APPLICATION NOTE

Mitochondrial Membrane Potential By Object Spot Counting

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MITO-ID® MEMBRANE POTENTIAL KIT (ENZ-51018)

INTRODUCTION

The mitochondrion is a highly dynamic organelle, often called the "powerhouse of the cell" for its ability to produce cellular energy in an efficient manner. Mitochondria are the primary manufacturers of ATP, but they also regulate iron homeostasis and the production of free radicals[1]. Mitochondria have a duality of function in that they are involved in the maintenance of viability and vitality, but also play a role in the regulation of apoptotic cell death. Studies have demonstrated that metabolic control through mitochondria is not only related to cell fate, but also plays an important role in differentiation[2]. These diverse functions of mitochondria are all at some point dependent on the mitochondrial membrane potential (MMP).

Mitochondrial respiration generates an electrochemical gradient of protons made up mostly of a negative electrical potential difference across the mitochondrial inner membrane[3]. During mitochondrial oxidative phosphorylation, the transfer of electrons through electron transport chain (ETC) complexes I-IV in the inner mitochondrial membrane provides the energy to drive protons against their concentration gradient across the inner mitochondrial membrane (out of the mitochondrial cytoplasm). The result of this process is an accumulation of H+ outside the membrane, which then flow back into the mitochondria through Complex V, thus producing ATP[4]. This accumulation of H+ results in an electrochemical gradient, otherwise known as MMP.

Mitochondria are present in most of the cells in a living organism, and as such, they are implicated in a wide variety of diseases. Defects in the transfer of electrons across the mitochondrial membrane can cause electrons to accumulate on the ETC complexes and enhance reactive oxygen species (ROS) production. This accumulation increases the potential for electrons to bind with free oxygen species and contributes to many pathological conditions including degenerative diseases, cancer, and aging[5]. Disruption of MMP is one of the earliest intracellular events to occur following induction of apoptosis. In mammalian cells, three responses of mitochondria following a death signal have been noted: a transient hyperpolarization of MMP, a subsequent substantial depolarization of MMP, and, in selected settings, the release of cytochrome c[6-7].

Thus, cell-based assays for analysis of MMP are extremely valuable in order to obtain insights into both cell disease and viability. Evaluating the functional status of mitochondria is critical to elucidating the role of mitochondrial activity in drug-induced toxicity, apoptosis, stem cells, and other cellular and biochemical processes. Quantitative microscopy of the intracellular distribution of membrane-permeant cationic fluorophores provides a means to measure MMP in live cultured cells[3]. To assess the effects of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and ethanol on MMP, such a fluorescent probe in combination with object-based spot counting analysis. To accurately and efficiently determine the number of MITO-ID Membrane Potential positive aggregates per cell analysis was performed using the Gen5™ 3.03 software with object spot counting capability.

MATERIALS AND METHODS

Cell Culture

HeLa cells were grown in Advanced Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) with 10% FBS (Gibco) and 1x PennStrep-Glutamine (Cellgro, Manassas, VA). Cells were seeded into black sided clear bottom 96-well microplates (Corning, Corning, NY) at 20,000 cells per well.

MITO-ID® Membrane Potential Detection Kit

The MITO-ID® Membrane Potential Detection Kit (Enzo Life Sciences, Farmingdale, NY) was used to assess the change in MMP in perturbed HeLa cells. The probe is a cationic carbocyanine dye with a polychromatic fluorescent emission in which the dye fluoresces green or red, depending upon MMP. In normal healthy cells with an established proton gradient across the mitochondrial membrane, the cationic probe is largely driven into the organelle where it exists as red fluorescent aggregates; yet a portion of the dye remains as a green fluorescent monomer in the cytosol. The relative proportion of these localizations, aggregations and resultant fluorescence is dictated by the magnitude of the MMP. Hyperpolarized cells tend to exhibit more red fluorescence. As the mitochondrial membrane de-polarizes (MMP reduces), the cationic probe's tendency to follow the electrical gradient is reduced and preferentially exists as a green-fluorescent monomer in the cytosol.

