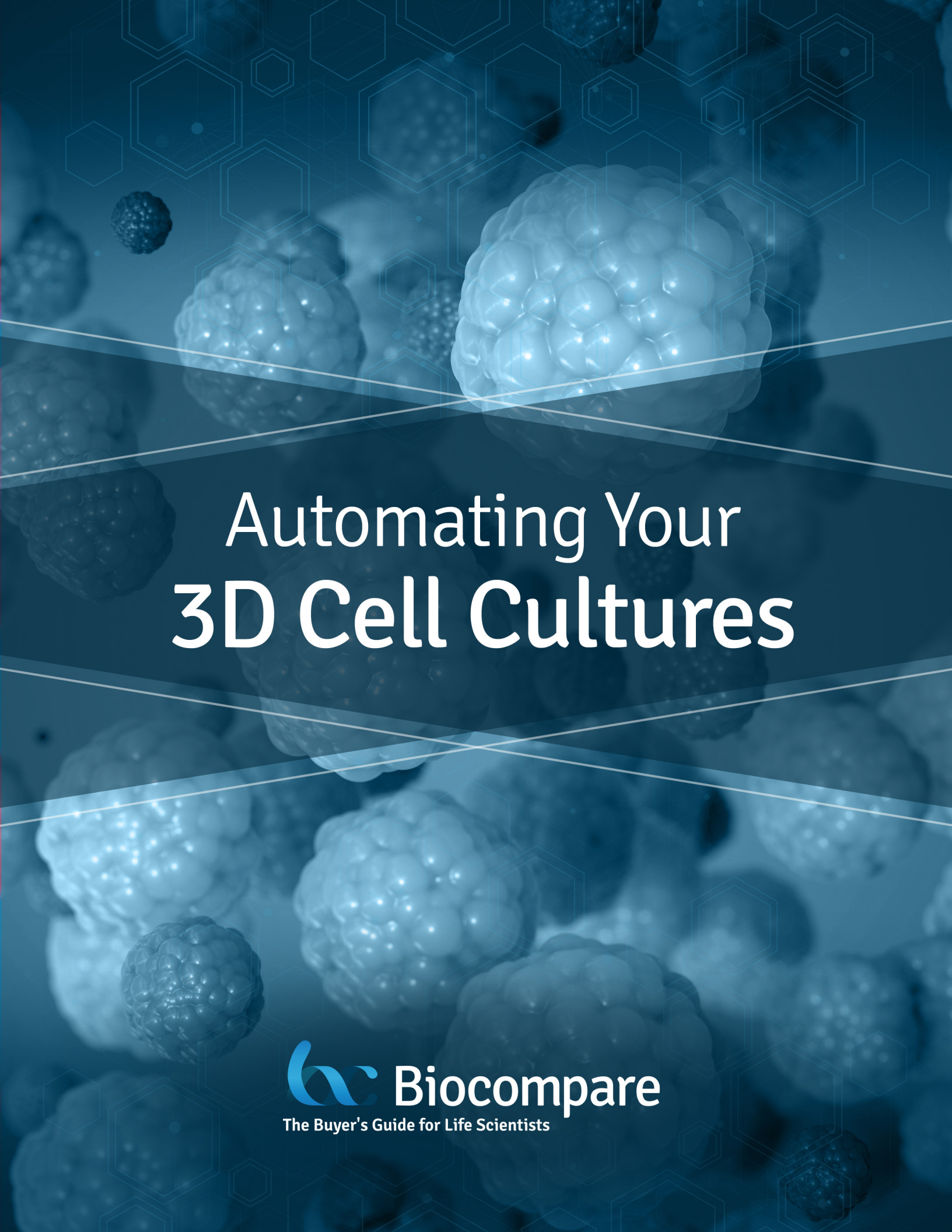

Automating Your 3D Cell Cultures

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Contents

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Adding High Throughput to 3D Cell Cultures

HT methods are becoming more efficient and widely available for 3D studies.

In June, Corning Life Sciences hosted the 2021 Virtual 3D Cell Culture Summit. As part of that event, four experts discussed challenges, solutions, and advances in using 3D cultures in a high-throughput environment.

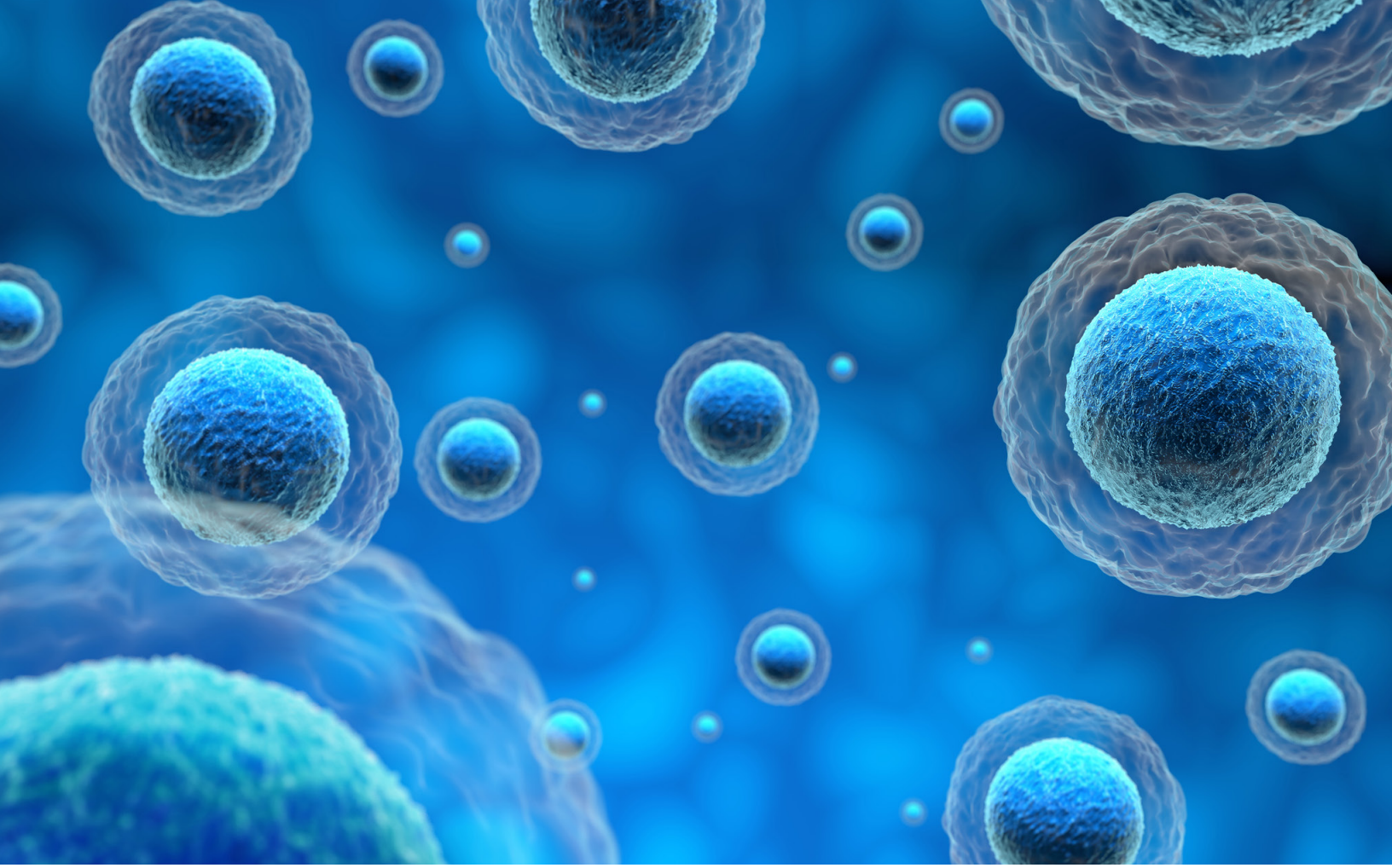
After moderator Elizabeth Abraham—business manager, advanced cell culture at Corning Life Sciences—introduced the experts, they revealed right away that different 3D model types have their unique challenges. For example, Hervé Tiriac, assistant research scientist at the University of California San Diego, works primarily with pancreatic cancer organoids developed from human tissue. He noted that organoids have "different growth rates depending on patients, different morphologies, and that there are different requirements for growth conditions and matrices." The nuances can make the path to moving your 3D cultures into a high-throughput environment more complex, he said, "but we believe the effort is worth it because of the ability of the system to predict outcomes."

Creating useful 3D cultures, though, depends on extreme attention to detail. "Every cell is different," explained Virneliz Fernández-Vega, scientific associate at Scripps Research. "It's a challenge every time we get a new project." When starting organoids in a 2D monolayer and then moving them to a 3D format using Corning® Ultra Low Attachment (ULA) plates, she noted, "you have to really pay attention to every little detail."

For 3D cell culture overall, scientists must address many details. Although the ones that matter the most vary with the specific kind of cell and structure, as well as the experimental questions being addressed, the list of parameters is always long. The path gets even more complicated when scientists move their 3D cultures into mid-throughput and high-throughput environments. Furthermore, the demand on cell numbers is higher to perform 3D cell culture, therefore assay miniaturization would be ideal.

Automating where possible

When scientists use high-throughput approaches to 3D cell culture, it also requires fast methods of data collection and analysis. For instance, Andrei Bonteanu, doctoral candidate at Rice University, works on patient-derived xenografts that model prostate



cancer. He uses automated image analysis to collect data on the resulting spheroids. Then, Bonteanu pointed out, the analysis is done with "batch processing so we're not spending a lot of man hours, if you will, looking at the data."

Other experts also noted the value of automation, even while pointing out some of the hurdles to implementing the technology. When discussing scaling a process to create enough material, Christopher Suarez, field application scientist manager at Corning Life Sciences, said, "you're going to hit some challenges potentially with automating and even sorting."

For instance, Fernández-Vega and her colleagues are working on large-format cell sorting across multiple cell lines. This includes scaling cells and putting them in wells for downstream drug screening analysis. She said, "I've seen a lot of interaction with customers trying to address that bottleneck, to really transition into a [high-throughput screening] environment." Here, some scientists will use a scaffold system, and some won't, and it can depend on the culture system and questions being asked.

When working with 2D and 3D cultures, scientists often grow cells in an extracellular matrix, like Corning® Matrigel® matrix. For example, Tiriac noted that he uses Matrigel matrix as a scaffold in most of his work. Given that it's a biological matrix, he said, there "is a little bit of variation from lot to lot, but it has given us very reproducible results over years of research, and it's been really easy to use for our purposes." Still, he notes the lack of automation to support his methods.

As these experts discussed ways to automate high-throughput uses of 3D cell cultures, Suarez pointed out that his team at Corning Life Sciences

can "help to facilitate a lot of those discussions" for scientists just starting in 3D cell culture or moving into high-throughput screening.

Prospects for more progress

When Abraham asked the panelists "to talk about blue-sky ideas," Bonteanu started off by describing the benefits that automated liquid handing could bring to his lab. He'd use this technology to regulate various parameters, such as pH and temperature. With that, he explained that he could "control the different ECM models that you're going to use and be more open to different types of formulations." Beyond automating liquid handling, Bonteanu would also like to automate some of the steps in spheroid production, such as moving spheroids into media with or without a scaffold.

Tiriac's lab is aiming to co-culture cancer organoids with immune, endothelial, and other cells. "We would like to get the readout on those cells on a cell-specific level," he explained. "So, it is a technical challenge in the laboratory to put all these components together, and a technical challenge to make sure that you get cell compartment–specific readouts."

All along scientists intended to create more physiological conditions with 3D cultures. These experts plan to do even more with these tools. For example, Vega said, "I'm excited to keep expanding our scope of 3D cell models, and definitely looking into co-cultures, angiogenesis, and stem-cell cancer models." She also hopes to isolate cells from patients to expand and grow in 3D cultures, and then use those cells to develop personalized medicine to possibly reduce the time for drug discovery.

