Oridonin Perfusion Causes Cytotoxicity in U-2 OS Cells
Using the ONIX2 System in Conjunction with the Lionheart™ FX Imager

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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. Here we describe the use of the Lionheart™ FX Automated Live Cell Imager 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Cytotoxicity is a leading cause of drugs failing during lead optimization and clinical trials and in some cases being withdrawn from the market.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 R&D 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 multi-well plates 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 of time. During the experiment, the test compound may be undergoing any number of chemical transformations, such 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.

Lionheart™ FX Automated Live Cell Imager
The Lionheart FX is an automated digital inverted fluorescence microscope capable of a number of 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, CO₂ (0-20%) and O₂ (1-19%) 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 a number of 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 6 objectives in an automatic rotating turret, with available air objectives ranging from 1.25x to 60x and oil ranging from 60x-100x. Images are captured with a 16-bit gray scale camera to provide true 16-bit TIFF raw image files, which can be saved in a number of 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).

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 via manually with a pipette and cultured prior to the experiment or with an automated software routine immediately prior to a run.

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% CO₂. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into CellASIC ONIX switching plate for mammalian cells (cat # M04S-03-5PK) at 1.5 x 10⁶ cells per mL.

Imaging

Cultures were imaged using a Lionheart™ FX Automated Live Cell Imager (BioTek Instruments, Winooski, VT) Configured with DAPI, CY5 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 cube
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-2OS 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 (2 hours of dye exposure) only shows minimal green staining. Cells exposed for 8 hours have an intermediate number of green stained nuclei.

These image data can be quantitated using the automated threshold mask object cellular analysis tools in BioTek’s Gen5 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 µM) resulting in a more rapid plateau than that observed with 10 µM. Note that both concentrations will eventually cause cytotoxicity in all the cells in the chamber.
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.

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 9).

Discussion

These data demonstrate that the Lionheart FX in conjunction with the CellASIC ONIX2 systems is capable of automating 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 of time require considerable labor by researchers or some degree of automation; particularly true when the time span of the assay exceeds 16-24 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 [3]. 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 ONIX2 to image cellular cytotoxicity assays.

References

