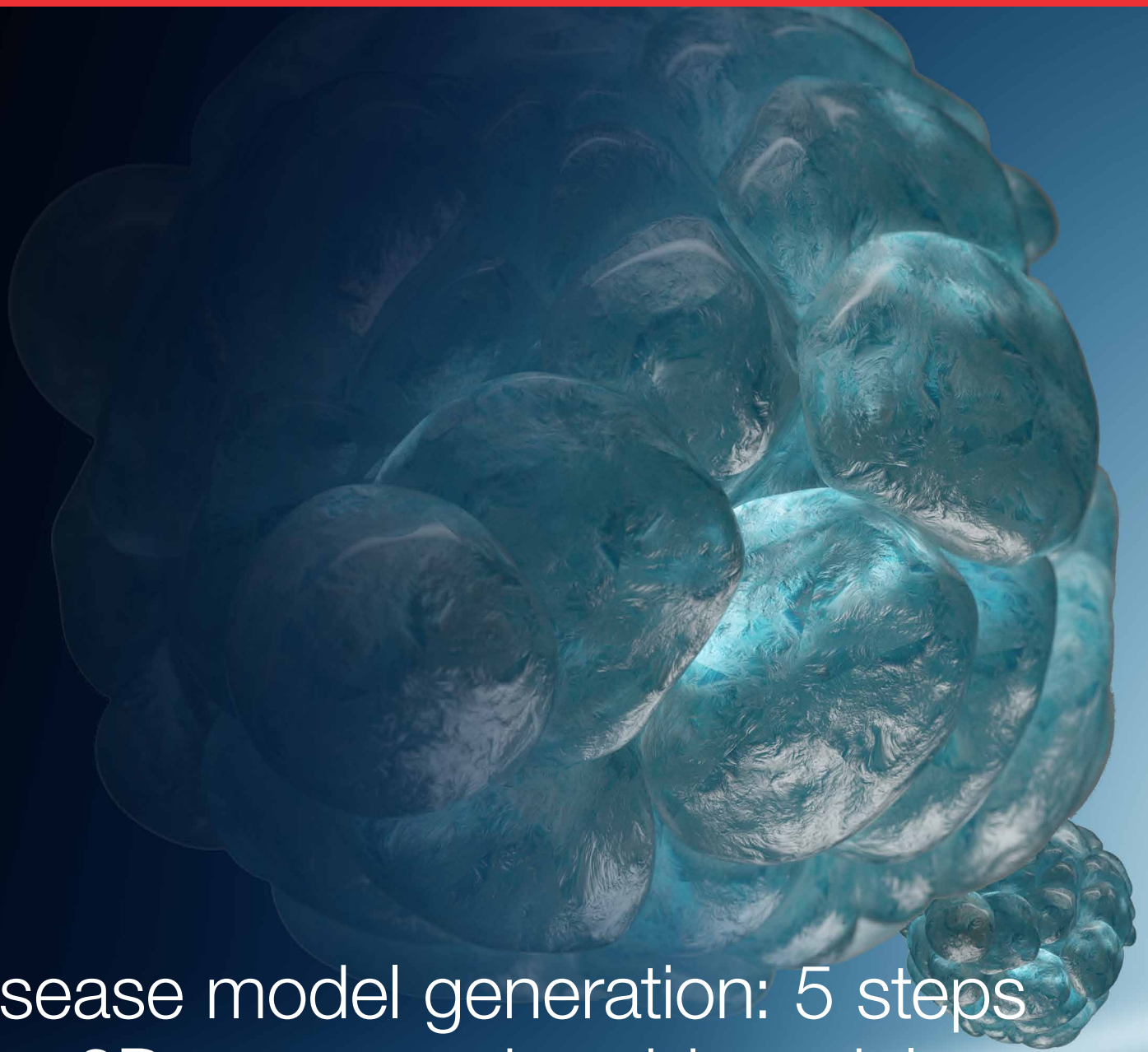




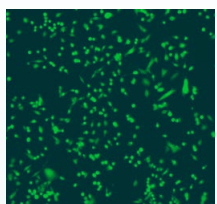
Disease model generation: 5 steps to a 3D cancer spheroid model

Follow this proven guide to easily build 3D cancer
spheroids from a breast cancer cell line

