APPLICATION NOTE

Elucidating the Mechanism of Cell Death Through the use of High Content Analysis

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Abstract

Cell catabolism occurs primarily by one of three mechanisms: Apoptosis, Autophagy and Necrosis. Each of these mechanisms has a number of specific morphological and biochemical characteristics from which it can be identified. In addition, apoptosis, autophagy and necrosis serve specific functions in both normal and diseased cellular states. Autophagy is one of the major intracellular protein degradation pathways that is essential during cell starvation, differentiation, aging and death. Apoptosis is the process of programmed cell death in which a tightly controlled cascade of events occurs in order to maintain tissue homeostasis. Necrosis is defined as nonapoptotic and non-autophagic cell death where the cell is destroyed and its components leak into the extracellular space. Identifying the mechanism by which cells are dying, can potentially lead to the ability to pharmacologically or genetically modulate that process. Here, we present several qualified biochemical and functional assays that can be used to understand the process of cell death *in vitro*.

Introduction

Cell death is a process that occurs as both a part of normal tissue homeostasis and can be aberrantly regulated in some disease states, specifically cancer. Many cancer therapies function by initiating cancer cell death by affecting changes to critical cell cycle pathways or by inducing cellular trauma as in radiation treatments. Many types of cell death have been previously described; however, the most prevalent mechanisms are apoptosis, autophagy and necrosis. The processes have certain morphological and biochemical hallmarks that allow them to be differentiated from one another (Table 1).

	Early Apoptosis	Late Apoptosis	Necrosis	Autophagy
Phosphatidylserine "flipping"	✓			
Mitochondrial membrane potential flux	✓			
Cytochrome c release	✓			
Caspase-3 activation	✓			
Membrane permeability		✓	✓	
DNA fragmentation		✓	✓	
Cytoskeletal collapse		✓		
LC3B expression				✓

Table 1: Examples of morphological and biochemical changes associated with cell death that can be measured with High Content Imaging.

Apoptosis is the process of programmed cell death in which a tightly controlled cascade of events occurs in order to maintain tissue homeostasis. An insufficient amount of apoptosis results in uncontrolled cellular proliferation, such as cancer. Current clinical techniques include attempting to inhibit the ability of tumor cells to evade apoptosis. Cancer researchers are continuously screening for better chemotherapeutic compounds all with the hope of disrupting tumor cell proliferation while minimizing changes to normal cells. A host of morphological and biochemical changes are associated with the process of apoptosis. Caspase activation through one of two major pathways, Fas/TNF or Bcl-2 family members, results in cell shrinkage, DNA fragmentation and phagocytosis by neighboring cells,

cytochrome c release and mitochondrial depolarization. Autophagy is one of the major intracellular protein degradation pathways that is essential during cell starvation, differentiation, aging and death. Autophagy is a protective mechanism that may prevent cell death. During cell starvation, autophagy is induced to generate energy and nutrients via degradation of organelles and proteins. LC3B is an ubiquitin-like protein that is necessary for the formation of the autophagosome, and its localization forms discrete puncta as the protein is incorporated into the lipid layers of the autophagic vesicles. Necrosis is characterized by rapid permeabilization of the plasma membrane that is accompanied by cytosolic proteins being extruded into the extracellular space. This often invokes an inflammatory response. Necrosis was once thought to be an unregulated process; however, there is an increasing amount of evidence that this process is tightly controlled. Thermo Scientific™ products offer a number High Content Assay templates designed to study cell death. They have been optimized for common *in vitro* cell death assays (Figure 1).

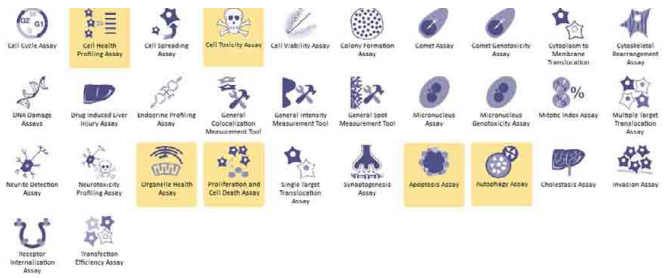


Figure 1: Thermo Scientific™ HCS Studio™ Cell Analysis Software assay templates. Assays that specifically measure aspects of cell death are highlighted in yellow.

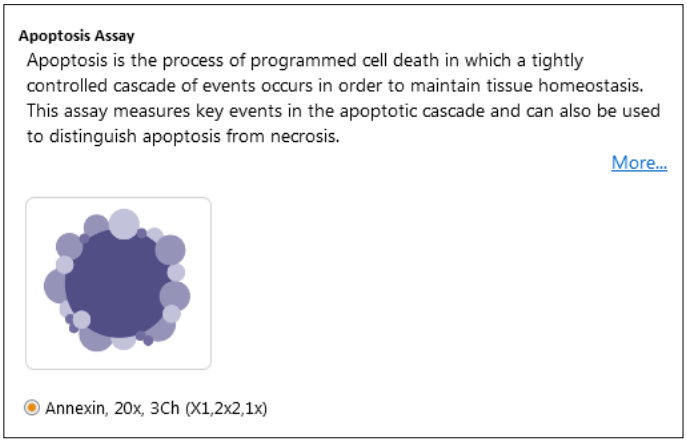


Figure 2: An example cell death assay optimized for measuring Annexin V and YOYO™-1 staining in Jurkat cells. Assay descriptions and example images and data are provided within HCS Studio Cell Analysis Software.

