Imaging-based Cytotoxicity Assays

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Abstract

Cell death occurs throughout the life of an organism, and this is critical for developmental plasticity and organismal health, in part by eliminating unneeded and unhealthy cells in a timely and effective manner. However, dysfunctional programmed cell death leads to diseases such as cancer, neurodegeneration, and ischemic damage. Two classic pathways of cell death are apoptosis and necrosis. Apoptosis is an active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation. Necrosis is a more passive process with uncontrolled release of inflammatory cellular content. Healthy cells respond to death-inducing stimuli by imitating a variety of molecular pathways leading to cell death. Completion of the proper pathway is a critical cellular function to ensure that the appropriate outcome is ultimately achieved.

The quantification of the cell death response is an integral component of exploring cell biology, responses to cellular stress and performing high-throughput drug screens. One classic method of cell death detection is flow cytometry, which requires extensive handling of cells and only provides end-point data. Kinetic imaging, in contrast, is a critical application for studying dynamic biological processes in real time. Kinetic analysis of cell death analysis allows for sensitive, real-time determination of the accumulation of both apoptotic and necrotic events within the cellular population.

Here we present the use of apoptosis and necrosis dyes in combination with automated kinetic imaging to quantitatively assess the effects of known inducers of cell death in multiple cell lines. We use high contrast, label-free brightfield imaging to assay for total number of cells, and cellular dyes to label both apoptotic and necrotic cells concurrently. This allows for determination of percent apoptosis and necrosis in each population over forty-eight hours of drug treatment.

BioTek Instrumentation

Figure 1. BioSpa 8 Automated Incubator and Cytation 5 Cell Imaging Multi-Mode Reader.

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 is a modular multi-mode 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 Gen5™ Microplate Reader and Imager Software.

BioSpa™ 8 Automated Incubator: The BioSpa 8 links BioTek readers or imagers together with washers and dispensers for full workflow automation of up to 8 microplates. Temperature, CO_2/O_2 and humidity levels are controlled and monitored through the BioSpa software to maintain an ideal environment for cell health during all experimental stages.

Materials and Methods

Cell Culture

HT1080 cells were grown in Advanced Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) with 10% FBS (Gibco) and 1x PennStrep-Glutamine (Cellgro, Manassas, VA). SKOV3 cells were grown in McCoy's 5A Medium Modified (Gibco) with 10% FBS (Gibco) and 1x PennStrep-Glutamine (Cellgro). Cells were seeded into black-sided, clear bottom 96-well microplates (Corning, Corning, NY) at a density of 2000 (HT1080) or 4000 (SKOV3) cells per well and were allowed to adhere over night. Environmental conditions, including temperature (37 °C), gas (5% CO_2), and humidity (90%) were maintained during the two day incubation by a BioSpa 8 Automated Incubator.

Cell Death Induction and Component Addition

Cell death was induced by treating the cells with dilutions of camptothecin or staurosporine with concentrations ranging from 10,000-2.44 nM and a no compound control. The media also contained reagents from the Kinetic Apoptosis Kit (Abcam, Cambridge, MA). The polarity sensitive indicator of viability and apoptosis (pSIVA), a marker of apoptosis, was added to the media at a concentration of 10 µL/mL. Propidium Iodine (PI), a necrosis indicator, was added to the media at a concentration of 5 µL/mL.

Cell Imaging

Cell culture plates were transferred by the BioSpa 8 to a Cytation 5 Cell Imaging Multi-Mode Reader every two hours. Environmental conditions were maintained at 37 °C and 5% CO_2 within the Cytation 5 throughout the imaging steps. Images were captured at 4x in the PI, GFP, and brightfield channels. Two high contrast brightfield images were captured at each time point: an in-focus image used for reference, and a defocused image for cell counting. Briefly, cells were brought into focus using the high contrast brightfield kit. The view line profile tool was then used to draw a line that crossed cells and background sections of the imaging field. The focal height was then decreased while observing the line profile to determine the focal height at which maximum contrast between cell and background brightness was achieved. This value was then input as an offset from the reference high contrast brightfield image.

