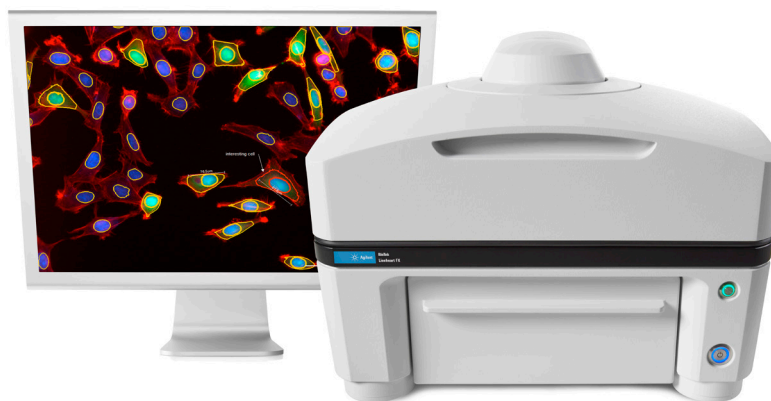


# Oridonin Perfusion Causes Cytotoxicity in U-2 OS Cells

Using the ONIX2 system in conjunction with the Agilent BioTek Lionheart FX automated microscope



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## Abstract

The imaging and analysis of fluorescently stained cells has traditionally been accomplished using manual microscopic methods with low numbers of samples. Unfortunately, much of the work has been performed under static fluidic conditions that allow compound degradation products and cellular metabolites to build up. This application note describes the use of the Agilent BioTek Lionheart FX automated microscope and the CellASIC ONIX2 perfusion micro-incubator to rapidly image and analyze perfused tissue culture cells in multiple fluorescent colors and brightfield.

## Introduction

Cytotoxicity is a leading cause of drugs failing during lead optimization and clinical trials and in some cases being withdrawn from the market. Implementing an *in vitro* cell-based predictive assay early in the drug discovery process would help improve early compound attrition (reducing research and development costs) 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 such 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.

ADME/Tox drug compound testing with live cells is typically performed in microplates. These microplates allow for numerous test variables to be assessed simultaneously, saving time and reagents. However, these types of assays are by definition performed under static hydraulic conditions and do not truly mimic cells *in vivo*. In these experiments, cells are treated with compounds in microplate wells and efficacy or toxicity is assessed after a defined period. During the experiment, the test compound may be undergoing any number of chemical transformations, such as hydrolysis or oxidation that can alter its potency. In addition, cellular metabolites increase in concentration over time. These perturbations can only be rectified in bulk with periodic media exchanges, whereas *in vivo* mammalian cells exist in a vascularized tissue or in a body fluid with a controlled environment where nutrients are provided and wastes are removed on a continual basis.

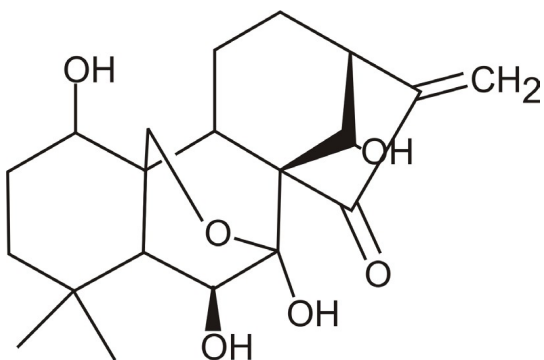


Figure 1. Oridonin structure.

## Agilent BioTek Lionheart FX automated microscope

The Lionheart FX is an automated digital inverted fluorescence microscope capable of several different imaging modes including fluorescence, brightfield, color brightfield, and phase contrast. The imager can accommodate many different vessel types, including slides, counting chambers, cell culture flasks, perfusion chambers, and microplates, while providing for complete environmental control. An optional environmental control cover provides temperature control up to 40 °C, and with an optional gas controller module, CO<sub>2</sub> (0 to 20%) and O<sub>2</sub> (1 to 19%) levels can be maintained.

