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# All About Organoids

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# Contents

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# Organoids: Changing Basic Research and Medicine

*These 3D, organ-like collections of cells create innovative approaches to drug development.*

**by Mike May**

An organoid is a 3D cluster of cells with structural and functional features of an organ. Scientists can start with induced pluripotent stem cells (iPSCs) or adult stem cells acquired from a specific patient and generate organ- and disease-specific organoids. Consequently, organoids make it possible to study the impact of a drug on a specific disease, even a person's own disease.

"The whole point of an in vitro system is to model a patient as closely as possible without having the patient in the lab," says Rob Vries, CEO at the Netherlands-based HUB (Hubrecht Organoid Technology), a nonprofit company. "With organoids, we can take cells from a patient and grow them—expanding genetically stable cells—in the lab in virtually unlimited amounts to directly study disease." He should know, as he was one of the authors on the first report of organoid development, which was created in Hans Clevers's lab from intestinal cells that express Lgr5—a gene that turns on division on these cells.[1]

The real key is what scientists can discover with organoids. For example, organoids generated from patient tumors can "be exposed to varying drugs in vitro to identify the best treatment to fight a particular cancer—thus personalizing medicine to treat a disease," says Elizabeth Abraham, senior product manager at Corning. "Taking this idea even further is the ability to repair genes in cells that can form organoids, then using those organoids to understand treatment regimens."

## Expanding opportunities

Beginning with the first organoid developed in Clevers's lab, scientists wanted to show that an organoid developed from a patient's cell replicates what occurs in the patient. Now, Clevers and his colleagues have done that with cystic fibrosis[2] and cancer.[3] Based on this research and work by other scientists, Vries says, "There's an extremely high correlation between how an organoid and a patient behaves." That was a fundamental requirement in moving ahead with organoids in clinical work.

To date, organoids have been made from many tissues, including brain, breast, intestine, kidney, liver, lung, pancreas, and prostate.[4] Some of the next steps in using organoids will involve creating even more

complex systems, and some examples already exist. One comes from Takanori Takebe, assistant professor of pediatrics at the University of Cincinnati, and his colleagues, who created the first connected tri-organoid system—the human hepato-biliary-pancreatic organoid.[5]

"This is a remarkable achievement since it moves the field from individual organoid research to connected organoid systems, which more physiologically mimic the interplay between human tissues," says Roxanna Ghadessy, technical marketing manager at Corning.

## Creating a cluster

Scientists generate organoids in a way that works best for a specific application. The key is finding a process that nurtures an organoid to form and grow as needed, and then allows a researcher to access the organoids.

For example, organoids can be grown in a drop of Matrigel® matrix that forms a dome. At the Medical College of Wisconsin, scientists grew patient-derived pancreatic organoids in Matrigel domes, and they reported: "These studies provide the first report of novel and disease-relevant 3D in-vitro models representing pancreatic tumor, stromal and immune components using primary organoid co-cultures representative of the tumor-microenvironment."[6] These domes can be created in various types of labware, including the Corning spheroid microplates.

At the University of Washington, assistant professor Benjamin Freedman, Ph.D., grows kidney organoids. "We start out in a very thin layer of Corning® Matrigel matrix, and we put the pluripotent stem cells on top," he explains. "Then, we sandwich them in another thin layer of Matrigel." He uses Corning ultra-low attachment plates in his organoid work. "These have been very helpful in growing our organoids in suspension," he says. "What we've seen is that the organoids can grow in a disease state to a very large size—centimeters in size—using these plates, and they couldn't do that when they were growing attached to the dish or even surrounded by Matrigel matrix." The plates also prove helpful in experiments that last for weeks, when Freedman needs the cells to resist attachment to the plate.[7]

## Organoid applications

Scientists can use organoids in many ways. For example, Freedman is looking for new kidney disease treatments from his work on organoids, but he also envisions other uses. "In the long term, we'd like to be able to generate new grafts from people's own bodies that can recreate the function of the kidney," he says. "Being able to do this in 3D culture could be a way of developing those kinds of transplant modalities."

From the NCI Center for Systems Biology of Small Cell Lung Cancer at Vanderbilt University, scientific center manager Amanda Linkous, Ph.D., says, "Our main focus is to improve the preclinical models of glioblastoma." She adds, "We want to model the human brain using cerebral organoids." She and her colleagues create "mini-brains" from a patient's iPSCs and, subsequently, co-culture these cerebral organoids with the patient's glioma stem cells.[8] "So, you have the patient's tumor cells invading into the patient's mini-brain, and then we can monitor the growth of that tumor," she says. "Our hope is to ultimately screen those tumors growing in the mini-brains against a library of a variety of different combinations of drug therapies, and be able to predict which combination or just the reagent or drug alone that may be most beneficial to inhibiting that patient's tumor growth."

## Moving forward

Organoid-based research is not easy. "Working with organoids requires a certain level of precision, working in a very structured manner that is more complex," Vries says. "Plus, two to three different patients can give two to three answers to a question about one kind of disease."

However, dealing with that complexity and heterogeneity allows scientists to learn more at earlier stages of drug development. "We move some clinical work to the preclinical phase," Vries says, "and that reduces the time of a project and makes it cost less." Plus, organoids can be used to study the basics of biology in many organs, the development and growth of a wide range of diseases, and much more.

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## About the Author


Mike May earned an M.S. in biological engineering from the University of Connecticut and a Ph.D. in neurobiology and behavior from Cornell University. He worked as an associate editor at American Scientist, and he is the author of hundreds of articles for clients that include Nature, Science, Scientific American, and many others.


## Additional Resources

**The Ultimate Guide to Corning® Matrigel® Matrix**

# Attacking Liver Disease through Organoids

*Organoids drive advances in transplantation research as well as personalized medicine.*

**by Mike May**

*Every year, liver disease kills about two million people around the world, but Bart Spee—an expert in liver biology at the Utrecht University in The Netherlands—hopes to change that through his work with organoids and other techniques. We talked to Spee about his work, and here's what we learned.*

## How did you get interested in the biology of the liver?

**Bart Spee:** I first learned about the great regenerative power of the liver from the story of Prometheus in Greek mythology. His liver was eaten by an eagle every day, and it grew back every night. Although the liver has this large regenerative capacity, there are still a lot of liver diseases. That's why I became interested in the liver—to try to understand these diseases better and ultimately find a cure for them.

There are not many treatments for end-stage liver disease. When liver diseases progress into the cirrhotic stage, where excess fibrosis hampers liver function, the only option those patients have is receiving a donor liver. That's why our group is investigating alternatives to that.

