Characterizing Cellular Responses in 2-D and 3-D Culture Conditions with EarlyTox Cell Viability Assays

Cathy Olsen, Yen-Wen Chen, Hoang Ha, Jayne Hesley
Molecular Devices, LLC, 1311 Orleans Drive, Sunnyvale, CA 94089

OVERVIEW

Apoptosis is a highly regulated cellular program that causes cell death in normal processes such as embryonic development, as well as certain disease conditions. Components of apoptotic signaling pathways are also targets for drug discovery. To distinguish apoptosis from other mechanisms of cell death, one needs tools that to identify the signaling pathways involved. These should function in a wide variety of cell types and modes of growth, i.e. two-dimensional monolayer cell cultures and the more biologically relevant three-dimensional spheroids formed by many cell types under specific culture conditions. We present the results of cellular analysis performed on a number of cell lines relevant to drug discovery, using a new suite of EarlyTox™ cell viability assay kits optimized for use on SpectraMax® fluorescence microplate readers and ImageXpress® Micro imaging systems.

EARLYTOX CELL VIABILITY ASSAYS

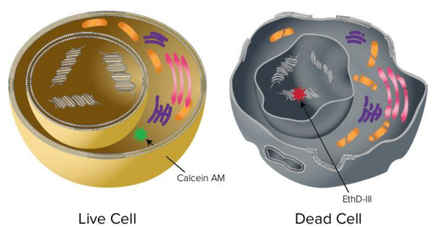
EarlyTox Cell Viability Kits are a family of reagents for assessing cellular conditions, e.g., viability and apoptosis. Optimized primarily as homogeneous assays for fluorescence microplate readers, some of the assay kits can be used with cellular imaging systems.

Each assay follows the generalized work flow depicted below. Cells are seeded in microplates and treated experimentally. Reagents are then added and the plate is incubated. The results are detected using a fluorescence microplate reader or (for some assays) an imaging system.

Following are examples of how the different EarlyTox cell viability assays were used to assess viability and apoptosis in HeLa and other cells.

EarlyTox Live/Dead Assay Kit

This assay uses separate markers for live and dead cells. Calcein AM is a green-fluorescent live-cell marker. Ethidium homodimer-III (EthD-III) is a red-fluorescent dye that stains the nuclei of dead cells. The kit is suitable for plate readers and imaging systems.



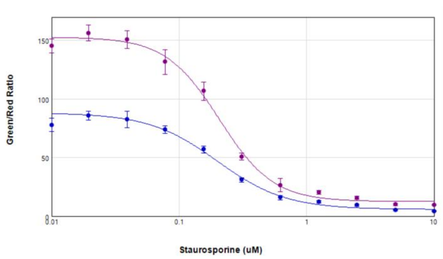


Figure 1. HeLa cells treated with staurosporine for 24 hours. Cells were incubated in calcein AM and EthD-III for 50 minutes (blue) or 2.5 hours (purple). 4-parameter curves were plotted in SoftMax® Pro Software using the ratio of green (530 nm emission) over red (645 nm emission) RFUs on the Y-axis. EC_{50} = 200 nM for the 50-minute and 207 nM for the 2.5-hour incubation.

APOPTOSIS ASSAYS

EarlyTox Caspase-3/7 NucView™ 488 Assay Kit

This assay enables detection of apoptosis in intact cell populations through the use of NucView 488 Caspase-3 substrate, which consists of a fluorogenic DNA dye coupled to the caspase-3/7 DEVD recognition sequence. Initially non-fluorescent, it permeates the cell membrane, and if the cell is apoptotic, the substrate is cleaved by caspase-3/7, releasing a dye that enters the nucleus and binds to DNA, resulting in bright green fluorescence. This reagent is non-toxic to cells and can be used for kinetic studies of apoptosis on plate readers or imaging systems. A Masking Reagent provided in the kit reduces background to enable more accurate EC_{50} determination on plate readers.

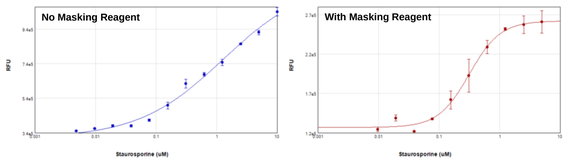


Figure 2. HeLa cells treated with staurosporine for 20 hours to induce apoptosis. NucView 488 substrate (DMSO formulation) was added at a final concentration of 5 µM. Fluorescence corresponding to caspase activity was measured using a SpectraMax i3x Multi-Mode Microplate Reader. Left: Assay without Masking Reagent. Right: Assay with Masking Reagent added to wells to reduce background, enabling more accurate EC_{50} determination (EC_{50} = 0.32 µM).