A high-resolution lead screw driven stage with open access allows for precise localization with repeated imaging. High-powered LEDs, available in several different wavelengths work with numerous filter pair/dichroic mirror combinations in order to provide a multitude of fluorescent color choices. The imager holds up to six objectives in an automatic rotating turret, with available air objectives ranging from 1.25 to 60x and oil ranging from 60 to 100x. Images are captured with a 16-bit grayscale camera to provide true 16-bit TIFF raw image files, which can be saved in several different formats, including video formats.

## CellASIC ONIX2

The CellASIC ONIX2 Microfluidic System uses microfluidics to enable continuous live cell imaging with media flow. The design allows cells to be exposed to different solutions and conditions via pressurized flow channels controlled by user-specified time intervals and flow rates. The system connects to the microfluidic plate via a pneumatic manifold that uses pressurized air to pump cells and liquids from the plate wells into the microfluidic cell culture chambers (Figure 2).

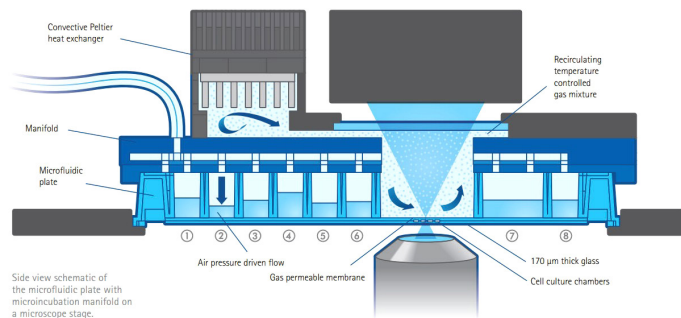
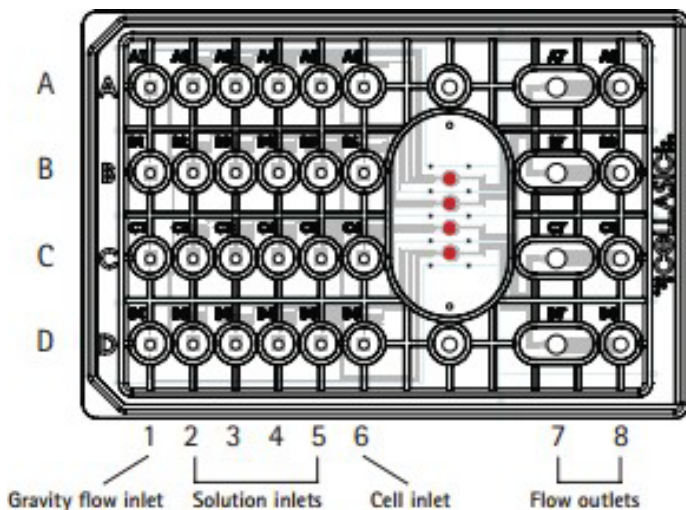
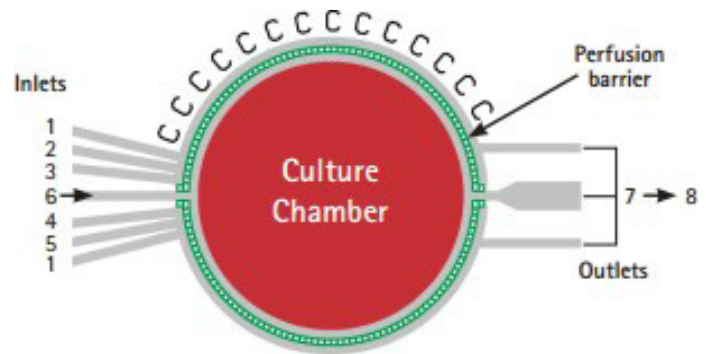


Figure 2. CellASIC plate with ONIX2 heated manifold.

