Simultaneous analysis of cell death mechanisms and oxidative stress using Molecular Probes® next generation reagents for imaging and flow cytometry

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ABSTRACT

Cell death can occur through multiple pathways, such as apoptosis, autophagy, and necrosis. Although necessary for proper growth and development, dysregulation of apoptosis has been associated with a variety of diseases including cancer and neurodegenerative disorders. Increased oxidative stress has also been associated with these diseases and has been shown to lead to apoptosis and autophagy. Importantly, cell death can occur through a single pathway, or in concert with multiple pathways. Staurosporine has been shown to induce apoptosis; chloroquine is known to promote autophagy; and nefazodone results in both apoptosis and autophagy.

In this study we utilized multi-parametric high content imaging and flow cytometry to differentiate between apoptotic and autophagic cell death after induction by different agonists. In addition, we simultaneously examined levels of oxidative stress to determine the relationship between oxidative stress and cell death. We used the fluorogenic CellEvent™ Caspase 3/7 Green Detection Reagent as an indicator of apoptosis, LC3B RFP and an antibody specific for LC3B to measure autophagy, and CellROX™ Deep Red Reagent, a near infrared fluorescent ROS probe to evaluate oxidative stress. Furthermore, loss of mitochondrial membrane potential was also observed in apoptotic cells. By using a multi-parametric approach to high content imaging or flow cytometry we were able to characterize the mechanism of cell death by discriminating between cells which were apoptotic (active caspase-3/7), autophagic (LC3B-postive autophagosomes), or both. This multi-parametric approach provided detailed information at both the cellular and population level enabling correlation between oxidative stress and different mechanisms of cell death.

INTRODUCTION

- Increased oxidative stress has been associated with a variety of diseases including cancer, Alzheimer's disease, and other neurodegenerative diseases.
- High levels of oxidative stress can also lead to cell death through several mechanisms including apoptosis and autophagy.
- Understanding the relationship between oxidative stress and cell death is essential to our understanding of disease progression and the development of therapies to combat these diseases.
- Live cell microscopy enables temporal resolution between cellular events such as increased oxidative stress and apoptosis signaling.
- Multi-parametric imaging allows for several parameters to be analyzed simultaneously within the same cell providing key information at the cellular level.

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

1A(i) Apoptosis – CellEvent™ Caspase-3/7 Green Detection Reagent

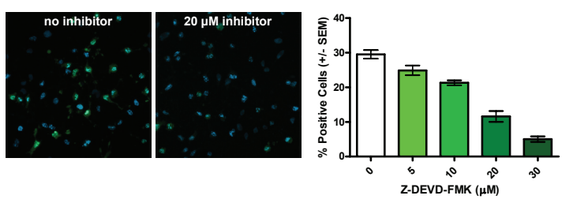


Figure 1A(i): HeLa cells were treated with 0.5 µM staurosporine in the presence of 0 – 30 µM of the Z-DEVD-FMK caspase inhibitor for 4 hours. Cells were then labeled with 5 µM CellEvent™ Caspase-3/7 Green Detection Reagent (green) for 30 minutes, followed by Hoechst 33342 (blue) for 15 minutes in complete media. Images were acquired and analyzed on a Thermo Fisher Cellomics ArrayScan® VTI. A decrease in the percent of cells positive for active caspase 3/7 was observed in cells incubated in the presence of the caspase inhibitor, indicating specificity of the probe for active caspase 3/7.

Figure 1A(ii): Acoustic focusing flow cytometry based easy detection of activated caspase 3/7 positive apoptotic cells

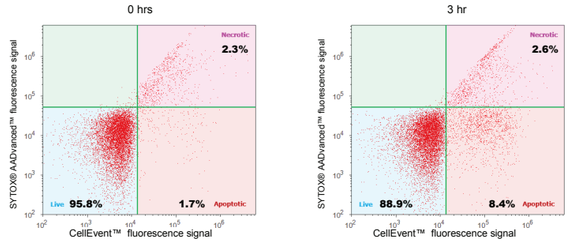


Figure 1A(ii): Jurkat cells were treated with 10 µM camptothecin from 0 - 3 hours to induce apoptosis. To detect activated caspase 3/7 the cells were labeled with 5 µM CellEvent™ Caspase-3/7 Green Detection Reagent 30 minutes prior to harvesting. After caspase labeling, cells are spun to remove drug, and resuspended in dPBS then labeled with SYTOX® AADvanced™ Dead Cell Stain. Data acquisition and analysis was performed on an Applied Biosystems® Attune™ Acoustic Focusing Cytometer. An increase in apoptotic cells as measured by caspase activity can be seen within 3 hours following treatment.