Figure 3. NucView 488 assay of HeLa cells, imaged on ImageXpress Micro Confocal High-Content Imaging System. Left, control (untreated) cells with low level of apoptosis. Right, cells treated with 25 µM hydroxyurea to induce apoptosis.

EarlyTox Caspase-3/7 R110 Assay Kit

This kit provides a single-step, homogenous assay that is specifically optimized for microplate readers. The fluorogenic substrate (Ac-DEVD)2-R110 contains two DEVD consensus target sequences and is completely hydrolyzed in cell lysate by the enzymes in two successive steps. Hydrolysis of both DEVD peptides releases the green fluorescent dye rhodamine 110 (R110), resulting in a substantial fluorescence increase.

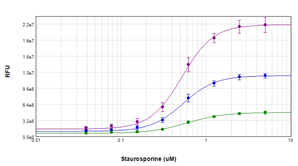


Figure 4. HeLa cells treated with staurosporine for four hours. Cells were incubated in R110 substrate for 35 minutes (green), 1.5 hours (green) or 3 hours (purple). The assay plate was read on a SpectraMax i3 Multi-Mode Microplate Reader, and results were graphed in SoftMax Pro Software. The resulting EC_{50} values were 0.61 µM, 0.54 µM, and 0.52 µM, respectively.

EarlyTox Glutathione Assay Kit

This assay uses monochlorobimane (MCB) to detect cellular GSH levels, which decline in the early stages of apoptosis. This blue fluorescent assay works in a homogeneous format on live cells and is suitable for both plate readers and imaging.

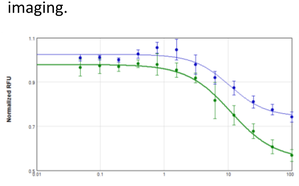


Figure 5. PC-12 cells treated with L-butyl-sulfoxomine (BSO). BSO is known to reduce intracellular GSH levels. PC-12 cells were treated with BSO overnight. BSO-containing medium was then removed, and the cells were stained with DRAQ5 nuclear stain (optional, for imaging) and assayed using the EarlyTox Glutathione Kit. The plate was read on a SpectraMax i3x reader and imaged on an ImageXpress Micro High Content Imaging System.

HEPG2 SPHEROID ASSAY

HepG2 cells were seeded in a 96-well spheroid plate (Corning cat. #4520) at 1500 cells per well and allowed to grow and form spheroids for 48 hours, reaching an approximate diameter of 500 µm. They were treated with compounds for an additional 4 days. Spheroids were assayed for cell viability using the EarlyTox Live/Dead kit and imaged on the ImageXpress Micro Confocal High-Content Imaging System. Whole spheroids were acquired in a single field of view using confocal optics and a 10X objective.

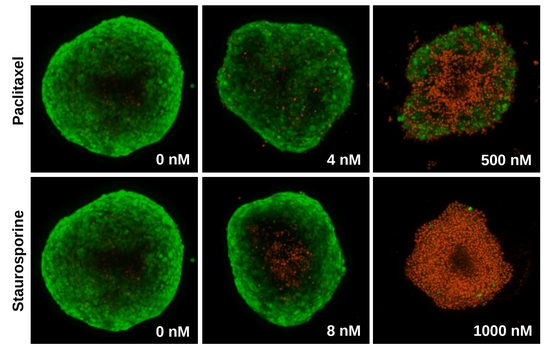


Figure 6. EarlyTox Live/Dead assay on HepG2 spheroids treated with paclitaxel (top row) or staurosporine (bottom row).

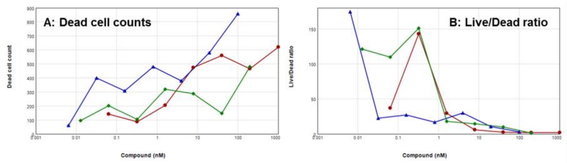


Figure 7. Spheroid analysis using MetaXpress® High-Content Image Acquisition and Analysis Software. A, Dead cell counts vs. compound concentration for antimycin A (red circles), paclitaxel (blue triangles), and staurosporine (green diamonds). B, Live/dead ratio calculated from integrated density sum for signal from live (calcein AM) and dead (ethidium D-III) fluorescence for the same 3 compounds.