A vacuum seal created between the manifold and the microfluidic plate ensures that each well is independent and that flow rates and fluid switching are accurate. Flow control is managed through a computer software program. The M04S plate has four independent culture chambers connected to separate source inlets and waste flow outlets by microfluidic channels embedded in the plate (Figure 3). Each bank of source inlets can be used independently to provide reagents to its corresponding culture chamber. The M04S plates enable continuous perfusion culture for long-term, live mammalian cell analysis. Continuous perfusion of medium and steady removal of waste ensures a suitable growth environment for long-term experiments. A perfusion barrier surrounding the culture chamber separates the chamber from the reagent channels, preventing cells from leaving (Figure 4). Cells are easily loaded manually with a pipette and cultured prior to the experiment or with an automated software routine immediately prior to a run.



**Figure 3.** Schematic of CellASIC M04S plate. The M04S microfluidic plate has four independent culture units (A to D), each with a gravity flow inlet (1), four solution inlets (2 to 5), a cell inlet (6), and two shared outlets (7 and 8). Each row of wells (A to D) addresses the corresponding culture chamber. All four culture chambers (RED) are located under a single viewing window to minimize travel distance for imaging.



**Figure 4.** Perfusion culture chamber of a CellASIC M04S-03 plate. Each culture chamber is connected to a corresponding source well by a separate microfluidic channel. A perfusion barrier surrounds the chamber to separate it from the flow channels.

## Materials and methods

### Cell culture

U-2 OS were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO<sub>2</sub>. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluency. For experiments, cells were plated into CellASIC ONIX switching plate for mammalian cells (part number M04S-03-5PK) at 1.5 x 10<sup>6</sup> cells per mL.

### Imaging

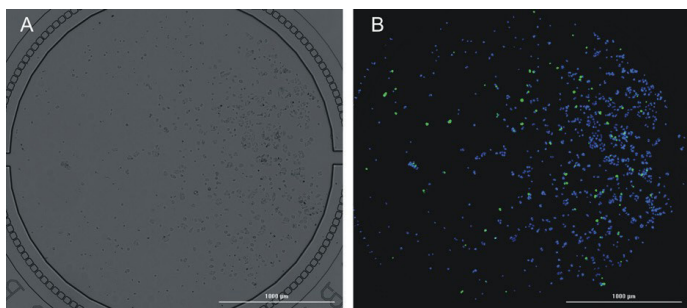
Cultures were imaged using the Agilent BioTek Lionheart FX automated microscope configured with DAPI, CY5, and GFP cubes. The imager uses a combination of LED light sources in conjunction with bandpass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cube uses a 377/50 nm excitation filter and a 447/60 nm emission filter, the CY5 light cube uses a 628/40 nm excitation and a 685/40 nm emission filter, while the GFP light cube uses a 469/35 nm excitation filter and a 525/39 nm emission filter.

## Perfusion

Cells and reagents were loaded into CellASIC M04S switching plates for mammalian cells according to the manufacturer. Briefly, image chambers were preloaded with complete media (10  $\mu$ L per chamber) through capillary action. After preloading, 10  $\mu$ L of cell suspension was added manually to the load chamber, while media with dye and oridonin compound was loaded into source wells. Typically, 300  $\mu$ L of reagent was loaded into source wells. After plate loading the plate was sealed in the ONIX2 manifold. Cells were injected into the image chamber using a dedicated software routine to apply pressure to the loading chambers forcing the cells through the microfluidic channels. Perfusion routines for various source wells and time were programmed using the CellASIC software. When engaged, pressure was typically set to 6.9 kPa.

## Image analysis

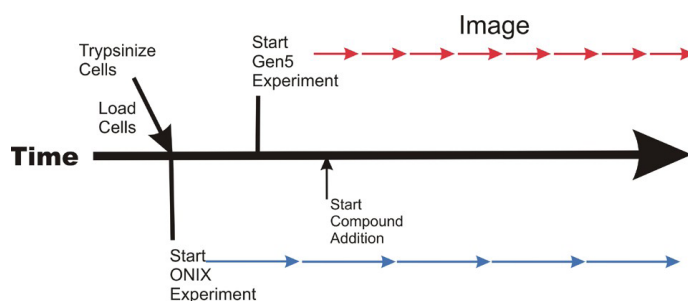
Two-color digital images were overlaid using Agilent BioTek Gen5 microplate reader and imager software. Object cell counting of the DAPI or CY5 channel was used to identify cell nuclei with Hoechst 33342 or DRAQ5 dyes, respectively. These data were false-colored blue, while the GFP channel was colored green. The intensity of coloration was based on the magnitude of the raw grayscale signal. Subpopulation analysis was used to determine the mean fluorescence intensity of the GFP channel as a means to assess cytotoxic positive cells (Figure 5).



