Application Note

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Use of Phase Contrast Imaging to Track Morphological Cellular Changes due to Apoptotic Activity

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Introduction

Phenotypic screening, or the determination of the effects (phenotypes) that a molecule has on a cell, tissue, or whole organism, dates back to the earliest drug discovery efforts. Due to advances in molecular biology and biochemistry in the 1990s, this approach was de-emphasized in favor of a more reductionist target-based approach.[1] Through mounting evidence, however, the shift appears not only to have failed in accelerating discovery of new first-in-class medicines, but has also led to higher attrition rates of new lead molecules.[2] Therefore, a more balanced, holistic approach, which incorporates both discovery methods, is now being implemented.

One of the most important and widely studied phenotypic responses is apoptosis; particularly in oncology research. Understanding apoptosis as it relates to a particular disease helps in understanding its pathogenesis, as well as how it can be treated. In cancer, the normal course pursued by a cell towards death via specific stimuli is lost, leading to uncontrolled cell division. This represents a major causative factor in the development and progression of the disease.[3] A variety of methods exist to track apoptotic activity in whole cells or specific organelles, including antibodies, fluorescent stains, and proluminescent substrates. These methods, while easy to use, result in changes to the original state of the cell by means of foreign material introduction, in addition to lysis or fixation and permeabilization, and can also diminish the ability to perform multiplexed analyses. Through the incorporation of cellular imaging, morphological changes that are the hallmark of apoptosis, including shrinkage of the cell and fragmentation into membrane-bound apoptotic bodies[4], can be monitored in a label-free manner.

This application note demonstrates a method to incorporate automated, digital widefield phase contrast microscopy to monitor potential apoptotic effects of lead molecules. The ability to control conditions within the imaging chamber at 37 °C/5% CO_2 enabled kinetic images to be captured on an hourly basis throughout the entire incubation period. Agilent BioTek Gen5 cellular analysis software also allowed calculation of rounded apoptotic cells. Determination of apoptotic activity was also performed with a fluorescent live-cell assay. Results confirmed the validity of the image-based method to provide accurate analysis of apoptotic induction.

Materials and methods

Materials

Cells: MDA-MB-231 cells (part number 92020424) were purchased from Sigma-Aldrich (Saint Louis, MO). The MDA-MB-231 cells were propagated in Advanced DMEM Medium (part number 12491-015) plus Fetal Bovine Serum (FBS), 10% (part number 10437-028) and Pen-Strep-Glutamine, 1x (part number 10378-016) each from Life Technologies (Carlsbad, CA).

Reagents: The Kinetic Apoptosis Kit (part number ab129817) was purchased from Abcam (Cambridge, MA). Oridonin (part number O9639) and Hoechst 33342 (part number 14533) were purchased from Sigma-Aldrich.

Agilent BioTek Cytation 5 cell imaging reader: Cytation 5 combines automated digital microscopy and conventional multimode microplate detection providing rich phenotypic cellular information and well-based quantitative data. 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. The phase contrast and GFP imaging channels were used to monitor morphological changes, as well as changes in fluorescence from the kinetic apoptosis reagent, respectively.

Agilent BioTek Gen5 data analysis software: Gen5 software controls the operation of the Cytation 5 for both automated digital microscopy and PMT-based microplate reading. Image analysis and subpopulation calculations allow the counting of apoptotic cells meeting a predetermined signal threshold and circularity requirements.

Methods

Cell preparation and dispensing into microplates: Cells were harvested and diluted to a concentration of 5.0×10^4 cells/mL. A volume of 100 µL was then dispensed to appropriate wells of a 96-well clear bottom, black TC-treated microplate (part number 3904) from Corning Life Sciences (Corning, NY).