Prior to MITO-ID Membrane Potential (MP) detection, HeLa cells were treated either with 0.5 – 16 µM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 30 minutes or 0.375% - 6% ethanol (EtOH) for 6 hours. Following treatment, cells were washed 2x with 100 µL 1x assay solution prepared according to the manufacture's protocol. Next, the assay solution was replaced with 100 µL detection reagent (1mL assay solution + 2 µL Hoechst + 15 µL MITO-ID Membrane Potential Detection Reagent) for 15 minutes at room temperature in the dark. The detection reagent was prepared immediately prior to use. Finally, the cells were washed once with assay solution and were imaged right away.

Cell Imaging

Images were acquired using a 20x objective on the Lionheart™ FX Automated Microscope (BioTek Instruments, Winooski, VT) configured with DAPI, GFP, and Texas Red light cubes. The DAPI light cube is configured with a 377/50 excitation filter and a 447/60 emission filter. The GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter. The Texas Red light cube uses a 585/29 excitation filter and a 624/40 emission filter. Images were taken by automatically focusing on the DAPI channel, with an offset of 10-12 on the GFP and Texas Red channels.

Image Analysis

Object pre-processing was used to ensure the best possible detection of nuclei and the best separation between individual aggregates. Imaging pre-processing parameters are described in detail in Table 1. The Texas Red channel of all the images was pre-processed with a 1 µm rolling ball in order to obtain the best separation between individual aggregates (**Figure 1**).

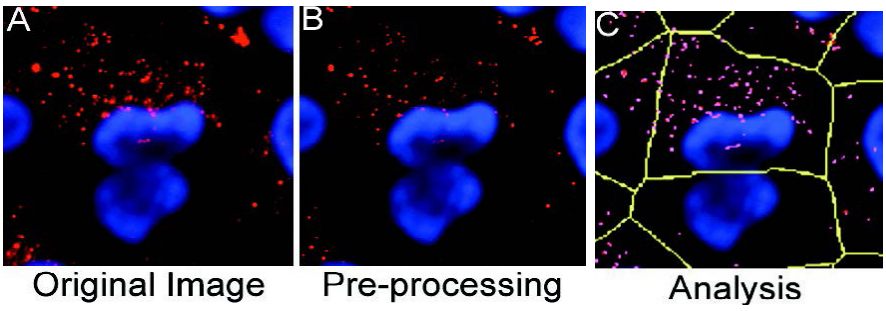

Figure 1. MITO-ID Membrane Potential spot counting workflow. (A) Original image. (B) Pre-processed image. (C) Object Masks highlighting cell area in yellow and spots in pink.

Automatic pre-processing parameters were applied to the DAPI channel to facilitate masking of the nuclei. Cell object counting analysis was then performed on the transformed DAPI channel to highlight each individual cell and object spot counting was performed on the Texas Red channel to determine the number of MITO-ID Membrane Potential aggregates per nuclei according to the parameters outlined below in **Table 1**.

IMAGING PRE-PROCESSING	
Image Set	DAPI
Background	Dark
Rolling Bar Diameter	Auto
Image Smoothing Strength	0
Image Set	Texas Red
Background	Dark
Rolling Bar Diameter	1 µm
Priority	Fast Speed
Image Smoothing Strength	0
CELLULAR ANALYSIS	
Detection Channel: Primary Mask and Count	Tsf[DAPI 377,447]
Threshold	5000
Secondary Mask	Tsf[Texas Red 586,647]
Measure within a Secondary Mask	Checked
Expand Primary Mask	30 µm
Threshold	Unchecked
Count Spots	Checked
Size	0.5-5 µm
Advanced Options	Count Spots Options
Rolling Ball Size	Smaller
Threshold	1000

Table 1. Gen5™ Microplate Reader and Imager Software Settings. Image analysis parameters for generating a cellular mask in the DAPI channel and an object mask in the Texas Red channel in order to count MITO-ID Membrane Potential positive aggregates.

RESULTS

HeLa cells were treated with 0.5 – 16 µM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 30 minutes in order to determine the effect of increasing concentrations of CCCP on the number of MITO-ID Membrane Potential aggregates per cell. CCCP is a proton ionophore and un-coupler of oxidative phosphorylation in mitochondria. CCCP leads to a dissipation of the electrochemical potential across the inner mitochondrial membrane caused by its ability to translocate protons across membranes[8]. As such, it is used here for depolarizing mitochondrial membranes. In this assay, decreasing red MITO-ID Membrane Potential fluorescence aggregates indicate decreasing MMP, as the red MITO-ID Membrane Potential aggregates leave the mitochondria during depolarization. There is a decrease in the number of MMP positive aggregates as a result of increasing CCCP concentration (**Figure 2**).

