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The Next Generation of Cell-Based Imaging Assays for Apoptosis, Autophagy and Oxidative Stress from Molecular Probes®

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ABSTRACT

Cell death occurs through multiple pathways from multiple origins. While necessary for natural growth and development, dysregulation of apoptosis has been associated with a variety of diseases, including cancer and neurodegenerative disorders. Increased oxidative stress associated with these diseases has also been shown to lead to apoptosis and autophagy, which are important indicators of cell death pathways that ultimately shed light on mechanism. Importantly, cell death can occur through a single pathway, or in concert involving multiple pathways. For example, staurosporine has been shown to induce apoptosis, while chloroquine is known to promote autophagy, and nefazodone results in both apoptosis and autophagy.

Here, we utilized a novel series of cellular probes in a high content imaging analysis to differentiate between apoptotic and autophagic cell death after induction by different agonists. To accomplish this, we simultaneously examined levels of reactive oxygen species (ROS), autophagosome formation and apoptosis to determine relationships between oxidative stress, autophagy and apoptotic cell death. To this end, we used the fluorogenic CellEvent™ Caspase 3/7 Green Detection Reagent as an indicator of apoptosis together with an antibody specific for LC3B to measure autophagosome formation. We also studied oxidative stress with CellROX™ Deep Red Reagent, a near infrared fluorescent ROS probe developed for multiplexed, live-cell studies of oxidative stress. Further, loss of mitochondrial health was measured in apoptotic cells with the mitochondrial membrane potential dye TMRM.

Using a multi-parametric approach in combination with high content imaging we were able to characterize mechanisms of cell death by discriminating between cells which were apoptotic (active caspase-3/7), autophagic (LC3B-postive autophagosomes), or both. This approach provided detailed information at the cellular level enabling correlations between oxidative stress and different cell death mechanisms.

Finally, we demonstrate here that our CellEvent™ Caspase 3/7 Reagent is fully amenable to a high throughput, homogenous and cell based configuration, and can be multiplexed effectively with PrestoBlue™ Cell Viability Reagent, allowing flexibility across scale, platform and research application in the discovery workplace.

Figure 1: Validation of probes used for oxidative stress, apoptosis, and autophagy by fluorescence microscopy

1A. Apoptosis – Fluorogenic CellEvent™ Caspase 3/7 Substrate Assay

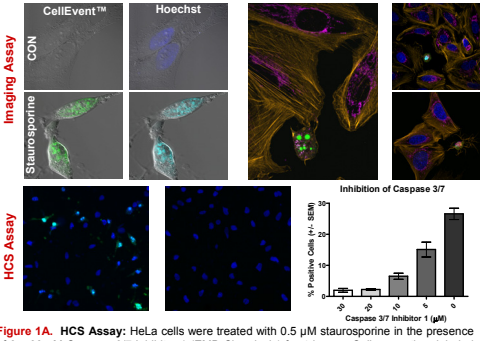


Figure 1A. HCS Assay: HeLa cells were treated with 0.5 μM staurosporine in the presence of 0 – 30 μM Caspase 3/7 Inhibitor 1 (EMD Chemicals) for 4 hours. Cells were then labeled with CellEvent™ Caspase 3/7 Substrate (green) for 30 minutes, followed by Hoechst 33342 (blue) for 15 minutes in complete media. Images were acquired and analyzed on a Thermo Fisher Cellomics ArrayScan® VTI.

1B. Oxidative Stress – CellROX™ Deep Red ROS sensor

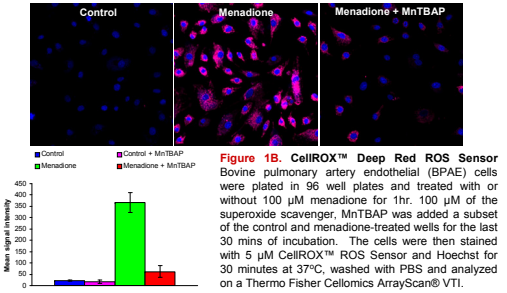
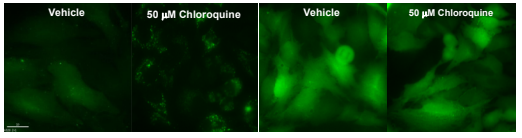


Figure 1B. CellROX™ Deep Red ROS Sensor Bovine pulmonary artery endothelial (BPAE) cells were plated in 96 well plates and treated with or without 100 μM menadione for 1hr. 100 μM of the superoxide scavenger, MnTBAP was added a subset of the control and menadione-treated wells for the last 30 mins of incubation. The cells were then stained with 5 μM CellROX™ ROS Sensor and Hoechst for 30 minutes at 37°C, washed with PBS and analyzed on a Thermo Fisher Cellomics ArrayScan® VTI.

