Guide to CellCarrier Spheroid ULA microplates

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Generating 3D cultures in Revvity CellCarrier Spheroid ULA microplates

Introduction

Over the past 20 years, 3D cell culture has become more widely used and integrated into a variety of workflows due to the intrinsic advantages it holds over 2D cultures. Growing cell cultures in 3D can better simulate natural cellular interactions and mimic *in vivo* microarchitecture for more physiologically- and biologically-relevant information. This is achieved by allowing cells *in vitro* to grow in all directions, as compared to only two dimensions in traditional flat, adherent cultures. The addition of 3D cell culture techniques into discovery workflows can reduce downstream costs, such as secondary assay testing and *in vivo* animal testing, by providing biologically relevant and predictive data.

Revvity's CellCarrier Spheroid ULA 96-well microplates have a unique ultra-low attachment (ULA)-coated surface that enables the formation of consistently round spheroids from numerous different cellular models. It's important to note that Revvity's CellCarrier Spheroid ULA microplate coating also helps to eliminate satellite spheroid growth, which in turn allows for easier data acquisition and analysis.

This guide describes a basic method for generating and culturing different cellular models in 96-well spheroid (ULA) microplate format. The timing of 3D spheroid formation, subsequent growth profile, and the development of specific morphological characteristics vary with cell type and initial seeding density and are factors that can affect downstream applications.

Therefore, we recommend these variables be optimized for your specific downstream assay. To illustrate how varying these factors influence spheroid formation, we titrated four different cancer cell lines and seeded a range of up to 11 different cell numbers into a single 96-well CellCarrier Spheroid ULA microplate and visually examined their growth profile and morphological characteristics over time.

Materials and methods

HeLa (human cervical cancer cells), Hep G2 (human liver cancer cells), DU 145 (human prostate cancer cells), and MCF7 (human breast cancer cells) were used in this experiment and were purchased from ATCC®, (respective catalog numbers CCL-2™, HB-8065™, HTB-81™, and HTB-22™). They were all grown and maintained in culture in Eagle's Minimum Essential Medium (EMEM; ATCC Cat. No. 30-2003). All growth media contained 10% Fetal Bovine Serum (FBS; ThermoFisher Cat. No. 26140079) and media for MCF7 cells contained an additional 0.01 mg/mL human recombinant insulin (Sigma Cat. No. I9278-5ML).

Cell lines were maintained according to standard cell culture practices and within specific densities as indicated by ATCC in larger volume T-75 and T-150 flasks prior to harvesting and seeding into microplates.

CellCarrier Spheroid ULA 96-well microplates are sold in two quantities. Catalog number 6055330 is a pack of 10 individually pouched plates with lids and 6055334 is a case of 40 lidded plates packed as two bags with 20 plates each. A sample pack size of two individually pouched plates may be requested through your local Revvity account representative.

Figure 1: Revvity's CellCarrier Spheroid ULA 96-well microplate.

Seeding cells into spheroid microplates

Cells used for seeding may originate from fresh cultures or from frozen vials. For larger-scale studies, expand your cell line(s) of interest in large culture volumes or flask cultures. Seeding cell density (initial number of cells plated per well) for spheroid formation depends on factors such as cell-type, time spent growing in culture, and the specific size of spheroid desired for assessment.

Depending on the cell lines to be cultured and your subsequent application, starting volume per well can range from 75 µL to 200 µL per well (with a maximum working volume of no more than 250 µL). For most purposes, a plating volume of 100 µL per well is sufficient.

Basic protocol (see Figure 2 for a model overview):

1. Grow and expand cell cultures in larger flask cultures and separate cells or prepare cells directly from frozen vials using standard practices.
    - For our experiments, the four cell lines were expanded in T-75 flasks and harvested to single cell suspension by first rinsing with PBS and applying 0.25% Trypsin-EDTA (ThermoFisher Cat. No. 25200056) to separate cells.
2. Harvest cells and ensure a healthy, single cell suspension.
    - Cells can be passed through a 40 µM cell strainer (StemCell Technologies, Cat. No. 27305) using centrifugation into a 50 mL conical tube to achieve a single cell suspension and to remove any additional debris.
3. Prepare a sufficient volume of each cell seeding density in order to dispense the required volume per well multiplied by the number of wells at each cell concentration and cell type. For our experiment, each well received 100 µL of cell suspension. The top seeding density prepared for each cell line was 40,000 cells/well with 10 subsequent dilutions at 2X increments down to 39 cells / well so that each column of the plate had a different cell density and the 12th column contained media only (no cells, negative control).
    - To prepare eight wells at 40,000 cells per well (or 40K cells/100 µL), a minimum of 800 µL is needed plus some excess volume. For example, for one plate 3 mL of suspension may be prepared at 400,000 cells/mL. One half of that volume (1.5 mL) may be diluted with equal volume (1.5 mL) of media and so forth for each subsequent dilution to prepare all 11.
    - Cell seeding dilutions can be prepared or poured into individual or divided (sterile) trays or deep well plates (e.g., 2.0 mL StorPlates, Revvity Cat. No. 6008490) plates for easier pipetting.

