**Introduction**

Z-projection is a digital image processing method, which combines multiple images taken at different focal distances (z-stacking) to provide a composite image with a greater depth of field (i.e. the thickness of the plane of focus) than any of the individual source images. It is particularly useful for capturing in-focus images of objects under high magnification as depth of field (DOF) decreases with magnification primarily because microscope objectives with higher magnification have typically higher numerical apertures (NA). According to the Shillaber equation, DOF relates to NA for a given wavelength of light ($\lambda$) and medium refractive index ($n$):

$$DOF = \frac{\lambda}{2(n-1)}(\frac{NA}{n})^2$$

Table 1 illustrates this concept for a series of commercially available microscope objectives using 500 nm light and air as the medium ($n = 1.00$) between microscope objective and object.

<table>
<thead>
<tr>
<th>Magnification</th>
<th>Numerical Aperture</th>
<th>Depth of Field (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x</td>
<td>0.10</td>
<td>50</td>
</tr>
<tr>
<td>10x</td>
<td>0.25</td>
<td>7.7</td>
</tr>
<tr>
<td>20x</td>
<td>0.40</td>
<td>2.9</td>
</tr>
<tr>
<td>40x</td>
<td>0.65</td>
<td>0.9</td>
</tr>
<tr>
<td>60x</td>
<td>0.85</td>
<td>0.36</td>
</tr>
<tr>
<td>100x</td>
<td>0.95</td>
<td>0.17</td>
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</tbody>
</table>

**Materials and Methods**

**Materials**

**Cells**

Colorectal carcinoma HCT 116 cells (Catalog No. CCL-247) were obtained from ATCC (Manassas, VA). The cells were propagated in McCoy’s 5A Medium (Catalog No. 15600) plus Fetal Bovine Serum, 10% (Catalog No. 10437) and Pen-Strep, 1x (Catalog No. 15140) from Life Technologies (Carlsbad, CA). The cells were plated at a final density of $2.5 \times 10^5$ cells/mL for 72 hours prior to performing the assay.
Fluorescent Probes

Hoechst 33342 (Catalog No. 14533) was purchased from Sigma-Aldrich Corporation (Saint Louis, MO). Alexa Fluor® 488 phalloidin (Catalog No. A12379), and CellMask™ Orange plasma membrane stain (Catalog No. C10045) were purchased from Life Technologies (Carlsbad, CA).

RAFT™ Reagents and Plates

96-well RAFT Plate and 96 -well Culture Plate are part of the 4 x 96 RAFT™ Plate Kit (Catalog No. 016-0R92). Collagen Solution, 10x Minimum Essential Medium, and RAFT™ Neutralising Solution are part of the RAFT™ Reagent Kit (Catalog No. 016-0R94). All RAFT™ components were supplied by TAP Biosystems (Hertfordshire, UK), now exclusively distributed by Lonza BioScience.

Instrumentation

Cytation™ 5 Cell Imaging Multi-Mode Reader

Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides high resolution microscopy in fluorescence, brightfield, color brightfield and phase contrast. Cytation 5 has temperature control to 65 °C, CO₂/O₂ gas control and dual injectors for kinetic assays. The instrument was used to image spheroids, as well as individual cell invasion through the Matrigel matrix.

Gen5™ Image+ Data Analysis Software

Gen5 Image+ software controls the operation of the Cytation™ 5 for both automated digital microscopy and microplate reading. Image acquisition is completely automated from sample translation, focusing and exposure control. Users can also optimize and automate acquisition of images through 3D cellular structures or tissue, as well as creation of the final Z-Projection.

3D Cell Culture Components

RAFT™ 3D Cell Culture System

Figure 1. Creation of 3-Dimensional Cell/Collagen Hydrogel using RAFT™ System. (A) Cell/collagen mix dispensed to wells of 96-well plate. (B) 96-well RAFT™ plate containing individual sterile absorbers. (C) Absorber insertion into plate well. (D) Absorption of medium, concentrating collagen and cells to in vivo strength. (E) Completion of absorption process creating 120 µm thick hydrogel. (F) Removal of absorber prior to dispense of fresh cell medium.

