Automated Cell Dispensing and Image-Based Spheroid Formation Tracking

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Introduction

Scientists using cell culture 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, and tracking spheroid formation. Dispensing into the plates can be manual or automated. Automated dispensing is faster than manual pipetting, which is the preferred method, especially with the 384-well plate. Cell imaging in the hanging drop is possible with a 4x or 10x objective, or a long working distance 20x objective.

Here we demonstrate how dispensing and imaging procedures were accomplished to facilitate higher throughput in 3D HDP processing. 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.

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 Plates.

Cytation™3 Cell Imaging Multi-Mode Microplate 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

Figure 1. Perfecta3D Hanging Drop Plate Assembly.

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 drop begins to form on the bottom side of the well. Tips are then removed (C), while the drop fully forms below each well with the cells inside.

3D Biomatrix's Perfecta3D HDPs (Figure 1) facilitate 3D spheroid formation in 96- or 384-well formats. A drop of cell suspension is pipetted into the top of each well (Figure 2A and B), and the plate geometry causes it to hang stably below the well (Figure 2C). Spheroid diameter can be controlled with the type and number of cells added. Access holes at the top of each well allow for media exchange and the addition of compounds, reagents or additional cells or spheroids to establish co-cultures. Without contact with any surfaces or matrices, cells aggregate together to form one spheroid per well.

Materials and Methods

Cells: MCF-7 breast adenocarcinoma cells stably expressing GFP (Catalog No. AKR-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-Strep, 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. cAP 0008RFP) were purchased from Angio-Proteomie (Boston, MA). The fibroblast cells were propagated in Dulbecco's Modified Eagle Medium, High Glucose (Catalog No. 11995-065) plus Fetal Bovine Serum, 10% (Catalog No. 10437) and Pen-Strep, 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 $6.25x10^5$, $2.5x10^5$, $1.25x10^5$, and $2.5x10^4$ 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 $2.5x10^4$, $1.25x10^4$ and 6.2^5x103 cells/mL. The cells were combined together in a single final volume to create spheroids containing 2000, 1000, or 500 cells per spheroid and equal numbers of each cell type.

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's primary or secondary peristaltic pump position, while a 10 µL Cassette Type 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.

Instrument Setup	
Dispensing Method	Peristaltic Pump
Peri Pump Cassette Used	5 µL
LHC Programmed Peri Pump	10 µL
Dispense Step Settings	
Flow Rate	Low
LHC Programmed Volume	80 µL
Actual Dispensed Volume	40 µL
Z-Axis Position	229
X-Axis Position	10
Y-Axis Position	0

Table 1. MultiFlo FX Perfecta3D HDP Dispense Parameters.

A "Peri-pump Dispense Step" was then selected. In order to dispense 40 µL using the specialized method, 80 µL was entered as the dispense volume. Further dispense settings included selecting a "Low" Flow Rate, in addition to positioning coordinates of Z: 249; X: 10; and Y: 0. The X coordinate shifts the dispense tips to the right of center, while the Z coordinate further ensures that drops consistently form beneath each well. In this manner, 40 µL of cell suspension was slowly dispensed as a droplet without falling through the well bottom, and with optimal contact between the well sides and the droplet.

Cell/Spheroid Imaging

Preceding cell imaging, Cytation 3's temperature control was set to 37 °C, and the gas control module was set to 5% CO_2. 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 Expose and Auto 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.

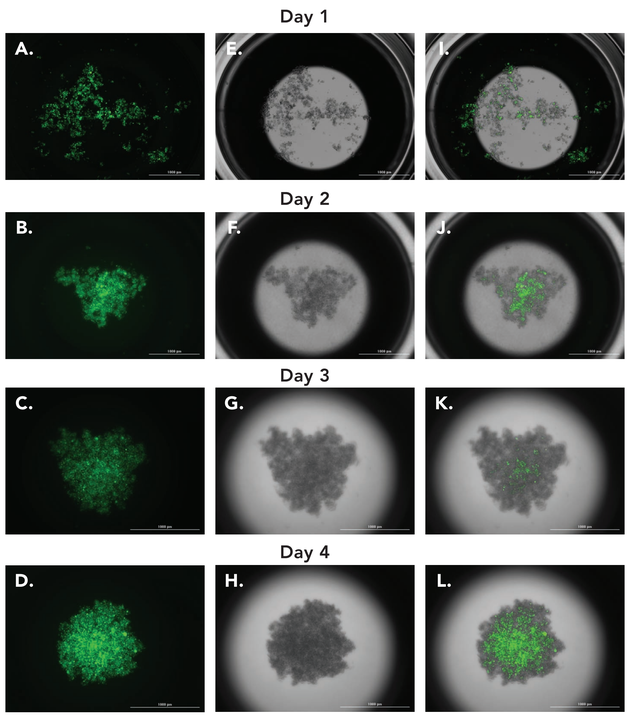

Figure 3. 5000 Cell Spheroid Formation Tracking. MCF-7 spheroid formation tracking over a four day period using GFP fluorescent channel **(A-D)**; or brightfield imaging **(E-H)**, in addition to 2.5x magnification. Gen5 software used to create final overlaid composite images **(I-L)**.

