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, reproducibly generating single intestinal organoids 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 iPSC Cell Culture: Normal Human iPSCs (iXCell Biotechnologies Cat. No. 39HU-002) were cultured on Corning Matrigel™-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. 130-104-995), 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 Biotech MACSQuant®.
- iPSC Embryoid Body Formation: iPSCs were harvested to single cells with Accutase® cell detachment solution (Corning Cat. No. 20-058-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 dihydropyrrolidine (Sigma Cat. No. 10-0503).
- Definitive Endoderm Differentiation: Following definitive endoderm formation, 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 3-7: RPMI with glutagro containing 100 ng/ml of Activin A and 2% defined FBS.
- Day 7: 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 RmPMI 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 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 37°C for 30 minutes to the addition of 100 μL intestinal medium, which consisted of Advanced DMEM (Invitrogen®/Thermo Fisher Cat. No. 12363-010) 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. Orginoids 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).

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. 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 images taken with 4X objective using Thermo Fisher CellInsight™ CX7. Scale bar is 400 µm and 100 µm, respectively.

Intestinal Organoids

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

SUMMARY/CONCLUSIONS

- Following this protocol it is possible to set up an intestinal organoids screen for high throughput analysis.

REFERENCES


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.

Histological confirmation. Representative photomicrographs of paraffin embedded and sectioned 4-week old intestinal organoids. Immunofluorescent images show presence of Muc2 (goblet cells), vimentin (mesenchymal cells), villin (enterocytes), chromogranin 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 SPS 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 mid/hindgut marker expression via flow cytometry. Representative histograms demonstrating positive expression of CDX2 compared to isotype control from dissociated spheroids.

Intestinal Organoids

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