1B. Oxidative Stress – CellROX™ Deep Red Reagent

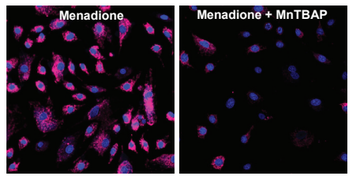


Figure 1B(i): Bovine pulmonary artery endothelial (BPAE) cells were plated in 96 well plates and treated with or without 100 µM menadione for 1hr. 100 µM of the superoxide scavenger, MnTBAP was added to a subset of the control and menadione-treated cells for the last 30 mins of incubation. The cells were then stained with 5 µM CellROX™ Deep Red Reagent and Hoechst 33342 for 30 minutes at 37ºC, washed with PBS and analyzed on a Thermo Fisher Cellomics ArrayScan® VTI. MnTBAP treatment inhibited ROS caused by menadione confirming that the signal was due to ROS induced by this compound.

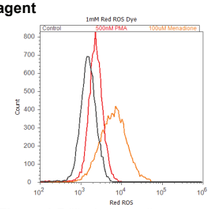


Figure 1 B(ii): Jurkat cells were treated with 500 nM PMA or 100 µM menadione for 1hr. The cells were then stained with 5 µM CellROX™ Deep Red Reagent and Hoechst 33342 for 30 minutes, washed 3x with PBS and analyzed on a BD Biosciences BD™ LSRII flow cytometer using red laser excitation and 650-670 nm emission.

1C. Autophagy – Premo™ Autophagy Sensor LC3B-GFP

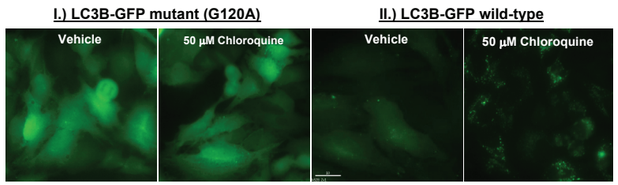


Figure 1C. HeLa cells were transduced with BacMam based - Premo™ Autophagy Sensor LC3B-GFP encoding either mutant (I) or wild-type (II); 24 hours post-transduction, cells were treated with 50 µM chloroquine and imaged 16 hours later. Cells expressing the non-cleavable mutant LC3B-GFP showed no difference in LC3B-GFP distribution after treatment with chloroquine or vehicle. In cells expressing wild type LC3B-GFP, chloroquine caused a pronounced labeling of autophagosomes. In subsequent experiments, LC3B-RFP was used.

RESULTS

Figure 2: Multiplex imaging of oxidative stress and activated caspase 3/7

A.

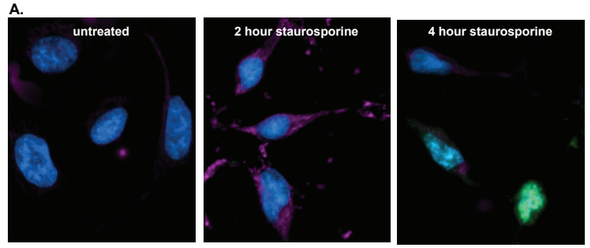


Figure 2A: Traditional fluorescence imaging of oxidative stress and apoptosis: HeLa cells were treated with 0.5 µM staurosporine for 0, 2 or 4 hours in the presence of 7.5 µM CellEvent™ Caspase-3/7 Green Detection Reagent . Cells were then stained with 5 µM CellROX™ Deep Red Reagent and Hoechst 33342 for 30 minutes at 37ºC, then washed with warm DPBS. Cells were imaged immediately on a Zeiss Axiovert® inverted microscope using a 40x objective. Increased oxidative stress was observed at 2 hours after treatment (magenta) while caspase 3/7 activation was not observed until 4 hours after treatment (green) as shown in representative cells above.

B.