As scientists make 3D cultures increasingly realistic, more diseases will be explored and better treatments will be developed. Increasing the pace of that work relies of making high-throughput methods more efficient and more widely available. Plus, scientists need assistance in some cases to find the best available tools to address ongoing problems.

To view the full panel discussion, or any other content from the Corning 3D Cell Culture Summit, visit **www.corning.com/3Dsummit** to watch on demand.

Corning® Matrigel® Matrix 3D Plates for High-Throughput Organoid Assays

Plates were successfully used to screen pancreatic cancer organoids to identify chemotherapeutic treatments.

Hilary Sherman and John Shyu

Introduction

The use of organoids as research tools has become more common due to their ability to better recapitulate disease as compared to more traditional models.[1] Additionally, organoids show great promise in personalized medicine, as biopsies can be used to generate organoids that maintain many functional and genomic characteristics of the donor patient.[2] In order to use organoids as a model, it is essential to maintain proper morphology and polarity. In the body, basement membrane is responsible for helping cells to establish and maintain polarity.[3] This process can be modeled and assayed in vitro by culture of epithelial cells in an extracellular matrix. Corning Matrigel matrix has been demonstrated to enable polarized epithelial structures to form in vitro and can be used to create and maintain a wide variety of organoid models.[2] To increase the throughput for screening with 3D models, Corning has developed 96- and 384-well microplates pre-dispensed with Matrigel matrix specifically for 3D applications. The current study highlights the use of pre-coated Matrigel matrix-3D plates to screen pancreatic cancer organoids.

Materials and Methods

Organoid Culture

Pancreatic cancer organoids HCM-CSHL-0094-C25 (ATCC® Cat. No. PDM-41™) were cultured as recommended by the vendor. In brief, organoids were resuspended in Corning Matrigel matrix for organoid culture (Corning Cat No. 356255) and AdDF complete medium (Table 1) at 60% Matrigel matrix:cell volume. 24-well plates (Corning Cat. No. 3524) were incubated at 37°C in a humidified incubator for a minimum of 24 hours prior to use. Five to seven microliter domes of pancreatic cancer organoids were placed in several wells of a 24-well multiwell plate using pre-chilled Axygen® Maxymum Recovery® 200 µL tips (Corning Cat. No. T-200-C-LR- S). Once domes were plated, plates were inverted in the laminar flow hood for 5 minutes. Plates were then transferred to a 37°C incubator for another 15 minutes in the invert-

Table 1. AdF Complete Medium			
Description	**Vendor**	**Cat. No.**	**Final Concentration**
Advanced DMEM/F-12 (Dulbecco's Modified Eagle Medium/Ham's F-12)	Thermo Fisher	12634	1X
Corning glutagro™ solution	Corning	25-015-CI	2 mM
HEPES buffer solution	Corning	25-060-CI	10 mM
Penicillin-Streptomycin solution	Corning	30-002-CI	1X

Table 2. Pancreatic Organoid Medium			
Description	**Vendor**	**Cat. No.**	**Final Concentration**
WNT3a Conditioned medium			50%
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	1.25 mM
Noggin	Peprotech	50-399-007	100 ng/mL
Rspondin-1	R&D Systems	46-45RS-100	250 ng/mL
B27 Supplement	Invitrogen	17-504-044	1X
Gastrin	Sigma-Aldrich	G9145	10 nM
Nicotinamide	Sigma-Aldrich	N0636	10 nM
hEGF	Peprotech	AF-100-15	50 ng/mL
FGF-10	R&D Systems	345FG025	100 ng/mL
TGFb type I Receptor Inhibitor	Tocris	29-395-0	500 nM
Primocin	Invivogen	NC9392943	0.5X
AdDF+++			Remaining

ed position. Once domes had fully polymerized, 500 µL of pancreatic organoid medium (Table 2) with a final concentration of 10 µM Rock inhibitor (MilliporeSigma Cat. No. Y0503) was added to each well. Medium was changed every 2 to 3 days for fresh pancreatic organoid medium without Rock inhibitor. When organoids were ready for passage, domes were collected by pipetting with Axygen Maxymum Recovery 1000 µL tips (Corning Cat. No. T-1000-C-LR-S). Organoids were resuspended in 2°C to 8°C AdDF complete and centrifuged at 450 x g for 5 minutes. Precipitated organoids were resuspended in 500 µL of AdDF complete and transferred to autoclaved Costar® 1.7 mL low binding microcentrifuge tubes (Corning Cat. No. 3207). Organoids were sheared by triturating with a 20-gauge blunt needle (SAI Infusion Technologies Cat. No. B20-100) attached to a 1 mL syringe (Fisher Scientific Cat. No. 14-955-456). Sheared organoids were centrifuged at 90 x g for 5 minutes. Organoids were then resuspended in AdDF complete with 60% Matrigel matrix volume at a dilution between 1:4 and 1:8. Organoids were re-plated as previously described until ready for assay set up.

Assay Set Up

The day prior to assay set up, Corning® Matrigel® matrix-3D plates (Corning Cat. No. 356257) were placed at 4°C to thaw overnight. On the day of seed, Corning Matrigel matrix-3D plates were placed at 37°C to

polymerize for at least 1 hour. While polymerizing, organoids were collected and centrifuged as previously described. Organoids were dissociated to single cells by incubating with Accutase® (Corning Cat. No. 25-058-CI) for approximately 15 minutes with gentle pipetting. Pancreatic cancer organoids were diluted to 50,000 cells/mL in pancreatic organoid medium containing 10 µM Rock and 0.09 mg/mL Matrigel matrix for organoid culture. Twenty microliters of pancreatic cancer organoid cell suspension were added to each well of the polymerized Corning Matrigel matrix-3D plate. Organoids were incubated for 48 hours prior to treating with drugs.

Screen

After 48 hours, 20 µL of 20 µM compounds from the Enzo Cancer Library (Enzo Cat. No. ENZ-LIB102), 20 µM paclitaxel (Enzo Cat. No. BML-T104-0005), or media control with matched DMSO concentration were added to the wells. Pancreatic cancer organoids were cultured with compounds for 5 days. On day 5, 40 µL of CellTiter- Glo® 3D (Promega Cat. No. G9683) was added to each well. Plates were shaken for 5 minutes and then incubated at room temperature for another 25 minutes prior to reading on Perkin-Elmer EnVision® Multimode Plate Reader.

Follow Up Doses

After 48 hours of culture on Corning Matrigel matrix-3D plates, 20 µL of paclitaxel, daunorubicin (MilliporeSigma Cat. No. D8809), gemcitabine (MilliporeSigma Cat. No. G6423), 5-fluorouracil (Acros Cat. No. 228440010), oxaliplatin (AdipoGen Cat. No. AG-CRI-3592-M005), bortezomib (Selleckchem Cat. No. S1013), or media matched with DMSO were added to pancreatic cancer organoids. Pancreatic cancer organoids were cultured for 5 additional days and assayed with CellTiter-Glo 3D, as previously described.

Results and Discussion

For high throughput assays to provide referenceable data, it is necessary to reduce or control as many variables as possible. For organoid assays, one of the major contributors to variability is the extracellular matrix coating process. Extracellular matrices tend to be temperature-sensitive and viscous which can make uniform coating problematic. Figure 1 demonstrates typical pancreatic cancer organoid morphology after 2 days of culture on Corning Matrigel matrix-3D plates. After 2 days, drugs were added, and organoids were cultured for an additional 5 days. Figure 2 shows representative images of organoids after culture with 10 µM paclitaxel or media with DMSO control. Z' was calculated to deter-

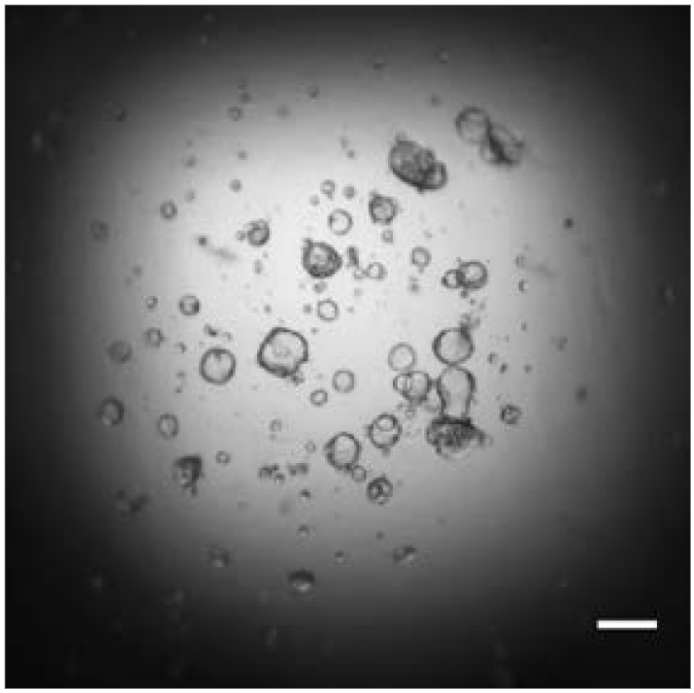


Figure 1. Typical pancreatic cancer organoid morphology. Representative image of typical pancreatic cancer organoid morphology after two days cultured on Corning® Matrigel® matrix-3D plates. Scale bar 200 µm.

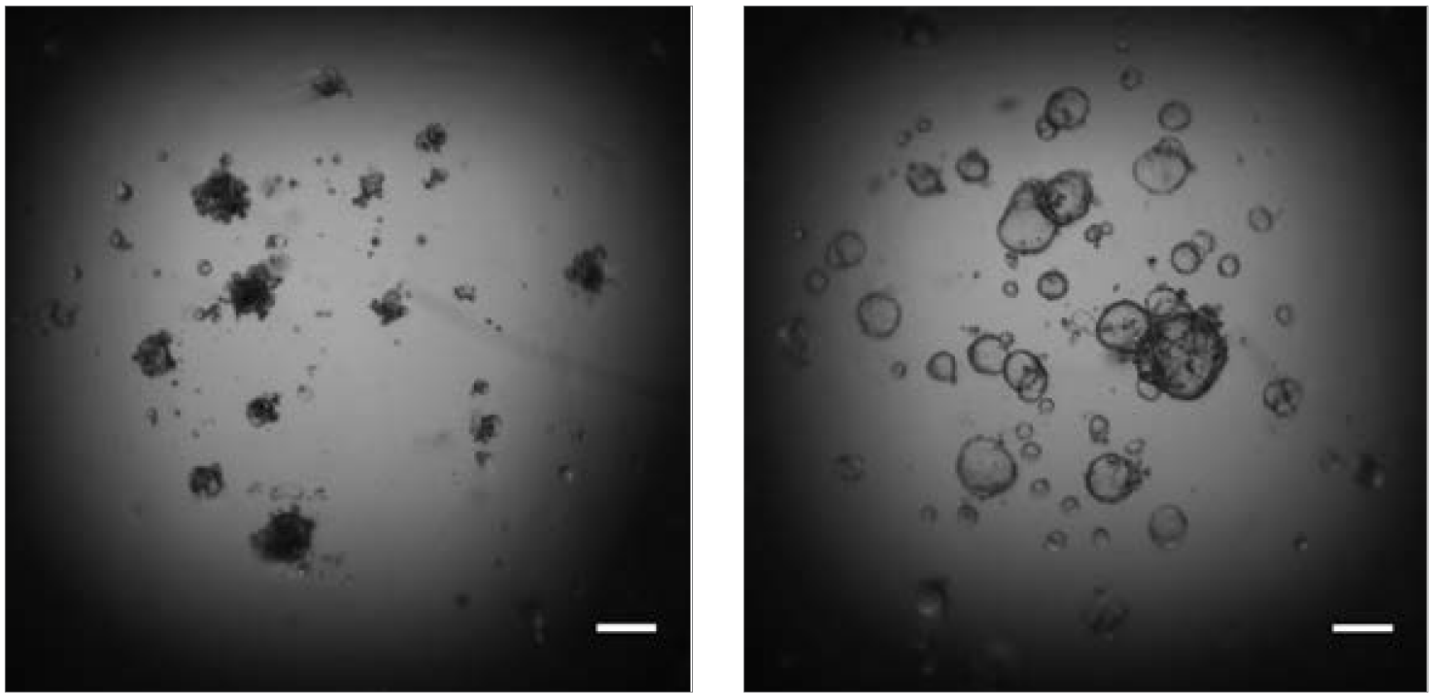


Figure 2. Typical morphology of organoids after exposure to paclitaxel or media control. Representative image of pancreatic cancer organoids after five days of exposure to 10 µM paclitaxel (left) or media with matched DMSO (right). Scale bar 200 µm.

