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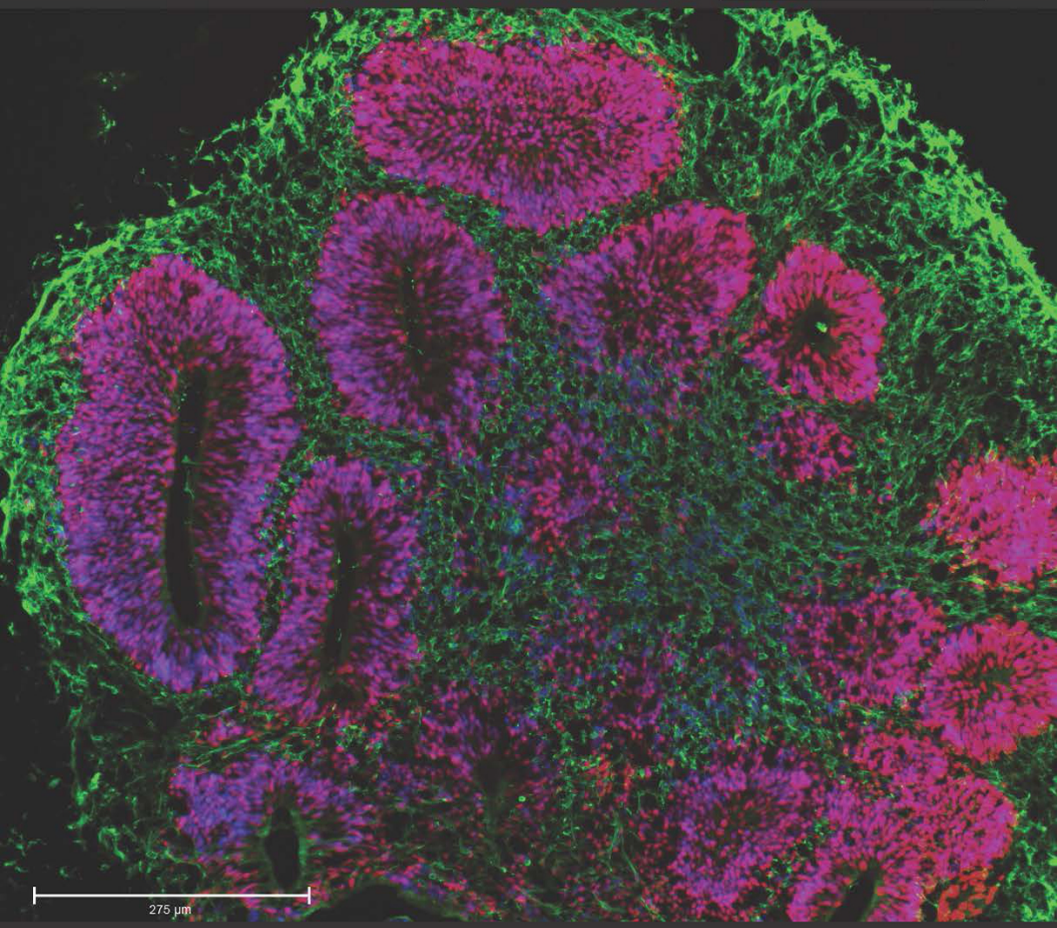

3D Cell Culture and Analysis

Evolution and Applications

Essential Knowledge Briefings

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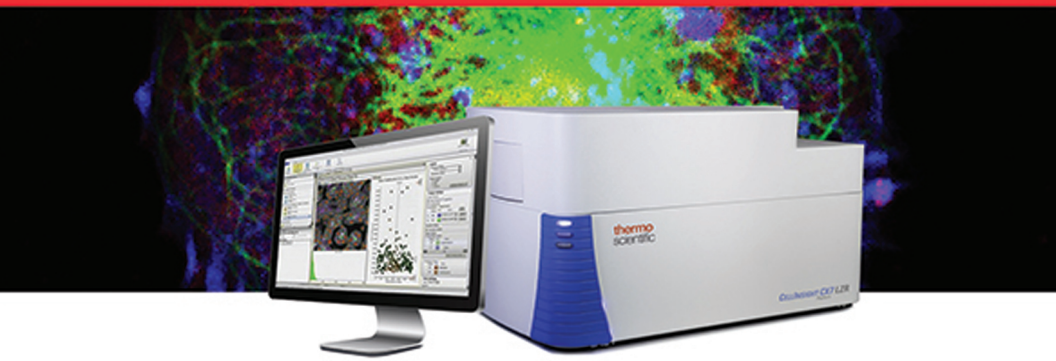

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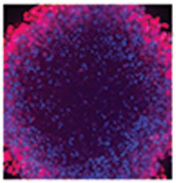
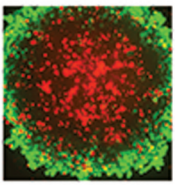


Confocal image of live spheroids of A549 cells stained with an Invitrogen™ LIVE/DEAD™ viability kit (Cat. No. L3224) taken at 10x magnification on the CX7 LZR high-content analysis platform.



