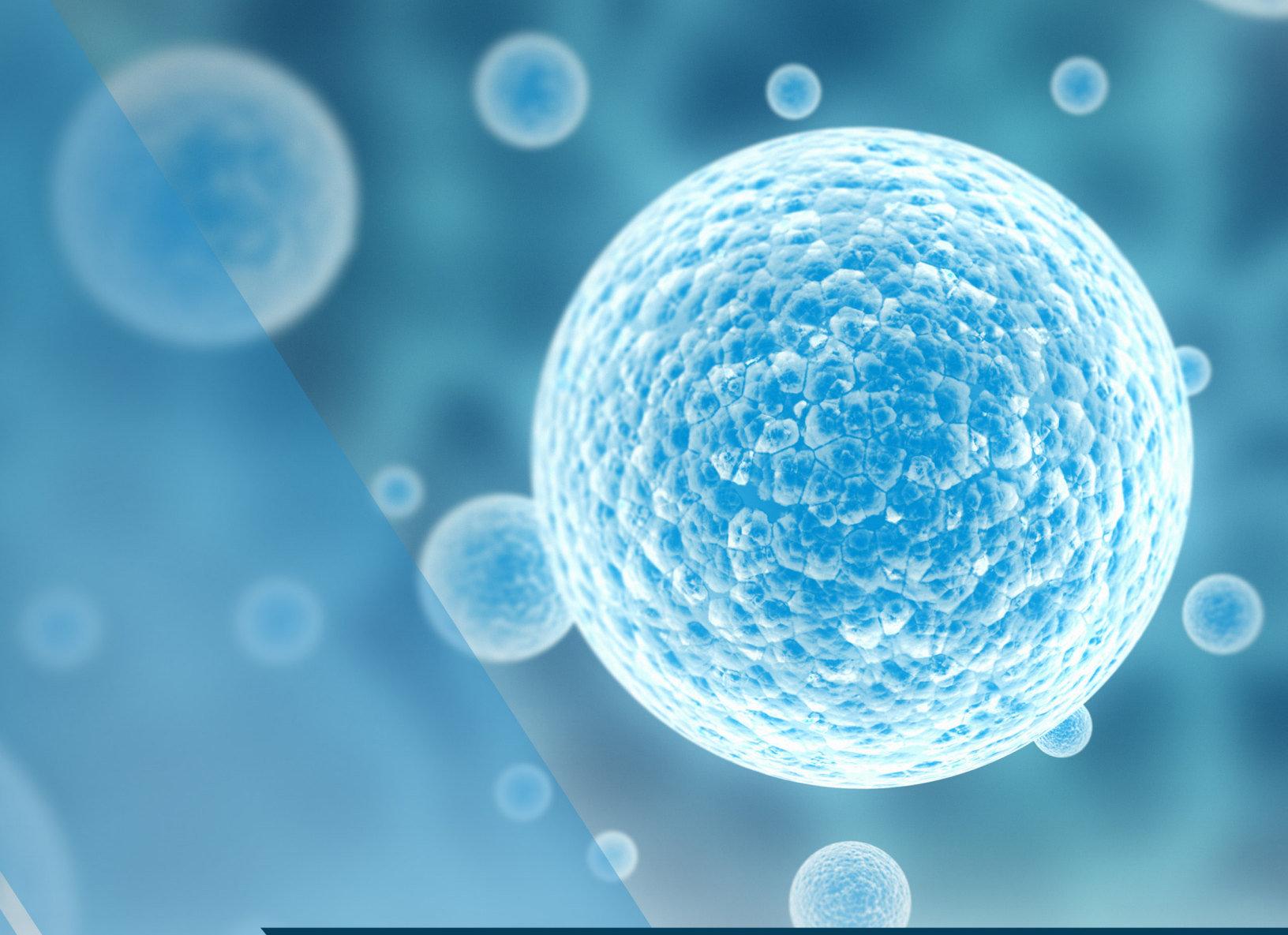

Expert Guide to Advanced 3D Cell Culture

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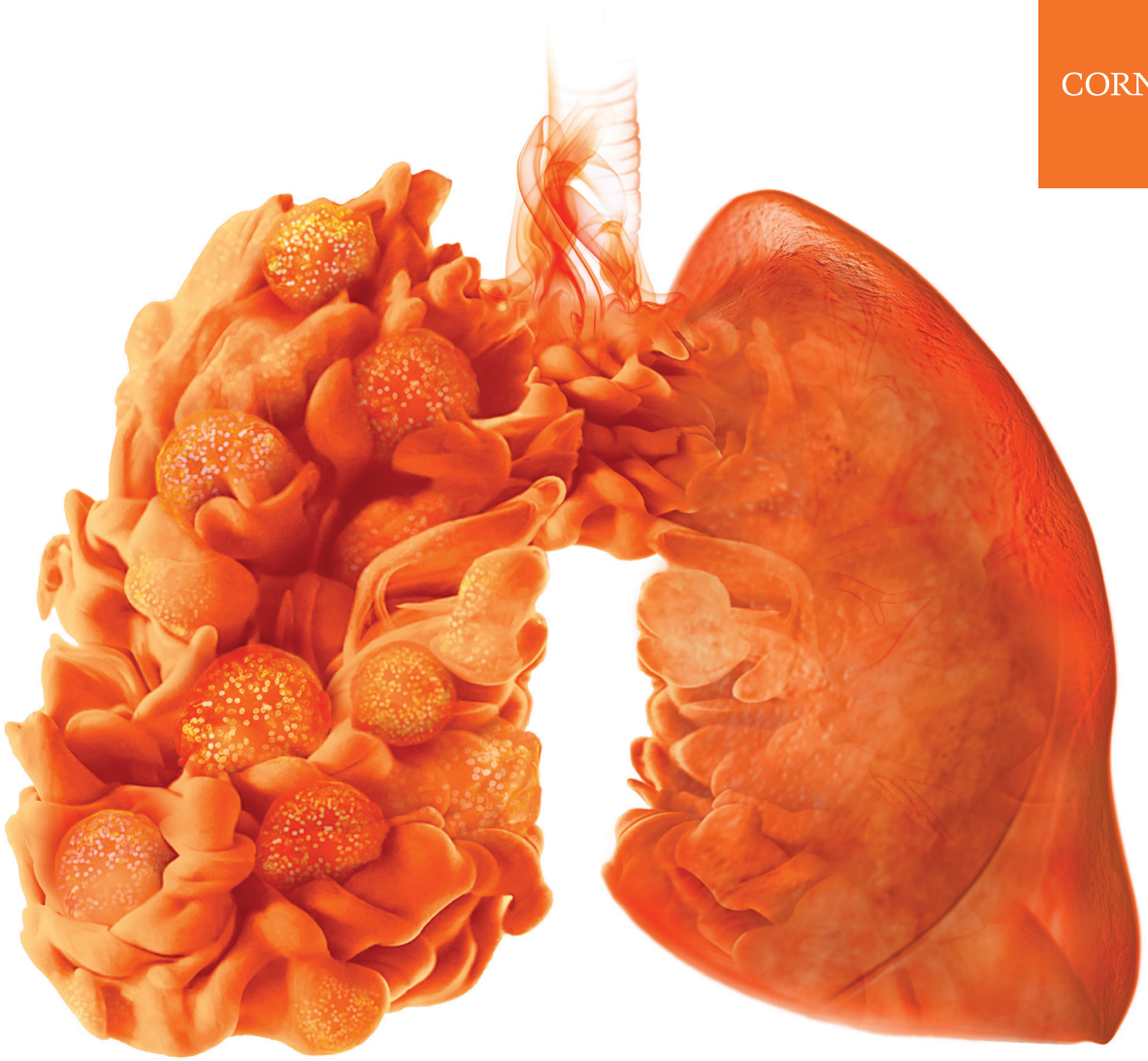