4. Dispense the cell suspension(s) into wells of the 96-well Spheroid plate. This step can be achieved by using either a manual multichannel pipettor or an automated liquid handling system.
   - During seeding, make sure that pipette tips don't scratch the bottom or sides of the wells to avoid damaging the ULA surface coating.
   - **Note:** A centrifugation step is not necessary to promote spheroid formation and most cell lines will form nice spheres within 24 hours. If working with a cell line that shows slower spheroid development and spheroids are needed sooner, a quick pulse spin in a centrifuge may help cells congregate faster. This procedure would need to be optimized by the end user.
5. Following seeding, the plates may be lidded and moved to an incubator. (For most mammalian cultures, standard humidified incubators set to 37 °C and 5% CO_2 are sufficient.)

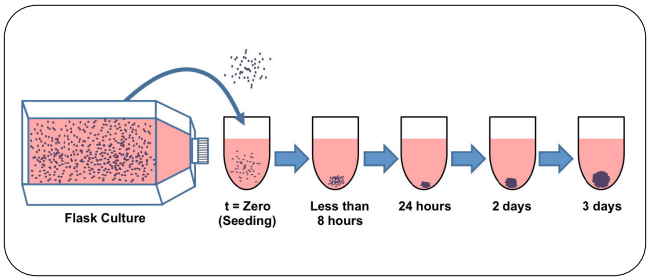


Figure 2: Workflow model for generating and growing spheroid cultures in spheroid microplates. This diagram illustrates spheroid formation within an individual well of a 96-well CellCarrier Spheroid ULA microplate.

6. Daily monitoring of spheroid formation and growth can be done using any microscope equipped with brightfield optics.
7. For imaging and quantification of growth, fluorescent live cell stains may be included and imaging achieved using a high content imager (see section below for more on this).
8. Depending on the cell line being maintained and the length of culture time, a feeding step may be required (e.g., every two - three days is common). Spheroids may be fed by adding fresh media to wells or by removing some of the old and dispensing fresh media into the wells.
   - Pipetting of media can be done manually or by using an automated liquid handling system.
   - In order to prevent disturbing spheroidal tissue during media changes, angle pipettes towards the sides of wells to remove and add media and pipette at average or below average speeds.
9. Treatment with compounds or drugs may be achieved by direct addition to wells or during media changing steps by removing and adding half volumes to each well with 2X the final concentration of compound.

Live-cell staining

For many imaging applications, a live cell stain is needed. In our experiment CellTracker™ Green CMFDA dye (Life Technologies, Cat. No. C7025) was used, allowing for fluorescence and brightfield imaging and automated size analysis based on fluorescence intensity thresholding. CMFDA dye passes freely through cell membranes where it is transformed inside the cell into a cell-impermeant reaction product that demonstrates excellent retention, strong fluorescence, and relatively uniform cytoplasmic staining. Also, CellTracker dyes are inherited by daughter cells and not transferred to adjacent cells in a population. This makes them useful in identifying separate cellular populations in co-culture experiments by pre-loading cells with different colors before seeding them into the ULA microplates. For our purposes, cells were loaded with CellTracker green dye by direct addition to media in cell culture flasks for a final concentration of 5 µM (1:2000 dilution from 10 mM stock). After at least 30 minutes, cultures were rinsed with fresh media and used later in the day for cell seeding (see Figure 3 for an example of CellTracker Green expression).