Materials and Methods *(Cont.)*

Image Analysis

Image preprocessing was employed to obtain the best possible enhancement of contrast; reducing each cell to a single bright spot. In order to achieve this, the high contrast image was processed with a black background and a 30 µm rolling ball. This resulted in a dark image with bright spots delineating cells. Automatic background flattening parameters were used to remove background fluorescence from the GFP and PI channels. (Figure 2). Object masking thresholds were then set to identify each cell for counting. Images were analyzed by masking on the high contrast brightfield images and expanding the brightfield mask to capture the PI and GFP signal (Figure 4). The PI or GFP integral in the secondary mask was calculated for each cell. In order to determine which cells were positive for apoptosis or necrosis, a threshold was set depending on the negative control well (Figure 3). This threshold was set at two standard deviations above the mean of the average value of the negative control well. The percent of total apoptotic/necrotic cells per time point was determined using the ratio transformation function in the data reduction tool box.

Results

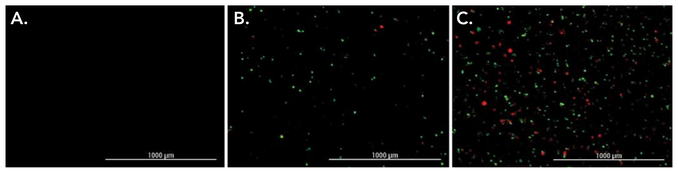

Figure 2. Camptothecin Apoptosis Necrosis HT1080 Images. HT1080 cells were treated with camptothecin in order to determine the effect of camptothecin on the apoptotic and necrotic response of HT1080 cells. 4x **(A)** 0, **(B)** 12, and **(C)** 24 hours of HT1080 cells treated with 2500 nM of camptothecin and stained with PI (red, necrosis) and pSIVA (green, apoptosis) are shown. Apoptosis and necrosis both increase over time and these results demonstrate an increase over time in the percentage of GFP positive apoptotic cells and PI positive necrotic cells.

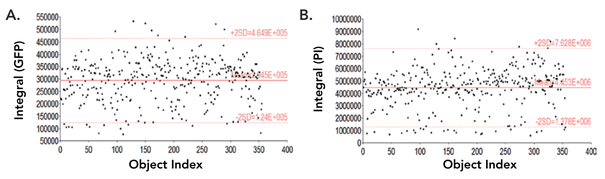

Figure 3. Scatter plots for apoptosis necrosis cut-offs. (A) GFP integral and **(B)** PI integrals from negative control wells were used to determine cutoffs for apoptotic and necrotic cells in subpopulation analysis. The average plus two standard deviations was used as the basis for determining positive apoptotic and necrotic cells.

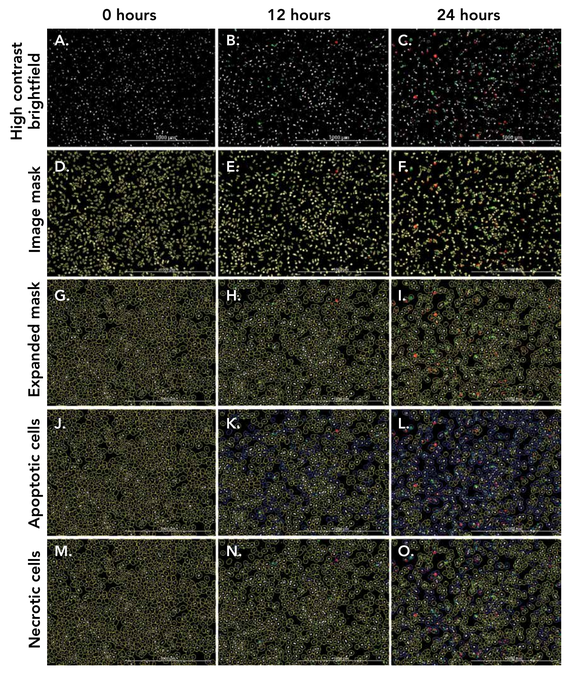

Figure 4. Image analysis of apoptotic and necrotic cells. HT1080 cells were treated with camptothecin in order to determine the effect of the drug on the apoptotic and necrotic response of HT1080 cells. Row 1 **(A-C)** shows pre-processed high contrast brightfield images along with GFP and PI at **(A)** 0, **(B)** 12 and **(C)** 24 hours after treatment. Row 2 **(D-F)** shows the primary mask surrounding each individual cell as delineated by the pre-processed high contrast brightfield image. Row 3 (**G-I)** shows the expanded mask which captures more of the cell area and encompasses the GFP and PI signal. Row 4 **(J-L)** shows the apoptotic cells highlighted in blue. Row 5 **(M-O)** indicates necrotic cells highlighted in blue.