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3D Cell Cultures Today and Tomorrow

Expert panel discusses how 3D techniques are impacting translational and clinical applications as well as basic research.

We talked to a panel of experts about the many ways that three-dimensional techniques in cell culture impact translational and clinical applications, as well as basic research, and how that will expand in the future. Their responses shed light on the burgeoning use of 3D techniques as well as the most promising applications, and improvements on the horizon.

Roxana Ghadessy, Ph.D.
Technical Marketing Manager
Corning

Shabana Islam, Ph.D.
Product Line Manager
Corning

Kevin Kelly
Senior Technical Support
Corning

M. Laura Martin, Ph.D.
Ex Vivo Models Director, Englander Institute for Precision Medicine
Weill Cornell Medicine

Austin Mogen, Ph.D.
Senior Field Application Scientist
Corning

Hilary Sherman
Senior Scientist
Corning

Catherine Siler, Ph.D.
Field Application Scientist
Corning

How have three-dimensional techniques impacted cell culture, especially in translational/clinical applications?

Kevin Kelly: 3D cell culture has made a huge impact. For example, a 2004 **paper** from Mina Bissell's lab at Lawrence Berkeley looked at cultures of breast cancer cells and found that EGFR and β1 integrin were suppressed by LY294002 in 3D cultures with lrBM from Englebreth-Holm-Swarm tumors (Matrigel matrix), but not 2D—polarity and proliferation are controlled

by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. So, it's already been effectively used, and it's only going to grow in the future.

Catherine Siler: One good indicator is to look at the number of publications, on a resources website such as PubMed. Within the past several years, the incidence of 3D cell culture techniques has increased dramatically. That's a great way to get a sense of the effect that 3D techniques are already having on basic science. You can imagine the impact that basic science is going to have on more applied techniques.

M. Laura Martin: There have been some papers lately on approaches to cancer drug screening and how well they represent the response of a patient in 2D models versus 3D models. The evidence is accumulating that the 3D systems are more accurate at representing a tumor and the drug response.

Shabana Islam: 3D cell culture has the potential to provide a more predictive preclinical model for drug target identification and screening studies. As a result, there has been a great interest in the development of targeted therapies.

Roxana Ghadessy: A long-term challenge in clinical trials has been high drug failure due to lack of efficacy and safety issues, particularly in cancer research. 3D *in vitro* techniques, which can better replicate *in vivo* biology and microenvironment dynamics, have spurred progress in reducing attrition rates in drug discovery.

Austin Mogen: These 3D methods provide more robust disease models. That's become particularly relevant recently with SARS-CoV-2, which is the virus that causes COVID-19. These 3D organoids are providing more realistic models to study areas like viral attachment, infection, and other novel diseases.

In the next 5 to 10 years, what improvements in translational/clinical applications are likely to arise from 3D culturing?

Hilary Sherman: Models like organoids can be used to screen personalized treatments for patients, instead of just offering the standard-of-care procedure or chemotherapeutic.

Martin: More research labs will try to have a clinical application of an organoid platform for drug screening, so they will become a routine tool or assay. We grow organoids from patients, and we plan to use information from those to inform the treatment once we are clinically certified. With this approach, you treat each patient as its own entity.

Kelly: Patient-derived tumor organoids allow you to take a more direct approach. You will be able to determine how sensitive a patient's tumor is to a particular anti-cancer drug.

Islam: The failure of drug candidates during clinical development is usually due to a lack of efficacy or exhibiting toxicity profiles. Advances in 3D cell culture will give a better idea of how the drug is working in the model that you are studying. Organoids show great promise to validate a drug with a more complex system.

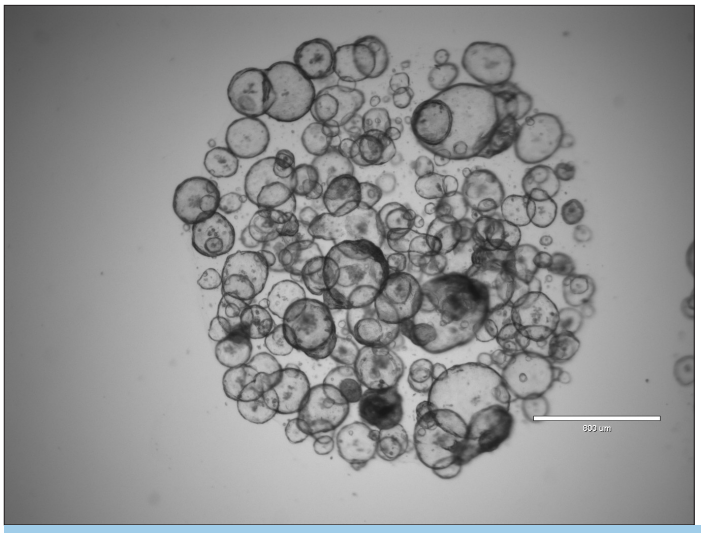
Mogen: We would probably all agree that one of the powerful aspects of organoids is the ability to use patient-derived organoids for drug screening, modeling, and understanding of a patient's individual cancer. One of the challenges with patient-derived organoids is around long-term storage. In the next five to ten years, some of the major improvements will be focused on optimizing some of those freezing and banking protocols to make sure that the tissue can be utilized in a more reasonable and long-term way.

Martin: One thing that might be developed in the next five years is the incorporation of different components of the microenvironment into the organoids, like T cells, endothelial cells, or fibroblasts. This would create a more accurate representation of the patient's tissue.

What advances or improvements in technology will be required to drive these advances from 3D culturing?

