

Monitoring Cytotoxicity by Oridonin in U-2 OS Cells in Real Time Using the BioSpa™ 8 to Manage Kinetic Image-based Analysis of Live Cells

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The imaging and analysis of fluorescently stained live cells has traditionally been accomplished using complete manual microscopic methods with exceptionally low numbers of samples. The analysis of higher sample numbers required the use of very expensive dedicated microscopes. Here we describe the use of the BioSpa™ 8 to automate the assay workflow of a kinetic live cell assay using fluorescent probes to monitor cytotoxicity with an automated digital fluorescence microscope.

Introduction

Cytotoxicity is a leading cause of drugs failing during clinical and pre-clinical trials. Implementing an *in vitro* cell-based predictive assay early in the drug discovery process would improve early compound attrition and develop safer drug candidates. The high content imaging approach increases the sensitivity and specificity for predicting cytotoxicity by simultaneously detecting cellular targets and properties associated with cell loss, DNA content, cellular redox stress, and mitochondrial stress. High content assays have further progressed to the extent that many of these assays are performed with live cells rather than fixed and antibody stained specimens. The use of live cells can provide critical temporal information that static fixed cell staining techniques cannot. The trend towards live cell high content has in turn driven the need for automation of these types of assays.

Affordable live cell automation has been elusive in drug discovery biomedical research. Modular robotic systems, historically designed primarily for high throughput screening, often consist of crane or articulating arm that picks and places microplates from a number of process specific instruments to carry out the assay's process steps. By their nature these systems are generally quite large and expensive, requiring specialized rooms or custom chambers with HEPA filters to maintain sterility when running live cell assays. Additionally, highly skilled individuals with extensive robotics experience are mandatory. Compact automated low throughput systems have been developed primarily with ELISA reactions in mind. These systems generally are limited with regard to assay flexibility, with minimal capacity for reagent addition and fixed incubation temperatures and no environmental gas control. While suitable for ELISA they make a poor choice for automated live cell assays.

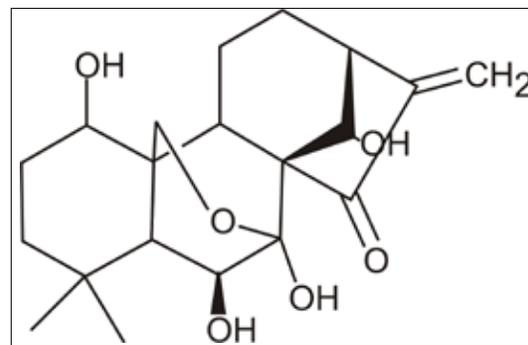


Figure 1. Oridonin Structure.

BioSpa™ 8

BioTek Instruments has developed the BioSpa 8 Automated Incubator to address the need for an assay workstation that is both flexible and affordable, while providing the means to run long term kinetic live cell assays. The BioSpa 8 links BioTek microplate readers or imagers together with washers and dispensers for full workflow automation of up to 8 microplates. Real time control and continuous temperature, CO₂/O₂ and humidity level monitoring; along with lid handling ensure an ideal environment for cell cultures during all experiment stages, with minimal manual intervention. BioSpa software, which features customizable text or email notifications and alerts, also relieves the need for onsite monitoring. The software's session timelines and environmental reports allow quick scrutiny of the process and system status. BioSpa 8 automates assay workflows by repeated manipulation and storage of up to 8 microplates or other vessels containing live cells or temperature sensitive reagents.

Its size is such that it, along with a liquid handler and reader/imager, can be placed inside a conventional 6-foot biosafety cabinet.

To demonstrate the value of this system, we have evaluated the effects of Oridonin on U-2 OS cells using the reagent CellTox™ Green. Oridonin, a natural tetracycline diterpenoid (Figure 1) isolated from Chinese herb *Rabdosia rubescens*, has been reported to be a potent cytotoxic agent against a wide variety of tumors. Oridonin has been shown to induce potent growth inhibition on human breast cancer cells MCF-7 and MDA-MB-231 in a time- and dose-dependent manner though cell cycle arrest at the G2/M phase [1]. The compound has also been shown to induce apoptosis in a variety of cell lines [2]. In order to demonstrate the utility of the BioSpa™ 8, we've designed a series of experiments that use the BioSpa 8 to transfer microplates to a liquid handler and a microplate imager as well as to provide environmental control in between process steps.

Materials and Methods

Cell Culture

U-2 OS cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in 5% CO₂. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black sided clear bottom 96-well microplates at 2,500 to 10,000 cells per well depending on the experiment.

Imaging

Cultures were imaged using a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) configured with DAPI, and GFP cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cubes uses a 337/50 excitation filter and a 447/60 emission filter, while the GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter.

