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# Multiplexed approaches correlating mitochondrial health to cell health using microcapillary cytometry

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ABSTRACT

Mitochondria are critical cellular organelles that play a fundamental role in cellular metabolism and oxidative stress and are well known to trigger multiple cell death pathways. The study of sequence of mitochondrial events as it relates to apoptotic/cell death events can provide critical insights into mechanism of cellular homeostasis, stress and death. Availability of rapid and simplified cytometric testing methods for evaluating mitochondrial changes, apoptosis and cell death in parallel can greatly enhance our understanding of mechanism of compound action. In this study, we investigated a series of compounds to evaluate apoptotic/cell death effects in context of mitochondrial changes using plate-based assays on Guava® easyCyte systems. Studies utilized multiplexed assays for mitochondrial membrane potential changes and apoptosis/cell death markers and allowed for easy identification of hit compounds. Dose and time response studies with Niclosamide, an anti-helmintic drug and comparison of effects with Gambogic acid and celastrol demonstrated early and significant mitochondrial impacts for niclosamide and gambogic acid. No apoptotic or cell death impacts were observed in parallel at low doses/short times of incubation for niclosamide, while increased time with niclosamide caused increase in mitochondrial, apoptotic and cell death response. The method demonstrates great power in being able to distinguish between potency of compounds and conditions in modulating mitochondrial/apoptotic changes. The simplicity of the assays described coupled with the ease of use of plate based microcapillary cytometry can provide researchers valuable tools to obtain a more comprehensive insight into how compounds modulate mitochondria and its relationship with subsequent apoptosis/cell death pathways.



## 1. Introduction

Evaluations using multiplexed flow cytometry assays are powerful in providing enriched information from the same cellular sample, minimizing variation, and providing greater confidence in results by analyzing a large number of cells quickly. Traditionally, this has used expression markers to tease out subpopulations in heterogenous populations. Equally important, is the fact that, multiplexing parameters allows for the assessment of the relationship between different aspects of the same cell, at the same time [1,2]. This relationship in combination with time and dose response provide an important insight into the sequence of events in a response to compound or condition. Further, the multiparametric information that can be obtained from intact cells is what makes flow cytometry a more powerful method for compound/condition impact than other methods that lyse the cells or provide single parameter data.

The central role of mitochondria in cellular bioenergetics and metabolism is well understood alongside with their role in proliferation, differentiation, and adaptation to stress [3]. Recent years have clearly elucidated the crucial and central role mitochondria play in cellular stress mechanisms and multiple cell death pathways such as apoptosis, autophagy and necrosis, and more recently oxytosis and ferroptosis [4–13]. The role of the mitochondria in both extrinsic and intrinsic apoptotic pathways has been demonstrated in a number of studies. Cells undergoing apoptosis have been shown to involve early depolarization of the inner mitochondrial membrane electrochemical gradient, release of key mitochondrial proteins such as cytochrome *c* into the cytosol [5,6], and activation of specific proteases termed caspases [2], blebbing of cytosolic vesicles from the cell surface and loss of plasma membrane asymmetry, condensation of nuclear material, and finally, DNA cleavage and ruptures of the plasma membrane. Necrosis has also been shown to progress with mitochondrial swelling, loss of mitochondrial potential, impaired oxidative phosphorylation

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(OXPHOS) and ATP generation, and release of apoptogenic proteins from the mitochondria. Proteins such as AIF and its liberation from mitochondria has been shown to be an important part of Caspase-independent death [7]. More recently BID mediated mitochondrial damage has been linked to death pathways of ferroptosis for compounds such as Erastin [13]. Mitochondrial health and cell health are intimately related and mitochondrial dysfunction due to disease or compound/conditions can have dire consequences for the cell [14–17].

Traditionally, cell screening assays for compound action typically assess apoptosis by phosphatidylserine externalization [1] or cell death by membrane permeabilization, fewer studies incorporate evaluate mitochondrial perturbations or caspase activations in these early screens [2]. Given the fact that cell death may be as a result of death pathways other than or in addition to apoptosis, screening using multiple indicators of cell health is essential and provide insights into sequence of stress and death for the cell. One such example is studying mitochondrial perturbations in context of apoptosis and/or cell death. Traditionally, this has been a challenge because many of the dyes used for mitochondrial depolarization, such as JC-1 [17], have provided data that has been difficult to interpret and/or have been challenging to combine with commonly used cell death markers such as 7-AAD or blue laser based probes. This has complicated both assay setup and data interpretation and limited use of such multiplexed studies. The assessment of mitochondrial toxicity [18–20] is particularly important in drug development and evaluation of safety and toxicity of hit compounds. In addition, there is also substantial interest in specifically developing drugs that modulate mitochondrial activity [21].

