An Automated Approach to Kinetic, Live Cell Multi-Parametric Cell Death Analyses

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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 treatment therapies. In fact, many programmed and non-programmed cell death pathways are being studied for the development of 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.

Here, we demonstrate an automated, multiplexed method to assess real-time cell death. Three common cell death biomarkers are measured: mitochondria membrane potential, phosphatidylserine (PS) externalization, and cell membrane integrity, using fluorescent probes from Abcam. 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 non-polar environment of the cell's membrane lipid bilayer, and detects irreversible and transient phosphatidylserine exposure that is characteristic of apoptosis and necroptosis. Finally, the far-red fluorescent dye, DRAQ7™ is impermeant in healthy cells, while it stains nuclei in dead and permeabilized necrotic and necroptotic cells. Combining these dyes into a single, multiplexed method with real-time morphological analysis provides major advantages when characterizing cell death systems. Fibrosarcoma target cells and dyes were combined in a microplate along with a known inhibitor 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 forty-eight hours, where fluorescent imaging was performed to assess cellular activity, as well as high-contrast brightfield imaging to allow for accurate cell counting over the entire incubation period.

BioTek Instrumentation

Figure 1. BioSpa 8 and Cytation 5 integrated as the BioSpa Live Cell Imaging System.

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 is a modular multi-mode 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 Gen5™ Microplate Reader and Imager Software, which also automates image capture, analysis and processing. The instrument was used to kinetically monitor cellular activity.

BioSpa™ 8 Automated Incubator: The BioSpa 8 Automated Incubator links BioTek readers or imagers together with 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 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.

Materials and Methods

Cell Preparation

A total of 2000 HT-1080 fibrosarcoma cells (Catalog No. CCL-121, ATCC, Manassas, VA) in prepared media were added to wells of a 96-well TC 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 from Abcam (Cambridge, MA) were diluted to 1X working concentrations and added to media as follows: 10 µL/mL pSIVA-IANDB green fluorescent probe (part of the Kinetic Apoptosis Kit, Catalog No. ab129817); 200 nM TMRE (as part of the TMRE-Mitochondrial Membrane Potential Assay Kit, (Catalog No. ab113852)); 3 µM DRAQ7 far-red fluorescent dye (Catalog No. ab109202). Camptothecin (Catalog No. 208925, EMD Millipore, Billerica, MA) was diluted in media containing reagents to create an eight-point titration from 10 µM to 0 µM using serial 1:4 dilutions.

Assay Procedure

Following incubation, media was removed from the microplate and replaced with media containing the TMRE, pSIVA, and DRAQ7 reagents and compound dilutions. The plate was then placed into the BioSpa 8, with atmospheric conditions previously set to 37 °C/5% CO_2. Water was also 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 (high contrast brightfield, GFP, RFP, Cy5) 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.

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 2A), while an additional image was captured approximately 300 µm below the original focal plane (Figure 2B) and automatically pre-processed to increase cellular contrast (Figure 2C). Quantitative analysis was then performed, placing object masks around cells (Figure 2D), to determine total cell numbers in each test well.

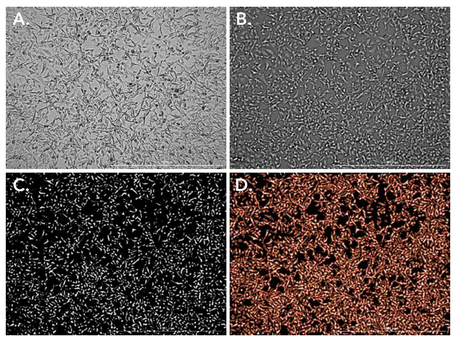

Figure 2. High contrast brightfield image capture, pre-processing, and analysis. (A) 4x in-focus image of total HT-1080 cells. **(B)** 4x off-focus image of total HT-1080 cells. **(C)** Pre-processing of off-focus image. **(D)** Cellular analysis of pre-processed image. Object masks (in red) placed around total cells within image.

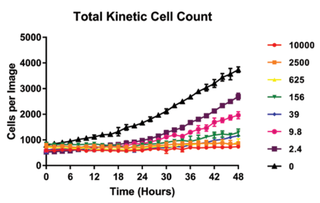

Figure 3. Kinetic HT-1080 total cell dose response curves. Average cell number per image was calculated from three replicate wells of each compound treatment at each timepoint. Camptothecin concentrations were as follows: 10,000 nM, 2500 nM, 625 nM, 156 nM, 39 nM, 9.8 nM, 2.4 nM, 0 nM.

Results from the kinetic total cell analysis confirm 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.

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 accumulates in negatively charged mitochondria, whereas depolarized mitochondria have decreased membrane potential and do not sequester the dye. Cytation 5's RFP imaging channel was used to capture signal from the TMRE probe. Cells at time 0 (Figure 4A) contain active mitochondria leading to large areas of cytoplasmic probe signal. Loss of mitochondrial membrane integrity leads to diminished probe expression and smaller areas of cytoplasmic signal (Figure 4B). Secondary cellular analysis placed object masks around the TMRE probe RFP signal (Figure 4C and D). By setting minimum object area criteria, the number of active mitochondrial cells per image over time was then determined (Figure 4E). This number was then normalized by dividing by previously determined total cell numbers to calculate kinetic active mitochondria cell percentages (Figure 4F).