## Currently, what are your goals in studying liver disease?

**Bart Spee:** On the one hand, we're trying to create models for liver and liver diseases in the lab that we can use to study the diseases, even on a personalized level, and that's what we do with organoids. So, we can have adult stem cells cultured as organoids from healthy tissue, but also from patients. Then, we use biofabrication technologies to try to mimic liver physiology the best we can. That also means using co-culture of organoids with supporting cell types, bioreactors, microfluidics—those kinds of technologies. That is for the in vitro part.

The second part is that we also want to use the organoids for whole-organ engineering. But making an entire liver from scratch using stem cells really re-

quires a different set up from the bottom up and is much more challenging.

## What are the top challenges in working with organoids?

**Bart Spee:** I can think of two major challenges. First, although we have these adult stem cells—which you can, in our case, differentiate to hepatocyte-like cells—they still remain immature. With biofabrication technologies, such as bioprinting, we want to create more-differentiated organoids so that they better reflect the in vivo function. So, the maturation is one of the key things that we really need to improve.

If we really need to mature the cells further in a particular application, then we have to incorporate other cell types, like supporting cells. With standard differentiation, though, an organoid is limited in terms of function, but it's still enough to study metabolic diseases.

*Image: At the Utrecht University in the Netherlands, Bart Spee uses organoids in his research aimed at better understanding and treating liver disease. Image courtesy of Utrecht University, Veterinary Medicine.*

The second challenge is that upscaling the organoid culture requires a lot of plates, and a lot of Matrigel®. Upscaling is necessary if we want to use these cells for multiple testing in our in vitro models or for clinical applications, such as whole-organ engineering. We've started using Corning spinner flasks for the upscaling of organoids. This turns out to be a great thing to alleviate both problems: You get more cells more quickly, and they also differentiate better.

## Did you expect organoids to differentiate better when using the spinner flasks or was that a surprise?

**Bart Spee:** It was a surprise, I would say, but it's probably simply due to better oxygenation. If you consider that human liver has about 240 billion cells, hepatocytes, we can create that with these spinner flasks from two and a half million cells in just six weeks. That is really a massive achievement.

Plus, the adult human stem cells that we start with can proliferate that fast and stay genetically stable. Otherwise, with such a high proliferation speed, I would guess that you would really create tumors.

## Overall, how would you assess the impact of organoids on your work in liver disease?

**Bart Spee:** I would say this is the first cell type that I would consider a viable option for whole-organ engineering, due to the fact that you could use them to create entire new livers and still be genetically stable. In fact, our liver organoids could turn into an alternative for liver transplantation. I don't really see any other cell type doing that.

In the Netherlands alone, roughly 20% of the people who are on a waiting list to receive a donor liver die before they get one. So, there is a big need for an alternative to donor organs. We just don't have enough of them. This is one of the main places that organoids are really contributing.

In addition, we can use stem cells from the patients themselves, which allows us to take the personalized-medicine approach. That's something that is up and coming. We have created biobanks of adult stem cells from patients with different liver diseases, and that gives us the chance to really study those patients in great detail. Usually these are orphan diseases, which don't occur that much, and there's not a lot of investigation going on. Now, we really have the tools to study these diseases.

## About the Author

Mike May earned an M.S. in biological engineering from the University of Connecticut and a Ph.D. in neurobiology and behavior from Cornell University. He worked as an associate editor at American Scientist, and he is the author of hundreds of articles for clients that include Nature, Science, Scientific American, and many others.

## Additional Resources

**Citations on Corning® Matrigel® Matrix and Organoid Culture**

# Culture of Mouse Intestinal Organoids in Corning Matrigel Matrix for Organoid Culture

*Novel extracellular matrix supports organoid growth and differentiation while saving reagents and time used for lot testing.*

**by Katie Slater and Himabindu Nandivada**

## Introduction

Organoids are defined as complex three-dimensional (3D) structures that can mimic functionality of the in vivo organ counterparts.[1,2] They can be formed by the expansion and subsequent self-organization of stem cells and progenitor cells and their differentiated offspring. Organoids recapitulate numerous interactions such as cell-cell, cell-matrix, and tissue-specific physiological functions.[3] They have a wide range of applications in the fields of basic biology, disease modeling, drug discovery, personalized medicine, and regenerative medicine.

In adult mammals, the intestinal epithelium is a rapidly renewing tissue and is controlled by intestinal stem cell maintenance and self-renewal.[4] In the small intestine, self-renewing stem cells reside at the bottom of crypts and differentiate into different cell types such as enterocytes, goblet cells, paneth cells, and enteroendocrine cells. After studying the growth requirements for intestinal epithelium in vivo, a robust culture system has been described wherein stereotypical structures resembling the intestinal crypts (i.e., organoids) were formed and maintained in longterm culture.[5] This culture method involved the use of basement membrane extract that mimics the Laminin-rich crypt base, as well as the addition of factors such as Wnt-agonist R-spondin-1, epidermal growth factor (EGF), and Noggin.

Corning Matrigel matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins including Laminin (a major component), Collagen IV, heparan sulfate proteoglycan, entactin/nidogen, and several growth factors that are found in normal EHS tumors.[6,7] Corning Matrigel matrix has been used in a wide range of applications such as stem cell culture, cell attachment and differentiation, angiogenesis assays, tumor growth, and tissue engineering.[8,9] We present Corning Matrigel matrix for organoid culture, an optimal extracellular matrix that supports organoid growth and differenti-

ation while saving reagents and time used for lot testing. Each lot of Corning Matrigel matrix for organoid culture has a specific elastic modulus value indicating the stiffness of the gel formed and is qualified to form stable 3D domes commonly used in organoid culture protocols.

Using HUB Organoid Technology methods, we demonstrated that Corning Matrigel matrix for organoid culture supported the culture of mouse intestinal organoids (MIO) for more than 7 passages in serum-free IntestiCult™ organoid growth medium (mouse). The MIOs cultured in Corning Matrigel matrix for organoid culture domes showed a typical budding morphology and contained differentiated intestinal cells that expressed specific surface markers.

## Materials and Methods

### Corning Matrigel Matrix Handling

Corning Matrigel matrix for organoid culture (Corning Cat. No. 356255) was thawed by submerging a vial in an insulated bucket of ice and kept in the refrigerator (2°C to 8°C) overnight according to Guidelines for Use. Corning Matrigel matrix for organoid culture was aliquoted and stored at -80°C for long-term storage. Aliquots of Corning Matrigel matrix for organoid culture were always kept on ice and thawed as needed just before use with pre-chilled tips and tubes. In this study, organoids were cultured in three lots of Corning Matrigel matrix for organoid culture.