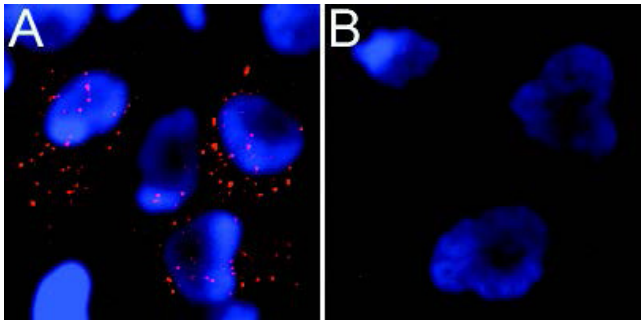

Figure 2. MMP positive aggregates decrease after treatment with CCCP. (A) 0 µM CCP (B) 16 µM CCCP.

In order to determine the percent responders, or percent MMP positive cells, we used the scatter plot function in Gen5™ 3.03. Anything above the mean plus two standard deviations (SD) is considered a "responder." We use the spot count from the negative control sample to define what constitutes a responder. We determined that anything above 1.5 spots per cell was a responder (**Figure 3A**). **Figure 3B** shows a responder cell highlighted in green and non-responders highlighted in yellow. We plotted the percent MMP positive cells against CCCP concentration and, as expected, see a decrease in percent MMP positive cells with an increase in CCCP concentration (**Figure 3C**).

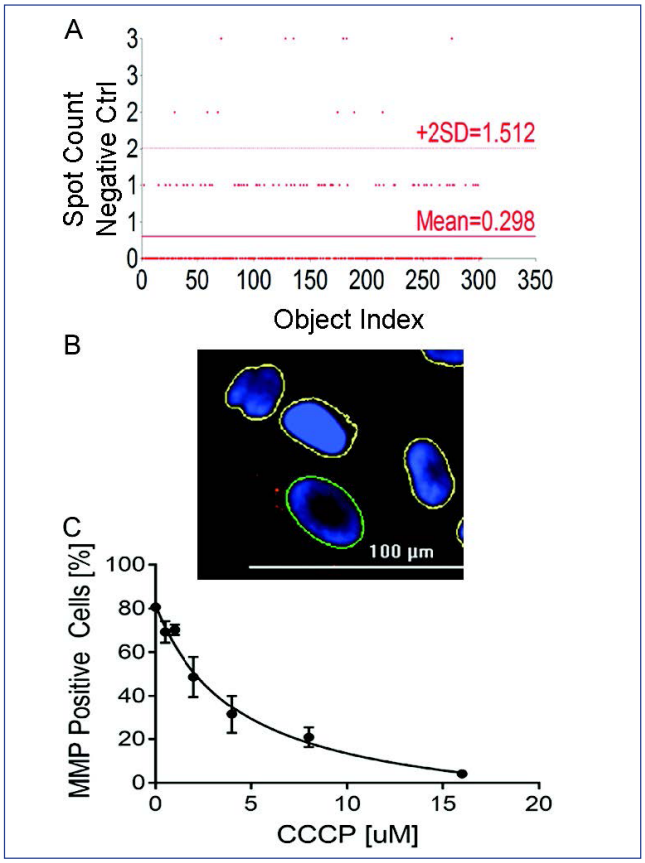

Figure 3. Percent MMP positive cells following treatment with CCCP.(A) Scatter plot showing mean + 2 SD = 1.5 spots per cell. (B) Nonresponders shown in yellow, responders shown in green. (C) Percent Responders decrease in a concentration dependent manner.

HeLa cells were treated with 0.375 – 6 percent EtOH for 6 hours in order to determine the effect of increasing concentrations of EtOH on the number of MMP positive aggregates per cell. Changes in MITO-ID Membrane Potential fluorescence indicated an initial hyperpolarization of the mitochondrial membrane followed by a decrease in polarization with the highest concentration of EtOH (**Figure 4**). This is consistent with previous studies where ethanol at lower concentrations caused hyperpolarization of the mitochondrial membrane in ARPE-19 cells and higher concentrations of ethanol resulted in membrane depolarization[9].