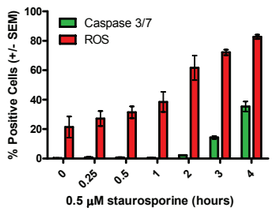


Figure 2B: High content imaging reveals details progression of oxidative stress and apoptosis: HeLa cells were treated with 0.5 µM staurosporine for 0 – 4 hours in the presence of 7.5 µM CellEvent™ Caspase-3/7 Green Detection Reagent. Cells were then stained with 5 µM CellROX™ Deep Red Reagent and Hoechst 33342 for 30 minutes at 37ºC, 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: Multiplex time-lapse imaging showed loss of mitochondrial membrane potential followed by caspase 3/7 activation

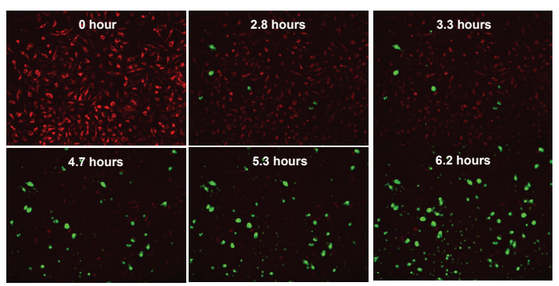
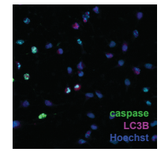


Figure 3: HeLa cells were loaded with 50 nM TMRM followed by 5 µM CellEvent™ Caspase-3/7 Green Detection Reagent . Cells were then treated with 0.5 µM staurosporine and images were acquired every 5 minutes over 7 hours on a Molecular Devices ImageXpress® Micro (high content screening system) at 10x. Over the 7 hour time course, staurosporine induced a loss of mitochondrial membrane potential followed by activation of caspase 3/7 as evidenced by a decrease in TMRM signal (red) and an increase in caspase 3/7 signal (green), respectively.

Figure 5: Simultaneous detection of apoptosis and autophagy by fluorescence microscopy

A.



B.

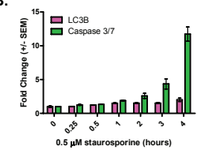
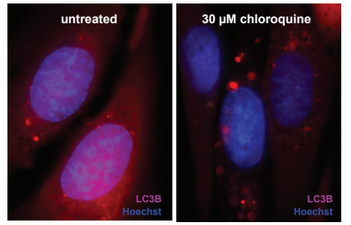


Figure 5A-B, HeLa cells treated with staurosporine undergo apoptosis through activation of caspase 3/7: HeLa cells were treated 0.5 µM staurosporine for 0 – 4 hours in the presence of 7.5 µM CellEvent™ Caspase-3/7 Green Detection Reagent (green). Cells were then fixed, permeabilized and labeled with an anti-LC3B antibody (Life Technologies) and subsequently detected with a goat anti-rabbit Alexa Fluor® 647 secondary antibody (red). 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.

C.



D.

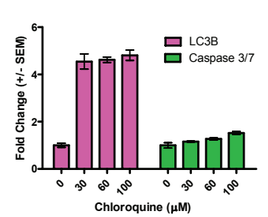


Figure 5C-D: U-2 OS cells treated with chloroquine undergo autophagy but not apoptosis: **Figure 5C** U-2 OS cells transduced with Premo™ Autophagy Sensor LC3B-RFP (red) were treated next day ± chloroquine for ~ 24 hours. Cells were then stained with the 7.5 µM CellEvent™ Caspase-3/7 Green Detection Reagent (green) for 30 minutes at room temperature, Images were acquired on a Zeiss Axiovert® inverted microscope using a 63x water objective. Cells treated with chloroquine showed increased punctate staining (red spots) indicating formation of LC3B positive autophagosomes. In figure **5D**, U-2 OS cells were treated with 30 µM chloroquine for ~ 24 hours prior to staining with 7.5 µM CellEvent™ Caspase-3/7 Green Detection Reagent for 30 minutes at room temperature, followed by fixed, permeabilized and labeled with an anti-LC3B antibody (Life Technologies), and subsequently detected with a goat anti-rabbit Alexa Fluor® 647 secondary antibody, followed by Hoechst 33342 stain. Cells were analyzed on a Thermo Fisher Cellomics Arrayscan® VTI. Signal intensity for caspase 3/7 (nuclear) and LC3B (spots within the cytosol) were plotted as fold change relative to untreated cells. Chloroquine treatment increased signal intensity from autophagosomes, which was associated with a small increase in activated caspase 3/7.

CONCLUSIONS

- A rapid and easy-to-use caspase 3/7 fluorogenic reagent is demonstrated in imaging and flow cytometry for detection of apoptotic cells.
- 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.
- This multi-parametric approach was also amenable to time-lapse imaging which enabled temporal resolution of oxidative stress relative to cell death mechanisms.