Application Note

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Automated, Multiparameter, Kinetic Methods to Quantify Cell Death

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Abstract

Automated, multiparameter methods, using fluorescent probes and an integrated live-cell analysis system, can streamline cell death pathway analyses. In this study, three fluorescent probes were used together to quantify the effect of camptothecin over time on fibrosarcoma cells. Each probe was specific to a phenotype associated with various stages of cell death: mitrochondrial membrane depolarization, phosphatidylserine exposure, and plasma membrane rupture. It was found in each case that camptothecin induced these phenotypes in a dose-dependent manner.

Introduction

Deep insight into regulation and dysregulation of cell death processes is critical towards understanding disease states such as cancer, and developing effective, well tolerated therapies. In fact, many programmed and nonprogrammed cell death pathways are being studied to develop more effective and less toxic chemotherapeutic regimens.[1] However, the diversity of cell death modalities is complicated by shared signaling elements, overlapping mechanisms, and complex crosstalk among various cell death pathways.[2] Differentiating the morphological hallmarks of cell death pathways can be labor-intensive, and when incorporating end point assays, can often miss critical yet transient events.

This application note demonstrates an automated, multiplexed method to assess real-time cell death using a live-cell analysis system. Three common cell death phenotypes are measured: mitochondria membrane potential, phosphatidylserine (PS) externalization, and cell membrane integrity, using fluorescent probes. The fluorescent, positively charged tetramethylrhodamine ethyl ester (TMRE) dye readily passes through cell membranes and accumulates in healthy, active mitochondria, where it produces a red-orange signal. If the mitochondria membrane is depolarized or inactive, as in apoptotic and necrotic cells, the dye diffuses throughout the cell. The green fluorescent probe, pSIVA-IANBD binds to the nonpolar environment of the cell's membrane lipid bilayer, and detects irreversible and transient PS exposure that is characteristic of apoptosis and necroptosis. Finally, the far-red fluorescent dye, DRAQ7 is plasma membrane impermeant, and only with plasma membrane rupture consistent with necrotic and necroptotic cells will the nuclei be stained. Combining these dyes into a single, multiplexed method with real-time morphological analysis provides major advantages when characterizing cell death systems.

In these automated experiments, fibrosarcoma target cells and dyes were combined in a microplate along with a known cytotoxic compound, and incubated in an automated benchtop incubator. The plates were automatically transferred from the incubator to a combined microplate reader and automated digital imager every two hours for a total of 48 hours, where fluorescent imaging was performed to assess cellular activity, as well as high-contrast brightfield imaging to allow accurate cell counting over the entire incubation period.

Materials and methods

Materials

Cells and media

HT-1080 fibrosarcoma cells (part number CCL-121) were obtained from ATCC, Manassas, VA. Advanced DMEM (part number 12491-015), fetal bovine serum, (part number 10437-036), and penicillin-streptomycin-glutamine (100x) (part number 10378-016) were purchased from ThermoFisher Scientific (Waltham, MA).

Assay and experimental components

The TMRE fluorescent dye, as part of the TMRE-Mitochondrial Membrane Potential Assay Kit (part number ab113852), pSIVA-IANDB green fluorescent probe, as part of the Kinetic Apoptosis Kit (part number ab129817), DRAQ7 far-red fluorescent dye (TM, BioStatus, Ltd.) (part number ab109202), and the known topoisomerase I inhibitor, camptothecin (part number ab120115) were provided by Abcam (Cambridge, MA).

Agilent BioTek BioSpa live-cell analysis system

The BioSpa live-cell analysis system offers complete workflow automation for live-cell kinetic assays running up to two weeks at a time, and consists of the Agilent BioTek Cytation 5 cell imaging multimode reader and Agilent BioTek BioSpa 8 automated incubator.

Cytation 5 is a modular multimode microplate reader combined with an automated digital microscope. 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 shaking, 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 microplate reader and imager software, which also automates image capture, analysis, and processing. The instrument was used to kinetically monitor cellular activity.