Some other fluorescent cellular stains we have used for staining and analyzing 3D spheroids are: Hoechst 33342 (Life Technologies, Cat. No. H3570), Calcein AM (Life Technologies, Cat. No. C3100MP), Wheat Germ Agglutinin (WGA) Alexa® 350 Conjugate (Life Technologies, Cat. No. W11263), Tetramethylrhodamine (TMRM, Life Technologies, Cat. No. T-668), CellEvent™ (Life Technologies, Cat. No. C10423), SYTOX® Red (Life Technologies, Cat. No. S34859), and CellTox™ Green (Promega, Cat. No. G8741).

In addition to traditional fluorescence stains, another option for quantifying cellular activity in living tissue is to use an *in vivo* near-infrared (NIR) agent. Revvity's NIR agents are designed to monitor and quantify biological events such as inflammation and cancer progression. They are composed of two parts – the biological part, which mediates interaction with a target molecule or gets cleaved by a target protein, and the near-infrared dye with an emission wavelength of 700 nm, which allows for visualizing the biological event in an imager equipped with the right optics, like Revvity's Opera Phenix® Plus or Operetta® CLS™ high-content imaging systems. Some agents that we have tested and found useful in a 3D cancer tissue model are: ProSense® 680 (Cat. No. NEV10003), MMPSense® 680 (Cat. No. NEV10126) and HypoxiSense® 680 (Cat. No. NEV11070).

For more information on the use of *in vivo* agents with 3D spheroids, see our application note titled "Quantitative Analysis of 3D Microtissue Growth and Biomarker Intensity."

Image acquisition and evaluation

Images were acquired daily in all microplate wells using the10X long WD (working distance) objective on the Revvity Operetta® high-content analysis system to image both brightfield and CellTracker Green Fluorescence channels. In order to collect images of the entire plate faster, a single imaging height (or depth) was selected for imaging all spheres in the plate based on stacks of images collected in representative wells in confocal mode (see Figure 3 of a composite image developed from a stack of images). The images were analyzed using Harmony® high-content imaging and analysis software to measure spheroid size based on threshold cut-offs for the green fluorescence signal. These threshold cut-offs were determined by eye to select the entire spheroid region for each cell type and density. Due to larger seeding densities producing spheroids that exceed the imaging field, images for seven seeding dilutions were analyzed, with the largest spheres generated from 10,000 cells. Spheroid area was quantified and average area calculated across wells of the same seeding density.

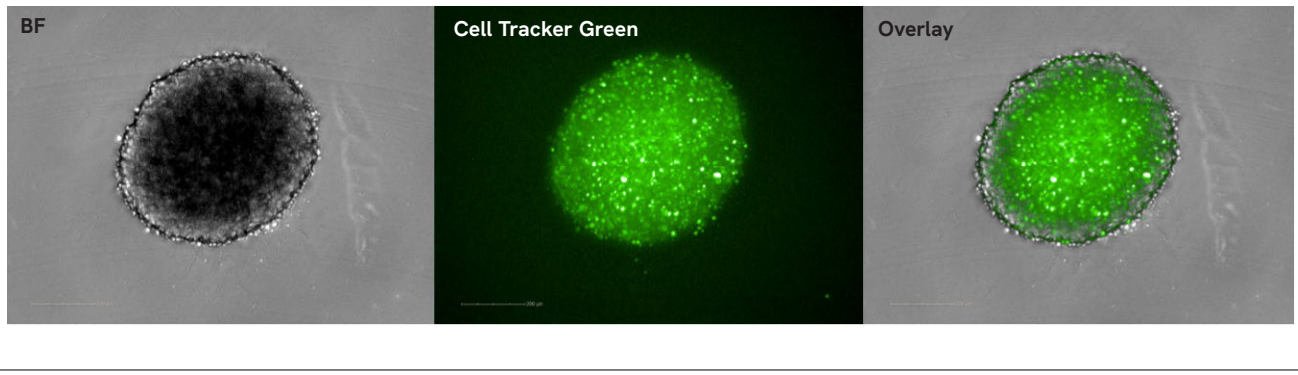


Figure 3: Representative image of a HeLa cell sphere formed from 10,000 cells seeded after 48 hours showing expression of Cell Tracker Green dye. Images were obtained using the 10X long WD objective on the Revvity Operetta high-content analysis system using brightfield and green fluorescence channels (140 µM stack, 10 µM plane distance, confocal mode). The maximum intensity projection images are shown here. Scale bar = 200 µm.

