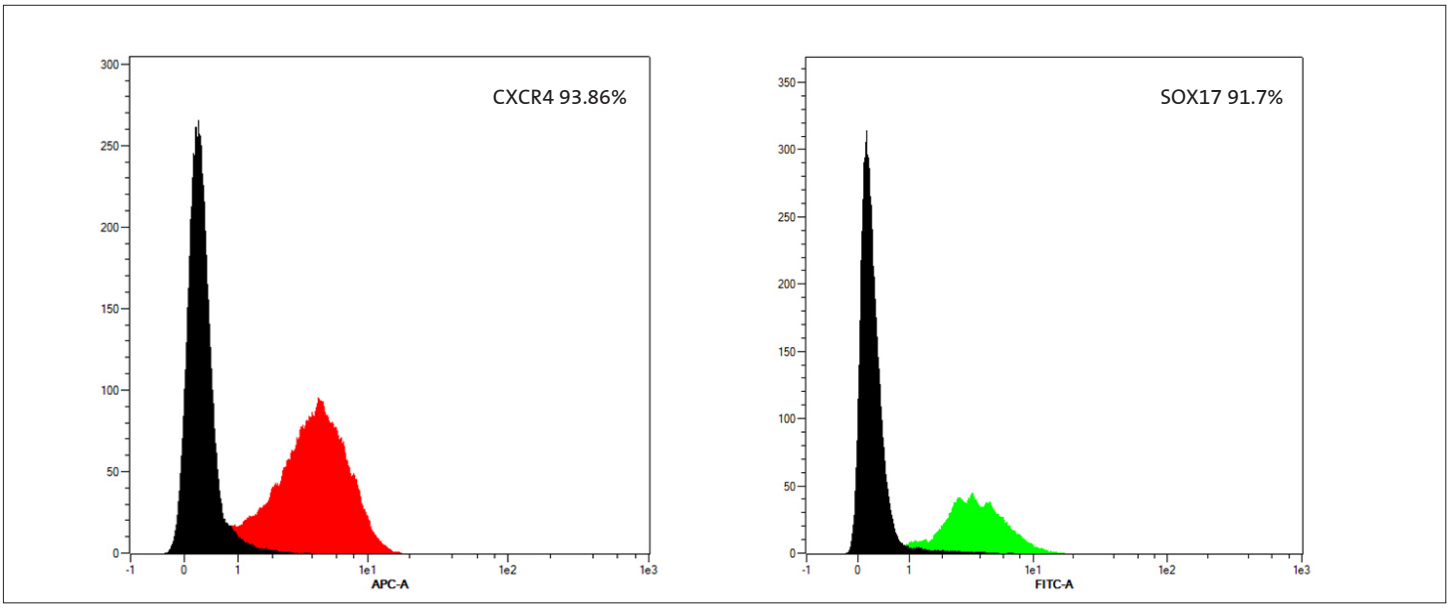


Figure 2. Representative definitive endoderm marker expression via flow cytometry. Representative histograms demonstrating positive expression of SOX17 and CXCR4 compared to isotype controls from dissociated spheroids.

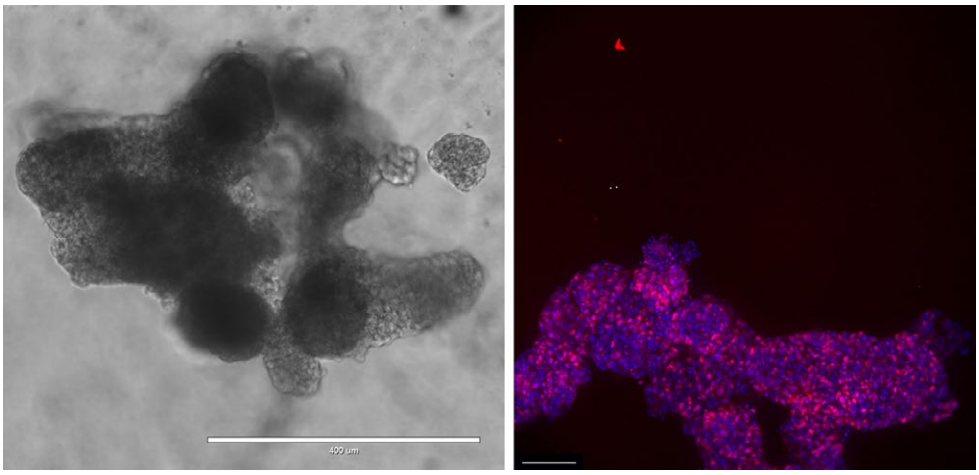


Figure 3. Representative photomicrographs of mid/hindgut spheroids. Bright field image taken with 10X objective (left) and composite image of 15 confocal Z-stacked images with 30 µm slices of CDX2 stained spheroid with Hoechst nuclei counterstain (right). Confocal image taken with 4X objective using Thermo Fisher CellInsight CX7. Scale bars are 400 µm and 100 µm, respectively.

fixed, embedded, sectioned and stained for marker expression to confirm the presence of cells types associated with the intestine. Figure 6 demonstrates positive staining for muc2, vimentin, villin, chromogranin A, and lysozyme marker expression indicating the presence of goblet cells, mesenchymal cells, enterocytes, entero-endocrine cells, and paneth cells, respectively.