Introduction

Breast cancer is the most common form of cancer in women. It is estimated that 1 out of 8 women in the US will develop invasive breast cancer during her lifetime. Since R&D initiatives provide a lot of impetus to drug development and growth of the cancer therapeutics market, it is important to have relevant model systems to assess drug response. As such, three-dimensional (3D) cell cultures of cancer have become the models of choice for basic as well as preclinical research in cancer biology (1). The generation of a 3D model for cancer includes choosing the appropriate cell types reflective of the cancer

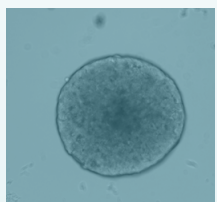
type, optimizing growth and maintenance conditions for the 3D cultures, characterizing the models, and assessing drug responses on such models to better understand the physiological effects of such drugs. The following 5 steps outline the development of a 3D spheroid model for breast cancer as an example. These guidelines can be extrapolated to cells of other cancer types as well.



1. Culture

Choose and propagate representative cell lines for 3D spheroid culture.

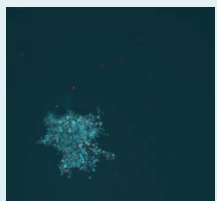
Key reagents and tools: Thermo Scientific™ EasyFlask™ cell culture flasks, Gibco™ DMEM, RPMI 1640, FBS, TrypLE™ reagent



2. Generate

Optimize growth conditions and cell seeding density for spheroid formation.

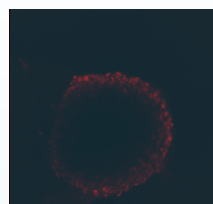
Key reagents and tools: Thermo Scientific™ Nunclon™ Sphera multiwell plates, reservoir, Thermo Scientific™ Finnpipette™ multichannel pipettes, Sorvall™ centrifuge, Invitrogen™ Countess™ II Automated Cell Counter



3. Optimize

Consider adding extracellular matrix (ECM) to media to facilitate spheroid formation for difficult cell lines.

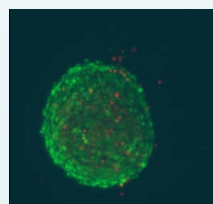
Key reagents and tools: Sorvall centrifuge, Invitrogen™ EVOS™ family of microscopes, Gibco™ Geltrex™ matrix, rat tail collagen I



4. Characterize

Check for spheroid size, sphericity, compactness, and viability.

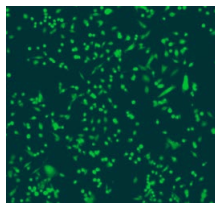
Key reagents and tools: Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader, CellInsight™ CX7 HCS platform, Invitrogen™ DiI, LIVE/DEAD™ viability kit, NucBlue™ Live ReadyProbes™ Reagent, PBS, PrestoBlue™ HS Cell Viability Reagent



5. Measure

Run disease-relevant assays on the spheroids and analyze the response.

Key reagents and tools: Thermo Scientific™ CellEvent™ Caspase-3/7 Green Detection Reagent, CellInsight CX7 HCS platform, Varioskan LUX Multimode Microplate Reader, PrestoBlue HS Cell Viability Reagent, Click-iT™ EdU Cell Proliferation Kit



Step 1: Culture

Culture cell lines in appropriate media

The phenotype of breast cancer can be manifested in different forms such as ductal carcinoma *in situ*, invasive ductal carcinoma, inflammatory carcinoma, and metastatic carcinoma. For each subtype, representative cell lines are available [2,3]. In this example, T47D and MDA-MB-231 cell lines have been used. T47D is an estrogen receptor–positive cell line belonging to the invasive ductal carcinoma subtype. It is cultured in RPMI medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin. MDA-MB-231 is a triple-negative breast cancer cell type belonging to the adenocarcinoma type. It is regularly cultured in DMEM supplemented with 10% FBS and 1% antibiotics. Cells are maintained in tissue culture–treated flasks for at least 1 passage before seeding them for 3D culture in ultralow-attachment plates (Figure 1).

Product highlight

Nunclon EasyFlask cell culture flask: These polystyrene flasks receive the proprietary Thermo Scientific™ Nunclon™ Delta surface treatment, which maximizes adhesion of a broad range of cell types, from cell lines to primary and stem cells. The neck design also allows for easy access with pipettes and cell scrapers.