Component preparation and addition: Hoechst 33342 was diluted to a final 1x concentration of 5 µM in medium. Following removal of the plating medium, 100 µL of the diluted dye was added per well and incubated for 15 minutes at 37 °C/5% CO_2. The pSIVA-IANBD apoptosis reagent was then diluted in medium at a concentration of 10 µL/mL, followed by an oridonin dilution into medium containing apoptosis reagent or medium alone to a concentration of 100 µM. Serial 2.5x dilutions were carried out to create two 12-point titration curves (including a no-compound control), with concentrations ranging from 100 to 0.01 µM. Following the 15-minute incubation, medium was again removed and replaced with 100 µL of compound with or without apoptosis reagent.

Kinetic image-based monitoring of apoptotic induction: The 96-well plate containing cells and compound was immediately placed into the Cytation 5, with temperature and gas control having been previously set to 37 °C/5% CO_2. Imaging of each well was completed using 4x and 20x objectives. The phase contrast imaging channel was used to capture images from wells containing nonlabeled cells, while the fluorescent signal from the pSIVA-IANBD reagent was imaged using the GFP channel. A discontinuous kinetic procedure was chosen where imaging was carried out with each designated well once every hour over a 24-hour incubation period.

Agilent BioTek Gen5 cellular analysis: Cellular analysis was performed using Gen5 software on the 4x phase contrast and GFP images captured. The number of apoptotic cells per image was counted morphologically through changes in contrast, and increases in circularity exhibited by this cell type, and confirmed through fluorescent tracking of external phosphatidylserine exposure. Tables 1 and 2 describe the parameters used to count cells with the phase contrast and GFP channels.

Table 1. 4x Phase contrast image cellular analysis parameters.

Phase Contrast Detection Channel Cellular Analysis Parameters	
Threshold	6,000 RFU
Minimum Object Size	10 µm
Maximum Object Size	40 µm
Bright Objects on a Dark Background	Unchecked
Split Touching Objects	Checked
Advanced Options	
Evaluate Background On	5% of Lowest pixels
Image Smoothing Strength	0
Background Flattening Size	Auto
Subpopulation Analysis	
Object Metric	Circularity
Condition	>0.4

Table 2. 4x GFP image cellular analysis parameters.

GFP Detection Channel Cellular Analysis Parameters	
Threshold	3,000 RFU
Minimum Object Size	10 µm
Maximum Object Size	100 µm
Bright Objects on a Dark Background	Checked
Split Touching Objects	Checked
Advanced Options	
Evaluate Background On	5% of Lowest pixels
Image Smoothing Strength	0
Background Flattening Size	Auto

Results and discussion

Analysis of apoptotic induction using phase contrast imaging

From a visual analysis of the phase contrast images captured using a 20x objective (Figure 1), numerous characteristic morphological features of apoptotic cells can be identified. Cell rounding due to shrinkage and cytoplasm condensation, indicators of early apoptosis[4], can be seen in Figure 1B. Separation into apoptotic bodies, or blebs, a marker of later-stage apoptosis[4], is also witnessed in Figures 1C and 1D.