**Figure 5.** Representative 10x montage image of U-2 OS cells in read chamber M04S stained with Hoechst 33342 and CellTox Green. Cells were treated with 30  $\mu$ M oridonin for six hours, then stained with Hoechst 33342 (1  $\mu$ g/mL) and CellTox Green for 30 minutes. Digital microscopic images (10x) were made using Agilent BioTek Lionheart FX automated microscope with the montage stitched using Agilent BioTek Gen5 microplate reader and imager software. Hoechst 33342 signal is colored blue, while CellTox Green signal is colored green.

## Experimental design

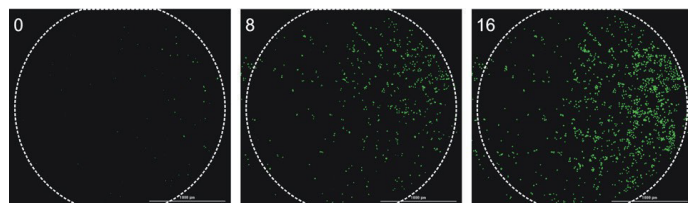
The ONIX2 and Gen5 software packages work independently of one another. The ONIX software is responsible for perfusion timing while the Gen5 software records kinetic images. Cells, media, and oridonin are loaded in specific identified wells of the CellASIC microplate and reagent addition is controlled using the ONIX2 software. After a period to allow cells to attach to the bottom surface of the chamber, imaging with the Lionheart FX automated microscope was begun. Care was taken to note the imaging start time relative to when drug reagent was scheduled to be added (Figure 6).



**Figure 6.** Time line representation of Agilent BioTek Gen5 microplate reader and imager software and ONIX2 processes. Cells are loaded into CellASIC plates and the ONIX2 experiment started. After cells have had time to attach the Gen5 kinetic experiment is then begun.

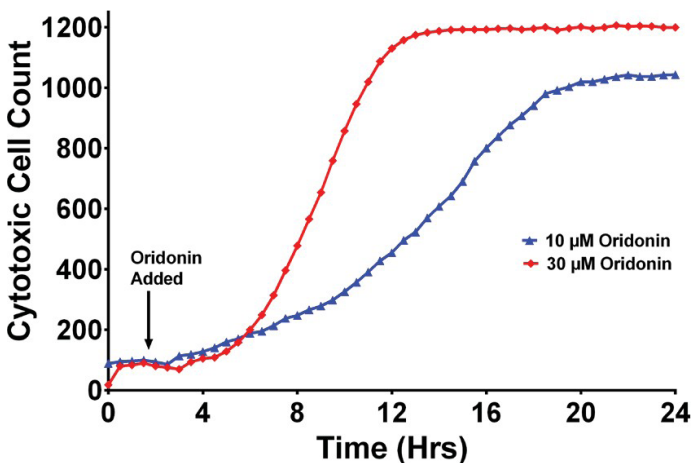
## Results and discussion

Initial experiments used CellTox Green to identify cells that had compromised cellular membranes. Permeable membranes allow the stain access to nuclear DNA where it becomes highly fluorescent. As shown in Figure 7, U-2 OS cells uptake CellTox Green over time as oridonin causes cytotoxicity. This effect is time-dependent, with virtually all cells exhibiting green nuclear fluorescence by 16 hours. The image for time 0 (two hours of dye exposure) only shows minimal green staining. Cells exposed for eight hours have an intermediate number of green stained nuclei.