Mogen: Moving into the clinic and translational applications, I see people utilizing 3D cell culture to do 3D bioprinting of tissues. Extracellular Matrix—ECM—proteins such as rat tail collagen or a basement-membrane extract, like Corning Matrigel matrix, can be used as bio-ink in these workflows. One of the challenges with the current substrates and ECM components for bioprinting of tissues for implantation is that they are animal-derived. If the actual 3D culture or tissue itself is being used as a therapy, typically it's not acceptable to use animal-derived products. So, the move to more of a synthetic ECM substrate over time is important, so the organoid research can translate into the clinic.

Siler: One of the main challenges of organoids is trying to make them suitable for a high-throughput screening environment. Being able to use large quantities of organoids, for example in drug screening, you need high-throughput methods and tools. A step in the right direction to addressing this challenge is the Corning Matrigel matrix-3D plates, a precoated ready-to-use option, available in 96-well and 384-well formats, so you get consistency across a screen. You may also create a different environment by combining a basement-membrane matrix, like Corning Matrigel matrix and the Corning spheroid microplate, which we have done for intestinal organoids.



Patient derived pancreatic cancer organoids cultured in Corning® Matrigel® matrix for organoid culture (2x objective).

Kelly: 3D live-cell imaging including confocal stack where people can do high-throughput screening and look at the different levels of an organoid over time would really help. Sometimes the changes aren't necessarily in intensity but in shape, and imaging is the only way to look at changes in space and conformation.

Ghadessy: 3D techniques are gaining traction, in particular organ-on-chip technology whereby improvements in microfabrication and microfluidic techniques coupled with computer technology has enabled *in vitro* organ models that mimic whole-body responses in terms of drug-toxicity predictivity.

Martin: The more standardized the technology gets, the more people can use organoids. Right now, not everyone has the resources to work with organoids. Academia and industry are rapidly moving with tools and techniques to make them more accessible across all labs.

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Corning® Matrigel® Matrix-3D Plate

Move your 3D models into a high throughput environment with new Corning Matrigel matrix-3D plates. A convenient and consistent plate format that boosts productivity in homogenous assays.

Convenient

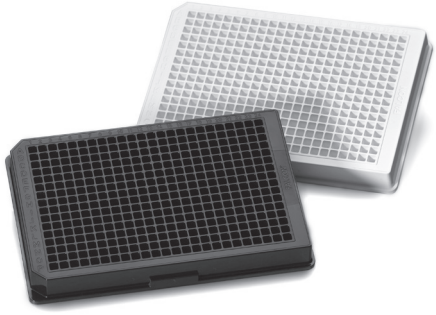
Reduces workflow steps and manual inaccuracies, while providing the convenience to grow and assay 3D structures within a plate.

Consistent

Matrigel matrix volume consistency across wells, resulting in robust and consistent plate-to-plate performance[1].

Certain

Shown to support drug discovery applications using organoids, cancer spheroids, and polarized epithelial 3D structures[2].



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)

Expanding the Applications of Organoids

Perspectives from an expert user on drug discovery applications as well as the importance of the tools and materials used.

Robert Vries—the chief executive officer at Hubrecht Organoid Technology (HUB) in Utrecht, The Netherlands—talked to us about using organoids for drug discovery, better understanding COVID-19, and more.

How do you use organoids in drug discovery?

Vries: Organoids as a model that is directly derived from patient samples are used in the drug discovery field, which means they can be used in the preclinical field to support development of new drugs. Recently, we found that adult stem cell derived organoids are actually so closely related to the patient that we can also use them to predict the individual's response to drug treatment. So, we are using this as a predictive diagnostic tool to see which patient requires what drugs, in for instance, cystic fibrosis or in cancer.

Because of those clinical studies, we now know that when we test new drugs against organoids we have grown from a specific organ that many of the responses that we see are reflective of what we can expect to see in the patient. We generate organoids of normal and diseased tissue from many different patients—in what we call a living organoid biobank. In colon cancer, for example, we have samples from about 150 patients in a biobank, and when industry comes to us with questions on drugs or targets that they are developing, we look at the target patient population, select an appropriate group of organoids, and use that organoid selection in screening and mechanistic studies.

Please briefly describe a successful application of organoids in drug discovery.

Vries: We license our HUB Organoid Technology to many drug discovery companies, and we know that many compounds screened against our organoid biobanks have gone from research into clinical applications. One that stands out is a drug from Eloxx that is used for cystic fibrosis. In this case, organoids were used to identify patient groups of several rare mutations, which were very responsive to their new drug treatment. It is simply not possible to achieve this with normal cell lines; you need the epigenetic complexity of organoids. With these experiments we were able to identify several new patient groups that respond to their drug, in addition to the groups for which they are now in Phase II clinical trials.

How have you used organoids to study COVID-19?

Vries: Viruses are so specific in their evolution that they can only infect cells that are *in vivo*-like. That means that traditional cell lines are very difficult to use for viral studies. Animal models, for the same reason—because a mouse is not a human—are difficult to use in these studies. Because of this, virology is typically difficult to study preclinically. However, when you introduce organoids to this area of research, they are a good solution because the protein expression—such as proteins presented on the membrane—is exactly like a patient. In the case of something like SARS-CoV-2, it means that all of the receptors are there that are required for the infection. We have shown that we can match the lifecycle of viruses, such as Coronavirus, in our lung and intestinal organoids, which are derived from adult stem cells (ASC).

In your application to COVID-19, how did it help that you already had experience using organoids?

Vries: We had previously worked on several other viruses, such as respiratory syncytial virus, hepatitis B virus, and several others. When COVID-19 came along, we knew we had to try that as well. We see now how difficult making a COVID-19 vaccine is going to be. This is one of those fields where you need advanced models and, hopefully, our technology. With complicated diseases, like COVID-19 and cancer, our ASC-derived organoids are the new modern system for the future of this type of research.

What improvements would you like to see in organoids?

Vries: We have come a long way since we made the first organoid in 2007, but there is still a lot of undiscovered road ahead of us. In fact, I think that our collaboration with Corning is very important. We are the biologists and we develop the cells and the systems, but we need the materials that go from pipettes to organ-on-a-chip. In all of the systems and materials around making organoids, there is a lot that works well, but there's a lot that we can still develop. By improving the tools and materials we use, we can make these systems even more complex, for instance an organ-on-a-chip model. Because the organoids by nature are more finicky, the tools and materials used are very important. Some of the next improvements will be focused on combining the biology with all of the other materials that we use in the lab.

How would those improvements change your application of organoids?

Vries: We want to increase the throughput of the screening. Organoids are grown in Corning® Matrigel® matrix and through a specific combination with Corning Ultra Low Attachment (ULA) 384-well plates. We can make these models suitable for higher throughput screening. So now, we can screen 50,000 to 100,000 compounds, whereas three or four years ago we had very low throughput, maybe 100 or 200 compounds maximum when using organoids.