1C. Autophagy – BacMam Premo™ Autophagy Sensor LC3B-GFP and Anti LC3B



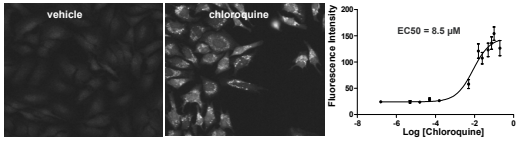


Figure 1C. BacMam Premo Autophagy Sensor: HeLa cells were transduced with BacMam - Premo™ Autophagy Sensor LC3B-GFP encoding mutant (I) or wild-type (II); 24 hours post-transduction, cells were treated with 50 μM chloroquine and imaged 16 hours later.

Anti LC3B HCS: HeLa cells were incubated with chloroquine for 16 hours. Following incubation, cells were fixed, permeabilized and LC3B was labeled with an anti-LC3B antibody at 0.5mg/ml (Life Technologies) and subsequently detected with a goat anti-rabbit Alexa Fluor® 647 secondary antibody. Autophagosomes were measured by peri-nuclear spot intensities. Imaging analysis was performed on a Thermo Fisher Cellomics Arrayscan® VTI.

Figure 2: Imaging Oxidative Stress Together with Caspase 3/7 Activity

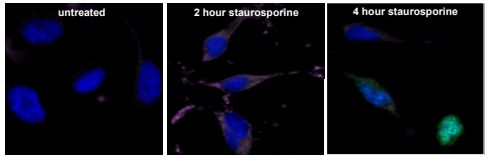


Figure 2A. Traditional fluorescence imaging of oxidative stress and apoptosis: HeLa cells were treated with or without 0.5 μM staurosporine for 2 or 4 hours in the presence of 7.5 μM CellEvent™ Caspase 3/7 Reagent. Cells were then stained with 5 μM Far Red ROS Sensor and Hoechst 33342 for 30 minutes at 37°C, then washed with warm DPBS. Cells were imaged immediately on a Zeiss Axiovert® inverted microscope using a 40x objective.

2B. Multiplexed High Content Imaging of Redox Stress and Apoptosis

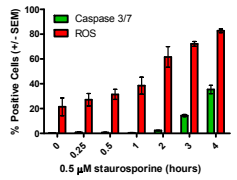


Figure 2B. Simultaneous imaging of oxidative stress and caspase activation: HeLa cells were treated with 0.5 μM staurosporine for 0 – 4 hours in the presence of 7.5 μM CellEvent™ Caspase 3/7 Reagent. Cells were then stained with 5 μM Far Red ROS Sensor and Hoechst 33342 for 30 minutes at 37°C, then washed with warm DPBS. Images were acquired and analyzed on a Thermo Fisher Cellomics Arrayscan® VTI. Increased oxidative stress began ~ 2 hours after treatment (red bars), while caspase 3/7 activation was observed 3-4 hours after treatment (green bars) with staurosporine.

Figure 3: Multiplexed time-lapse imaging of mitochondrial membrane potential and caspase 3/7 activation

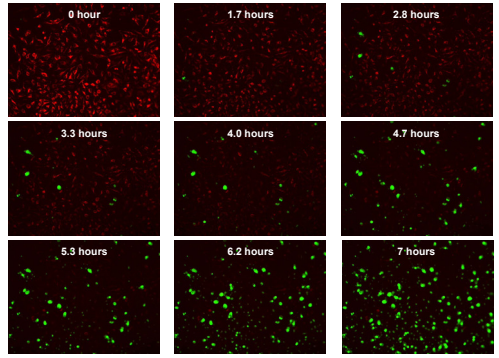
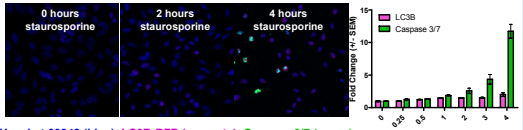


Figure 3. Time lapse of TMRM decrease with CellEvent™ Caspase Substrate increase: HeLa cells were loaded with 50 nM TMRM followed by 5 μM CellEvent™ Caspase 3/7 Reagent. Cells were then treated with 0.5 μM staurosporine and images were acquired every 5 minutes over 7 hours on a Molecular Devices ImageXpress® Micro (high content screening system) at 10x.