Results *(Cont.)*

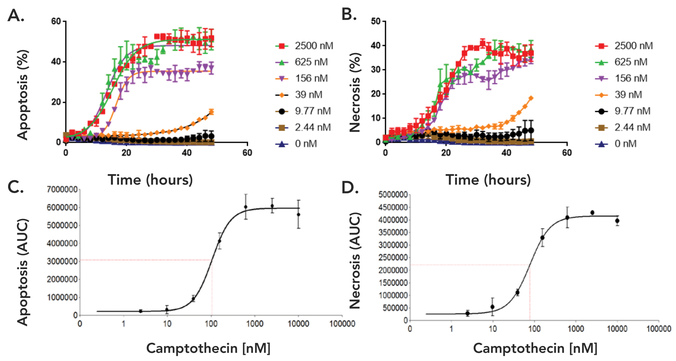

Figure 5. HT1080 cells treated with camptothecin. HT1080 cells were plated at a density of 2000 cells/well in 96 well plates and treated with dilutions of camptothecin in order to determine the effect of the drug on the apoptotic and necrotic response of HT1080 cells. Apoptosis and necrosis increase in a dose dependent manner as demonstrated by **(A,B)** time course analysis over 48 hours; and **(C,D)** dose response at 48 hours.

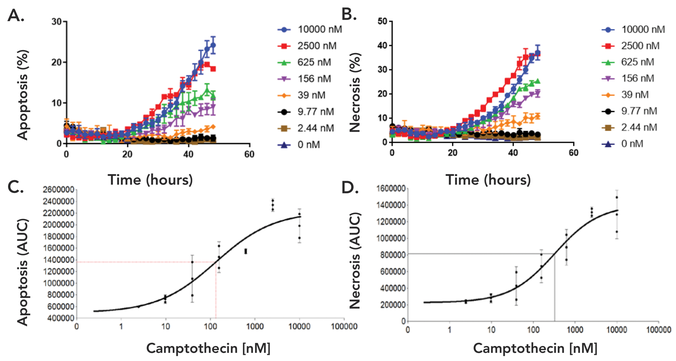

Figure 6. SKOV3 cells treated with camptothecin. SKOV3 cells were plated at a density of 4000 cells/well in 96 well plates and treated with dilutions of camptothecin in order to determine the effect of the drug on the apoptotic and necrotic response of SKOV3 cells. Apoptosis and necrosis increase in a dose dependent manner as demonstrated by **(A,B)** time course analysis over 48 hours; and **(C,D)** dose response at 48 hours.

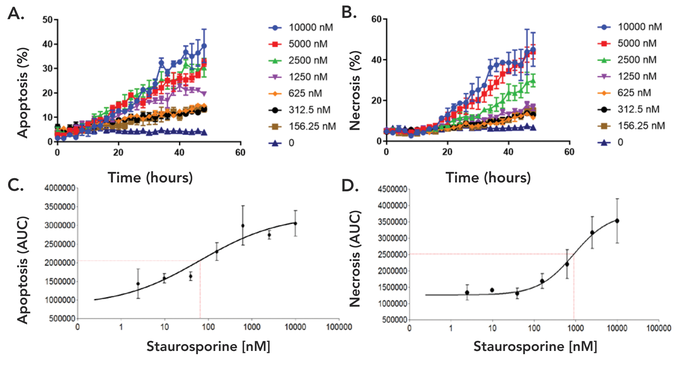

Figure 7. SKOV3 cells treated with staurosporine. SKOV3 cells were plated at a density of 4000 cells/well in 96 well plates and treated with dilutions of staurosporine in order to determine the effect of the drug on the apoptotic and necrotic response of SKOV3 cells. Apoptosis and necrosis increase in a dose dependent manner as demonstrated by **(A,B)** time course analysis over 48 hours; and **(C,D)** dose response at 48 hours.

Conclusions

1. The use of apoptosis and necrosis dyes in combination with automated kinetic imaging allows for quantitative assessment of the effects of known inducers of cell death in multiple cell lines.
2. This assay uses high contrast, label-free brightfield imaging to assay for total number of cells and cellular dyes to label both apoptotic and necrotic cells concurrently. This allows for determination of percent apoptosis and necrosis in each population over 48 hours of drug treatment without use of a nuclear dye.
3. When HT1080 or SKOV3 cells are treated with dilutions of camptothecin or staurosporine, apoptosis and necrosis are induced in a dose-dependent manner over time.
4. Kinetic analysis of cell death analysis allows for sensitive, real-time determination of the accumulation of both apoptotic and necrotic events within the cellular population.