In the next five to ten years, what do you envision as the biggest organoid-driven changes in drug discovery?

Vries: Now that we can generate organoids from all these different patients, selecting a good clinical trial is where we can see a major conceptual change. We can take 100 organoids or 200 organoids—basically clinical-trial size samples—and in a very cheap and fast way assess which drug is creating a response and at what frequency. That can significantly change the clinical trial phase, because you can really preclinically make a more accurate clinical assessment. That integration of late-stage preclinical and an early-stage clinical or maybe even a later-stage clinical trial is where I think the biggest conceptual phase is going to be.

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

Method allows for maintenance of human intestinal organoids for multiple passages.

Hilary Sherman and John Shyu

Introduction

During the last decade, the number of research articles on organoids has steadily increased.[1] This trend can be attributed to properties of organoids, such as their similarity to primary tissue, genomic stability, and that they are comprised from parallel cell types to the original tissue.[2] Although a great tool for research, cultured organoids can be inconsistent and divergent during growth and maintenance due to inconsistency in sample sourcing and reagents used.[3] Corning offers an extracellular matrix (ECM) specifically for culturing organoids to lessen potential variability. Each lot of Corning Matrigel matrix for organoid culture is tested for elastic modulus and ability to form a stable dome; both of which are important for organoid culture.[4] In this article, we demonstrate use of Corning Matrigel matrix for organoid culture to maintain human intestinal organoids (HIO) for multiple passages.

Materials and Methods

Human intestinal organoids with a cystic fibrosis phenotype were purchased from Hubrecht Organoid Technology (HUB; Utrecht, Netherlands). Organoids were cultured per HUB methodologies.[5] In brief, organoids were resuspended in Corning Matrigel matrix for organoid culture (Corning 356255) and AdDF complete medium (Table 1) at 55% to 65% Matrigel: cell volume. At least 24 hours prior to plating HIO, 24-well plates (Corning 3524) were incubated at 37°C in a humidified incubator. Four to five domes of 5 to 7 µL of HIO were placed in several wells of a 24-well multiple well plate using pre-chilled Axygen® 200 µL Maxymum Recovery® tips (Corning T-200-C-L-R-S). Once domes were plated, plates were inverted in the laminar flow hood for 5 minutes. Inversion of the plates allows for organoids to settle away from the plate surface where they might attach and differentiate. Plates were then transferred to a 37°C incubator for another 15 minutes in the inverted position. Once domes had fully polymerized, 500 µL of colon medium (Table 2) with a final concentration of 10 µM

Table 1. AdDF complete Medium

Item	Vendor	Cat. No.	Final Concentration
Advanced DMEM with F-12 Hams	Thermo Fisher	12634010	1X
Corning glutagro™	Corning	25-015-CI	2 mM
Hepes	Corning	25-060-CI	10 mM
Penicillin/Streptomycin	Corning	30-002-CI	1X

Table 2. Colon Medium

Item	Vendor	Cat. No.	Final Concentration
WNT3a Conditioned Medium			50%
N-Acetylcysteine	MilliporeSigma	A9165-5G	1.25 mM
Noggin	Peprotech	50-399-007	100 ng/mL
Rspondin-3	R&D Systems	3500RS025CF	250 ng/mL
B27 supplement	Invitrogen	17-504-044	1X
Gastrin	MilliporeSigma	G9145	5 nM
Nicotinamide	MilliporeSigma	N0636	10 mM
hEGF	Peprotech	AF-100-15	50 ng/mL
p38 MAPK inhibitor (p38i) SB202190	MilliporeSigma	S7067	10 µM
TGFb type I Receptor inhibitor	Tocris	29-395-0	500 nM
Primocin	Invivogen	NC9392943	0.5X
AdDF complete Medium			Remaining

Rock inhibitor (MilliporeSigma Y0503) was added to each well. Medium was changed every 2 to 3 days for fresh colon medium without Rock inhibitor. When organoids were ready for passage, domes were collected by pipetting with Axygen 1000 µL Maxymum Recovery tips (Corning T-1000-C-L-R-S). Organoids were resuspended in 2°C to 8°C AdDF complete and centrifuged at 450 x g for 5 minutes. Pelleted HIO were resuspended in 500 µL of AdDF complete and transferred to autoclaved Costar® 1.7 mL low binding microcentrifuge tubes (Corning 3207). HIO were sheared by triturating with a 20-gauge blunt needle (SAI Infusion Technologies B20-100) attached to a 1 mL syringe (Fisher Scientific 14-955-456).

Once organoids were sheared to the desired size, the larger non-sheared organoids were allowed to settle to the bottom of the tube while the smaller organoids were transferred to a new tube. Sheared HIO were centrifuged at 90 x g for 5 minutes. Organoids were then resuspended in AdDF complete with 55-65% Matrigel matrix volume at a dilution between 1:4 and 1:6. Organoids were re-plated and cultured as previously described until ready for passage.

Representative images were taken from 3 different lots of Matrigel matrix for organoid culture preceding each passage. Prior to the fourth passage, organoids were collected for histology with Axygen 1000 µL Maxymum Recovery tips. Organoids were washed twice with 1 mL of 2°C to 8°C phosphate buffered saline (PBS; Corning 21-040-CM) per microcentrifuge tube.

After aspirating PBS, 1 mL of 2°C to 8°C Corning® Cell Recovery solution (Corning 354253) was added to each tube. Organoids were incubated at 2°C to 8°C for 5 minutes. HIO were washed with cold PBS twice more. Once all Matrigel matrix was removed, organoids were fixed with 4% paraformaldehyde for 1 hour on a shaker at room temperature. After fixation, organoids were washed two additional times with PBS prior to being given to the University of New England for histology. HIO were processed per Spheroid Processing and Embedding for Histology Guidelines for Use (Corning Lit. Code CLS-AN-043) with markers listed in Table 3.

Results and Discussion

HIO were passaged into 3 lots of Matrigel matrix for organoid culture and then subsequently passaged 3 additional times in each Matrigel matrix

Table 3. Cell Specific Markers

Antibody	Vendor	Cat. No.	Concentration
Villin	Abcam	ab130751	1:400
MUC2	Abcam	ab11197	1:1000
Lysozyme	Abcam	ab108508	1:500

lot. Throughout the entire study, HIO demonstrated typical budded morphology of those derived from a donor with cystic fibrosis (Figure 1). Upon completion of the 3-passage study, HIO were fixed and sectioned to confirm the presence of appropriate intestinal cells. To assess their similarity to primary tissue, HIO from the large intestine should contain goblet cells, Paneth cells, and enterocytes.[6] Figure 2 confirms positive staining of all three cell types from the organoids cultured in three lots of Matrigel matrix for organoid culture (Corning 356255).

