Application Note

Cell Health and Viability

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Using Automated Imaging and Advanced 3D Cell Culture Techniques to Quantify Apoptosis Activity

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Introduction

Apoptosis, or programmed cell death, is essential to normal development and homeostasis of all multicellular organisms, and is a key research tool in the fight against cancer. Yet a challenge remains when culturing cell models, including those of human origin, for use in apoptosis studies. Traditional two-dimensional (2D) culture methods lack a biomimetic environment, and can result in a loss of differentiated cellular function and metabolic capacity. This may, in turn, suggest that 2D cultured tumor cells do not respond to cancer therapeutics/compounds in the same fashion as they would *in vivo*. Newer three-dimensional (3D) methods encourage cell-cell and cell-matrix interactions, and allow cell morphology and behavior to closely mimic that found in the body. These 3D cell culture models are particularly beneficial for investigating mechanistic processes and drug resistance in tumor cells.

This application note demonstrates the utility of a novel 3D spheroid cell culture model, Elplasia, used to elucidate the apoptotic potential of two compounds in two different cell lines. The cells were populated onto the nonadherent micropatterned plates, allowing cell spheroids to form and self-assemble per microwell. The microwell geometry aids spheroid formation in the center of each well, while the optically clear round bottom allows cellular imaging, and the opaque body prevents cross-talk. Spheroid proliferation was initially validated visually, and induced apoptosis levels within the spheroids were then quantified, using a cell imaging multimode reader.

Materials and methods

Materials

Cells: HT-1080 fibrosarcoma cells (part number CCL-121) and HCT116 colorectal carcinoma cells (part number CCL-247) were obtained from ATCC (Manassas, VA).

Elplasia 3D discovery tools: Elplasia 384-well black, clear-bottom microplates (part number SQ 200 100 NA 384) were donated by Kuraray Co. Ltd. (Tokyo, Japan).

Assay components: Kinetic Apoptosis Kit (part number ab129817) was purchased from abcam (Cambridge, MA). Doxorubicin HCl (part number BML-GR319-0005) was donated by Enzo Life Sciences (Farmingdale, NY). Oridonin (part number O9639) and Hoechst 33342 (part number 14533) were purchased from Sigma-Aldrich (St. Louis, MO).

Agilent BioTek Cytation 5 cell imaging multimode reader: Cytation 5 is a modular multimode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield, and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control, and dual injectors for kinetic assays, and is controlled by integrated Agilent BioTek Gen5 data analysis software. The instrument was used to image spheroids using brightfield and fluorescence microscopy, as well as individual differentiated cells plated in two dimensional format.

Methods

Cell preparation and spheroid formation: HCT116 and HT-1080 cells were harvested, and each resuspended at a concentration of 2.25×10^5 cells/mL. Next, 50 µL of suspended cells were added to separate test wells in the Elplasia 384-well microplate, for a total of approximately 50 cells per microspace. The plates were incubated at 37 °C/5% CO_2 for approximately 48 hours to allow the cells to aggregate into spheroids within each microspace.

Component preparation and addition: Doxorubicin was resuspended in 100% DMSO at a concentration of 10 mM, and oridonin was resuspended in 100% DMSO at a concentration of 20 mM. Serial titrations of both compounds were then created, ranging from 20 to 0 µM (2x), using 1:4 dilutions, in medium containing Hoechst 33342 and the Kinetic Apoptosis Reagent, pSIVA-IANDB, contained within the Kinetic Apoptosis Kit. After spheroid creation, 25 µL of medium was removed from each well, and replaced with an equal amount of either the doxorubicin or oridonin compound titration.

Spheroid apoptosis analysis: The plates were placed into Cytation 5, previously set to to 37 °C/5% CO_2, where kinetic imaging was performed every four hours over a 48-hour period. A 4x objective was used to image the entire well using the brightfield imaging channel, with a 2 × 2 image montage incorporated to visualize the entire well. The same objective was used, along with DAPI and GFP imaging channels, to image all spheroids, and apoptotic spheroids, respectively.

Results and discussion

Image-based spheroid monitoring

HCT116 spheroid proliferation and location within the microspace was confirmed using Cytation 5 and Gen5 data analysis software. Using brightfield imaging, four images were captured in 2 × 2 configuration to cover the well. The images were stitched together, using Gen5 software, to create a final, single image of all the microspaces in a well (Figure 1A), while the cell permeable fluorescent stain, Hoechst 33342 allowed identification of spheroid location by staining all nuclei blue (Figure 1B) using the DAPI channel.