Image Analysis

Two-color digital images were overlaid using Gen5™ Microplate Reader and Imager Software. Object cell counting of the DAPI channel was used to identify cell nuclei. Subpopulation analysis was used to determine the mean fluorescence intensity of the GFP channel as a means to assess cytotoxic positive cells (Figure 2).

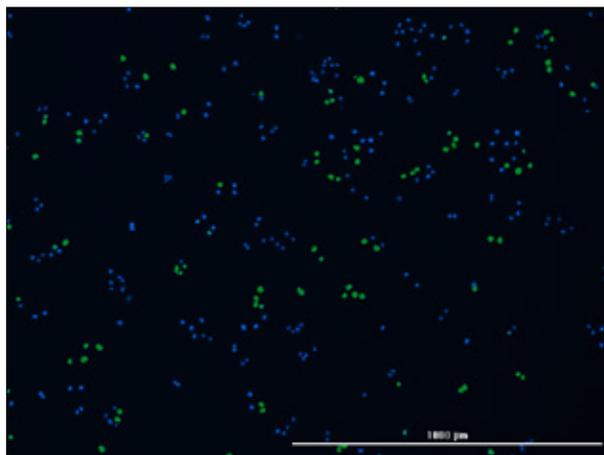


Figure 2. Representative 4x image of U-2 OS cells stained with Hoechst 33342 and CellTox™ Green. Cells were treated with 30 μM oridonin for 6 hours, then stained with Hoechst 33342 (1 μg/mL) and CellTox™ green for 30 minutes. Digital microscopic images (4x) were made using Cytation 5.

Experimental Design

Oridonin is added to the plate using the MultiFlo FX peripump such that different concentrations of drug are added with each of the eight separate dispense tubes in rows A-H (Figure 3).

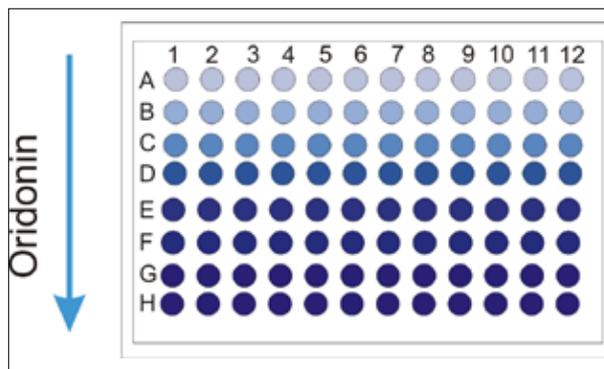


Figure 3. Plate map configuration of Image Centric Oridonin cytotoxicity experiments. Oridonin is added to the plate using the MultiFlo FX peripump such that different concentrations of drug are added with each of the eight separate dispense tubes in rows A-H. Row H would have the highest drug concentration, while row A has the lowest drug concentration.

After Oridonin is added, the plates are imaged periodically over 24 hours. Image analysis is performed repeatedly within that period (Figure 4). Between process steps, the plates are housed in the BioSpa 8, which provides environmental controls such as temperature, humidity and CO₂ gas levels.

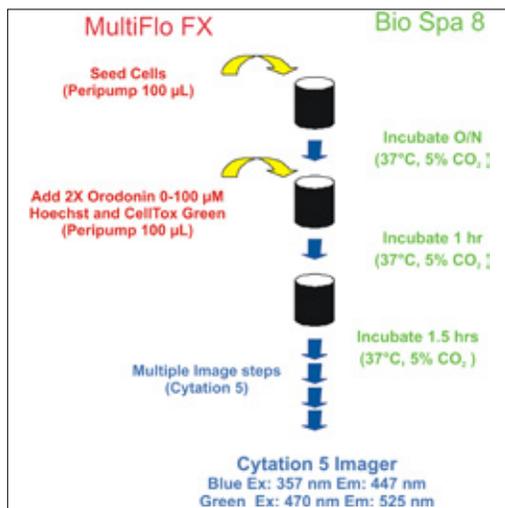


Figure 4. Assay Process steps of a Cytotoxicity Assay with Multiple Imaging Steps.

The BioSpa™ software schedules each batch based on the expected time for each process step and the number of plates in conjunction with the assay process. With scheduling, a graphical Gantt chart depicting the estimated time and duration for each process for all of the plates programmed is developed. The process steps of a three-plate experiment is depicted in Figure 5.