### Mouse Intestinal Organoid Culture and Passaging

Cryopreserved mouse intestinal organoids (STEMCELL Technologies) were thawed, cultured, and passaged in IntestiCult organoid growth medium (STEMCELL Technologies) according to manufacturer's instructions. Briefly, a vial of cryopreserved MIO segments was thawed, centrifuged, and the organoid pellet was resuspended in cold IntestiCult organoid growth medium (100 µL). Then, cold Corning Matrigel matrix for organoid culture (100 µL) was added to the MIOs and the suspension was pipetted up and down. Using a prewetted 200 µL pipet tip, domes were formed in each well by adding 50 µL of the 1:1 MIO/Corning Matrigel matrix suspension to a preincubated Costar® 24-well plate (Corning). The plate, with organoid containing domes, was then placed in a humidified incubator (37°C and 5% $CO_2$) for 10 minutes after which IntestiCult organoid growth medium (750 µL) was added gently to each well. The culture medium was changed 3 times per week. The morphology of the MIOs was observed daily using an EVOS® XL Cell Imaging System (Thermo Fisher). Organoids were passaged after 5 to 7 days using Gentle Cell Dissociation Reagent (STEMCELL Technologies) and cultured in Corning Matrigel matrix for organoid culture domes as described above.

### Organoid Immunohistochemistry

Corning Matrigel matrix domes containing MIOs were washed with Dulbecco's phosphate buffered saline (DPBS, Thermo Fisher) 3 times. Paraformaldehyde (4% in DPBS; EM Scientific) was added to the wells and incubated for 1 hour at room temperature. The wells were then washed with DPBS (3 times) to remove the paraformaldehyde. After the final rinse, DPBS containing bovine serum albumin (1%; Sigma Aldrich) was added to the wells (to prevent non-specific binding of the organoids to the wells and pipet tips), and the domes were mechanically disrupted by pipetting up and down. The organoids and organoid fragments (in 1% BSA solution) were transferred to a 96-well clear round bottom Ultra-Low Attachment microplate (Corning), covered with 96-well sealing mats, and transported for embedding at the University of New England (Biddeford, ME).

The MIOs were embedded into agarose, then paraffin-embedded and sectioned at 5 to 8 µm. Sections were stained using hematoxylin & eosin (H&E; VWR) and immunohistochemistry (IHC) against 6 markers [lysozyme (Abcam), MUC2 (Genetex), chromogranin-A, villin, vimentin (Abcam), and E-cadherin (BD Biosciences)]; plus, isotype and primary antibody omission controls (Abcam) via multiplex immunofluorescence. H&E slides were imaged using brightfield microscopy (Keyence BZ-X700 microscope); IHC slides were assessed/imaged on an epifluorescence microscope (Leica DM2500), then final images were collected using a laser scanning confocal microscope (Leica TCS SP5).

## Results and Discussion

Mouse intestinal organoids (MIOs) were cultured in domes containing a 1:1 mixture of Corning® Matrigel® matrix for organoid culture, using the HUB Organoid Technology method and IntestiCult™ organoid growth medium. The MIOs cultured in three different lots of Corning Matrigel matrix for organoid culture showed typical intestinal organoid morphology with buds (Figure 1A). Furthermore, H&E staining of embedded sections of MIOs cultured in three lots of Corning Matrigel matrix for organoid culture (Lot 1: passage 7; Lot 2: passage 9; Lot 3: passage 7) showed highly organized structures with a lumen and a budding morphology (Figure 1B).

Immunohistochemistry revealed that the MIOs cultured in all three lots of Corning Matrigel matrix for organoid culture contained differentiated intestinal cells such as mesenchymal cells, goblet cells, enterocytes, enteroendocrine cells, and paneth cells as demonstrated by the presence of vimentin, mucin2, villin, chromogranin- A, and lysozyme respectively (Figure 2).

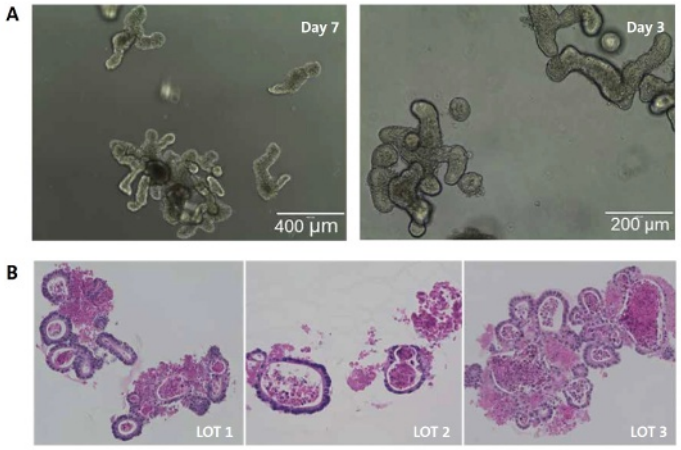


*Figure 1: Morphology of mouse intestinal organoids cultured in Corning Matrigel matrix for organoid culture. (A) Representative images of MIOs in culture. (B) Representative photomicrographs of paraffin embedded and sectioned Day 7 MIOs cultured in three lots of Corning Matrigel matrix for organoid culture (Lot 1: passage 7; Lot 2: passage 9; Lot 3: passage 7).*

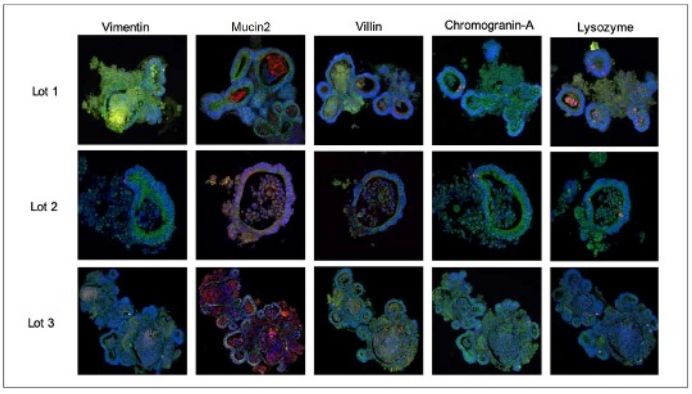


*Figure 2: Representative immunofluorescent images of MIOs in three different lots (Lot 1: passage 7; Lot 2: passage 9; Lot 3: passage 7) of Corning Matrigel matrix for organoid culture show presence of vimentin, mucin2, villin, chromogranin-A, and lysozyme, demonstrating the presence of mesenchymal cells, goblet cells, enterocytes, enteroendocrine cells, and paneth cells, respectively. In each image – Green: E-cadherin; Blue: DAPI, and Red: Specific marker.*

## Conclusions

- Corning Matrigel matrix for organoid culture is an optimal extracellular matrix that saves reagents and time used for lot testing. Each lot of Corning Matrigel matrix for organoid culture has a specific elastic modulus value indicating the stiffness of the gel formed and is qualified to form stable 3D domes commonly used in organoid culture protocols.
- Corning Matrigel matrix for organoid culture supported the culture and expansion of mouse intestinal organoids in IntestiCult organoid growth medium for more than 7 passages. Throughout the culture period on Corning Matrigel matrix for organoid culture, the organoids displayed typical morphology. The MIOs contained differentiated intestinal cells as demonstrated by the expression of surface markers via immunohistochemistry.

