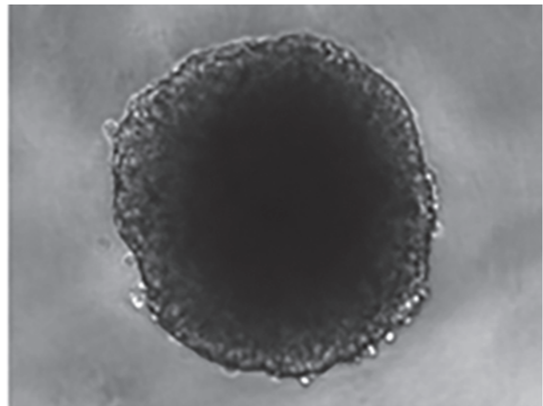
Harnessing new dimensions in your research: coming ’round to spheroid culture

Introduction

Cells cultured in 2D can differ in terms of both physiology and cellular responses compared with cells *in vivo*. These differences have led to a surge in the popularity of using 3D culture techniques. Mounting evidence suggests that culturing cells in 3D is more representative of the *in vivo* environment, creating more physiological cell models, even to the extent that the gene expression profiles of cells from 3D cultures more accurately reflect clinical expression profiles than those observed in 2D cultures [1,2]. Spheroids, or sphere cultures, have become an especially exciting area of 3D *in vitro* culture due to their great potential for use in studies that investigate growth and function of both malignant and normal tissues. These sphere cultures have contributed considerably to our knowledge of cellular responses thanks to the accuracy with which they reflect the *in vivo* system. This is primarily because cells do not normally grow or interact in isolation, but instead form complex interactions with other cells and the surrounding microenvironment. Thus, the creation of a 3D environment that incorporates spheroids more closely mimics *in vivo* conditions, allowing researchers to incorporate cell-cell interactions, nutrient gradients, and diffusion kinetics in their *in vitro* models.



Spheroids offer particular benefits in cancer biology, where they contribute immense value in examining the growth and behavior of tumors since they share several key histomorphological and functional traits that include the formation of cell-cell contacts, decreased proliferation, increased survival rates, and a hypoxic core [3,4]. As more researchers recognize the benefits that spheroid cultures provide as a cell model, development efforts have increased to better aid spheroid generation, culture, and scale-up. Researchers are now moving toward advanced culture methods, employing hypoxic conditions, or co-culturing with different cell types to develop increasingly accurate *in vitro* models of disease and physiology.

Brief history of spheroid development

Researchers have cultured cells in aggregates since the 1950s [5], but it wasn’t until 1971 when the term “spheroid” was coined in work using Chinese hamster V79 lung cells as a model of nodular carcinomas, which happened to form perfect spheres [6]. Robert Sutherland’s early research provided some of the first glimpses into not only the effects of nutrition and oxygenation on cell growth, but also allowed for the determination of the growth fraction following treatment with drugs or radiation.

By the 1980s, Mina Bissell and her team at Lawrence Berkeley National Laboratory began pioneering the use of 3D techniques for more accurate *in vivo* cell models. This shift away from traditional 2D culture systems was first published in a paper highlighting the importance of the extracellular matrix (ECM) along with the crucial role of the microenvironment [7]. These observations were critical for driving the uptake of spheroid culture as a widespread and biologically relevant system with obvious advantages over the widely used monolayer culture methods.

Since then, the field has expanded rapidly to investigate a number of topics from small-scale disease modeling to large-scale, high-throughput screening (HTS) platforms attempting to combat the rising attrition rates seen in existing drug discovery programs.

The ECM: an influential network

Industry has responded to these changes and supported spheroid culture in research through the development of specialized equipment and protocols for culture and maintenance, including plates, synthetic coatings, and cellular scaffolding. There are several common methods used in the generation of spheroids. These include the liquid overlay technique [8], spinner flask [9], gyratory [10], and hanging drop methods [11], or more recently, using suspension culture in individual wells for high-throughput analysis [12]. Following the initial generation of spheroids, the task of maintaining and culturing them can make use of a wide selection of techniques. Depending on the intended application, spheroid culture can involve extracellular matrices or scaffolds, modified surfaces, rotating bioreactors, microcarriers, magnetic levitation, hanging drop plates, or magnetic 3D bioprinting.