Confocal image of live spheroids of A549 cells stained with Invitrogen™ MitoTracker™ Orange dye (Cat. No. M7510) and Invitrogen™ Hoechst™ 33342 stain (Cat. No. H3570), taken at 10x magnification on the CX7 LZR high-content analysis platform.

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Front cover image courtesy of Mark Kennedy, Ph.D. R&D Scientist at Thermo Fisher Scientific.

Front cover caption: Neural Organoid cultured in Thermo Scientific Nunclon Sphera 6-well microplates (10um thick cryo-section), stained with Donkey anti-Rat Alexa Fluor 488, Donkey anti-mouse Alexa Fluor 594, Goat anti-rabbit Alexa Fluor 647, taken on the Invitrogen EVOS FL Auto 2 Cell Imaging System.

Prepared for Wiley and Thermo Fisher Scientific, Inc. by EKB Editor, Mike May, Ph.D.

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CONTENTS

INTRODUCTION

The key to cell culture is creating the most realistic environment possible, but in a way that cells can still be analyzed. At the Centre de Biophysique Moléculaire in Orléans, France, molecular biologist Claudine Kieda and her colleagues generate cultures that replicate cancer cells and grow them in a microenvironment, including the surrounding tissues and cells found in our complex bodies. These scientists want to understand how cancer grows and how to arrest or reverse that growth. Their research depends on three-dimensional (3D) cell cultures.

Scientists cultured cells in two-dimensional (2D) monolayers for nearly a century before turning to three-dimensional cell culture. 2D cell cultures can be set up more quickly and controlled more easily, but the many benefits of culturing cells in a 3D format often outweigh the inconveniences. For many scientists like Claudine Kieda and her colleagues, who have trained in the world of 2D cell culture, the biggest challenge is learning how to adopt a 3D approach. In this Essential Knowledge Briefing, scientists will cover a brief history of 3D cell culture, learn about applications, basic protocols, troubleshooting tips and explore future directions.

HISTORY AND BACKGROUND

In 1885, German zoologist Wilhelm Roux reported on the first example of 2D cell culture, when he kept chicken embryonic cells alive for a few days in a saline solution.

More than 70 years later, in 1957, at the Rockefeller Institute for Medical Research, American developmental biologist Aron Moscona showed that dissociated cells cultured together created aggregates – 3D structures. In the early 1970s, Robert Sutherland and his colleagues at the University of Western Ontario in Canada coined the term spheroids for the structures that Moscona described. Sutherland and his colleagues cultured Chinese hamster lung cells in suspensions that created spheroids, and the scientists suggested that these 3D structures could be used in many ways, including studying the growth of cell clusters and developing drug treatments.

Still, much remained to be learned about culturing cells in 3D. For example, in 1982, Mina Bissell, now a distinguished scientist in the biological systems and engineering division at Lawrence Berkeley National Laboratory, showed that the extracellular matrix (ECM) – the molecules outside of cells that give them structural and biochemical assistance – impacts the gene expression of cells. That discovery started a very strong trend toward culturing in 3D instead of 2D.

The trend to culture cells in 3D has continued for many reasons. Even the appearance of cells differs in 2D versus 3D cultures. In 2D systems, cells tend to be stretched out, making them flatter than *in vivo*. That abnormal morphology can impact many processes, including proliferation, differentiation, and cell death. In general, 3D cell cultures replicate *in vivo* conditions more accurately than 2D systems. That is, cells cultured in 3D provide data that are physiologically more relevant. For example, the gene expression of cells cultured in 3D is closer to that of cells in their natural environment than cells cultured in 2D. Nonetheless, 3D approaches face some challenges. In some examples,

more cells in 3D cultures die over time, perhaps due to lack of adequate exchange of nutrients, oxygen and accumulated cellular waste between the core of cellular aggregates and the external milieu.

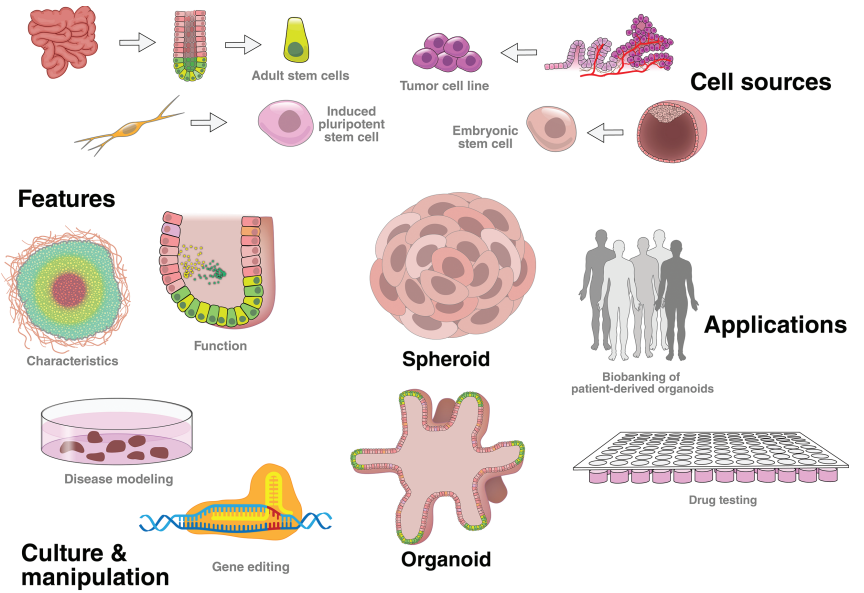

Fig 1. A variety of cell sources can be used to make different 3D cultures that can be used in diverse applications.

