**Introduction**

Scientists using cell cultures for drug discovery, toxicology, stem cell biology, and basic research realize the critical importance of 3-dimensional (3D) models. Data from cells cultured in a non-physiologic, monolayer format on plastic surfaces has long been suspected to differ from true in vivo physiology, and evidence supporting how this difference is slowing the pace of scientific discovery is mounting. Much of a candidate drug’s early discovery and screening is performed using 2-dimensional (2D) cell monolayers that clearly do not recapitulate the 3D complexity seen within the human body. The most cost-effective solution is to obtain better targets and initial toxicological results using relevant cell culture models. In the past, there were few affordable, reliable choices for 3D culture and almost none that were amenable to high-throughput screening. Spheroids, self-assembled microscale cell aggregates, are superior models of avascular in vivo microtumors. Using hanging drop plate (HDP) technology, a drop of cell suspension is pipetted into the top of each well. The cell suspension forms a stable drop below where the cells aggregate into spheroids.

Two critical steps must be accomplished to facilitate spheroid formation for 3D culture in the HDPs. These include dispensing cells and medium into the wells of the Hanging Drop Plate. Cytation™3 Cell Imaging Multi-Mode Reader: Cytation 3 combines optimized automated digital widefield microscopy and conventional plate detection. The instrument was used to image the cells and spheroids using fluorescence and brightfield imaging, while maintaining optimal temperature and environmental conditions through software-based temperature control and a gas control module.

**BioTek Instrumentation**

MultiFlo™FX Microplate Dispenser. The MultiFlo™FX Microplate Dispenser was used to dispense cells and medium into the wells of the Hanging Drop Plate. Cytation™3 Cell Imaging Multi-Mode Reader: Cytation 3 combines automated digital widefield microscopy and conventional microplate detection. The instrument was used to image the cells and spheroids using fluorescence and brightfield imaging, while maintaining optimal temperature and environmental conditions through software-based temperature control and a gas control module.

**Perfecta3D® 96-Well Hanging Drop Plates**

![Image: Hanging Drop Plate Assembly](Image)

Figure 1. Perfecta3D Hanging Drop Plate Assembly

![Image: Manual Hanging Drop Formation Procedure](Image)

Figure 2. Manual Hanging Drop Formation Procedure. Pipette tips, filled with 30-50 μL of cell suspension, are inserted halfway into the well (A). Cell suspension is slowly dispensed (B), and a spheroid begins to form on the bottom side of the Well (C). Tips are then removed (D), while the drop forms below each well with the cells inside.

3D Biomatrix’s Perfecta3D HDPs (Figure 1) facilitate 3D spheroid formation in 96- or 384-well plates with a 4x or 10x objective, or a long working distance 20x objective. Automating each of these processes provides a 3D solution that is less labor intensive and more reproducible than previous methods, and further promotes the use of this spheroid formation method.

**Materials and Methods**

**Cells:** MCF-7 breast adenocarcinoma cells stably expressing GFP (Catalog No. ARK-211) were purchased from Cell Biolabs, Inc. (San Diego, CA). The MCF-7 cells were propagated in Minimum Essential Medium α (Catalog No. 12561-056) plus Fetal Bovine Serum, 10% (Catalog No. 10437), Pen-Strp, 1x (Catalog No. 15140-122), and Human Recombinant Insulin (Catalog No. 12585-014) from Life Technologies (Carlsbad, CA). Human Neonatal Dermal Fibroblast cells stably expressing RFP (Catalog No. 6009MRP) were purchased from Angio-Protein (Boston, MA). The fibroblast cells were propagated in DMEM/F12 Opti-MEM® High Glucose (Catalog No. 11995-065) plus Fetal Bovine Serum, 10% (Catalog No. 10437) and Pen-Strp, 1x (Catalog No. 15140-122) from Life Technologies (Carlsbad, CA).

**Cell Preparation:** For spheroids containing a single cell type, MCF-7 GFP cells were harvested and diluted to concentrations of 0.25x10⁶, 2.5x10⁶, 1.25x10⁷, and 2.5x10⁷ cells/mL. When dispensed to the HDP in 40 μL volumes, final cell concentrations equaled 25,000, 10,000, 5000, and 1000 cells per spheroid. For co-cultured spheroids, MCF-7 GFP and fibroblast cells were harvested and diluted to concentrations of 2.5x10⁶ and 6.25x10⁶ cells/mL. The cells were then combined to form a single final volume to create spheroids containing 2000, 1000, 500, or 200 cells per spheroid and equal numbers of each cell type.