Successfully generating and culturing spheroids has a lot to do with the ECM. The ECM is generally composed of soluble proteins and insoluble collagen fibers. While collagen forms the rigid structures that allow tissues to tolerate mechanical stresses like stretching, the proteins within the ECM are involved in a variety of other processes. Proteoglycans, for example, can aid in signaling, binding growth factors, and binding hormones, while multiadhesive matrix proteins like laminin and fibronectin can bind both collagen and other ECM components.

The points at which the ECM makes contact with a cell's plasma membrane are known as focal adhesions. These vary between tissues but generally consist of integrin molecules that associate with both the intracellular and ECM components—making these ECM components functional units of intracellular signaling.

The ECM is also important when it comes to adhesion not only between cells, but also to the culture vessel. When culturing spheroids, the ECM proteins mediating adhesion will automatically adhere to the surface of a culture vessel. This can interfere with complete spheroid formation and may possibly result in the formation of multiple spheroids or satellite colonies. In an attempt to optimize spheroid formation, manufacturers have developed a number of synthetically modified culture vessel surfaces that specifically inhibit the adsorption of ECM proteins from initiating adhesion between the cell and the culture vessel, thereby prompting cell–cell aggregation and spheroid formation *in vitro*.

The Nunclon Sphera surface is superior for culturing cancer spheroids

The Thermo Scientific™ Nunclon™ Sphera™ hydrophilic polymer-coated surface has been shown to minimize surface variability. This polymer coating discourages ECM adsorption to the surface, thereby supporting the formation of consistent spheroids (Figure 1).

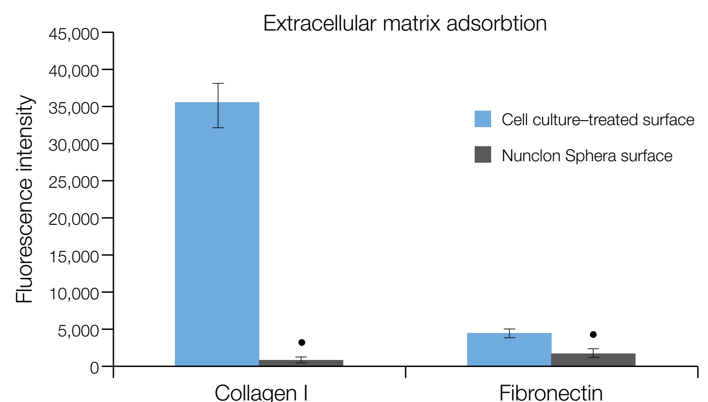


Figure 1. Extracellular matrix adsorption. The adsorption of collagen I and fibronectin to the Nunclon Sphera surface is extremely low compared to the standard cell culture–treated surface. Student's *t*-test, $P < 0.01$.

By combining a hydrophilic polymer coating with U-bottom–shaped wells, it is possible to culture spheroids without the production of satellite colonies. HCT 116 human colon carcinoma cells were seeded into Nunclon Sphera 96-well U-bottom plates in complete DMEM. Similarly, cells were seeded into 96-well U-bottom nontreated plates in complete DMEM containing 3% methylcellulose. Using different seeding densities of HCT 116 human colon carcinoma cells, it was shown that single spheroids with well-defined edges can be consistently generated in each individual well (Figure 2).

To demonstrate spheroid growth, A549 human adenocarcinoma cells and HCT 116 human colon carcinoma cells were cultured at different densities in Nunclon Sphera plates for 2 weeks. Both cell types displayed adequate spheroid growth as demonstrated by size measurements (Figure 3A). Additionally, the cell health of A549 and HCT 116 spheroids were assessed by Invitrogen™ PrestoBlue™ cell viability assay (Figure 3B). Data was normalized against spheroid size for better quantitative comparison—a higher ratio indicates healthier spheroids. Cell viability of cancer spheroids was further confirmed by Invitrogen™ LIVE/DEAD™ fluorescence staining assay (Figure 3C). All parameters indicated that cancer spheroids grown on Nunclon Sphera plates were healthy and robust, and that the Nunclon Sphera 96-well U-bottom plate is a reliable and convenient tool for both routine and high-throughput cancer spheroid applications.