## Acknowledgments

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NOTE: Should you intend to use the HUB Organoid Technology methods for commercial purposes, please contact HUB at info@hub4organoids.nl for a commercial use license.

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## About the Authors

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# High Throughput Gene Expression Analysis of 3D Airway Organoids

*New approach facilitates analysis in healthy and disease-model characterization and screening.*

**by Hilary Sherman, Nathan Elliott, Andrew White, and Ann Rossi, Ph.D.**

## Introduction

For over 30 years, researchers have been using permeable support systems for air-liquid interface culture to study the airway epithelium. Utilizing this traditional technique, primary airway cells are cultured on permeable supports and differentiated into ciliated, goblet, and basal cells to model lung tissue for research in toxicology and respiratory disorders, such as asthma and cystic fibrosis. There is continued need for research in these areas to identify treatments, necessitating an increase in throughput for airway models. Three-dimensional (3D) airway organoids pose a solution for modeling airway tissue. Airway organoids produced from primary airway cells have the same ability to differentiate into polarized structures consisting of ciliated, goblet, and basal cells without the need for a permeable support system.[1] This allows for increased throughput of airway studies beyond the currently available 96-well format to 384-well and potentially larger formats. Importantly, advances in characterization of these model airway cultures is also critical to understanding these pathologies and facilitates high throughput cell-based assays for therapy research and development. An essential aspect of studying respiratory disorders is comparison of gene expression of healthy versus diseased tissue, as alterations in the composition of the differentiated cells or cytokines produced are often associated with various pathologies. We use the nCounter® PlexSet™ assay to characterize the gene expression of airway organoids generated from healthy and asthmatic primary bronchial cells. The PlexSet assay provides a simple and cost-effective solution for multiplex sample analysis of up to 96 genes in 96 samples per run. The assay is compatible with whole cell lysate, without the need for RNA purification and was simple to incorporate into the airway organoid workflow. The result is high throughput gene expression data without amplification, cDNA conversion, or library prep.

## Materials and Methods

### Airway Organoid Culture

Normal and Asthmatic human bronchial epithelial cells (HBEC; Lonza Cat. Nos. CC-2540S and 00194911, respectively) were cultured in PneumaCult™-Ex Plus medium (STEMCELL Technologies Cat. No. 05040) per manufacturer's protocol. Cells were harvested with Accutase® (Corning Cat. No. 25-058-CI) and

seeded onto 50 µL of undiluted, polymerized Corning® Matrigel® matrix for Organoid Culture (Corning Cat. No. 356255) per well of a 96-well microplate (Corning Cat. No. 353219). Cells were seeded at a density of 1.4 x $10^4$ cells per well in a volume of 80 µL per well of assay medium. Assay medium consisted of complete PneumaCult-ALI medium (STEMCELL Technologies Cat. No. 05001) containing 0.45 mg/mL Matrigel matrix. Medium was exchanged 3 times per week for 20 to 23 days.

### Airway Organoid Staining

Organoids were collected from Matrigel matrix by pipetting up and down with Axygen® wide bore tips (Corning Cat. No. TF-205- WB-R-S) and incubating with Corning Cell Recovery Solution (Corning Cat. No. 354253) at 4°C for 20 minutes. Organoids were washed several times with cold phosphate buffered saline (PBS) before fixing with cold 4% paraformaldehyde for 15 minutes at 4°C. Organoids were permeabilized with 0.5% Triton X™-100 for 20 minutes prior to washing with PBS and staining. One microliter of primary conjugated antibody was added to each well containing 49 µL of PBS for overnight incubation at 4°C. Airway organoids were immunolabeled with Abcam antibodies for beta IV tubulin, mucin 5AC, cytokeratin 5, and isotype control (Abcam Cat. Nos. ab204034, ab218714, ab193894, and ab199091, respectively). The next day, organoids were washed with PBS and nuclei were stained with 10 µg/mL of Hoechst 34580 (Thermo Fisher Cat. No. H21486). Images were captured with the Thermo Fisher Scientific CellInsight™ CX7 Hight-Content Screening Platform using a 40X objective.

### Drug Exposure

Twenty-four hours prior to lysing organoids, media was replaced with assay medium containing 1 µM Dexamethasone (Sigma Cat. No. D2915) or media.

### Gene Expression Analysis

Samples were sent to NanoString Technologies for analysis via their nCounter platform (Figures 1 and 2). Prior to analysis, organoids were removed from Corning Matrigel matrix for Organoid Culture as previously described. After organoids were separated from Matrigel matrix, they were lysed with iScript™ buffer (Bio-Rad Cat. No. 1708898) per manufacturer's protocol. Samples were immediately frozen at -80°C and shipped on dry ice for analysis. Five microliters of each lysate were hybridized with nCounter® Plex-Set™ reagents for 16 hours at 67°C. Data was collected on the nCounter MAX instrument system. Digital counts were normalized to the geometric mean of internal positive control and reference gene counts, and log2 transformed. Reference genes ABCF1, GUSB, HPRT1, LDHA, POLR1B, and RPLP0 are commonly used by NanoString Technologies in many of their commercially available panels.