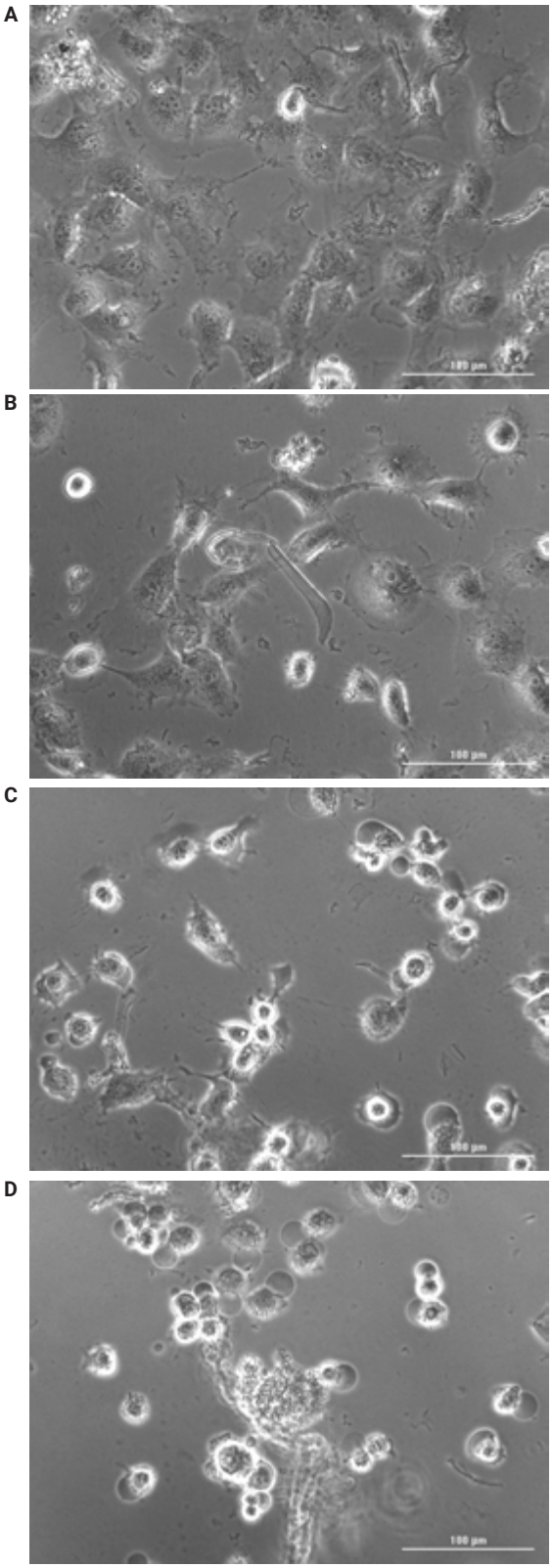

Figure 1. Phase contrast image-based monitoring of apoptosis induction. 20x phase contrast images of MDA-MB-231 cells after a seven-hour incubation with (A) 0, (B) 1, (C) 40, or (D) 100 µM oridonin.

Label-free quantification of apoptotic activity can also be performed using a 4x objective to sample a larger portion of the total cell population within the well (Figure 2A), and Gen5 data analysis software. Using the primary cellular analysis parameters previously described in Table 1, object masks are drawn around cells within the image (Figure 2B). However, not all cells within the image are apoptotic. Therefore a sub-population criteria is also applied which takes advantage of the round appearance of apoptotic cells (Figure 2C). Healthy cells remain spread out on the bottom of the well in a noncircular manner. This secondary criteria allows a more accurate count of cells undergoing apoptosis.

Determination of the apoptotic effect of a test molecule at various endpoints can then be performed by comparing the number of cells identified from treated versus untreated wells (Figure 3).

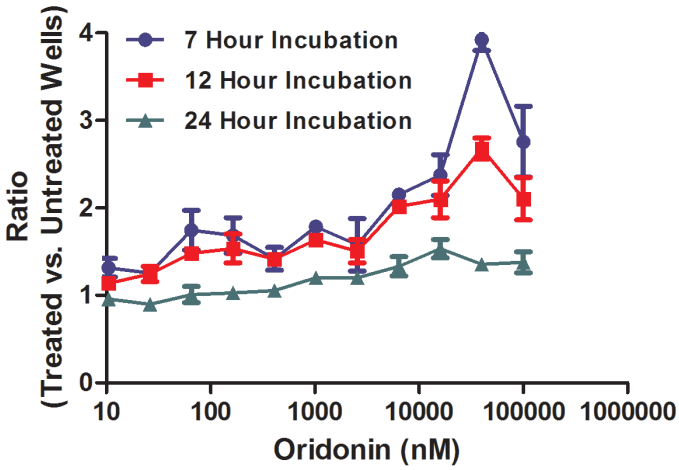

Figure 3. Quantification of apoptosis induction. Ratio of cell counts from wells containing 10 to 100,000 nM oridonin compared to untreated wells after 7-, 12-, and 24-hour incubation periods.