Results

Visualizing 3D spheroid development for four different cancer cell lines

Spheroid formation and growth was assessed using the Operetta high-content analysis system to photograph all spheroids in each plate at each time-point. Representative images of individual spheroid cultures taken over three days, encompassing six different seeding densities and four cell types are shown in Figure 4.

Observations from 3D culture images

- By 24 hours, cells have come together to form aggregated multicellular spheroids.
- After 48 hours, the wells containing DU 145, Hep G2, and HeLa cells have all formed tight, clearly defined spheroid tissue cultures, whereas the MCF7 cells are showing a loose association, though roughly spherical in shape.
- DU 145 spheres appear to decrease in size over time, whereas HeLa spheres appear to mostly maintain their size between 24 and 72 hours in the larger spheres.
- MCF7 cells form what appear to be more loosely-attached clumps of cells that are roughly spherical in shape and continue to grow in size over 3 days.
- The Hep G2 cells begin to form more distinct morphological zones within 24 hours in wells where more than 5,000 cells were seeded which appear to differentiate the inside from outer layers of the sphere. These morphological differences are also observed to a lesser extent in the largest spheres formed from DU 145 and Hep G2 cells.

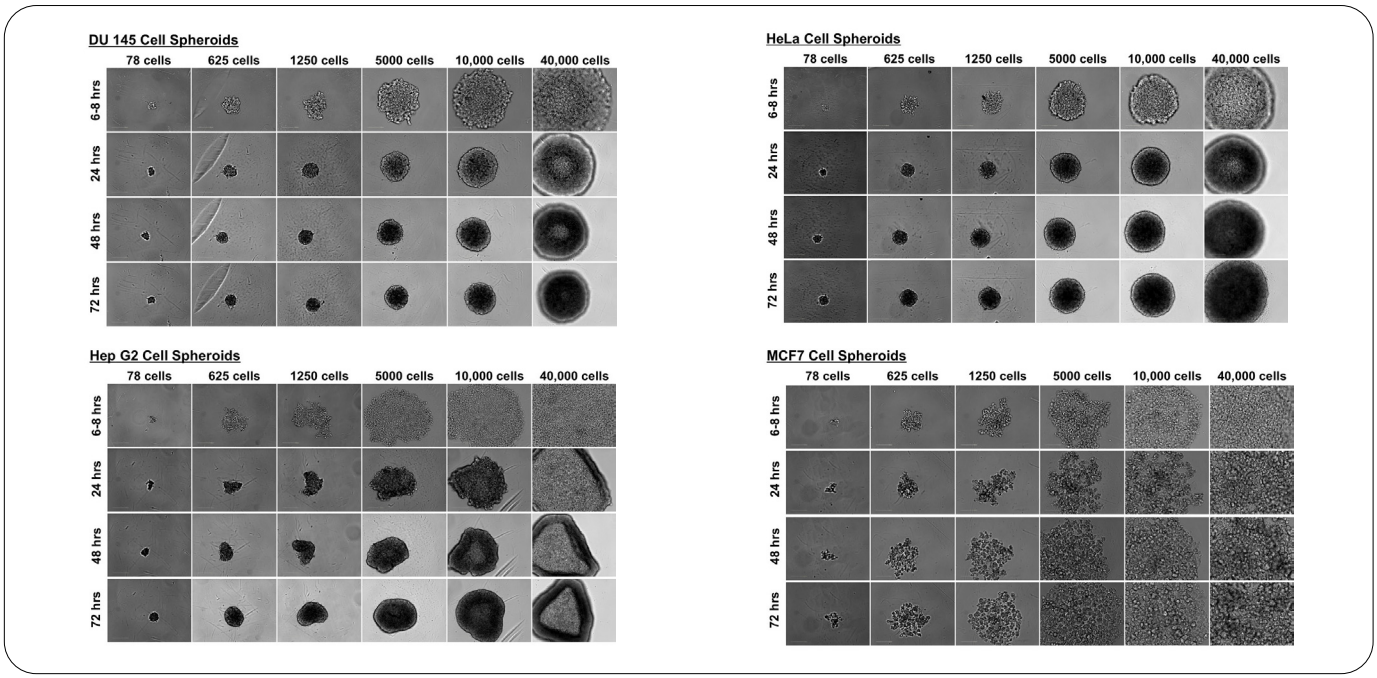


Figure 4: Representative images of 3D spheroid cultures generated from six different seeding densities and four different cancer cell types captured over three days. Images were acquired daily in all microplates using the 10X long WD Objective on the Revvity Operetta high-content analysis system. Scale bar = 1 mm.

