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Application Note

Fluorescent Detection of Drug-Induced Mitochondrial Toxicity

Using the EL406™ Combination Washer Dispenser to Automate the Processing of the Mito-ID™ Membrane Potential Cytotoxicity Assay

Paul Held, BioTek Instruments, Winooski, VT
Kheng Newick, University of Vermont, Burlington VT
Dee Shen and Wayne Patton, Enzo Life Sciences, Farmingdale, NY

The screening of potential drug compounds for deleterious effects on cells is a key aspect of in vitro toxicology testing. Damage to cellular mitochondria and interruption of the respiratory electron transport chain is of particular concern. Drug accumulation in the mitochondria can result in a compromised membrane potential with a resultant disruption of electron transport. Here we describe the use of the EL406™ Combination Washer Dispenser to automate the liquid processing steps of the Mito-ID™ Membrane Potential Cytotoxicity assay from Enzo Life Sciences.

Introduction

The identification of cytotoxic effects is a critical element of the small molecule drug discovery process. Equally important is the ability to automate as many aspects 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, a diverse range of approved drugs have subsequently been shown to be detrimental to mitochondrial function, requiring them to receive Black Box warnings from the US FDA or to be withdrawn from the market altogether. 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, particularly the liver and heart.

The majority of ATP production occurs via oxidative phosphorylation by the mitochondrial respiratory chain, which is essentially driven by the transmembrane electrical potential. The mitochondrial membrane potential provides much of the cell's energy needs and as such is very tightly regulated. Mitochondrial permeability transition, during which the electrochemical gradient across the mitochondrial membrane is lost, is a key step in the induction of apoptosis. Thus the ability to assess mitochondrial membrane potential can provide information regarding mitochondrial and cellular health, as well as the state of the respiratory chain.

The Mito-ID™ Membrane Potential Cytotoxicity assay uses a cationic dye that accumulates throughout the cell's cytosol as a monomer, emitting primarily green fluorescence. In healthy, energized cells, the mitochondria's negative charge established with an intact membrane potential allows the lypophilic dye to enter the mitochondrial matrix and accumulate. With increasing mitochondrial concentration the dye forms J-aggregates that exhibit orange fluorescence (Figure 1).

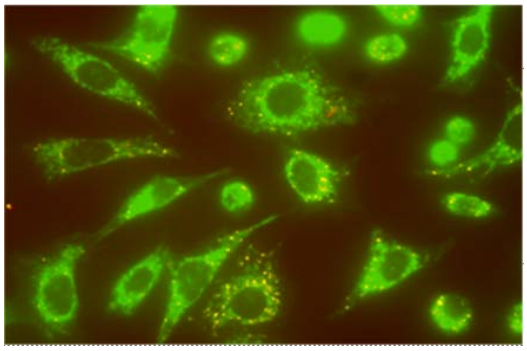

Figure 1. The mitochondria of HeLa cells were stained with Mito-ID™membrane potential dye, and visualized by epifluorescence microscopy. Orange fluorescent aggregates are localized in the mitochondria, while green fluorescent monomers mainly stain the cytosol.

With mitochondrial damage or loss of membrane potential, the dye cannot accumulate in the mitochondria, which is indicated by a lack of orange fluorescence. Using a single excitation wavelength both monomers and aggregates can be measured with dual wavelength fluorescence emission determination (Figure 2).

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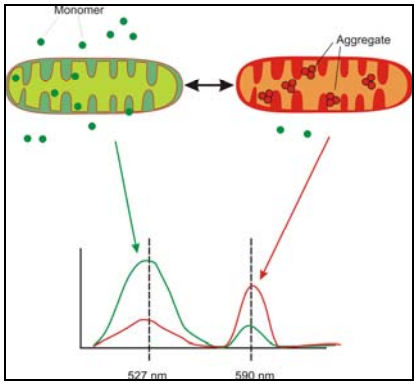

Figure 2. Schematic illustration of Mito-ID™ Membrane Potential dye aggregation in the mitochondria as a result of membrane polarization.

The Mito-ID™ Membrane Potential Cytotoxicity Kit enables the monitoring of mitochondrial membrane potential changes using a fluorescence microplate reader. Applications of this kit are of particular importance in aiding the drug development process, particularly the preclinical drug safety assessment (*in vitro* toxicology) process.

The EL406 Combination Washer Dispenser is well suited to the Mito-ID™ cytotoxicity assay workflow. It 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.

Materials and Methods

H-mesothelioma (H-Meso) cells were seeded at 20,000 cells per well and allowed to attach overnight. The following morning the cells were treated with increasing doses of the uncoupler, CCCP. After a 30 minute exposure to the drug, the media was aspirated and Mito-ID™ Membrane Potential reagent was added using the EL406 (BioTek Instruments). After a 30 minute incubation the fluorescence was then determined using a Synergy™ Mx reader. Using Synergy™ Mx Multimode Microplate Reader (BioTek Instruments), orange fluorescence was measured with an excitation wavelength of 480 nm and an emission wavelength of 590 nm, while green fluorescence was determined with an excitation wavelength of 480 nm and an emission wavelength of 530 (Figure 3).

