ThermoFisher SCIENTIFIC

NUNCLON SPHERA 3D CULTURE SYSTEM APPLICATION NOTES

A collection of application notes for 3D cell culture supported by the Nunclon Sphera low-attachment surface

Make the connection

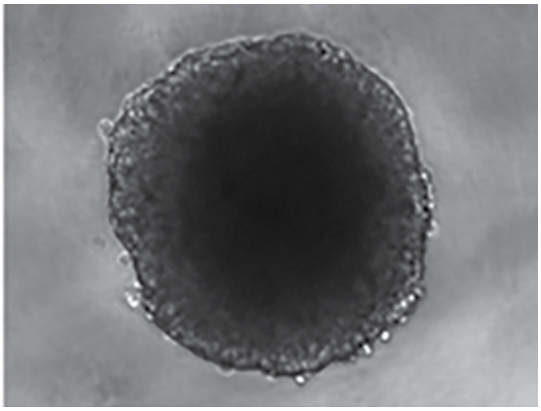
Contents

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).

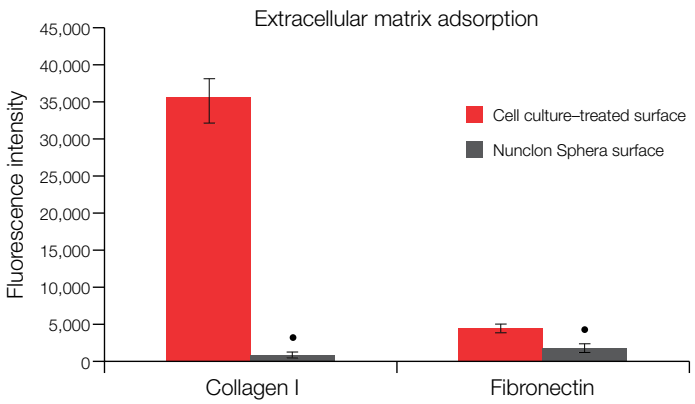


Figure 1. Extracellular matrix adsorption. The adsorption of collagen I and fibronectin to the Nunclon Sphera surface is extremely low compared to the standard cell culture–treated surface. Student's *t*-test, $P < 0.01$.

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.

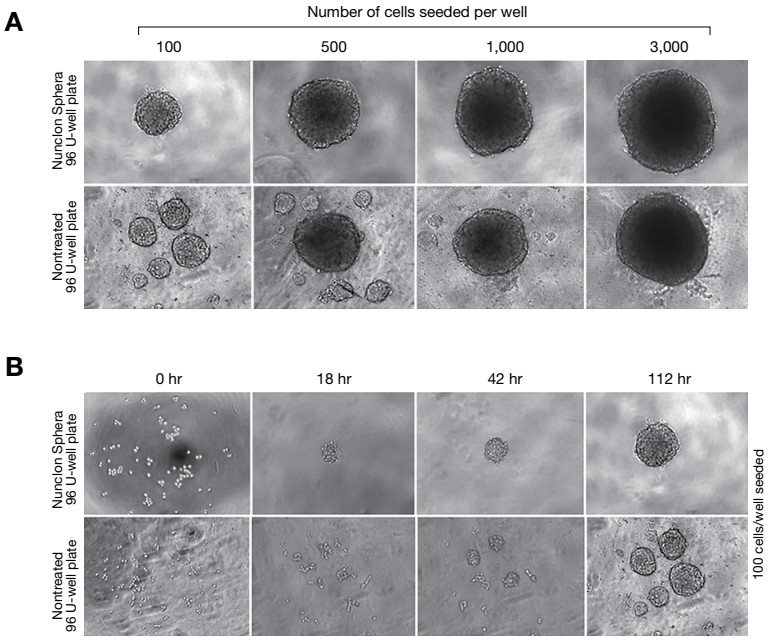


Figure 2. Advantages of Nunclon Sphera plates over nontreated plates and methylcellulose-containing medium. (A) High and consistent quality of cancer spheroids grown in the Nunclon Sphera plate. **(B)** Early formation of single cancer spheroids in the Nunclon Sphera 96-well U-bottom plate. (Courtesy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

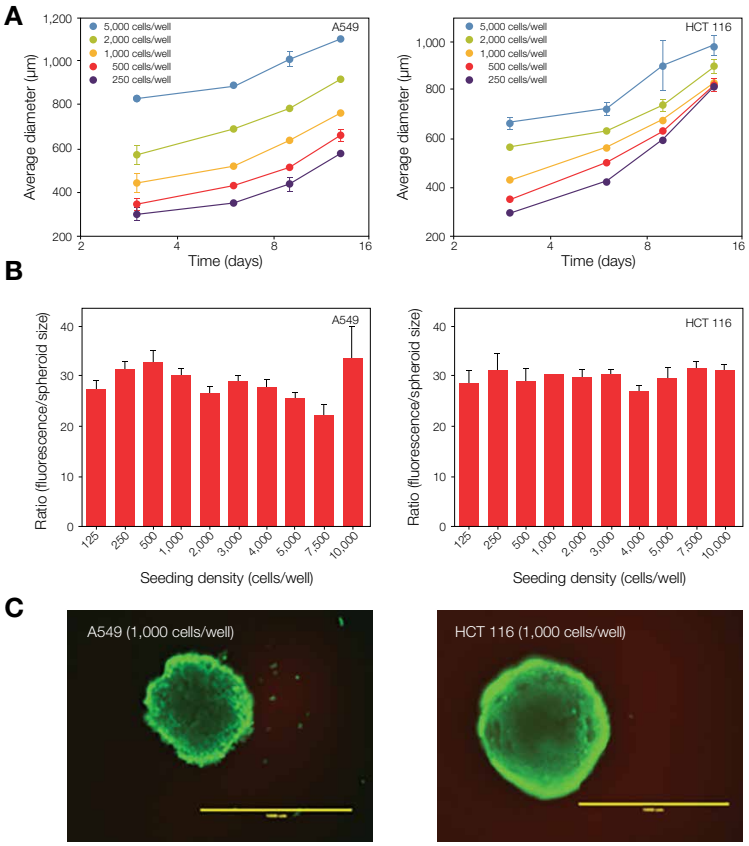


Figure 3. Assessments of spheroid growth, cell health, and viability on Nunclon Sphera plates. (A) Growth kinetics of A549 and HCT 116 cancer spheroids on Nunclon Sphera plates were evaluated over period of 13 days. Data represents the mean ± SD of 3 replicates for each cell number. **(B)** Spheroid 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].

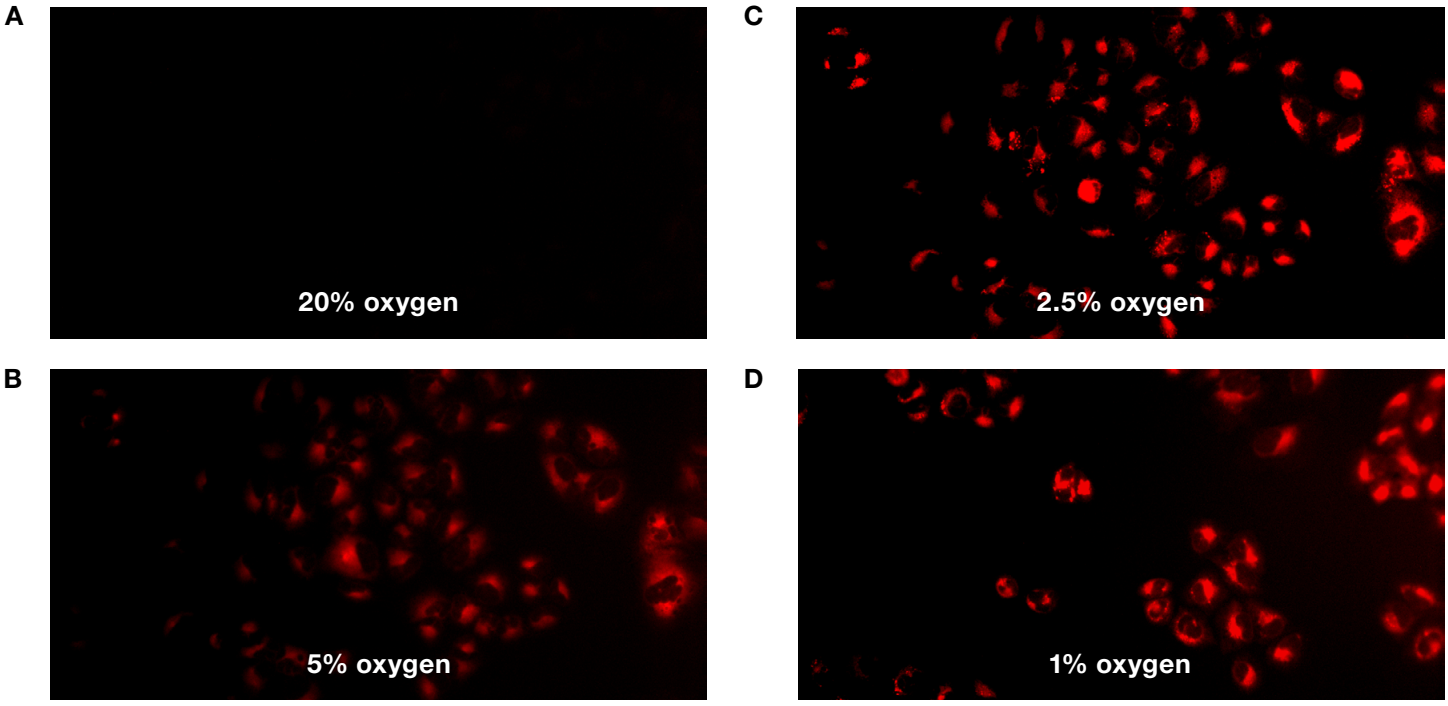


Figure 4. Detection of hypoxic conditions. A549 cells were grown on Thermo Scientific™ Nunc™ 35 mm glass-bottom dishes in complete medium at a density of 10^5 cells/dish. The cells were incubated in Gibco™ FluoroBrite™ DMEM with 5 µM Image-iT Hypoxia Reagent (red) at **(A)** 20%, **(B)** 5%, **(C)** 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].

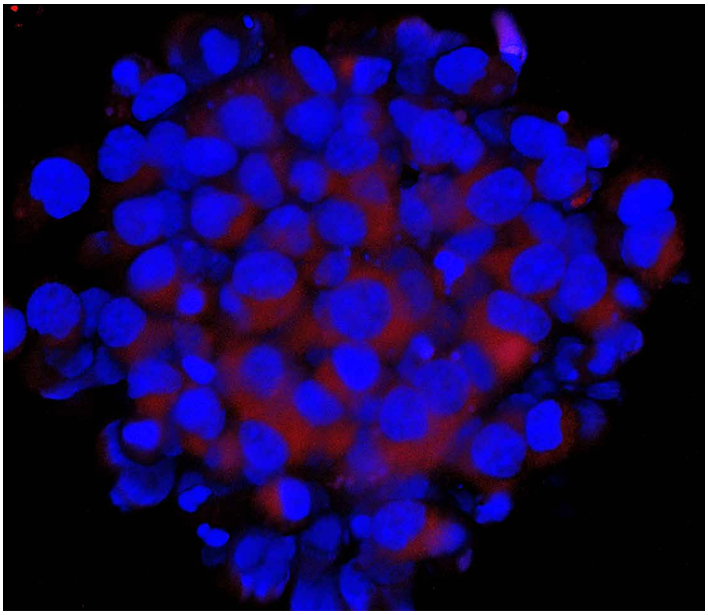


Figure 5. A single HeLa spheroid used in the assessment of hypoxic cores. HeLa cells were plated at a density of 1,000 cells/well. After two days of culture on Nunclon Sphera 96-well U-bottom plates, HeLa spheroids were stained with Image-iT Hypoxia Reagent (red) and Invitrogen™ NucBlue™ Live ReadyProbes™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

References

1. Fennema E, Rivron N, Rouwkema J et al. (2013) Spheroid culture as a tool for creating 3D complex tissues. *Trends Biotechnol* 31(2):108–115.
2. Hirschhaeuser F, Menne H, Dittfeld C et al. (2010) Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 148(1):3–15.
3. Heylman C, Sobrino A, Shirure VS et al. (2014) A strategy for integrating essential three-dimensional microphysiological systems of human organs for realistic anticancer drug screening. *Exp Biol Med* 239(9):1240–1254.
4. Tanner K, Gottesman MM (2015) Beyond 3D culture models of cancer. *Sci Transl Med* 7(283):283ps9.
5. Moscona A (1957) The development *in vitro* of chimeric aggregates of dissociated embryonic chick and mouse cells. *Proc Natl Acad Sci USA* 43(1):184–194.
6. Sutherland RM, McCredie JA, Inch WR (1971) Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J Natl Cancer Inst* 46(1):113–120.
7. Bissell MJ, Hall HG, Parry G (1982) How does the extracellular matrix direct gene expression? *J Theor Biol* 99(1):31–68.
8. Carlsson J, Yuhas JM (1984) Liquid-overlay culture of cellular spheroids. *Recent Results Cancer Res* 95:1–23.
9. Sutherland RM, Durand RE (1984) Growth and cellular characteristics of multicell spheroids. *Recent Results Cancer Res* 95:24–49.
10. Moscona A (1961) Rotation-mediated histogenetic aggregation of dissociated cells: a quantifiable approach to cell interactions *in vitro*. *Exp Cell Res* 22:455–475.
11. Kelm JM, Timmins NE, Brown CJ et al. (2003) Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng* 83(2):173–180.
12. Ivascu A, Kubbies M (2006) Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. *J Biomol Screen* 11(8):922–932.
13. Richter A, Sanford KK, Evans VJ (1972) Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells. *J Natl Cancer Inst* 49(6):1705–1712.
14. Mandavilli BS, Chen A, Robinson V et al. (2015) Intracellular detection of hypoxia in live cells. Presentation at American Association for Cancer Research (AACR), Poster 3007.
15. Zhang S, Hosaka M, Yoshihara T et al. (2010) Phosphorescent light-emitting iridium complexes serve as a hypoxia-sensing probe for tumor imaging in living animals. *Cancer Res* 70(11):4490–4498.
16. Thoma CR, Zimmerman M, Agarkova I et al. (2014) 3D cell culture systems modeling tumor growth determinants in cancer target discovery. *Adv Drug Del Rev* 69–70:29–41.
17. Das V, Bruzzese F, Konecny P et al. (2015) Pathophysiologically relevant *in vitro* tumor models for drug screening. *Drug Disc Today* 20(7):848–855.
18. Dufau I, Frongia C, Sicard F et al. (2012) Multicellular tumor spheroid model to evaluate spatio-temporal dynamics effect of chemotherapeutics: application to the gemcitabine/CHK1 inhibitor combination in pancreatic cancer. *BMC Cancer* 12:15.
19. Mead S, Foster R (2015) A multicellular tumor model: Applications for evaluating drugs and signaling pathways in a 3D system. Application note available from http://www.horizondiscovery.com/
20. Benien P, Swami A (2014) 3D tumor models: history, advances and future perspectives. *Future Oncol* 10(7):1311–1327.

Ordering information

Product	Cat. No.
DMEM with GlutaMAX Supplement	10569010
MEM Non-Essential Amino Acids Solution	11140050
FluoroBrite DMEM	A1896701
Penicillin-Streptomycin	15070063
HEPES	15630080
Fetal Bovine Serum, dialyzed	26400036
TrypLE Express Enzyme	12605010
Nunc EasYFlask Cell Culture Flasks	156499
Nunclon Sphera Microplates	174925
Nunc Glass Bottom Dishes	150680
PrestoBlue Cell Viability Reagent	A13261
LIVE/DEAD Viability/Cytotoxicity Kit	L3224
Image-iT Hypoxia Reagent	H10498
NucBlue Live ReadyProbes Reagent	R37605
EVOS FL Auto Imaging System	AMAFD1000
EVOS Onstage Incubator	AMC1000

Generation of cancer spheroids—tips and tricks

Introduction

Tumor cells grown as spheroids offer an intermediate complexity between cancer cells grown in 2D monolayers and *in vivo* tumors. This potentiates their use as model systems to study tumor progression as well as to perform high-throughput screening of cytotoxic therapies, including chemotherapies and cell-based treatments.

Cancer spheroids are formed when cells are allowed to grow in suspension, as a result of which they aggregate, either on their own or with the aid of extracellular matrices. There are two factors critical in limiting variation in high-throughput assays with cancer spheroids. First, it is essential to have one spheroid per well in a multiwell plate, to reduce variability in readouts. Second, it is important that spheroids be of uniform shape and size—otherwise there can be variability between experiments. In our lab, we have tested spheroid generation conditions in nine human cell lines belonging to six cancer types. To summarize the results, we have compiled a general workflow and a few tips and tricks that would help in high-throughput generation of uniform and reproducible spheroids in Thermo Scientific™ Nunclon™ Sphera™ multiwell plates. The tips are specific to the cell type tested but can also be referred to for troubleshooting spheroid generation in other cell types.