mine the robustness of the assay. Z' greater than 0.5 were achieved in all 3 independent studies (Figure 3). Enzo Life Sciences SCREEN-WELL® Cancer Library was used to determine compound cytotoxicity for the specific organoid model being tested. The resulting cytotoxicity from 3 studies was averaged and sorted by effect. The results are shown in Figure 4. Six compounds were selected for follow up of dosing ei-

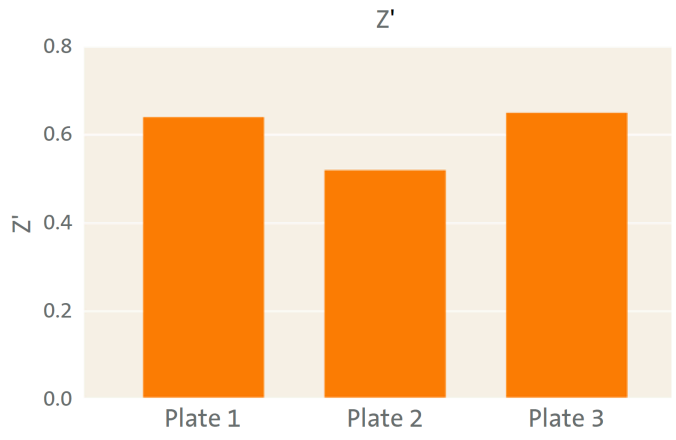


Figure 3. Assay is robust. Z' calculated from comparing viability of pancreatic cancer organoids exposed to paclitaxel or media control. All values were above 0.5 indicating a robust assay.

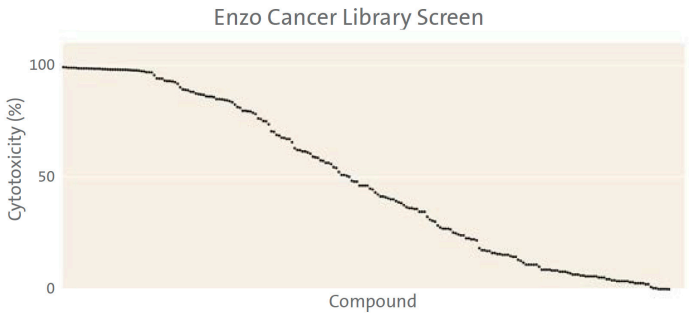


Figure 4. Enzo cancer library screen. Average percent reduction in pancreatic cancer organoid viability compared to media with DMSO response. Data is average of 3 independent screens and sorted by effect on viability.

ther because they were a hit in the screen or because they are traditionally provided as treatment for pancreatic cancer. Figure 5 shows varying degrees of sensitivity between the compounds. Gemcitabine and paclitaxel are often standard recommended treatments for pancreatic cancer.[4] Our study found these compounds to be highly effective at inducing cytotoxicity with the pancreatic cancer organoids tested. (EC_{50} values of 0.5139 nM for paclitaxel and 3.887 nM for gemcitabine). Other traditional chemotherapeutics offered for pancreatic cancer, 5-fluorouracil and oxaliplatin, have been shown to be less effective for pancreatic cancer which corresponded with our data.[5,6] We also found Bortezomib, a proteasome inhibitor that has shown promise as a therapeutic for pancreatic cancer, to be highly effective.[7]

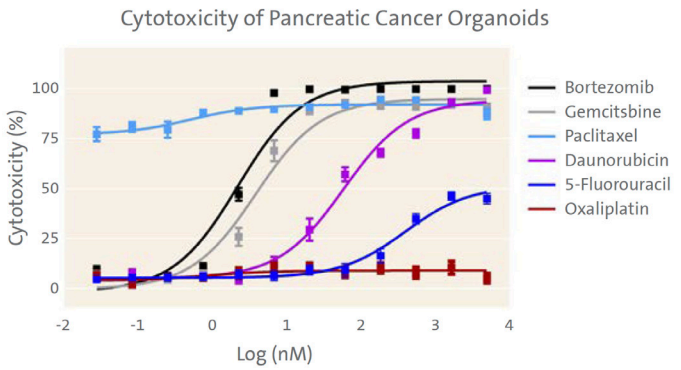


Figure 5. Follow up doses. Follow up cytotoxicity of selected compounds from library. Data is shown as average from 3 independent studies with standard error bars. N = 12 for each dose.

Conclusions

For organoids to become commonplace in personalized medicine or drug discovery, it is essential that the methods used become amenable to high throughput. Corning® Matrigel® matrix-3D plates eliminate one of the typical challenges encountered when self-coating microplates with extracellular matrices (i.e., Matrigel matrix) by providing a ready-to-use option. Corning Matrigel matrix-3D plates provide the convenience and consistency required for 3D high throughput applications such as organoid screening. Our results demonstrated how Corning Matrigel matrix-3D plates can be used to screen pancreatic cancer organoids in order to identify ideal chemotherapeutic treatments. 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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Hilary Sherman and John Shyu are with Corning Life Sciences.

Automation of Forskolin-Induced Swelling Assay of Human Intestinal Organoids

Dispensed organoids were found to be functional and usable in a wide variety of assays.

Hilary Sherman, John Shyu, and Holly Hung

Introduction

Over the last several years, there has been increased interest around the use of patient-derived organoids for personalized medicine and drug discovery.[1] The unique ability of organoids to recapitulate the organ structure, model disease, and maintain the genetic diversity of the donor tissue makes them a promising tool.[2] In order to reach their full research potential, organoids need to be amenable to more automated and high throughput methods. Automating the handling of organoids has been problematic due to the required manipulation of viscous basement membrane extracts. Here, we demonstrate the use of the SPT Labtech dragonfly® discovery to accurately and evenly dispense small (3 µL) droplets of human intestinal organoids mixed with Corning® Matrigel® matrix for organoid culture, followed by a forskolin-induced swelling (FIS) assay.

Materials and Methods

Human intestinal organoids (HIO) from a healthy and cystic fibrosis (CF) donor carrying F508del/S1251N mutation, were purchased from Hubrecht Organoid Technology (HUB; Utrecht, Netherlands). Organoids were cultured per HUB methodologies.[3] In brief, organoids were resuspended in Corning Matrigel matrix for organoid culture (Corning 356255) and growth medium was replaced every 2 to 3 days. When organoids were ready for passage, domes were collected by pipetting with Axygen® Maxymum® Recovery 1000 µL tips (Corning T-1000-C-L-R-S).

Organoids were sheared by triturating with a 20-gauge blunt needle (SAI Infusion Technologies Cat. No. B20-100) attached to a 1 mL syringe (Fisher Scientific 14-955-456), a modification of the HUB method. For a more detailed protocol and materials list for culturing HIO please refer to "Culturing Human Intestinal Organoids with Corning Matrigel Matrix for Organoid Culture" (Corning Application Note CLS-AN-569). At least 24-hours prior to assay setup, Corning 96-well cell culture microplates (Corning 3596) were pre-warmed by placing at 37°C. Addi-

tionally, SPT Labtech reservoirs (SPT 4150-07203, 4150-07204), cool block (SPT 3152-02011), and syringes (SPT 4150-07209) were placed at -20°C. On the day of seeding, organoids were sheared to the desired size and combined with Corning Matrigel matrix for organoid culture to a ratio of 50% Matrigel matrix:cell volume. Then, the dragonfly discovery was used to dispense a single 3 µL drop into the center of each well of a 96-well microplate. There was no pipetting performed to mix while dispensing into a single plate. A full 96-well microplate took less than 1 minute to seed. Next, Matrigel matrix domes were polymerized for 15 minutes at 37°C before adding 100 µL of complete medium containing 10 µM Rock inhibitor (MilliporeSigma Y0503) with or without 3 µM VX809 (Selleckchem S1565) to each well. Sixteen to twenty-four hours later, dragonfly discovery was used to add 10 µL per well of 0.04 µg/mL Calcein AM (Corning 354216) diluted in medium. Organoids were incubated for 30 minutes until completely stained. After staining, medium was removed and replaced with 100 µL of medium containing 0.128 µM Forskolin (MilliporeSigma F6886) with or without 3 µM VX770 (Selleckchem S1144). Images of organoids were taken just prior to addition of compounds and every 10 minutes after for 60 minutes using a 2X objective Thermo Scientific CellInsight™ CX7.

Results and Discussion

In order to automate organoid assays, such as the FIS assay, it is essential that liquid handlers can accurately dispense organoids. Additionally, if the end point of the assay requires image capture, the location of dispensed volumes need to be consistent in order to keep imager scan times manageable. Figure 1 shows a typical 96-well microplate of stained human intestinal organoids dispensed in 3 µL drops using dragonfly discovery. Each well is a single and centered field of view demonstrating the ability of

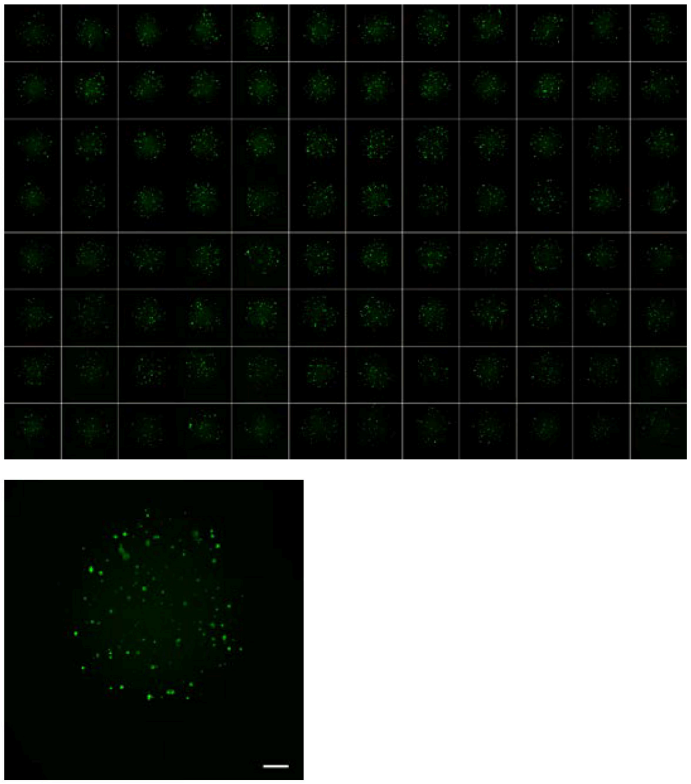

Figure 1. Uniform domes dispensed in the center of each well. Representative image of uniform, single domes dispensed in each well of a 96-well microplate with one well digitally zoomed in. Images were taken with Thermo Scientific CellInsight CX7 High-Content Screening Platform using a 2X objective. Scale is 200 µm.

the instrument to precisely dispense drops of the same size in a consistent location. Further, the instrument dispenses a consistent number of organoids in each drop as shown by coefficients of variation (CV) (in Figure 2). The FIS assay was chosen as proof of the capability to automate a functional organoid assay. The FIS assay is used to detect the presence of mutations in the encoding cystic fibrosis transmembrane conductance regulator (CFTR) and potential therapies to correct the disfunction. The premise is that mutations in CFTR cause improper regulation of fluid and electrolytes in epithelial cells of organs, such as the intestine and the lung.[4] Healthy HIO swell in size when stimulated with forskolin due to an increase