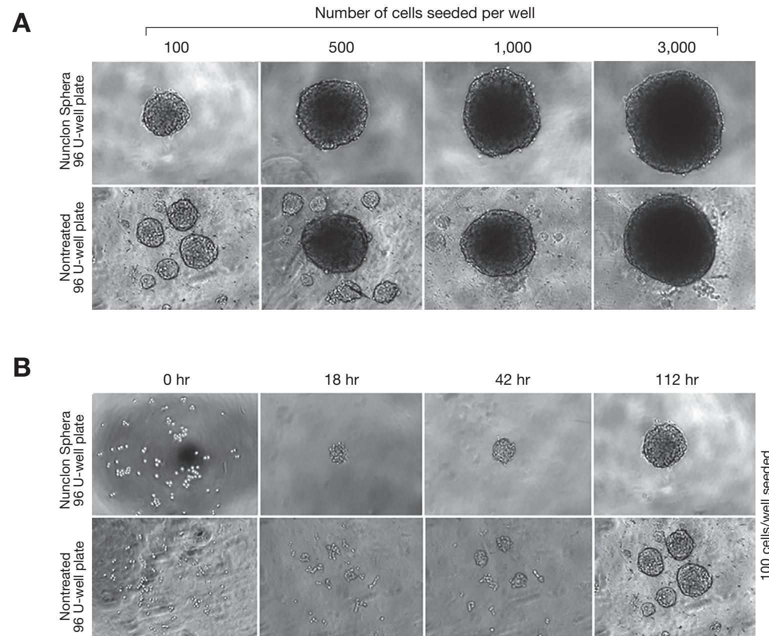


Figure 2. Advantages of Nunclon Sphera plates over nontreated plates and methylcellulose-containing medium. (A) High and consistent quality of cancer spheroids grown in the Nunclon Sphera plate. **(B)** Early formation of single cancer spheroids in the Nunclon Sphera 96-well U-bottom plate. (Courtesy of Professor Dolznig from the Institute of Medical Genetics at the Medical University of Vienna.)