General workflow

1. On the day of experiment, dissociate cells using **Gibco™ TrypLE™ Express Enzyme** and then neutralize the enzyme using 4 volumes of complete medium (medium will vary depending on cell line chosen).
2. Count cells using the **Invitrogen™ Countess™ II FL Automated Cell Counter**. Cell viability should be >90%.
3. Dilute the suspension at a ratio of 1:10–1:20 in complete medium or a medium containing required additives. Seed the required number of cells in respective wells of **Nunclon Sphera 96-well plates** using **Thermo Scientific™ Finnpipette™ F2 Multichannel Pipettes**.
4. Centrifuge plates at the required speed (250–450 x *g*) for 5–10 min at room temperature or 4°C, based on the use of additive (e.g., for **Gibco™ Geltrex™ matrix** addition, 4°C is necessary, and for collagen I, a temperature below 18°C is required).
5. Change the medium as necessary until spheroids are ready. Add the medium slowly along the side of the wells without touching the spheroids.

Considerations for growing cancer spheroids

Spheroid size

Depending on the cell line, spheroids differ in compactness. Figure 1 shows cancer spheroids generated from 5,000 cells of four different cell lines. As is evident, the seeding cell number does not correlate to spheroid size. Thus, to obtain spheroids of a specific diameter for use in a particular downstream assay, the seeding cell density for the respective cell line needs to be standardized. All brightfield images were captured using the Invitrogen™ EVOS™ M7000 Imaging System with a 4x objective unless stated otherwise.

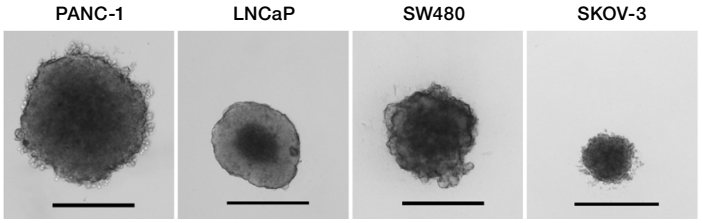


Figure 1. Spheroids generated from cancer cell lines on a Nunclon Sphera plate. PANC-1: pancreatic cancer; LNCaP: prostate cancer; SW480: colorectal cancer; SKOV-3: ovarian cancer. A total of 5,000 cells were seeded in each case. Scale bar: 500 µm.

Time

Cells have been shown to proliferate more slowly in 3D culture than in 2D culture [1]. Based on our observations, depending on the doubling time of the cells, some cancer spheroids are ready within 24 hr (for example, A549 and SKOV-3), while some might require 4–9 days (PC-3 and T47D). An ideal spheroid is translucent with a defined boundary and minimal dark core. However, certain cells, especially those that require an extracellular matrix for spheroid formation (see next section, "Extracellular matrices"), do not exhibit the ideal morphology. Figure 2 shows the morphological changes of T47D and SKOV-3 spheroids over time in culture. T47D spheroids grew in size and their cores became progressively darker over time; spheroids were ready by day 5. In contrast, SKOV-3 spheroids were ready on day 1; with increasing time in culture, the compactness increased (Figure 2B), and the cells seemed to be diverging from the spheroid.

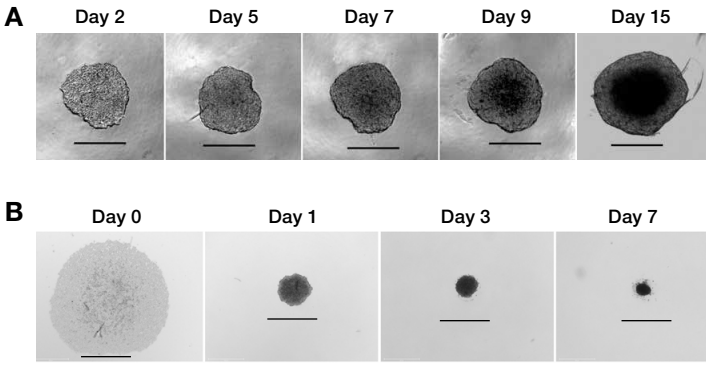


Figure 2. Morphological change in different spheroid types. **(A)** 4,000 T47D cells and **(B)** 10,000 SKOV-3 cells were seeded on Nunclon Sphera 96-well plates and observed on the indicated days. Scale bar: 500 µm.

Extracellular matrices

Some cell lines form spheroids on their own, while others form loose or tight cellular aggregates. Finicky cell lines require the assistance of various extracellular matrices (ECMs) to form spheroids. For example, PC-3 cells require Geltrex matrix (Figure 3). In order to optimize conditions for spheroid formation by MDA-MB-231 cells, various ECM components were tested in Nunclon Sphera 96-well plates with 1 x 10^4 cells seeded per well. The day after plating, complete medium containing various ECMs was added to the spent medium, and cells were observed on day 5. As depicted in Figure 4, we found that collagen I worked best in this case to form a spheroid with a defined boundary. In all other cases, the cells formed aggregates.

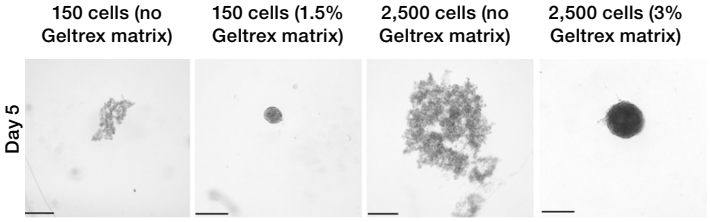


Figure 3. PC-3 cells seeded for spheroid formation with and without Geltrex matrix. Scale bar: 650 µm.

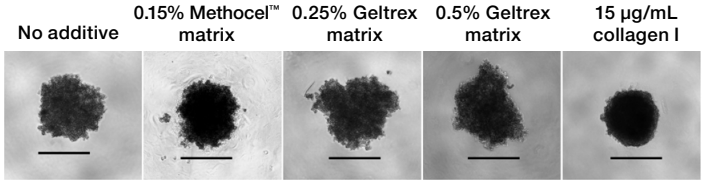


Figure 4. Effect of ECMs on spheroid growth. 10,000 MDA-MB-231 cells were seeded in medium supplemented with different ECMs. Scale bar: 500 µm.

We further standardized the collagen I concentration required for spheroid formation. Subsequent testing indicated that a final concentration of 3 µg/mL collagen I worked best, with higher concentrations leading to a disrupted spheroid morphology with cells diverging from the spheroid (Figure 5).

To verify spheroid formation, we stained the cellular entities with Invitrogen™ DiI, a lipophilic membrane stain. All cells in the aggregate were easily accessible to the dye, whereas the compactness of the spheroid prevented the dye from entering the core (Figure 6).

While a single concentration of ECM worked for MDA-MB-231 cells, some cell lines such as SW480 required different ECM concentrations based on the seeding density. As seen in Figure 7, a concentration of 3 µg/mL of collagen I worked for 625–2,500 cells only. However, increasing the collagen I concentration for higher cell densities formed better spheroids than those formed using a single lower concentration of collagen I for those cell densities.

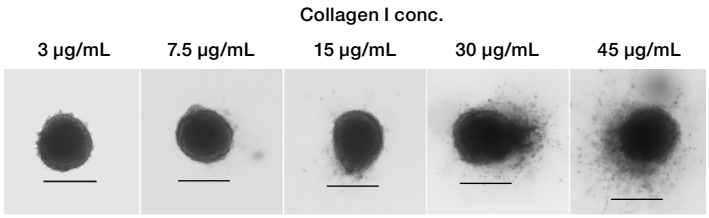


Figure 5. Standardizing collagen I concentration. 10,000 MDA-MB-231 cells were seeded in medium supplemented with different concentrations of collagen I. Scale bar: 500 µm.

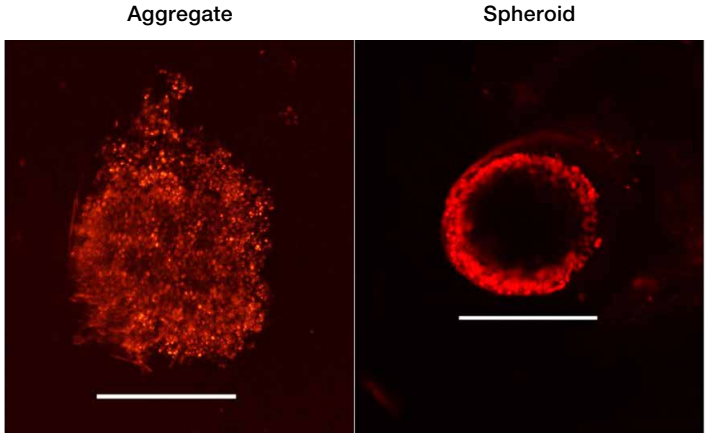


Figure 6. Verification of spheroid formation. 5,000 MDA-MB-231 cells in medium with or without collagen I were seeded onto Nunclon Sphera plates and stained with DiI 4 days later. Images were captured using the Thermo Scientific™ CellInsight™ CX7 High Content Analysis Platform with a 4x objective. Scale bar: 400 µm.

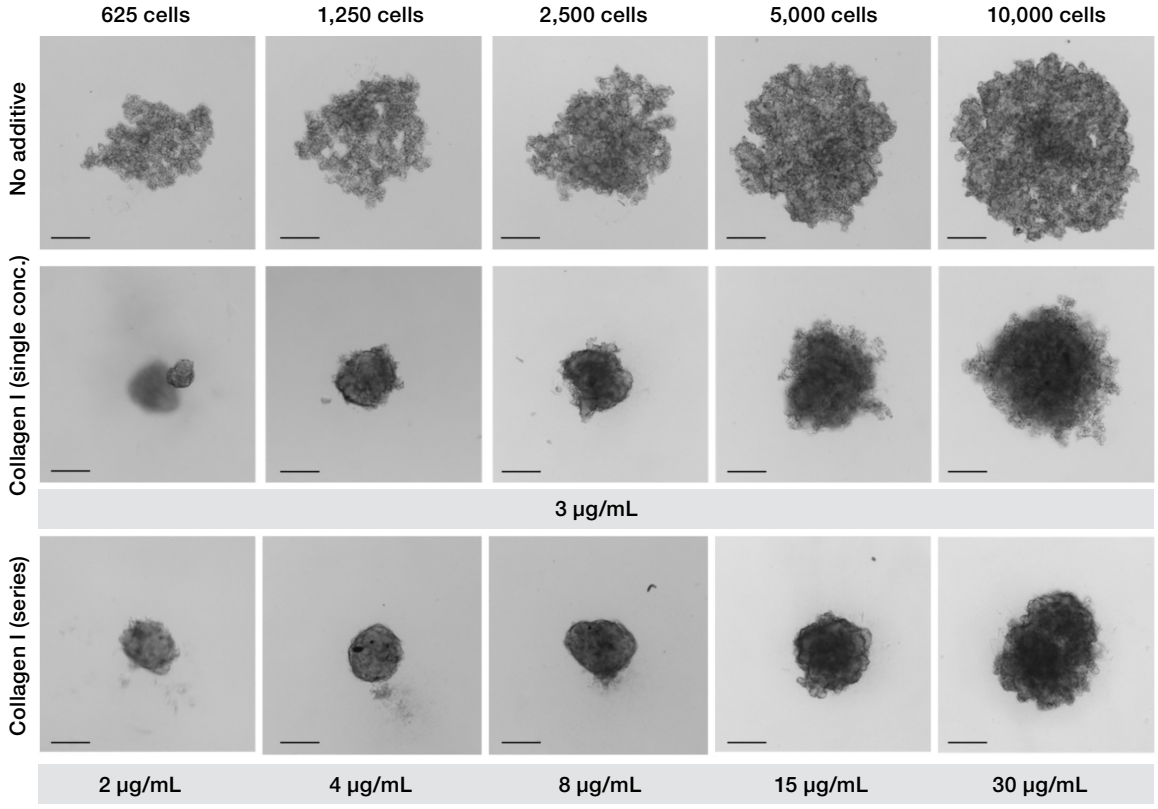


Figure 7. Higher seeding densities may require more concentrated ECM. SW480 cells were seeded in medium supplemented with either a single concentration or a concentration series of collagen I with increasing cell seeding density. Scale bar: 650 µm.

Plastic surface

The primary requirement for spheroid formation is a non-adherent surface. The Nunclon Sphera plates help to repel the cells from settling at the bottom and facilitate uniform spheroid formation upon centrifugation. We compared this surface to Corning™ ULA plates for spheroid formation. In our observations, out of nine cell lines tested, approximately 50% of cell lines formed satellite colonies around the spheroids on the Corning ULA surface. This was more evident at higher cell densities, as in the case of HepG2 (Figure 8A, lower panel). On the other hand, Nunclon Sphera plates helped in consistent formation of a single spheroid per well for all cell lines tested.

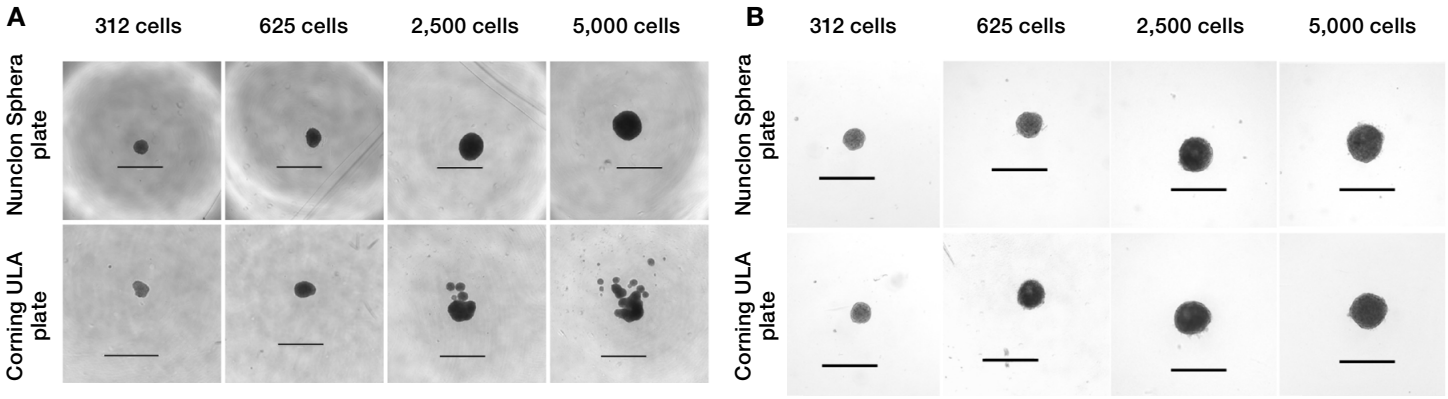


Figure 8. Effect of surface on spheroid formation in different cell lines. (A) HepG2 cells were seeded for spheroid formation on a Nunclon Sphera plate (top panel) and a Corning ULA plate (bottom panel). **(B)** PC-3 cells were seeded in medium supplemented with Geltrex matrix on a Nunclon Sphera plate (top panel) and a Corning ULA plate (bottom panel) for spheroid formation. Scale bar: 1,000 µm.

Conclusion

By using the right plastic surface, medium, and extracellular matrix, and following the tips and tricks, uniform and reproducible cancer spheroids can be generated easily. In our observations, cells that have a circular morphology and cells that grow in clusters can form spheroids on their own. However, cells that don't grow in clusters require ECM support. Cells with elongated morphology vary in their requirement for ECM, so spheroid-generating conditions need to be optimized for each cell line. Our portfolio supports robust generation, characterization, and high-throughput applications and analyses of 3D cancer spheroids.

Ordering information

Product	Cat. No.
Plastics	
Nunc EasYFlask Cell Culture Flasks	156499
Nunclon Sphera 96-well plate	174925
Matrix Reagent Reservoirs	8094
Nunc 15 mL and 50 mL Conical Sterile Polypropylene Centrifuge Tubes	339650, 339652
Instruments	
EVOS M7000 Imaging System	AMF7000
CellInsight CX7 High Content Analysis Platform	CX7A1110
Countess II Automated Cell Counter	AMQAX1000
Finnpipette F2 Multichannel Pipettes	4662030, 4662060
Reagents	
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413301
Collagen I, Rat Tail	A1048301
DiI Stain	D3911

Reference

1. Anna C et al. (2013) Impact of the 3D microenvironment on phenotype, gene expression, and EGFR inhibition of colorectal cancer cell lines. *PLoS One* 8(3):e59689.