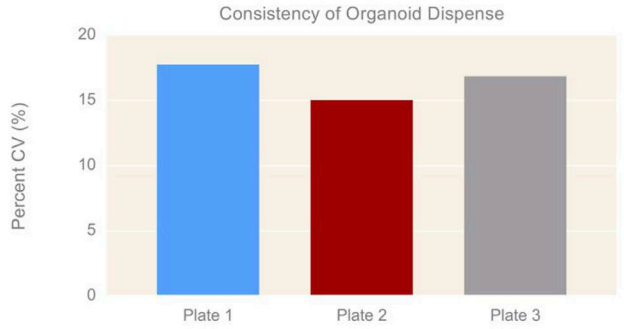

Figure 2. Consistency of dispense within a plate. The number of organoids dispensed in each dome from three 96-well microplates was enumerated in order to calculate consistency across each plate. Each plate resulted in a CV of less than 18%.

of fluid secretion in the lumen of the organoid. CF organoids, depending on the extent of the mutation, demonstrate more limited or no swelling when stimulated with forskolin. Part of the challenge with finding treatments for CF is that there are many different CFTR mutations and the mutation will dictate the best drug combination for each patient.[3]

Figure 3 shows representative images of organoids prior to stimulation and 60 minutes after with or without the addition of treatment(s). The images show the difference in response of healthy organoids compared to CF organoids with DMSO and no additional drugs. The healthy organoids increase in size with a noticeably larger lumen in many of the organoids after 60 minutes. There seems to be little to no noticeable change in CF organoid size when DMSO or VX809 (a CFTR corrector that partially restores CFTR function with some CFTR mutations) is added.[5] VX770 (a CFTR potentiator that has been shown to increase the activity CFTR proteins) appears to have a positive impact on CF organoid swelling. Figure 4 is the average measured change in organoid size from 2 independent studies. The data shows that the combination of VX770 and VX809 is

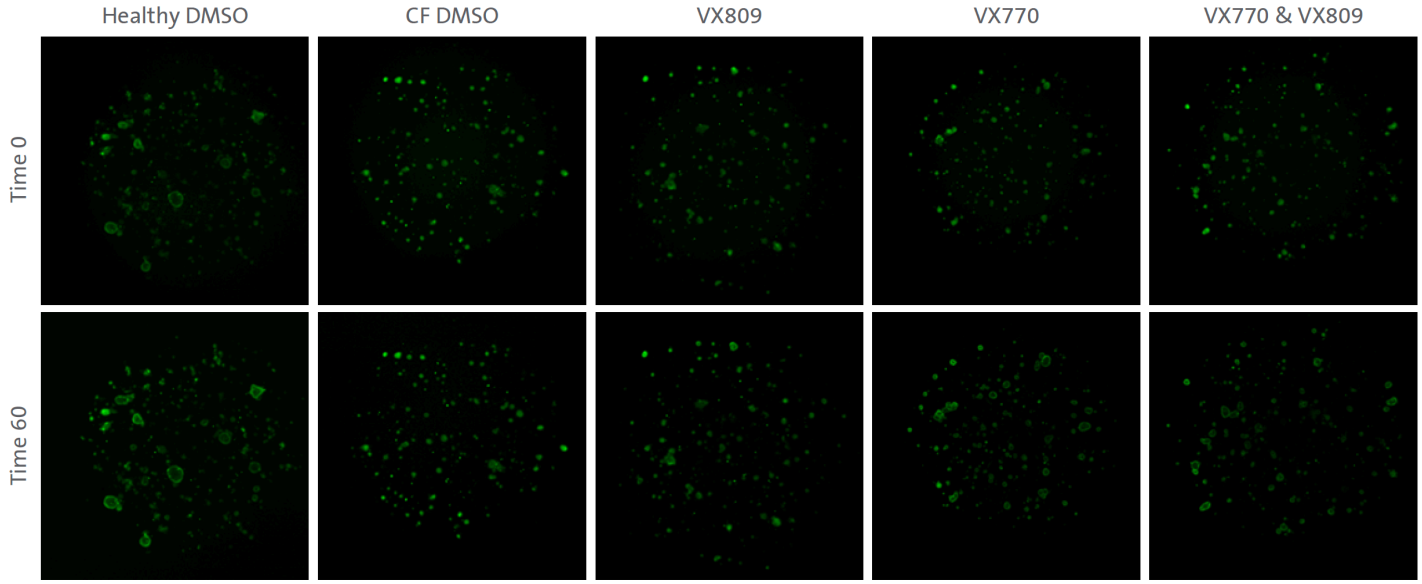

Figure 3. Representative images before and after forskolin addition. Representative photomicrographs demonstrating healthy and CF organoid swelling response under different conditions. Images were taken with Thermo Scientific CellInsight CX7 High-Content Screening Platform using a 2X objective.

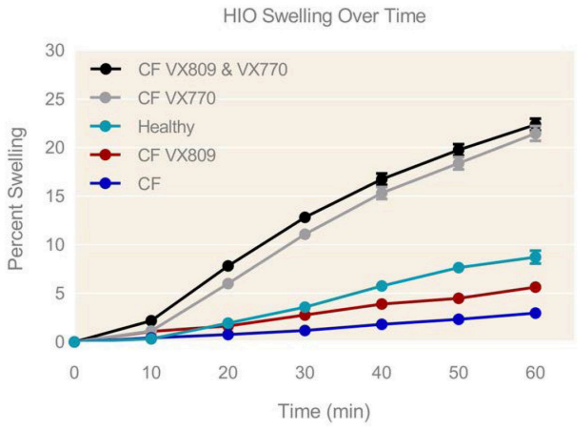

Figure 4. Forskolin-induced HIO swelling over time. Average percent swelling compared to time 0 of healthy and CF HIO. Data is the average from two independent studies shown with standard error bars. N = 38 for healthy HIO and 40 for CF HIO conditions.

a potential treatment for this patient's specific CFTR mutation. Additionally, the data is consistent with what has been previously reported for CF lines with

Conclusions

Automating the handling of organoids is essential for organoids to become a feasible tool for personalized medicine and drug discovery. The dragonfly discovery is a positive displacement, non-contact dispenser that can accurately and precisely dispense low volumes. By pre-chilling all consumables, we were able to dispense 3 µL droplets of human intestinal organoids suspended in Corning® Matrigel® matrix for organoid culture into 96-well microplates. The dispensed organoids are functional and can be used in a wide variety of assays such as the FIS assay demonstrated in this application note.

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

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Hilary Sherman and John Shyu are with Corning Life Sciences. Holly Hung is with SPT Labtech.

Applications Using Human Liver Spheroids

Drug safety assessment and liver disease modeling are increasingly popular applications.

Feng Li, Li Cao, Jie Wang, Sweta Parikh, and Joanne Bourgea

Introduction

3D human PHH liver spheroids are emerging as a more physiologically and pathologically relevant preclinical human liver models for drug safety assessment and liver disease modeling.[1-2] 3D spheroid culture using primary human hepatocytes (PHHs) have been described in recent years. We and others have previously demonstrated the advantages of using PHH liver spheroids in liver toxicity tests over the traditional 2D monolayer culture of PHHs.[3-5] In this application note, we describe the consistent production of Corning® HepGo™ Assay-ready Liver Spheroid Kit using Corning 3D Spheroid-qualified PHHs in 96-well Corning Spheroid Microplates. Since each PHH liver spheroid is made of a limited number of cells, a great number of spheroids will be needed to generate materials such as RNA for routine molecular biology analysis. With slightly modified conditions, PHH liver spheroid culture in Corning Elplasia® 24-well Plates can provide an easy solution for this need. Development of methods and protocols for high content imaging-based applications and other quantitative assays can greatly expand the use of PHH liver spheroids for *in vitro* liver toxicity and disease modeling such as steatosis as showcased in this application note.

Materials and Methods

Chemicals and Reagents

Unless otherwise specified, all the reagents were purchased from MilliporeSigma which include: troglitazone (Cat. No. T2573), lipopolysaccharide (LPS, Cat. No. L4391), oleic acid (OA, Cat. No. O1008), and palmitic acid (PA, Cat. No. P5585).

Liver Cells – Thawing and Cell Suspension Preparation

Corning 3D Spheroid-qualified Primary Human Hepatocytes (Corning Cat. No. 454552; Lots 348a, 397, and 404) were used in this study.

Cryopreserved hepatocytes were thawed using Corning Hepatocyte Thawing Medium (Corning Cat. No. 454671), centrifuged at 100g for 10 minutes and cell pellet was suspended in pre-warmed plating medium (containing 10% fetal bovine serum).

3D Liver Spheroid Culture in Corning 96-well Spheroid Microplates and 24-well Corning Elplasia Plates

For initial plating, 10% fetal bovine serum (FBS) was supplemented to Corning HepGo™ Hepatocyte Medium (Corning Cat. No. 464670) as described above. Corning 96-well Spheroid Microplates (Corning Cat. No. 4515 or 4520) or 24-well Elplasia Plates (Corning Cat. No. 4441) were used to generate liver spheroids for all the assays described in this study. After spheroid formation, Corning HepGo Hepatocyte Medium (Cat. No. 464670) was used for compound treatment and spheroid culture maintenance.

For PHH spheroid culture on 96-well spheroid microplates, 1000 PHH cells/well were seeded on a 96-well spheroid microplate as determined in previous studies.[5] The initial cell mix plating volume is 100 µL/well on a 96-well spheroid microplate. After seeding, the plates were centrifuged at 100g for 2 minutes and then transferred to the tissue culture incubator at 37°C and 5% CO_2.

For liver spheroid culture in 24-well Elplasia plates, PHHs were plated (Day 0) at 290 x 10^3 cells/well in total 1 mL plating media (i.e., approximately 500 cells/microwell for spheroid formation. 1 mL media was added on Day 2. Half medium change was performed daily to maintain the liver spheroid culture. On the day before sample collection for albumin analysis, 1.5 mL of used media was removed and replenished with 1.5 mL fresh media. 1 mL of used media was collected weekly for albumin measurement using the Human Serum Albumin SimpleStep® ELISA Kit (Abcam Cat. No. ab179887).

Steatosis Induction, Mitochondrial Toxicity Assay, and High Content Imaging Using 3D Liver Spheroids

A mixture of monounsaturated fatty acid oleic acid (160 µM) and saturated fatty acid palmitic acid (80 µM) were prepared in the medium to induce steatosis in liver spheroid cultures. For the 4-day steatosis induction in PHH liver spheroids, two media formulas were tested. The first medium (pathological medium) contained high glucose (25 mM) and high insulin (1720 nM). The second medium (physiological medium) had low glucose (5.5 mM) and low insulin (0.1 nM). For the 11-day steatosis induction, the steatosis induction was performed in the physiological media.

Mitochondria staining was done using MitoTracker™ Orange (Thermo Fisher Cat. No. M7510) at final concentration of 200 nM for 1 hour in a tissue culture incubator at 37°C, 5% CO_2. Nuclear staining was performed with Hoechst 33342 (Thermo Fisher Cat. No. H3570) at final concentration 10 µg/mL prepared in 4% PFA (Paraformaldehyde). Stained and fixed spheroids were washed 3 times with PBS before imaging. Images were taken and subsequently analyzed on a PerkinElmer Operetta imager. Dose responsive curves were generated using the normalized fluorescent signal intensity measurement.

ELISA and Bioluminescent Assays

For albumin secretion measurement, culture supernatant samples were collected for analysis. The concentrations of human albumin were determined by ELISA assays using the Human Serum Albumin SimpleStep ELISA Kit (Abcam Cat. No. ab179887).

Following fatty acid treatment on liver spheroid culture, bioluminescent assays were performed using liver spheroids to measure the lipid accumulation. The concentration of glycerol was determined using the Triglyceride-Glo™ Assay Kit (TGA, Promega Cat. No. J3161). Standard curves were prepared accordingly to calculate the concentrations of glycerol in the samples.

Results and Discussion

Corning® HepGo Assay-ready Liver Spheroid Kit made from Corning 3D Spheroid-qualified PHHs

PHH liver spheroids can be consistently produced using Corning 3D Spheroid-qualified PHHs (Lot 348a) in 96-well Corning spheroid microplates following standard procedures (Figure 1). Plating 1000 cells/well of Lot 348a PHHs generated liver spheroids around Day 6. With fourteen 96-well microplates for each production batch, the average spheroid diameter was 235.1 ± 9.2 µm (%CV 3.9) and 230.5 ± 4.1 µm (%CV 3.9) for batch 1 and batch 2 production, respectively. These pre-made PHH liver spheroids in the assay-ready format can be shipped directly using Corning proprietary package design to user's sites.