Source: Thermo Fisher Scientific eLearning Module: experimental approaches.

Today, there are a variety of cultures that can be created. Histotypic cultures include just one cell type. A spheroid is a 3D group of cells that includes structural and functional features of a biological material, such as a tumor, and spheroids can include more than one kind of cell, such as multicellular spheroids. Tumor spheroids have become an increasingly important tool in studying basic tumor biology and cancer therapy. Organoids are comprised of multiple cell types that mimic the microanatomy of an organ. For instance, various types of epithelial cells can be cultured in 3D to make human intestinal enteroids (organoids made from small-intestine cells), which can be used to

study intestinal physiology. These are just a few examples in a multitude of possibilities in biomedical research. In general, most cell types, as well as a wide range of combinations, could be cultured in 3D to analyze cell structure, interactions, cellular communication, and more.

Methodology

Various methods can be used to culture cells in a 3D environment. For example, Sutherland's methods of suspension cultures are still being used, largely because it is inexpensive and easy to adopt. Scientists can use this technique with vessels such as Thermo Scientific™ Nunclon™ Sphera™ Microplate, which has a synthetic polymer-coated surface that discourages ECM adsorption.

Other 3D methods include variants of existing methods. In mid- to large-scale suspension culturing, spinner flasks – that is, stirrer bottles – improve the gas exchange in a culture, which allows more cells to aggregate, although controlling the size and number of the spheroids with this method is nearly impossible. 3D cell culturing can also take place in a low cell binding vessel (e.g. Nunclon Sphera) that is rotated in a gyratory pattern. In the tedious hanging drop method, a drop of cells in suspension actually hangs from a culture structure. For cells that do not tend to aggregate very well on their own, microcarrier beads can be added to a culture, so the cells attach to the beads, which leads to 3D aggregation. The beads can be purchased with different coatings for different kinds of cells, even if little is known about the precise impact of these microcarrier beads on cellular functions.

To grow cells in space, NASA researchers developed a technology that rotates cells. Such rotating bioreactors also work well for some applications in Earth-bound labs, where the device simulates microgravity by keeping the cells in a state of free fall. This gentle technique can quickly produce spheroids.

Instead of reducing gravity, 3D cell cultures can also be maintained with increased magnetism. Here, cells are treated with magnetic

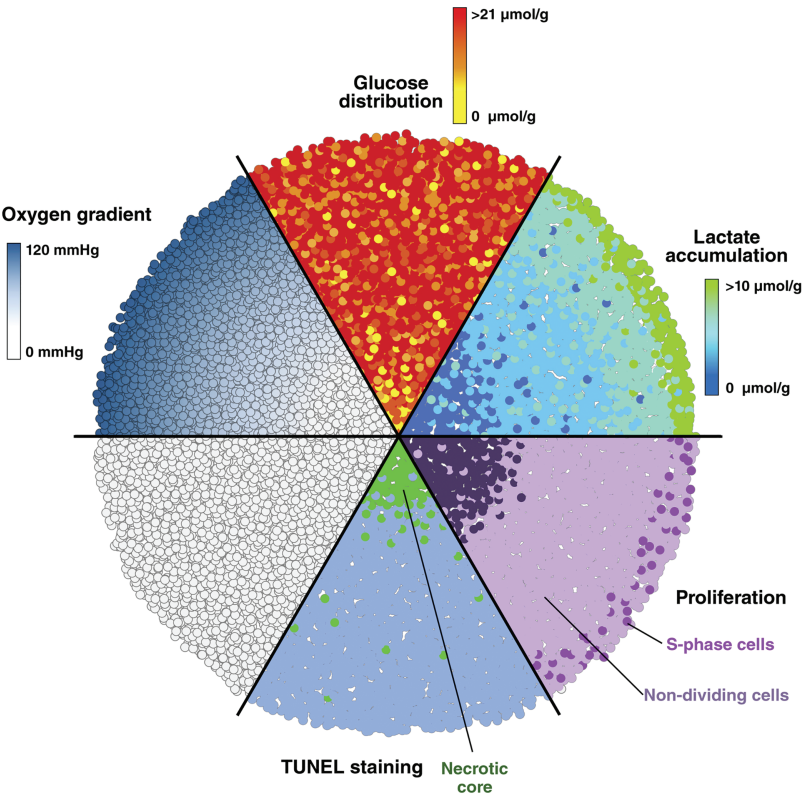

Fig 2. Analyzing spheroids with various techniques—autoradiography, the tunnel assay, bioluminescence imaging, and probing with oxygen microelectrodes—is depicted here in a combined image that shows how scientists can study specific features, such as cell proliferation and viability.