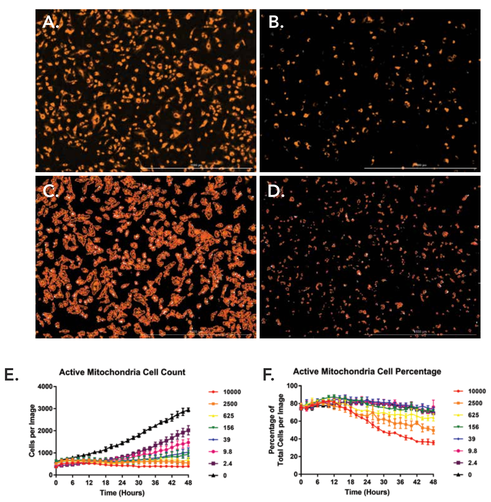

Figure 4. Active mitochondria imaging and analysis. Images captured with a 4x objective and RFP imaging channel following incubation periods of **(A)** 0 hours; and **(B)** 48 hours with 2500 nM camptothecin. Cellular analysis performed to place secondary masks around active mitochondria signal from cells treated with 2500 nM camptothecin for **(C)** 0, and **(D)** 48 hours. White: pre-processed high-contrast brightfield cellular areas; orange: TMRE cytoplasmic signal; Red: object masks. Kinetic curves then plotted for **(E)** total active mitochondrial cells, and **(F)** mitochondrial cell percentages per image.

The plotted curves in Figures 4E-F validate the known phenomenon, that camptothecin induces cell death by negatively impacting mitochondrial membrane integrity[3]. Furthermore, these results confirm the ability of the BioSpa Live Cell Imaging System and Gen5 to deliver accurate results using the TMRE live cell mitochondrial membrane potential probe.

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 5A-B). Secondary cellular analysis placed object masks around cytoplasmic pSIVA probe GFP signal (Figure 5C and D). As apoptotic induction is independent of area, minimum object mean criteria identified apoptotic cells over time (Figure 5E) which was again normalized to total cell counts (Figure 5F).

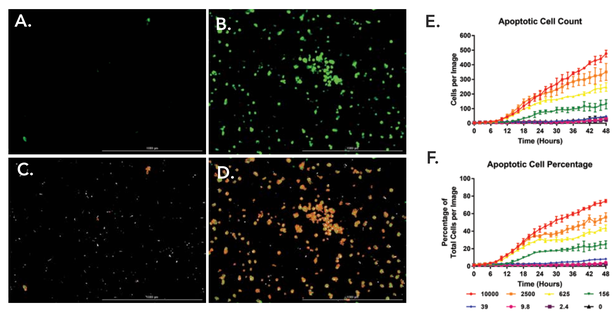

Figure 5. Apoptosis imaging and analysis. Images captured with a 4x objective and GFP imaging channel following incubation periods of **(A)** 0 hours; and **(B)** 48 hours with 2500 nM camptothecin. Cellular analysis performed to place secondary masks around pSIVA signal from cells treated with 2500 nM camptothecin for **(C)** 0, and **(D)** 48 hours. White: pre-processed high-contrast brightfield cellular areas; green: pSIVA cytoplasmic signal; Red: object masks. Kinetic curves then plotted for **(E)** total apoptotic cells, and **(F)** apoptotic cell percentages per image.

The graphs in Figures 5E-F confirm the ability of the imaging and analysis to detect and quantify camptothecin induced pSIVA apoptotic signal in a multiplexed setting.

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 is captured using the CY5 imaging channel. Similar to pSIVA, DRAQ7 fluorescent signal increases upon camptothecin incubation with cells (Figures 6A-B). Secondary cellular analysis placed object masks around nuclear probe CY5 signal. Minimum object mean criteria again identified necrotic cells over time and was normalized to total cell counts (Figure 6C).

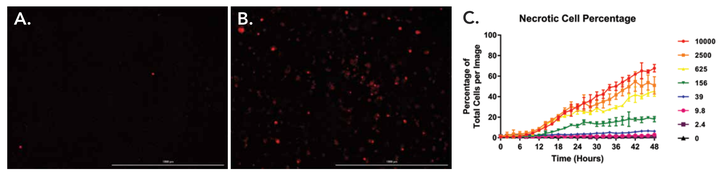

Figure 6. Necrosis imaging and analysis. Images captured with a 4x objective and CY5 imaging channel following incubation periods of **(A)** 0 hours; and **(B)** 48 hours with 2500 nM camptothecin. **(C)** Necrotic cell percentages per image following primary and secondary cellular analysis.

The graph in Figure 6C further validates the ability of the imaging and analysis to detect and quantify camptothecin induced changes in TMRE, pSIVA, and DRAQ7 signals from a single well in a multiplexed setting.

Variable Incubation Analysis Comparison

An advantage of incorporation of the BioSpa Live Cell Imaging 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 7, 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 illustrate 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.

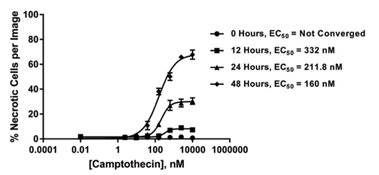

Figure 7. Camptothecin percent necrotic cell per image dose response curves. Induced necrotic cell percentage per image curves following camptothecin incubations of 0, 12, 24, and 48 hours.

Conclusions

1. Live cell fluorescent probes from Abcam may be combined to provide a simple and robust method to measure multiple cell death pathways in a multiplexed fashion.
2. The BioSpa Live Cell Imaging System provides proper environmental conditions when automating multi-parametric cell death assays.
3. Automating assay procedures with robotic instrumentation yields an efficient, robust method to increase throughput and repeatability while decreasing the time required for manual manipulations.
4. Incorporation of kinetic imaging allows for accurate determination of the effect test molecules can have to induce cell death.

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. https://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.; Zhang, X-J.; Lin, K-Y.; Ye, H.; Feng, S-Y.; Zhang, H.; Chen, Y-Q. Camptothecin Induces Apoptosis in Cancer Cells via MicroRNA-125b-Mediated Mitochondrial Pathways. *Mol. Pharma.* **2012**, *81*(4), 578-586.