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

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ABSTRACT

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. As organoids are self-organizing and more accurately resemble the morphological and molecular biology of the tissue they are derived from, there is higher demand for methods to form reproducible organoids for high throughput screening applications. Currently, many high throughput screens are conducted utilizing 2D monocultures that may not accurately reflect *in vivo* conditions. Here we demonstrate the formation of gastrointestinal organoids derived from human induced pluripotent stem cells (hiPSC) in the Corning® 96-well spheroid microplate in conjunction 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.

METHODS/MATERIALS

Differentiation protocols adapted from references 1-3.

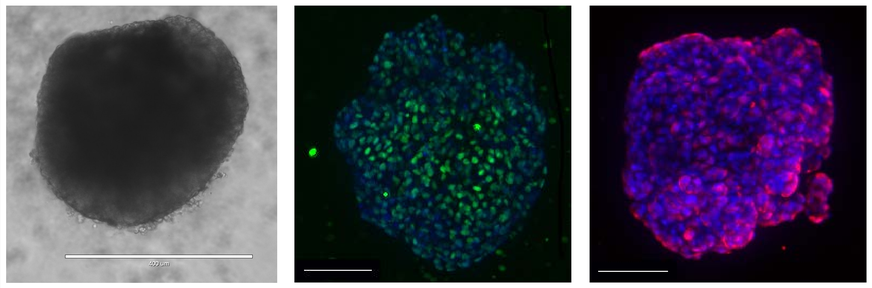
- **Human iPS Cell Culture:** Normal Human iPS Cells (iXCells Biotechnologies Cat. No. 30HU-002) were cultured on Corning Matrigel hESC-qualified matrix (Corning Cat. No. 354277) coated plates using mouse embryonic feeder (MEF) conditioned medium (iXCells Biotechnologies Cat. No. MD-0015). Pluripotency was routinely confirmed by flow analysis via Tra-1-60 (Miltenyi Cat. No. 130-100-350), Anti-Sox2-FITC (Miltenyi Cat. No. 130-104-993), Nanog (Miltenyi Cat. No. 130-105-080), Anti-Oct 3/4-PE (Miltenyi Cat. No. 130-109-763), and isotype controls (Miltenyi Cat. No. 130-104-611 and 130-104-613) utilizing the Miltenyi Biotec MacsQuant®.
- **iPSC Embryoid Body Formation:** iPSC were harvested to 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)
- **Definitive Endoderm Differentiation:** After overnight incubation, single spheroids formed in each well. Medium was exchanged daily as follows:
  - Day 1: RPMI with Corning glutagro™ (Corning Cat. No. 10-104-CV) with 100 ng/mL of Activin A (eBioscience/Thermo Fisher Cat. No. 34-8993-82).
  - Day 2: RPMI with glutagro containing 100 ng/mL of Activin A and 0.2% defined FBS (Hyclone Cat. No. SH30070.02).
  - Day 3-5: RPMI with glutagro containing 100 ng/mL of Activin A and 2% defined FBS.
  - Day 6: Spheroids were either dissociated using TrypLE™ Select Enzyme (10X) (Thermo Fisher Cat. No. A1217701) and analyzed via flow cytometry or fixed as whole spheroids for confocal imaging in order to confirm definitive endoderm with SOX17 and CXCR4 expression.
- **Mid/Hindgut Differentiation:** Following definitive endoderm formation, medium was exchanged daily for four additional days to contain RPMI with glutagro 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.
  - Day 10 spheroids were either dissociated using TrypLE Select Enzyme or fixed as whole spheroids for confocal imaging to determine mid/hindgut differentiation via CDX2 expression.
- **Intestinal Differentiation:** Day 10 spheroids were embedded in Corning Matrigel basement membrane matrix (Corning Cat. No. 354234) by aspirating mid/hindgut medium and replacing with 50 µL per well of undiluted Matrigel matrix. Matrigel matrix was allowed to gel at 37ºC for 30 minutes prior to the addition of 100 µL intestinal medium, which consisted of Advanced DMEM (Invitrogen/Thermo Fisher Cat. No. 12634-010) supplemented to 2X B27 (Invitrogen/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-Spondin-1 (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 2-4 days as needed. After 24 days in culture, an additional 50 µL of Matrigel matrix was added to each well prior to addition of fresh medium. Organoids were processed on day 38 by fixing in 4% paraformaldehyde prior to paraffin embedding and staining. Histological preparations and imaging were performed by the Histology and Imaging Core at the University of New England (Biddeford, Maine USA)