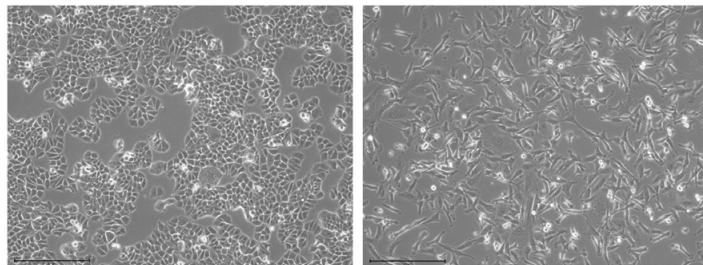
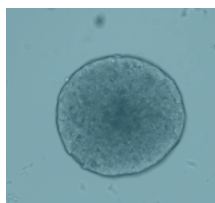


Figure 1. Phase contrast images showing the morphology of T47D (left) and MDA-MB-231 (right) breast cancer cell lines. Images were captured using the Invitrogen™ EVOS™ M7000 Imaging System. Scale bar: 275 µm.

Tips

- To maintain healthy cells, subculture them when they reach 70–80% confluency.
- Incubating cells for too long in cell-dissociating reagents compromises viability and cell health.



Step 2: Generate

Use Nunclon Sphera surface to form uniform spheroids

After cells have reached splitting confluency, the monolayer is lifted off

the cell culture surface using a cell dissociation agent like TrypLE reagent and made into a single-cell suspension. Following this, the cells are diluted 1:1 in Invitrogen™ Trypan Blue Solution, and cell counts and viability are obtained using the Countess II Automated Cell Counter. Based on the cell-seeding density, the required number of cells are diluted in complete medium and seeded onto Nunclon Sphera ultralow-attachment plates. For high-throughput drug screening assays, which require one spheroid per well, cells are seeded into 96- or 384-well plates using multichannel pipettes. The cell suspension is added along the sides of the wells to prevent scratching the bottoms of the wells with the pipette tips. Once the cells are seeded, the plate should be centrifuged to help the cells clump at the bottom of the well, facilitating uniform spheroid formation.

The plate is then left undisturbed in a 37°C incubator with 5% CO₂. The growing spheroids are fed with new medium at a 1:1 ratio with spent medium at regular intervals until the spheroids are ready (Figure 2). The time required for spheroid formation is dependent on cell type [4-6]. Some cell lines, like the cervical cancer cell line HeLa and lung cancer cell line A549, form spheroids within 24 hours, while others like the prostate carcinoma cell line PC-3 take 5–9 days to form spheroids. It takes 3–4 days for both T47D and MDA-MB-231 cells to form spheroids. In the case of T47D, seeding >8,000 per well of a 96-well plate does not yield uniform spheroids. But MDA-MB-231 cells can be seeded at a density as high as 20,000 cells per well of the same well size, for uniform and reproducible spheroid formation.

Product highlights

Nunclon Sphera cell culture surface: To get reliable and consistent assay results, uniform spheroids need to be used. The Nunclon Sphera platform provides an ultralow-attachment surface that facilitates cell growth in suspension. It supports the growth of multiple cell types, including stem cells, as well as their ability to form uniform spheroids or embryoid bodies consistently. For different customer needs, the surface comes in multiple plate formats as well as in flasks.

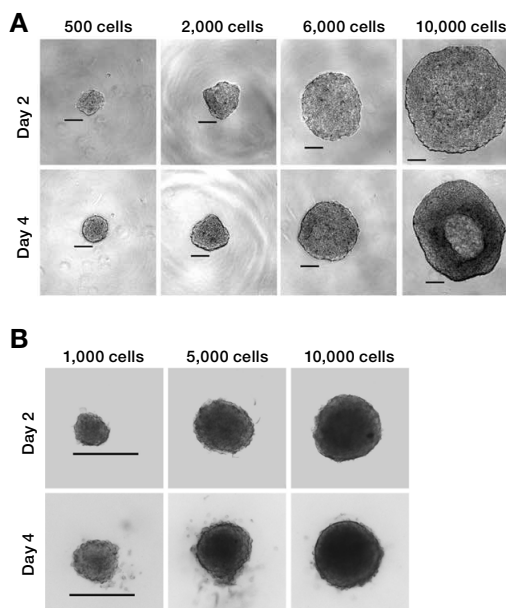
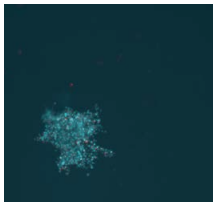


Figure 2. Spheroid formation with different cell types. (A) T47D spheroids seeded at different cell densities were observed over days. Images were captured using the EVOS XL microscope. Scale bar: 200 µm. (B) MDA-MB-231 spheroids seeded at different cell densities were observed over days. Images were captured using the EVOS M5000 microscope. Scale bar: 400 µm.