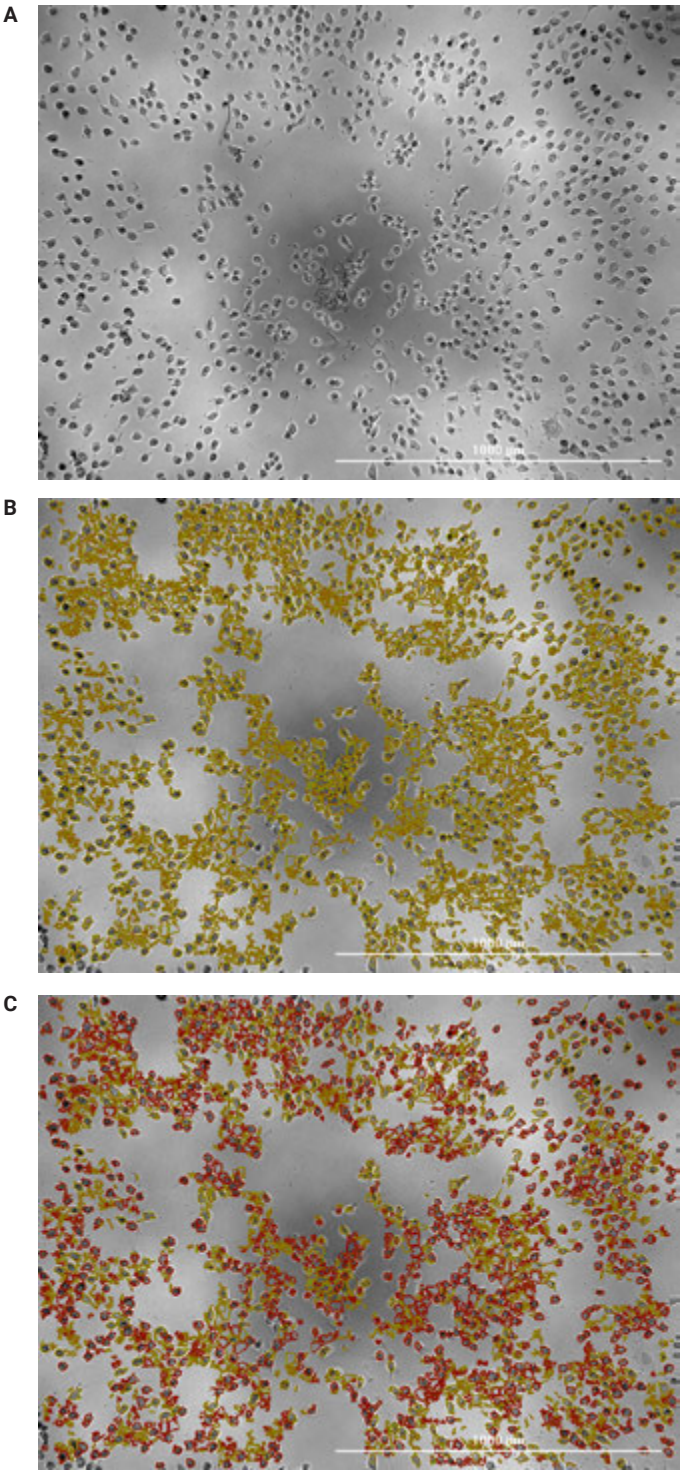

Figure 2. Apoptotic cell determination. (A) 4x phase contrast image of MDA-MB-231 cells following 40 µM oridonin incubation. (B) Total object count using initial cellular analysis parameters. (C) Apoptotic cell count following subpopulation parameter application.