PHH Liver Spheroids Made in Corning Elplasia Plates

PHH liver spheroids can also be made in a large number using Corning Elplasia plates that support the growth of a large number of spheroids within each well. For instance, each well of the 24-well Elplasia plate contains approximately 550 microwells for in-

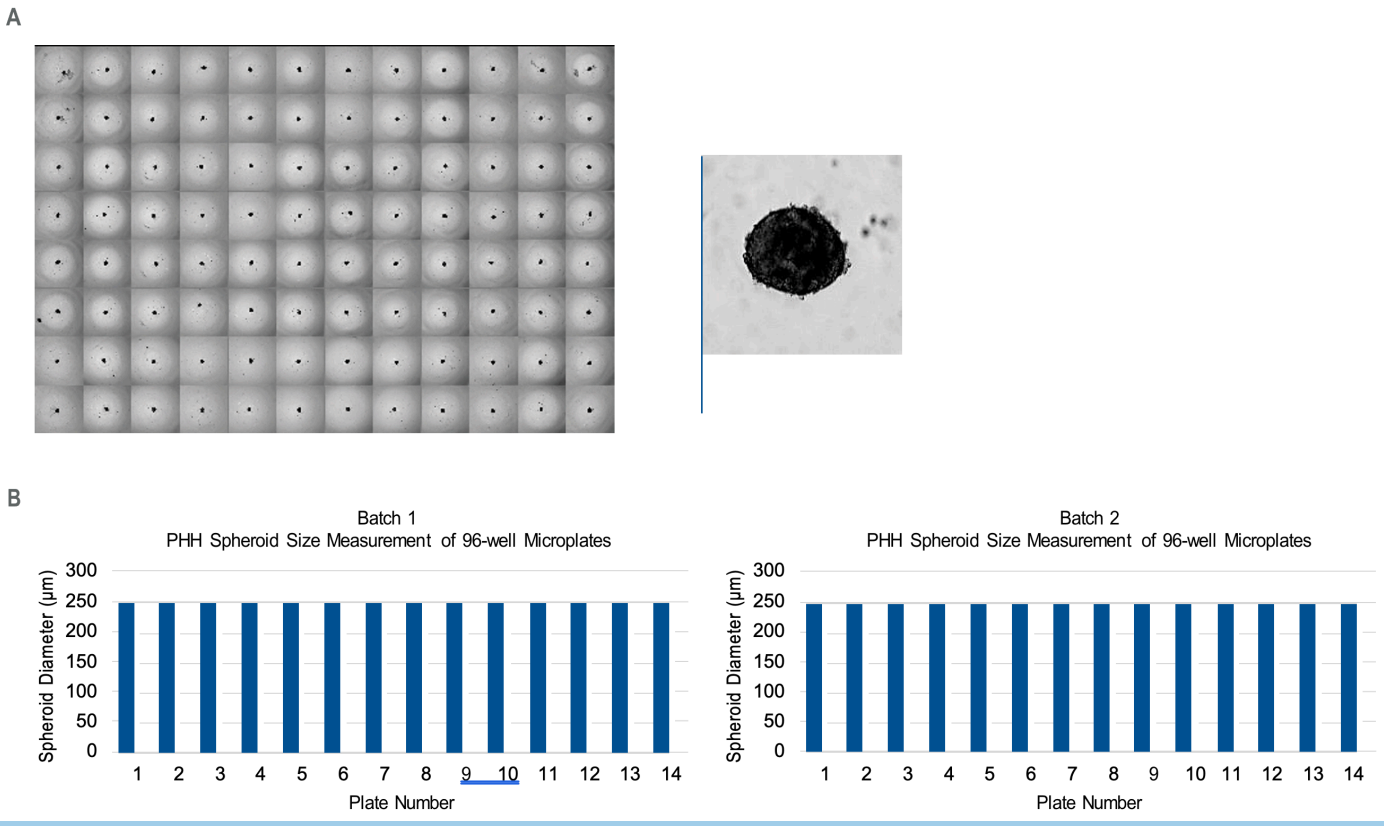


Figure 1. Consistent production of Corning HepGo Assay-ready liver spheroids kit. (A) PHH Lot 348a was used for liver spheroid culture and 1000 cells/well were plated in a Corning 96-well spheroid microplate. A representative image of a 96-well microplate of assay-ready PHH liver spheroids was taken on Day 6 after spheroid formation showing a single PHH spheroid within each well. (B) Bar graphs show consistent PHH liver spheroid size measurement (diameter, µm) on Day 7 from two separate production rounds of fourteen 96-well microplates each. More than 60 spheroids were measured for each plate to calculate the average size of spheroids.

dividual spheroid to form within each microwell. As a single liver spheroid only provides limited amount of materials such as protein, DNA or RNA molecules, this type of liver spheroid culture format is ideal for assays that require more materials. As shown in Figure 2, 3 lots of Corning 3D spheroid-qualified PHHs (Lots 348a, 397, and 404) were plated in a 24-well Elplasia plate to grow liver spheroids. On average, approximately 500 cells/microwell were plated into each well of the 24-well Elplasia plate. The spheroid formation was monitored over time and PHH liver spheroid formed in 5 days in the Elplasia plate. Homogenous spheroid formation across the microwells was observed within the same well.

In Elplasia plates, PHH liver spheroids remained relatively stable over 3 weeks as measured by spheroid size and albumin secretion (Figures 2B and 2C). At the end of the 3-week culture, spheroids made from Lots 348a and 404 were harvested and total RNA were isolated. The yields of total RNA from PHH liver spheroids ranged from 354 to 570 ng/well, or average of approximately 460 ng/well under these conditions (Figure 2D). This amount of RNA should be enough to support routine molecular analysis such as real-time RT-PCR or genome wide gene expression.

Image-based Mitochondrial Toxicity Measurement Using 3D PHH Liver Spheroids

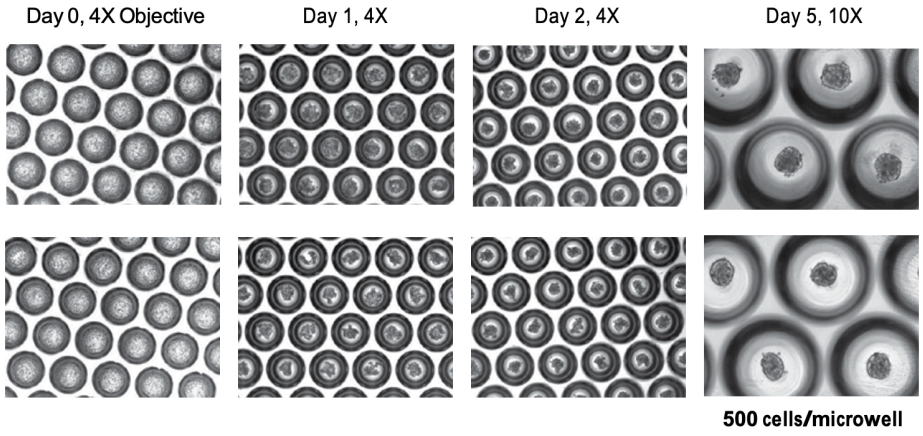
High content image-based analysis can offer sensitive and multiplexed endpoints for liver toxicity testing. PHH liver spheroids can be adopted for image-based mitochondrial toxicity assays. Mitochondrial damage is a major mechanism of hepatotoxicity induced by troglitazone. After 48 hours exposure to a single dose treatment of troglitazone in serial dilutions, liver spheroids were stained with the MitoTracker orange and the fluorescent signals of this dye were detected to measure mitochondria function (Figure 3A). A dose dependent mitochondrial toxic responses was shown as diminishing mitochondria staining at higher doses of troglitazone. An imaging analysis protocol was established to quantitatively measure the changes of total fluorescent intensity. Using liver spheroids made from 3 lots of Corning 3D spheroid-qualified PHHs (Lots 348a, 397, and 404), dose response curves were generated to calculate the IC_{50} values of troglitazone in mitochondrial toxicity. Some extent of donor lot-to-lot variations were detected among these three PHH lots in this assay as the IC_{50} values were at 11.9, 42.1, and 18.0 µM for PHH Lots 348a, 397, and 404, respectively (Figure 3B).

Steatosis Induction Using 3D PHH Liver Spheroids

Steatosis is the accumulation of excessive fatty acid within the liver hepatocytes. As described in the Materials and Methods section, a mixture of oleic acid and palmitic acid (OA/PA) was used to induce steatosis in PHH liver spheroids for 4 days and bioluminescent assay was used to detect the triglyceride lipid accumulation in the liver spheroids. At the same time, two different media with different insulin and glucose concentrations were compared for their impact or steatosis induction (Figure 4A). In this 4-day short-term steatosis induction test, PHH liver spheroids using physiological media showed 2.2- to 2.5-fold increase among 4 different PHH lots in comparison to the DMSO control samples. In contrast, PHH liver spheroids using pathological media showed higher basal levels of triglyceride and little induction of steatosis during the same 4-day treatment. Lot-to-lot variations were also observed at the basal levels of triglyceride in the control (DMSO) samples and in the induced samples (OA/PA).

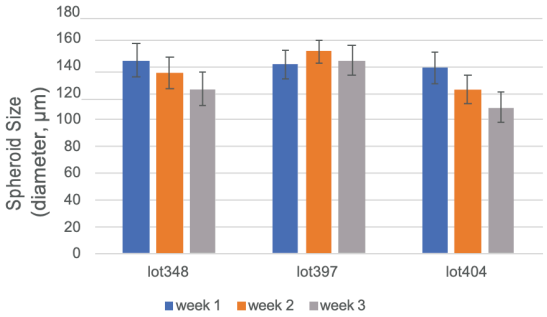
In Figure 4B, two PHH lots were treated for longer (11-day induction) steatosis induction in PHH liver

A

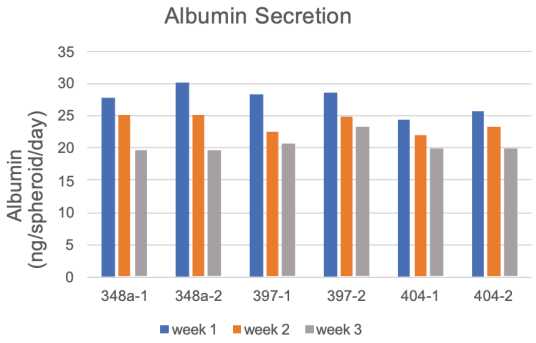


Corning Elplasia Plate (round bottom)	24-well
Approx. number of microcavities/well	554

B



C



D

RNA Yield/Well

Sample ID	RNA Yield (ng)
348a-1	354
348a-2	570
404-1	570
404-2	522

Figure 2. PHH liver spheroid culture in 24-well Corning Elplasia plates. (A) PHH Lots 348a and 397 used for liver spheroid culture at the seeding density of approximately 500 cells/microwell were plated in a Corning 24-well Elplasia plate. Representative images were taken on different days after seeding (Day 0). (B) Bar graphs show comparable PHH liver spheroid size measurement (diameter, µm) over three weeks in culture for PHH Lots 348a, 397, and 404. Twenty-four spheroids were measured at each time point to calculate the average size of spheroids. (C) Albumin secretion over 3 weeks was measured by ELISA assay in duplicate for each lot of PHH. (D) Total RNA yields from PHH spheroids (Lots 348a and 404) in duplicate after 3 weeks of culture.