Results

APOPTOSIS

Annexin V Apoptosis Assay

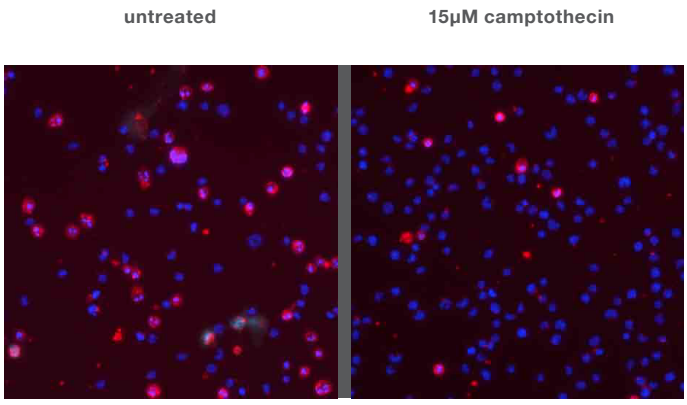
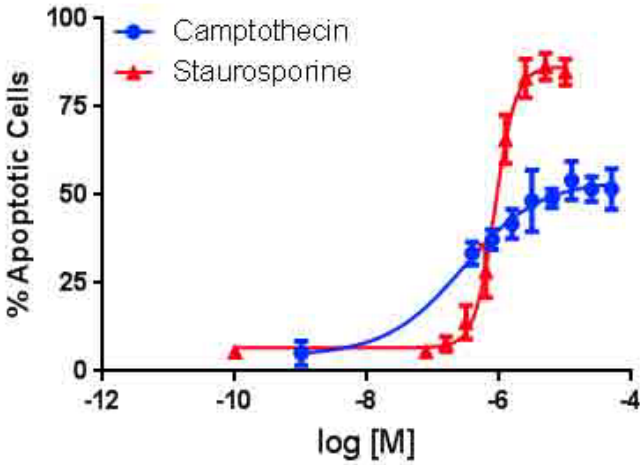


Figure 3: Representative images from Jurkat cells plated in 96- well plates were incubated with and without Camptothecin for four hours then were stained with Yoyo-1, Annexin V and Hoechst. Apoptotic cells exhibit binding of annexin V to Phosphatidylserine in the absence of Yoyo-1 staining.

Dose response



	Camptothecin	Staurosporine
IC50	2.805e-007	8.652e-007

Figure 4: Dose-response curves for Jurkat cells treated with Camptothecin and Staurosporine for four hours. Exposure to these compounds results in a dose-dependent increase in the number of cells in early Apoptosis. This was achieved by determining the proportion of cells with Phosphatidylserine extrusion in the absence of membrane permeability.

Cytochrome C Assay

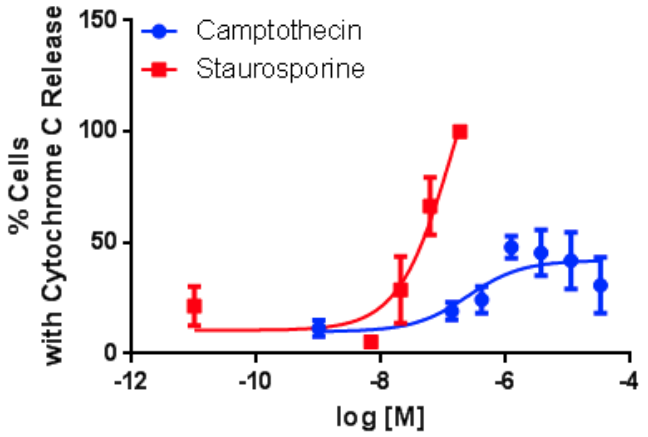


Figure 5: Cytochrome c release from mitochondria was measured in HK2 cells by indirect immunofluorescence. HK-2 cells were treated with Camptothecin and Staurosporine for 72 hours. Exposure to these compounds results in dose-dependent cytochrome c release from mitochondria.