Source: Thermo Fisher Scientific eLearning Module: experimental approaches.

nanoparticles, and then a magnetic field keeps the cells at the medium–air interface, which prevents cells from attaching to the culture vessel and puts cells in proximity to each other to encourage speedy aggregation. An air–liquid interface (ALI) approach can also be used in cell culture vessels with porous membrane. For example, Thermo Scientific Nunc Carrier Plates with precise hanging height for Cell Culture Inserts make ALI culture much easier to achieve and less labor intensive.

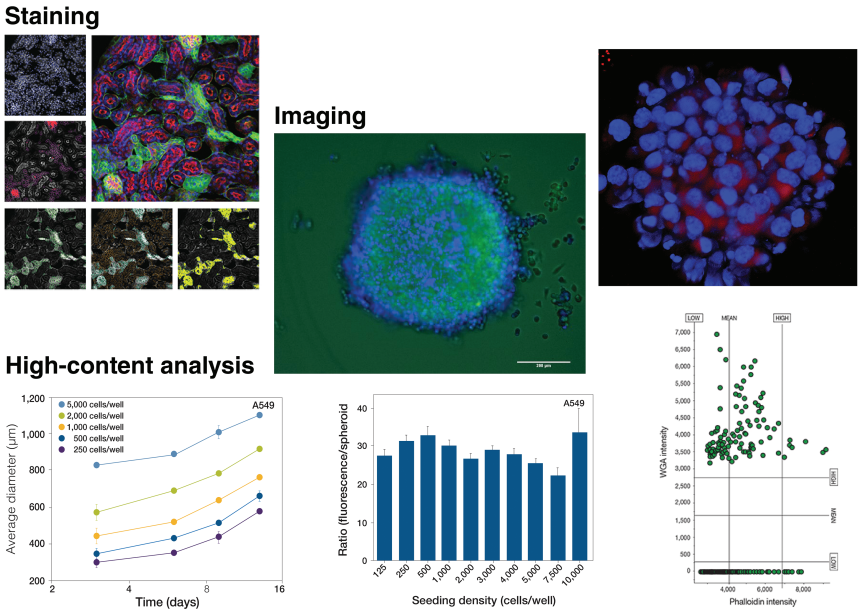

Fig 3. 3D cultures can be used for cell-based analysis, stained and imaged, or used in high-throughput or high-content screening.
Source: Thermo Fisher Scientific eLearning Module: experimental approaches.

In some cases, when physical structures are needed in 3D culture providing the cells something to grow on, such as a scaffold, helps to maintain a culture by mimicking the ECM. In fact, some bioengineered scaffolds have been shown to encourage cell maintenance and the production of ECM.

With 3D cell cultures created and maintained, scientists need ways to analyze the results. Microscopy is one approach. 2D cultures provide thin samples for observation, but that is not the case with 3D cultures. The latter's structure is thicker and scatters light more, which reduces the capabilities of some microscopic methods, such as bright-field or other techniques that transmit light through a sample for imaging. Nonetheless, 3D cell cultures can be analyzed with some forms of microscopy, including confocal, multiphoton, and optical coherence tomography.

With confocal microscopy, scientists can image 3D structures around 200 µm deep in a sample—depending on the microscope, lens and the sample itself—with or without the addition of fluorescent reagents that stain certain proteins. Multiphoton microscopy can analyze structures to a depth of about 1 mm. To go deeper, as much as several micrometers, scientists can use optical coherence tomography. To select the best imaging for a specific application, scientists should consider the required depth and resolution of imaging and if fluorescence is needed for marking specific structures. For instance, confocal microscopy is the most limited in depth, but can include fluorescence, and some commercial platforms allow submicron resolution.

Other analytical methods provide useful features. With microcomputer tomography, X-rays scan the sample nondestructively with no staining required. To examine a collection of experiments quickly and automatically, many researchers – especially in the pharmaceutical industry – use high-throughput screening (HTS). HTS requires a comprehensive robotic platform for the full automation of cell-based and biochemical assays. At its core is a high-throughput

liquid handling system connected to ancillary instrumentation, such as an optical plate reader for more typical HTS assays and an automated microscope for HCS. HTS is especially useful for drug screening. Some commercial systems for HTS work with a range of micro-well plates and provide enough depth of field to analyze a complete spheroid in one image. Plus, fluorescent assays – such as cell-viability dyes and calcium-flux dyes – can be used to analyze populations of spheroids. High-content screening (HCS) simultaneously analyzes multiple features of a 3D culture. Using a collection of fluorescent markers and lasers, an automated microscope captures images in which specific features, such as cell movements or interactions, can be tracked and analyzed. Such systems require advanced optics and computation for data analysis. The expanse of data collected, however, makes HCS useful in many 3D applications, such as exploring the cellular basis of diseases.

IN PRACTICE

Covering the entire range of 3D culture applications would take a book, and the content is a moving target. As more scientists opt for 3D methods and labware and analytical platforms evolve, applications will grow in parallel. Here, several broad applications will be briefly described.