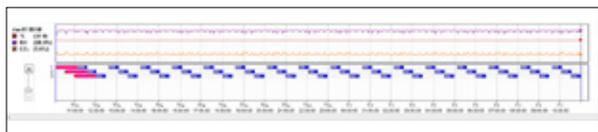


Figure 5. Gantt chart of a BioSpa 8 cytotoxicity assay session with Multiple Image Steps. Oridonin (0-100 µM), along with Hoechst 33342 and CellTox® Green dyes, is added to U-2 OS cells in 3 separate plates with the MultiFlo™ FX. After incubating for 1.5 hours to enable dye loading plates are imaged every 1.5 hours using a Cytation™ 5 Cell Imaging Multi-Mode Reader. Plates are incubated in the BioSpa 8 at 37 °C, with a humidified 5% CO₂ atmosphere between imaging steps.

Results

Treatment with oridonin is known to cause apoptosis and cell death in mammalian cells. Hoechst 33232 is a membrane permeable dye that will bind nucleic acids of live and dead cells, while CellTox™ green can only stain nucleic acids from membrane incompetent dead cells. This combination of dyes allows for subpopulation analysis of image data that can determine the percentage of cytotoxic cells through image analysis of DAPI and GFP images (Figure 2).

When time sequential images from the same wells treated with oridonin are analyzed, a concentration dependent spike in cytotoxicity is observed at 5 hours after the addition of 100 µM oridonin (Figure 6). Treatment with 30 µM showed a spike in the percentage of cells determined to be cytotoxic, which is delayed slightly relative to 100 µM. The lower concentration of 10 µM only begins to produce cytotoxicity near 24 hours of exposure to oridonin.

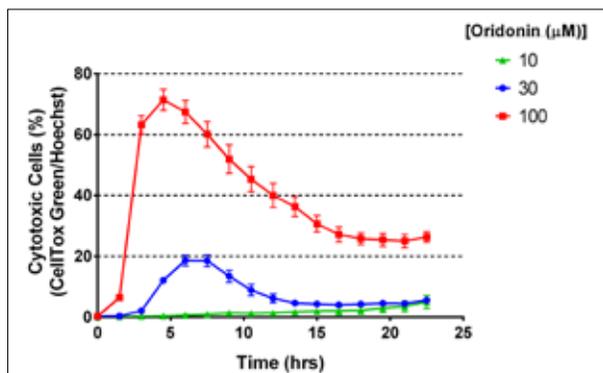


Figure 6. Effect of oridonin on the percentage of cytotoxic cells as a function of time. Cell were exposed to either 30 or 100 µM oridonin and imaged at various points in time. Data points represent the mean and standard deviation from 12 replicates from a single microplate.

The apparent decrease in the % of cytotoxic cells for the two higher oridonin concentrations corresponds with the data that shows a loss of cell number in the oridonin treated wells, while the untreated wells demonstrate an increase during the same time period (Figure 7).

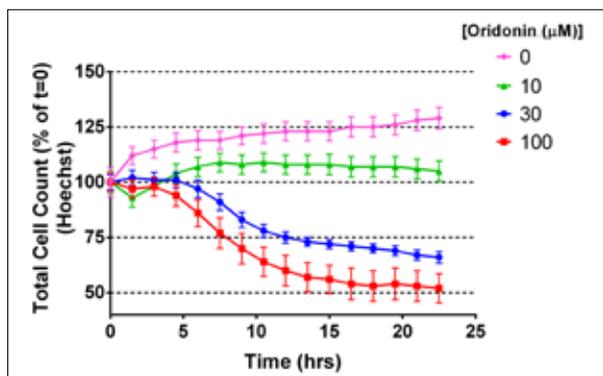


Figure 7. Cell counts with Oridonin treatment. Cell number was determined by object counting Hoechst 33342 stained U-2 OS cell nuclei. Data is expressed as the percent change from the initial determination. Each data point represents the mean and standard deviation of 12 data points from a single plate.

Oridonin cytotoxicity is dose dependent as demonstrated in Figure 8. A 7.5 hour oridonin treatment with increasing concentrations of drug results in greater percentages of cytotoxic cells in a dose dependent manner. The repeatability of the assay can also be seen as the plot for three separate plates are very similar.

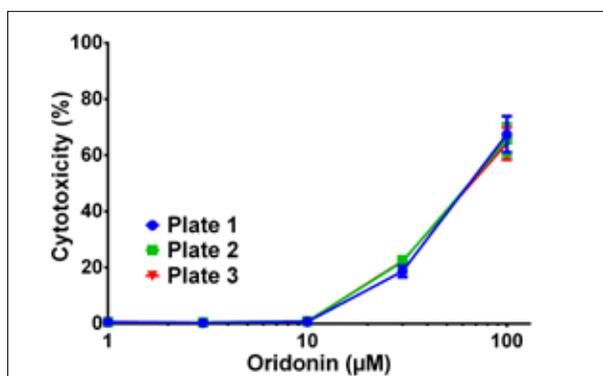


Figure 8. Cytotoxicity Oridonin Dose response. The percent age of cytotoxic cells was calculated for various concentrations of oridonin after a 7.5 hour treatment. Each data point represents the mean and standard deviation of 12 data point. Three separate plates were plotted independently.