Phosphatidylserine is a cytosolic-facing cell membrane component, and its exposure on a cell's extracellular surface, either persistently or transiently, is an indicator of early apoptosis. The cell membrane-impermeant fluorescent probe, pSIVA-IANDB, binds to phosphatidylserine, creating a strong green fluorescent signal to allow apoptosis monitoring over time. Using this probe, HCT116 and HT-1080 spheroid apoptotic activity was tracked over the compound concentrations tested. As shown in Figure 2, when imaging HT-1080 spheroid apoptotic activity treated with 400 nM doxorubicin, little to no signal is seen at 24 hours, but at 48 hours, high levels of green fluorescence are exhibited, indicating increased apoptosis levels.

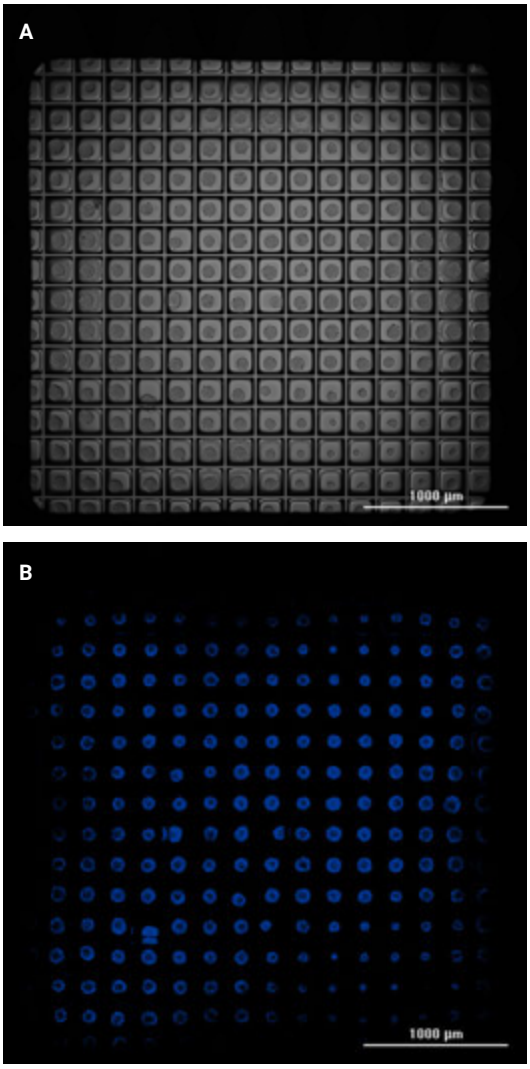

Figure 1. Microspace imaging at 4x magnification. (A) Stitched 2 × 2 montage images of HCT116 spheroids in a well microspace. (B) DAPI channel imaging of spheroid location.

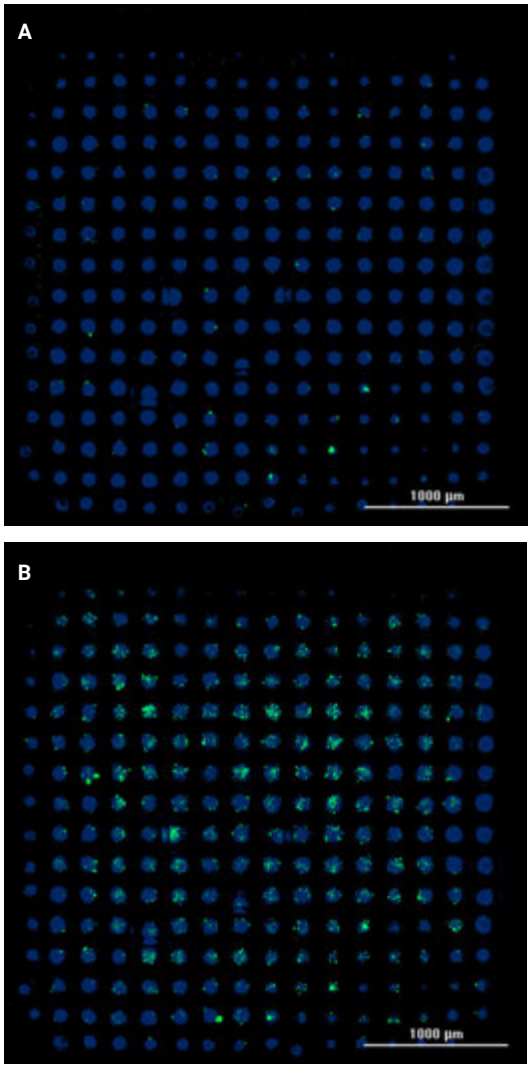

Figure 2. Apoptotic activity in HT-1080 cells treated with 400 nM doxorubicin. 4x DAPI and GFP channel imaging (A) 24 hours post treatment and (B) 48 hours post treatment.