Generation of MCF7 spheroids in serum-free conditions

Introduction

It is widely accepted that spheroid cultures recapitulate tumor microenvironments far better than traditional monolayer cultures do [1-3]. As opposed to cells in monolayers, cells in spheroids have more physiological cell–cell and cell–matrix interactions. In addition, larger spheroids (200–500 µm in diameter) have nutritional gradients that are common in solid tumors, with a pathophysiological necrotic core forming in spheroids larger than 500 µm [1]. These attributes are important features of spheroids, as cell–cell, cell–matrix, and chemical gradients have all been implicated in driving both tumor progression and therapy resistance [1-3]. Therefore, spheroids are regarded as an important preclinical tool for assessing drug efficacy and have provided key insights into the physiology of tumor progression [1-3].

For a variety of applications, it may be better to grow cells in a phenol red–free and serum-free environment. This is especially relevant for MCF7 cells, given that they are estrogen receptor (ER)-positive and have an increase in ER signaling in response to phenol red, an estrogen mimic, and fetal bovine serum (FBS), which contains estrogen [4]. In addition to reducing the exogenous estrogen, the absence of FBS also allows for studying the effect of specific growth factors on MCF7 cells; FBS contains many types and variable amounts of growth factors which, if present, may confound the effects of specific growth factors on MCF7 cells. For these reasons, we developed a serum-free growth medium for the generation and maintenance of MCF7 spheroids. We utilized this medium to examine the impact of heat-stable basic fibroblast growth factor (HS bFGF) signaling on spheroid growth dynamics. Since native bFGF (also known as FGF2) is not stable at 37°C, media requiring bFGF supplementation are typically changed every 2–3 days. Changing medium is a well-known obstacle for spheroid culture, as spheroids may be aspirated in the process. HS bFGF offers a solution to this problem as it maintains >80% bioactivity even after 72 hr at 37°C [5].

In this application note, the materials and methods we used for the formation of spheroids as well as the spheroid growth that resulted are discussed. A step-by-step protocol can be found in Appendix A. Tips and considerations for spheroid generation, including a list of other media systems in which we have previously generated MCF7 spheroids, can be found in Appendix B.

Suggested workflow

Prior to the experiment, stocks of MCF7 cells were grown in Thermo Scientific™ Nunc™ EasYFlask™ Cell Culture Flasks in Gibco™ DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), supplemented with 10% FBS (Cat. No. 16000069), referenced below as standard culture medium.

For each experiment, two types of media were used:

- Standard culture medium: DMEM/F-12, HEPES, no phenol red, supplemented with 10% FBS
- Experimental medium: DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), with 1X Gibco™ B-27™ Plus Supplement (Cat. No. A3582801), which was supplemented with bFGF based on the desired experimental condition. Our experimental conditions were differentiated by:
  - Gibco™ HS Recombinant Human bFGF (Cat. No. PHG0360)
  - Gibco™ FGF-Basic (AA 1-155) Recombinant Human Protein (native) (Cat. No. PHG0266)
  - No bFGF (negative control)

On the day of the experiment (Figure 1) the medium was aspirated, and MCF7 cells were washed with Gibco™ DPBS (no calcium, no magnesium) (Cat. No. 14190250), then dissociated using Gibco™ TrypLE™ Express Enzyme (Cat. No. 12604021; alternatively, Cat. No. 12563029). After the cells had lifted, the standard culture medium was added to the flask and cells were counted.

Spheroids were generated using a low-adhesion U-bottom microplate, the Thermo Scientific™ Nunclon™ Sphera™ 96U-well microplate (Cat. No. 174929). Prior to cell seeding, the microplate was prepared by:

1. Adding 0.1 mL of experimental medium (containing either 20 ng/mL HS bFGF, 20 ng/mL native bFGF, or no bFGF) to all wells that were to be seeded with cells. We prepared (and later seeded) the inner 36 wells of the plate.
2. Adding 0.2 mL DPBS (no calcium, no magnesium) to all wells where there were to be no cells seeded.

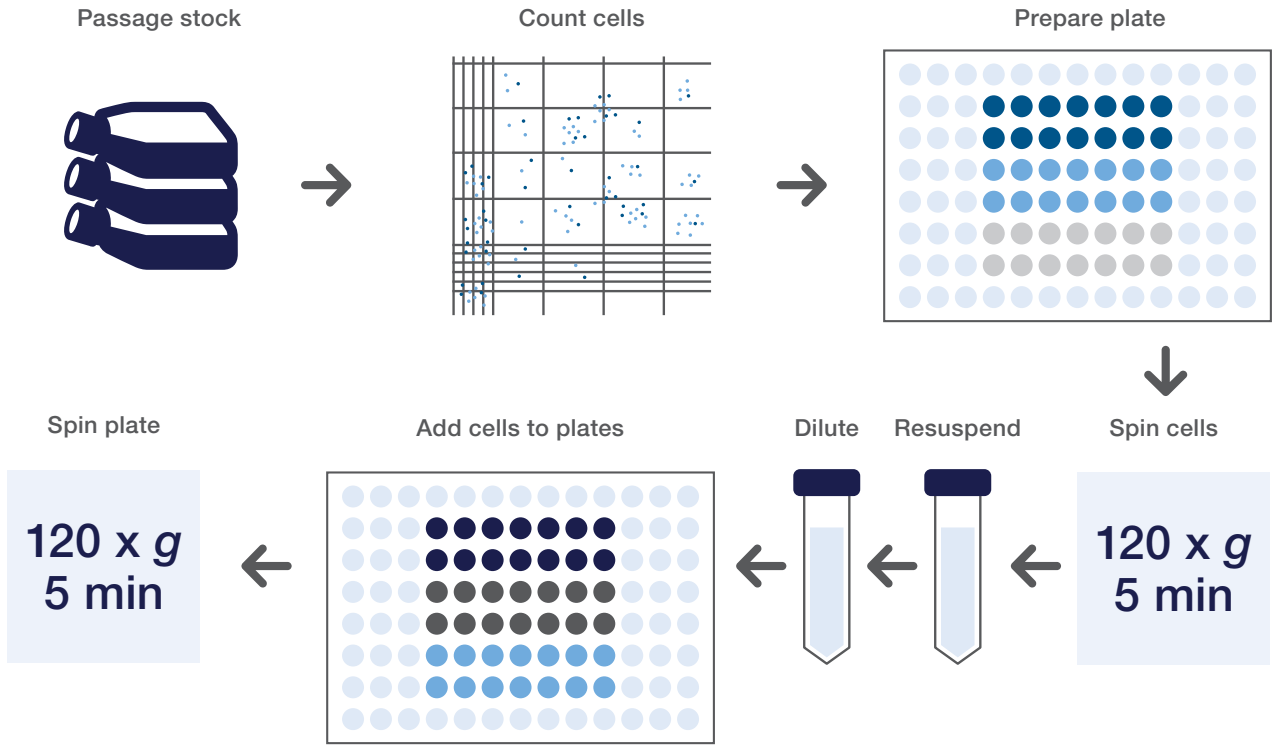


Figure 1. Suggested workflow for spheroid generation. On the day of the experiment, the MCF7 stock is passaged, counted, centrifuged, and resuspended in the serum-free experimental medium. The prepared wells of a Nunclon Sphera microplate are seeded at a density of 500 cells/well. The seeded plate is then centrifuged again to encourage spheroid development.

To prepare the cell seeding suspension, 7.2 x 10^5 cells* from the stock cell suspension were transferred to a new 15 mL conical tube. The new 15 mL conical tube was centrifuged at 120 x *g* for 5 min and the supernatant was aspirated off. The cell pellet was then resuspended in 12 mL of the experimental medium without bFGF and mixed thoroughly. From that 12 mL, 1 mL was transferred to another 15 mL conical tube containing 11 mL of the experimental medium without bFGF, for a 1:12 dilution.

This new diluted cell seeding suspension was mixed thoroughly and used to seed the prepared wells in the Nunclon Sphera microplate; 0.1 mL of cell seeding suspension was added to each well already containing 0.1 mL medium, for a total volume of 0.2 mL/well and a total of 500 cells/well.** The plate was then centrifuged at 120 x *g* for 5 min before being placed in a 37°C, 5% CO_2 incubator. The spheroids were incubated for 8 days without changing the media before analysis, but the spheroids were observed to form overnight after seeding.

Each data set comprises at least three separate experiments. Statistical analysis was completed across all spheroids per experimental condition. Any wells in which multiple spheroids were observed to have formed were eliminated from further analysis. For statistical analyses, Student's *t*-test and analysis of variance (ANOVA) with post-hoc Tukey honestly significant difference (HSD) test were completed, as appropriate for the data set.

Results and discussion

We found that MCF7 spheroids formed in all experimental conditions after overnight incubation. There was no significant difference in the size of the spheroids one day after seeding. In all conditions, a seeding density of 500 cells/well resulted in ~200 µm diameter spheroids (Figure 2).

However, after 8 days in culture, there were significant differences in the size and morphology of spheroids (Figure 3). Endpoint live/dead staining and subsequent confocal imaging confirmed that all spheroids were viable (Figure 3A). Spheroids exposed to bFGF expanded significantly more over the course of 8 days than those that were not exposed to any bFGF (Figure 3B). In addition, Invitrogen™ PrestoBlue™ viability reagent revealed that the spheroids grown in HS bFGF were not only larger in area but also contained a higher number of viable cells than the spheroids grown in native bFGF (Figure 3C).

Qualitatively, there were notable morphological differences between the spheroids exposed to bFGF and those that were not (Figure 3). Even though only wells with a single spheroid grown after one day of incubation were included for further analysis (Figure 2), small and self-contained spheroids (separated from the original bulk of the spheroid) were observed to have formed in all conditions after 8 days in culture (Figure 3A). These smaller spheroids were more numerous and widespread in the bFGF-treated conditions (white arrows, Figure 3A), contributing to what may be described as a different bulk morphology for spheroids in those conditions.

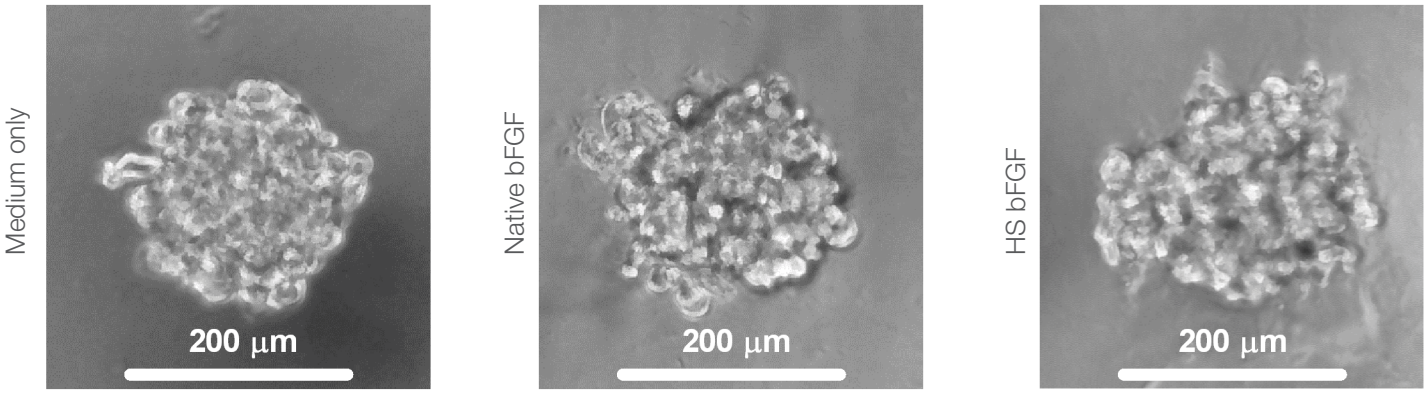


Figure 2. MCF7 cells form ~200 µm diameter spheroids after one day of incubation in all experimental conditions. Regardless of the bFGF supplementation condition, the 500 cells/well seeding density resulted in spheroids that were ~200 µm in diameter after only one day post-seeding.

* This will need to be adjusted depending on the number of wells you would like to seed. This determination is also based on the desired final seeding density, which was 500 cells/well for this study.

** A density of 500 cells/well was found to be optimal for our purposes, but spheroids also formed at 1,000 cells/well in our experience. See Appendix B for more information on how to choose seeding density.

These results are significant in several ways. First, while bFGF is not necessary for MCF7 spheroid generation and growth, it does contribute to greater proliferation. This effect is supported not only by the spheroids' size differences between the bFGF-free and bFGF-containing conditions, but also by the increased cell number observed with the use of stabilized bFGF vs. native bFGF. This is significant given that the effect of bFGF on MCF7 proliferation has been debated in the literature [6].

Second, the morphology observed in the bFGF-containing conditions may have important implications. A different bulk morphology, as opposed to the mass-like morphology that is typical of MCF7 cells, is associated with an invasive phenotype and enhanced ability of cells to break away from the primary tumor *in vivo* [7,8]. Morphological changes may be indicative of bFGF inducing a more invasive phenotype in the MCF7 cells, as has been demonstrated for the ductal carcinoma line T-47D when exposed to exogenous bFGF [9]; however, more work needs to be completed in order to confirm this hypothesis.

It must be noted that these observations would have been impossible without the use of HS bFGF. HS bFGF directly enabled the evidence of induction of MCF7 proliferation with stable bFGF signaling. In addition, if only native bFGF was used, frequent media changes would have disturbed the spheroids and likely confounded any attempts to make observations on the effect of bFGF on MCF7 spheroid morphology. Therefore, we highly recommend HS bFGF to be used in any media formulation that calls for bFGF.

Conclusions

In lieu of a phenol red– and serum-containing media system in which estrogen signaling in MCF7 cells may occur, we demonstrate that DMEM/F-12 (HEPES, no phenol red) with 1X B-27 Plus Supplement may be used in Nunclon Sphera 96U-well microplates to generate MCF7 spheroids. A seeding density of 500 cells/well generated ~200 µm diameter spheroids after one day of incubation. The utility of this serum-free system was demonstrated in an effort to understand the effect of bFGF on MCF7 spheroid growth. However, this method of MCF7 spheroid generation may be applicable more broadly for any study in which the inclusion of FBS may be confounding (e.g., hormone and growth factor studies).

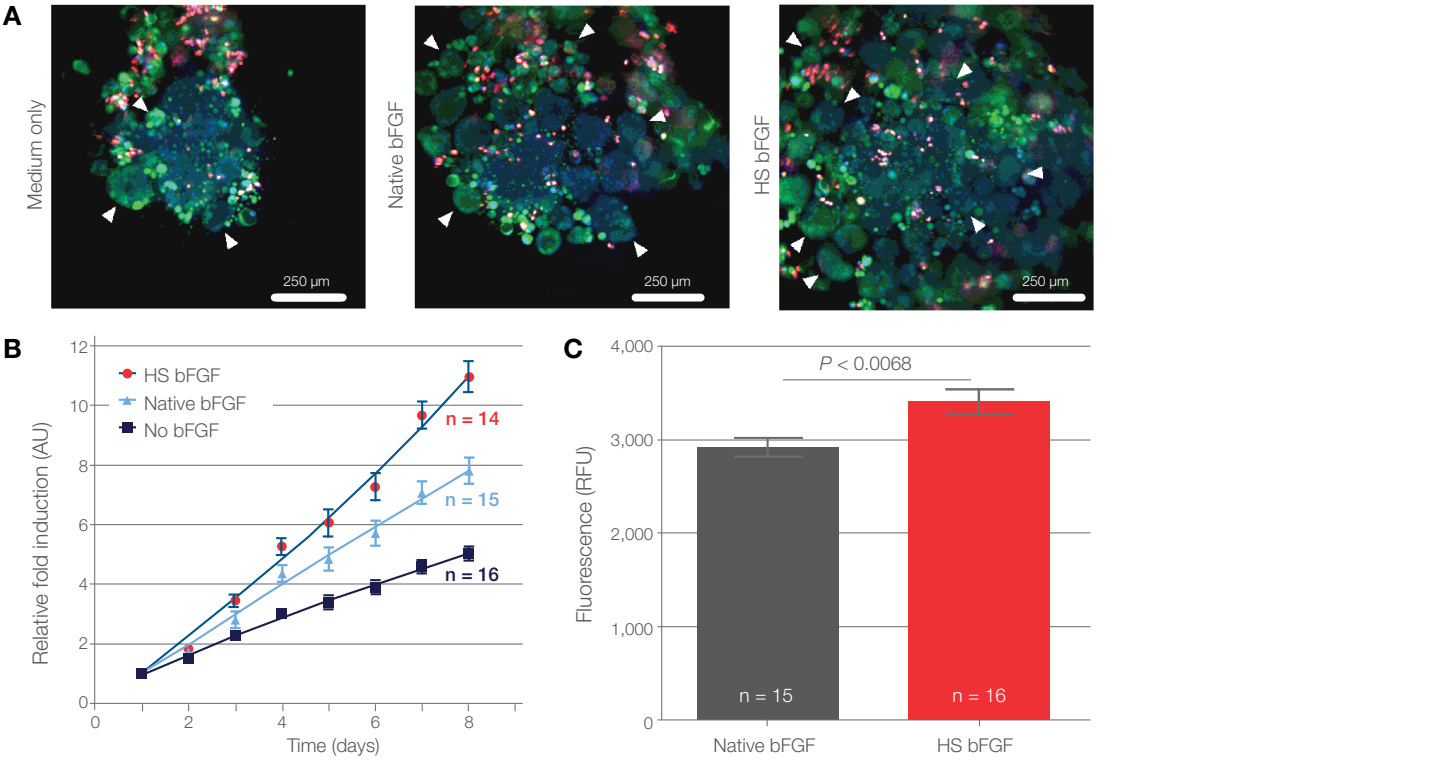


Figure 3. bFGF induced larger spheroids and an altered spheroid morphology. (A) Eight days post-seeding, spheroids in all conditions were viable, as shown by live/dead staining (live = green, dead = red, cell nuclei = blue). By visual inspection, it was evident that spheroids exposed to bFGF were larger and had a different bulk morphology than the spheroids grown in the non-bFGF condition. **(B)** Analysis of relative growth (change in spheroid size, normalized to the initial size) over the course of 8 days revealed that HS bFGF supported the largest increase in size, and spheroids grown in both bFGF conditions maintained faster growth than spheroids in the non-bFGF condition; $P < 0.0001$ at day 8. **(C)** The PrestoBlue assay on spheroids 8 days post-seeding showed a higher number of viable cells in spheroids in the HS bFGF condition vs. those in the native bFGF condition.