Improved cell growth and interactions make 3D culture especially useful in developmental biology studies. As cells grow ***in vivo***, they respond to different developmental cues in the microenvironment of surrounding cells and tissues. So, 3D culturing could be used to study cell differentiation, migration, tissue and organ formation, and many other developmental processes. Stem cells are excellent examples of the value of moving from 2D to 3D cultures. More cells can be grown and differentiated in 3D environments, such as by expanding a population by a billion-fold, and some stem cells need the ***in vivo***-like environment to function properly or differentiate.

Beyond basic research, 3D cell culture has widespread applications in translational medicine including disease modeling. With spheroids composed of cancer cells, for instance, scientists can study the interactions between a tumor and its microenvironment or how cancer cells grow, migrate, and invade other tissues. Using a patient-derived xenograft (PDX) – a sample of a tumor that is cultured or even grown in mouse models – cancer cells can be analyzed and challenged with various treatments. The groundbreaking value of 3D culturing in cancer research has been known for some time. In 2002, cancer-research pioneers Tyler Jacks and Robert Weinberg wrote in ***Cell***: "Suddenly, the study of cancer cells in two dimensions seems quaint, if not archaic."

Since then, many scientists have used 3D culturing to study cancer. In ***Cell*** in 2018, for instance, cancer biologist Hans Clevers at the University Medical Center Utrecht (Utrecht, Netherlands) and

his colleagues created more than 100 primary and metastatic breast cancer organoid lines. They found that the organoids "matched the histopathology, hormone receptor status, and HER2 status of the original tumor." As a result, the scientists noted that these organoids could be used in breast cancer research and drug development.

Various lines of research have revealed limitations of 2D cultures in drug research. Monolayers of cells lack the typical tissue architecture, cell-to-cell interactions, and so on, that could impact a potential chemical's performance as a treatment. In fact, some cancer cells resist a drug ***in vivo*** and in 3D cell cultures – for example, colon cancer cells responding to fluorouracil – but not in 2D culturing methods. In general, cells in 3D models make better tools for discovering and testing new medicines.

To explore a few applications in more detail, here are five specific case studies.

CASE STUDY 1

At Ehime University in Japan, Hidemasa Kato, a professor at the Graduate School of Medicine, uses 3D cell culturing in hopes of improving the pluripotency of stem cells. In particular, he wants to make human-induced pluripotent stem cells (iPSCs), which can be generated from adult human cells, able to retain more of their embryonic capabilities. Creating iPSC with broader potential for developing into a greater variety of mature cell types will provide more options for medical use, including disease modeling and replacement of lost or damaged cells.

Embryonic stem cells from mice remain more pluripotent than the ones from humans and, in turn, can be more easily manipulated in cell differentiation. Kato wants to know what causes that difference and the key pathways that impact the developmental fate of human iPSCs. He works with iPSCs that grow spheroids in Thermo Scientific Nunclon Sphera vessels. With enough of these cells aggregated in a spheroid, they start to differentiate. In ordinary culture, these human iPSCs spheroids develop along a mesendodermal line, thought to be controlled by SMAD signaling. When Kato introduced the TET1 gene (ten-eleven translocation methylcytosine dioxygenase 1) in the cells, his human iPSCs matured along a line of neurogenesis. Doing that, however, appears to depend on a spheroid's size, and the medium must be adjusted prior to any cellular differentiation.

Kato's research helps scientists learn to control the processes for creating specific cells from iPSCs that could be used to model diseases for drug discovery and screening.

1. Bioprocess., *2015, 3:199–213. (DOI: 10.4155/pbp.15.9)*

CASE STUDY 2

At the Creighton University School of Medicine (Omaha, NE), Xian-Ming Chen, professor of medical microbiology and immunology, and his colleagues used enteroids developed from mice to study gastrointestinal infections caused by the protozoan parasite Cryptosporidium. *In adults with a normally functioning immune system, this parasite might not cause any symptoms, but it can be deadly in people who are immunocompromised, such as HIV/AIDS patients.*

Using immunocompromised mice – that is, mice that lack an immune system which would reject the human cells – Chen and his colleagues created enteroids, which were tested against Cryptosporidium parvum. *The researchers compared the level of infection and gene expression in enteroids developed from adult and neonatal mice. Infection inhibited the propagation of the enteroids, and it also decreased the expression of intestinal stem cell markers. In addition, a* C. parvum *infection increased the expression of some antagonists of the Wnt signaling pathway and decreased expression of several Wnt proteins. Chen and his colleagues also saw increased levels of cell death in infected enteroids. The researchers concluded "that enteroids from adult mice are susceptible to* ex vivo *infection, suggesting that a systemic immune response and/or a mature intestinal microbiome may" account for the resistance to infection* in vivo *in adult mice.*

In comparing enteroids from adult and neonatal mice, the scientists found similar results in infection burden and changes in expression. As Chen and his colleagues wrote, "This suggests to us that the epithelial response to infection in neonatal and adult mice, in the absence of a systemic immune response and a mature intestinal microbiome, may be similar. However, a slight higher infection burden was detected in enteroids from neonatal mice than that in enteroids from adult mice,