To demonstrate the robustness and reproducibility of intestinal organoid formation using the 96-well Corning® spheroid microplate, 4-week-old intestinal organoids were imaged in the 96-well spheroid microplate and subjected to a commonly used ATP assay for live cell assessment. Using a 2X objective from the Thermo Fisher Scientific CellInsight™ CX7, the presence of a single intestinal organoid in each well of the spheroid microplate was confirmed (Figure 7). The microplate was then used to demonstrate the compatibility of this system with HTS applications. Figure 8 shows a representative ATP study with a luminescence signal to background ratio of 90, demonstrating the large signal window that this screening assay is capable of achieving with a single intestinal organoid per well.

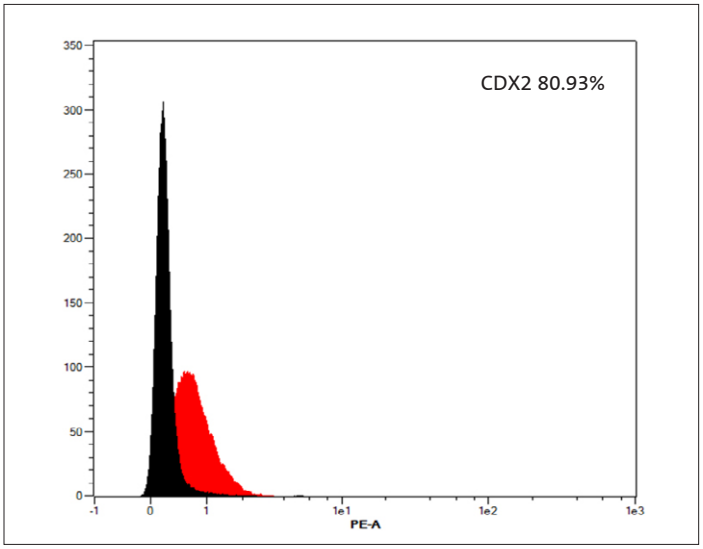
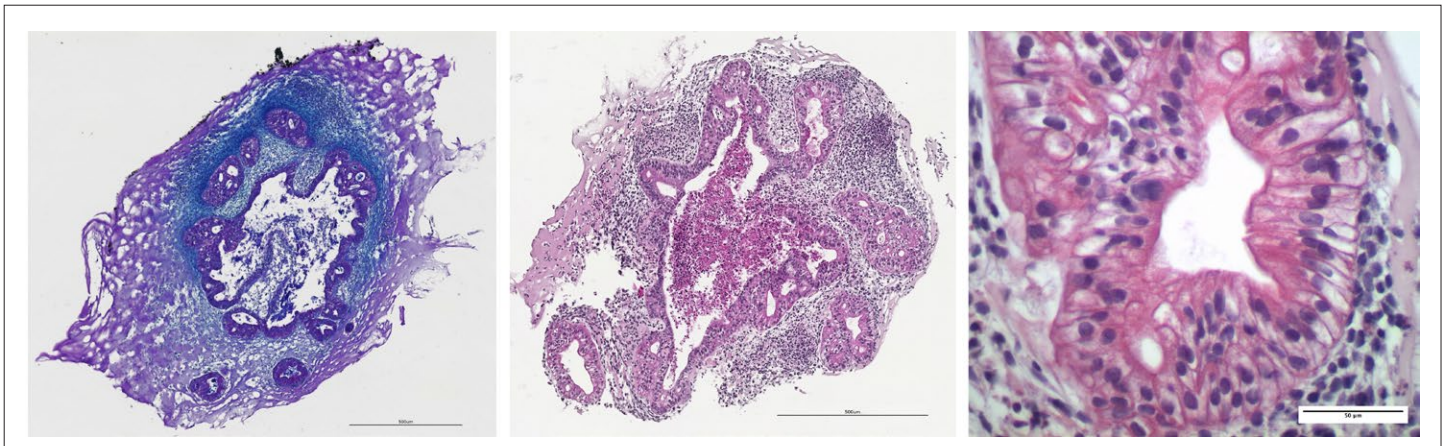


Figure 4. Representative mid/hindgut marker expression via flow cytometry. Representative histogram demonstrating positive expression of CDX2 compared to isotype control from dissociated spheroids.



