ABSTRACT

Three-dimensional (3D) systems of cell culture can provide a more representative model of solid tumors and more physiologically relevant outcomes from drug screens than two-dimensional cell cultures. A diverse array of 3D models exist, each with challenges to establish and maintain at the high throughputs required for screens. Cancer spheroids are large clusters of cells formed in suspension to replicate the growth of parent tumors, and drugs are assayed by their ability to penetrate the tumor and to elicit a response. Matrigel® or other hydrogel cultures are often used to replace native cell culture matrix and to enable or to study cell structure formation or interactions. To overcome manual challenges and improve reliability, we automated the drug treatment, and analysis of these 3D models. We were able to form consistent cancer spheroids in both hanging drop and low adherence plates and used imaging and flow cytometry to gain a more complete, unbiased understanding of the cytotoxicity of the drug repressor in the model. Cells were also plated on top of and embedded within Matrigel and cell growth and apoptosis metrics were measured with standard and confocal imaging approaches. Both 3D models were miniaturized to 384-well format, thereby enabling the throughputs required for large-scale screens to identify novel cancer treatments.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

iC3DTE colorectal carcinoma cells (ATCC) were cultured in McCoy’s 5A Modified Medium with 10% Fetal bovine serum, 3D cultures were treated with serial dilutions of staurosporine, 5-Fluorouracil (both from Sigma Aldrich) , and camptothecin (HDQ Milipore). Cultures were assayed for cell death by staining with a EarlyTox™ Cell Integrity Kit (Molecular Devices). We also utilized the SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices) to measure initial cell growth for Matrigel surface cultures, 100 μL XTT reagent was added to wells and incubated for three days. Day 4 %CV

AUTOMATED 3D CULTURES

A BioHeath® enclosure (Figure 1, Beckman Coulter) was used to automate all cell plating, drug dilution and treatment, and sample preparation for analysis. Spheroids: 4000 cells were plated in 40 μL of medium in well 96 PerfectQ® Hanging Drop Plates (“HDP”, 96-well 3D Biomatrix) or 384-well ultra-low attachment plates (“ULA”, Corning). Spheroid formation in HDPs was accelerated by the addition of 0.25% polyvinyl alcohol to the media. After three days of culture, spheroids were assayed for consistency and treated with compounds for 24 hours prior to analysis for cell death.

Matrigel® (Thermo Fisher Scientific) was added to the media of 48 well plates at a 0.25% concentration. After three days of culture, spheroids were analyzed for consistency and treated with compounds for 24 hours prior to analysis for cell death.

HIGH-CONTENT IMAGING

2-D images of spheroids or HDP cultures were acquired with a Czia Imager (Micro (Figure 6) or Imaging/Probes Micro Confocal High-Content Imaging System (Figures 2, 3, 7 in Table 1). Spheroids were imaged with a 60X magnification water immersion lens or a 100X oil immersion lens (top/5 dilutions shown) and stained for cell nuclei (blue) and apoptosis (green) with Hoechst 33342 and Propidium iodide (Figure 7). For immunofluorescence imaging, spheroids were stained with a fluorescent viability dye (blue) and ApoAlert® Caspase-3/7 Green (Figures 4, 6) and propidium iodide (Figure 4). For fluorescent imaging assays, cell nuclei were stained with NuBlue® Live/DeadBrothers® Reagent (Figures 2-4) and 7 for 24 hours. All reagents were from Thermo Fisher Scientific unless otherwise indicated.

FLOW CYTOMETRY

Flow cytometry preparations required the transfer and subsequent disaggregation of spheroids into single cell suspensions by addition of Accutase® (HDQ Milipore) and mechanical disruption through repetitive pipetting. Dissociated cells were then stained and analyzed on a Gallus flow cytometer (Figure 4, Beckman Coulter). Cells were acquired as a pure sample and fluorescence was detected in the FL1 and FL3 channels. Data were analyzed in Kaluza software (Beckman Coulter).

CELL GROWTH ASSAYS

To measure initial cell growth for Matrigel surface cultures, 100 μL XTT reagent was added to wells and incubated for four days. Absorbance was measured at 450 nm using a SpectraMax 3D Imaging Cytometer (Molecular Devices) to measure the percentage of the cell well occupied by spheroids or in embedded colonies (“field analysis” setting: Figure 6).

CANCER SPHEROIDS

Spheroids were imaged at 4X for cytotoxicity assays, images were acquired with FITC, Cy3 or Cy5, and DAPI filters. NucBlue-stained spheroids were used to count cell nuclei and calculate spheroid volume. Matrigel: Threedimensional (3D) Matrigel cultures were treated with serial dilutions of staurosporine, 5-fluorouracil (both from Sigma Aldrich) , and camptothecin (HDQ Milipore). Cultures were assayed for cell death by staining with an EarlyTox™ Cell Integrity Kit (Figure 3, Molecular Devices). We also utilized the SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices) to measure initial cell growth for Matrigel surface cultures, 100 μL XTT reagent was added to wells and incubated for three days. Day 4 %CV

Figure 3. Consistent size and shape of automated spheroids. A) Microscope images showing the three-dimensional colonies stained with a fluorescent viability dye (blue) and ApoAlert® Caspase-3/7 Green (Figures 4, 6) and propidium iodide (Figure 4). For fluorescent imaging assays, cell nuclei were stained with NuBlue® Live/DeadBrothers® Reagent (Figures 2-4) and 7 for 24 hours. All reagents were from Thermo Fisher Scientific unless otherwise indicated.

Table 1. Spheroid measurements

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
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<tbody>
<tr>
<td>Shape factor</td>
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<td>Volume (μm³)</td>
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</tbody>
</table>

MATRIGEL CULTURES

To measure initial cell growth for Matrigel surface cultures, 100 μL XTT reagent was added to wells and incubated for four days. Absorbance was measured at 450 nm using a SpectraMax 3D Imaging Cytometer (Molecular Devices) to measure the percentage of the well occupied by spheroids or in embedded colonies (“field analysis” setting: Figure 6).

Figure 4. Comparison of spheroid size and shape by flow cytometry (Day 3). Spheroid growth on day 3 was measured using the FlowJo software. Data were analyzed in Kaluza software (Beckman Coulter).