Appendix A

Spheroid generation protocol

Prior to the experiment, stocks of MCF7 cells were grown in Nunc EasYFlask Cell Culture Flasks in DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), supplemented with 10% FBS (Cat. No. 16000069), referenced below as the standard culture medium.

For each experiment, two types of media were used:

- Standard culture medium: DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), supplemented with 10% FBS (Cat. No. 16000069)
- Experimental medium: DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), and 1X B-27 Plus Supplement (Cat. No. A3582801), to which HS bFGF (Cat. No. PHG0360) or native bFGF (Cat. No. PHG0266) was added as needed for the experiment. Based on our results, a final concentration of 10 ng/mL HS bFGF is recommended for long-term (~1 week) growth of a spheroid without medium change.

1. Cells in the flask were washed twice with DPBS (no calcium, no magnesium) (Cat. No. 14190250), then dissociated using TrypLE enzyme (Cat. No. 12604021; alternatively, Cat. No. 12563029) for no more than 5 min.
2. After the cells had lifted, the standard culture medium was added to the flask. The cell suspension was transferred to a 15 mL conical tube, and the viable cell density was determined.
3. A Nunclon Sphera 96U-well plate was prepared:
   - To all wells that were to be seeded with cells, 0.1 mL of experimental medium (containing either 20 ng/mL HS bFGF, 20 ng/mL native bFGF, or no bFGF) was added.
   - To all other wells of the plate, 0.2 mL DPBS (no calcium, no magnesium) was added.
4. To prepare the seeding suspension:
   - From the stock cell suspension, 7.2 x 10^5 cells* were transferred to a new 15 mL conical tube.
   - The new 15 mL conical tube was centrifuged at 120 x *g* for 5 min, the supernatant was aspirated off, and the cell pellet was resuspended in 12 mL of experimental medium and mixed thoroughly (7.2 x 10^5 cells/12 mL = 6 x 10^4 cells/mL).
   - From that 12 mL, 1 mL was transferred to another 15 mL conical tube containing 11 mL of experimental medium (1:12 dilution); this new cell seeding suspension was then mixed thoroughly (6 x 10^4 cells/12 mL = 5 x 10^3 cells/mL) before it was used to seed the plate.
   - The cell seeding suspension (5 x 10^3 cells/mL) was used to seed the prepared wells in the Nunclon Sphera 96U-well plate; 0.1 mL of the seeding suspension was added to each well already containing 0.1 mL medium, for a total volume of 0.2 mL/well and a total of 500 cells/well.**
5. The plate was then centrifuged at 120 x *g* for 5 min before being placed in a 37°C, 5% CO_2 incubator.
6. The spheroids were incubated for 8 days without changing the media before analysis, but the spheroids were observed to form overnight after seeding.

* This will need to be adjusted depending on the number of wells you would like to seed. This determination is also based on the desired final seeding density, which was 500 cells/well for this study.

** A density of 500 cells/well was found to be optimal for our purposes, but spheroids were also formed at a density of 1,000 cells/well in our experience. See Appendix B for more information on how to choose seeding density.

Appendix B

Tips and considerations for MCF7 spheroid generation using Nunclon Sphera plates

- If you are using bFGF in your serum-free formulation, the HS Recombinant Human bFGF is highly recommended. Native bFGF is not stable in standard culture conditions (i.e., 37°C); we have demonstrated a loss of ~80% bFGF bioactivity after 72 hours at 37°C [5]. Although changing the medium on your spheroids is not impossible, care should be taken to minimize the risk of unintended spheroid aspiration. HS bFGF maintains >80% bFGF bioactivity after 72 hours at 37°C [5] and thus eliminates the need to change the medium.

- Final spheroid size may be optimized by varying the seeding density (number of cells per well):

  – For this study, we tested seeding densities of both 500 cells/well and 1,000 cells/well, and continued with 500 cells/well due to the relatively long length of our study.

  – The seeding density will be dependent on the application, especially with regard to drug testing. Although spheroids ≤200 µm in diameter are more representative of physiological cell–cell and cell–matrix interactions than 2D culture, they will likely not represent the hypoxia gradient and necrotic core associated with many solid tumors; spheroids of 200–500 µm, or larger, are needed in order to have these pathophysiological attributes [1].

- We have found that centrifugation of the Nunclon Sphera microplate post-seeding, in combination with the U-bottom configuration of the plate, best encourages spheroid development.

- In our experience, MCF7 spheroids were formed overnight in all of the following media conditions:

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018) and 10% FBS (Cat. No. 16000069)

  – DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047) and 1X B-27 Plus Supplement (Cat. No. A3582801)

  – DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL HS bFGF (Cat. No. PHG0360)

  – DMEM/F-12, HEPES, no phenol red (Cat. No. 11039047), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL bFGF (Cat. No. PHG0266)

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018) and 1X B-27 Plus Supplement (Cat. No. A3582801)

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL HS bFGF (Cat. No. PHG0360)

  – DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018), 1X B-27 Plus Supplement (Cat. No. A3582801), and 10 ng/mL bFGF (Cat. No. PHG0266)

Ordering information

Product	Cat. No.
DMEM/F-12, HEPES, no phenol red	11039047
FBS, certified, US origin	16000
B-27 Plus Supplement	A3582801
DPBS, no calcium, no magnesium	14190
Heat Stable Recombinant Human bFGF Protein	PHG0360
TrypLE Express Enzyme (1X)	12604
Nunc 15 mL Conical Sterile Polypropylene Centrifuge Tubes	339651
Nunc EasYFlask Cell Culture Flasks	156499
Nunclon Sphera 96U-Well Microplate	174929
CellInsight CX7 High Content Analysis Platform	CX7A1110

References

1. Hirschhaeuser F et al. (2010) Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 148:3–15.
2. Nath S and Devi GR (2016) Three-dimensional culture systems in cancer research: Focus on tumor spheroid model. *Pharmacol Ther* 163:94–108.
3. Edmondson R et al. (2014) Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol* 12:207–218.
4. de Faria AN et al. (2016) Estrogen and phenol red free medium for osteoblast culture: study of the mineralization ability. *Cytotechnology* 68:1623–1632.
5. Gibco Heat Stable Recombinant Human bFGF. Available from: **thermofisher.com/heatstablebfgf**
6. Liu JF et al (1998) Fibroblast growth factor-2 has opposite effects on human breast cancer MCF-7 cell growth depending on the activation level of the mitogen-activated protein kinase pathway. *Eur J Biochem* 258:271–276.
7. Kenny PA et al. (2007) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 1:84–96.
8. Nijkamp MM et al. (2011) Expression of E-cadherin and vimentin correlates with metastasis formation in head and neck squamous cell carcinoma patients. *Radiother Oncol* 99:344–348.

Analysis of cancer spheroids through high-throughput screening assays

Introduction

Cancer cells grown as spheroids resemble human tumors more closely than cells grown in monolayers, with respect to morphology, structural complexity, phenotype, and sensitivity to chemotherapeutics. Being more physiologically relevant model systems, they can be more predictive of drug profiling and cytotoxicity. So, early screens of drugs have become more dependent on 3D cell culture. In recent years, tumor-derived spheroids have been utilized to optimize cancer therapeutics for ovarian and hepatocellular carcinoma [**1**,**2**,**3**]. However, there are some challenges to using spheroids for drug screening: primarily, the number of spheroids per well, and the shape and size of spheroids, need to be uniform in order to reduce variability between replicates. To address this challenge, we compiled tips and tricks on how to generate uniform and reproducible cancer spheroids for high-throughput screening (HTS) assays in an application note titled "**Generation of cancer spheroids—tips and tricks**". Additionally, we have outlined a workflow for robust 3D cancer spheroid generation in Figure 1.

Graphical workflow of 3D cancer spheroid generation

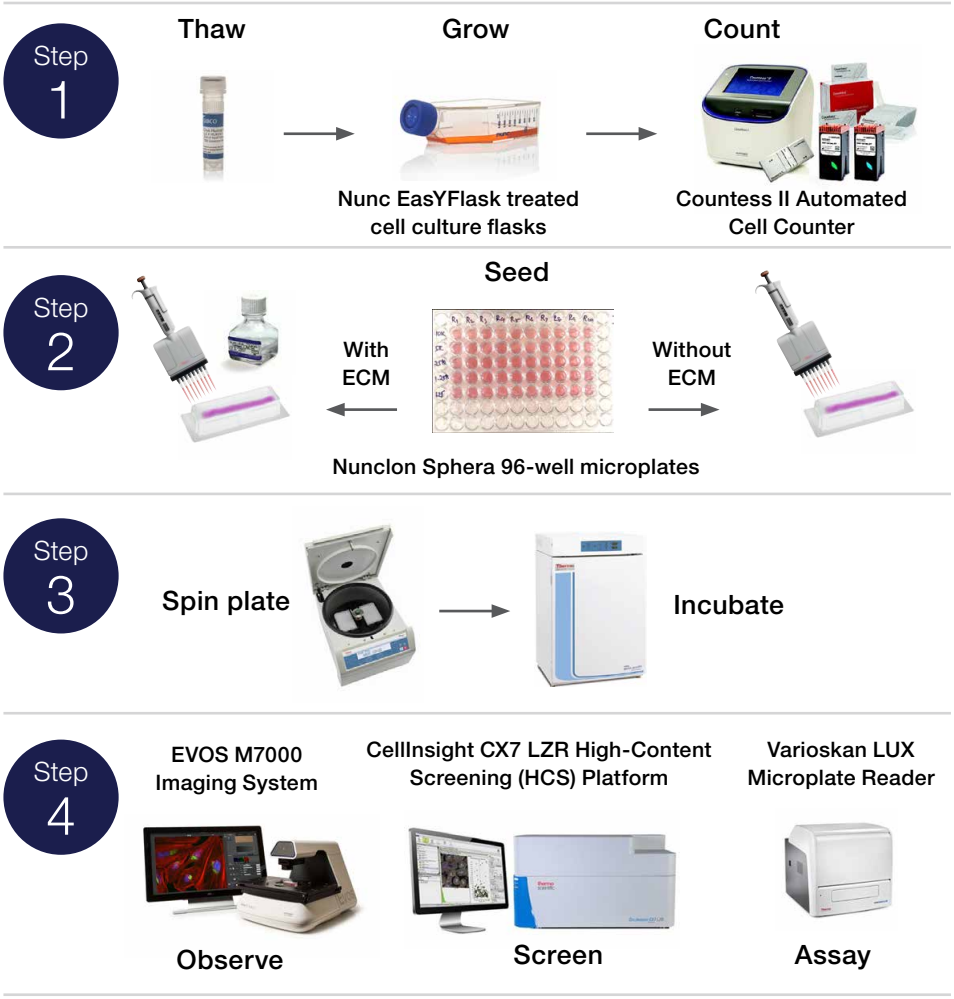


Figure 1. Schematic representation of the process of spheroid generation on Thermo Scientific™ Nunclon™ Sphera™ plates.

Another challenge in working with spheroids is determining penetration of drugs to optimize treatment times. Moreover, the use of spheroids can complicate experimental design and interpretation, but this can be overcome by using the right kinds of reagents, equipment, and protocols. Here we outline different kinds of HTS assays that can be performed on cancer spheroids to assess drug response. We also provide some useful guidelines for handling spheroids and acquiring data to get the most meaningful results. All spheroids were generated on Nunclon Sphera plates using the appropriate Gibco™ cell culture medium.

Assays with plate reader–based readouts

Cell viability and cytotoxicity assays using Invitrogen™ PrestoBlue™ HS Cell Viability Reagent

This straightforward assay utilizes resazurin as a cell health monitor. Upon entering healthy cells, resazurin is reduced in the mitochondria to resorufin, resulting in fluorescence (Ex/Em 560/590 nm). Using this assay, we compared the response of cells in 2D and 3D cultures to doxorubicin, a chemotherapeutic agent. Two different types of cancer cells (HepG2 and PANC-1) were considered. Spheroids and monolayers were treated with doxorubicin either 4 (HepG2) or 7 days (PANC-1) after plating and allowed to incubate for 72 hours. PrestoBlue HS reagent was then added 1:10 (v/v) to the spent medium, and spheroids were incubated at 37°C for 6 hours.

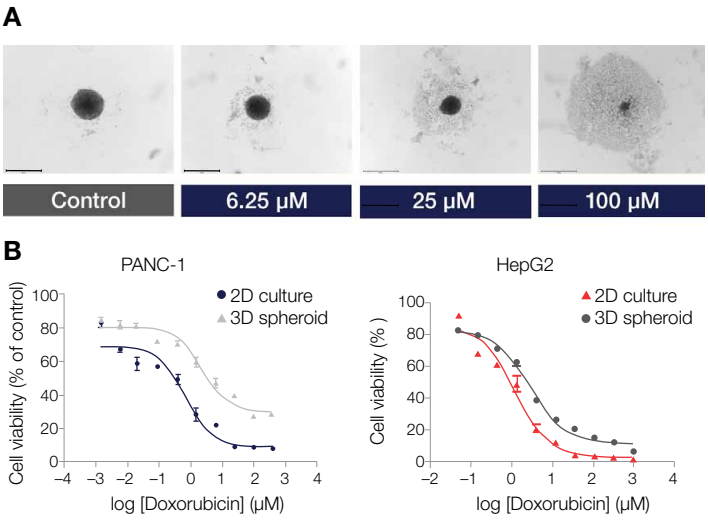


Figure 2. Morphology and effectiveness of doxorubicin treatment on spheroids in 2D and 3D cultures. (A) Morphology of control and doxorubicin-treated PANC-1 spheroids 72 hours posttreatment. Images were captured using the **Thermo Scientific™ EVOS™ M7000 Imaging System** under a 4x objective. Scale bar: 650 µm. **(B)** Dose response curves for doxorubicin-treated PANC-1 (left) and HepG2 (right) spheroids in 2D and 3D cultures.

Following this, high-throughput readouts were obtained using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader. We recommend taking a top read for homogeneity between experimental repeats. Nonlinear regression analysis was performed for variable slope of log (inhibitor) vs. response to calculate the IC_{50} using GraphPad Prism 5.01. As seen with PANC-1 (Figure 2A), doxorubicin treatment caused disintegration of spheroids with increasing dose, indicating cytotoxicity. For both cell lines, the IC_{50} of doxorubicin for 3D culture was at least twice that for the 2D culture (Figure 2B), suggesting increased sensitivity of 2D cultures towards the drug.

Analyzing PSA levels using the Invitrogen™ PSA (Total)/KLK3 Human ELISA Kit

Prostate-specific antigen (PSA) in serum is a known biomarker for prostate cancer diagnosis. The PSA (Total)/KLK3 Human ELISA Kit has been successfully used to detect PSA in cell culture supernatant from 2D culture [**4**]. Using the manufacturer's instructions, we compared PSA secretion in 2D and 3D cell cultures. We chose the LNCaP cell line that expresses the *KLK3* gene (which in turn encodes PSA) endogenously. Medium from PC-3 cells, which do not produce PSA endogenously, was used as a negative control. LNCaP monolayers and spheroids were treated with 2 nM dihydrotestosterone (DHT, which enhances PSA expression) or 80 µM cisplatin (represses PSA expression) 4 days after plating, and incubated for 48 hours. Culture supernatant was collected, diluted 1:20 in diluent buffer, and assayed for secreted PSA using the PSA (Total)/KLK3 Human ELISA Kit and the Varioskan LUX Multimode Plate Reader for colorimetric reading. The colorimetric readings were used to calculate relative PSA levels according to the kit instructions.