Kinetic apoptotic analysis

As a result of a label-free analysis method being used, which does not require the addition of an endpoint reagent, kinetic analysis of apoptotic induction can also be performed through the incorporation of temperature and gas control within the imaging chamber (Figures 4A to 4C). This enables a more definitive investigation of all concentrations being examined.

The cell count and subsequent cell ratio graphs (Figures 4D to 4E) illustrate that apoptosis is induced at a faster rate with higher oridonin concentrations. However, the level of induction does not reach that seen with lower concentrations. This is most likely due to the higher level of overt toxicity within the cells causing necrosis, rather than apoptosis.

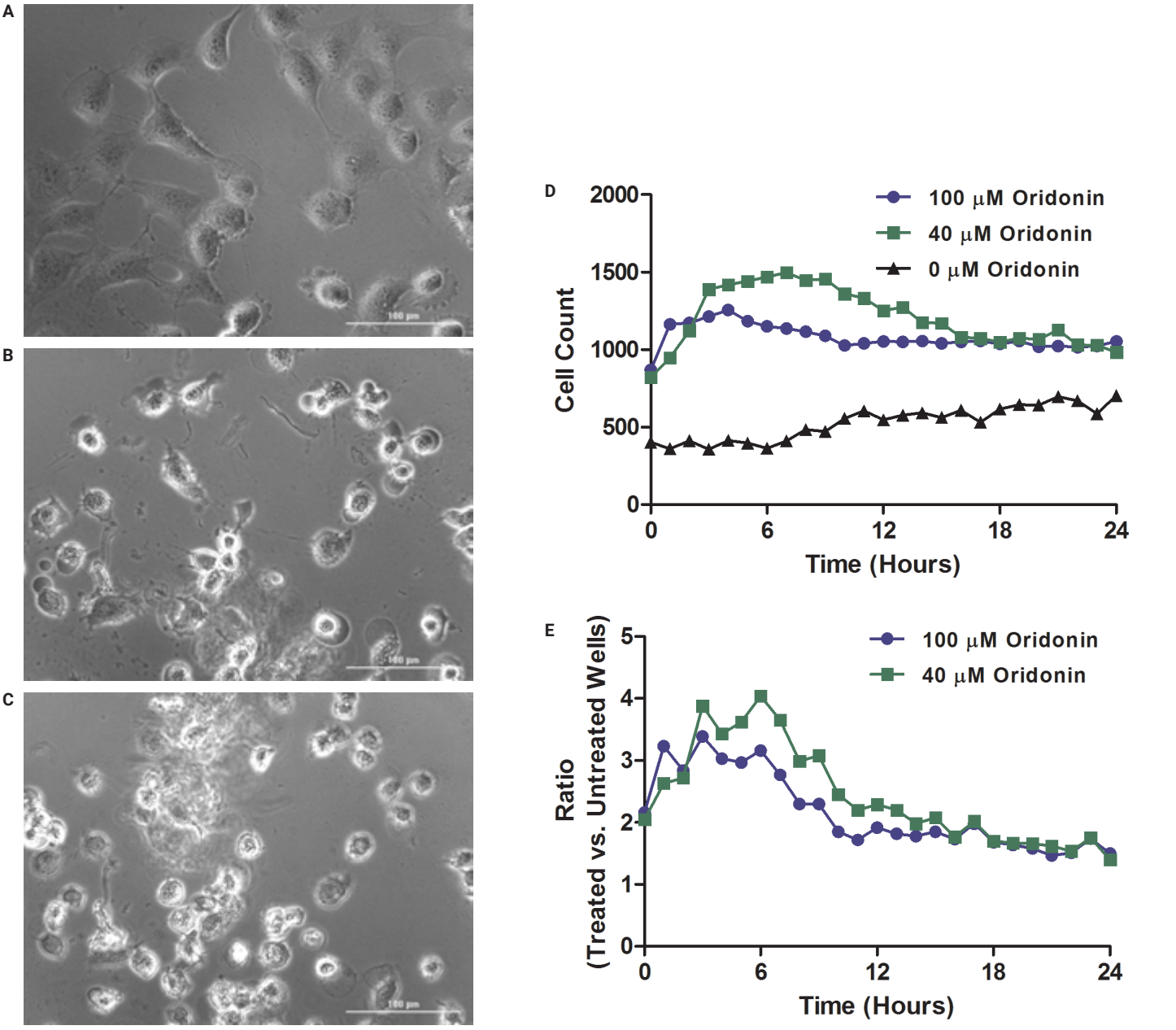

Figure 4. Kinetic monitoring of apoptosis induction. 20x phase contrast images of MDA-MB-231 cells captured after (A) 0-, (B) 7-, and (C) 24-hour incubation with 40 µM oridonin. (D) Cell counts from kinetic 4x images of wells containing cells treated with 0, 40, and 100 µM oridonin. (E) Cell number ratios from treated versus untreated wells.

Fluorescent live-cell assay validation

Validation of the data generated using phase contrast imaging was completed by performing cellular analysis on oridonin treated and untreated wells, having been preincubated with Hoechst 33342 and the kinetic apoptosis reagent (Figure 5). Table 2 outlines the parameters used for the fluorescence-based analysis.