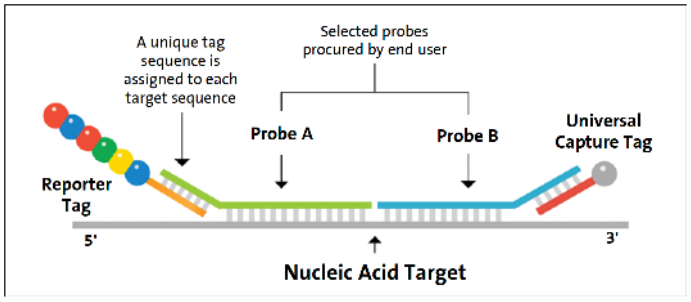


*Figure 1. nCounter barcoding technology for target counting. nCounter barcoding technology uses color-coded molecular barcodes to hybridize and count nucleic acids, and is applied to analysis of RNA, DNA, and protein.*

## Results and Discussion

### Organoid Confirmation

Within 3 days of culture, 3D structures began to appear in the wells followed by lumen formation in the

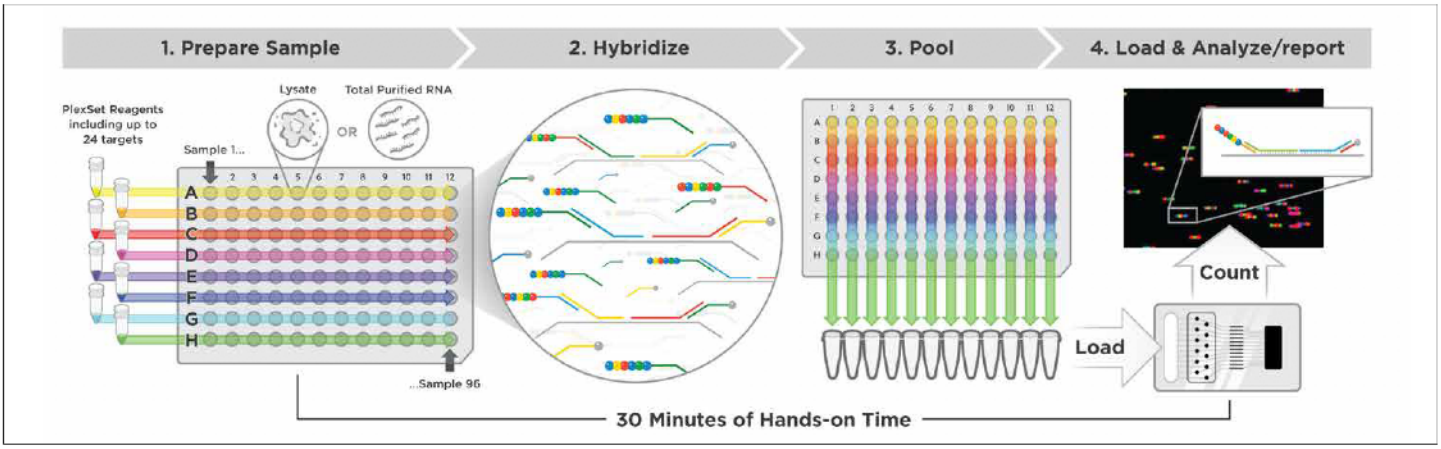


*Figure 2. nCounter technology workflow. Schematic of workflow for high throughput gene expression analysis directly from cell lysates.*

center of the structures by day 17 (Figure 3). After 23 days, many of the structures had moving cilia facing the lumen that were visible via bright-field imaging at 200X. Immunofluorescence staining revealed the presence of basal cells, ciliated cells, and mucus producing cells in both normal and asthmatic organoids (Figure 4). There was more positive mucin 5AC staining with the asthmatic organoids (Figure 4), which is consistent with primary literature reports.[2]

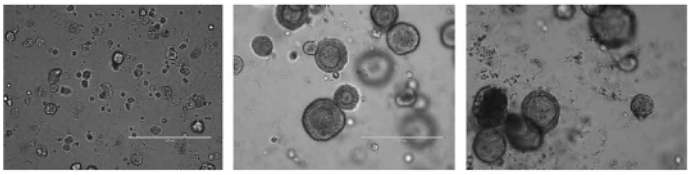

*Figure 3. Airway Organoids. Representative photomicrographs of 3- (left), 17- (middle), and 29-day-old (right) airway organoids on Corning Matrigel matrix for organoid culture. Images were capture at 100X magnification with an EVOS® FL microscope. Scale bars are 400 µm.*

## Gene Expression Analysis

Reproducibility of nCounter data was demonstrated by generating a correlation plot of counts from

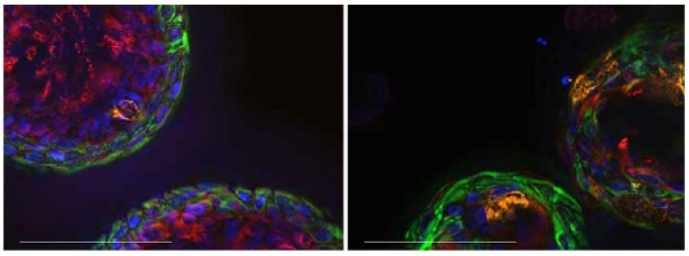

*Figure 4. Normal versus asthmatic airway organoids. Representative photomicrographs of 23-day-old airway organoids from normal (left) and asthmatic (right) donors. Multicolor fluorescent labels indicate specific cells types: basal cells (green), ciliated cells (red), mucus production from goblet cells (orange), nuclei (blue). Images were captured at 40X magnification. Scale bars are 100 µm.*

two different wells of the same condition (Figure 5). The resulting R2 value was 0.977 which indicates strong correlation,[3] and thus, highly reproducible data from independent samples. The nCounter expression data was then utilized to compare expression of cell type specific genes for differentiated airway organoids relative to expression in the source HBECs prior to differentiation. Figure 6

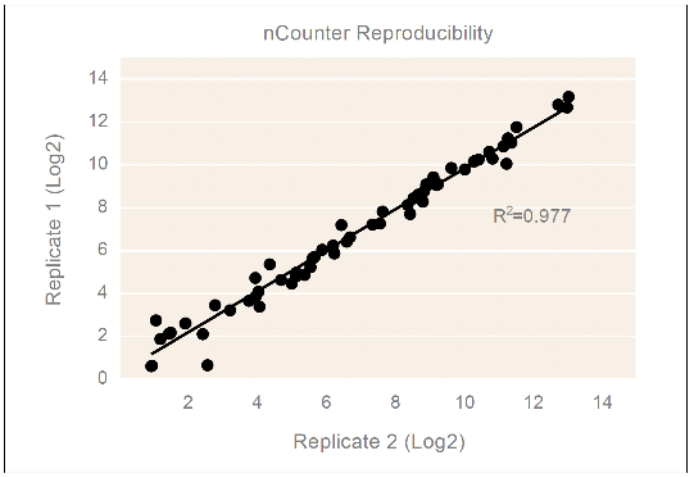


*Figure 5. Reproducibility of gene expression data from replicates. Representative correlation plot demonstrating reproducibility of counts from two different wells of the same condition.*