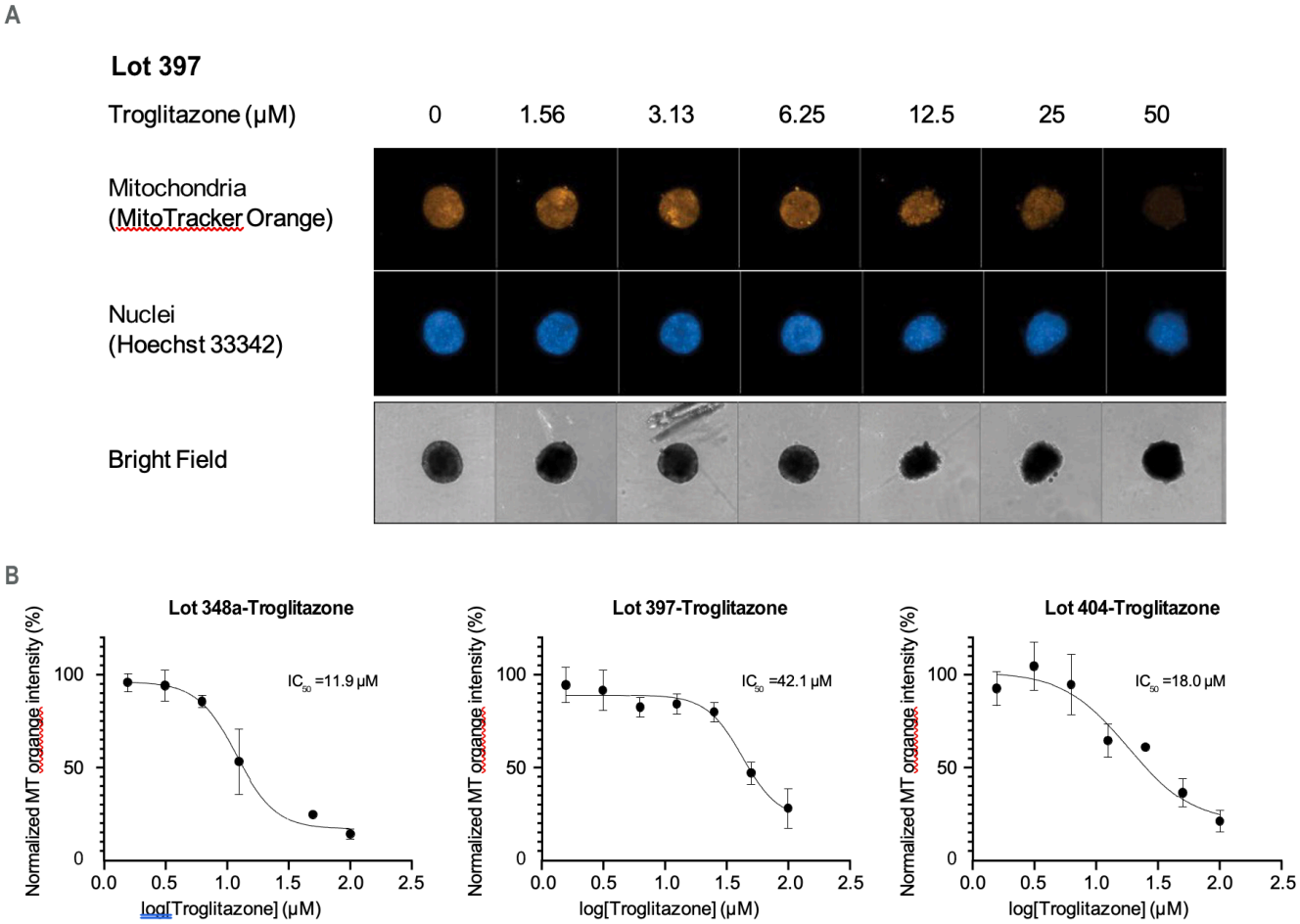


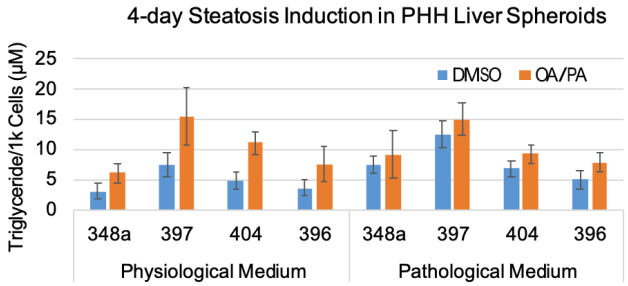
Figure 3. High content imaging-based analysis for troglitazone induced mitochondria toxicity in PHH liver spheroids. (A) PHH liver spheroids (Lot 397) were treated for 48 hours with serial dilutions of troglitazone. After treatment, PHH liver spheroids were stained with MitoTracker Orange and Hoechst 33342 as described in the Materials and Methods section. (B) Dose-responsive curves were generated and IC_{50} values were calculated with GraphPad Prism from normalized MitoTracker total fluorescent intensity. Dose-dependent mitochondrial toxicity was observed in PHH liver spheroids made from the three PHH lots (348a, 397, 404).

spheroids using physiological media, higher fold changes at 11.3 and 19.6 were detected for lots 309 and 404, respectively under these conditions. From these results, we conclude both the duration of steatosis induction and the medium composition (glucose and insulin concentrations) could make a significant impact on the *in vitro* steatosis tests using PHH liver spheroids. In addition, different PHH lots have differences in their steatosis induction fold changes under the same conditions.

Conclusions

In summary, the Corning HepGo assay-ready liver spheroid kit is shipped to customer site for immediate end-user applications and can be produced robustly and consistently for immediate end-user applications such as quick liver toxicity testings. PHH liver spheroids made from Corning 3D spheroid-qualified PHHs can also be easily grown in large quantities in

A



Fold Change	2.4	2.2	2.5	2.4	1.3	1.2	1.4	1.7

B

	11-day Induction		
PHH Lots	Triglyceride DMSO	Triglyceride OA/PA	Fold Change
309	30.0	337.5	11.3
404	14.0	274.2	19.6

Figure 4. Steatosis induction in 3D PHH spheroids. (A) Four PHH Lots (348a and 397, 404 and 396) were used for liver spheroid culture in Corning 96-well spheroid microplates in either physiological (low insulin and low sugar) or pathological medium (high insulin, high sugar). Following the 4-day treatment of free fatty acid (OA/PA) or DMSO control treatment, the lipid accumulation in PHH liver spheroids was analyzed using bioluminescent triglyceride assay. Fold changes in lipid induction were calculated comparing to DMSO control, (n = 6). (B) Longer term steatosis induction was performed for 11 days with OA/PA mixture or DMSO control treatment in PHH liver spheroids made from two PHH lots (309 and 404). Average triglyceride levels were calculated from measured samples (6 to 11 spheroids each).

Corning Elplasia plates that easily produce sufficient materials such as total RNA for molecular analysis. Methods of high-content imaging and quantitative bioluminescent assays can be adapted for studying drug-induced liver toxicity or *in vitro* steatosis with PHH liver spheroids as well. Further development and optimizations are currently underway by Corning and others in the field to advance the utilization of PHH liver spheroids as a novel tool for liver diseases and drug safety assessment.

References

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Feng Li, Li Cao, Jie Wang, Sweta Parikh, and Joanne Bourgea are with Corning Life Sciences.

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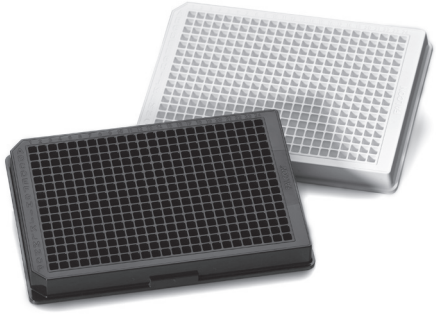

Visit **www.corning.com/Matrigel3DPlates** to learn more.

References
1. Corning Matrigel Matrix-3D Plates for High Throughput 3D Assays (Corning Application Note CLS-AN-572)
2. Implementation of Rapid, Reproducible, Homogeneous, and Cost-effective 3D Matrix-based Models on Primary Human Pancreatic Cancer (Corning Application Note CLS-AN-607)

Reproducible Production of Well-Defined and Uniform Embryoid Bodies Using Corning® Elplasia® Plates

High-quality, reproducible EBs can now be produced for drug discovery and functional screening applications.

Zhu Mingfei and Wang Xuebin

Introduction

Human induced pluripotent stem cell (hiPSC)-derived embryoid bodies (EBs) are three-dimensional multicellular aggregates that show significant advantages in hiPSC differentiation efficiency and organoid establishment.[1] When hiPSCs are cultured in a suspension environment with relevant media, cells spontaneously differentiate and form EBs comprising the three developmental germ layers: endoderm, mesoderm, and ectoderm. Compared with conventional 2D planar hiPSC culture, this multicellular structure better recapitulates in vivo cell-cell interactions, improves intercellular communication, and enhances substance exchange.[2] 3D cell culture systems such as spheroids and organoids are gaining traction in drug discovery applications as improved models of disease. iPSC-derived organoids are invaluable resources that can provide more predictive screens to aid in personalized medicine. It has been demonstrated that the quality of EBs can impact the stability and feasibility of iPSC- derived organoids.[3,4] Therefore, to further advance organoids for functional screening, the need for high throughput production of homogenous EBs with uniform shape and size has emerged.

A variety of techniques can be used to form EBs from iPSCs with EB-specific medium. Conventional methods such as static suspension culture in dishes or hanging-drops can lead to variable EB production and unstable EB-derived differentiation.[5,6] Newer platforms based on size-defined micropatterned wells, also known as “micro-space” or “microcavity” culture, have been developed to generate EBs with uniform size and morphology using single hiPSCs.[7] The Corning Elplasia plate is an example of a microcavity plate that can produce replicate spheroids of uniform size in mass quantities. The plate is black with a clear bottom and features the Corning

Ultra-Low Attachment (ULA) surface, a biologically inert hydrogel that supports the self-aggregation of cells to form 3D structures. Corning Elplasia plates are available in 6- to 384-well formats enabling reproducible scale-up of EBs to support basic research to screening applications. In this study, we demonstrate how the Corning Elplasia plate can enable spontaneous formation of spheroids from hiPSCs and subsequent reproducible production of uniform differentiated EBs (Figure 1).

Materials and Methods

Spheroid Formation

Corning Elplasia 24-well round bottom plates with ULA surface (Corning Cat. No. 4441) were pre-wet prior to seeding cells by adding 1 mL mTeSR™1 medium (STEMCELL Technologies Cat. No. 85850) per well and centrifuging at 500 x g for 1 minute to remove trapped air. Human iPSCs (DYR0100; ATCC® Cat. No. ACS- 1011™) were harvested from a Corning Matrigel® matrix-coated 6-well plate (Corning Cat. No. 354671) and cells counted using a Corning Cell Counter (Corning Cat. No. 6749). Cells were seeded into plates at increasing densities of 100, 250, 500, and 1000 cells per microwell in a volume of 1 mL mTeSR1 medium containing 10 µM Y27632 (R&D Systems Cat. No. 1254) per well. Cells were incubated and media (mTeSR1 + Y27632) was changed daily for 3 days. The study was independently repeated three times.

EB Formation

On day 3, the media was changed to 1 mL AggreWell™ EB Formation Medium (STEMCELL Technologies Cat. No. 05893) containing 10 µM Y27632. Cultures were incubated for an additional 3 days with daily media (EB Formation Medium + Y27632) change.

Spheroid and EB Consistency

On days 3 and 6, 1 µL of 1 mM Calcein AM (Corning Cat. No. 354216) was added to each well and incubated for 15 minutes. Once cells were completely stained, the 3D structures were imaged with the Olympus IX53 microscope to assess size and morphology (M). Spheroid and EB diameters were measured with Olympus cellSens Standard software.