In this paper, we describe a series of assays that correlate mitochondrial health with cell health and provide important information on the action of compounds. In particular, the MitoDamage assay provides a triplexed assay for studying mitochondrial depolarization, annexin based apoptosis detection, and cell death in parallel on a single cellular sample, that allow for easy assay setup and interpretation. Assays such as Cytochrome *c* release provide information on key mitochondrial proteins that triggers the apoptotic cascade. Evaluating caspase 8 or 9 activation while simultaneously assessing cell death provides additional information on the impact and role of particular caspases in the death pathway being studied. The combination of the assays with plate based screening and the heat map features of analysis software allow for identification of hits and the pathway being effected among a large number of compounds analyzed in parallel. Further, when used in combination with time and dose response studies, these assays and approaches provide important and quick mechanistic insights into the role of mitochondria in the action of compounds as shown here in example for niclosamide, an approved anti-helminth now being evaluated for diabetes and cancer, [22] and gambogic acid, currently being evaluated as an anti-tumor agent [23].

## 2. Screening of cytoactive compounds using plate-based cytometry

Often, drug screening and compound discovery experiments evaluate a large number of cytotoxic, immunosuppressive, anti-proliferative, and anti-inflammatory compounds to determine "hits" and evaluate both drug potency and mechanism of action using cellular assays. Multiparametric assays using a minimum of two markers during such evaluations to investigate mitochondrial and cell health can be used to determine impacts on autophagy, mitochondrial stress, apoptosis, and cell death in samples and allow for further understanding of the compounds mechanism of action. Here we discuss evaluation of a minimum of 3 cell health markers, in combination with microcapillary cytometry in a plate based format with powerful analysis tools. These assays and methods provide advantages in allowing for powerful multiplexing of multi-color assays and conditions while using a small number of cells leading to enriched information in a single experiment.

To evaluate differential effects two plates of 160 compounds was evaluated with 3 cell health assays at two time points on Jurkat cells, a non-adherent human T cell line. Six cell health parameters were evaluated; mitochondrial stress shown by loss of mitochondrial membrane potential, mitochondrial stress as shown by loss of the cytochrome *c* from the mitochondria, apoptosis as shown by annexin V binding, activation of caspase 8 and or 9, and plasma membrane permeabilization as indicator of cellular death. Three assays allowed for the evaluation of these parameters, the FlowCellect® MitoDamage Kit, the FlowCellect® Cytochrome *c* Kit, and combining probes for Caspase 8 and Caspase 9. All assays were performed within 4 h of completion of compound treatment and were performed without compensation, allowing for simplified assay setup and analysis. Using as few as 6 96-well plates, allowed for the full assessment as described below.

The FlowCellect® MitoDamage Kit (MilliporeSigma) is a cytometry based kit that can simultaneously provide information on mitochondrial potential changes, as assessed by decrease in fluorescence of MitoSense Red, apoptosis as measured by an increase in Annexin V binding, and cell death, as measured by an increase in 7AAD staining, in a single simplified assay. The kit includes the; MitoSense Red Dye (1,1′,3,3,3′,3′ – Hexamethylindodicarbocyanine iodide), a fluorescent cationic dye that accumulates in the mitochondria and is responsive to mitochondrial potential changes, Annexin V conjugated to CF488A which binds to phosphatidylserine on surface of apoptotic cells, and 7-AAD a membrane impermeant dead cell dye. The simultaneous use of the reagents allows researchers to obtain information on connecting mitochondrial health to apoptosis and other death pathways in one simple assay.

The FlowCellect® Cytochrome *c* Kit (MilliporeSigma) evaluates the level of Cytochrome *c* in mitochondria of cells which can be a key step during the intrinsic process of apoptosis. This allows for the quantitation of percentage that have undergone a shift in the level of mitochondrial Cytochrome *c*. The assay utilizes an optimized and specialized permeabilization and fixation protocol with a FITC conjugated anti-cytochrome *c* antibody to evaluate the loss of mitochondrial cytochrome *c*.

Caspases are intracellular proteases actively involved in the degradation of a wide range of cellular components during the apoptotic cell death. The Caspase 8 and Caspase 9 reagents (ImmnoChemistry Technologies) were used here, each employ a fluorescently-tagged cell permeable caspase specific FLICA (Fluorescent Inhibitor of Caspase Activation) reagent which specifically binds to active caspase molecules. The FLICA reagent is used in addition to the dead cell stain 7-AAD to allow for distinction between cell death and caspase activity.

### 2.1. Methodology of screening cytoactive compounds

#### 2.1.1. Compound screening of Jurkat cells

The night before induction Jurkat cells were split at $5 \times 10^6$ cells/mL to allow for log phase growth. 16 h later, Jurkat cells were spun at 300 × g for 5 min, and resuspended at $5 \times 10^5$ cells/mL in fresh complete growth medium (RPMI with 5% FBS, 5 mM L-Glutamine, 5 mM D-Glucose, and 5 mM Sodium Pyruvate). 200μL of cells were then pipetted to each well microtiter plates. A panel of 160 cytotoxic, immunesuppressive, anti-proliferative, and anti-inflammatory compounds were obtained from Microsource Discovery Systems Inc. Compounds were received from the vendor at a concentration of 10 mM in DMSO. For initial screening,