

Abstract

Almost all compounds will elicit deleterious effects on cells at some concentration. Anticancer agents typically have cytotoxicity of tumor cells as a primary function, while medicinal agents can have cytotoxicity as an unwanted side effect. Drugs elicit cytotoxic effects through several different mechanisms, which can be discerned by a variety of assays. Drug accumulation in the mitochondria can result in a compromised membrane potential with a resultant disruption in electron transport. Lysosome perturbation, as a result of ion trapping of amine containing compounds has been demonstrated to cause the formation of autophagosomes and autophagic cytopathology. Additionally numerous compounds have been shown to initiate the apoptotic process in cells. Here we describe the use of the EL406 Washer Dispenser to automatically aspirate media, wash cells and dispense reagents for three different CELLestial™ cytotoxicity detection assays from Enzo Life Sciences. H-Meso cells have been treated with agents know to perturb lysosomes, such as chloroquine and verapamil and their effects monitored using the Lyso-ID™ Red Detection Kit. Likewise, the effect of staurosporine in regards to the induction of apoptosis has been assessed using the Nuclear-ID™ Green Chromatin Condensation Kit. Mitochondrial membrane toxicity has been monitored using the Mito-ID™ Membrane Potential Cytotoxicity Kit. Assay work flow along with performance results will be provided.

Introduction

The identification of cytotoxic effects is a critical element of the drug discovery process. Equally important is the ability to automate as many processes of the drug discovery process as possible. Cytotoxic effects of drug molecules are often first observed as perturbations of normal cellular organelle functionality. For example, mitochondria, which play a central role in cellular oxidative respiration, can have a compromised membrane potential resulting from drug accumulation. This toxicity has been shown to contribute to the toxicity of various organs.

Cationic amphiphilic compounds (i.e. small molecule drug compounds) can be absorbed by cells by simple diffusion and accumulate inside the acidic cellular organelles, a process referred to as lysosomotropism. While many drugs require the presence of a cationic moiety for bioactivity, their accumulation into subcellular organelles can lead to many undesirable effects [Ikeda et. al. : BBRC 377:268-274 (2008)]. Numerous cationic amphiphilic drugs are known to trigger phospholipidosis, which is typified by excessive intracellular accumulation of phospholipids as lamellar bodies [Anderson and Borlak; FEBS Lett. 580:5533-5540 (2006)]. While the origins of these lamellar bodies remain unknown, they appear to be generated by autophagic or heterophagic processes.

Apoptosis or programmed cell death can be caused by a number of different factors involving two basic pathways. The extrinsic pathway involves the binding of "death inducing ligands" to cell surface receptors or the induction by cytotoxic T-lymphocytes by granzyme. This pathway results in the activation of Caspase-8 [Stennicke and Salvesen; BBA. 1477:299-306 (2000)]. The intrinsic pathway is initiated by cellular stress and generally involves changes to the mitochondria that release Cytochrome c, which interacts with Apaf-1, dATP and multiple molecules of pro-caspase-9 to generate an active apoptosome complex that activates caspase-9. [Stennicke and Salvesen; Cell Death and Dif. 6:1054-1059 (1999)] During the process chromatin undergoes a phase change from a heterogeneous genetically active network to a highly condensed form that is subsequently fragmented and packaged into apoptotic bodies.

Using three different CELLestial™ cell cytotoxicity assays we demonstrate the utility of the EL406 washer dispenser to automate the liquid handling steps required to perform routine compound toxicity testing.

CELLestial™ Nuclear-ID™ Green Assay

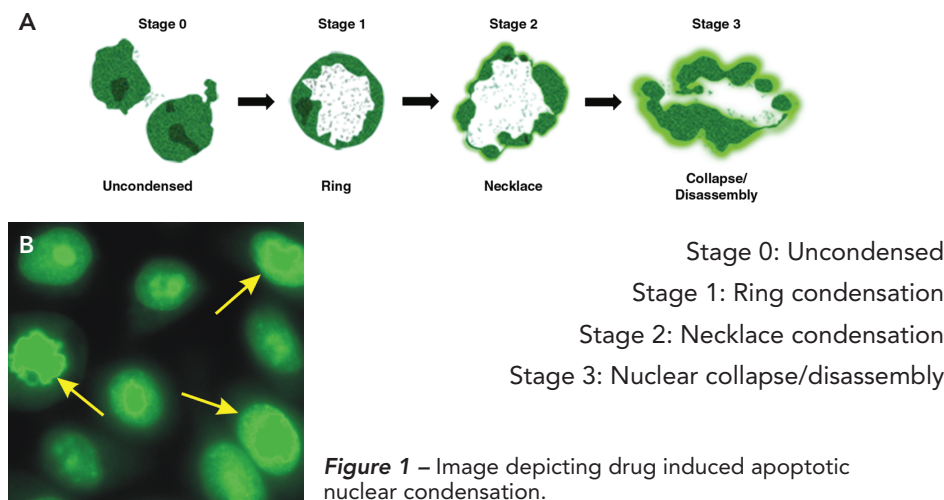


Figure 1 – Image depicting drug induced apoptotic nuclear condensation.