The BioSpa 8 automated incubator links Agilent BioTek readers or imagers together with Agilent BioTek washers and dispensers for full workflow automation of up to eight microplates. Temperature, CO_2/O_2, and humidity levels are controlled and monitored through the Agilent BioTek BioSpa software to maintain an ideal environment for cell cultures during all experimental stages. Test plates were incubated in the BioSpa to maintain proper atmospheric conditions for a period of forty-eight hours and automatically transferred to the Cytation 5 every two hours for high-contrast brightfield and fluorescent imaging.

Methods

Cell preparation

A total of 2,000 HT-1080 fibrosarcoma cells in prepared media were added to wells of a 96-well tissue culture-treated microplate. The microplate was incubated at 37 °C/5% CO_2 overnight to allow the cells to attach to the wells.

Reagent and inhibitor dilutions

Assay reagents were diluted in media to the following 1x working concentrations: 200 nM TMRE; 10 µL/mL pSIVA-IANDB; 3 µM DRAQ7. Camptothecin was diluted in media containing reagents to create an eight-point titration from 10 to 0 µM using serial 1:4 dilutions.

Assay procedure

Following overnight cell attachment, media was removed from the microplate and replaced with media containing the TMRE, pSIVA, and DRAQ7 reagents in addition to compound dilutions. The plate was then placed into the BioSpa 8, with atmospheric conditions previously set to 37 °C/5% CO_2. Water was added to the pan to create a humidified environment. The BioSpa 8 software was programmed such that the plates were automatically transferred to Cytation 5 for high-contrast brightfield and fluorescent imaging of the test wells every two hours for a total of 25 imaging iterations over the 48-hour incubation period. A single 4x image was taken with each channel (Table 1) to capture a representative population of cells per well. Laser autofocus was incorporated to ensure proper focusing on the target cell layer as well as the most efficient focusing procedure.

Table 1. Cells imaged per channel.

Imaging Channel Target	
HCBF	All cells
GFP	Apoptotic cells
RFP	Active mitochondrial membrane potential
CY5	Necrotic cells

Image processing

Following capture, images were processed prior to analysis to remove background signal from each channel using the settings in Table 2.

Table 2. Image preprocessing parameters.

2D Image Preprocessing Parameters			
Channel	Apply Image Preprocessing	Background	Rolling Ball Diameter
HCBF	Yes	Dark	25
GFP	Yes	Dark	Auto
RFP	Yes	Dark	150 µm
CY5	Yes	Dark	Auto

Cellular analysis of processed images

Cellular analysis was carried out on the processed images to determine the total cell number per image, in addition to the cell number meeting the criteria for active mitochondria with polarized membranes, apoptotic, and necrotic activity using the criteria in Tables 3 and 4.

Table 3. Primary and secondary analysis criteria.

Total Cell Primary Analysis	
Channel	Tsf[Brightfield]
Threshold	10,000
Background	Dark
Split Touching Objects	Checked
Fill Holes in Masks	Checked
Minimum Object Size	5 µm
Maximum Object Size	100 µm
Include Primary Edge Objects	Unchecked
Analyze Entire Image	Checked
Advanced Detection Options	
Rolling Ball Diameter	25 µm
Image Smoothing Strength	0
Evaluate Background On	5% of lowest pixels
Active Mitochondria Cell Secondary Analysis	
Channel	Tsf[RFP]
Measure Within a Secondary Mask	Checked
Type	Exclude primary mask
Distance from Primary Mask	1 µm
Ring Width	25 µm
Threshold	5,000
Smoothing	0
Method	Propagate mask
Metric of Interest	Area_2[Tsf[RFP]]
Apoptotic Cell Secondary Analysis	
Channel	Tsf[GFP]
Measure Within a Secondary Mask	Checked
Type	Exclude primary mask
Distance from Primary Mask	1 µm
Ring Width	25 µm
Threshold	10,000
Smoothing	0
Method	Propagate mask
Metric of Interest	Mean_2[Tsf[GFP]]
Necrotic Cell Secondary Analysis	
Channel	Tsf[CY5]
Measure Within a Primary Mask	Checked
Expand Primary Mask	15 µm
Metric of Interest	Mean[Tsf[CY5]]

Table 4. Image preprocessing parameters.