Tips

- Start with cells that are >90% viable.
- Use a multichannel pipette for ease of cell seeding in multiwell plates.
- Do not touch the bottoms of the Nunclon Sphera plate wells with pipette tips; this makes scratches and produces background in assays.
- Always centrifuge the plate after seeding cells to bring all cells together; this facilitates uniform spheroid formation.



Step 3: Optimize

Optimize growth conditions for individual cell types

Some cell types can readily form spheroids, while some finicky types

do not. For the latter, a few modifications need to be made in the growth medium or cell seeding conditions to aid in spheroid formation. In general, cells that have a roundish morphology and grow in clusters tend to form spheroids readily. On the other hand, cells that are elongated and do not grow in clusters need additional medium supplements to form spheroids. For example, T47D cells form spheroids on their own, while MDA-MB-231 cells do not. Several ECM products that mediate cell–cell interactions, such as methylcellulose, Geltrex matrix, and collagen I, are available to facilitate self-assembly and spheroid formation from finicky cell types. For example, addition of a small quantity of collagen I in the growth medium of MDA-MB-231 significantly improves its ability to form uniform spheroids (Figure 3). At the same time, Geltrex matrix and methylcellulose do not work for this cell line (Figure 4). Different cells respond to ECMs differently. Thus, the best conditions for spheroid formation need to be optimized for each cell type.

Product highlights

Rat tail collagen I: Collagen I is the most abundant protein of the ECM. Rat tail collagen I comes as a ready-to-use solution of 3 mg/mL, which can be diluted to desired concentrations. Apart from spheroid formation, collagen I can also be used to coat tissue culture–treated surfaces to help in the attachment and growth of primary cells.

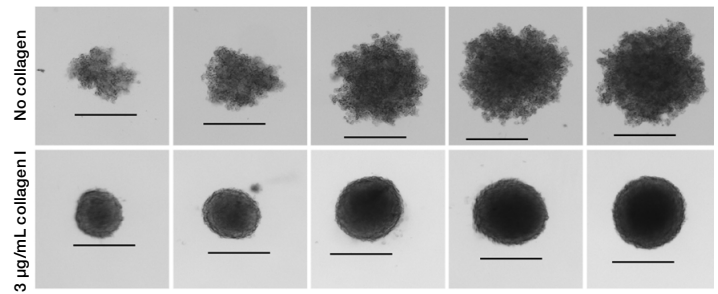


Figure 3. Brightfield images of MDA-MB-231 cell aggregates and spheroids on day 4. Images were captured using the EVOS M5000 microscope under 4x magnification. Scale bar: 400 µm. The addition of collagen I was found to dramatically improve the formation of spheroids.

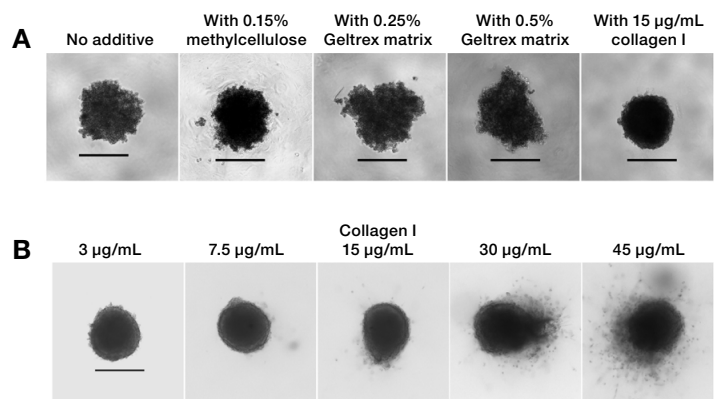
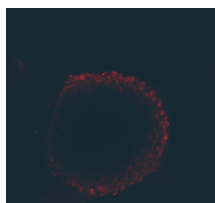


Figure 4. Cell growth optimization under different conditions. (A) 10,000 MDA-MB-231 cells were seeded for spheroid formation in complete medium containing different ECMs. **(B)** 10,000 MDA-MB-231 cells were seeded for spheroid formation in complete medium containing different concentrations of collagen I. All images were captured using the EVOS M7000 microscope under 4x magnification. Scale bar: 500 µm. A collagen I concentration of 3 µg/mL worked best for this cell line.