Using the primary cellular analysis parameters in Table 1, Gen5 automatically placed masks around the objects in the microspace meeting the designated criteria (Figure 3A). Upon further analysis, two phenomena were identified. First, small variations can be seen in the size of aggregated spheroids in the well due to the number of cells settling into each microspace. Second, the emission signal from fluorescent probes can reflect off the plastic in the microspaces, causing these areas of the well to appear as spheroids. By using size and circularity subpopulation criteria identified in Table 1, nonspheroidal objects, in addition to smaller spheroids not meeting minimal size criteria, are eliminated from analysis. This serves to increase the accuracy of calculated results (Figure 3B). Finally, a second subpopulation filter was applied to identify the number of apoptotic spheroids from the number of previously identified true spheroids (Figure 3C). The same two criteria were again used as with the first subpopulation analysis, in addition to a minimal mean GFP setting to identify increases in signal above background levels. By incorporating the object masks and multiple subpopulation criteria, only fluorescence emanating from actual spheroids is quantified, and any background noise is made insignificant, yielding apoptotic spheroid results with a high degree of accuracy and consistency. It is important to note that cellular analysis parameters may vary depending on cell type, so parameter optimization should always be performed when working with untested cell models.

Table 1. Agilent BioTek Gen5 fluorescent spheroid analysis primary, advanced, and subpopulation parameters.

Spheroid Primary Cellular Analysis Parameters	
Channel	DAPI
Threshold	Auto-2
Background	Dark
Minimum Object Size	25 µm
Maximum Object Size	150 µm
Fill Holes in Masks	Checked
Split Touching Objects	Checked
Analyze Entire Image	Checked
Include Primary Edge Objects	Unchecked
Advanced Options	
Evaluate Background On	5% of Lowest pixels
Image Smoothing Strength	0
Background Flattening Size	1,000 µm
True Spheroid Subpopulation Parameters	
Size	>85 µm
Circularity	>0.4
Apoptotic Spheroid Subpopulation Parameters	
Size	>85 µm
Circularity	>0.4
Mean GFP	>6,000

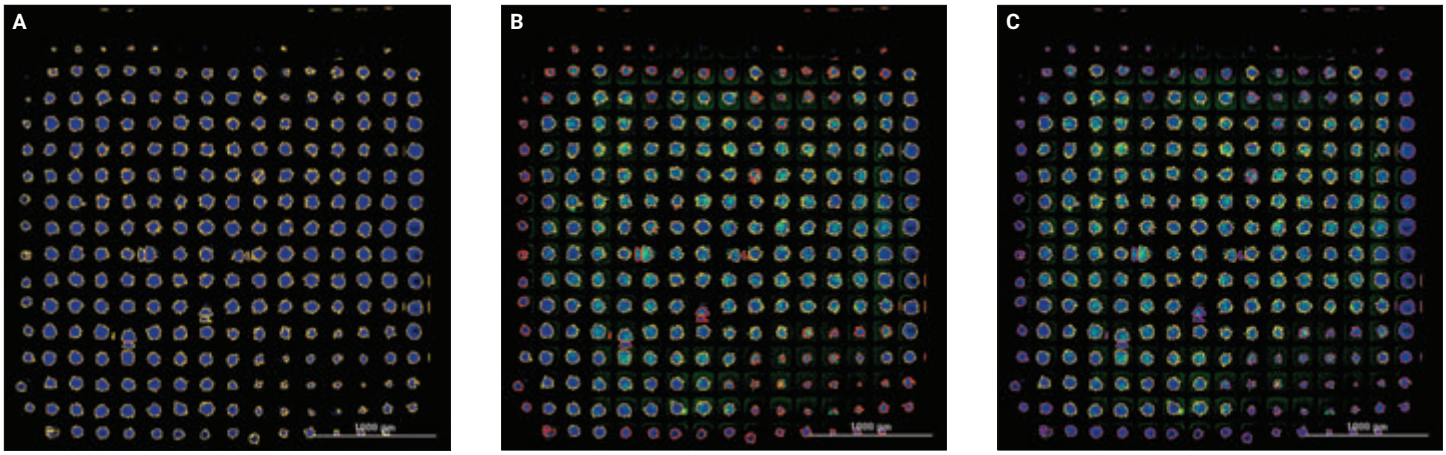

Figure 3. Cellular analysis procedure to determine apoptotic spheroid number per well. (A) Agilent BioTek Gen5 masks automatically drawn around objects meeting primary and advanced cellular analysis criteria using DAPI channel captured Hoechst 33342 signal, (B) red object masks indicating eliminated artifacts and spheroids not meeting minimal size and circularity subpopulation requirements, and (C) purple object masks denoting objects not meeting initial subpopulation criteria in addition to not meeting apoptotic spheroid criteria.

Spheroid apoptosis level determination

Using the selected subpopulation, Cytation 5 determined the apoptotic spheroid number per well over time using integrated Gen5 data analysis software. As shown in Figure 4, variations are seen between compound treatments and cell types, confirming that induction of apoptotic activity is due to the specific compound effect on the cells, and not due to spheroid incubation time in the microspaces. The Y-axis demonstrates the number of spheroids meeting the apoptotic spheroid subpopulation criteria.