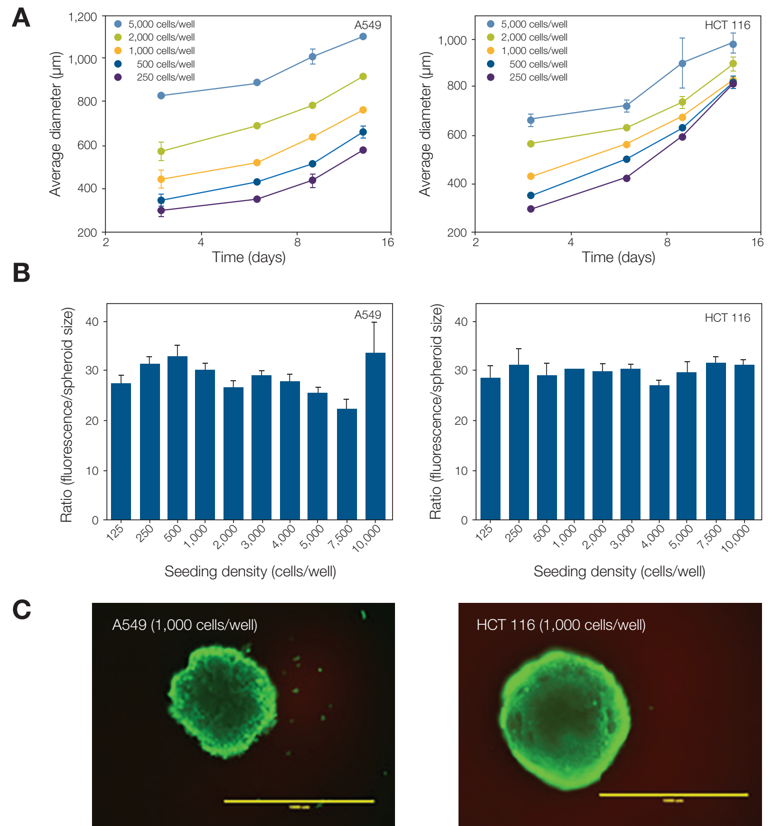


Figure 3. Assessments of spheroid growth, cell health, and viability on Nunclon Sphera plates. (A) Growth kinetics of A549 and HCT 116 cancer spheroids on Nunclon Sphera plates were evaluated over period of 13 days. Data represents the mean ± SD of 3 replicates for each cell number. **(B)** Spheroid cell health assessments on Nunclon Sphera plates were performed using the PrestoBlue cell viability assay with data normalized by spheroid size. **(C)** Spheroid cell viability was evaluated by LIVE/DEAD staining assay, where live cells are stained green and dead cells are stained red. Scale bar = 1,000 µm.

The hypoxic culture condition

In addition to specialized culture vessels, culturing spheroids requires precisely controlled abiotic conditions such as temperature, humidity, and pH. Gas condition is another vital requirement of cell culture, and typically this has meant mimicking atmospheric oxygen tension supplemented with 5–10% carbon dioxide. Yet, while atmospheric levels of oxygen are approximately 20%, the levels within the human body range from 12% to as low as 1%. In light of this, some researchers have taken to culturing their cells under hypoxic conditions.

The role of oxygen was seen as early as 1972 when Alan Richter and colleagues improved plating efficiency of mouse and rat embryonic tissues by cultivating in 1–3% oxygen [13]. The 21st century is seeing cell culture truly coming of age, taking positions in everything from routine cell culture to cell therapy and the development of personalized medicines. These applications have rekindled an interest in the levels of oxygen used in cell culture, and over the past decade or so, the hypoxic element came to the forefront of spheroid culture.

Cells cultured under hypoxic conditions grow faster, live longer, and show lower stress. A cell culture incubator that controls nitrogen gas, in addition to carbon dioxide, is the best way to achieve hypoxic conditions. So-called tri-gas incubators, such as the Thermo Scientific™ Heracell™ VIOS Incubator, optimize low-oxygen cultures to offer optimal growth and culture stability. However, the term "tri-gas" is a misnomer as only carbon dioxide and nitrogen are supplied, thereby reducing the internal oxygen levels to as low as 1%.

Detecting hypoxic conditions in real time is often carried out using a chemical that generates a fluorescent signal under specific conditions. A specialized hypoxia probe, in the form of a fluorogenic compound that is live-cell permeable and begins to fluoresce when oxygen levels fall below 5%, provides robust and reproducible measurements of hypoxia in cells (Figure 4). This reagent is preferable to using pimonidazole adducts that only respond to very low levels of oxygen (at a partial pressure of ≤10 mHg), below levels at which hypoxia may occur, potentially yielding false negative results. The Invitrogen™ Image-iT™ Hypoxia Reagent has a greater range of sensitivity and responds quickly to changing levels of oxygen, making it ideal for detecting hypoxic conditions in 3D cultures, spheroids, or neurons, for example [14,15].