Subpopulation Criteria			
Cell Subpopulation	Channel	Metric of Interest	Positive Cell Cutoff Criteria
Active Mitochondria	RFP	Area_2[Tsf[RFP]]	>100 µm^2
Apoptosis	GFP	Mean_2[Tsf[GFP]]	>10,000 RFU
Necrosis	CY5	Mean[Tsf[CY5]]	>2,000 RFU

Results and discussion

Label-free total cell counting

High-contrast brightfield, label-free live-cell imaging was performed to quantify total cell numbers throughout the 48-hour camptothecin incubation period. The focal plane of cells was automatically determined via laser autofocusing (Figure 1A), while an additional image was captured approximately 300 µm below the original focal plane (Figure 1B) and automatically preprocessed (Table 2) to increase cellular contrast (Figure 1C). Quantitative analysis was then performed using the total cell primary analysis parameters in Table 3, placing object masks around cells (Figure 1D), to determine total cell numbers per image.

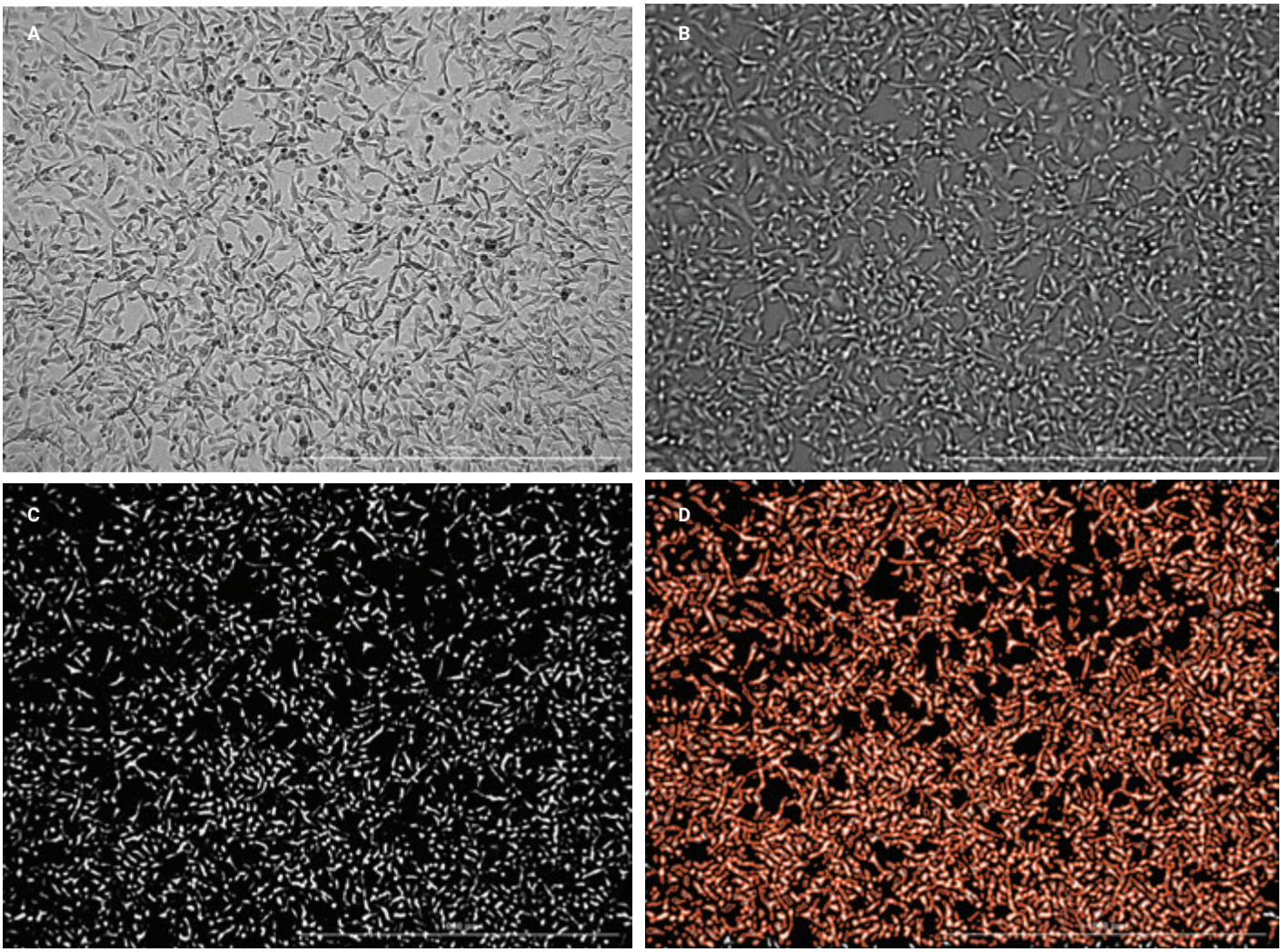

Figure 1. High-contrast brightfield image capture, preprocessing, and analysis. (A) 4x in-focus image of total HT-1080 cells. (B) 4x off-focus image of total HT-1080 cells. (C) Preprocessing of off-focus image. (D) Cellular analysis of preprocessed image. Object masks (in red) placed around total cells within image.

Results from the kinetic total cell analysis (Figure 2) confirmed the effect that increasing camptothecin concentrations have on HT-1080 cell proliferation. The accurate cell counts also demonstrate how primary cell analysis using high-contrast brightfield can be used as a normalization of positively affected cell counts.