The RAFT™ (Real Architecture for 3D Tissue) cell culture technique developed by TAP Biosystems, now exclusively distributed by Lonza BioScience, allows researchers to culture cell type(s) of their choice in an in vivo like collagen hydrogel environment. The technology uses the most abundant matrix protein in the body, type I collagen. The RAFT™ process raises the collagen concentration to physiological levels quickly and reproducibly. It takes less than 1 hour to generate cell cultures which are ~120µm thick, biomimetic, dimensionally stable and transparent with high cell viability.
Methods

3D Tumoroid Formation Process

Day 1

HCT116 cells were added manually to the prepared collagen solution. The cell suspension was then dispensed to the 96-well plate in a volume of 240 μL per well. The final cell concentration equaled 25,000 cells/well. The cell plate was then incubated at 37 °C/5% CO₂ for 15 minutes, followed by manual addition of the absorbers in the RAFT™ plate, and an additional 15 minute incubation at 37 °C/5% CO₂ during which the RAFT™ process increases the collagen density to a physiologically relevant strength. The absorbers were then removed and 100 μL of new medium was then added to the concentrated cell/collagen hydrogel. The plate was once again incubated at 37 °C/5% CO₂ for three days to allow the tumoroids to form.

Day 4

Following the incubation period, the spent medium was removed and the tumoroids were stained with the Hoechst 33342, Alexa Fluor® 488 phalloidin, and CellMask™ Orange plasma membrane fluorescent probes.

Creation of Z-Stacked Images

In the imaging procedure read step, selection of objective, imaging channel and exposure settings is performed in a manner similar to that for single image set capture. “Image Z-Stack” is then selected (Figure 2). The number of slices, or images taken through the structure can be manually chosen depending on the definition desired. “Step size” is the distance in μm that the objective will move in the z-axis between each captured image. The default value is the depth of field for the objective chosen; 2.5x: 68 μm; 4x: 53 μm; 10x: 9 μm; 20x: 4 μm; 40x: 2 μm; 60x: 1 μm, which can also be manually adjusted should a higher number of slices be desired.

To determine the sample thickness and number of slices required to image through the complete structure, it is recommended to select manual imaging with one of the channels to be used. “Auto Focus” is selected to allow the Cytation to focus on a point within the structure. The focal height is then changed manually in each direction of the z-axis to the point where a portion of the spheroid or tissue remains in focus (Figure 3).

Z-Projection Creation

Following capture of the z-stacked images, z-projection can be completed by performing a “Z-Projection” Data Reduction step (Figure 4). Individual imaging channels can be chosen for inclusion in the projected image. The top and bottom image slices to use can be optimized to guarantee that the most in-focus image is created. Multiple projection methods exist which incorporate different algorithms for selecting the most in-focus portion of the z-stacked images. The method providing the desired projection may vary depending on the images captured and the final analysis required.
If additional imaging channels are to be included in the projection (Figure 5), subsequent channel tabs can be selected. The parameters can be kept consistent with those used for the initial channel (Figure 5A), or can also be changed if necessary (Figure 5B).

A. B.

Results and Discussion

Tumoroid Image Deconvolution and Cellular Analysis

The cells in the aggregated three dimensional tumoroid structures can be found on multiple z-planes within the RAFT™ hydrogel. Experimental analysis of the effects that a potential drug candidate has on these structures, depending on the assay and test being performed, can at times be accomplished using single plane imaging. However, this is not always the case, and a final image showing improved cellular definition may be necessary. Upon visualization of the images displayed in Figure 6, it was apparent that the z-projection of the z-stack (Figure 6D) allowed the cells to be seen with more detail and greater clarity in each tumoroid structure compared to the single plane images (Figures 6A-C).
Conclusions

3D cellular models continue to be incorporated into a growing number of research areas; including dermatology and drug discovery. Here we have shown that the Cytation™ imagers, in conjunction with Gen5™ Image Analysis Software, can provide improved depth of fields of cells aggregated into tumoroid structures within the RAFT™ hydrogel in a rapid, automated fashion, while providing accurate evaluation and conclusions from z-projected images.

References


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