Tips

- Always dilute stock solutions of ECM products in cold medium, as they tend to polymerize quickly when the temperature rises.
- Allow ~24 hours for MDA-MB-231 cells to clump before adding medium containing collagen I. This facilitates uniform spheroid formation.



Step 4: Characterize

Check for spheroid size, compactness, cell health, and viability

Forcing cells to grow in suspension for spheroid formation puts them under stress. Thus, it is important to assess cell health and viability before using the spheroids for downstream assays. Moreover, since every cell type has a different shape, spheroid size does not correlate with seeding cell density. Usually, cancer spheroids 300–500 μm in diameter are used in drug-response assays. T47D cells yield bigger spheroids than MDA-MB-231 cells. For example, 8,000 T47D cells seeded per well of a 96-well plate will yield spheroids ~600 μm in diameter in 4 days, whereas the same seeding number for MDA-MB-231 forms spheroids of 300–350 μm within the same number of days (Figure 5). The automated imaging setup on the EVOS M7000 microscope allows for quick capturing of brightfield images of spheroids over days for the measurement of spheroid size and roundness. Also, several reagents are available for assessing cell viability and health via imaging as well as microplate readers. Two reagents commonly used to assess cell health of spheroids are PrestoBlue HS viability dye and the LIVE/DEAD imaging kit. These assays require incubation of the spheroids with the respective reagents, followed by confocal imaging using the Thermo Scientific™ CellInsight™ CX7 LZR HCS platform, or taking fluorescence/absorbance readings using a microplate reader like the Varioskan LUX Multimode Microplate Reader. For example, using the PrestoBlue HS reagent, it was observed that the viability of MDA-MB-231 spheroids increased over time, indicating that cells were healthy and dividing (Figure 6). However, it did not give a measure of dead cells. The LIVE/DEAD imaging kit helped visualize the measurement over days, as seen in the increased accumulation of dead cells at the core of the spheroids with higher cell-seeding densities (Figure 7). Other important reagents include hypoxia imaging dyes, cell proliferation detection reagents such as that of the Invitrogen™ CyQUANT™ direct assay, oxidative stress detection dyes such as Invitrogen™ CellROX™ reagent, and mitochondrial superoxide detection reagents such as Invitrogen™ MitoSOX™ Red reagent. In addition, spheroid compactness can also be assessed using stains like Invitrogen™ Dil dye.

For more information, go to thermofisher.com/cellhealth

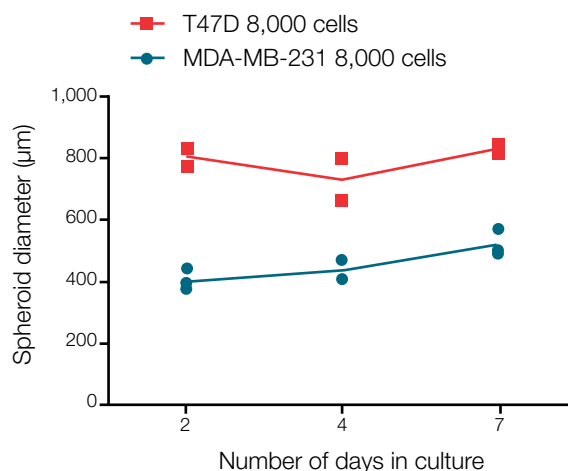


Figure 5. Correlation between number of days of spheroid culture vs. spheroid diameter. Spheroids were generated from 8,000 T47D and MDA-MB-231 cells. At least 6 spheroids were considered per experiment. N = 2.

Tips

- Spheroids are more resistant to dye penetration than monolayers, so increase dye incubation time for 3D cultures.
- Depending on whether the assay is cell-based or spent media-based, use the “top reading” (media-based) or “bottom reading” (cell-based) function on the Varioskan LUX plate reader.
- Cell behavior and health can be measured over time using an instrument that has an on-stage incubator.