HEPG2 VIABILITY & APOPTOSIS IN 2-D

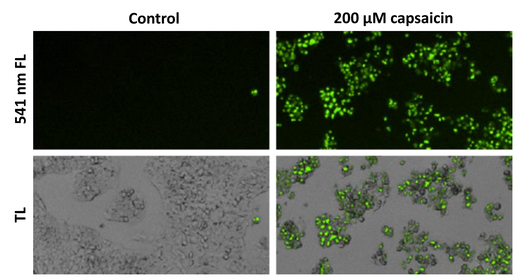


Figure 8. Capsaicin-induced apoptosis in HepG2 cells. Cells in monolayer (2-D) culture were treated with media control (left panels) or 200 µM capsaicin (right panels) for 22 hours and assayed using the EarlyTox Caspase-3/7 NucView 488 Assay Kit. They were imaged using the SpectraMax MiniMax 300 Imaging Cytometer with green fluorescent and transmitted light (TL) channels.

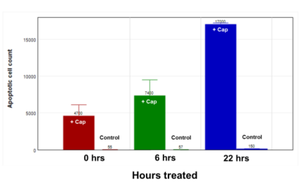


Figure 9. EarlyTox Caspase-3/7 NucView 488 Assay (time course). HepG2 cells were treated with 200 µM capsaicin (left bar of each pair), or media control (right bar of each pair), for 4.5 hours (red bars), 6 hours (green bars), or 22 hours (blue bars). Apoptosis was assessed using the EarlyTox Caspase-3/7 NucView 488 Assay Kit on the SpectraMax MiniMax 300 Imaging Cytometer at 0, 6, and 22 hours. System.

HEPG2 R110 & GLUTATHIONE ASSAYS

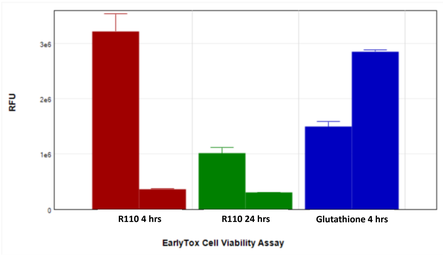
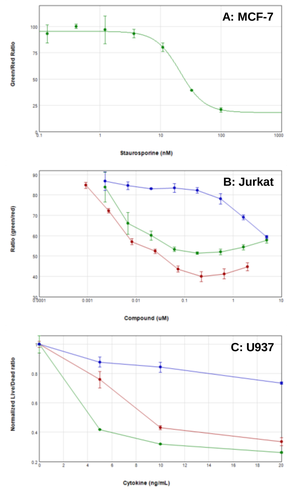


Figure 10. EarlyTox Caspase-3/7 R110 Assay Kit and EarlyTox Glutathione Assay Kit. HepG2 treated with 200 µM capsaicin (left bar of each pair) or media control (right bar of each pair) were assayed using the EarlyTox Caspase-3/7 R110 Assay Kit (red and green bars), or EarlyTox Glutathione Assay Kit (blue bars). Both assays were detected on the SpectraMax i3x reader.

MCF-7, U937, JURKAT CELL VIABILITY

Below are several examples of how the EarlyTox Live/Dead Assay was used to determine compound IC_{50} values, as well as assess the effects of cytokine treatment on cell viability, in a variety of different cell lines. Both adherent (MCF-7) and suspension (Jurkat, U937) cells were tested.

Figure 11. EarlyTox Live/Dead assays.
A) MCF-7 cells were treated with staurosporine for 24 hours and assayed using the EarlyTox Live/Dead Assay Kit and the SpectraMax i3x reader. Results were plotted as a ratio of green/red vs. compound concentration (IC_{50} = 21.2 nM).
B) Jurkat cells were treated with staurosporine (red), camptothecin (green), or etoposide (blue) for 24 hours.
C) U937 cells were treated with concentrations of GM-CSF (blue plot), TNFα (red plot), or TNFα plus GM-CSF (green plot) ranging from 0 ng/mL to 20 ng/mL, for 48 hours. A loss of cell viability was observed in treated cells that was particularly severe when TNFα and GM-CSF were combined.



SUMMARY

The EarlyTox Cell Viability suite of assay kits:

- Enables homogeneous work flows for faster data generation
- Works in a wide variety of cell types and culture conditions, including adherent monolayers, suspension cultures, and 3-D spheroids
- Are all optimized for use on SpectraMax fluorescence plate readers, with most also compatible with imaging systems (Live/Dead, NucView 488, and Glutathione)
- Includes pre-configured protocols with built-in analysis and curve plotting in SoftMax Pro Software

MOLECULAR DEVICES