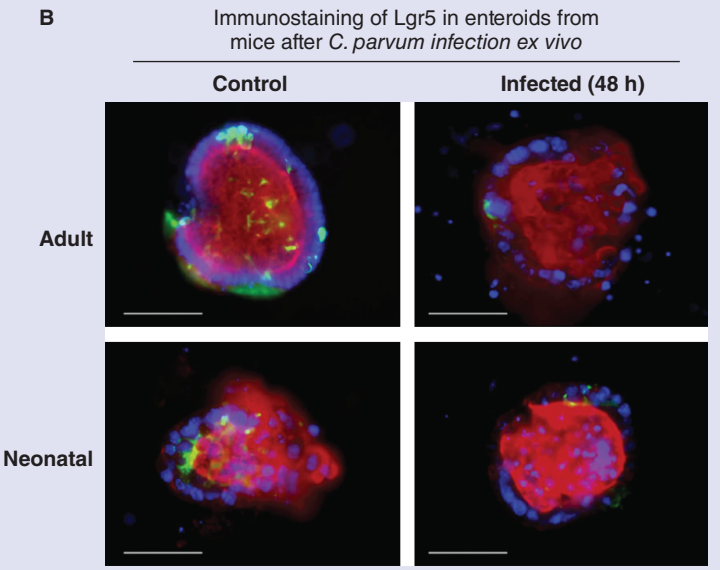

Fig 4. In enteroids made from neonatal and adult mice, infections with *Cryptosporidium parvum* decreased the expression of Lgr5 (green), which is an intestinal stem cell marker.

Source: Cryptosporidium parvum infection attenuates the ex vivo propagation of murine intestinal enteroids. Xin-Tian Zhang et al. *Physiological Reports*, 2016, 4: e13060 (DOI: 10.14814/phy2.13060).

given the fact that the same amount of parasite sporozoites were initially added to the culture. Therefore, immature intestinal epithelium, such as in neonatal mice, may have deficiency in clearance of C. parvum *infection."*

This case study shows how enteroids can be used to explore detailed cellular aspects of disease, as well as age-related differences.

1. *From* Cryptosporidium parvum *infection attenuates the* ex vivo *propagation of murine intestinal enteroids,* Physiological Reports*, Volume: 4, Issue: 24, First published: 30 December 2016, DOI: 10.14814/phy2.13060.*

2. Physiological Reports*, 2016, 4:e13060. (DOI: 10.14814/phy2.13060)*

CASE STUDY 3

Airway organoids can be used to study basic biology, disease development and progression, and treatment. Marc Hild and Aron Jaffe of the Novartis Institutes for BioMedical Research (Cambridge, MA) described a method for developing bronchospheres, which are organoids that represent part of a human airway.

"In contrast to existing methods used for the culture of well differentiated human airway epithelial cells, bronchospheres do not require growth on a permeable support and can be cultured in 384-well assay plates," Hild and Jaffe wrote. Consequently, these bronchospheres, the scientists pointed out, can be used to "model growth, repair, and differentiation of the human airway epithelium without the need for expensive and time-consuming air-liquid interface (ALI) cultures that require the use of a permeable insert." This system can also be used for HTS of potential drugs.

To make bronchospheres, Hild and Jaffe started with human bronchial epithelial cells, which can be purchased and used as the source of basal cells that will grow into the desired organoid. The scientists note, however, that "Details relating to the isolation and purification of the cells are rarely made available by vendors, and, as such, their expression of airway basal cell markers (p63, NGFR, ITGA6) should be checked routinely." The authors added: "The starting material and subsequent culturing of cells is critical for obtaining reproducible, well-differentiated 3-D bronchospheres."

The development of the bronchospheres can be tracked by testing for specific cell markers after seeding the cells into a vessel. For example, because the process starts with basal cells, their markers – p63 and ITGA6 – get expressed from start to finish in a culture. Goblet cell markers – MUC5AC, MUC5B, and FOXA3 – start being expressed after about five days, and markers for ciliated cells can be found after seven days. Ultimately, this process creates 3D organoids that include multi-ciliated cells; goblet cells, which produce mucin; and airway basal cells.

1. Curr. Protoc. Stem Cell Biol., *2016, 37:IE.9.1–IE.9.15. (DOI: 10.1002/cpsc.1)*

CASE STUDY 4

Creating and maintaining 3D cell cultures provides a more realistic experimental environment, but none of that matters if the resulting structures cannot be adequately analyzed. Although the 3D shape enhances in vivo*-like conditions, that geometric feature hinders a scientist's ability to study the results. With stem cells, in particular, one challenge is confirming the developmental lineage of cells inside the structure. To help scientists address that challenge, Luke Wiley, a research assistant professor of ophthalmology and visual sciences at the University of Iowa's Carver College of Medicine, and his colleagues explained how to embed and section a spheroid and then analyze it with immunocytochemical methods.*

This team of scientists took a visual-system approach, working with iPSCs created from cells in the human eye's retinal cups. The researchers embedded the iPSCs in blocks of low-melt agarose and then sliced the blocks – in 50–100-μm-thick sections – with a vibratome. After that, the sections were ready for immunohistochemical analysis.