Product highlights

- PrestoBlue HS Cell Viability Reagent:** This is a resazurin-based solution used as a cell health indicator. The mitochondria of healthy cells reduce resazurin to resorufin, which gives out a fluorescence signal. PrestoBlue HS reagent allows monitoring of cell viability and health over time. It is a relatively simple assay to perform. The reagent is added to spent medium at a 1:10 (v/v) ratio and incubated at 37°C for 4–6 hours, depending on the cell type. Following this, fluorescence readings are taken using the Varioskan LUX multimode plate reader with an Ex/Em of 560/590 nm. As an example, the relative viability of 7-day-old MDA-MB-231 spheroids was more than that of 4-day-old spheroids, indicating that the cells were proliferating over time. However, this reagent does not give information on the dead cell population. Nevertheless, this is a useful reagent for quick assessment of the effect of cytotoxic drugs.
- LIVE/DEAD imaging kit:** This is a kit based on Invitrogen™ calcein AM dye and ethidium homodimer-1 (EthD-1), used for visualizing as well as assessing live (calcein AM) and dead (EthD-1) populations in spheroids. The live cells convert calcein AM to calcein, which gives a green fluorescence signal. On the other hand, EthD-1 can enter dead cells and bind to their DNA, resulting in red fluorescence. The kit is compatible with imaging as well as a fluorescence plate reader, but via imaging it gives spatial information on population characteristics. As an example, compared to 4-day-old MDA-MB-231 spheroids, 7-day-old spheroids were found to have more dead cells in the core. Similarly, for T47D cells, spheroids with high cell density had more dead cells in the core. This indicates more dead cells might accumulate in the spheroid core over time, since there is less access to nutrients and oxygen.
- Dil dye:** Dil is a lipophilic membrane stain that is weakly fluorescent until incorporated into a cell membrane. This property can also be used to assess the compactness of spheroids. A compact spheroid would limit entry of the stain towards its core, resulting in a ring-like staining pattern, as was seen for MDA-MB-231 cells (Figure 8).

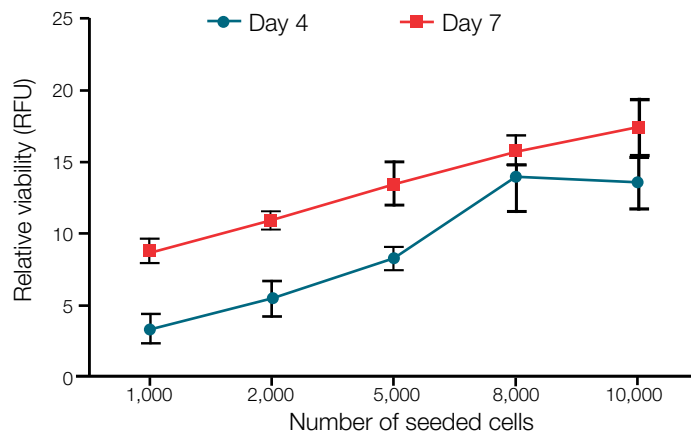


Figure 6. Relative viability over days of MDA-MB-231 spheroids. N = 3. At least 6 spheroids were considered per experiment. Readings were acquired using the Varioskan LUX Multimode Microplate Reader using Ex/Em of 560/590 nm and bottom reading.

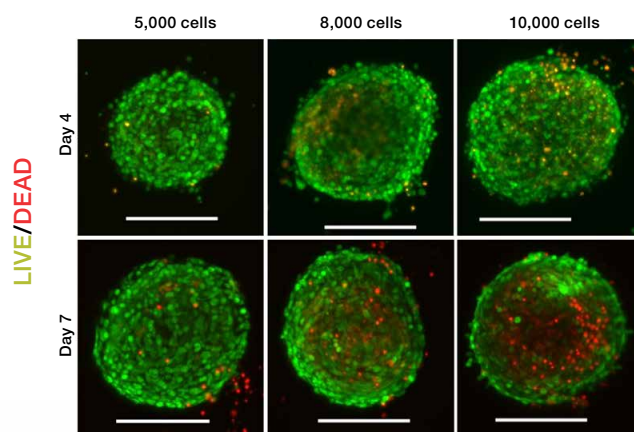


Figure 7. Visualization of MDA-MB-231 spheroids using the LIVE/DEAD HCS staining kit on days 4 and 7. Images were acquired using the CellInsight CX7 HCS platform with a 4x objective. Spheroids at 7 days have more dead cells than at 4 days. Scale bar: 400 µm.