Conclusions

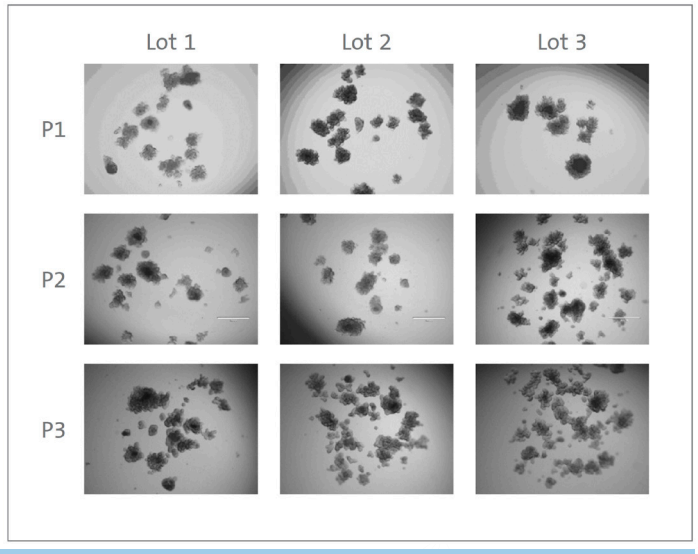


Figure 1. Typical budded morphology of CF HIOs.

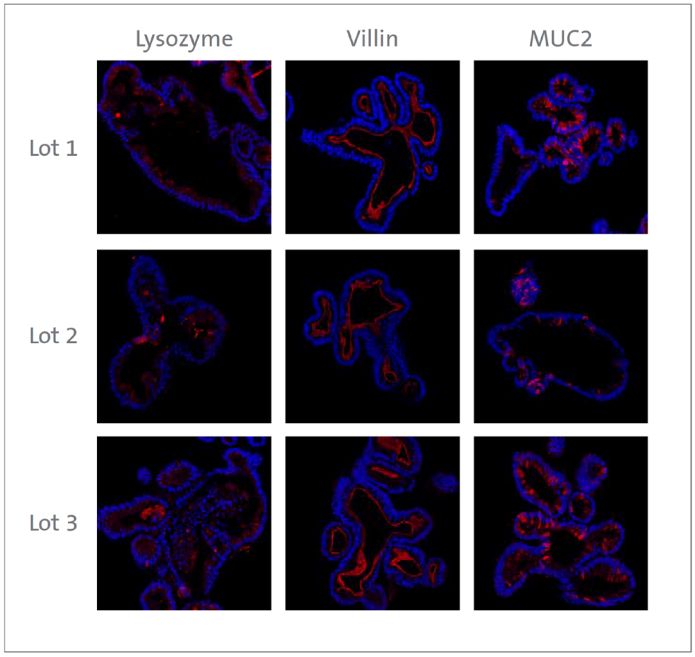


Figure 2. Positive staining of organ-specific cell types.

Culturing and maintenance of human organoids is complex. One of the greatest challenges is reproducibility, Corning Matrigel matrix for organoid culture aims to reduce some of this inherent variability by offering an ECM for culturing organoids. This article demonstrates that we were able to maintain similar HIO cultures from 3 different lots of Matrigel matrix for organoid culture.

Acknowledgments

We are grateful to Peter Caradonna at the COBRE Histology and Imaging Core (University of New England, Biddeford, ME) for the histology and IHC work. Financial support to COBRE is provided by NIGMS (grant number P20GM103643). 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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2. Fatehullah A, Tan SH, and Barker, N. Organoids as an in vitro model of human development and disease. Nature cell biology 18.3 (2016): 246-254.
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4. Broguiere N, et al. Growth of epithelial organoids in a defined hydrogel. Advanced Materials 30.43 (2018): 1801621.
5. Boj SF, et al. Forskolin-induced swelling in intestinal organoids: an in vitro assay for assessing drug response in cystic fibrosis patients. JoVE (Journal of Visualized Experiments) 120 (2017): e55159.
6. Sato T, et al. Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche. Nature 459.7244 (2009): 262.

About the authors

Hilary Sherman and John Shyu are scientists at Corning Incorporated, Life Sciences

Additional Resources

1. **The Ultimate Guide to Corning Matrigel Matrix (Corning Guide CLS-DL-AC-016)**
2. **Culture of Mouse Intestinal Organoids in Corning Matrigel Matrix for Organoid Culture (Corning Application Note CLS-AN-542)**
3. **High Throughput Gene Expression Analysis of 3D Airway Organoids (Corning Application Note CLS-AN-534)**
4. **A Novel Method for Generating Single, Intestinal Organoids for High Throughput Screening (CLS-AN-464)**
5. **All About Organoids e-book available on www.corning.com/matrigelfororganoid**

Visit www.corning.com/3D to access all Corning scientific resources.

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

Matrigel matrix-3D plates have been shown to support a robust HTS assay format.

Virneliz Fernández-Vega, Louis Scampavia, Elizabeth J. Abraham, Lynsey C. Willetts, and Timothy P. Spicer

Introduction

Pancreatic cancer is among the most lethal forms of metastasis and the large majority of these are exocrine in nature. Pancreatic ductal adenocarcinoma (PDAC), an exocrine neoplasm, is the most common and aggressive form of this disease. Lack of effective treatment options is one of the many contributors to mortality. To study PDACs, human tumor tissue grown in 2D culture conditions does not support growth of non-transformed cells. Therefore, 3D models are being explored such as neoplastic pancreatic organoids that better mimic *in vivo* physiology and are thought to be better predictors for compound profiling. Corning® Matrigel® matrix has been the gold standard for organoid cultures providing the necessary physiological milieu to support these 3D structures. Here, we evaluated growth of human pancreatic ductal tumor organoids using Corning Matrigel matrix-3D plates. We found that these pre-coated, high throughput formats were convenient and easy to use as Matrigel matrix was already pre-dispensed into each well. The Matrigel matrix-3D plates are potential tools for drug discovery efforts using organoids as demonstrated by the ability to support human pancreatic organoid cultures.

Materials and Methods

Human pancreatic ductal adenocarcinoma organoids (hT3) were generated from tissues of pancreatic cancer patients in the laboratory of Dr. Tuveson, M.D.[1] The Tuveson lab has established a solid platform to generate organoid models from normal and neoplastic

murine and human pancreas tissues.[2] The pancreatic organoids can be rapidly generated from resected tumors and biopsies, surviving through the cryopreservation process and exhibiting ductal- and disease stage-specific characteristics.[1,2] This specific organoid line, hT3 has always been maintained in 3D cell culture conditions and never manipulated using 2D methods.

