Automation and miniaturization of 3D tumor models for compound screens

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ABSTRACT

Three-dimensional (3D) systems of cell culture can provide a more representative model of solid tumors and more physiologically relevant outputs from drug screens than two-dimensional cell cultures. A diverse array of 3D models have been used to investigate tumor physiology and susceptibility to chemotherapeutics but all of these models present challenges to establish and manipulate at the high throughputs required for screens. Cancer spheroids are large clusters of cells formed in suspension to replicate the gradients of gases, nutrients, and drugs seen by solid tumors. In contrast, Matrigel® or other hydrogel cultures are often used to replicate native cell morphology and polarity or to enable studies of 3D structure formation or cell migration. To overcome manual challenges and improve reliability, we automated the plating, drug treatment, and analysis of these 3D models. We were able to form consistent cancer spheroids in both hanging drop and low attachment plates and used imaging and flow cytometry to gain a more complete understanding of the cytotoxic drug response in this model. Cells were also plated on top of and embedded within Matrigel and cell growth and apoptosis induction was measured with standard and confocal imaging approaches. Both 3D models were miniaturized to 384-well format, thereby enabling the throughput required for large-scale screens to identify novel cancer treatments.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

HCT116 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 (EMD Millipore). Cultures were assayed for cell death by staining with an EarlyTox™ Cell Integrity Kit (Figure 3, Molecular Devices) or CellEvent® Caspase-3/7 Green (Figures, 4, 7) and propidium iodide (Figure 4). For fluorescent imaging assays, cell nuclei were stained with NucBlue® Live ReadyProbes® Reagent (Figures 2-4 and 7) for 24 hours. All reagents were from Thermo Fisher Scientific unless otherwise indicated.

AUTOMATED 3D CULTURES

A Biomek FX^P Workstation within a HEPA-filtered 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 96-well Perfecta3D® Hanging Drop Plates ("HDP", 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 analyzed for consistency and treated with compounds for 24 hours prior to analysis for cell death.

Matrigel: Phenol red-free Matrigel (Corning) was diluted to 6 mg/mL with FluoroBrite DMEM medium and for embedded cultures, HCT116 cells were added to a concentration of 100,000 cells/mL. The Matrigel (+/- cells) was added to a single column of a deep well plate that was maintained at 4°C on a shaking Peltier device. 25 µL of Matrigel was added to wells of a 384-well plate. This assay plate was then heated to 37°C on a static Peltier for 30 minutes to solidify the Matrigel. 75 µL FluoroBrite DMEM medium containing 10% FBS was then added to each well. For cell cultures grown on top of the Matrigel surface, HCT116 cells were added to the supplemental media rather than to the Matrigel. Final cell plating numbers were 2500 cells/well for embedded cultures and 8000 cells/well for surface cultures. Cell growth was monitored for three days after plating and day 5 cultures were treated with apoptosis inducers for 24 hours prior to analysis.

HIGH-CONTENT IMAGING

Z-stack images of spheroids or Matrigel cultures were acquired with an ImageXpress® Micro (Figure 4) or ImageXpress® Micro Confocal High-Content Imaging System (Figures 2, 3, and 7, Molecular Devices). Spheroids were imaged at 10X magnification under transmitted light and analyzed for their size (perimeter and area) and circularity (shape factor and elliptical form factor). NucBlue-stained spheroids were used to count cell nuclei and calculate spheroid volume. Matrigel cultures were imaged at 4X for cytotoxicity assays, images were acquired with FITC, Cy3 or Cy5, and DAPI filters.

FLOW CYTOMETRY

Flow cytometry preparation required the transfer and subsequent dissociation of spheroids into single cell suspensions by addition of Accumax® (EMD Millipore) and mechanical disruption through repeated pipetting. Dissociated cells were then stained and analyzed on a Gallios flow cytometer (Figure 4, Beckman Coulter). 2500 events were acquired per 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 hours. Absorbance was measured at 475 nm and 660 nm using the SpectraMax i3X Multi-Mode Detection Platform (Figure 6, Molecular Devices). We also utilized the SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices) to measure the percentage of the well occupied by surface or embedded colonies ("field" analysis setting, Figure 6).