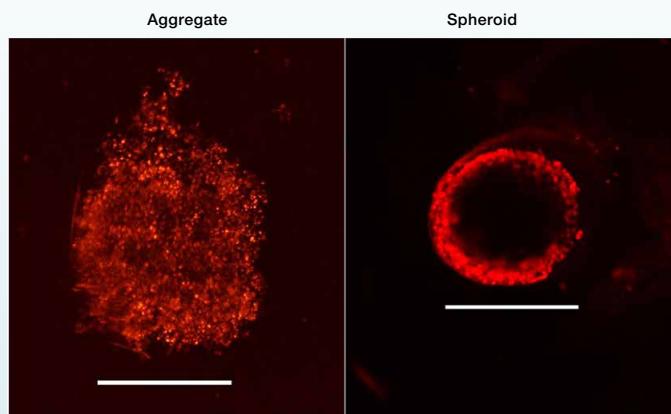
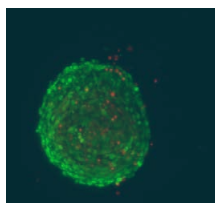


Figure 8. Verification of spheroid formation. 4-day-old spheroids and aggregates from 5,000 MDA-MB-231 were stained with Dil dye. Images were captured using the CellInsight CX7 HCS platform under a 4x objective. Scale bar: 400 µm.



Step 5: Assay/measure

Measure dose-response of chemotherapeutic drugs

After assessment for cell health and viability of spheroids, healthy spheroids can be used in various downstream assays [7,8]. A wide variety of assays can be performed, which may include but are not limited to: LDH release assay to measure cell toxicity, apoptosis assays with Invitrogen™ CellEvent™ caspase-3/7 detection reagent, cell proliferation assays with Click-iT EdU detection or the CyQUANT Direct assay, as well as assays to evaluate gene and protein expression changes in response to drug treatment. The reagents in combination with instruments like the Varioskan LUX multimode plate reader, CellInsight CX7 LZR HCS platform and Invitrogen™ QuantStudio™ real-time PCR system support high-throughput readouts of drug response. For example, using the CellInsight CX7 microscope and the accompanying Thermo Scientific™ HCS Studio™ analysis software, it was possible to perform real-time live-cell imaging and analysis of the response to the apoptosis-inducing drug etoposide on MDA-MB-231 spheroids (Figure 9). Also, the effect of colchicine on cell proliferation in T47D spheroids was quantified in a high-throughput manner using the Click-iT EdU detection kit in combination with the CellInsight CX7 HCS imaging platform (Figure 10).

Product highlight

- **CellInsight CX7 LZR HCS platform:** This is a laser-based automated cell imaging and analysis platform for high-content quantitative imaging. The 7-channel confocal imaging feature, along with laser-based illumination, helps to image thick specimens like tissues, spheroids, and organoids. In addition, the EurekaSkan “seek and find” feature helps locate the spheroid at lower magnification, and image it at higher magnification, thus reducing the scan time significantly. HCS Studio analysis software allows for real-time analysis of more than 30 cell-based assays. Using this system, high-throughput imaging analysis of the effect of chemotherapeutic drugs on cell proliferation and apoptosis was performed.

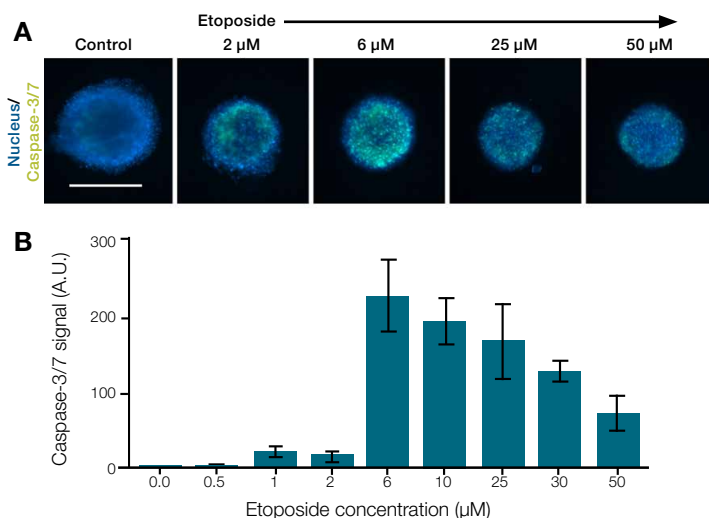


Figure 9. Real-time, live-cell imaging of MDA-MB-231 spheroids in response to etoposide. 4-day-old MDA-MB-231 spheroids were treated with increasing concentrations of etoposide for 72 hours and visualized using CellEvent caspase-3/7 detection reagent. Images were (A) acquired using the CellInsight CX7 HCS platform and (B) analyzed using HCS Studio analysis software. Scale bar: 400 μm.