Quantifying 3D spheroid size and growth based on spheroid 2D area

Spheroid formation and growth was assessed using the Operetta high-content analysis system as described in the Methods section. Data from this evaluation illustrates the variability in spheroid size and growth dynamics for four different cell lines. Graphical representations of the data are presented in Figures 5 and 6.

Observations and conclusions from Figures 5 and 6:

- Spheroid culture size increases with cell number seeded in a linear fashion for all four cell lines over one day in culture.
- Different cell lines result in different sized spheroids.
- Spheroid sizes change over time.
- The rate, magnitude and direction (increase or decrease as seen in DU 145 cells) of change in spheroid size varies with each cell line.

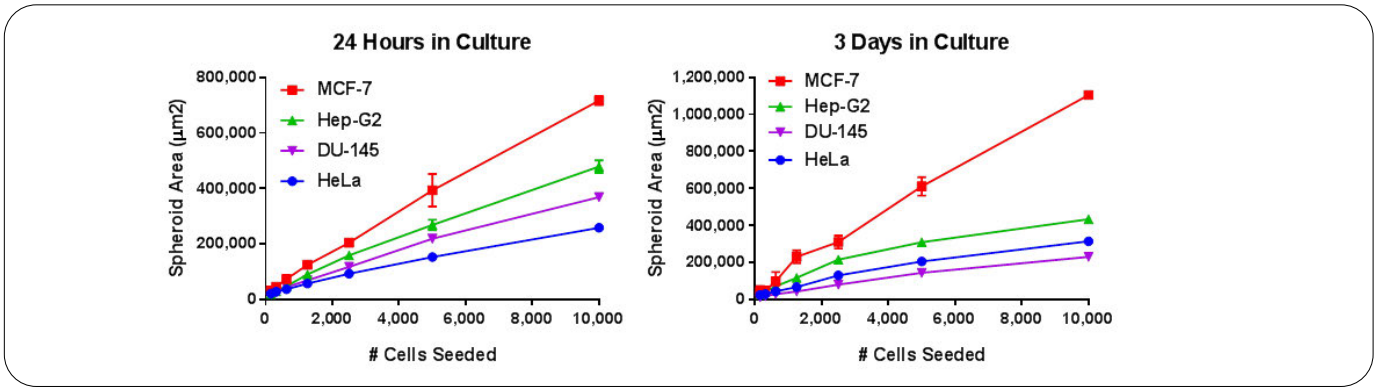


Figure 5: Graphs illustrating spheroid size variability. The graph on the left shows how different cell lines produce different sized spheroids and spheroid size increases with cell number seeded. Each data point is an average of three wells.