To test the Matrigel matrix-3D plates (Corning 356256) a cell titration was performed using disaggregated hT3 organoids and seeded at different cell densities (0 to 10,000 cells/well) following manufacturer's instructions for the 'overlay' method, with minor modifications. The experiments were conducted using 7 replicates per each condition for a period of 5 days of total incubation. The 3D organoids were monitored using bright-field microscopy to visualize the formation of spheres over time. The Matrigel matrix-3D plates used were a 384-well, black/clear square bottom format pre-coated with Matrigel matrix in each well. The plates were incubated prior to seeding the cells to polymerize the Matrigel matrix. Cells were grown in human complete feeding media containing supplements and growth factors specially formulated for human organoids as previously described.[2] Shown below is an outline of the protocol that was followed:

Thaw Corning Matrigel matrix-3D plates for ≥6 hours at 4°C prior to use.

Polymerize Matrigel matrix by incubating the microplates for 1 hour at 37°C prior to cell seeding. This results in gelation of the matrix layer.

Seed cells onto the polymerized Matrigel matrix by carefully adding 25 µL/well of cell suspension in the growth media, taking care to not disturb the gelled layer of Matrigel matrix.

Incubate culture in a humidified incubator at 37°C for a period of 5 days and image as needed.

96 hours post-cell seeding, add 25 µL/well of 3D CellTiter-Glo® reagent (Promega), and incubate for 60 minutes at room temperature.

Read luminescence.

Results and Discussion

We observed human pancreatic organoids (hT3) formation within 48 hours of plating using standard bright-field microscopy with 4X and 10X objectives as shown in Figure 1. It also appears that the number of organoids per well was proportional to initial cell seeding density.

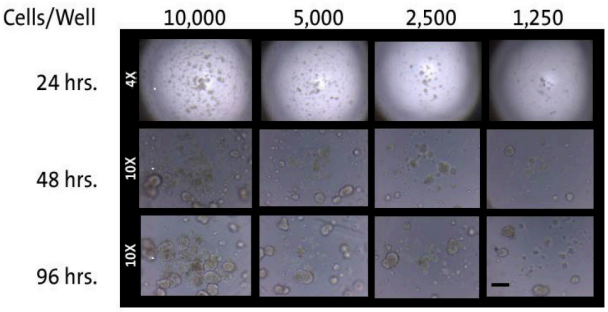
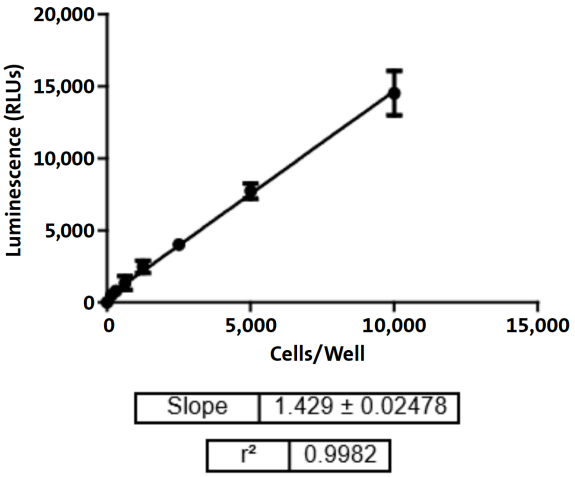


Figure 1. Assessment of 3D sphere formation of the hT3 organoids seeded at different cell densities in Corning Matrigel matrix-3D plates using 4X and 10X objective at different time points. Bar scale in lower right is 100 µm.

Conclusions

- Corning® Matrigel® matrix-3D plates were able to support pancreatic organoid culture.
- The Corning Matrigel matrix-3D plates were able to support a homogenous assay format, wherein both the organoid culture and end-point viability read out (luminescence) were achieved simultaneously.
- The Z' values achieved using these plates show that they can support a robust HTS assay format.



Cells/Well	Z'	S/B
156.25	0.21	75.29
312.5	0.17	115.58
625	0.14	177.58
1,250	0.48	360.22
2,500	0.74	584.72
5,000	0.79	1127.27
10,000	0.68	2118.97

Figure 2. Correlation plot demonstrating linearity of growth and detection based on the cell number of hT3 organoids seeded per well in the Corning Matrigel matrix-3D plate versus the luminescence readout after 5 days of incubation (x and y axes, respectively, n = 7 replicates per data point; error bars in SD).

Acknowledgments

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About the authors

Virneliz Fernández-Vega, Louis Scampavia, and Timothy P. Spicer are from The Scripps Research Institute. Elizabeth J. Abraham and Lynsey C. Willetts are with Corning Incorporated, Life Sciences.

Additional Resources

1. **Corning Matrigel Matrix-3D Plate (Corning FAQ CLS-AN-571)**
2. **Organoid Master Class Webinar Series, available on www.corning.com/lifesciences under "Events".**
3. **Automation of Forskolin-induced Swelling Assay of Human Intestinal Organoids (Corning Application Note CLS-AN-614)**
4. **Matrigel Matrix-3D plates (www.corning.com/matrigel3dplateguidelines)**
5. **Corning Matrigel Matrix-3D Plates for High Throughput 3D Assay (CLS-AN-572)**

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Analysis of RNA Transcript Levels Reveals Upregulation of Hypoxia Markers for Pancreatic Cancer Cells Cultured in 3D

High-throughput, automated system facilitates RNA isolation from reproducibly formed spheroids.

Audrey Bergeron, Sarah Teter, and Chris Suarez

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a notoriously aggressive tumor type due to its high levels of metastasis and recurrence, and is the fourth leading cause of cancer-related deaths in the Western world.[1] Pancreatic tumors typically lack vasculature and are hypoxic due to oxygen diffusion limitations. Hypoxia can lead to the transcriptional induction of a series of genes that participate in angiogenesis, tumor growth, invasion, and metastasis through hypoxia-inducible factor-1 (HIF-1), an oxygen-sensitive transcriptional activator.[2] These genes, which include glucose transporter protein type 1 (GLUT-1) and carbonic anhydrase IX (CA IX), are associated with a decreased survival in PDAC patients.[2-4] To model PDAC *in vitro*, three dimensional (3D) cell culture models can be used as they have been shown to develop gradients of diffusion for oxygen and glucose at diameters >200 µm, and can develop hypoxic cores with low pH.[5,6] In this study, the PDAC cell line PANC-1 was seeded at densities ranging from 500 to 5,000 cells in Corning® spheroid microplates. Corning spheroid microplates are round bottom plates with a hydrophilic, biologically inert Ultra-Low Attachment surface coating that enables the formation of a single spheroid in each well. The cells were cultured for 7 days to form spheroids with diameters ranging from 430 to 850 µm. RNA was isolated from spheroids at each seeding density, as well as from PANC-1 cells cultured in 2D, using the Promega Maxwell® Rapid Sample Concentrator (RSC) instrument with the Maxwell RSC miRNA Tissue Kit. The Promega Maxwell RSC instrument is an automated nucleic acid purification platform that allows for hands-free processing of up to 16 samples simultaneously. Relative expression of hypoxia markers HIF-1α, GLUT-1, and CA IX were analyzed relative to 2D culture using RT-qPCR to assess the correlation between spheroid size and hypoxia marker

expression. As spheroid density increased, the gene expression of HIF-1α did not change; however, the expression level of HIF-1 targeted genes GLUT-1 and CA IX increased in direct relation to spheroid density.