Apoptosis or programmed cell death can be caused by a number of different factors. During the process chromatin undergoes a phase change from a heterogeneous genetically active network to a highly condensed form that is subsequently fragmented and packaged into apoptotic bodies. The Nuclear-ID™ Green assay kit identifies cells in late stage apoptosis. The basis of which is that cells with condensed compacted chromatin will bind greater amounts of the dye as compared to healthy cells.

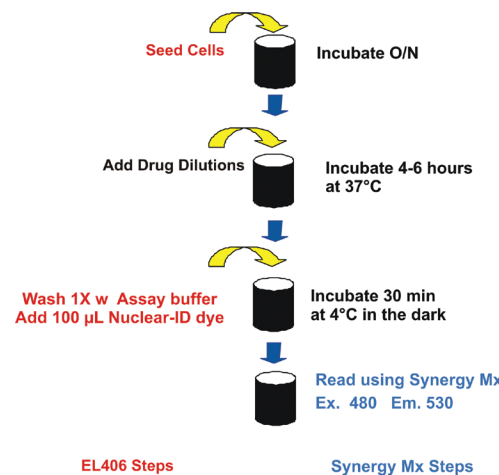


Figure 2 – Schematic of the automated Nuclear-ID™ Green Process carried out by the EL406 Washer Dispenser and Synergy Mx Reader.

Nuclear Condensation

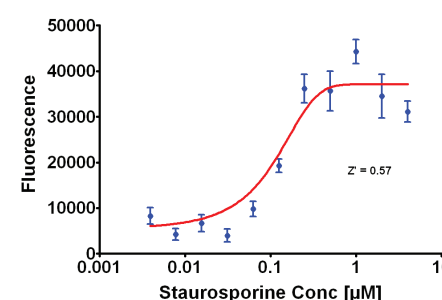
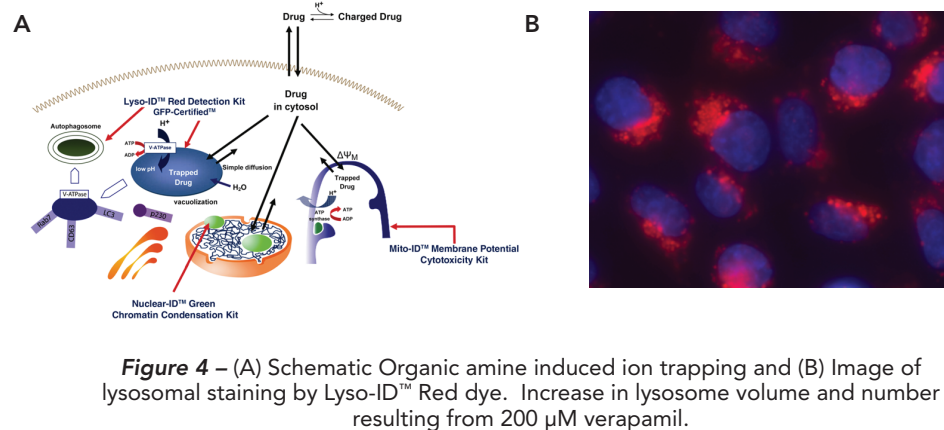
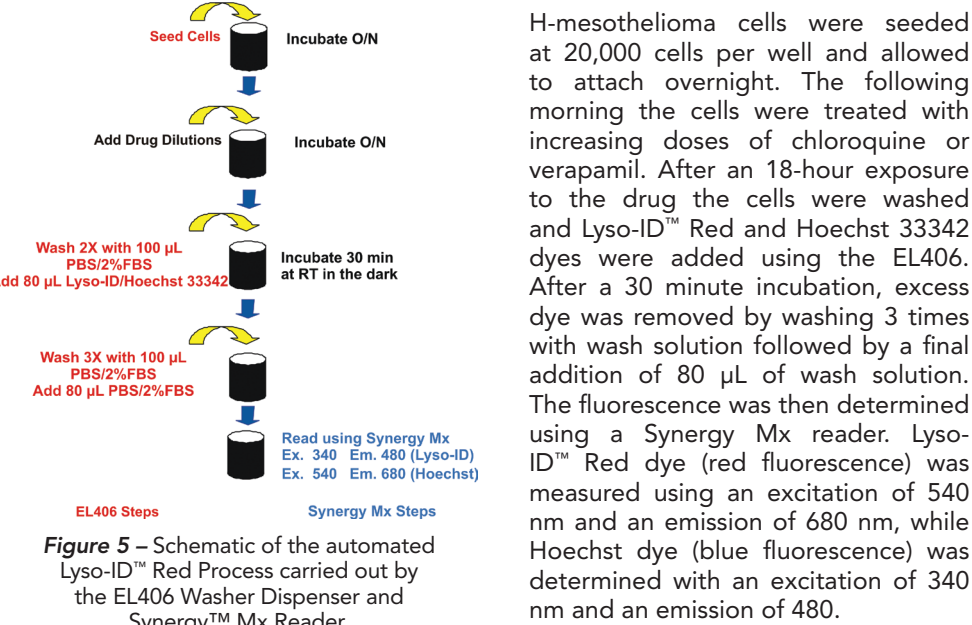