External exposure of phosphatidylserine (PS) is a seen as a hallmark of early apoptosis. However, increasing knowledge supports the idea that transient exposure occurs as the cell proceeds further along in the cell death process.[5,6] This phenomenon is also witnessed during oridonin treatment (Figure 5). Little to no signal is seen initially from the green pSIVA reagent. After seven hours of incubation with higher concentrations of the molecule, high levels of fluorescence are exhibited from binding of the reagent to PS (Figure 5B). The signal then wanes after extended exposure (Figure 5C), indicating later stages of apoptosis.

A comparison of ratios from treated versus untreated wells, calculated using phase contrast or fluorescence imaging, demonstrates that equivalent results can be seen when tracking changes to cellular morphology, as opposed to fluctuations in the signal from a fluorescent live-cell reagent (Figure 6).

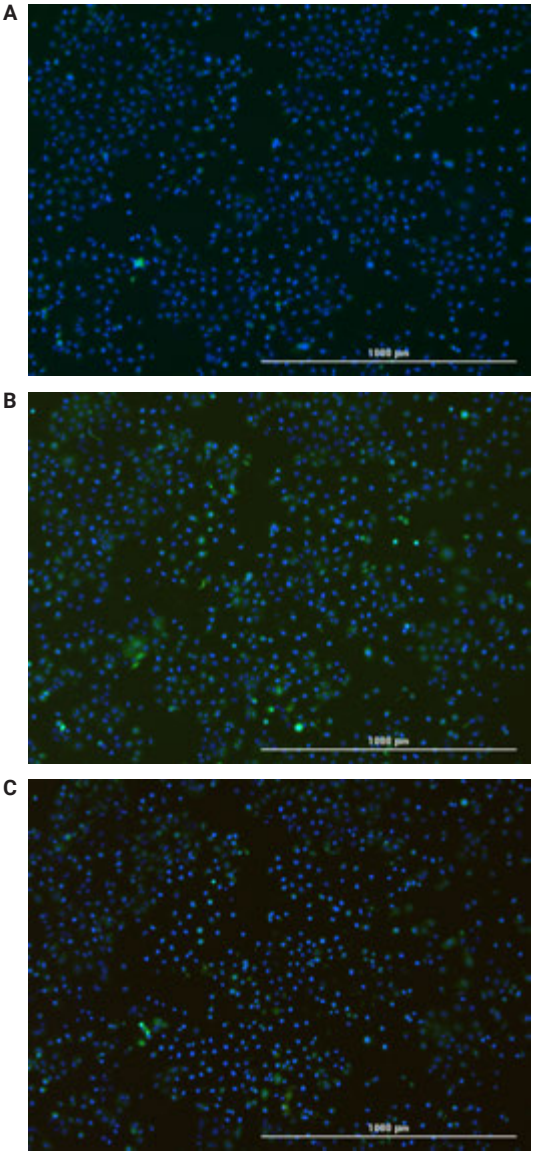

Figure 5. Fluorescent apoptotic activity monitoring. Kinetic 4x images taken of MDA-MB-231 cells stained with Hoechst 33342 (blue) and kinetic apoptosis reagent (green). Images captured after (A) 0-, (B) 7-, and (C) 24-hour incubation with 40 µM oridonin.