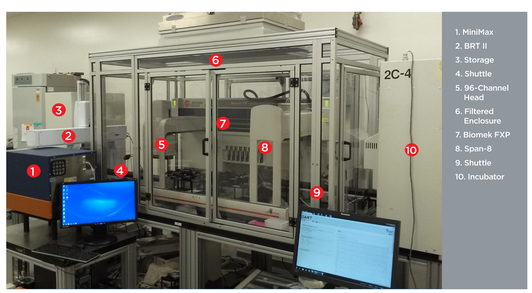


Figure 1. Cellular automation system. The Biomek FX^P Workstation was used to automate the plating, treatment, and sample preparation of spheroid and Matrigel cultures. Integrated incubators, plate and tip storage, and SpectraMax i3X with MiniMax Imaging Cytometer were used to automate all aspects of a 3D cell culture workflow. High-content imaging and flow cytometry analysis were completed offline.

CANCER SPHEROIDS

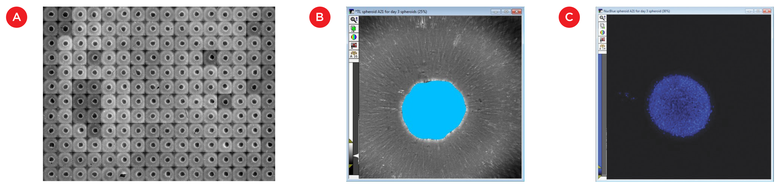


Figure 2. Consistent size and shape of automated spheroids. A) Montage of 192 spheroids imaged at 10X 3 days after automated cell addition into ultra-low attachment plates. B) Spheroids were analyzed for circularity (shape factor, elliptical form factor) and size (perimeter and area) to ensure consistency across wells. C) 32 wells were then stained for 24 hours with NucBlue so that cell nuclei could be counted and spheroid volume determined. Results are shown in Table 1 with low CVs demonstrating consistency across wells.

Table 1. Spheroid measurements

		Day 3 Avg (n=192)	Day 3 %CV	Day 4 Avg (n=32)	Day 4 %CV
Shape	Shape Factor	0.84	4.0%	0.81	0.8%
	Elliptical Form Factor	1.11	7.7%	1.06	3.5%
Size	Perimeter (µm)	1423	4.2%	1828	1.5%
	Area (µm^2)	134529	5.9%	216216	2.8%
	Volume (µm^3)	-	-	5.09x10^7	2.7%
	Nuclei	-	-	529	9.0%

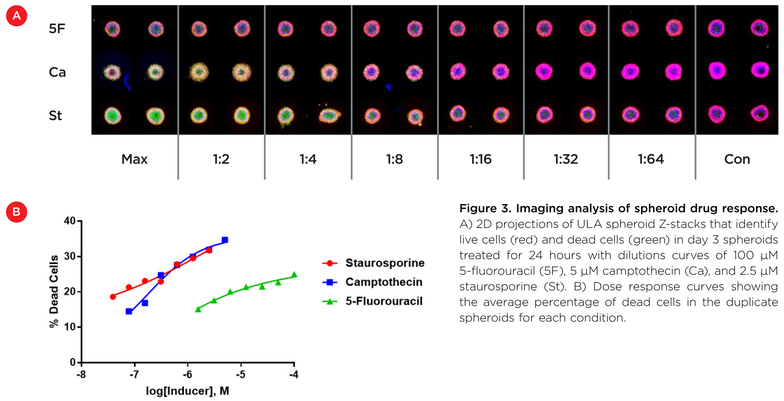


Figure 3. Imaging analysis of spheroid drug response. A) 2D projections of ULA spheroid Z-stacks that identify live cells (red) and dead cells (green) in day 3 spheroids treated for 24 hours with dilutions curves of 100 µM 5-fluorouracil (5F), 5 µM camptothecin (Ca), and 2.5 µM staurosporine (St). B) Dose response curves showing the average percentage of dead cells in the duplicate spheroids for each condition.

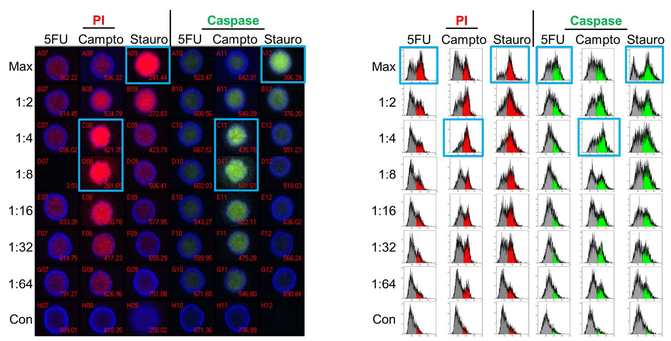


Figure 4. Comparison of spheroid analysis by imaging and flow cytometry. Day 3 hanging drop spheroids were treated with compounds and either stained and imaged (left) or dissociated, stained, and analyzed by flow cytometry. Maximal responses (blue boxes) correlate across the two methods for staurosporine and camptothecin but 5-fluorouracil treatment shows significant positive staining only by flow cytometry. This highlights that one may require multiple complementary analyses to obtain a complete picture of cancer spheroid drug response, including responding cell localization (imaging) and elimination of false negatives due to spheroid dye exclusion (flow cytometry).