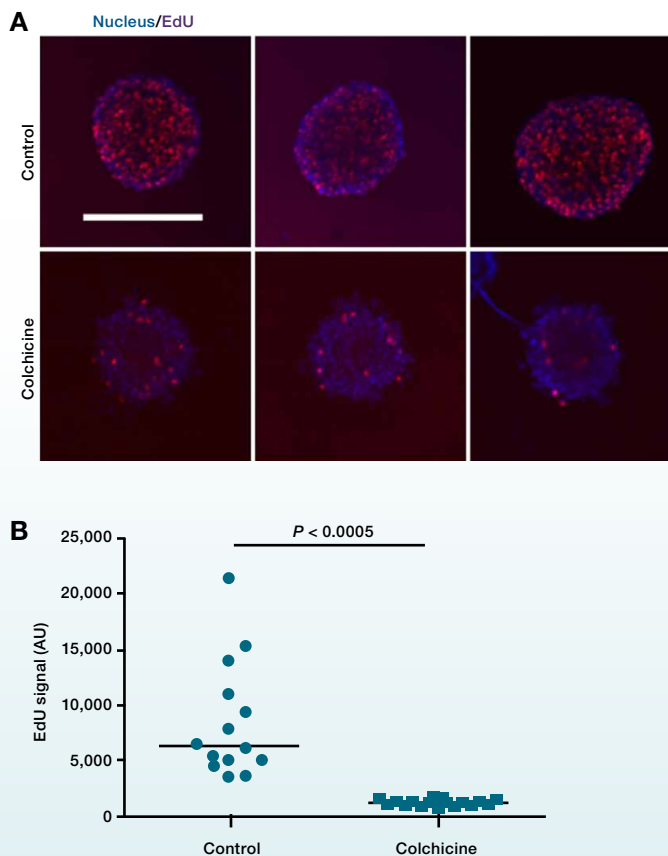


Figure 10. Effect of colchicine on cell proliferation. 4-day-old T47D spheroids were treated with 100 nM colchicine for 72 hours. Proliferating cells were visualized using Click-iT EdU detection reagent. Images were (A) acquired using the CellInsight CX7 HCS platform and (B) analyzed using HCS Studio analysis software. Scale bar: 200 μm.

New product highlight

- **Thermo Scientific™ Amira™ software:** This software is a new 3D analysis platform for visualizing, manipulating, and understanding data from many image modalities and is especially suited for tumor/spheroid biology. Amira software features Deep Learning for improved training and prediction, including intelligent de-noising functionality, and has been customized to recognize the native 3D image formats from the Cellinsight platforms. Collectively, Amira Software offers powerful tools to accurately and efficiently quantify immuno-oncology 3D models to facilitate the high-throughput demands of research.
- **CellEvent caspase-3/7 detection reagent:** Cellular apoptosis is an essential readout for studying the effect of cytotoxic drugs on cancer spheroids. Thermo Fisher Scientific offers the CellEvent caspase-3/7 detection reagent for direct assessment of caspase dependent apoptosis induction. An important event that happens during apoptosis is the cleaving of caspase-3 and caspase-7 to their active forms. Caspase-3/7 detection reagent is a 4-amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye. The peptide is nonfluorescent, but when DEVD is cleaved by active cleaved caspases the dye enters the nucleus and intercalates with DNA, emitting bright fluorescence. The readout can be obtained using an imaging or a plate-reader platform.
- **Click-iT EdU detection kit:** This kit uses “click” chemistry to identify proliferating cells in a population. This kit was used to study the effect of the chemotherapeutic agent called colchicine on the proliferation of T47D spheroids. During the synthetic (S) phase of the cell cycle, EdU present in the kit gets incorporated into newly formed DNA in place of thymidine. Following this, the EdU detection reagent

Tips

- Use confocal imaging with a laser light source and optical sectioning to obtain spatial information about drug responses of spheroids.
- Avoid centrifuging the plate multiple times during washes. This dislodges spheroids from the center of the wells and makes subsequent imaging difficult.

conjugated to a fluorescent probe helps identify the proliferating cells. For spheroids, this also helps in visualizing the spatial organization of the proliferating cells. For example, for T47D spheroids, more proliferating cells were found to be towards the periphery and less towards the core. This indicates that cells towards the periphery have more access to nutrients, resulting in increased cell proliferation rates.

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