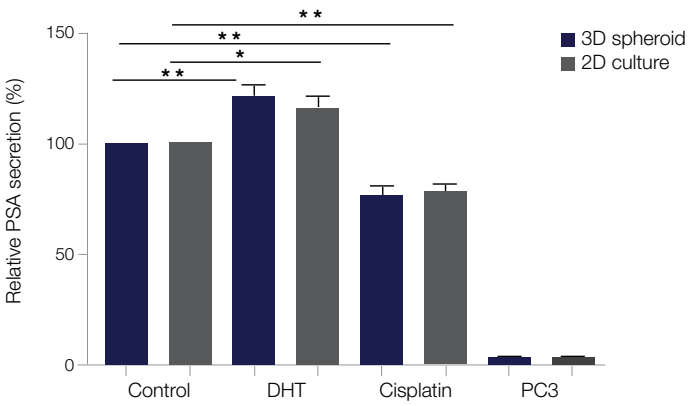


Figure 3. Quantification by ELISA of secretion of PSA following treatment with DHT and cisplatin. Error bars denote standard error of the mean. N = 2. *P < 0.01 and **P < 0.001 for difference from untreated control by one-way analysis of variance (ANOVA).

DHT treatment resulted in 17% and 21% increases in PSA secretion in 2D and 3D culture, respectively, while treatment with cisplatin reduced endogenous PSA secretion by 22–23% (Figure 3). However, 3D culture did not show any major difference in resting or induced PSA levels from 2D culture. This exemplifies how conditioned medium from spheroids can be used for high-throughput non–cell-based assays. In fact, using appropriate readouts, multiplexing of assays can also be performed.

Assays with image-based readouts

Cell viability/cytotoxicity assay using Invitrogen™ LIVE/DEAD™ kit

The LIVE/DEAD kit is a two-color assay that measures cell viability based on plasma membrane integrity and esterase activity. It discriminates live cells from dead cells by staining live cells with Invitrogen™ calcein AM, which is converted to green-fluorescent calcein by intracellular esterase activity, and dead cells with red-fluorescent ethidium homodimer 1 (EthD-1), indicating loss of plasma membrane integrity. After 1 day in culture, SKOV-3 spheroids were treated with various concentrations of the chemotherapeutic drug paclitaxel for 72 hours, followed by incubation with 1 µM each of calcein AM and EthD-1 at 37°C for 3 hours. Following this, spheroids were washed by exchanging half of the medium with 1X PBS, then imaged. We found that exchanging the medium gently from the sides of the wells works better than centrifuging the plates and helps the spheroids stay at the center of the wells, thus aiding in image acquisition (Figure 4A). Spheroids were autofocused using the DAPI channel (they were incubated with **Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent** along with calcein AM and EthD-1 staining), and the centered, maximum-intensity image projection was used to capture the z-stacks. Images were captured using the **Thermo Scientific™ CellInsight™ CX7 High-Content Screening Platform** and analyzed using the cell viability tool on Thermo Scientific™ HCS Studio Cell Analysis Software 4.0. Calcein fluorescence values in the treated samples were normalized to those of the control samples to calculate percentage of viable cells. Values were plotted against paclitaxel concentration using GraphPad Prism software. Increasing paclitaxel concentration led to concomitant reduction in cell viability (Figure 4B).

Apoptosis assay using Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent

The reagent is a four–amino acid peptide (DEVD) conjugated to a nucleic acid–binding dye. The dye is nonfluorescent unless DEVD is cleaved by active caspase-3/7. Following DEVD cleavage, the dye is able to bind to DNA and give a fluorescence signal, providing a means to detect cells undergoing caspase-3/7–dependent apoptosis. MDA-MB-231 spheroids were formed using collagen I as previously **described**, and on day 4 treated with various concentrations of the caspase-dependent, apoptosis-inducing drug etoposide for 72 hours. Spheroids were then incubated with 2 µM of the CellEvent Caspase-3/7 Green Detection Reagent and 1 drop of Invitrogen™ NucBlue™ reagent per milliliter of PBS at 37°C for 2 hours. If PBS is used at this stage, spheroids do not require additional washing. Images were captured on the CellInsight CX7 High-Content Screening Platform under a 4x objective in confocal mode and analyzed using the spot measurement tool of HCS Studio software 4.1.

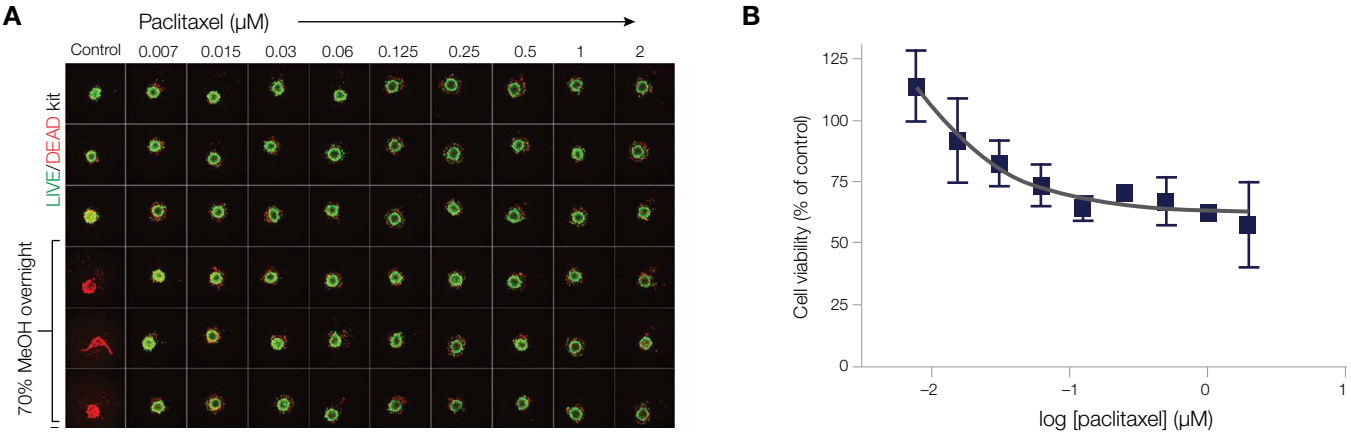


Figure 4. Cell viability assay analysis. (A) Image montage showing LIVE/DEAD staining of SKOV-3 spheroids following treatment with paclitaxel. Images were acquired using the CellInsight CX7 HCS Platform under a 4x objective and in confocal mode. Cells treated with 70% methanol (to kill the cells) in the specified wells served as a negative control for the assay. **(B)** Plot of percent viability of cells with increasing paclitaxel concentrations. The values obtained using HCS Studio software were plotted in GraphPad Prism software and were fit to scale using nonlinear regression. N = 2.

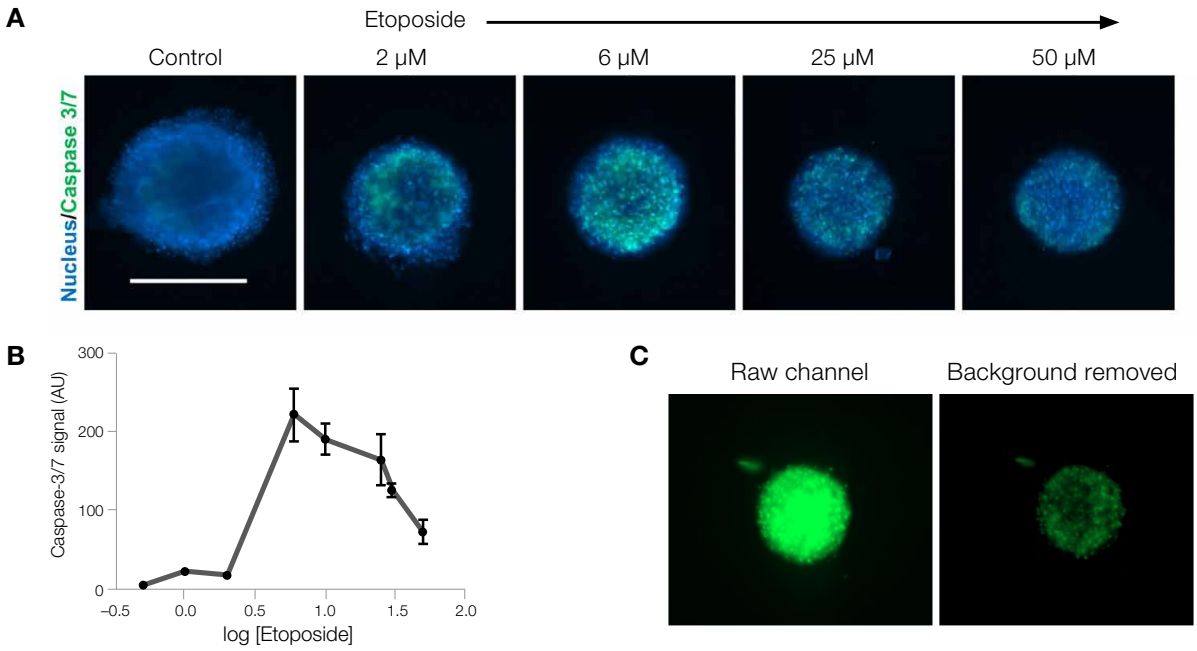


Figure 5. Apoptosis assay analysis. (A) Representative images of control and etoposide-treated MDA-MB-231 spheroids. Scale bar: 500 µm. **(B)** Plot of caspase-3/7 signal intensity against increasing concentrations of etoposide. Six spheroids were considered for every treatment concentration. The plot was generated using GraphPad Prism software from the data obtained from HCS Studio software. Error bars represent standard deviation; N = 2. **(C)** Representation of raw channel (left) and background-corrected channel image (right) for MDA-MB-231 spheroids.

Compared to the control, there was an increase in caspase-3/7 signal with increasing etoposide concentration. However, beyond 6 µM etoposide, the caspase-3/7 signal decreased gradually, possibly owing to an increase in cell death (Figure 5A, B). Another point to note is that the extracellular matrix created background in staining, but using the background removal function for the green channel in HCS Studio software removed it (Figure 5C).

Cell proliferation assay using the Invitrogen™ Click-iT™ EdU Cell Proliferation Kit

This kit uses "click" chemistry to detect cells undergoing new DNA synthesis. T-47D cells were allowed to form spheroids for 24 hours, after which they were treated with 100 nM colchicine, an inhibitor of the mitotic phase of the cell cycle. After approximately 30 hours of treatment, 50% of the spent medium was exchanged with fresh medium containing 20 µM EdU and incubated overnight at 37^{0}C. Proliferating cells that had incorporated EdU were detected using the Click-iT EdU Cell Proliferation Kit with slight changes in the manufacturer's protocol. Briefly, cells were fixed in 3.7% Thermo Scientific™ Pierce™ Formaldehyde for 30 minutes and permeabilized with 0.25% Thermo Scientific™ Triton™ X-100 detergent for 1 hour, followed by incubation with Click-iT EdU dye detection cocktail overnight (as opposed to 30 minutes at room temperature as stated in the kit instructions).

Due to multiple washes involved in the protocol, it is possible that spheroids get dislodged from the center of the well. As a result, they don't always fall completely in the path of light. This gives erroneous readings and variability between replicates. Thus, visualizing the spheroids followed by analysis gives more meaningful data. An example is shown in Figure 6A. Here, both spheroids have been dislodged from the center of the well, but the spheroid in the right panel (shown with arrow) is only partially captured in the field of view. Hence it was excluded from the analysis. Also, small spheroids (200–400 µm) had to be used in the assay to capture most of the spheroids on the Thermo Scientific™ CellInsight CX7 and CX7 LZR HCS Platforms. However, this challenge has been resolved with a new software technology, Thermo Scientific™ EurekaScan™ Finder. EurekaScan Finder has a "seek and find" feature for the CellInsight CX7 LED and LZR HCS Platforms aimed at accelerating discovery by automating the identification and capture of irregularly seeded biological samples, including spheroids, at progressively higher magnifications. With the EurekaScan Finder feature applied, specimens are identified during low-magnification "seek" operations and, once "found", efficiently scanned at higher magnifications for optimal resolution. EurekaScan Finder allows scientists to first identify samples using low magnification across large surface areas, capture them at intermediate magnification, then evaluate them for rare events or improved resolution at higher magnifications.

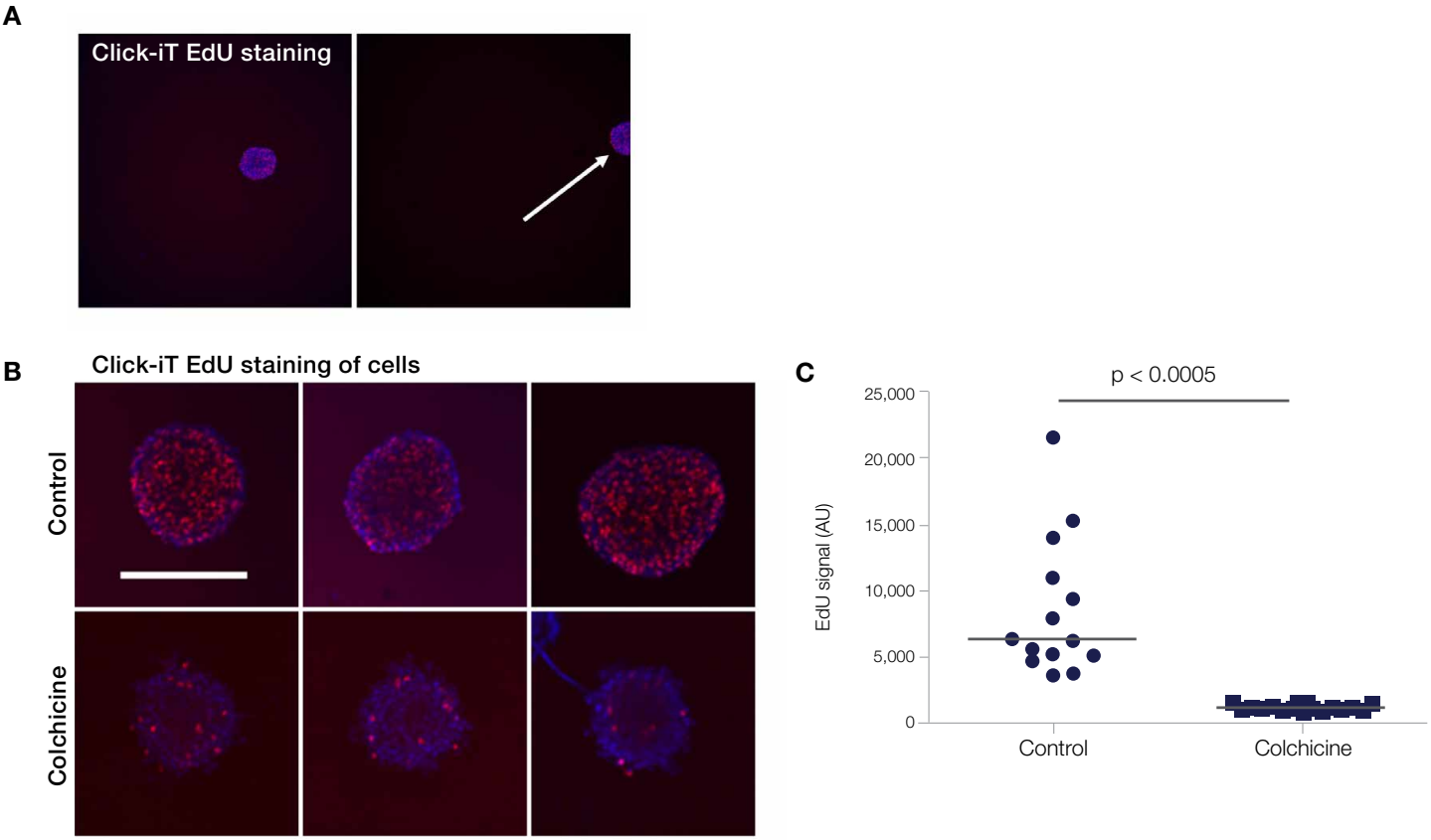


Figure 6. Cell proliferation assay analysis. (A) Field view of a fully captured (left) and a partially captured (right) spheroid as the latter got dislodged during washing. **(B)** Representative images showing Click-iT EdU staining (red) in T-47D spheroids without and with colchicine treatment. Images were acquired using the CellInsight CX7 HCS Platform under a 4x objective and in confocal mode. Scale bar: 200 µm. **(C)** Dot plot analysis of cellular proliferation in T-47D spheroids without and with colchicine treatment. The general intensity measurement tool in HCS Studio software 4.0 was used to analyze the Click-iT EdU signal (y-axis); N = 2. *P* < 0.0005 for difference from control by unpaired *t*-test.