MATRIGEL CULTURES

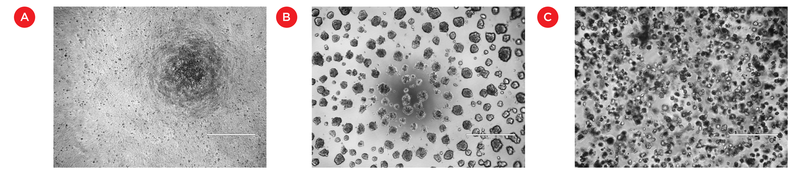


Figure 5. Automated Matrigel cultures. Images of HCT116 cells grown in standard monolayer cultures (A), on the surface of Matrigel (B), or embedded within a layer of Matrigel (C). Bar = 500 µm. Chilled positions maintained stock Matrigel in liquid form for automated transfers and heated positions induced Matrigel gelatinization in assay plates. Positional control of the pipette tips reduced the amount of Matrigel volume required to cover the 384-well bottom.

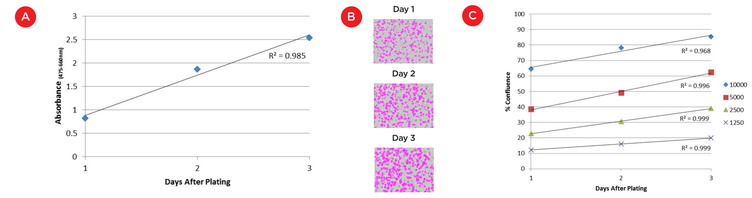
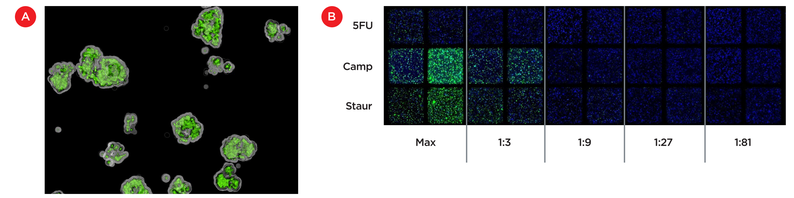


Figure 6. Analysis of cell growth on/in Matrigel. A) XTT assay of 8000 cells grown on the surface of Matrigel over three days. A linear growth rate (R^2 = 0.985) was seen indicating consistent growth over this time frame. B) Confluence measurements of cells plated within Matrigel. The images show the increase in the masked area of 2500 cells over three days. C) Graph of confluence measurements for various starting cell numbers. Change in confluence over three initial days of culture was highly linear for all cell numbers (R^2 = 0.968 to 0.999), indicating this approach is a viable label-free surrogate for cell growth.



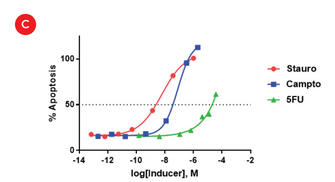


Figure 7. Induction of apoptosis in Matrigel-embedded cultures. A) Image illustrating the three-dimensional colonies stained with a fluorescent caspase 3/7 substrate as a marker of apoptosis. B) Replicate wells were treated with dilutions of staurosporine, camptothecin and 5-fluorouracil (top 5 dilutions shown) and stained for cell nuclei (blue) and apoptosis (green). C) Dose-response curves generated from imaging data for each compound, illustrating the suitability of this automated 384-well 3D culture model for drug screening.

CONCLUSION

Overcame 3D culture challenges with automation

- Minimized hanging drop or spheroid loss during plating and sample prep
- Enabled spheroid dissociation for flow cytometry analysis
- Temperature control positions prohibit/promote Matrigel polymerization
- Minimized Matrigel volume required for coating 384-well plates

Used 3D tumor models for compound screening

- Dose responses determined for multiple compounds and 3D culture systems
- Exclusion of stains may result in false negatives in intact spheroids
- Flow cytometry analysis eliminates false negatives but requires additional sample preparation and is an endpoint assay
- Stains requiring enzymatic activation (i.e. Caspase 3/7 substrate) reduced background staining in Matrigel cultures

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