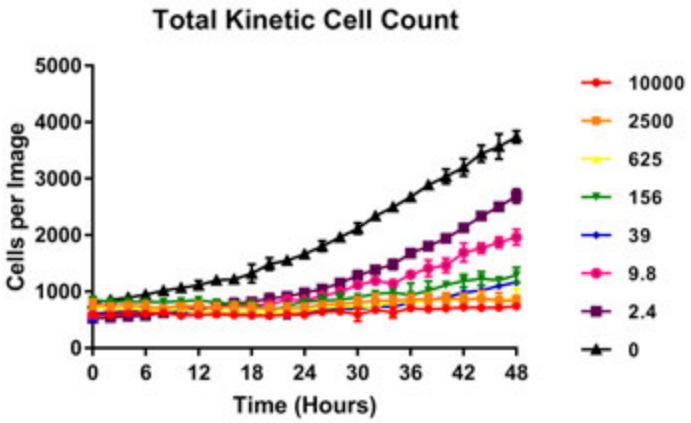

Figure 2. Kinetic HT-1080 total cell dose response curves. Average cell number per image was calculated from three replicate wells of each compound treatment at each timepoint. Camptothecin concentrations were as follows: 10,000, 2500, 625, 156, 39, 9.8, 2.4, and 0 nM.

Mitochondrial membrane potential

The TMRE cell permeant dye was added at time 0 to monitor mitochondrial membrane potential. As the dye is positively charged, it readily accumulated in negatively charged mitochondria, whereas depolarized mitochondria have decreased membrane potential and did not sequester the dye. The Cytation 5 RFP imaging channel was used to capture signal from the TMRE probe. Cells at time 0 (Figure 3A) contained active mitochondria leading to large areas of cytoplasmic probe signal. Loss of mitochondrial membrane integrity lead to diminished probe expression and smaller areas of cytoplasmic signal (Figure 3B). Active mitochondria cell secondary cellular analysis (Table 3) placed object masks around the TMRE probe RFP signal allowing the area criteria (Table 4) to compute the number of cells with active mitochondria. This number was then normalized by dividing by previously determined total cell numbers to calculate kinetic active mitochondria cell percentages (Figure 3C). It is evident that camptothecin negatively impacts mitochondrial membrane integrity in a dose-dependent fashion, agreeing with the literature.[3]