Periodic acid-Schiff/Alcian blue (20X) | Hematoxylin & Eosin (20X) | Hematoxylin & Eosin (40X)

Figure 5. Histological confirmation. Representative photomicrographs of paraffin embedded and sectioned 4-week-old intestinal organoids. Images were taken using a 20X or 40X objective.

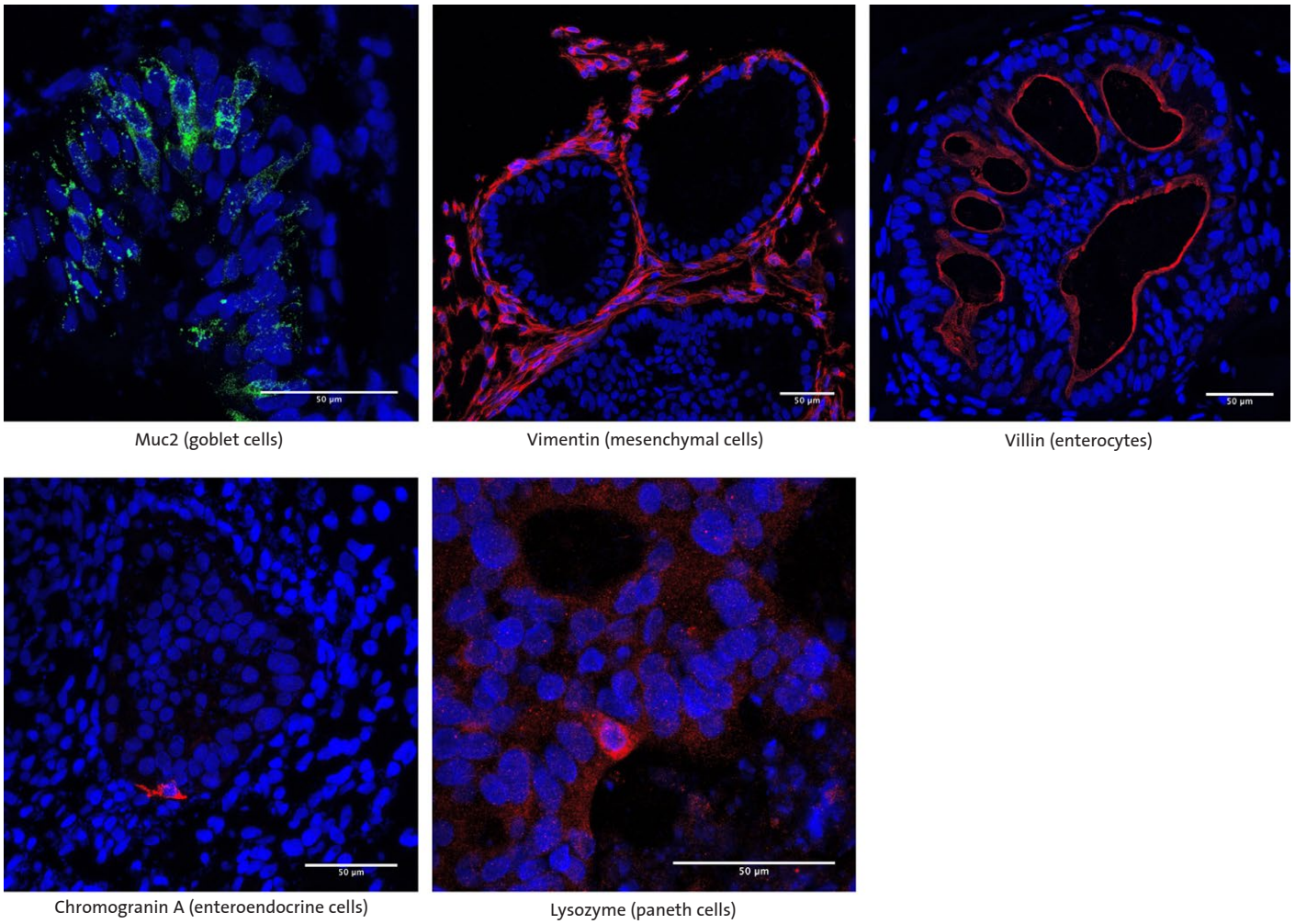


Figure 6. Histological confirmation. Representative immunofluorescent photomicrographs of paraffin embedded and sectioned 4-week old intestinal organoids. Immunofluorecent images show presence of muc2 (goblet cells), vimentin (mesenchymal cells), villin (enterocytes), chromagranin A (entero-endocrine cells), and lysozyme (paneth cells), positive staining, supporting the presence of goblet cells, mesenchymal cells, enterocytes, enteroendocrine cells, and paneth cells, respectively. Images were collected on a Leica TCS SP5 confocal laser scanning microscope with a 40X/1.3 NA plan apo objective between 1.0X and 4.0X digital zoom.