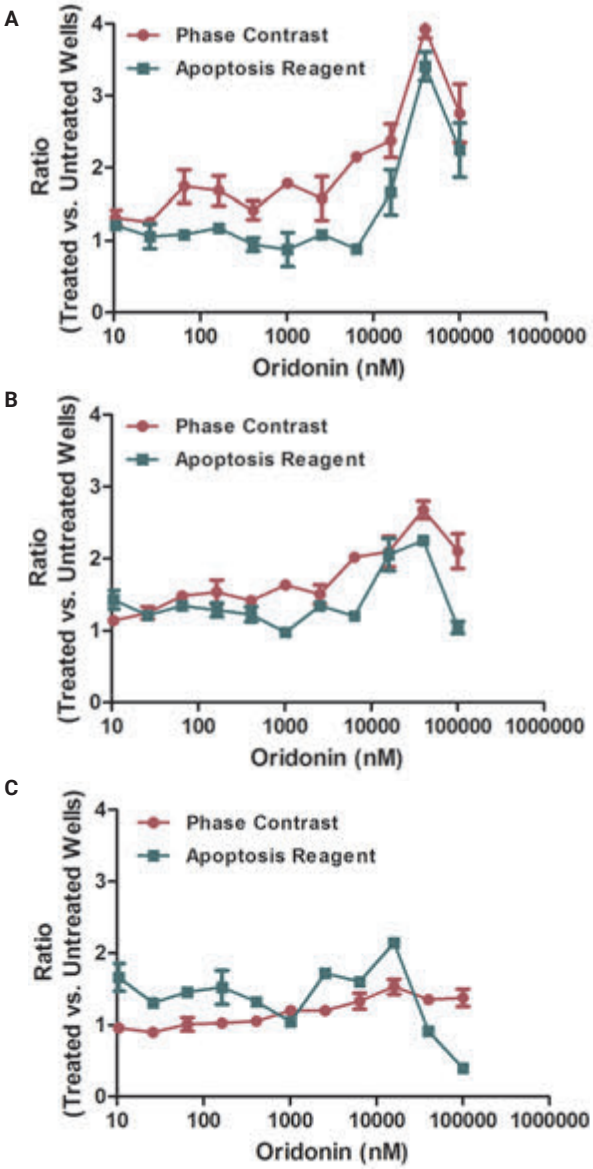

Figure 6. Cellular analysis method comparison. Cell count ratios calculated using 4x phase contrast or fluorescent images following a (A) 7-, (B) 12-, or (C) 24-hour oridonin incubation.

Conclusion

The use of changes in morphology is a desired method to track apoptotic activity in a target cell type, due to the process being label-free and multiplexing possibilities. Morphological changes which occur during this process are well characterized, and appear across all cell types, keeping analysis consistent. Any potential bias that might be seen when introducing an optical probe or other substrate is also eliminated. Through the incorporation of phase contrast imaging, this technique can be accomplished with the Agilent BioTek Cytation 5 cell imaging multimode reader. Higher magnification images taken with a 20x objective provide qualitative assessment, while the use of lower magnification, such as 4x, in addition to Agilent BioTek Gen5 microplate reader and imager software, allow quantitative, statistical determinations to be made. Temperature and gas control within the imaging chamber also enable automated, kinetic evaluations. The combination provides a simple, yet powerful method to study this important mechanism of cell death.

References

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