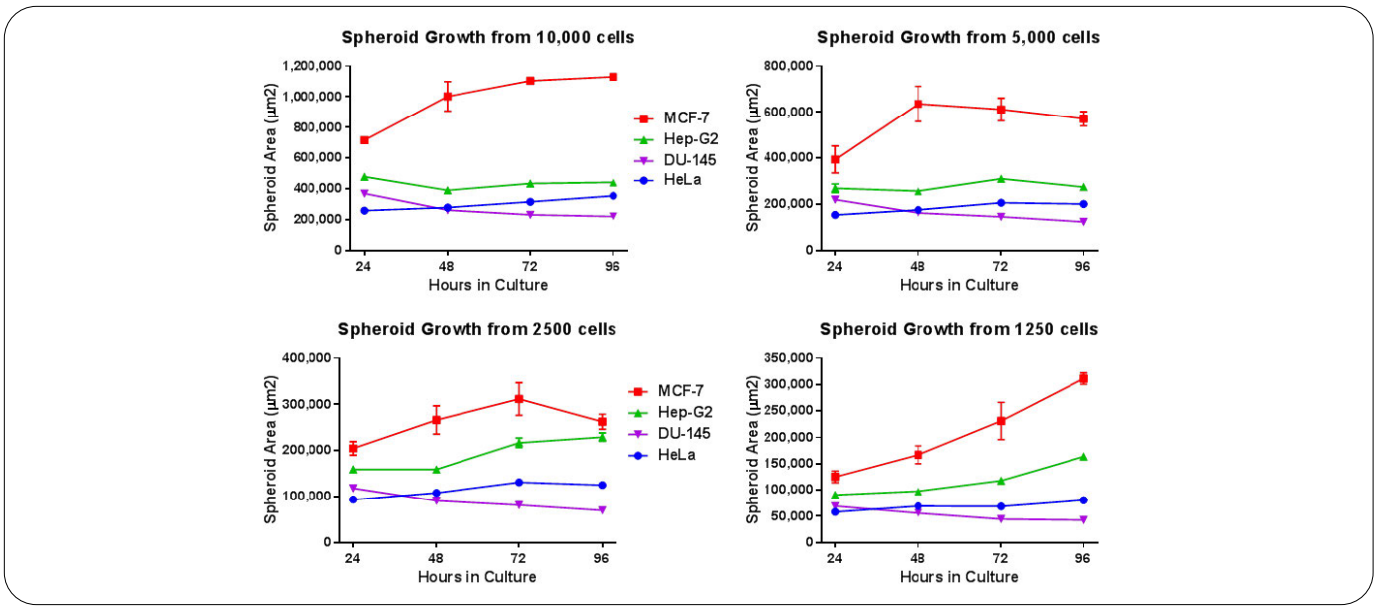


Figure 6: Spheroid growth varies based on cell type and initial cell seeding density. Four graphs of spheroid area changes over three days demonstrate how 3D spheroids produced from four different initial seeding densities and by different cell types grow at different rates. Each data point is an average of three wells.

Frequently asked questions

Q **What is the maximum volume that the wells can hold?**

A CellCarrier Spheroid 96-well plates can handle a total volume of up to 300 µL maximum, but a working volume of between 75 µL to 250 µL is sufficient for seeding and growing spheroid cultures.

Q **How do I change media to feed my cultures?**

A Since the spheroids in ULA-coated microplates are not attached to the well wall, it is not advised to remove all the media from the well for feeding cultures. For a media change, you need to remove a portion of the spent media from the wells and replace it with fresh media. To leave the spheroids undisturbed, we recommend performing this media change off-center using the sides of wells to remove and add media and to set electronic pipettors to a slower speed for removing media. Exchanging 50% of the media in each well is the simplest and most straightforward method. For example, to feed cultures with a starting volume of 100 µL, remove 50 µL of media from the sides of each well and add 50 µL of fresh media.

If a full media exchange or wash step is necessary, we recommend leaving behind a minimum of 10 µL to 20 µL per well. Use the plate dimensions listed at the end of this guide to program liquid handling instruments to be at least 2 mm to 3 mm above the bottom of the wells and slightly off-center and set it to a slow pipetting speed.

Q **What is the advantage of using a round-bottom vs. a flat-bottom microplate for forming spheroids?**

A The round well bottom design and ultra-low attachment coating of the Revvity CellCarrier Spheroid ULA microplate reliably produces single, uniform spheroids in each well. 3D cultures generated in flat bottom wells (also known as “clumpoids”) often generate several non-uniform sized clumps of cells that are not evenly distributed and may lack reproducibility across wells. Because of the variation from well to well, the technology does not lend itself to high throughput screening depending on the user’s application.

Q **What are the recommended storage conditions for CellCarrier ULA Spheroid Plates?**

A CellCarrier Spheroid ULA microplates with ULA coating are extremely stable and may be stored at room temperature.

Q **What cell lines have been validated as forming spheroids in CellCarrier Spheroid ULA 96-well microplates?**

A So far, the following cell lines have been used in house and observed to form spherical cultures: DU 145, HeLa, HEK293, Hep G2, A549, SKOV3, and BT474. MCF 7 cells on their own appear to form more loosely-associated clumps of tissue that overall are relatively spherical in shape.

Q How do I remove and transfer spheroid cultures to a slide for immunohistochemical staining or other microplate for higher-resolution imaging?

A Though many applications can be performed directly in the spheroid-producing plate, it may be necessary to remove the intact spheroids to another plate or surface for processing the tissue. This is a bit tricky but can be done with a manual pipette with a 200 µL wide-bore tip or, alternatively, one can clip a standard sized tip to make a hole big enough for aspirating the spheroid tissue without adding physical stresses. Set the pipette to a volume between 30 µL to 50 µL for aspirating and place the pipette tip just above or next to the tissue on the plate bottom (with the clarity of the plate, you can see larger spheres by eye) and aspirate at 'normal' speed.