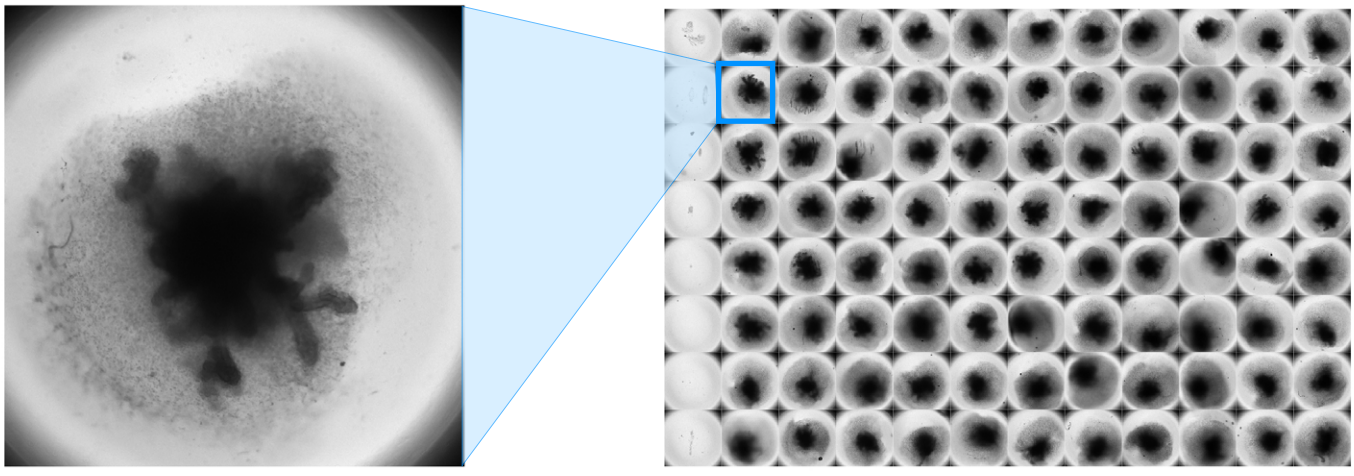


Figure 7. Representative 96-well spheroid microplate. Image of spheroid microplate containing one intestinal organoid per well, demonstrating consistency and reproducibility of organoid formation across spheroid microplate. Images taken with 2X objective from Thermo Fisher CellInsight CX7.

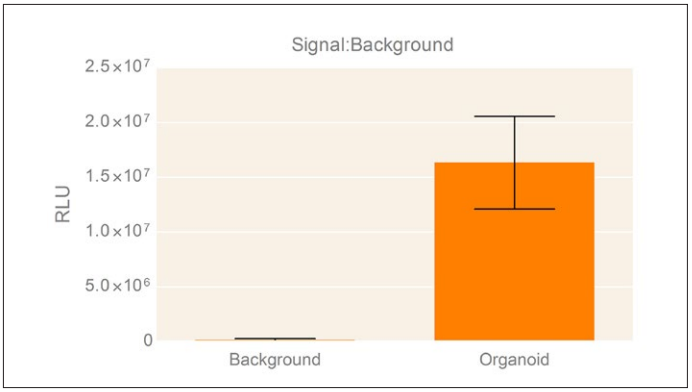


Figure 8. Representative 96-well organoid assay. Signal to background ratio of 90.6 was observed from a representative ATP assay. N=8 wells for background control, and N=88 wells for 4-week-old intestinal organoids. Data shown ± standard error.

Conclusions

- The Corning® spheroid microplate can be utilized to produce a single, uniformly-sized embryoid body in each well that can be further differentiated into definitive endoderm.
- Successful mid/hindgut differentiation can be achieved from definitive endoderm spheroids using in the Corning spheroid microplate.
- The addition of Corning Matrigel® matrix to mid/hindgut spheroids in the Corning spheroid microplate can be used to generate intestinal organoids that are compatible with HTS applications.
- Spheroids and organoids cultured in the Corning spheroid microplate are amenable to brightfield and confocal imaging, histology, and immunohistochemistry applications.

References

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