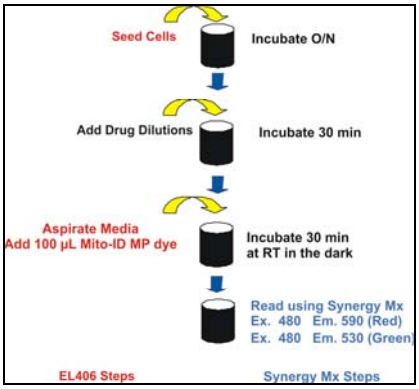

Figure 3. Automated Mito-ID™ Membrane Potential Cytotoxicity Procedure.

Results

Initial experiments used the compound carbonyl cyanide 3-chlorophenylhydrazone (CCCP). CCCP is a proton ionophore that destroys the membrane potential across the mitochondrial membrane. As demonstrated in Figure 4, with increasing CCCP drug concentration the ability of Mito-ID™ dye to form aggregates and emit orange fluorescence is diminished.

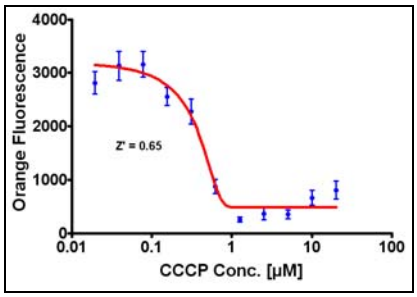

Figure 4. Mito-ID™ dye orange fluorescence response upon treatment with increasing CCCP concentrations.

The effect of CCCP on fluorescence is not the result of fluorescent quenching as the green fluorescence measured at the same time increased with drug concentration. The inability of the dye to form J-aggregates within the mitochondria, with accompanying orange fluorescence results in a greater concentration of cytoplasmic monomers (Figure 5). Note that the relatively large amount of monomer as compared to aggregates only results in a modest percentage increase in green fluorescence signal, despite the elimination of the orange signal.

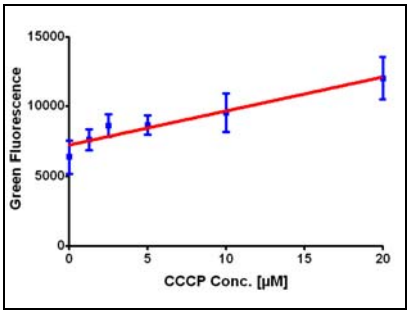

Figure 5. Mito-ID™ dye green fluorescence response upon treatment with increasing CCCP concentrations.

As part of a cytotoxicity profiling of novel compounds with potential activity on mitochondria, we tested the ability of thiostrepton to depolarize mitochondria in H-Meso cells. When the effect of thiostrepton on mitochondrial membrane potential is investigated, an increase in membrane potential is observed. The orange fluorescence emitted by aggregates of the Mito-ID™ stain increases 30% relative to the untreated control (Figure 6). Parallel experiments with the positive control CCCP showed an expected decrease in orange fluorescence.

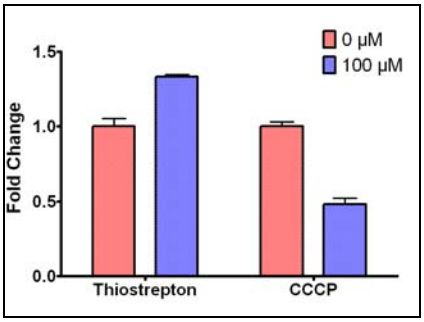

Figure 6. Effect of thiostrepton exposure to H-Meso cells on Mito-ID™ dye orange fluorescence signal.

Discussion

Identification of the loss of mitochondrial membrane potential through toxicity is a key piece of information when screening drug candidates. These data demonstrate the Mito-ID®™ Membrane Potential Cytotoxicity assay kit in conjunction with the EL406 plate washer can reliably automate mitochondrial toxicity testing. A known oxidative phosphorylation decoupler, the ionophore CCCP, was shown to decrease orange J-aggregate fluorescence relative to untreated cells, indicating that the loss of mitochondrial membrane potential can be identified with this assay. Interestingly, we found that the chemotherapeutic agent thiostrepton induces an increase in membrane potential as measured by Mito-ID™ dye orange fluorescence. The significance of this finding is not completely understood at this time, but might be related to the influence thiostrepton has on Nox1 production. Nox1 is an NADPH oxidase that acts as a proton transporter which constitutively produces intracellular ROS [1].

Over the years a number of different membrane permeable lypophilic cationic probes have been used to assess the electrochemical gradient ($\Delta\Psi$) of mitochondria. The most commonly employed ones have included 3',3-dihexyloxacarbocyanine iodide ($DiOC_6[_3]$) [2], nonylacridine (NAO) [2], safranine O [3], and rhodamine 123 (Rh123) [4]. These were designed for use with flow-cytometry rather than microplates and as such are not easily adapted to high throughput workflows. In addition, these systems have suffered from several different problems including a lengthy time to achieve equilibrium and the degree of non-specific binding to membranes. The compound JC-1 has solved many of the specificity problems, but suffers to some extent in regards to its sensitivity towards changes in $\Delta\Psi$ and tendency to precipitate in solution due to the very hydrophobic nature of the dye [5]. Mito-ID™ Membrane Potential dye provides a more robust method to monitor changes in mitochondrial membrane potential than JC-1. Mito-ID™ dye is more photostable, has a higher aqueous solubility, and is 10-fold more responsive to changes in mitochondrial membrane potential than JC-1.

The combination of the homogeneous cell-based Mito-ID™ Membrane Potential Cytotoxicity Kit and the EL406 washer dispenser provide an easy mechanism to automate the screening of compounds for mitochondrial toxicity.

References

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