Figure 3 – Dose responsive change in nuclear condensation caused by staurosporine.

CELLestial™ Lyso-ID™ Red Assay



Many drugs cause an accumulation of phospholipids and lysosomes in the cytoplasm. For example amioderone induces an abnormal accumulation of phospholipids that appear as vacuoles with multilamellar inclusions often referred to as autophagosomes. Other organic amines cause vacuolar-ATPase driven ion trapping, which has been associated with vacuolar and autophagic cytopathology. Lyso-ID™ Red is a fluorescent dye that accumulates in lysosomes. An increase in signal is indicative of an increase in the number or size of cellular lysosomes. In addition to the lysosomal specific dye the assay also uses Hoechst 33342 nuclear stain. A decrease of 30% or greater of the Hoechst signal is indicative of generalized cytotoxicity.



Increase in Lysosome Content

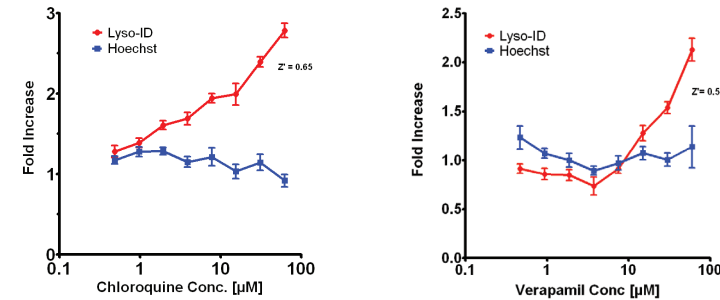
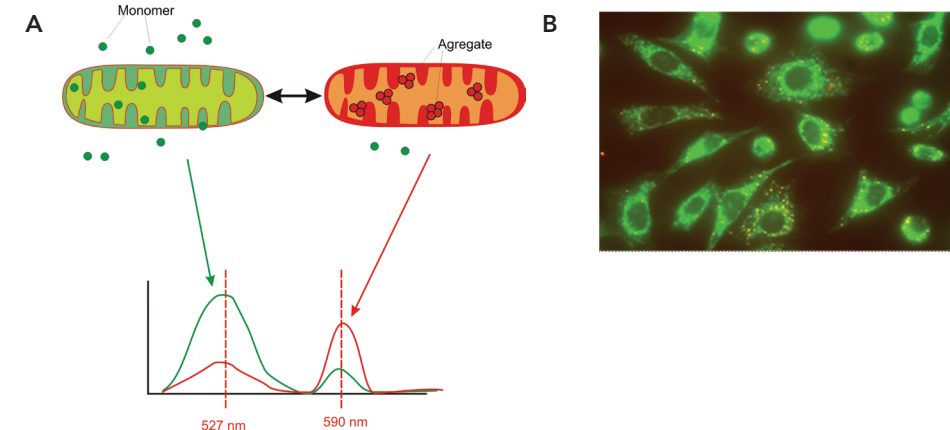


Figure 6 – Dose dependent drug induced increase in lysosomal content as measured by increase in Lyso-ID™ Red fluorescence.

CELLestial™ Mito-ID™ Membrane Potential Assay



Mitochondria play a central role in cellular oxidative respiration. Recently it has been discovered that compromised membrane potential caused by drug accumulation contributes to the toxicity of various organs. The Mito-ID™ Membrane Potential assay uses a cationic dye that accumulates in the cell cytosol as a monomer which primarily emits green fluorescence. In normal energized cells, the dye can also accumulate in the mitochondria as orange fluorescent aggregates. Mitochondrial damage or loss of membrane potential is indicated by a loss of orange fluorescence.