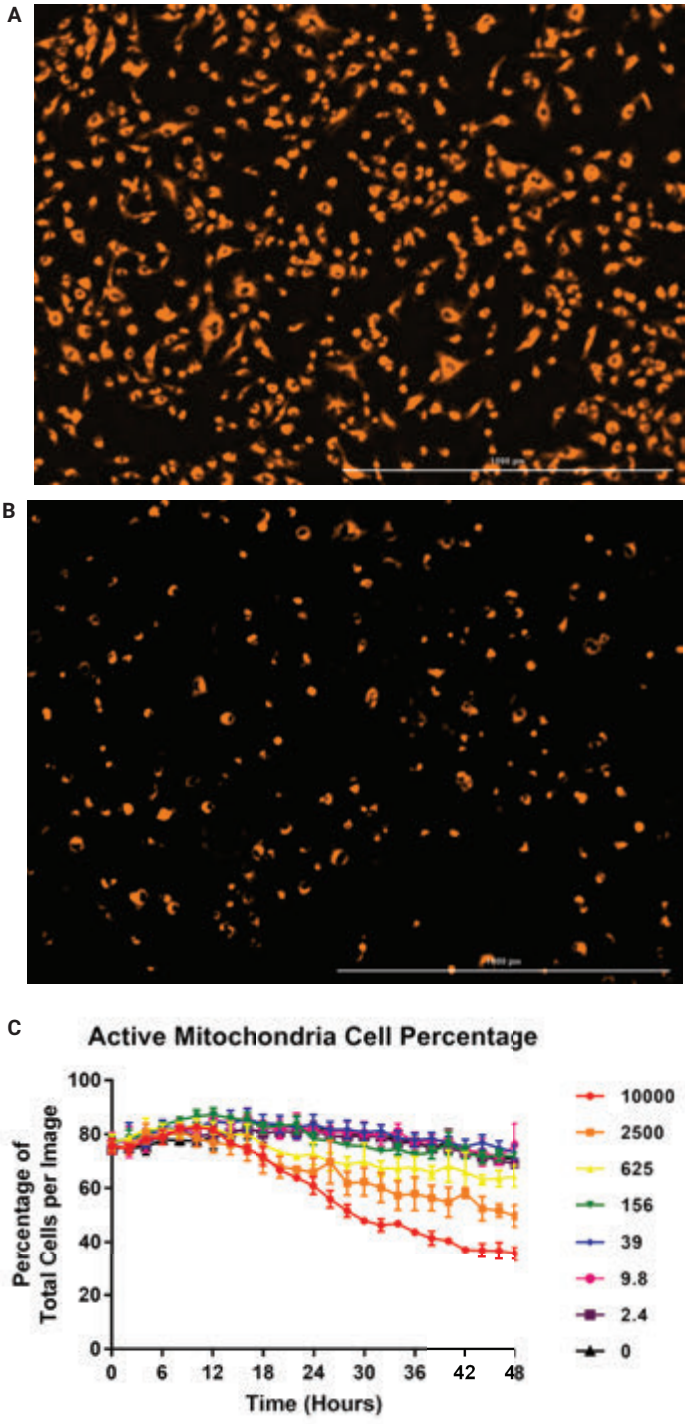

Figure 3. Active mitochondria imaging and analysis. Images captured with a 4x objective and RFP imaging channel following incubation periods of (A) 0 hours; (B) 48 hours with 2,500 nM camptothecin; and (C) Active mitochondrial cell percentages per image.

Apoptotic external phosphatidylserine exposure

PS is a phospholipid that resides on the cytoplasmic surface of healthy cell membranes, and translocates to the outer membrane in the early stages of apoptosis. The reversible binding probe, polarity sensitive indicator of viability and apoptosis (pSIVA), binds to externally exposed PS to create an intense green signal, and releases when PS internalizes. The GFP imaging channel was used to detect the pSIVA fluorescent signal as it increased upon camptothecin incubation with cells (Figures 4A and 4B). Apoptotic cell secondary cellular analysis (Table 3) placed object masks around cytoplasmic pSIVA probe GFP signal allowing object mean criteria (Table 4) to identify apoptotic cells over time. This was again normalized to total cell counts (Figure 4C). This figure demonstrates that phosphatidylserine exposure is induced by camptothecin in a time and dose-dependent manner.