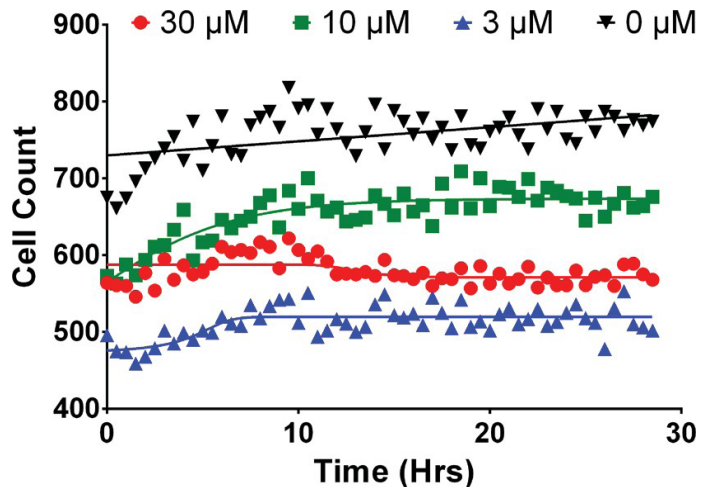
**Figure 7.** Fluorescent images of oridonin-treated U-2 OS cells. U-2 OS cells were treated with 30  $\mu$ M oridonin and imaged at 0, 8, and 16 hours after the initiation of treatment. CellASIC chamber was imaged with a 10x objective as a 4  $\times$  5 montage and the GFP tiles stitched. Scale bar indicates a distance of 1,000  $\mu$ m.

These image data can be quantitated using the automated threshold mask object cellular analysis tools in Gen5 microplate reader and imager software. This tool can define objects, such as nuclei, based on an area of higher fluorescence. In these experiments, a primary mask was developed using the nuclear stain, which was then used to determine cytotoxic cells using the GFP channel. Positive cells were determined using a fluorescence threshold set to 20,000. As demonstrated in Figure 8, oridonin causes cytotoxicity in U-2 OS cells that is both time and concentration dependent. The highest concentration tested (30  $\mu\text{M}$ ) resulting in a more rapid plateau than that observed with 10  $\mu\text{M}$ . Both concentrations will eventually cause cytotoxicity in all the cells in the chamber.



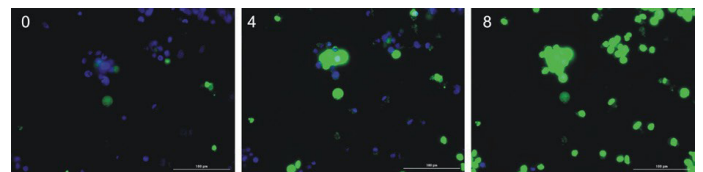
**Figure 8.** Change in cytotoxic cell count over time. CellASIC plates were imaged kinetically with a 10x objective in a 4  $\times$  5 montage using a GFP light cube. After stitching, automated threshold analysis was performed to count GFP positive nuclei for each time point. A threshold value of 20,000 was used to define a positive cell time point for the addition of oridonin is indicated.

Red fluorescent nuclear staining dyes such as DRAQ5 can also be used to quantitate the number of cells present in an image and are reported to result in less inherent toxicity than UV dyes such as Hoechst 33342. Using threshold masking data analysis of fluorescent images, cellular counts can be determined by identifying individual nuclei as done previously with Hoechst 33342. As seen in Figure 9, U-2 OS cells seeded into ONIX2 perfusion plates can be counted over time using DRAQ5. In these experiments, the number of cells remained constant over a period of 30 hours.