Images were captured of a well containing 5000 MCF-7 GFP cells after 1, 2, 3, and 4 days of incubation (Figure 3). Fluorescent imaging using the GFP channel (Figure 3A-D), as well as brightfield imaging (Figure 3E-H) was carried out to fully visualize cell configuration on each day. Brightfield and GFP images were then overlaid to create the final composite images (Figure 3I-L).

Variable Cell Spheroid Imaging

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.

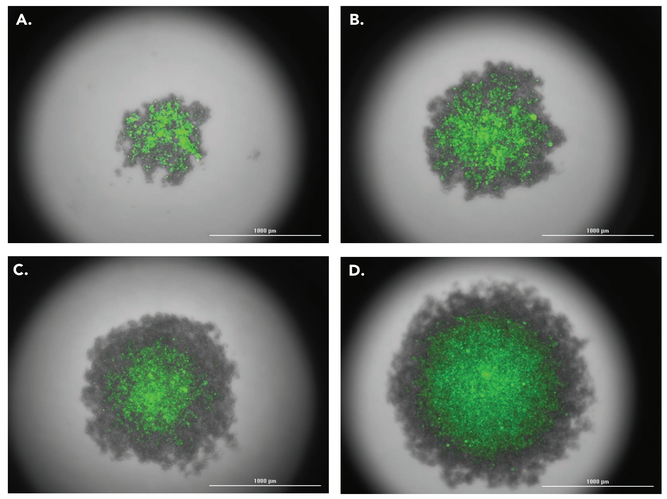

Figure 4. Variable Cell Spheroid Formation Tracking. Overlaid GFP fluorescence and brightfield images of MCF-7 spheroids containing **(A.)** 1000, **(B.)** 5000, **(C.)** 10,000, or **(D.)** 25,000 cells, using 2.5x magnification.

Fluorescent GFP and brightfield composite images demonstrated how consistently formed spheroids can be created with the MultiFlo FX using the hanging drop plates, and can also be imaged using the Cytation™ 3 Cell Imaging Multi-Mode Reader.

MCF-7 Cell/Human Dermal Fibroblast Spheroid Formation Imaging

Spheroids were also created containing a co-culture of MCF-7 and human dermal fibroblast cells. The important 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.

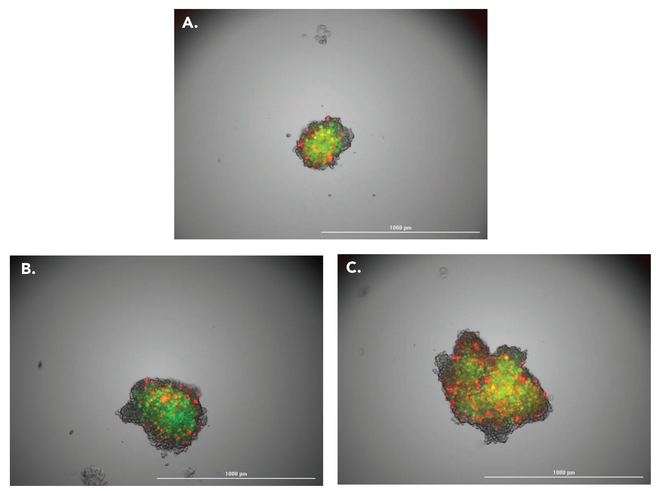

Figure 5. Co-cultured Cell Spheroid Formation Tracking. Overlaid fluorescence and brightfield images of MCF-7/human dermal fibroblast spheroids containing **(A.)** 500, **(B.)** 1000, or **(C.)** 2000 cells. Images were captured using 4x magnification. GFP and RFP expressed from MCF-7 and fibroblast cells, respectively.

Co-cultured MCF-7/fibroblast spheroids were also imaged following an aggregation time of four days. Individual images of each spheroid were once again captured using the GFP, RFP, and brightfield imaging channels. Overlaid images not only aid to determine the completion of the cell aggregation process, but also allow for the visualization of the dispersal and concentration of each cell type within the spheroid.

Conclusions

1. Cell suspensions can be rapidly dispensed in a uniform manner to 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 ensure 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