**Automated Cell Dispensing and Image-Based Spheroid Formation Tracking**

**Automated HDP Cell Suspension Dispensing Procedure**

Prior to dispensing, the plate and tray reservoirs (Figure 2) were filled with 3 and 5 mL of sterile Dulbecco’s phosphate buffered saline (DPBS). The buffer was added to provide additional humidity above and below the spheroids during incubation. A 30-50 μL volume of cell suspension is recommended to be dispensed into the 96-well hanging drop-plate wells. For the purposes of this application, a 40 μL dispense volume was chosen in order to reduce the dispense speed, and allow the cell suspension to dispense in a droplet rather than a stream of liquid, a 5 μL cassette was placed into the MultiFlo™FX primary or secondary peristaltic pump position, while a 10 μL cassette was selected in the Dispenser Utilities interface in the same position. This deviation also causes half the volume to move through the cassette tubing than is normally expected.

**Table 1. MultiFlo FX Perfecta3D HDP Dispense Parameters.**

<table>
<thead>
<tr>
<th>Setup</th>
<th>Flow Rate</th>
<th>Peri Pump Cassette Used</th>
<th>LHC Programmed Peri Pump</th>
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<td>Dispensing Method</td>
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<tr>
<td>Y-Axis Position</td>
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</table>

In addition to the 5000 cell spheroids, spheroids were also formed using 25,000, 10,000, and 1000 MCF-7 GFP cells and the procedure previously described.

**Conclusions**

1. Cell suspensions can be rapidly dispensed in a uniform manner into the Perfecta3D 96-Well Hanging Drop Plates using the peristaltic pump dispenser on the MultiFlo FX
2. Variable cell concentrations, volumes, or co-cultures are also possible using the single or dual peristaltic pump configurations
3. Consistent spheroid formation is easily accomplished in the hanging drop plates using a variety of cell types
4. The configuration of the plate assembly allows for image-based spheroid formation tracking without the need for removal of the bottom tray, therefore not jeopardizing the sterile environment around the cells
5. Temperature and gas control are consistent, proper atmospheric conditions during imaging
6. The combined features of instrumentation and plates create an ideal solution to perform automated dispensing into the Perfecta3D HDPs, consistent cell aggregation, and tracking of spheroid formation.

**Cell/Spheroid Imaging**

Precising cell imaging, Cytation 3’s temperature control was set to 37 °C, and the gas control module was set to 5% CO2. Following cell dispensing, the plate assembly was inserted into the Cytation 3 and Manual Imaging Mode was selected for Time 0 imaging. The imager can focus through the clear tray below the hanging drops, which comes with the HDPs; therefore it was not necessary to remove the tray before placing the plate onto the stage. Due to the fact that cells may be at multiple z-planes at Time 0, the Seek function was used to manually find the location where the largest number of cells were in focus. This can be carried out using brightfield imaging for unlabeled cells, or with the appropriate fluorescent imaging channel for labeled cells. Auto Exposure and Focus were then used to create the highest quality image.

On subsequent days, the plate assembly was once again placed into the Cytation 3. The cells moved to the bottom of the drop during the aggregation process, therefore manual focusing was once again performed to find the spheroid location. The typical focal height seen was approximately 4000 μm. The appropriate determined height was then entered as the bottom elevation for the plate height in the Gen5™ software, which then facilitated the use of subsequent automated imaging using a single, consistent focal height.

**Spheroids**

Spheroids were also created containing a co-culture of MCF-7 and human dermal fibroblast cells. The importance role that fibroblast cells play in tumor progression was previously shown (Li et al., 2011). Therefore forming 3D cell structures which contain co-cultured cancer cells, in addition to fibroblasts, further promotes the creation of an in vitro cell model which closer resembles a true in vivo tumor environment. Spheroids containing 2000, 1000, or 500 total cells, with equal numbers of cancer and fibroblast cells, were formed in the HDPs.