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

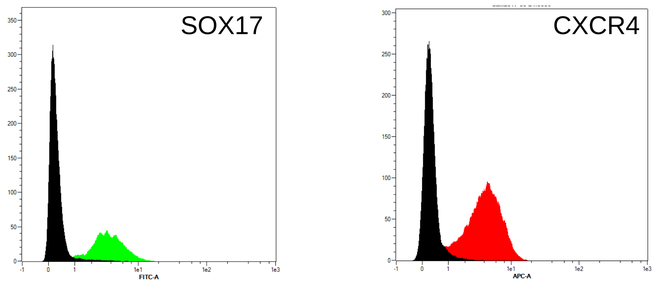
Flow cytometry (Flow); Immunofluorescence (IF)

RESULTS

Definitive Endoderm

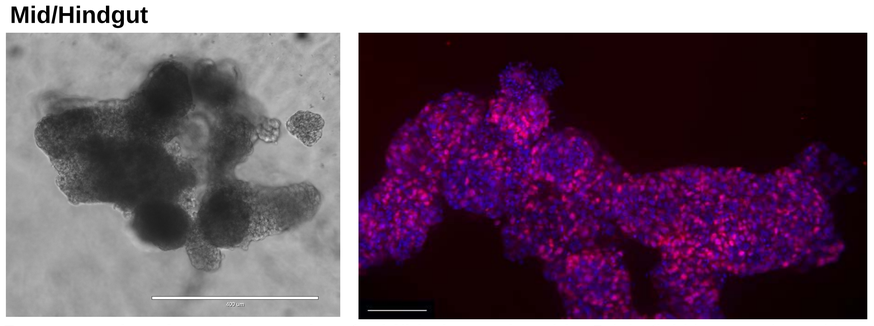


Representative photomicrographs of definitive endoderm spheroids. Bright field image taken with 10X objective (left) and composite image of 15 confocal Z-stacked images with 25 µm slices of SOX17 (middle); and CXCR4 (right) expression with hoechst nuclei counterstain. Confocal images obtained with Thermo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

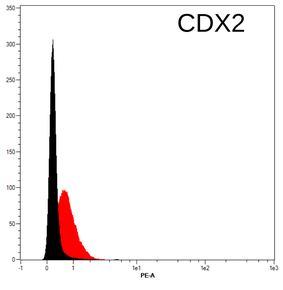


Representative definitive endoderm marker expression via flow cytometry. Representative histograms demonstrating positive expression of SOX17 and CXCR4 compared to isotype controls from dissociated spheroids.

Mid/Hindgut

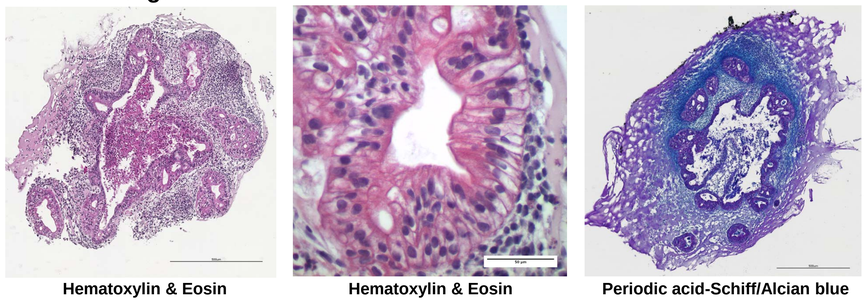


Representative photomicrographs of mid/hindgut spheroids. Bright field image taken with 10X objective (left) and composite image of 15 confocal Z-stacked images with 30 µm slices of CDX2 stained spheroid with hoechst nuclei counterstain (right). Confocal image taken with 4X objective using Thermo Fisher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