Nevertheless, buffer exchanges should be performed carefully, as scratches in the wells give background signal during imaging, leading to noise in analysis.

For cell proliferation analysis, the spheroid was masked to negate background signal intensity. As depicted qualitatively in Figure 6B and quantitatively in 6C, colchicine treatment led to a significant reduction in proliferating cells in spheroids, indicated by reduced EdU signals.

Conclusion

Though spheroids can be more complicated to analyze than cells cultured under standard 2D conditions, we have shown that a wide variety of cell-based as well as culture supernatant–based assays can be optimized to test drug responses in cancer cells grown in 3D. For the majority of cases, increasing the incubation time of drugs as well as detection reagents for 3D cultures helps reagents better penetrate the spheroids and results in more meaningful data. We recommend keeping washes to a minimum and instead using media exchanges. Based on our observations, centrifuging spheroid-containing plates multiple times does not help to settle spheroids at the bottom, especially if the spheroids are fixed. So, exchanging buffer carefully and gently along the sides of wells is recommended. For comparative studies where analysis can be done on the medium rather than the cells, e.g., PrestoBlue HS reagent or ELISA, a microplate-based readout is the preferred method. However, when the readout is cell based and involves multiple buffer exchange steps, such as the CellEvent Caspase-3/7 Green Detection Reagent for apoptosis studies or Click-iT EdU detection kit for cell proliferation studies, an image-based readout will yield more reliable and reproducible information about the cellular effect of drugs.

Ordering information

Product	Cat. No.
Plastics	
Nunclon Sphera 96-well plates	174925
Nunclon 96-well optical-bottom plates	164588
Matrix Reagent Reservoirs	8094
Media, serum, and antibiotics	
DMEM (PANC-1, MDA-MB-231)	31966021
MEM (HepG2)	11095080
RPMI (LNCaP, T47D)	72400047
McCoy's 5A (SKOV-3)	16600082
Fetal Bovine Serum (FBS)	10270106
Penicillin-Streptomycin	15140122
Reagents and kits	
Phosphate-Buffered Saline (PBS)	10010031
PrestoBlue HS Cell Viability Reagent	P50201
LIVE/DEAD Viability/Cytotoxicity Kit	L3224
NucBlue Live ReadyProbes Reagent	R37605
PSA (Total)/KLK3 Human ELISA Kit	EHKLK3T
CellEvent Caspase-3/7 Green Detection Reagent	C10423
Click-iT Plus EdU Cell Proliferation Kit	C10639
Instruments	
CellInsight CX7 LZR High-Content Screening (HCS) Platform	A46120
Varioskan LUX Multimode Microplate Reader	N16045
EVOS M7000 Imaging System	AMF7000
Pipettes	
Finnpipette F1 Multichannel Pipettes	4661020N, 4661030N

References

1. Ivanova E, Kuraguchi M, Xu M et al. (2020) Use of ex vivo patient-derived tumor organotypic spheroids to identify combination therapies for HER2 mutant non–small cell lung cancer. *Clin Cancer Res* 26(10):2393-2403.
2. Song Y, Kim JS, Kim SH et al. (2018) Patient-derived multicellular tumor spheroids towards optimized treatment for patients with hepatocellular carcinoma. *J Exp Clin Cancer Res* 37(1):109.
3. Shuford S, Wilhelm C, Rayner M et al. (2019) Prospective validation of an ex vivo, patient-derived 3D spheroid model for response predictions in newly diagnosed ovarian cancer. *Sci Rep* 9(1):11153.
4. Profumo V, Forte B, Percio S et al. (2019) LEADeR role of miR-205 host gene as long noncoding RNA in prostate basal cell differentiation. *Nat Commun* 10(1):307.

Differentiation of pluripotent stem cells into neural organoids

Introduction

Recent advances in cell culture techniques have focused on creating 3-dimensional (3D) systems in an attempt to represent *in vivo* cell–cell relationships and microenvironments *in vitro*. Various tissue engineering technologies such as bioprinting, microfluidics, and organs-on-chips have been used successfully to generate 3D cultures [1,2]. Remarkable progress has also been made utilizing adult and pluripotent stem cells (ASCs and PSCs) to generate 3D organ-like (i.e., organoid) cell models [3-5]. PSC-based methods frequently start by aggregating cells in suspension culture to form clusters called embryoid bodies (EBs). Cells in these clusters are capable of differentiating into many types and can undergo self-organization and self-morphogenesis to create a complex cell model that better mimics the *in vivo* cell–cell interactions and microanatomy of a given tissue type. Some PSC-based approaches also require the encapsulation of cells within a natural or synthetic extracellular matrix (ECM)-like substrate [6-8]. In all methods, the application of growth factors, small molecules, and other media supplements is used to guide the formation of organoid systems based on principles inferred from studies of embryogenesis and adult stem cell biology. There are now many published methods for generating a variety of organoid types that resemble different parts of the brain, as well as the liver, intestine, and kidney, to name a few.

The unknown compatibility of multiple reagents from different vendors that span the organoid workflow is an issue that many researchers experience. This issue can have dramatic consequences for the successful generation of the desired organoid system and its reproducibility between laboratories. Established workflows for generating neural organoids from PSCs typically follow a specific sequence of steps that begin with standard PSC culture followed by EB formation, neural induction, neural patterning, and organoid growth [7, 9-12] (Figure 1). The composition of the cell culture medium at each of these steps is critical for the successful differentiation of PSCs. Importantly, the differentiation capacity of a given PSC line must be determined empirically, and some optimization of the differentiation method may be needed for the PSC line of choice. In this application note, we demonstrate the use of feeder-free Gibco™ StemFlex™ Medium, Gibco™ Geltrex™ matrix, and Thermo Scientific™ Nunclon™ Sphera™ Microplates to create neural organoids and spheroids.

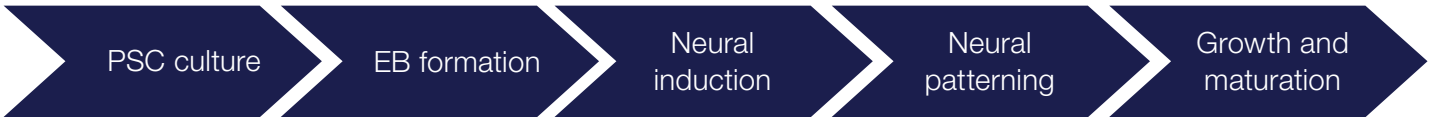


Figure 1. The essential steps of neural organoid formation from PSC cultures.

Experimental details and results

PSC culture

Prior to differentiation, H9 human embryonic stem cells (ESCs) and Gibco™ Human Episomal Induced Pluripotent Stem Cells (iPSCs, Cat. No. A18945) were maintained using StemFlex Medium and grown on Thermo Scientific™ Nunclon™ Delta tissue cultureware coated with a 1:100 dilution of Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301). PSC clumps were routinely passaged using Gibco™ Versene™ Solution (Cat. No. 15040066).

EB formation

PSCs were cultured in feeder-free conditions using StemFlex Medium (Cat. No. A3349401). When PSC cultures reached 70–80% confluency, they were dissociated into single-cell suspensions using Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501), Trypsin/EDTA Solution (Cat. No. R001100), or TrypLE™ Select Enzyme (Cat. No. 12563011). Cell counts and viability were determined using Gibco™ Trypan Blue Solution (Cat. No. 15250061) and the Invitrogen™ Countess™ II FL Automated Cell Counter (Cat. No. AMQAF1000). About 6–9 x 10^3 viable cells per well were seeded in StemFlex Medium with Gibco™ RevitaCell™ Supplement (Cat. No. A2644501) in Nunclon Sphera 96-well U-bottom microplates (Cat. No. 174925). Nunclon Sphera microplates exhibit virtually no cell attachment, promoting consistent formation of spheroids. EBs formed overnight equally well with all dissociation methods but most efficiently with the addition of RevitaCell Supplement (Figure 2). In the absence of RevitaCell Supplement, small EBs did form but with poor efficiency, as most cells either did not survive or did not self-aggregate (Figure 2). EBs were then cultivated for 3–4 days, with a 75% medium change every other day with StemFlex Medium with RevitaCell Supplement. The resulting EBs were of consistent size that was directly proportional to the number of cells seeded (Figure 3).

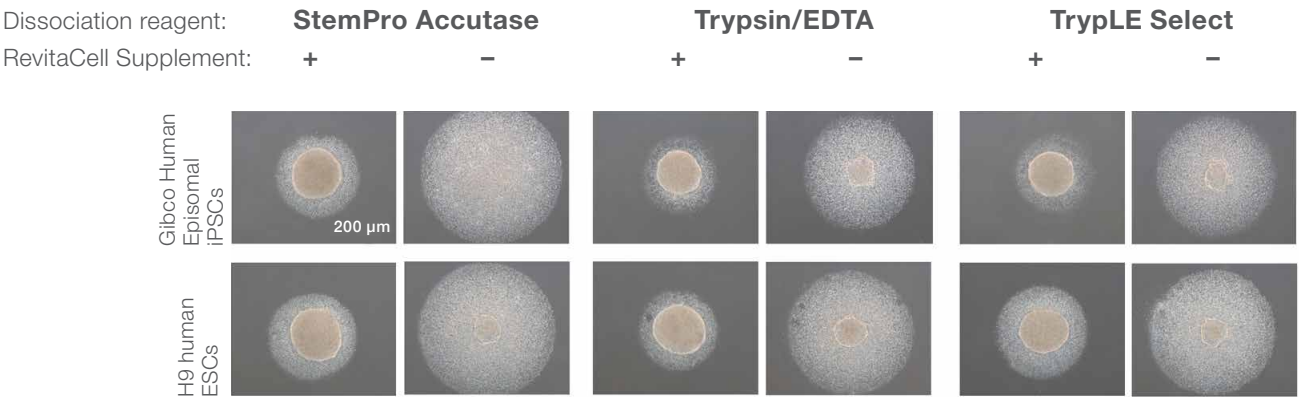


Figure 2. RevitaCell Supplement dramatically improves EB formation. A comparison of EB formation after isolation of PSCs by different methods demonstrated that EBs formed equally well with each dissociation reagent but only if RevitaCell Supplement was included in the culture medium. Cells that do not contribute to the EB are eventually washed away during media changes and do not typically interfere with subsequent steps; here they were not washed away, to illustrate the efficiency of EB formation.

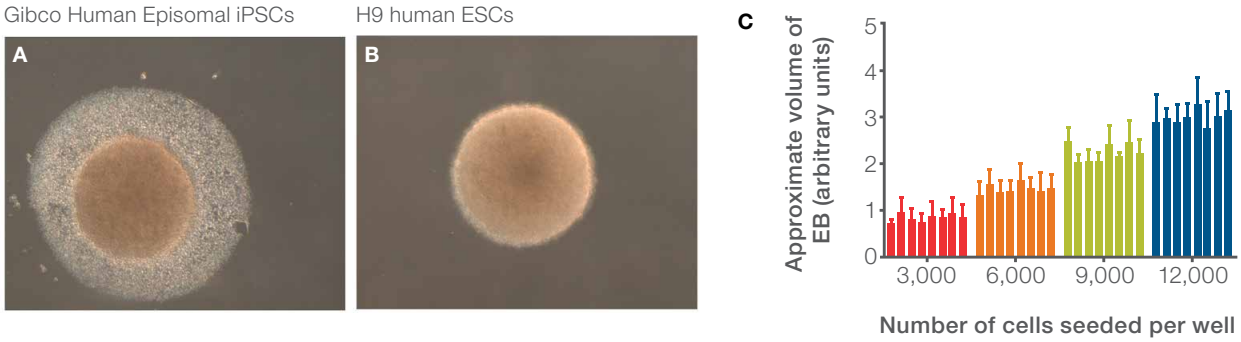


Figure 3. Evaluation of EBs formed in StemFlex Medium with RevitaCell Supplement. (A, B) These images show representative EBs from two different PSC lines after 4 days of culture. **(C)** EB size is directly proportional to the number of cells seeded. The graph shows the consistency in size between 8 replicates for each cell density that was evaluated. Data were calculated by measuring the area of each EB using ImageJ software. The area was then used to calculate the approximate EB volume, which is plotted on the y-axis.

Neural induction and patterning

Following EB formation, the cell aggregates were induced to differentiate into neural lineages by performing 3–4 successive 75% volume medium changes to serially dilute and remove the prior culture medium. Neural induction medium was composed of Gibco™ DMEM/F-12 with GlutaMAX™ Supplement (Cat. No. 10565018) and N-2 Supplement (Cat. No. 17502001). EBs were cultured for 8–9 days with a 75% volume medium change every other day until the outer layers of the EB formed a bright "ring" in contrast to the darker center (Figure 4). By day 10, each EB that displayed this phenotype was encapsulated in undiluted Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) and incubated at 37°C to gel. The use of Geltrex matrix for this application has been independently demonstrated elsewhere [13]. Droplets of Geltrex matrix containing EBs were then transferred to a differentiation medium consisting of DMEM/F-12 with GlutaMAX Supplement (Cat. No. 10565018) and Gibco™ Neurobasal™ Medium (Cat. No. 21103049) with GlutaMAX Supplement (Cat. No. 35050061), MEM with NEAA (Cat. No. 10370021), 2-mercaptoethanol (Cat. No. 21985023), insulin (Cat. No. 12585014), N-2 Supplement (Cat. No. 17502001), and B-27™ Supplement Minus Vitamin A (Cat. No. 12587010). Encapsulated samples were then transferred to Nunclon Sphera 6-well or 24-well plates (Cat. No. 174932, 174930) with a density of 3–5 or 1–2 droplets per well, respectively.

Growth and maturation

The samples were cultured in a growth and maturation medium of the same formulation as the previous incubation medium except this medium contained B-27 Supplement (Cat. No. 17504044). From this point on, neural organoids were cultured on an orbital shaker at 80–85 rpm and the medium was changed every 2–3 days. Neuroepithelia become easily visible within about a week. These samples can be continuously cultured for many weeks (Figure 5A, B) or until analysis is performed (e.g., cellular organization, marker expression). For example, Figure 5C indicates the presence of multiple neural cell types present at day 39 of culture. Gene expression analysis shows that these organoids still contain neural stem and progenitor cells, based on *SOX1*, *SOX2*, and *PAX6* expression, as well as immature neuronal markers such as *DCX* and *MAP2*. However, markers of specific neural regions such as *TBR1* (deep layer neurons), *FOXG1* (forebrain tissue), and *SLC6A1* (encodes GABA1 transporter expressed in cerebral cortical tissue, hippocampus, and other tissues) were also detected, indicating the presence of more differentiated cell types.

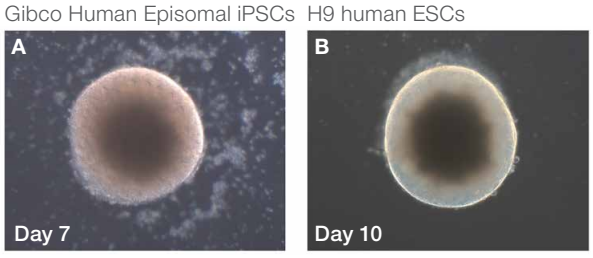
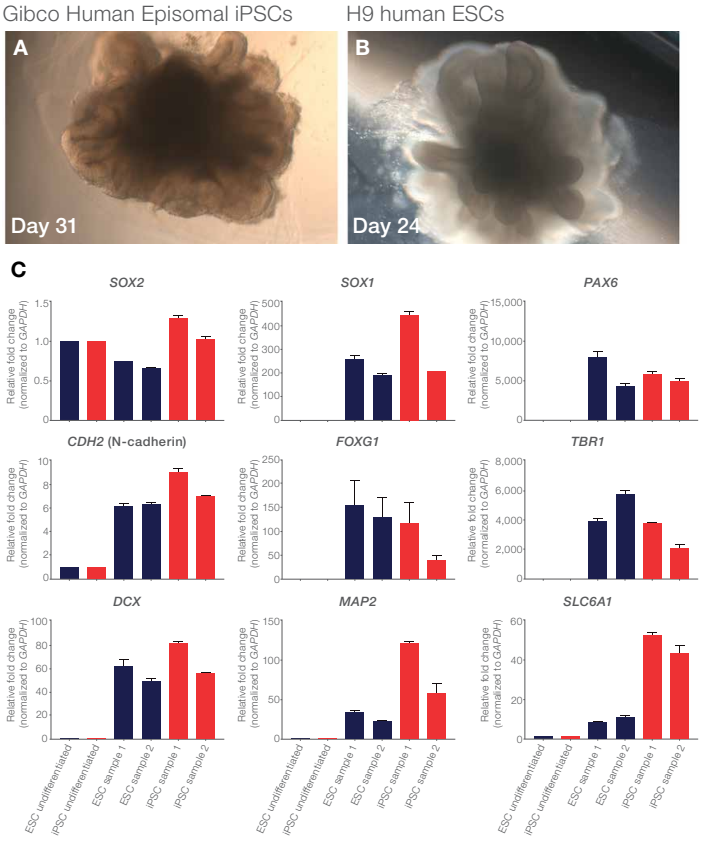


Figure 4. Neural induction and patterning. (A) Brightfield image showing a day 7 EB. **(B)** Day 10 neuralized EB immediately before encapsulation in Geltrex matrix.