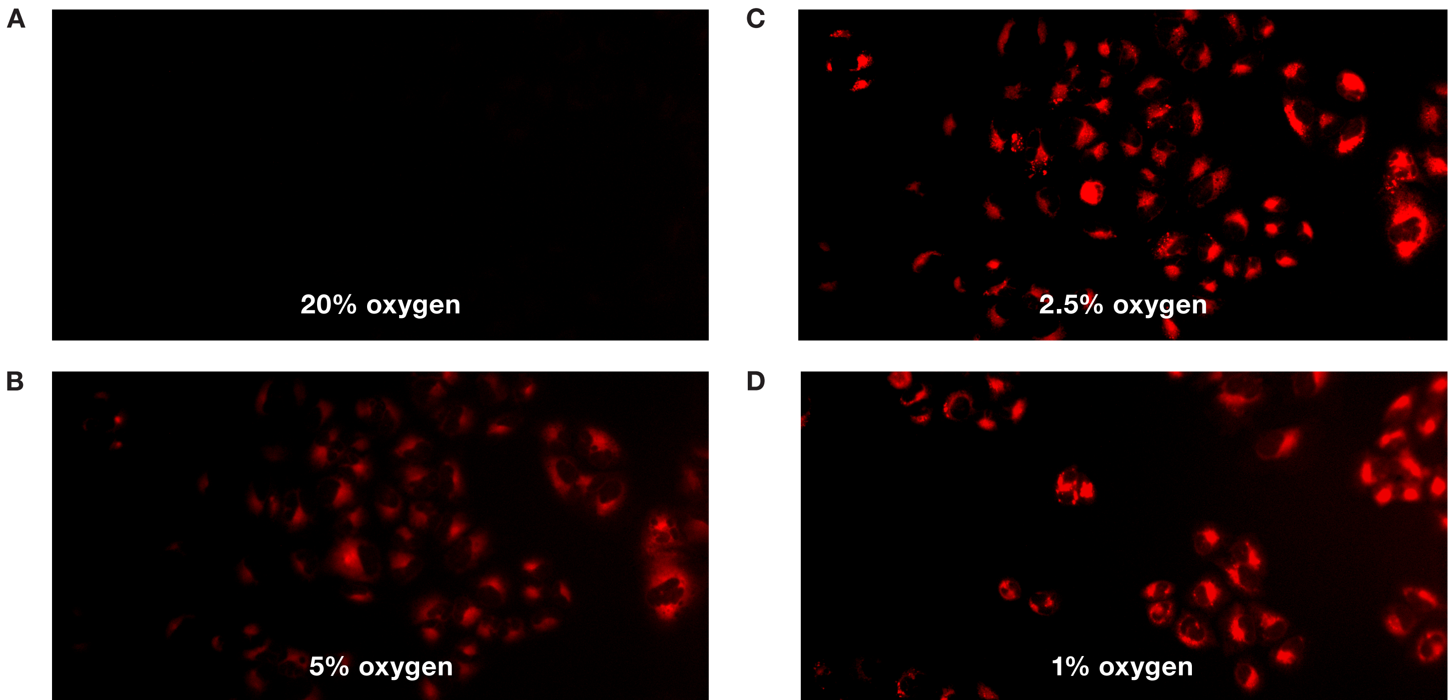


Figure 4. Detection of hypoxic conditions. A549 cells were grown on Thermo Scientific™ Nunc™ 35 mm glass-bottom dishes in complete medium at a density of 10^5 cells/dish. The cells were incubated in Gibco™ FluoroBrite™ DMEM with 5 µM Image-iT Hypoxia Reagent (red) at **(A)** 20%, **(B)** 5%, **(C)** 2.5%, and **(D)** 1% oxygen for one hour on an Invitrogen™ EVOS™ Onstage Incubator attached to an EVOS™ FL Auto Imaging System. The images were taken after one hour of incubation at each oxygen level. The hypoxia signal can be detected at oxygen levels as low as 5%, with increasing signal intensities at 2.5% and 1%.

Spheroids in cancer biology

Spheroid culture methods have made substantial contributions to the advances being made in our basic understanding of cell biology, as well as providing insights into cancer biology. The multicellular tumor spheroid (MCTS) model, using spheroids between 200–500 µm, has lent itself to cancer biology as it more accurately mimics the physiology of tumors, as mentioned earlier. Spheroids in this model develop chemical gradients of oxygen, nutrients, and catabolites just like a tumor *in vivo*, as well as possess similar histomorphological and functional features [16]. Internally, spheroids possess the same hypoxic core seen in solid tumors (Figure 5) where cells rapidly outgrow the blood supply, leaving the center of the tumor with an extremely low oxygen concentration. Chronically hypoxic regions of tumors are highly resistant to therapy as they are especially difficult to penetrate with chemotherapy [17].