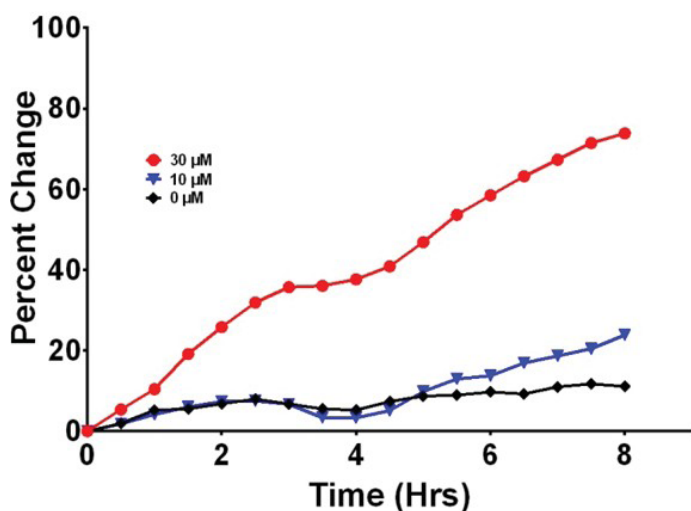
**Figure 9.** Cell counts of oridonin treated cells. DRAQ5 stained U-2 OS cells imaged using a 4x objective and a CY5 LED filter cube. Images were preprocess blank-subtracted and nuclear mask-identified using automated threshold cellular analysis. Cell counts for each time point were plotted. Time 0 reflects the initiation of oridonin dosing.

As shown previously, treatment with oridonin is known to cause cell death in mammalian cells. DRAQ5 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 (Figure 10).



**Figure 10.** Fluorescent images of oridonin-treated U-2 OS cells and stained with DRAQ5 and CellTox Green. U-2 OS cells were treated with 30  $\mu\text{M}$  oridonin and imaged at zero, four, and eight hours after the initiation of treatment. CellASIC chamber was imaged with a 20x objective using a CY5 and GFP light cubes. DRAQ5 staining is falsely colored blue and CellTox Green staining is colored green. Scale bar indicates a distance of 100  $\mu\text{m}$ .

Examination of the early time points after the initiation of oridonin exposure, shows that 30  $\mu\text{M}$  oridonin exposure results in significant increases in the percentage of nuclei identified with DRAQ5 also having green fluorescence (Figure 11). Lower concentrations of oridonin begin to demonstrate cytotoxicity at later time points and to a lesser extent, while untreated cells show minimal increases in cytotoxicity.



**Figure 11.** Change in percentage of cytotoxic cells with oridonin treatment. Using the cell nuclei previously identified and counted, subpopulation analysis was used to identify nuclei with a mean GFP signal greater than 2,000 after preprocessing background subtraction. Data plotted represents the percentage of cells designated as positive after initial dead cells subtracted. Time 0 reflects the initiation of oridonin.

## Conclusion

These data demonstrate that the Agilent BioTek Lionheart FX automated microscope in conjunction with the CellASIC ONIX2 systems can automate routine live cell cytotoxicity 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 require considerable labor by researchers or some degree of automation; particularly true when the time span of the assay exceeds 16 to 24 hours. These systems automate the procedure and continuous perfusion while doing so.

Oridonin is a natural diterpenoid compound previously shown to induce apoptosis in mammalian cancer cells.<sup>3</sup> It is an ideal drug candidate to demonstrate the utility of monitoring live cells for cytotoxicity in real time. This compound has been used as a model system in which to show the utility of the ONIX2 to image cellular cytotoxicity assays.

## References

1. Wang, S. *et al.* Oridonin Induces Apoptosis, Inhibits Migration and Invasion on Highly-Metastatic Human Breast Cancer Cells, *Am. J. Chin. Med.* **2013**, *41*, 177 DOI: 10.1142/S0192415X13500134
2. Gao, F-H. *et al.* Oridonin Induces Apoptosis and Senescence in Colorectal Cancer Cells by Increasing Histone Hyperacetylation and Regulation of p16, p21, p27 and c-myc. *BMC Cancer*, **2010**, *10*, 610, DOI: 10.1186/1471-2407-10-610
3. Tian, W.; Chen, S. Recent Advances in the Molecular Basis of Anti-Neoplastic Mechanisms of Oridonin, *Chin. J. Integr. Med.* **2013**, *19*(4), 315–320.

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