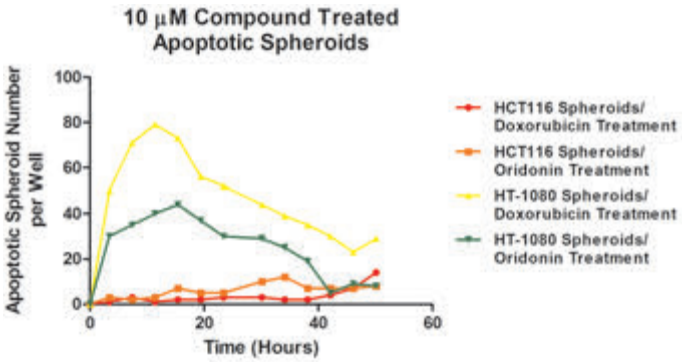

Figure 4. Apoptotic HCT116 and HT-1080 spheroid analysis over time after treatment with 10 µM doxorubicin or oridonin.

Percent apoptotic spheroids per well was then calculated to normalize the results to account for varying numbers of spheroids being present in each well (Figure 5). The value was calculated by dividing apoptotic spheroid numbers by the total identified actual spheroids per well at each time point. The resulting percent apoptotic spheroid kinetic graphs can be used to ascertain differences between compound effects on 3D spheroids.

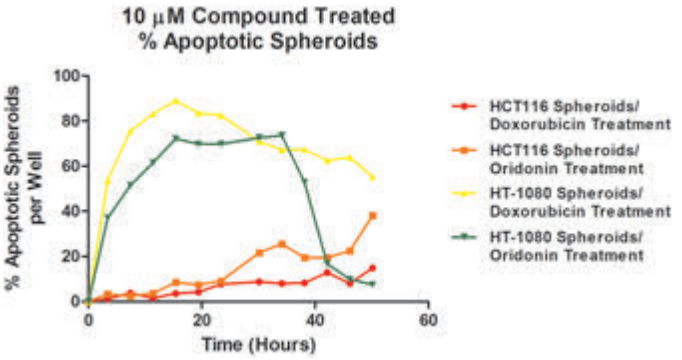

Figure 5. Kinetic graph showing percent apoptotic HCT116 or HT-1080 spheroids treated with a single 10 µM concentration of doxorubicin or oridonin.

Finally, end point analyses can be performed to compare apoptotic activity at specific compound concentrations and incubation times. As shown in Figure 6, the compounds exhibit a stronger apoptotic effect on HT-1080 spheroids as witnessed by the rapid rise in apoptotic spheroid percentage at low compound concentrations. Apoptotic activity then decreases at compound concentrations above 1,000 nM, most likely due to the cells within each spheroid becoming necrotic. HCT116 spheroids are more resistant to the compounds, as higher compound concentrations are needed to elicit an apoptotic response. The combined results illustrate that specific phenotypic apoptotic effects are able to be detected from individual cell type (e.g., primary cell, stem cell, and cancer cell line) and drug combinations using cellular imaging and the Elplasia spheroid microplates.

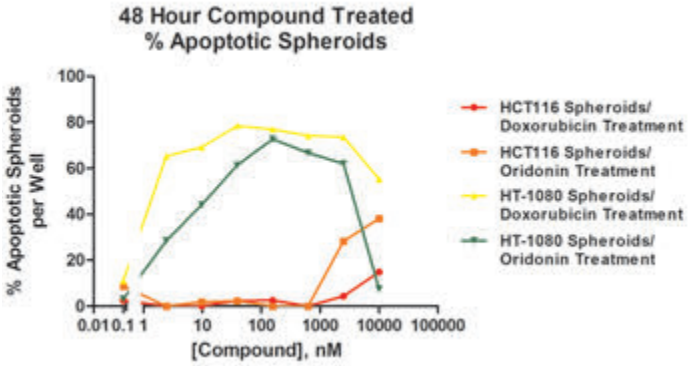

Figure 6. Percent of apoptotic HCT116 and HT-1080 spheroids after 48-hour incubation with various concentrations of doxorubicin or oridonin.

Conclusion

Use of spheroids may provide increased biological relevancy versus 2D cultured cells in cancer studies. Spheroid proliferation, using the novel Elplasia 3D Discovery Tool microplates, represent a simple and viable cell model that is robust and reproducible. Spheroid apoptosis levels were rapidly and easily detected using the Kinetic Apoptosis Kit from abcam. Finally, the Agilent BioTek Cytation 5 cell imaging multimode reader is a sensitive and flexible system when performing kinetic fluorescent and brightfield imaging of 3D spheroids using a wide magnification range. The combination together presents an accurate, easy-to-use method to assess target-based and phenotypic effects of anticancer drugs.

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