Materials and Methods

Spheroid Formation and 2D Cell Culture

PANC-1 cells (ATCC® CRL-1469™) were thawed and cultured following the vendor's protocols using DMEM (Corning 10-013-CM) supplemented with 10% fetal bovine serum (FBS; Corning 35-010-CV). The same lot of medium and FBS were used throughout the study. Early passage cells were harvested with 1X TrypLE™ (Thermo Fisher 12563029), filtered through a Falcon® 40 µm cell strainer (Corning 352340), and seeded into 96-well Corning spheroid microplates (Corning 4520) at 500, 1K, 2.5K, and 5K cells/well in 200 µL culture medium. Spheroids were incubated in a 37°C, 5% CO_2 humidified incubator for 7 days with half media changes performed every 2 to 3 days. On the fifth day, PANC-1 cells were also seeded in 2D at 5K cells/well into 96-well TC-treated plates (Corning 3603) in 200 µL culture medium. 2D cells were incubated in a 37°C, 5% CO_2 humidified incubator for 2 days. Cells were cultured following this protocol three independent times.

RNA Isolation

RNA was isolated from each culture condition using Maxwell RSC miRNA Tissue Kits (Promega AS1460) with a Promega Maxwell RSC instrument (Promega AS4500) using the miRNA Tissue Kit program. Samples were prepared following the vendor's recommended protocols and by pooling wells. 3D samples were pooled using 5 wells for 500 cells, 3 wells for 1K cells, and 1 well each for 2.5K and 5K cells. For 2D samples, 2 wells were pooled. Three replicates were prepared for each culture condition for each independent study. Isolated RNA was quantified using the QuantiFluor® RNA System (Promega E3310) and a Promega Quantus™ Fluorometer (Promega E6150) following the vendor's protocols. For each RNA sample, 2 µL of RNA eluate was combined with 200 µL of prepared dye solution for quantification.

RT-qPCR

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed for each sample using a GoTaq® Probe 2-Step RT-qPCR System (Promega A6110) following the vendor's protocols. For each independent study, a standardized amount of RNA (80 to 95 ng) was used to synthesize cDNA via reverse transcription with random hexamer primers. The amount of RNA was based on the purified sample with the lowest concentration for that independent study.

For qPCR assays, Thermo Fisher TaqMan® assays for HIF-1a, GLUT- 1 (SLC2A1), CA IX, and housekeeping gene TFRC were included (Table 1). TaqMan assays contained primer sets and FAM™ dye labeled probes. Samples were prepared in duplicate in 96-well PCR microplates (Corning PCR-96-FS-C) with ultra-clear sealing film (Corning UC-500), and qPCR was performed using a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. Cq analysis was completed using the Bio-Rad CFX Manager™ software. Results reflect ΔΔCq analysis for the expression of the target of interest normalized to that of the housekeeping gene (TFRC) under 2D and spheroid growth conditions.

Table 1. Thermo Fisher TaqMan® Assays

Item	Vendor	Cat. No.	Final Concentration
Advanced DMEM with F-12 Hams	Thermo Fisher	12634010	1X
Corning glutagro™	Corning	25-015-CI	2 mM
Hepes	Corning	25-060-CI	10 mM
Penicillin/Streptomycin	Corning	30-002-CI	1X

IF and Imaging

PANC-1 spheroids were fixed in cold 4% paraformaldehyde (Boston Bioproducts BM1552) for 1 hour at room temperature and were washed 3 times in Dulbecco's Phosphate Buffered Saline (DPBS; Corning 21-031-CM). For immunofluorescent [IF] labeling, fixed cells were embedded into paraffin through graded alcohols and xylene. Five-micron (5 µm) sections of spheroids were collected with a rotary microtome and immunolabeled with antibodies against HIF-1α (Novus Biologicals NB100-105), CA IX (R&D Systems AF2188), and GLUT-1 (Abcam ab652), and detected using fluorescently conjugated secondary antibodies. Sections were counterstained with DAPI and imaged using a laser scanning confocal microscope. Tissue embedding, sectioning, immunofluorescence labeling, and confocal microscopy were performed at the COBRE Histology and Imaging Core at the University of New England, Biddeford, ME, NIGMS grant number P20GM103643.

Results and Discussion

PANC-1 cells were seeded into 96-well Corning® spheroid microplates at seeding densities ranging from 500 to 5,000 cells/well and were cultured for 7 days with half media changes every 2 to 3 days. A single spheroid formed in each well, with spheroid size increasing with seeding density. To determine average spheroid diameter after 7 days of culture, representative brightfield images were taken of one spheroid per seeding density for each independent study using an EVOS® FL microscope with a 4X objective (Figure 1).

After 7 days of culture, representative PANC-1 spheroids were fixed, paraffin embedded, sectioned, and labeled with antibodies for known hypoxia markers. Spheroids of all sizes showed expression for HIF-1α, CA IX, and GLUT-1 proteins (Figure 2). However, the relative protein amounts were not quantified.

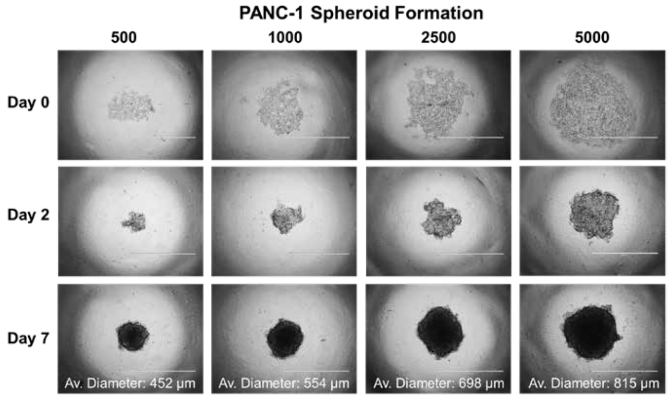

Figure 1. PANC-1 spheroid formation over 7 days. PANC-1 cells were seeded into 96-well Corning spheroid microplates in seeding densities ranging from 500 to 5,000 cells/well and were cultured for 7 days with half media changes every 2 to 3 days. A single spheroid formed in each well with spheroid size increasing with seeding density. Average spheroid diameters for each seeding density are displayed on Day 7 images. Representative brightfield images were taken with an EVOS FL microscope with a 4X objective. Scale bar = 1 mm.