Spheroid and EB Surface Marker Expression

On day 3, iPSC spheroids were stained with SSEA4 (BD Cat. No. 560128) and Oct4 (Abcam Cat. No. ab19857) antibodies for flow cytometry (FC) and immunofluorescence analysis (IF), respectively. For

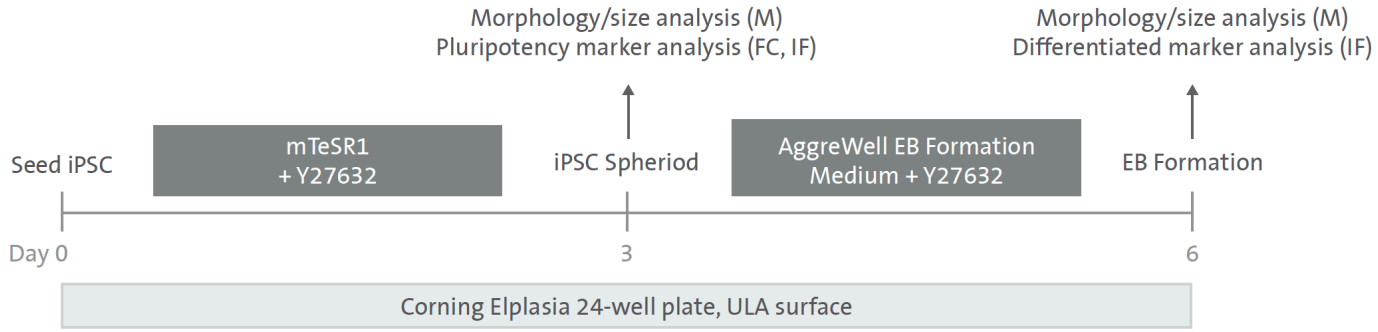

Figure 1. Schematic of the iPSC spheroid and EB formation protocol

flow cytometry analysis, spheroids were digested into single cells with Accutase® (Thermo Fisher Cat. No. A1110501). The BD Accuri™ flow cytometer was used. On day 6, EBs were stained with alpha- fetoprotein (C3; BD Cat. No. 563002), alpha smooth muscle Actin (Alexa Fluor® 647; Abcam Cat. No. ab196919), and Alexa Fluor 555 β-Tubulin, Class III (BD Cat. No. 560339) antibodies to characterize endoderm, mesoderm, and ectoderm layer formation, respectively. Immunofluorescence data was captured used the BioTek Cytation™.[5]

Results and Discussion

Uniform iPSC Spheroid Formation

To assess the ability of Corning® Elplasia® plates to generate uniform iPSC spheroids, DYR0100 cells were seeded at four different densities into plates and incubated for 3 days with daily media changes. Once iPSC spheroids were generated, they were stained with Calcein AM and viewed using brightfield and fluorescence microscopy to examine morphology and size. The data show single, circular iPSC spher-

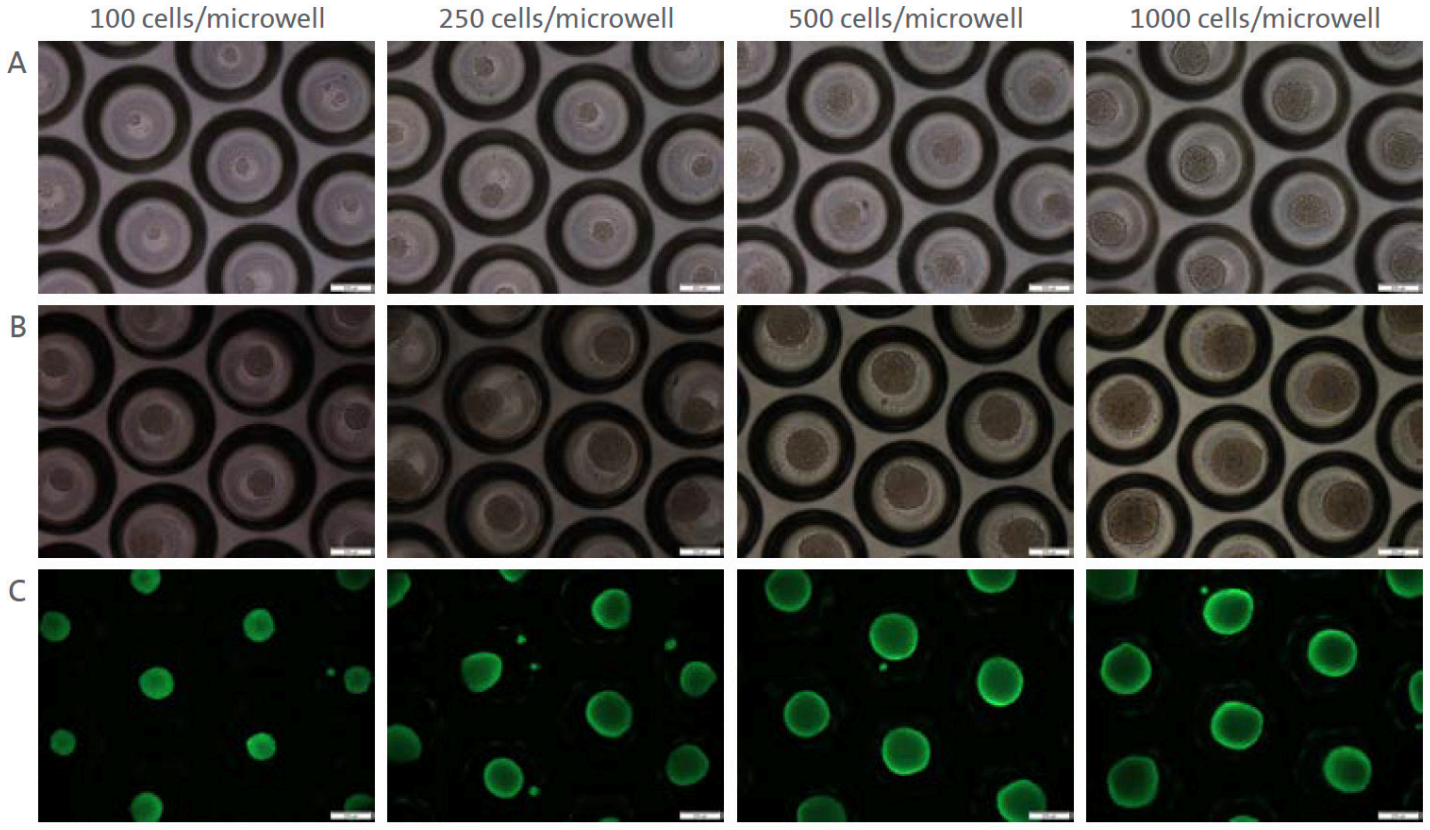

Figure 2. Uniform, single iPSC spheroids formed in each microcavity. Representative images of a digitally zoomed field from one well of a 24-well Corning Elplasia plate using brightfield (A = Day 1; B = Day 3) and fluorescence (C = Day 3) imaging. DYR0100 cells were initially seeded at four different densities into the plate (100 to 1000 cells/microwell). Images were taken with Olympus IX53 microscope using a 100X objective. Scale bar is 200 µm.

oids formed in each microcavity (Figure 2) that were consistent in size using diameter measurements (Figure 3). At each seeding density, spheroid diameter increased with longer culture duration from 1 to 3 days. The spheroids could also be size-controlled by adjusting the initial seeding density. Spheroid size ranged from 160 to 275 µm with seeding densities of 100 to 1000 cells per microwell at day 3 (Figure 3).

As the Corning® Elplasia® 24-well plate contains 554 microcavities per well, the average number of replicate iPSC spheroids that can be generated per plate is over 13,000 spheroids. This demonstrates the effective scale-up of uniform spheroids that can be cultured under a single culture condition.

iPSC Spheroid Characterization

On day 3, pluripotency-associated markers of iPSC spheroids generated on the Corning Elplasia plate were characterized using immunofluorescence and flow cytometry. Regardless of cell seeding density, iPSC spheroids demonstrated strong Oct4 tran-

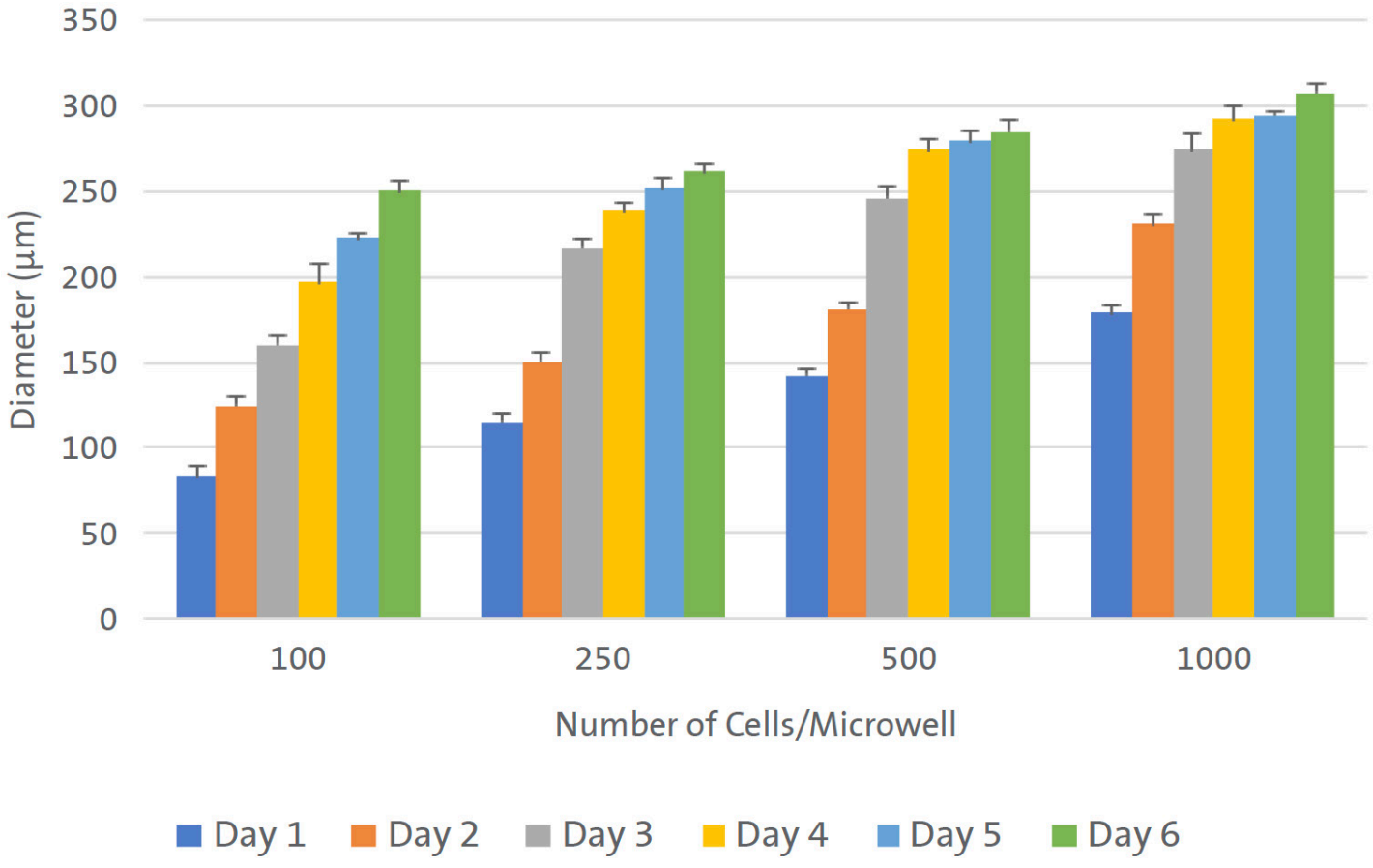

Figure 3. iPSC spheroids and EBs formed in each microcavity were consistent in size. DYR0100 cells were seeded at four different densities (100 to 1000 cells/microwell) into the Corning Elplasia plate and spheroid (days 1 to 3), and EB (days 4 to 6) diameters were measured using Olympus cellSens Standard software. Data shown with standard error of the mean from 20 independent microwells.

scription factor expression throughout the spheroid (Figure 4A) and a high percentage of SSEA+ cells (>96%; Figure 4B) indicating retention of pluripotency characteristics.

EB Differentiation and Characterization

On day 3, culture medium was changed to EB Formation Medium to initiate EB differentiation in the Corning Elplasia plate. After a further 3 days of differentiated culture, cells were stained and expressed protein markers characteristic of the 3 developmental germ layers (Figure 5). The differentiated EBs were stable and uniform in size as observed within the individual microcavities of a well (Figure 6). Similarly, to the iPSC spheroids, the EBs could also be size-controlled based on initial seeding density.