Using Image analysis of bright field images, cells were counted and assessed for roundness using object circularity (Figure 9). Because apoptotic cells typically exhibit a round shape phenotype, the circularity of cell objects can be used as an indicator for cell apoptosis analysis. The percentage of cells determined to be round can be a means to track apoptosis kinetically.

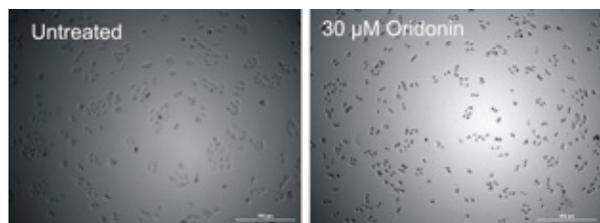


Figure 9. Bright field images of Untreated and Oridonin Treated U-2 OS cells. U-2OS cells were imaged using bright field (4x).

As demonstrated in Figure 10, oridonin exposure results in greater numbers of rounded cells. These cells are presumably undergoing apoptosis. High doses of oridonin (100 µM) demonstrate an increase in the percentage of apoptotic cells within the first 1.5 hours of exposure, with levels increasing further, reaching maximal levels at about 5-6 hours of exposure. Lower doses require slightly longer exposure times to achieve maximal levels and do not achieve as high a percentage. Using these analysis criteria, untreated cells have a constant percentage of cells exhibiting a round phenotype. These are presumably cells undergoing mitosis, as mitotic cells are known to become round immediately before cytokinesis [5]. The percentage observed is in line with reported times for G2 and M phases of the cell cycle relative to the total. These data are also in agreement with previous studies by Larson *et.al* using MDA-MB-231 cells, which showed an induction peak for apoptosis with 6-7 hour exposure. These studies used either phase contrast or the detection of phosphatidylserine on the cell surface to denote apoptosis [3].

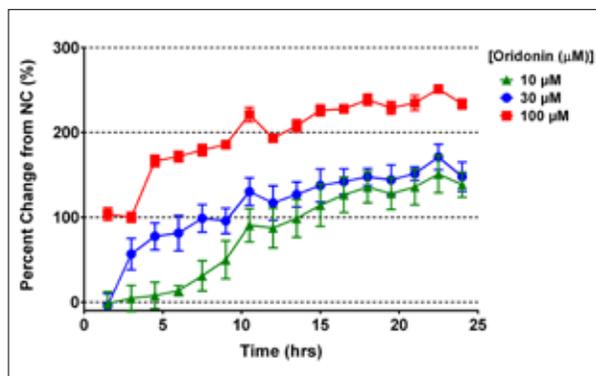


Figure 10. Effect of oridonin exposure to the change in the percentage of Apoptotic cells over time. U-2 OS cells were treated with oridonin and the percentage of rounded apoptotic cells determined. Cells were considered round if they had an object circularity greater than 0.6.

Conclusion

These data demonstrate that the BioSpa is capable of automating routine live cell based assays. The determination of the cytotoxicity of drug compounds is a routine ADME/Tox procedure for any potential therapeutic. Experiments where multiple measurements are made repeatedly over a period of time require considerable labor by researchers or some degree of automation; particularly true when the time span of the assay approaches 24 hours.

Oridonin is a natural diterpenoid compound previously shown to induce apoptosis in mammalian cancer cells [4]. As such it is an ideal drug candidate to demonstrate the utility of monitoring live cells for cytotoxicity in real time. We have used this compound as a model system in which to show the utility of the BioSpa 8 to automate typical live cell based assays.

Using the Biospa 8 to integrate a liquid handler, such as the MultiFlo FX with the Cytation 5 Cell Imaging Multi-Mode Reader has allowed the investigation of the temporal and concentration relationships of oridonin dosing and cytotoxicity of U-2 OS cells. By adding various concentrations of drug concurrently with specific nuclear stains and imaging at specific time intervals we have demonstrated that oridonin toxicity is both time and concentration dependent. In addition the two effects appear to be additive.

The Biospa 8 is an ideal vehicle to automate live cell assays of this nature. The robotic incubator design maintains temperature, gas control and humidity during incubation steps, while also transporting plates to and from liquid handling and detection instruments as needed. The robotic plate hotel is able to manage multiple plates and allow for a completely walk away solution to kinetic assays in a single experiment. Normally this would require extensive manual intervention or multiple imagers to accommodate multiple plates.

References

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