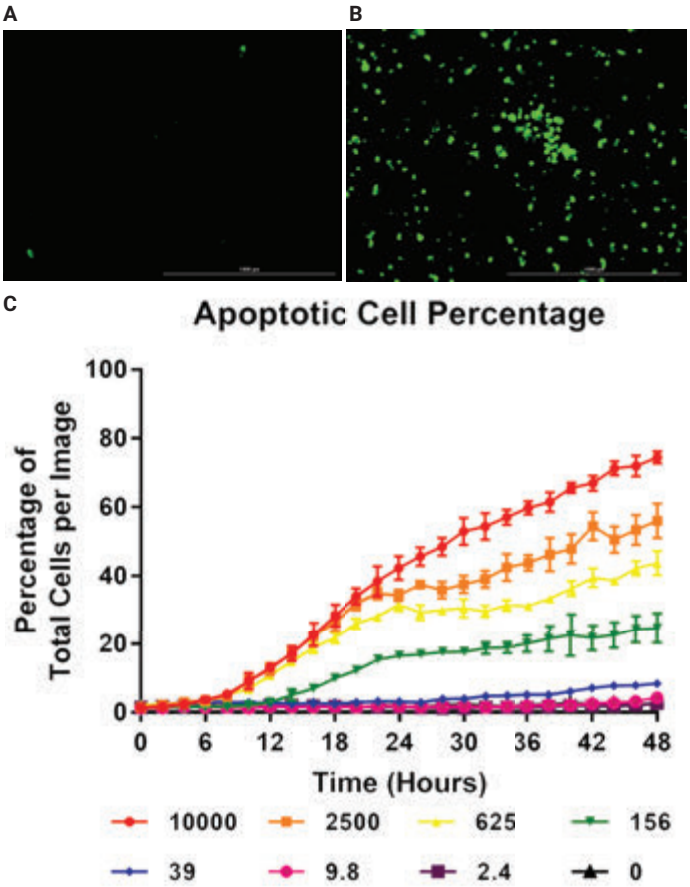

Figure 4. Apoptosis imaging and analysis. Images captured with a 4x objective and GFP imaging channel following incubation periods of (A) 0 hours and (B) 48 hours with 2,500 nM camptothecin; (C) apoptotic cell percentages per image.

Necrotic cell membrane integrity loss

The far-red cell impermeant dye, Deep Red Anthraquinone 7 (DRAQ7), binds to cell nuclei upon loss of cell membrane integrity, an indication of necrosis, to create a red fluorescent signal that was captured using the CY5 imaging channel. Similar to pSIVA, DRAQ7 fluorescent signal increased upon camptothecin incubation with cells (Figures 5A and 5B). Necrotic cell secondary cellular analysis (Table 3) placed object masks around nuclear probe CY5 signal, allowing minimum object mean criteria (Table 4) to identify necrotic cells over time and was normalized to total cell counts (Figure 5C). Similar to the pSIVA probe for apoptosis, DRAQ7 demonstrates increasing levels of plasma membrane rupture with increasing dose.