As Wiley and his colleagues described it, "This method includes an approach for antibody labeling that minimizes the amount of antibody needed for individual experiments and that utilizes large-volume washing to increase the signal-to-noise ratio allowing for clean, high-resolution imaging of developing cell types." The team adds that the method can be used for 3D organoids developed with any pluripotent stem cells.

With this method, scientists can assess the development of stem cells inside an organoid, as well as those on the surface. As Wiley and his colleagues noted, "This is essential for interrogation of disease pathophysiology and development of patient-specific cell replacement approaches when 3D differentiation methods are utilized."

Like other aspects of 3D culturing, this one comes with some trouble spots. Wiley and his colleagues pointed out that the process can be tedious and take some time to learn. "Those with prior experience with microdissections, particularly of smaller tissues and procedures requiring

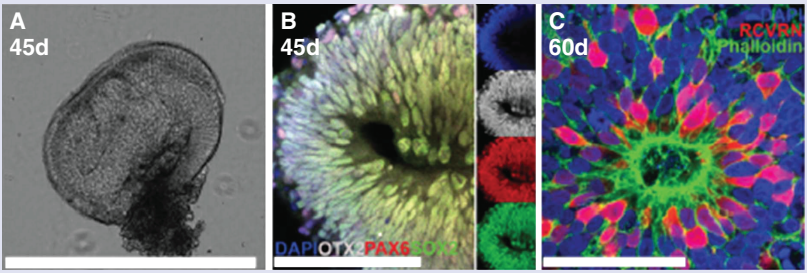

Fig 5. Confocal microscopy of thick-sectioned iPSC-derived 3D retinal organoids immunocytochemically labeled using the Netwells approach. (a) Light micrograph of a single agarose thick section of a retinal organoid differentiated for 45 days (45d). (b) At 45 days' post-differentiation, developing retinal organoids express the retinal progenitorspecific transcription factors – SOX2 (green), PAX6 (red), and OTX2 (gray). Smaller images to the right display each individual fluorophore. (c) Retinal organoids differentiated for 60 days develop polarized filamentous actin-positive (phalloidin, green) neural rosette structures that are composed of recoverin-positive (RCVRN, red) photoreceptor cells. Scale bars: a = 1000 µm; b and c = 50 µm.

Source: A Method for Sectioning and Immunohistochemical Analysis of Stem Cell–Derived 3-D Organoids. Luke A. Wiley et al. From *Curr. Protoc. Stem Cell Biol.* 2016, 37:1C.19.1–1C.19.11. (DOI: 10.1002/cpsc.3).

a steady and trained hand, will find this protocol very feasible," Wiley and his colleagues wrote. "That said, this approach has been demonstrated to be easily adopted by several previously inexperienced members within our lab and as such can be readily acquired."

1. *From a method for sectioning and immunohistochemical analysis of stem cell–derived 3-D organoids,* Curr. Protoc. Stem Cell Biol., *Volume: 37, Issue: 1, Pages: 1C.19.1–1C.19.11, First published: 12 May 2016, DOI: 10.1002/cpsc.3.*

2. Curr. Protoc. Stem Cell Biol., *2016, 37:1C.19.1–1C.19.11. (DOI: 10.1002/cpsc.3)*

CASE STUDY 5

At Columbia University Medical Center, geneticist Michael Shin and his colleagues studied bladder cancer with organoids. Although bladder cancer is the 9th most common cancer in the world, effective models for studying it remain difficult to create.

"To establish clinically relevant models for human bladder cancer," Shin and his colleagues wrote, "We have generated a biobank of patient-derived organoid lines that can be readily propagated in three-dimensional culture." To do that, the researchers took samples from patients' surgeries and divided the tissue into pieces for culturing. The scientists used such samples to develop organoids from 16 patients. They also cryopreserved the organoids for storage and later use. By analyzing the organoids, Shin and his team showed that they replicate "the histopathological and molecular diversity of human bladder cancer."

The organoids can be studied themselves or used in further forms of experimentation. For example, Shin's group also used samples of an organoid as a PDX, which the scientists implanted in immunodeficient mice.

After analyzing the organoids and PDXs, Shin and his team concluded: "Notably, organoid lines often retain parental tumor heterogeneity and exhibit a spectrum of genomic changes that are consistent with tumor evolution in culture."

One challenge with models for studying a disease is keeping a model consistent over time. For the most part, Shin's work showed that the mutational profiles stayed similar over time, but not exactly the same. As noted, the scientists "observed that a subset of mutations was either lost or gained during serial passaging in culture and/or during grafting or reestablishment of organoids from grafts." So, how a model is used can impact the consistency of its genetic profile.

Overall, Shin and his colleagues considered these bladder cancer organoids to be useful models. The authors concluded: "Our studies indicate that patient-derived bladder tumor organoids represent a faithful model system for studying tumor evolution and treatment response." Ultimately, that is just what a disease model should do.