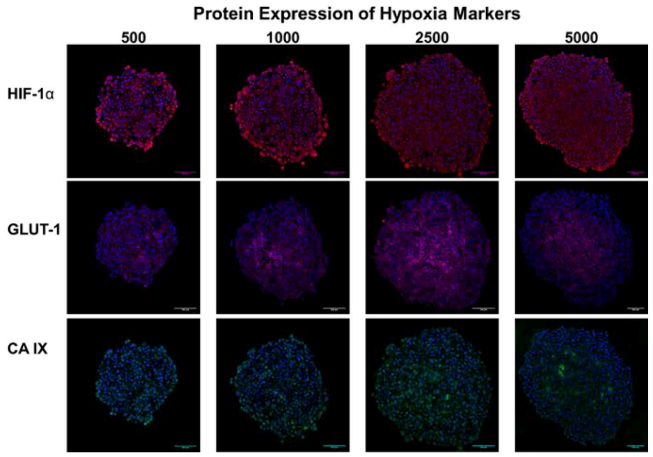

Figure 2. IF labeling reveals protein expression of hypoxia markers. PANC-1 spheroids were seeded at densities ranging from 500 to 5,000 cells/well, cultured for 7 days, and then fixed, paraffin embedded, and labeled with antibodies for known hypoxia markers. Spheroids of all sizes showed expression for HIF-1α, CA IX, and GLUT-1 proteins. Representative images were taken with a laser scanning confocal microscope with a 20X objective. Scale bar = 100 µm.

To quantify relative gene expression levels of hypoxia markers, RT-qPCR was performed using RNA that had been isolated from representative spheroids of each size (Figure 3). There were no differences in the expression of HIF-1α in any of the spheroids compared to 2D culture (Figure 3A), which is consistent with the literature.[7] Although hypoxic conditions stabilize the HIF-1α protein, which is a subunit of the transcription factor HIF-1, it has previously been reported that hypoxia does not affect mRNA levels of HIF-1α.[7] The expression of GLUT-1 in spheroids, however, increased in correlation with spheroid density, demonstrating approximately 4 times higher expression in the largest spheroid relative to 2D (Figure 3B). It has been shown previously that hypoxia-induced increases in HIF-1α protein result in increases in GLUT-1 mRNA levels.[8] Upregulation of GLUT-1 is associated with an increase in pancreatic cancer invasiveness and a decreased survival in PDAC patients.[3] Additionally, the expression level of CA IX in spheroids increased in correlation with spheroid density, with approximately 130 times higher expression in the largest spheroid relative to 2D (Figure 3C). Of the genes induced by hypoxia via HIF-1α, CA IX is known as one of the most uniformly induced genes and is associated with poor cancer prognosis.[4]

Conclusions

- IF labeling demonstrated protein expression of hypoxia markers HIF-1α, GLUT-1, and CA IX in PANC-1 spheroids. The relative gene expression of these markers was quantified using RT-qPCR analysis of isolated RNA and showed that GLUT-1 and CA IX increased in gene expression in correlation with cell seeding density and spheroid size.
- Combining the Corning® spheroid microplate with the Promega Maxwell RSC instrument demonstrates a high throughput, automated system for RNA isolation from reproducibly formed spheroids.

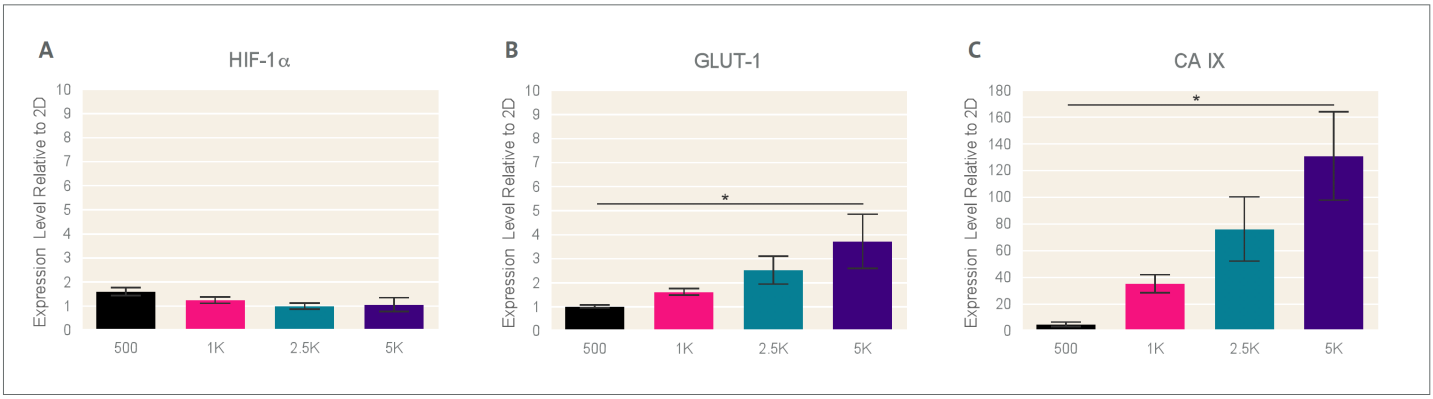

*Figure 3. Relative gene expression level of hypoxia markers. Gene expression for each spheroid size relative to 2D was determined in RT-qPCR with isolated RNA. (A) There were no differences in gene expression level of HIF-1α in any of the spheroids compared to 2D culture. (B) The expression level of GLUT-1 in spheroids increased in correlation with spheroid density demonstrating approximately 4 times higher in the largest spheroid relative to the 2D cells. (C) The expression level of CA IX in spheroids increased in correlation with spheroid density, demonstrating approximately 130 times higher expression in the largest spheroid relative to 2D. For all hypoxia markers tested, bars display average from 3 independent studies performed in triplicate (n=9). Error bars represent standard deviation (SD). A Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test was performed for each hypoxia marker, *p<.05.*

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About the authors

Audrey Bergeron and Chris Suarezare scientists at Corning Life Sciences. Sara Teter is with Promega Corporation.

Additional Resources

1. **Corning Spheroid Microplates (Corning User Guide CLS-AN-235)**
2. **Corning Spheroid Microplates Spheroid Formation (Corning Protocol CLS-AN-308) Corning Matrigel Matrix-3D Plate (Corning FAQ CLS-AN-571)**
3. **3D Model Systems: Spheroids, Organoids, and Tissue Models e-book available to download on www.corning.com/3D**
4. **Co-culturing and Assaying Spheroids in the Corning Spheroid Microplate (Corning Application Note CLS-AN-390)**
5. **Corning Elplasia Round Bottom Plates (Corning Guidelines for Use CLS-AN-536)**

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