Figure 4: Simultaneous Detection of Apoptosis and Autophagy

4A. Staurosporine Induces Potent Caspase Activation with Modest Autophagy



Hoechst 33342 (blue), LC3B-RFP (magenta), Caspase 3/7 (green)

Figure 4A. HeLa cells treated with staurosporine undergo apoptosis through activation of caspase 3/7: HeLa cells were treated 0.5 μM staurosporine for 0 – 4 hours in the presence of 7.5 μM CellEvent™ Caspase 3/7 Reagent (green). Cells were fixed, permeabilized and labeled with an anti-LC3B antibody (Life Technologies) and subsequently detected with a goat anti-rabbit Alexa Fluor® 647 secondary antibody (red). Cells were then stained with Hoechst 33342 (blue). Images were analyzed on a Thermo Fisher Cellomics Arrayscan® VTI. Images showed increased caspase 3/7 activation at 4 hours (A). Signal intensity for caspase 3/7 (nuclear) and LC3B (spots within the cytosol) were plotted as fold change relative to 0 hour treatment for each of the two parameters (B). Staurosporine significantly increased caspase 3/7 activation after 4 hours, which was associated with a small increase in autophagosome formation.

4B. Chloroquine Induces Potent Autophagy with Modest Caspase Activation

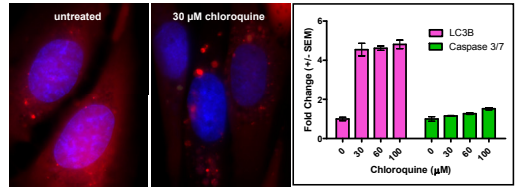
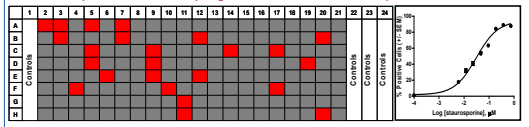


Figure 4B. U-2 OS cells treated with chloroquine undergo autophagy: U-2 OS cells were transduced with Premo™ Autophagy Sensor LC3B-RFP (red). The next day, cells were treated with or without chloroquine for ~ 24 hours. Cells were then stained with the 7.5 μM CellEvent™ Caspase 3/7 Reagent (green) for 30 minutes at room temperature, followed by Hoechst 33342 (blue). Images were acquired on a Zeiss Axiovert® inverted microscope. Cells treated with chloroquine showed increased punctate staining (red spots) indicating formation of LC3B positive autophagosomes. Tabulated data shows U-2 OS cells treated with 0 – 100 μM chloroquine.

Figure 5: CellEvent™ Caspase 3/7 Reagent in HCS and HTS

5A. Chloroquine Induced Autophagosome Formation with Caspase Activation



5B. HTS with CellEvent™ Caspase Reagent Alone and Multiplex with PrestoBlue™ Cell Viability Reagent

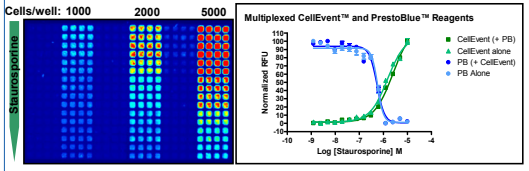


Figure 5. HCS determination of toxicity in a compound library and HTS multiplex of CellEvent™ Caspase 3/7 Detection Reagent with PrestoBlue™ Cell Viability Reagent:
5A. U-2 OS cells were treated with 160 different compounds from The Killer Collection (MicroSource Discovery Systems, Inc.) at 10 μM for 24 hours. Cells were then labeled with 7.5 μM CellEvent™ Caspase-3/7 Green Detection Reagent and Hoechst 33342 in HBSS for 30 minutes. Each square represents a different compound. Grey squares are <3 fold change, while red squares are >3 fold change. A dose-response curve of staurosporine, is plotted as a positive control. **5B.** Table: CHO-K1 cells were plated at 5,000 cells/well in a 384 well plate in the presence of a dilution series of staurosporine. The plate was incubated for 19 hours at 37oC/5% CO2. PrestoBlue™ reagent and CellEvent™ reagent were added directly to the wells and the plate was incubated for 30 minutes at 37oC/5% CO2 prior to reading out the fluorescence.

CONCLUSIONS

- In HeLa cells staurosporine induced an increase in oxidative stress followed by activation of caspase 3/7. Loss of mitochondrial membrane potential was also observed indicating cell death occurring by apoptosis.
- In U-2 OS cells chloroquine induced a significant increase in autophagosome formation with minimal change in caspase 3/7 activity.
- By using fluorescent markers for oxidative stress, active caspase 3/7 and LC3B we were able to determine levels of oxidative stress relative to activation of caspase 3/7, and distinguish between cells which were apoptotic or autophagic.
- **CellEvent™ Green Caspase 3/7 Detection Reagent: Catalogue # C10423**
- **BacMam Premo™ Autophagy Kits: LC3B-GFP # P36235; LC3B-RFP # P36236**
- **LC3B Antibody Kit for Autophagy: Catalogue # L10382**
- **CellROX™ Deep Red ROS Sensor: Catalogue # C10422**
- **PrestoBlue™ Cell Viability Assay: Catalogue # A13261**