D

Gene	TaqMan Assay	Cells or tissues
SOX2	Hs01053049_s1	Neural stem cells, radial glia
SOX1	Hs01057642_s1	Neural stem cells
PAX6	Hs01088114_m1	Neural stem cells, radial glia
CDH2 (N-cadherin)	Hs00983056_m1	Neural ectoderm
FOXG1	Hs01850784_s1	Forebrain
TBR1	Hs00232429_m1	Deep layer neurons
DCX	Hs00167057_m1	Neurons
MAP2	Hs00258900_m1	Neurons
SLC6A1	Hs01104475_m1	Cerebral cortex, hippocampus

Figure 5. Phenotypic characterization and gene expression analysis of neural organoids. (A, B) Brightfield images of neural organoids on day 31 or day 24 of culture show convoluted neuroepithelial structures. **(C)** Gene expression analyses of day 39 neural organoid cultures indicate the presence of multiple neural cell types, including neural stem cells and neurons. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, relative to undifferentiated H9 human ESCs or Gibco Human Episomal iPSCs. Samples from two experiments are shown. **(D)** Summary of Applied Biosystems™ TaqMan® Assays used for gene expression analysis.

Conclusions

Together, these data demonstrate the compatibility of feeder-free StemFlex Medium and Nunclon Sphera 96-well U-bottom microplates with EB formation and neural organoid differentiation. Furthermore, we demonstrate the effectiveness of Geltrex matrix for the encapsulation and morphogenesis of neural organoids. In all, the results indicate that these three products can be successfully integrated with existing Gibco basal media and supplements that are commonly used for studies involving neural organoids.

References

1. Bhatia SN, Ingber DE (2014) Microfluidic organs-on-chips. *Nat Biotechnol* 32:760–72.
2. Murphy SV, Atala A (2014) 3D bioprinting of tissues and organs. *Nat Biotechnol* 32:773–785.
3. Bartfeld S, Clevers H (2017) Stem cell–derived organoids and their application for medical research and patient treatment. *J Mol Med (Berl)* 95:729–738.
4. Clevers H (2016) Modeling development and disease with organoids. *Cell* 165: 1586–1597.
5. Drost J, Clevers H (2018) Organoids in cancer research. *Nat Rev Cancer* 18:407–418.
6. Sato T, Vries RG, Snippert HJ et al. (2009) Single Lgr5 stem cells build cryptvillus structures *in vitro* without a mesenchymal niche. *Nature* 459:262–265.
7. Lancaster MA, Renner M, Martin CA et al. (2013) Cerebral organoids model human brain development and microcephaly. *Nature* 501:373–379.
8. Gjorevski N, Sachs N, Manfrin A et al. (2016) Designer matrices for intestinal stem cell and organoid culture. *Nature* 539:560–564.
9. Mariani J, Simonini MV, Palejev D et al. (2012) Modeling human cortical development *in vitro* using induced pluripotent stem cells. *Proc Natl Acad Sci USA* 109:12770–12775.
10. Phillips AW, Nestor JE, Nestor MW (2017) Developing hiPSC derived serum free embryoid bodies for the interrogation of 3-D stem cell cultures using physiologically relevant assays. *J Vis Exp* 125:e55799.
11. Sloan SA, Darmanis S, Huber N et al. (2017) Human astrocyte maturation captured in 3D cerebral cortical spheroids derived from pluripotent stem cells. *Neuron* 95:779–790.e6.
12. Thomas CA, Tejwani L, Trujillo CA et al. (2017) Modeling of TREX1-dependent autoimmune disease using human stem cells highlights L1 accumulation as a source of neuroinflammation. *Cell Stem Cell* 21:319–331.e8.
13. Iefremova V, Manikakis G, Krefft O et al. (2017) An organoid-based model of cortical development identifies non-cell-autonomous defects in Wnt signaling contributing to Miller-Dieker syndrome. *Cell Rep* 19:50–59.

Ordering information

Product	Cat. No.
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A1413201
B-27 Supplement (50X), Serum Free	17504044
B-27 Supplement (50X), Minus Vitamin A	12587010
DMEM/F-12, GlutaMAX Supplement	10565018
N-2 Supplement (100X)	17502001
Neurobasal Medium	21103049
StemFlex Medium	A3349401
RevitaCell Supplement	A2644501
Nunclon Sphera Microplates, 96-Well U-Bottom	174925
Nunclon Sphera 24-Well Plate	174930
Nunclon Sphera 6-Well Plate	174932

In vitro evaluation of hepatic function using a primary human hepatocyte 3D spheroid culture system

Introduction

The conventional method of culturing **primary human hepatocytes** (PHH) in a 2-dimensional (2D) monolayer presents limitations in the study of hepatic biology, liver function, and drug-induced hepatotoxicity. Traditional 2D hepatocyte cultures dedifferentiate, resulting in the loss of specific hepatic function in approximately 5 days. We have developed and characterized a PHH 3-dimensional (3D) spheroid culture system that preserves hepatic function and promotes culture longevity.

Gibco™ PHH can easily be assembled into a 3D spheroid culture in 5 days using Thermo Scientific™ Nunclon™ Sphera™ low-attachment U-bottom 96-well microplates and Gibco™ plating medium and plating supplements. The 3D spheroid hepatocyte culture requires a significantly lower number of cells than its 2D counterpart, allowing this system to better support high-throughput assays. Moreover, the PHH in the 3D spheroid culture are functionally viable for at least 3 weeks, enabling long-term studies of hepatocyte function.

Application 1: PHH 3D spheroid formation

1. Plating medium was made by adding Gibco™ Primary Hepatocyte Thawing and Plating Supplements (Cat. No. CM3000) to Williams E Medium (Cat. No. A1217601). The plating medium and Gibco™ Hepatocyte Thaw Medium (HTM) (Cat. No. CM7500) were warmed in a 37°C water bath.
2. **Gibco™ 3D spheroid–qualified human hepatocytes (Cat. No. HMCPSQ**) were thawed quickly in a 37°C water bath, and the contents of the tube were transferred to the tube of HTM.
3. The cells were centrifuged at 100 x *g* for 10 min, and the cell pellet was gently resuspended in 3 mL of the plating medium.
4. After counting the hepatocytes, 1,500 cells/well were plated in the Nunclon Sphera 96-well microplate (Cat. No. 174925).
   Note: 1,500 cells in 200 µL of medium (7,500 cells/mL) can be added to each well; or, after pre-wetting the plate with 100 µL of plating medium, 1,500 cells in 100 µL of medium (15,000 cells/mL) can be added to each well.
5. After plating the cells, the plate was centrifuged at 200 x *g* for 2 min to pellet cells to the bottom of the plate.
6. The seeded cells were placed in a 37°C incubator with 5% CO_2 and allowed to incubate for 3–5 days undisturbed before changing the medium. **Note:** It is important to place the plate in an incubator that is not being used with frequent opening for other cultures, and to gently close the incubator door to avoid disturbing the spheroid formation.

7. The spheroids formed within 5 days. No earlier than day 7, biochemical assays and characterization were performed. Hepatocyte maintenance medium (prepared by adding **Hepatocyte Maintenance Supplement (Cat. No. CM4000**) to Williams E Medium (Cat. No. A1217601)) was used for 50% medium exchanges every 48–72 hours (Figure 1). Medium exchanges can be completed using the Thermo Scientific™ Wellwash™ Versa Microplate Washer (Cat. No. 5165010).

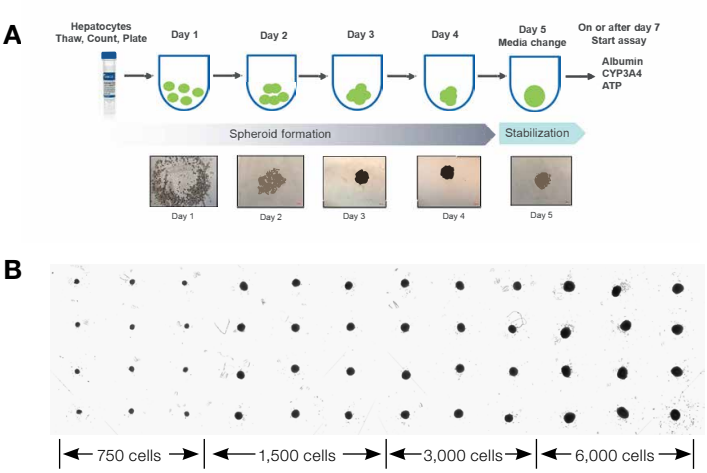


Figure 1. Workflow of assembly and characterization of primary hepatocytes in 3D spheroid culture. (**A**) Spheroids were imaged in phase at 10x magnification. These images show spheroid formation by day 5 of culture. (**B**) Spheroid size is directly proportional to the number of cells seeded. Spheroids were imaged using the Invitrogen™ EVOS™ FL Auto 2 Cell Imaging System (Cat. No AMAFD2000) at 4x. (**C**) Plating of hepatocyte spheroids in a Nunclon Sphera 96-well U-bottom microplate shows consistency in spheroid formation across the plate.

Application 2: Analysis of formation of bile canaliculi in 3D hepatic spheroids

1. Using Application 1, a 3D spheroid culture of hepatocytes was established.

2. A working solution of 5 µM 5-carboxyfluorescein diacetate (5-CFDA) (Cat. No. C1354), was prepared. 5-CFDA is used to visualize formation of bile canaliculi in the 3D spheroids. 5-CFDA permeates intact functional hepatocytes and is hydrolyzed to 5-carboxyfluorescein (5-CF), which is secreted out of the hepatocytes, accumulates in bile canaliculi, and exhibits strong fluorescence.

3. During week 1, the medium was removed from the 3D hepatic spheroids, and they were treated with 100 µL of 5 µM 5-CFDA stock solution and incubated for 1 hr at 37°C.

4. The wells were washed 3 times with hepatocyte maintenance medium, and the cells were imaged using transmission electron microscopy with GFP/FITC settings (Figure 2).

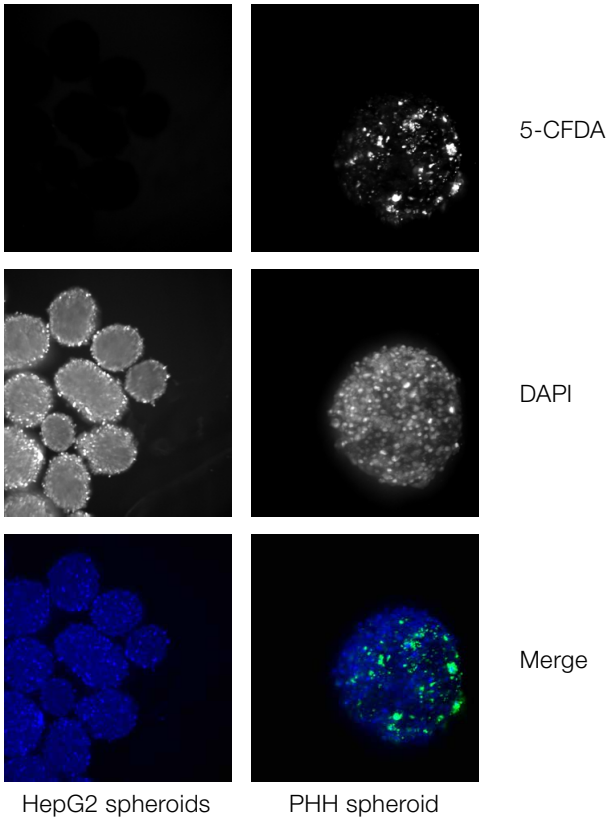


Figure 2. Evaluation of formation of bile canaliculi in hepatic spheroids. HepG2 spheroids during week 2 (left) and hepatic spheroids during week 1 (right) were stained with 5-CFDA and DAPI and imaged using the Thermo Scientific™ CellInsight™ CX7 platform at 10x magnification. Hepatic spheroids show clear formation of bile canaliculi in comparison to the HepG2 spheroids (used as the negative control).

Application 3: Measurement of albumin produced by 2D or 3D spheroid hepatic cultures

1. Using Application 1, a 3D spheroid culture of hepatocytes was established. Additionally, a 2D culture of hepatocytes was started.
2. On day 5 of the 2D hepatocyte culture and on various days of the 3D hepatic spheroid culture, 120 µL of the cell culture medium from each of the wells of the 2D and 3D cultures were collected for analysis of albumin secretion.
3. The cell culture medium was centrifuged at 3,000 x *g* for 10 min, and the supernatant was collected for an ELISA assay using the Abcam Human Albumin ELISA Kit (Figure 3).

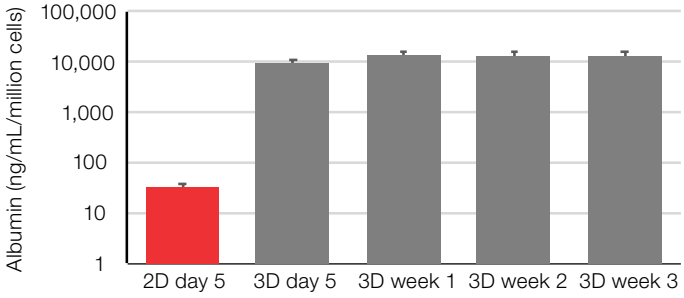


Figure 3. Albumin secretion in 2D and 3D spheroid hepatic cultures. The concentration of albumin secreted is normalized to the total number of cells per well.

Application 4: Activity in 2D or 3D spheroid hepatic cultures

1. Using Application 1, a 3D spheroid culture of hepatocytes was established.
2. On day 5 of the 2D hepatocyte culture and on various days of the 3D spheroid culture, a total of 8 hepatic spheroids were transferred to a single well on a **Thermo Scientific™ Nunclon™ Delta™ 24-well plate (Cat. No. 142475).**
3. The hepatocyte maintenance medium remaining in the 24-well plate after transfer of the spheroids was carefully removed by pipette and replenished with 500 µL of fresh hepatocyte maintenance medium.
4. Activity of the liver enzyme CYP3A4 was measured on the day of the culture indicated in Figure 4, using the protocol for the Promega P450-Glo™ CYP3A4 Assay with Luciferin-IPA.

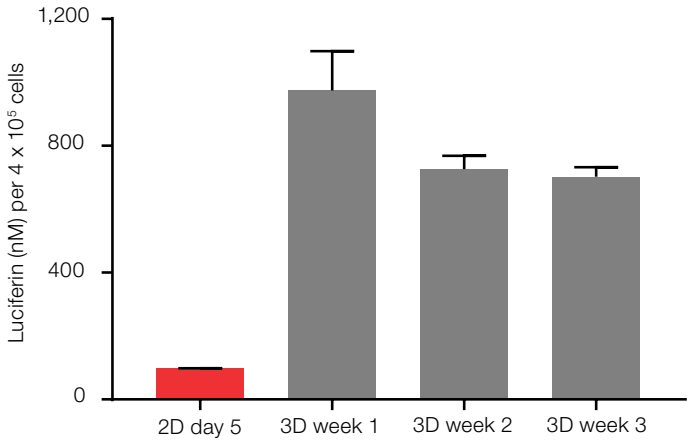


Figure 4. CYP3A4 activity in 2D and 3D spheroid hepatic cultures. CYP3A4 activity was measured using the Promega P450-Glo CYP3A4 Assay with Luciferin-IPA. CYP3A4 activity was found to be significantly higher in the 3D spheroids than in the 2D culture. The data presented are the mean ± SEM (n = 3 for the 2D culture, n = 8 for the 3D spheroids).

Application 5: ATP synthesis by 3D spheroid hepatic cultures

1. Using Application 1, a 3D spheroid culture of hepatocytes was established.

2. During week 1, ATP synthesis was measured in 3 replicates (Figure 5) using the Promega CellTiter-Glo™ 3D Cell Viability Assay.

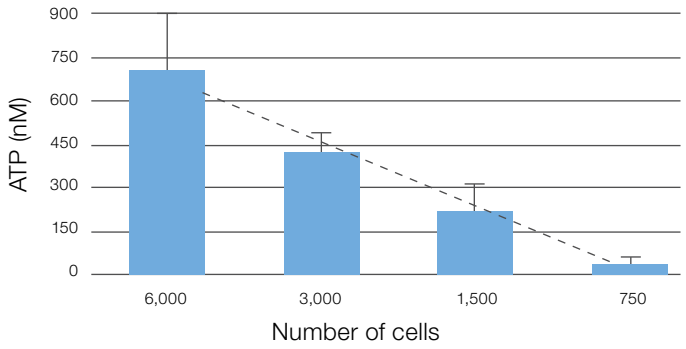


Figure 5. ATP synthesis by 3D spheroid hepatic cultures is proportional to the number of cells. Using the CellTiter-Glo 3D Cell Viability Assay in 3 replicates, ATP synthesis by individual spheroids was measured during week 1.