1. Cell, *2018, 173:515–528 (DOI: 10.1016/j.cell.2018.03.017)*

PROBLEMS AND SOLUTIONS

To get started with 3D cell culturing, a background in 2D comes in handy, but there is more to learn. It starts with selecting an approach or how to grow the cells. In some cases, this requires using the Nunclon Sphera plate that resists cells attaching to it so that they aggregate to form 3D spheroids. The beauty of this approach is that it requires very little new techniques and instruments to carry out the 3D cell culture. Not only is it one of the most cost-effective approach, but it also presents the largest selection of culture vessels, from the HTS-friendly microplates to various dishes and flasks, to suit your experimental needs. For some applications, scientists prefer to grow 3D cultures with a scaffold, which can mimic certain micro-environment in vivo, although selecting the right ECM scaffold is crucial to the success of downstream applications. When cells in 3D need continuous circulation of nutrients or other compounds, a 3D bioreactor might be the best choice.

To find the best approach to 3D cell culturing for a specific application, it can take some experimentation. In addition, one could benefit from culturing cells in more than one 3D method or using 3D cultured cells in different ways – as shown in Case Study 5. In part, this raises one of the hurdles that some see in 3D culturing, which is a belief that it is more art than science. That idea, however, is not new and not limited to 3D culturing. In fact, some scientists, even experts in cell culturing, fueled this philosophy. Although even a little experience in this field quickly reveals some of the thinking behind that idea, today's tools make 3D cell culturing more scientific than ever.

Despite the scientific advances that simplify 3D culturing, pitfalls remain. For one thing, different methods of culturing can produce different results with the same kinds of cells and seemingly similar conditions. In fact, one shortcoming of 3D culturing is the lack of a standardized system, but 2D culturing is easier to compare because of the relatively homogenous culture

conditions that can be more precisely controlled. Nonetheless, finding a standard form of 3D culturing is a continuous challenge.

A range of other issues must also be considered, such as how to extract cells from various ECM scaffolds for analysis or scaling up a process as needed. One of the key steps is selecting the right ECM. For 3D cell culture, some commercial options made from animals are available. Thermo Fisher Geltrex and Corning Matrigel come from Engelbreth-Holm-Swarm (EHS) mouse tumors. Geltrex can be used with human embryonic stem cells and human iPSCs. Some applications require xeno-free materials, which come from humans. Thermo Fisher CELLstart CTS is a xeno-free substrate that can be used for 3D culturing of human stem cells and for attachment and expansion. When a xeno-free bioscaffold is needed, Thermo Fisher AlgiMatrix is an option. This 3D structure is composed of a chemically defined material.

Whatever the application, a 3D model must be characterized in some way. For some features, microscopy can be used to analyze the health or changes in an aggregate of cells. Often, a more quantitative measurement will be required. In Case Study 5, for instance, the scientists used sequencing to track the mutational profile of a bladder cancer organoid. Similarly, scientists in Case Study 2 used gene expression to compare enteroids. No matter how 3D culturing is used, some method of characterization will be required, and the best one will depend on the cells and the intended use.

In drug discovery and development, scaling challenges arise. Structures like organoids must be scaled up – and done so in a manner that maintains the desired *in vivo* features – to provide enough material for drug toxicity and drug efficacy testing. This work demands the most accurate characterization of a 3D culture; otherwise, the value of a model is suspect for translational research. In fact, the wrong culture could produce exactly the opposite results for how a drug acts in humans.

A fundamental requirement of biomedical results from 3D cell cultures is the correlation to clinical outcomes. The more closely that a 3D sample represents a disease, the more likely it can provide accurate results. A patient-derived organoid (PDO) – an organoid formed from a sample of diseased cells from the patient – provides one approach to building very accurate models of a person's disease. Consequently, PDOs hold great promise as tools to model a patient's response to treatment, making personalised medicine a reality.

WHAT IS NEXT

Despite 60 years of history behind 3D cell culture, the future promises much bigger gains in technology and applications. Instead of letting cells form structures like organoids, some methods, such as 3D bioprinting, create the shape. Like other forms of 3D printing, bioprinting puts the material – cells in this case – in position. Some of these techniques can create spheroids in a few hours, although, as we know, the functioning of biological beings such as spheroids and organoids is a time-bound process. The impact of 3D bioprinting on tissue engineering as well as new drug and therapeutic development remains to be seen.

Cells can also be fashioned into organ-like structures through other methods. Using a microfluidic device – made up of very small channels – cells can be grown into an organ-on-a-chip. This technology combines cells in 3D organizations with mechanical and computational techniques, because the chip includes semiconductor technology.

Cancer research will certainly make significant advances from improvements in 3D culturing, such as creating more accurate tumor microenvironments. A key element to that environment is the level of oxygen around cells and modeling that requires vascularized organoids. Some work along these lines is underway – such as Kieda's work mentioned earlier – and more lies ahead, with the results creating a better cancer model for testing treatments.

To move ahead with 3D cell culture and analysis, education and training will play a crucial role. As part of its educational initiatives, Thermo Fisher Scientific have developed a 3D eLearning module. Tools like this encourage more scientists to adopt 3D techniques, adding new dimensions to their scientific discoveries.

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