Automated Cell Dispensing and Image-Based Spheroid Formation Tracking

Using the MultiFlo™ FX and Cytation™ to Dispense Cell Suspensions into Hanging Drop Plates and Track Spheroid Formation via Digital Widefield Imaging

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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 there is mounting evidence that this difference is slowing the pace of scientific discovery. It was recently estimated that the cost for bringing a new drug to market is nearly two billion dollars\(^1\). The primary reason for the exorbitant cost of new drug development is the extremely high 95% failure rate attributed to either lack of efficacy or unforeseen toxicity\(^2\). Much of a candidate drug’s early discovery and screening is performed using 2-dimensional (2D) cell monolayers that clearly do not mimic tissue physiology. The most cost effective solution is to obtain better targets and initial toxicological results using more relevant cell culture models before candidates progress to animal testing.

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, generated via Perfecta3D® Hanging Drop Plates (HDP)s from 3D Biomatrix, are superior models of avascular in vivo microtumors. Using this system, a drop of cell suspension is pipetted into the top of each well, which contains a small hole at the bottom. The cell suspension forms a stable drop below each well where the cells aggregate into spheroids (see Figure 1).

In both the 96- and 384-well plate formats, the spheroids form mass-transfer gradients of oxygen nutrients, wastes, and therapeutic drugs that are highly comparable to what is observed in tumors within the human body.

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, and 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. Tracking simultaneous formation of multiple spheroids can be accomplished by using the Cytation™ 3 Cell Imaging Multi-Mode Reader by BioTek Instruments.

In this application note we demonstrate how dispensing and imaging procedures were automated to facilitate higher throughput in 3D HDP processing. The MultiFlo™ FX Microplate Dispenser was used to consistently and gently dispense suspended cells into the 3D 96-well HDPs, while Cytation 3 Cell Imaging Multi-Mode Reader was used to easily visualize and track cells and spheroids in each hanging drop. 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.

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Figure 1. Hanging Drop Plate Assembly, consists of hanging drop plate (HDP), a lid and a tray. Both HDP and tray possess reservoirs. Together with the lid, they provide adequate humidity control when the reservoirs are filled with water.
Materials and Methods

Materials

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).

96-Well Hanging Drop Plates

Perfecta3D® 96-Well Hanging Drop Plates (Catalog No. HDP-1096) were provided by 3D Biomatrix, Inc. (Ann Arbor, MI).

Instrumentation

MultiFlo FX Microplate Dispenser

The MultiFlo FX Microplate Dispenser was used to dispense cells and medium to the wells of the Hanging Drop Plates.

Cytation 3 Cell Imaging Multi-Mode Reader

The Cytation 3 Cell Imaging Multi-Mode Reader was used to image the cells and spheroids using fluorescence and brightfield imaging, while maintaining optimal temperature and environmental conditions.

3D Cell Culture Components

Perfecta3D 96-Well Hanging Drop Plates

3D Biomatrix’s HDPs facilitate 3D spheroid formation in 96- or 384-well formats. A drop of cell suspension is pipetted into the top of each well, and the plate geometry causes it to hang stably below the well (see Figure 2). 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 cocultures. Without contact with any surfaces or matrices, cells aggregate together to form one spheroid per well.

Methods

Cell Preparation

For spheroids containing a single cell type, MCF-7 GFP cells were harvested and diluted to concentrations of 6.25x10⁵, 2.5x10⁵, 1.25x10⁵, and 2.5x10⁴ 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 2.5x10⁵, 1.25x10⁵ and 6.25x10⁴ 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 Cell Suspension Dispensing into Hanging Drop Plates

Prior to dispensing, the plate and tray reservoirs were filled with 3 and 5 mL of sterile Dulbecco’s phosphate buffered saline (DPBS). 3D Biomatrix recommends dispensing 30-50 µL of cell suspension 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.

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% 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 was in focus.

This can be carried out using brightfield imaging for unlabeled cells, or with the appropriate fluorescence 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.

MCF-7 imaging was performed using a 2.5x microscope objective. Co-cultures of MCF-7 and fibroblasts were imaged with a 4x microscope objective.

**Results and Discussion**

**MCF-7 Cell Spheroid Formation Imaging**

Images of a well containing 5000 MCF-7 GFP cells were captured after 1, 2, 3, and 4 days of incubation (Figure 3). Fluorescence 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).
In addition to the 5000 cell spheroids, spheroids were also formed using 25,000, 10,000, and 1000 MCF-7 GFP cells. Fluorescence GFP and brightfield composite images demonstrated how consistently formed spheroids can be created using the hanging drop plates, and can also be imaged using the Cytation 3 Cell Imaging Multi-Mode Reader.

Figure 4. Variable Cell Spheroid Formation Tracking. Overlaid GFP fluorescence and brightfield images of MCF-7 spheroids containing 1000, 5000, 10,000, or 25,000 cells.

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. 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 created and imaged using fluorescence GFP or RFP, as well as brightfield imaging channels.

Figure 5. Co-cultured Cell Spheroid Formation Tracking. Overlaid fluorescence and brightfield images of MCF-7/human dermal fibroblast spheroids containing 500, 1000, or 2000 cells. GFP expression from MCF-7 cells and RFP expression from fibroblast cells were measured.

Conclusions

The information provided here explains how the MultiFlo™ FX was used to dispense cell suspensions of various concentrations to the wells of the Perfecta3D® 96-well Hanging Drop Plates. Furthermore, the images shown illustrate how the spheroid formation process was tracked using fluorescence or brightfield imaging using the Cytation 3. The plate assembly remained intact, as the instrument is capable of focusing through the tray to the location of the suspended spheroid. Temperature and gas control ensure that proper cellular atmospheric conditions are maintained during imaging. These combined capabilities create an ideal solution to perform automated dispensing into the Hanging Drop Plates, as well as image-based tracking of spheroid formation.

References