Application 6: Assay of drug-induced cytotoxicity using 3D spheroid hepatic cultures

1. Using Application 1, a 3D spheroid culture of hepatocytes was established.

2. During week 2 of culture, 3D spheroids were treated with variable levels of the antipsychotic drug chlorpromazine and the anti-inflammatory drug diclofenac, in 4 replicates.

3. Cell viability was assayed 24 hours posttreatment using the protocol for the CellTiter-Glo 3D Cell Viability Assay. Nonlinear regression was performed for variable slope of log (inhibitor) vs. response using GraphPad Prism™ 7 Software (Figure 6). Table 1 shows that 2D and 3D spheroid hepatic cultures have comparable IC_{50} values.

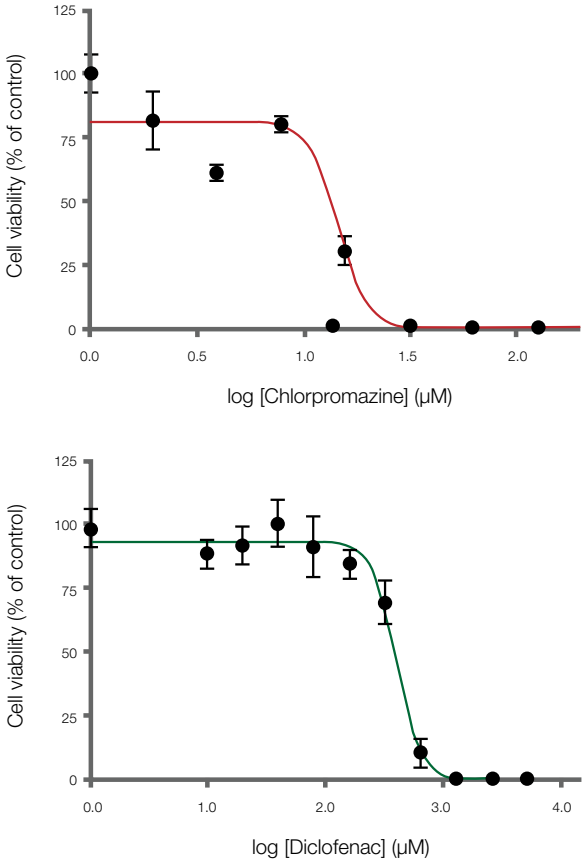


Figure 6. Drug-induced cytotoxicity assayed using 3D spheroid hepatic cultures.

Table 1. IC_{50} of drug-induced cytotoxicity for 2D hepatocytes and 3D spheroid hepatic cultures.

Drug	IC_{50} (2D culture)	IC_{50} (3D culture)
Chlorpromazine	34 µM	14 µM
Diclofenac	331 µM	396 µM

Conclusion

Collectively, these data confirm that cultures of 3D spheroid–qualified human hepatocytes have been characterized to show stable morphology, viability, and hepatocyte-specific functions for at least 3 weeks. We have demonstrated that our 3D spheroid–qualified hepatic cultures are functional, as indicated by formation of bile canaliculi as well as sustained albumin secretion. In comparing CYP3A4 activity on day 5 of 2D hepatic cultures and during week 1 of 3D spheroid hepatic cultures, we have shown that 3D spheroid cultures have significantly higher activity (Figure 4). We also show that this 3D spheroid hepatic culture system can be used to analyze drug-induced cytotoxicity in hepatocytes. Ultimately, these data indicate that the reduced number of cells required for 3D spheroid formation as well as the sustained longevity of these cultures may better support high-throughput assays and long-term studies of hepatocyte functions.

Ordering information

Product	Cat. No.
3D Spheroid–Qualified Primary Human Hepatocytes	HMCPSQ
Hepatocyte Thaw Medium	CM7500
Williams E Medium, no phenol red	A1217601
Primary Hepatocyte Thawing and Plating Supplements	CM3000
Primary Hepatocyte Maintenance Supplements	CM4000
Nunclon Sphera Microplates, low-attachment U-bottom 96-well	174925
Collagen I, Coated Plate, 24-well	A1142802
5-CFDA, AM (5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester)	C1354

Embryoid body formation in Nunclon Sphera plates

Introduction

The significance of stem cells lies in the ability of these cells to become different cell types. The formation of spheroids such as embryoid bodies (EBs) is an important milestone in this differentiation process.

Although several surfaces offering properties of low adhesion are commercially available, spontaneous stem cell differentiation resulting from random cell attachment is still a challenge to many stem cell researchers. The variability seen in spheroid culture has been linked to inconsistent performance of the culture surface in different culture media for different cell types. Here we show that Thermo Scientific™ Nunclon™ Sphera™ plates support EB formation of human embryonic stem cells (hESCs). The surface coating on Nunclon Sphera plates inhibits cell attachment to the culture dish by blocking the adsorption of extracellular matrix (ECM) proteins that usually mediate cell adhesion; the inhibition of attachment to the plate surface promotes cell–cell aggregation *in vitro*. The surface effectively and consistently allows stem cells to grow in suspension with virtually no attachment. The Nunclon Sphera U-bottom 96-well plate provides a specialized format to drive cellular aggregation and generate single EBs in each well (Figure 1).

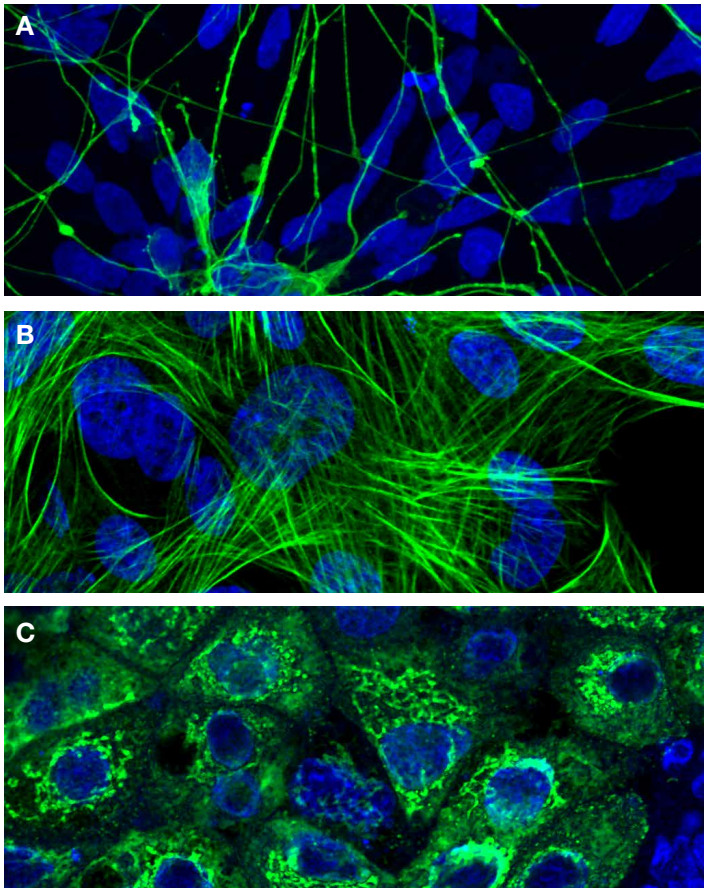


Figure 1. Differentiation of hESCs derived from EBs cultured in Nunclon Sphera plates. Cells were stained for the following markers (green) and counterstained with DAPI (blue): **(A)** ectoderm marker β-tubulin III; **(B)** mesoderm marker smooth muscle actin; **(C)** endoderm marker α-fetoprotein.

Materials

Product	Source	Cat. No.
Nunclon Sphera 96U-well plates	Thermo Fisher Scientific	174925
Essential 8 Medium	Thermo Fisher Scientific	A1517001
Essential 6 Medium	Thermo Fisher Scientific	A1516401
DMEM/F-12 with GlutaMAX Supplement	Thermo Fisher Scientific	10565018
KnockOut Serum Replacement (KSR)	Thermo Fisher Scientific	10828010
MEM Non-Essential Amino Acids (NEAA) Solution	Thermo Fisher Scientific	11140050
2-Mercaptoethanol	Thermo Fisher Scientific	21985023
FGF-Basic (AA 1-155) Recombinant Human Protein	Thermo Fisher Scientific	PHG0264
TGF-β1 Recombinant Human Protein	Thermo Fisher Scientific	PHG9204
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190136
StemPro Accutase Cell Dissociation Reagent	Thermo Fisher Scientific	A1110501
Thiazovivin	Fisher Scientific	38-451-0
PrestoBlue Cell Viability Reagent	Thermo Fisher Scientific	A13261
LIVE/DEAD Viability/Cytotoxicity Kit	Thermo Fisher Scientific	L3224

Protocol

1. Have ready a monolayer culture of hESCs. hESCs can be cultured under feeder-free conditions in either Gibco™ Essential 8™ Medium or hESC growth medium.
2. Prepare 100 mL hESC growth medium by mixing the following reagents:

Reagent	Volume	Final concentration
DMEM/F-12 with GlutaMAX Supplement	79 mL	1X
KSR	20 mL	20%
NEAA (10 mM)	1 mL	0.1 mM
2-Mercaptoethanol (55 mM)	100 µL	55 µM
FGF-Basic (10 µg/mL)	40 µL	4 ng/mL

   - If stored at 4°C, hESC growth medium can be kept for up to 1 week. Warm the medium at room temperature before use.
3. In a sterile biological safety cabinet, wash the monolayer culture of hESCs with DPBS.
4. Add Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent and incubate for 5–10 min.
5. Harvest and resuspend the cells in either Essential 8 Medium or hESC growth medium.
6. Centrifuge the cells at 250 x *g* for 5 min.
7. Resuspend the cells in either Gibco™ Essential 6™ Medium containing TGF-β1 (1.8 ng/mL final concentration) or hESC growth medium without FGF-basic.
8. Very important: Add thiazovivin to the cells (5 µM final concentration).
9. Seed cells into the wells of a Nunclon Sphera plate (Figure 2). To monitor the growth of EBs on the Nunclon Sphera surface in this study, cells were plated in 200 µL/well at different densities into a Nunclon Sphera 96U-well plate. Seeding density may need to be optimized for each cell line.
10. Centrifuge the plate at 250 x *g* for 5 min.
11. Incubate the plate at 37°C and 5% CO_2.
12. Monitor EB formation for up to 2 weeks.
13. Refeed as needed every 72 hr by carefully removing 100 µL of medium from each well and replenishing with 100 µL of fresh medium. Continue to incubate the plate at 37°C and 5% CO_2.
14. At the time of harvest, EBs can be collected from the plate by simply pipetting them out using wide-bore pipette tips. Alternatively, many fluorescence and colorimetric assays (e.g., Invitrogen™ PrestoBlue™ assay, LIVE/DEAD™ viability assay) can be easily performed on the Nunclon Sphera plates without the need to transfer the contents to another microplate.

Results

Growth kinetics of hESC EBs over a period of 12 days were evaluated by size measurement (Figure 3A, B). Data represent the mean of 3 replicates for each starting number of cells.

The PrestoBlue assay was also performed to assess hESC EB health (Figure 3C, D). The fluorescence reading was normalized against EB size for a better quantitative comparison; a higher ratio indicates healthier EBs. Briefly, 12–13 days after seeding, 20 µL of 10X PrestoBlue Cell Viability Reagent was added to each well. Plates were incubated at 37°C and 5% CO_2 for 2–5 hr before being read on a fluorescence-based microplate reader (excitation/emission at 560/590 nm).

The viability of hESC EBs was evaluated using the LIVE/DEAD Viability/Cytotoxicity Kit, which stains live cells green and dead cells red (Figure 3E, F). Briefly, 12–13 days after seeding, the spheroids were incubated with LIVE/DEAD staining solution (1 µM calcein-AM and 4 µM ethidium homodimer-1 in DPBS) at room temperature for 30–45 min. The EBs were rinsed 2–3 times by half-volume changes of DPBS before being imaged under a fluorescence microscope (scale bar of 1,000 µm).

Conclusions

- The Nunclon Sphera 96U-well plate format provides an excellent system to reproducibly generate single EBs in each well.
- The Nunclon Sphera surface, in combination with Essential 6 Medium, provides a completely defined solution for EB generation; the EBs grow faster and are healthier in Essential 6 Medium than in hESC growth medium.
- The Nunclon Sphera plate provides an easy solution for evaluating EB cultures using colorimetric or fluorescence assays such as PrestoBlue and LIVE/DEAD viability assays.

Figure 2. Seeding of hESCs for EB formation.

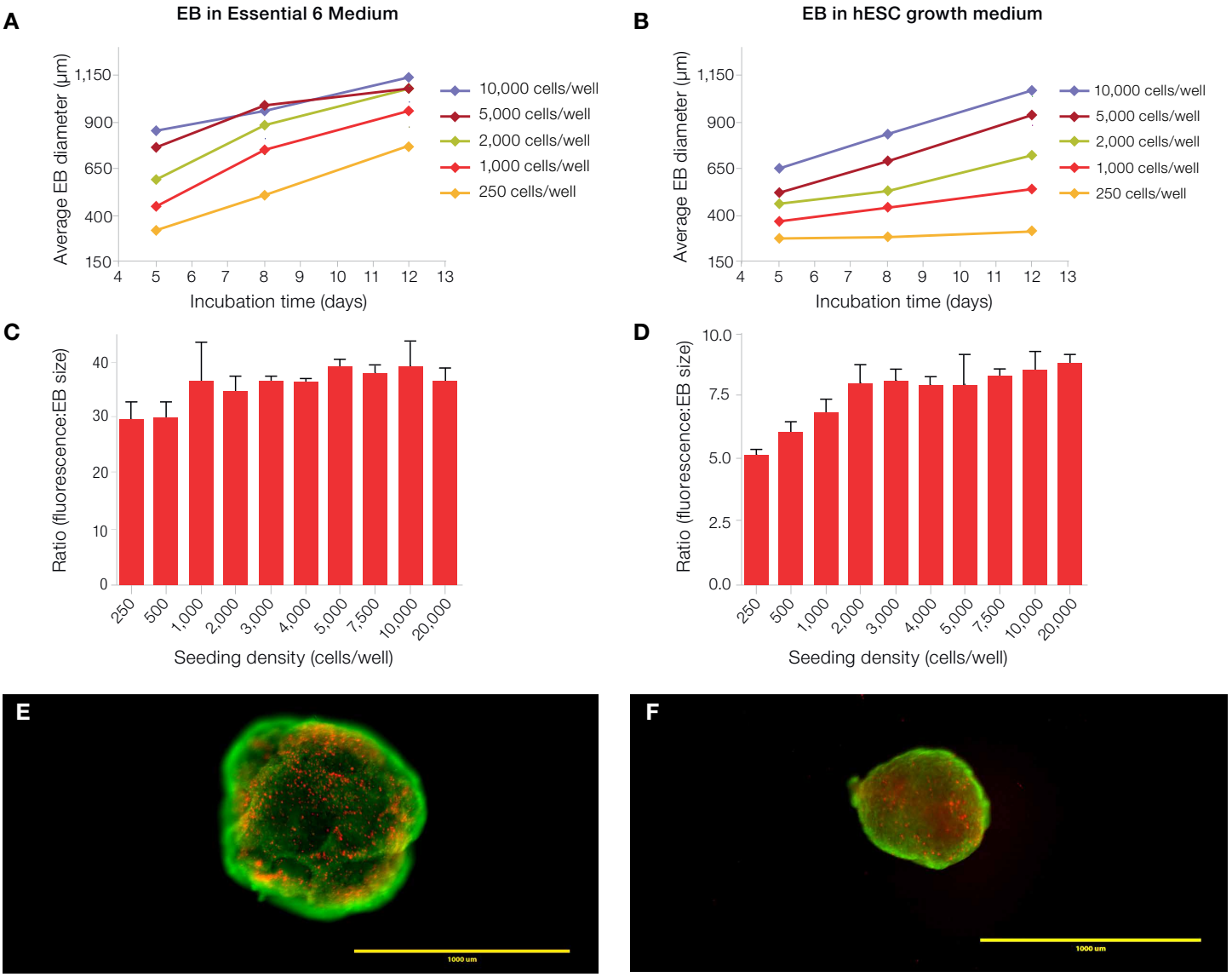


Figure 3. Assessment of human EB growth kinetics, health, and viability. EBs were grown on Nunclon Sphera plates in either Essential 6 Medium **(A, C, E)** or hESC growth medium **(B, D, F)**.

Find out more at **thermofisher.com/sphera**

ThermoFisher SCIENTIFIC