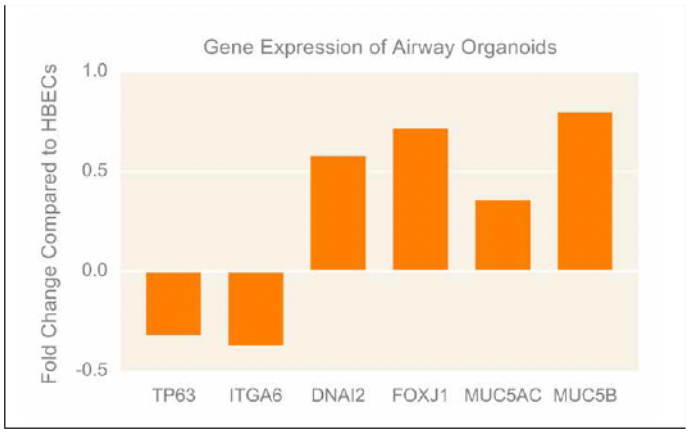


*Figure 6. Changes in expression of genes associated with airway organoid generation. Fold change of gene expression of airway organoids compared to normal HBECs. TP63 and IGA6 expression are associated with basal cells. DNAI2 and FOXJ1 expression are associated with ciliated cells. MUC5AC and MUC5B expression are associated with goblet cells.*

shows downregulation of genes TP63 and IGA6—the transcription factor tumor protein p63 and its downstream target integrin alpha 6, respectively—that are widely expressed in epithelial cells. Concurrently, there was an upregulation of genes associated with differentiated bronchial epithelial cells.[4] Specifically, expression of axonemal dynein intermediate chain 2 (DNAI2), which encodes for a protein that is part of the dynein complex in respiratory cilia, and forkhead box protein J1 (FOXJ1) transcription factor which is involved in ciliogenesis, were increased in the airway organoids relative to the source HBECs. MUC5AC and MUC5B that encode mucin proteins 5AC and 5B secreted by goblet cells were similarly upregulated in the organoids.

To demonstrate high throughput gene expression analysis of diseased organoids, we compared normal and asthmatic airway organoids (Figure 7). Of note, we observed increased expression of chemokine ligand 5 (CCL5), colony-stimulating factor 3 (CSF3), interleukin 6 (IL6), interleukin 10 (IL10), interleukin receptor-like 1 (IL1RL1), mucin 5AC (MUC5AC), and phospholipase A2 (PLA2G2A) in the asthmatic organoids. These genes encode for chemokines, receptors, and other proteins associated with inflammatory processes in asthmatic airway cells.[5-11] Finally, we were able to detect differential gene expression with the nCounter® technology between normal and asthmatic organoids (Figure 8). Generally, the subset of genes upregulated in the normal organoids are the same as those genes downregulated in asthmatic airway organoids and vice-versa. Exposure to the anti-inflammatory dexamethasone changed expression (up- and downregulation) of a greater subset of genes in the asthmatic organoids relative to the normal organoids. Taken together, the data demonstrates the feasibility of airway organoid culture with accompanying high throughput gene expression analysis for characterization and 3D screening assays.

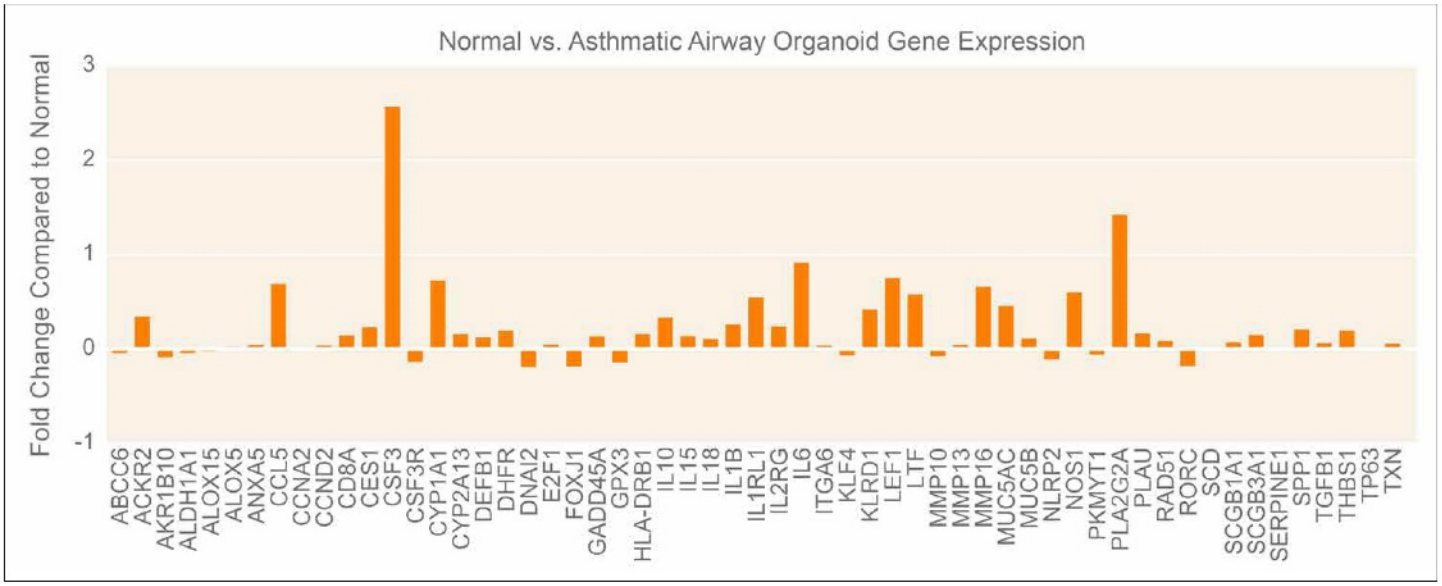


*Figure 7. Fold change of gene expression in asthmatic organoids. Fold change of asthmatic organoid gene expression relative to expression in normal organoids. N = 24 from 2 independent studies.*