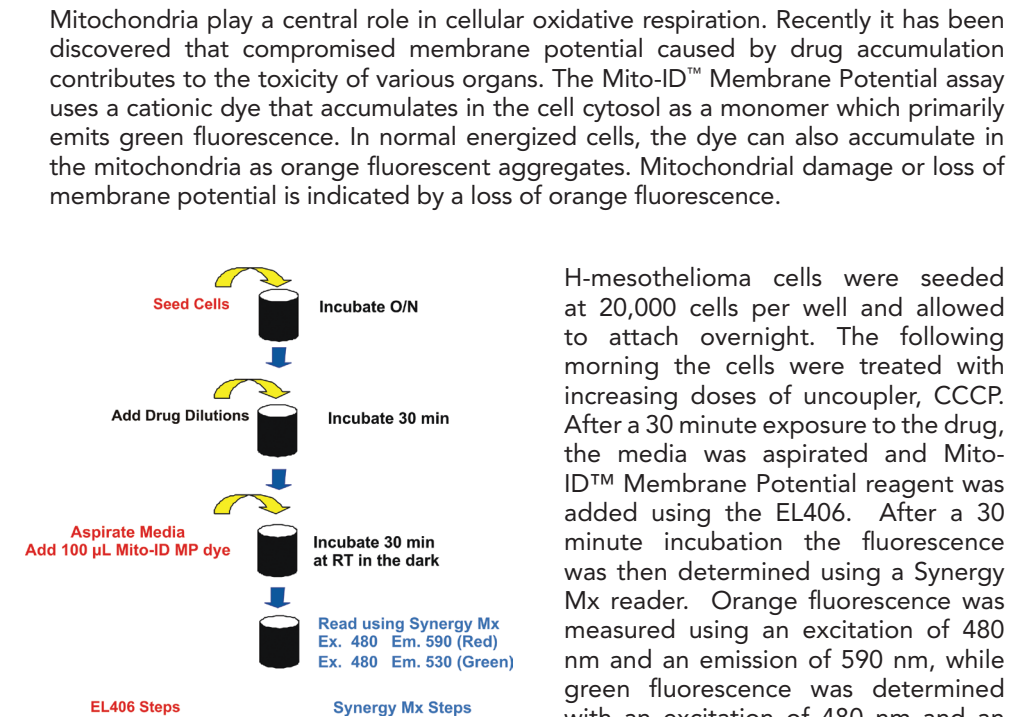


Figure 8 – Automated Mito-ID™ membrane potential cytotoxicity procedure.

Loss of Membrane Potential

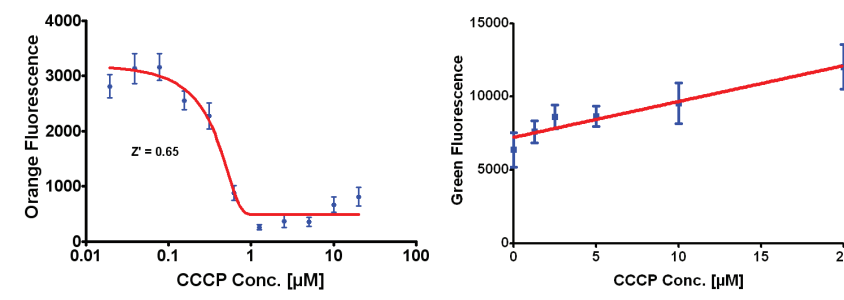


Figure 9 – Change from orange to green fluorescence of Mito-ID™ dye with loss of membrane potential.



Figure 10 – EL406 Combination Washer Dispenser.

The EL406 offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used to remove media, as well as dispense reagents to the 96-well cell plates.



Figure 11 – Synergy™ Mx Multi-Mode Microplate Reader.

The Ultra Fine-Tuned™ technology of the Synergy™ Mx incorporates a quadruple monochromator system which selects wavelengths with a repeatability of plus or minus 0.2 nm. The optical head can focus up and down on samples with a 100 µm resolution. It also uses a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. An ultra low noise digital photon integration system and high-quality optics ensure the best sensitivity available today.

Conclusions

- CELLestial™ kits provide an easy means to screen compounds for potential cytotoxic effects.
- The described assays are rapid, sensitive and specific. They are also compatible with standard high-throughput microplate-based screening workflows.
- The EL406 Washer Dispenser is capable of automating the different fluid handling steps of several different CELLestial™ assays.
- The Synergy™ Mx reader offers ease of use and wavelength flexibility for use in multiple assay determinations.
- Significant Z' values indicate excellent assay performance.