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

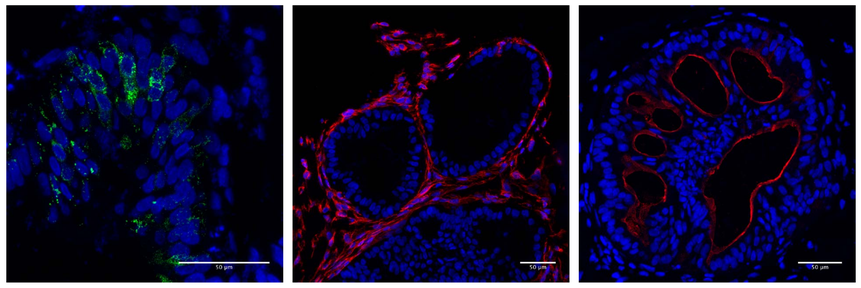
Intestinal Organoids



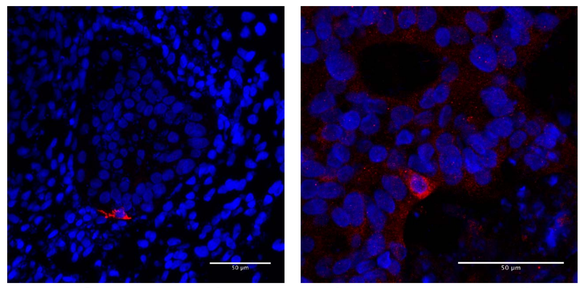
Hematoxylin & Eosin | Hematoxylin & Eosin | Periodic acid-Schiff/Alcian blue

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

Intestinal Organoids



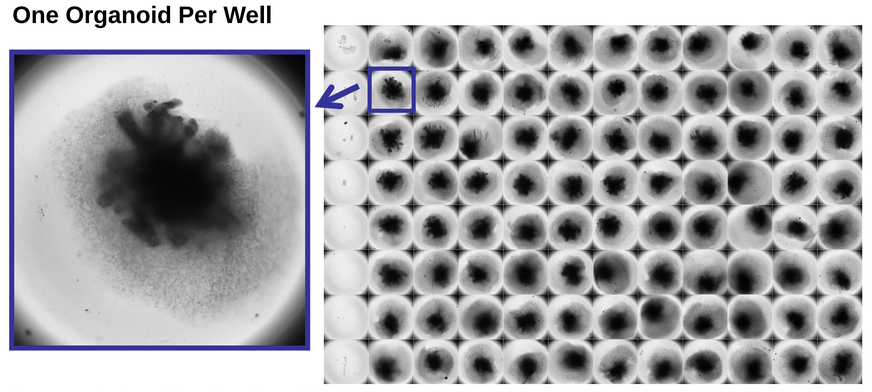
Muc2 (goblet cells) | Vimentin (mesenchymal cells) | Villin (enterocytes)



Chromogranin A (enteroendocrine cells) | Lysozyme (paneth cells)

Histological confirmation. Representative 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 (enteroendocrine 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.

One Organoid Per Well



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

SUMMARY/CONCLUSIONS

- Corning 96-well spheroid microplates allow for the formation of consistent sized, single spheroids in each well that are ideally suited for imaging.
- A single definitive endoderm spheroid can be achieved in each well of a spheroid microplate.
- A single mid/hindgut spheroid can be achieved in each well of a spheroid microplate.
- A single intestinal organoid can formed in each well of a spheroid microplate
- Following this protocol it is possible to set up an intestinal organoids screen for high throughput analysis.

REFERENCES

1. Spence J.R., Mayhew C.N., Rankin S.A. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature. 2011;470:105-109
2. McCracken K.W., Howell J.C., Wells J.M. Generating human intestinal tissue from pluripotent stem cells in vitro. Nat Protoc. 2011;6:1920-1928.
3. Tamminen K., Balboa D., Toivonen S., Pakarinen M. P., Wiener Z., Alitalo K., et al. (2015). Intestinal commitment and maturation of human pluripotent stem cells is independent of exogenous FGF4 and R-spondin1.

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