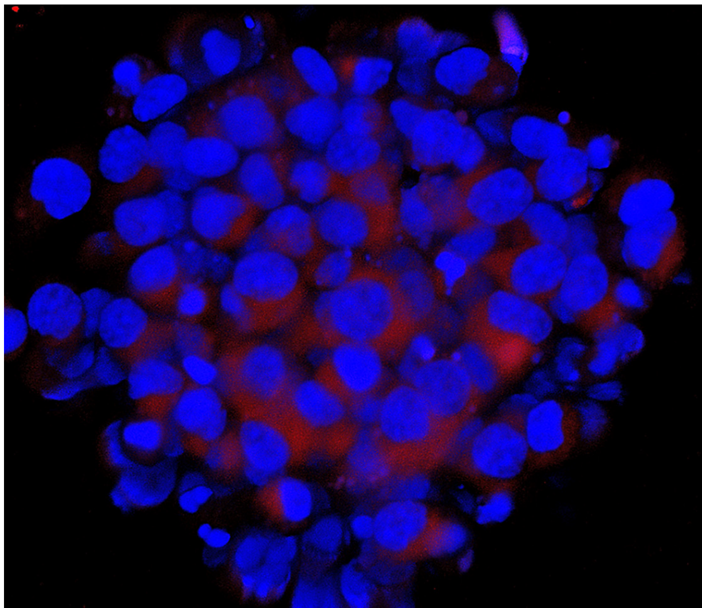


Figure 5. A single HeLa spheroid used in the assessment of hypoxic cores. HeLa cells were plated at a density of 1,000 cells/well. After two days of culture on Nunclon Sphera 96-well U-bottom plates, HeLa spheroids were stained with Image-iT Hypoxia Reagent (red) and Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (blue). Images were taken on a confocal microscope.

This gradient of oxygen in spheroids, progressing from normoxic cells at the periphery to hypoxic cells at the core, provides an excellent model for assessing novel pharmacological agents and drug delivery methods. MCTS models can be used to validate compounds that are activated under hypoxic conditions, thereby targeting the hypoxic core specifically, as well as evaluating drugs and signaling pathways [18,19].

While the ability of cancer spheroids to replicate key elements of tumors, such as hypoxia, necrosis, angiogenesis, and cell adhesion [20] is intriguing, 3D cell cultures have also been used for studies of viability, clonogenicity, LD_{50}, and metastatic potential under a broad spectrum of conditions. The versatility afforded by the spheroid system has been a game-changer in how we understand and develop treatments for cancer.

Conclusions

The spheroid system of cell culture has major implications not only for our fundamental understanding of how the interplay between cells, tissues, and the ECM affects pathological states such as cancer, but also for the development of more robust drug screening programs and improved organotypic models.

- The Nunclon Sphera surface demonstrates extremely low ECM binding properties; it therefore effectively discourages cell attachment and promotes spheroid formation
- Nunclon Sphera 96-well U-bottom plates support consistent formation and growth of cancer spheroids across commonly used cancer cell lines
- The evidence for hypoxic cores in cancer spheroids indicates that 3D cancer spheroid culture on Nunclon Sphera plates presents an ideal *in vitro* system for modeling tumor growth

Methods: cancer spheroid culture

Cancer cell lines were maintained in Thermo Scientific™ Nunc™ Cell Culture Treated EasYFlasks™ before they were subjected to spheroid culture. To form cancer spheroids, cells were seeded in Nunclon Sphera 96-well U-bottom plates at densities of 100–5,000 cells/well in 200 µL/well of Gibco™ DMEM with GlutaMAX™ Supplement and 10% FBS, 1X MEM Non-Essential Amino Acids, 100 U/mL Penicillin-Streptomycin, and 25 mM HEPES. Nontreated plates were similarly seeded in the complete DMEM medium containing 3% methylcellulose. The plates were briefly centrifuged at 250 x g for 5 minutes. The cells were then incubated at 37°C and 5% CO_2, and refed every 72 hr by carefully removing 100 µL of medium from each well and replenishing with 100 µL of fresh growth medium using a multichannel pipette. The formation and growth of spheroids were examined using an Invitrogen™ EVOS™ imaging system.

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