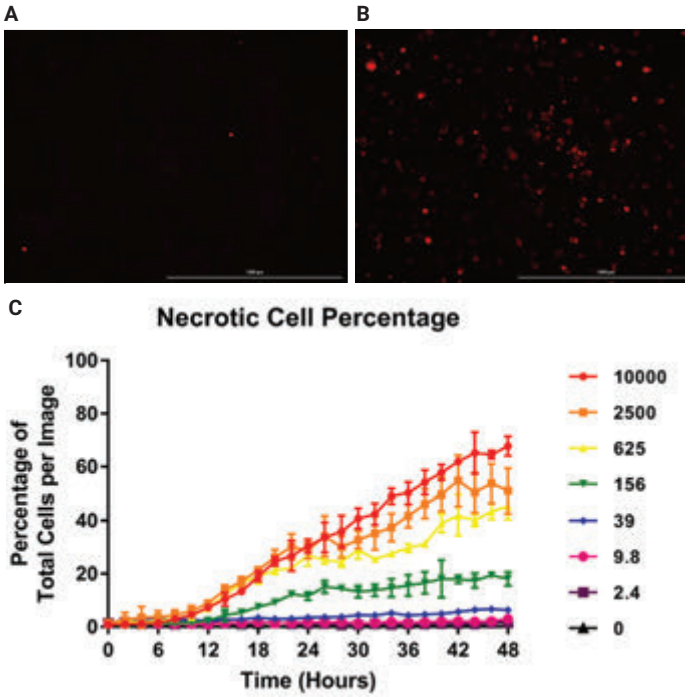

Figure 5. Necrosis imaging and analysis. Images captured with a 4x objective and CY5 imaging channel following incubation periods of (A) 0 hours and (B) 48 hours with 2,500 nM camptothecin. (C) Necrotic cell percentages per image following primary and secondary cellular analysis.

Variable incubation analysis comparison

An advantage of the BioSpa live-cell analysis system is the ability to monitor the effect of camptothecin treatment, not only at one particular timepoint, but at regular intervals kinetically over extended incubation periods. Per Figure 6, the calculated EC_{50} values generated from dose response curves plotted from necrotic cell percentages determined following 0-, 12-, 24-, and 48-hour camptothecin treatments illustrated that short-term incubations can lead to false assumptions about the true potency of test molecules. By incorporating kinetic analysis, accurate conclusions can be determined.

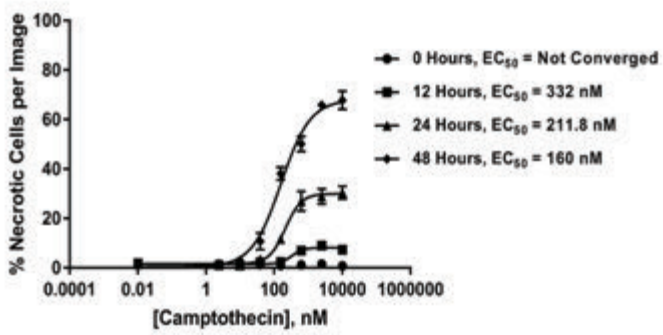

Figure 6. Camptothecin percent necrotic cell per image dose response curves. Induced necrotic cell percentage per image curves following camptothecin incubations of 0, 12, 24, and 48 hours.

Conclusion

The multiple fluorescent imaging detection channels on the Agilent BioTek Cytation 5 cell imaging multimode reader allow the live-cell TMRE, pSIVA, and DRAQ7 probes to be multiplexed within the same well, providing a simple and robust method to measure multiple cell death phenotypes. Proper environmental conditions provided by the Agilent BioTek BioSpa live-cell analysis system then allows images to be captured automatically over extended incubation periods, improving the ability to gain a more complete picture of the effect of each test molecule. Finally, by incorporating cellular analysis techniques with Agilent BioTek Gen5 multimode reader and imager software, informed conclusions can be deduced by viewing normalized kinetic induction curves, as well as the response from multiple test concentrations over time.

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References

1. Ricci, M. S.; Zong, W. Chemotherapeutic Approaches for Targeting Cell Death Pathways. *Oncologist*. **2006**, *11(4)*, 342–357.
2. Halonen, S. K. Modulation of Host Programmed Cell Death Pathways by the Intracellular Protozoan Parasite, Toxoplasma gondii – Implications for Maintenance of Chronic Infection and Potential Therapeutic Applications. In Cell Death – Autophagy, Apoptosis and Necrosis [Online]; Ntuli, T. M., Ed.; InTech: London, 2015; Chapter 17. www.intechopen.com/books/cell-death-autophagy-apoptosis-and-necrosis/modulation-of-host-programmed-cell-death-pathways-by-the-intracellular-protozoan-parasite-toxoplasma (accessed Oct 30, 2017).
3. Zeng, C. W. *et al.* Camptothecin Induces Apoptosis in Cancer Cells via MicroRNA-125b-Mediated Mitochondrial Pathways. *Mol. Pharmacol.* **2012**, *81(4)*, 578–586.

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