Q What are the plate dimensions to use in my plate reader, automated liquid handlers or other instrumentation?

A Revvity CellCarrier Spheroid ULA 96-well microplates adhere to standard SBS footprint dimensions for a 96-well microplate. Specific dimensions for programing instrumentation are listed below:

Working volume per well: 75 µL to 250 µL

MAXIMUM well volume: 300 µL

Well depth: 10.03 mm

Well diameter at top: 6.35 mm

Plate length: 127.75 mm

Plate width: 85.34 mm

Plate height: 14.15 mm

Stack height (with lid): 16.00 mm

A1 row offset: 11.00 mm

A1 column offset: 14.40 mm

Well to well (Center) spacing: 9.00 mm

Flange or skirt height: 2.40 mm

Well bottom thickness: 1.02 mm

Distance to bottom of plate: 3.10 mm

Q What specifications do I need to know in order to use Revvity's high-content imaging systems?

A For high content imaging of 3D tissue using Revvity's Operetta CLS and Opera Phenix Plus, the following specifications should be entered into the Plate Type Definitions Wizard in Harmony Software:

A: 14.40 mm

B: 11.00 mm

C: 127.75 mm

D: 85.34 mm

E: 113.50 mm

F: 74.15 mm

G: 14.15 mm

H: 3.10 mm

I: 1.02 mm

J: 6.35 mm

M: 0.60 mm

Working volume: 200 µL

Refractive index = 1.58; Polystyrol

Additional information

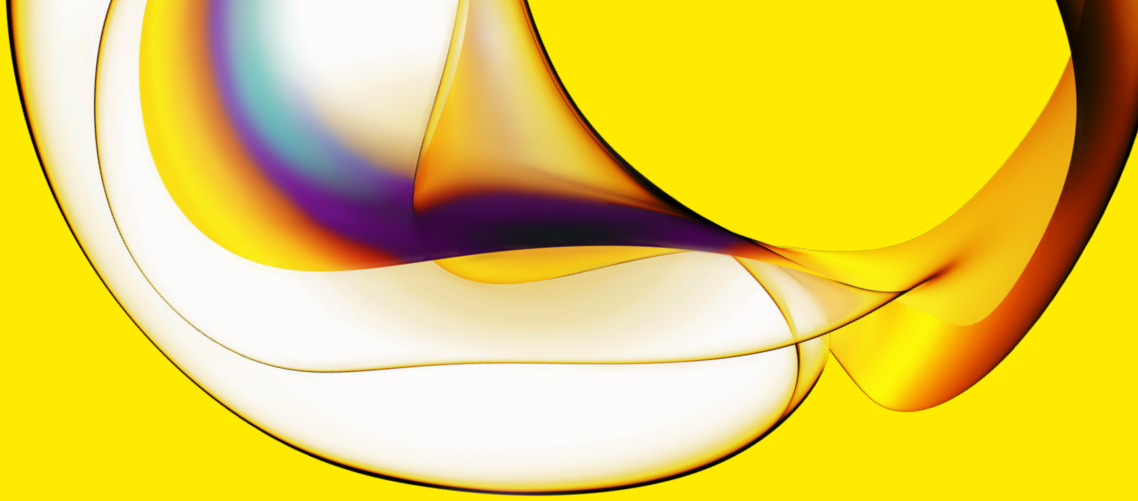
The following Revvity application notes illustrating multiple ways to assay spheroids are available at www.revvity.com:

- Imaging Bile Canaliculi in 3D Liver Microtissue using the Opera Phenix High Content Screening System, 2015.
- Cytotoxicity Studies on 3D Primary Liver Microtissues, 2014.
- Inhibition of MicroRNA Sensitizes 3D Breast Cancer Microtissues to Radiation Therapy, 2014.
- Imaging Microtissue Cores using the Opera High Content Screening System, 2013.
- Quantitative Analysis of 3D Microtissue Growth and Biomarker Intensity, 2012.

Authors

Jeanine Hinterneder, Ph.D.
Henk Koning
Anne Kwei
Richard Hellmer

Revvity, Inc.



www.revvity.com

Revvity, Inc.
940 Winter Street, Waltham, MA 02451 USA
(800) 762-4000 | www.revvity.com

For a complete listing of our global offices, visit www.revvity.com

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