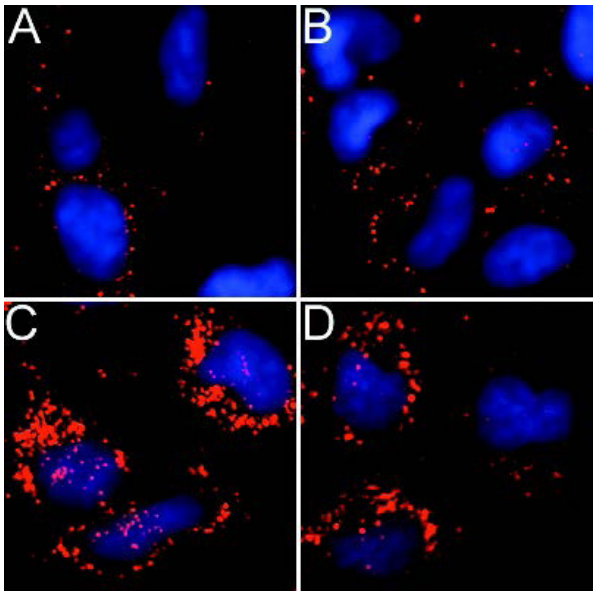

Figure 4. MMP positive aggregates in HeLa cells after treatment with EtOH. (A) 0 uM EtOH (B) 0.75% EtOH (C) 3% EtOH (D) 6% EtOH.

We again used the spot count from the negative control sample of this experiment to decide what constitutes a responder. We determined that anything above 37 spots per cell was a responder (**Figure 5A**). **Figure 5B** shows responder cells highlighted in green and a non-responder highlighted in yellow. We plotted the percent MMP positive cells against EtOH treatment and see an increase in percent MMP positive cells up to 3% EtOH and then a decrease in MMP positive cells at treatments above 3% (**Figure 5C**).

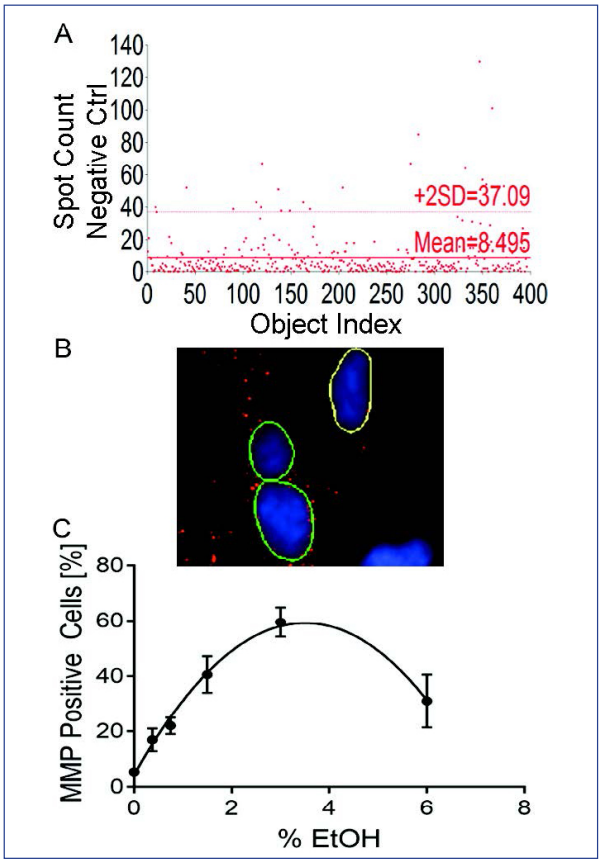

Figure 5. Percent MMP positive cells following treatment with EtOH. (A) Scatter plot showing mean + 2 SD = 37 spots per cell. (B) Nonresponders shown in yellow, responders shown in green. (C) Percent Responders show initial increase with EtOH treatment followed by a decrease at concentrations above 3%.

CONCLUSION

In this study, we employed MITO-ID® Membrane Potential Dye in combination with the Lionheart™ FX Automated Microscope and Gen5™ Microplate Reader and Imager Software to assess the effects of CCCP and EtOH treatment on the MMP of HeLa cells. MMP was quantified by analysis of MITO-ID Membrane Potential positive aggregates in cells using the Gen5 3.03 object spot counting feature. Spot counting allows for reliable and accurate measurements of object level MITO-ID Membrane Potential aggregate analysis in live cultured cells.

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