Mitochondrial Depolarization Assay

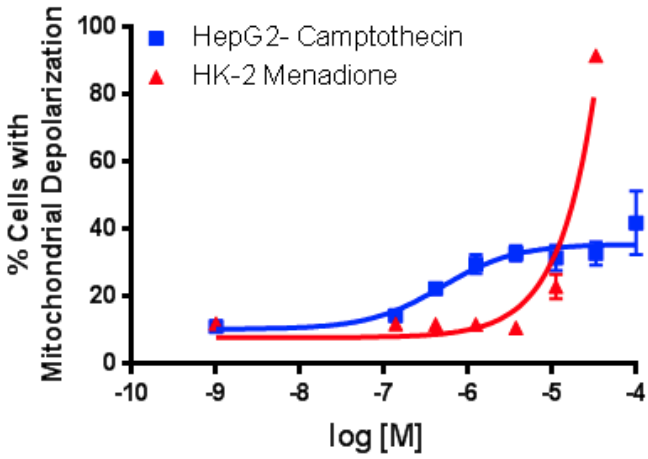


Figure 6: MitoTracker™ Orange CMTMRos, an orange fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential, was used to determine the extent of membrane depoplarization in HepG2 and HK-2 cells. HepG2 and HK-2 cells were treated with Camptothecin or Menadione for 72 hours. Exposure to these compounds results in dose-dependent mitochondrial depolarization.

Caspase 3 Activation Assay

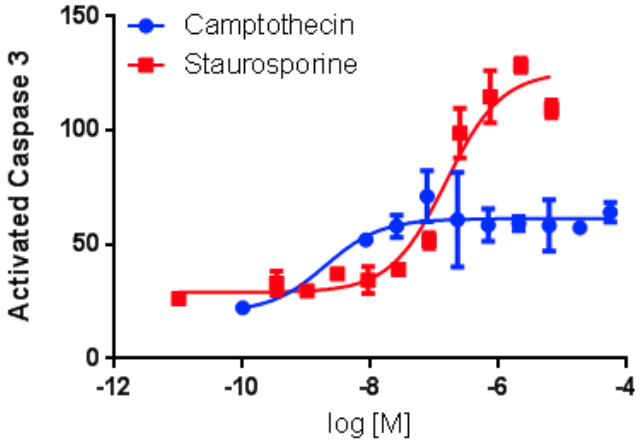


Figure 7: Caspase 3 activation, a key apoptotic event that indicates that the cell is committed to die, was measured in A549 cells. A549 cells were treated with Camptothecin and Staurosporine for 24 hours results in a dose-dependent increase in activated Caspase 3.

AUTOPHAGY

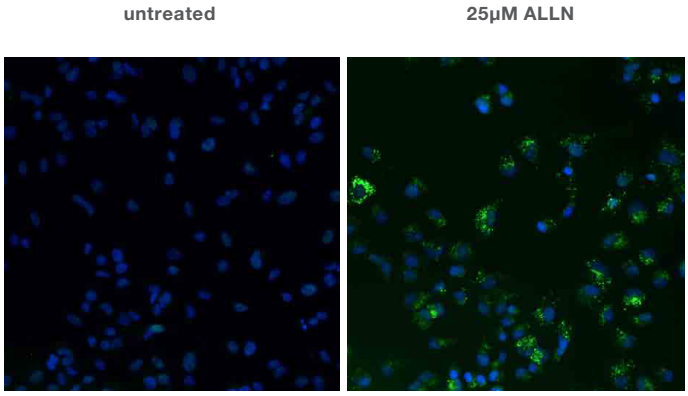


Figure 8: Autophagic vesicle formation. Representative images from HepG2 cells exposed to 25µM ALLN for 16 hours results in the formation of autophagic vesicles expressing the LC3B protein in the perinuclear region where they were visible as punctuate spots.

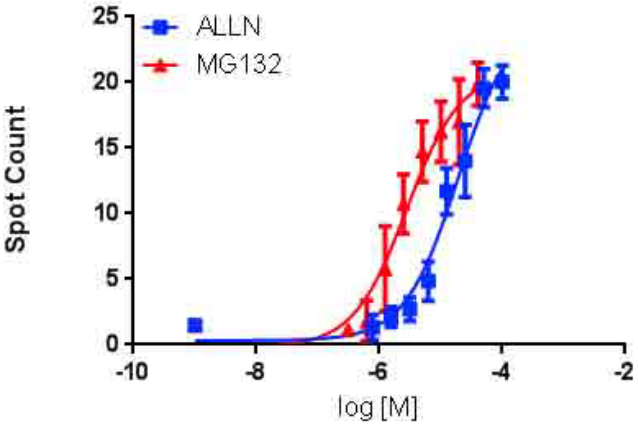


Figure 9: LC3B was measured in HepG2 cells by indirect immunofluorescence after the cells were treated with ALLN and MG132 for 16 hours. Exposure to these compounds results in a dose-dependent increase in autophagic vesicle formation.

Conclusion

- Quantitative, cell-based High Content Analysis assays enable measurement of multiple parameters involved in various cell death pathways. The markers can be used alone or in combination in multiple cell lines.

- In addition, HCS Studio Cell Analysis Software is capable of measuring many other hallmarks of cell death such as nuclear condensation, cell shrinkage and DNA fragmentation. These parameters can be combined with other cell death assays to help differentiate one type of cell death from another.

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