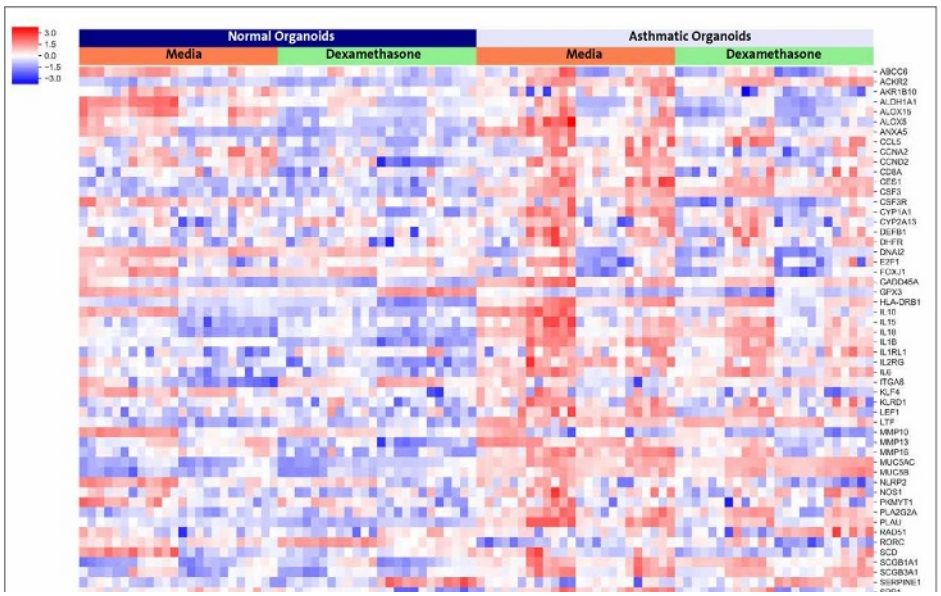


*Figure 8. Fold change of gene expression of normal and asthmatic organoids in response to drug exposure. Heat map demonstrating relative gene expression of normal (left side) and asthmatic (right side) organoids, following normalization to reference genes ABCF1, GUSB, HPRT1, LDHA, POLR1B, and RPLP0 (not shown) after 24-hour exposure to DMSO control or dexamethasone. N = 24 from 2 independent studies.*

## Conclusions

- Corning® Matrigel® matrix for organoid culture provides an ideal environment for the differentiation of bronchial epithelial cells to airway organoids.
- Corning Matrigel Matrix for organoid culture enables 3D cell culture of primary, patient-derived bronchial epithelial cells for high throughput 3D organoid research.
- Sample processing for nCounter analysis requires only cell lysis, eliminating the conventional steps

for gene expression analysis (i.e., gene amplification, cDNA conversion, or library prep).

- The nCounter platform enables high throughput gene expression analysis of 3D organoid cultures for healthy and disease model characterization and screening.

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

*Organoid model allows for the study of in vivo developmental and biological processes, including tissue renewal, stem cell functionality, and drug responsiveness.*

**by Hilary Sherman and Hannah J. Gitschier, M.S.**

## 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

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References:
1. Application Note: (CLS-AN-542), Culture of mouse intestinal organoids in Corning® Matrigel® Matrix for Organoid Culture
2. Application Note: (CLS-AN-534), High Throughput Gene Expression Analysis of 3D Airway Organoids.

(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.

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

| 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 1. Media Formulations Utilized for Definitive Endoderm Differentiation*

| 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

*Table 2. Antibodies: Flow Cytometry and Immunofluorescence*

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).

Further differentiation of definitive endoderm spheroids into mid/hindgut lineage, was similarly

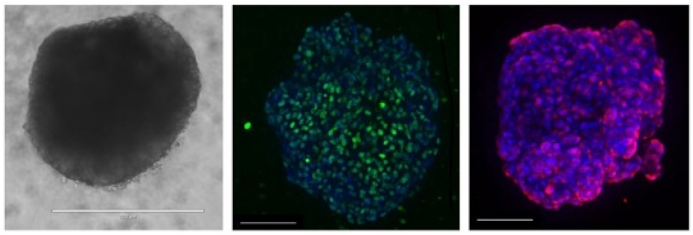

*Figure 1. Representative photomicrographs of definitive endoderm spheroids. Bright field image taken with 10X objective (left) and composite of 15 confocal Z-stacked images with 25 µm slices of SOX17 (middle) and CXCR4 (right) expression with Hoechst nuclei counterstain. Confocal images obtained with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

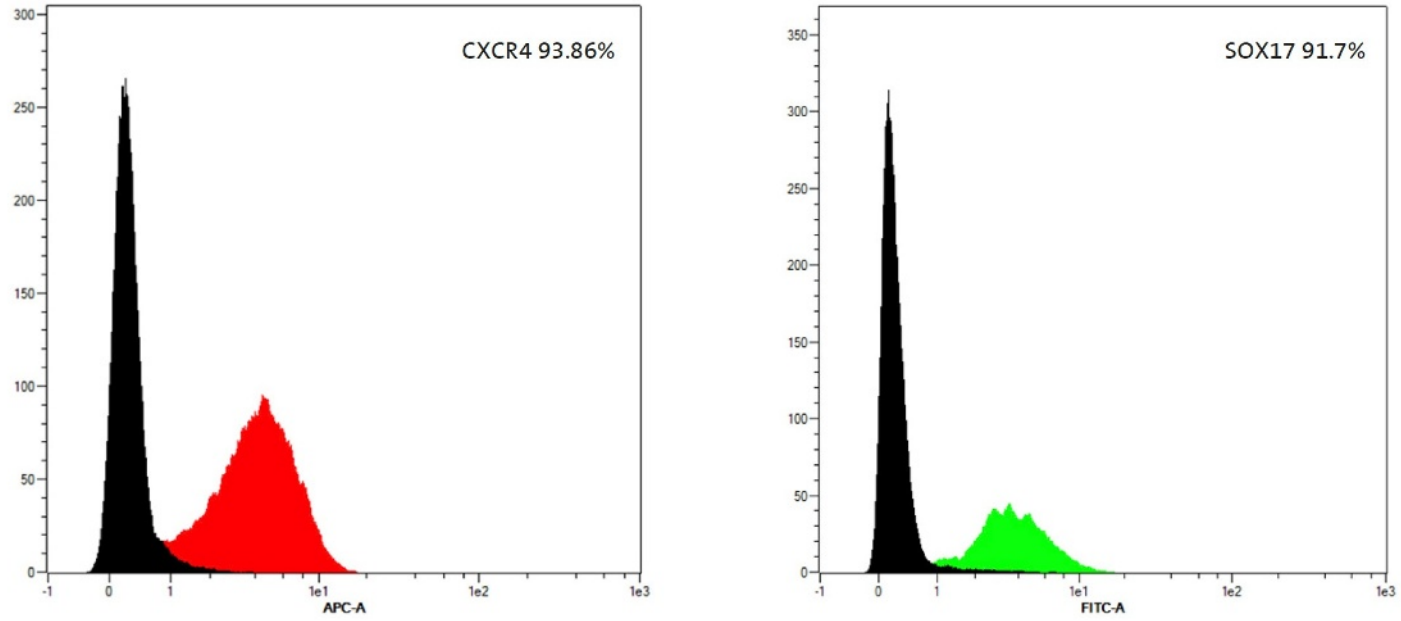


*Figure 2. Representative definitive endoderm marker expression via flow cytometry. Representative histograms demonstrating positive expression of SOX17 and CXCR4 compared to isotype controls from dissociated spheroids.*

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.