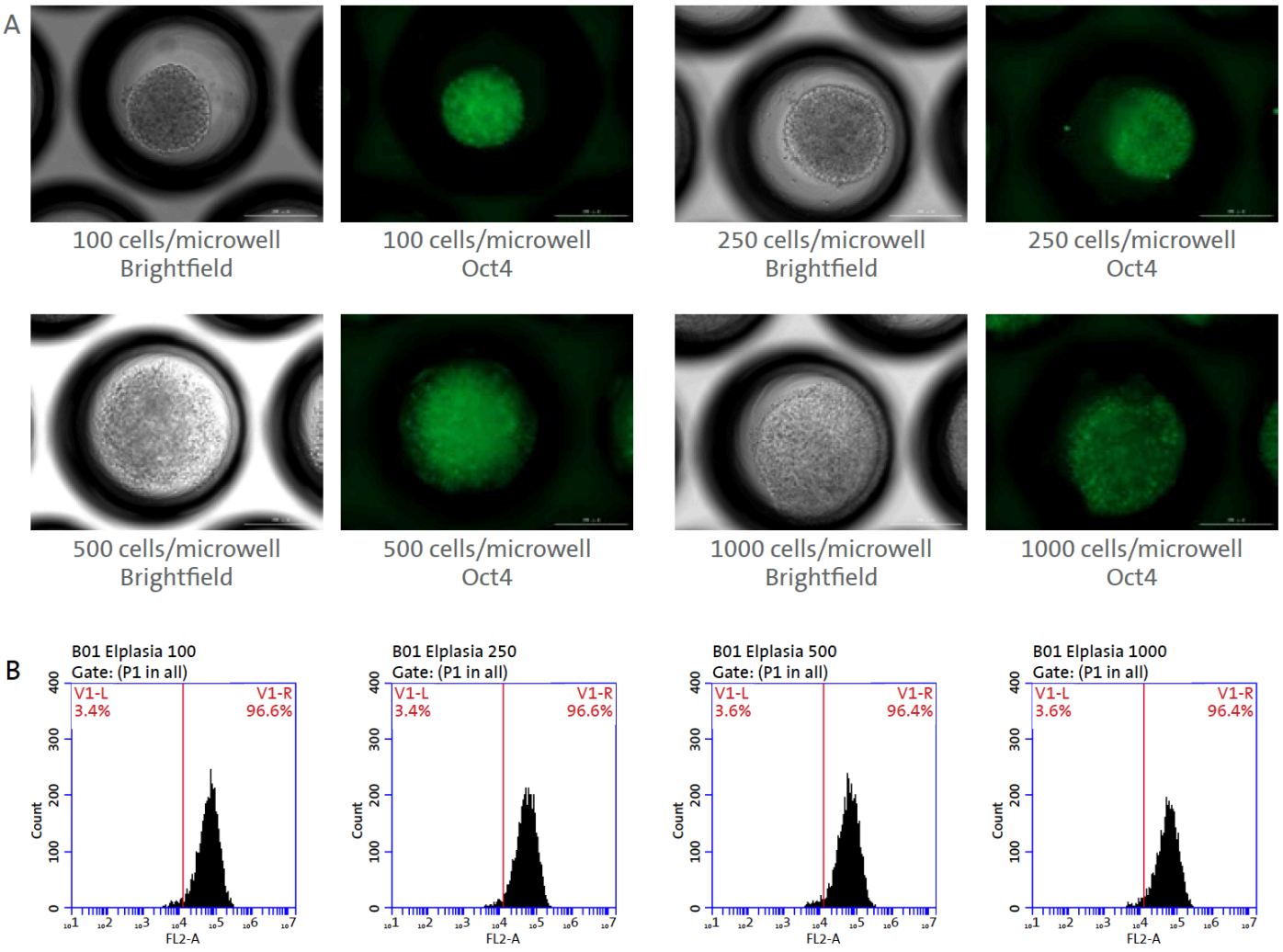

Figure 4. iPSC spheroids retained pluripotency marker expression. (A) Representative images of Oct 4-stained spheroids in individual Corning Elplasia plate microcavities. Images were taken using the BioTek Cytation 5 with a 400X objective. Scale bar is 200 µm. (B) Representative profile of flow cytometry analysis of SSEA+ cells in the iPSC spheroids using the BD Accuri flow cytometer. Data shown is for iPSC spheroids generated with 4 different cell seeding densities (100 to 1000 cells/microwell).

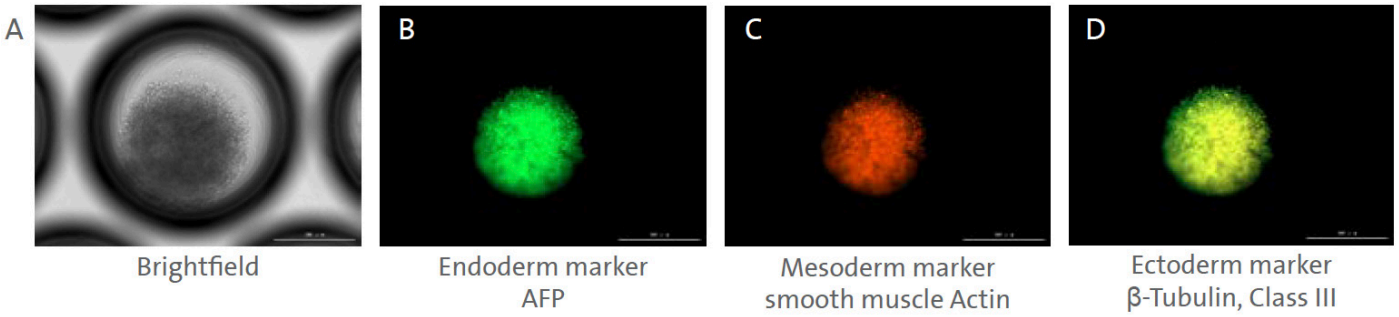

Figure 5. iPSC-derived EBs expressed typical germ layer-specific markers on day 6. (A) Brightfield, (B) Alphafetoprotein (endoderm), (C) smooth muscle Actin (mesoderm), (D) β-Tubulin, Class III (ectoderm). Images were taken using the BioTek Cytation 5 with a 400X objective. Scale bar is 200 µm.

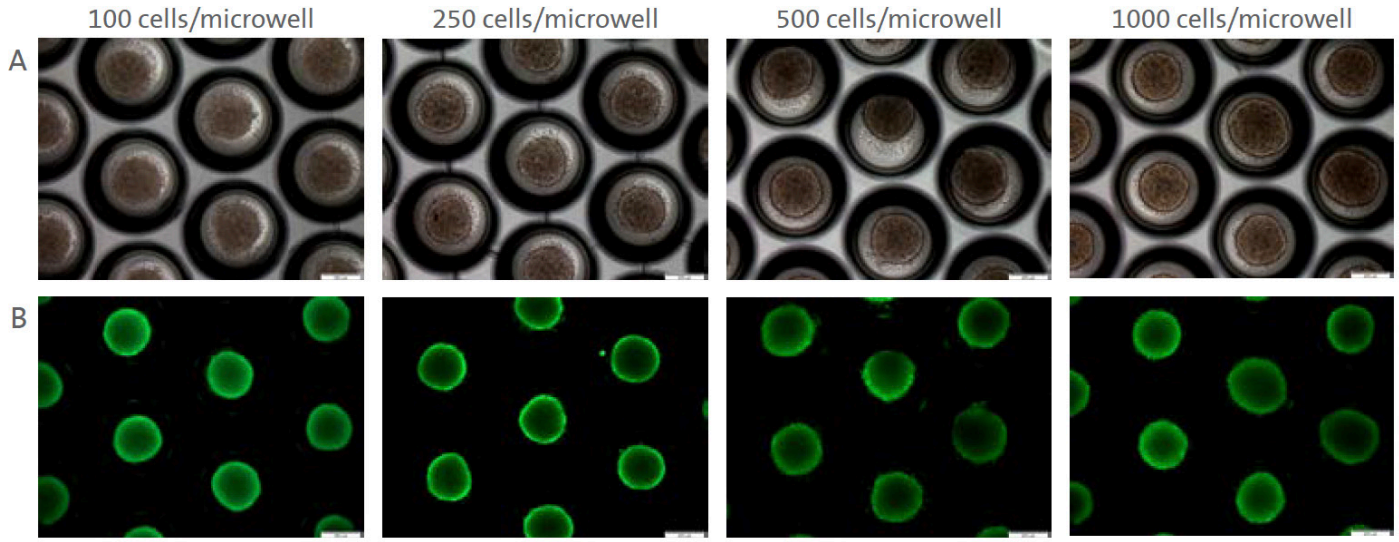

Figure 6. Uniform, single EBs formed in each microcavity. Representative images of a digitally zoomed field from one well of a 24-well Corning Elplasia plate using brightfield (A) and fluorescence (B) imaging on day 6. Images were taken with the Olympus IX53 microscope using a 100X objective. Scale bar is 200 µm.

EB size ranged from 250 to over 300 µm in diameter with seeding densities of 100 to 1000 cells per microwell at day 6 (Figure 3).

Following the same logic as earlier, the average number of reproducible differentiated EBs that can be generated per Corning® Elplasia® 24-well plate is over 13,000 EBs under a single culture condition.

Conclusions

- Corning Elplasia plates can support spontaneous uniform iPSC spheroid formation and progression to reproducible differentiated EBs using a simple 6-day protocol.

- The iPSC spheroids and differentiated EBs can be size-controlled in the Corning Elplasia microcavities depending on initial cell seeding density.
- The iPSC spheroids demonstrated strong pluripotency marker expression; EBs expressed endoderm, mesoderm, and ectoderm markers indicating differentiation of the specific cell lineages.
- The Corning Elplasia plates can be used for scale-up production of high quality, reproducible EBs, and as a tool in drug discovery and functional screening.

References

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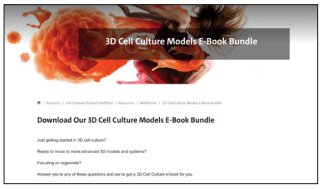
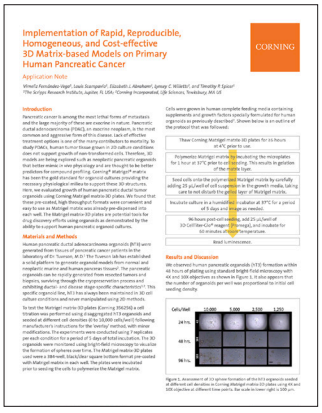
Zhu Mingfei and Wang Xuebin are with Corning Life Sciences.

Resources

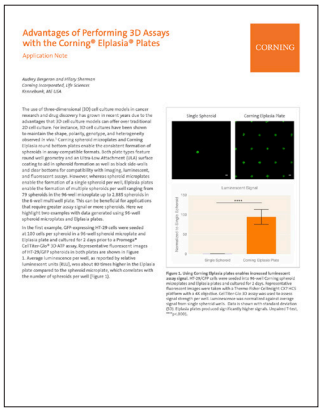


3D Cell Culture Models E-Book Bundle

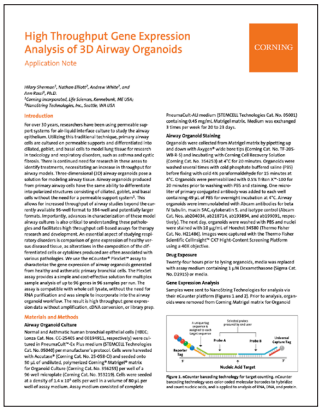


Implementation of Rapid, Reproducible, Homogeneous, and Cost-Effective 3D Matrix-based Models on Primary Human Pancreatic Cancer

Corning® Matrigel® Matrix-3D Plates for High Throughput 3D Assays



Advantages of Performing 3D Assays with the Corning® Elplasia® Plates



High Throughput Gene Expression Analysis of 3D Airway Organoids

Culturing Human Intestinal Organoids with Corning® Matrigel® Matrix for Organoid Culture