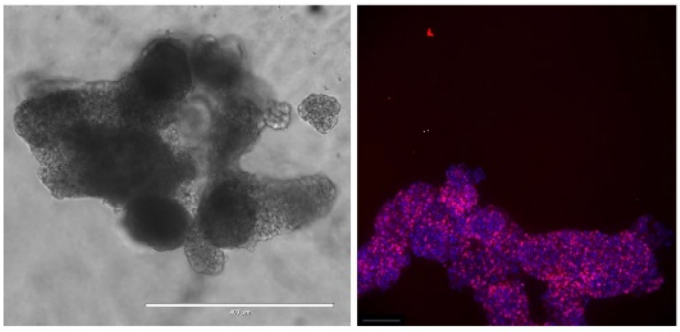

*Figure 3. Representative photomicrographs of mid/hindgut spheroids. Bright field image taken with 10X objective (left) and composite image of 15 confocal Z-stacked images with 30 µm slices of CDX2 stained spheroid with Hoechst nuclei counterstain (right). Confocal image taken with 4X objective using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

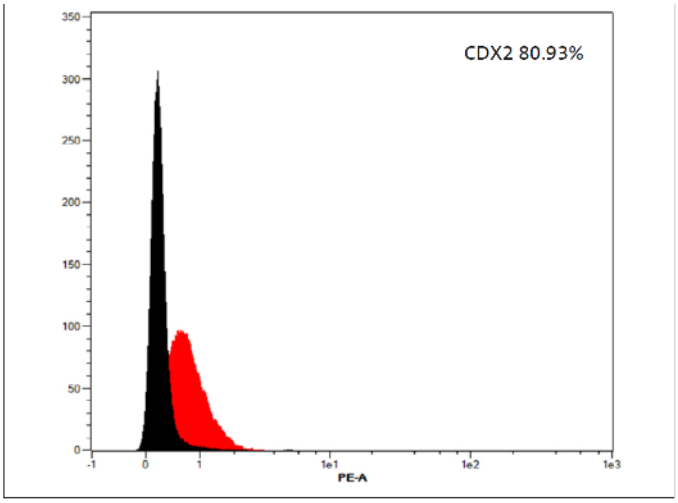


*Figure 4. Representative mid/hindgut marker expression via flow cytometry. Representative histogram demonstrating positive expression of CDX2 compared to isotype control from dissociated spheroids.*

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 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, enteroendocrine cells, and paneth cells, respectively.

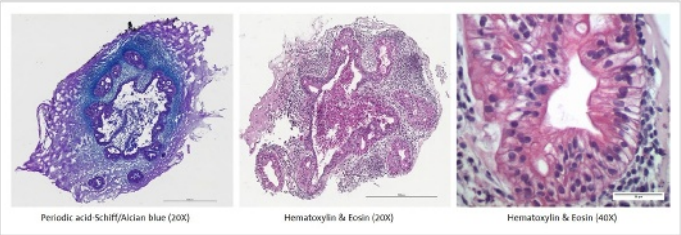


*Figure 5. Histological confirmation. Representative photomicrographs of paraffin embedded and sectioned 4-week-old intestinal organoids. Images were taken using a 20X or 40X objective.*

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.

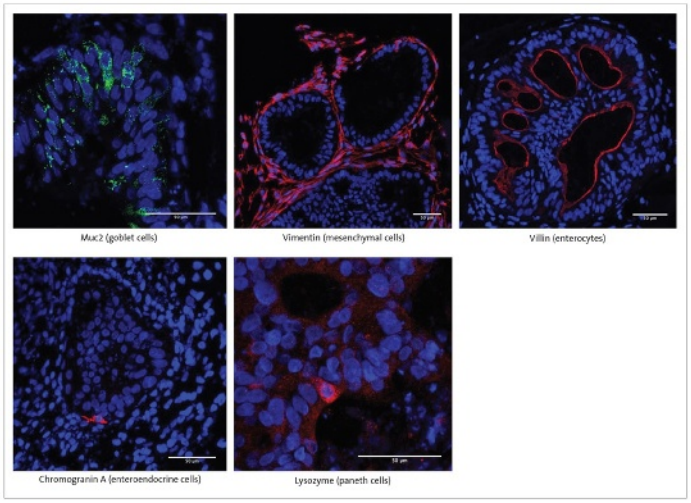


*Figure 6. Histological confirmation. Representative immunofluorescent photomicrographs of paraffin embedded and sectioned 4-week old intestinal organoids. Immunofluorecent images show presence of muc2 (goblet cells), vimentin (mesenchymal cells), villin (enterocytes), chromagranin A (enteroendocrine 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.*

## 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.

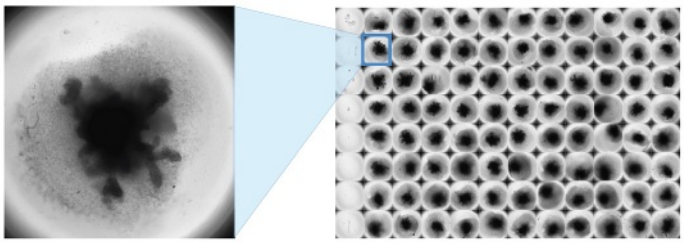

*Figure 7. Representative 96-well spheroid microplate. Image of spheroid microplate containing one intestinal organoid per well, demonstrating consistency and reproducibility of organoid formation across spheroid microplate. Images taken with 2X objective from Thermo Fisher CellInsight CX7.*

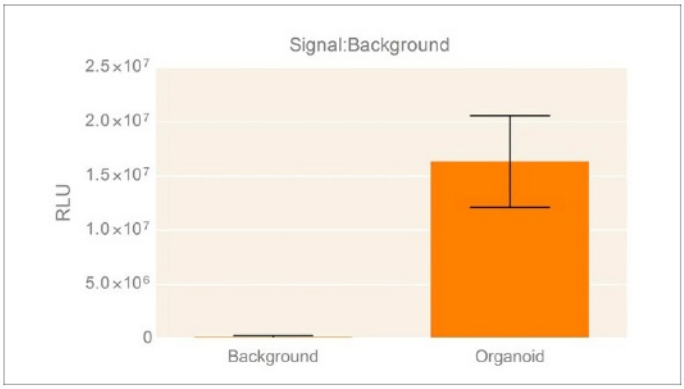


*Figure 8. Representative 96-well organoid assay. Signal to background ratio of 90.6 was observed from a representative ATP assay. N=8 wells for background control, and N=88 wells for 4-week-old intestinal organoids. Data shown ± standard error.*

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.

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