Monitoring Mitochondrial Membrane Potential with Orflo's Moxi GO – Next Generation Flow Cytometer

Introduction/Background

Mitochondria are the principle organelles underlying cellular metabolism, serving as the "energy factories" for the cell. Through the process of oxidative phosphorylation, mitochondria utilize a series of redox reactions to oxidize pyruvate and NADH, generating stored energy in the form of ATP molecules. The driving force behind the associated reactions is the maintenance of an electrochemical proton gradient across the mitochondrial membrane. As that gradient provides the "fuel" for the oxidative phosphorylation, it has been identified as a valuable metric for the overall health of cells as well as a attribute that can be quantitatively monitored in studying the effects of environmental insults, e.g. toxins, on cellular metabolism.

One commonly used dye applied towards this purpose is Tetramethylrhodamine ethyl ester perchlorate (TMRE). TMRE is a cell-permeant and mitochondria membrane-permeant, red-orange fluorescent (540/595nm excitation/emission) cationic dye that rapidly accumulates in the negatively-charged mitochondria in a potential-dependent fashion. Because of the dependency of the mitrochondrial membrane potential on dye loading, TMRE can be used to quantitatively assess changes in the mitochondrial membrane potential[1].

In this application note, we show how Orflo's newest, "Next Generation Flow Cytometer", the Moxi GO (Figure 1), can be applied towards the tracking of cellular mitochondrial potential in response to the applications of two pharmacological agents, sodium azide ("azide") and camptothecin. Azide is a known mitochondrial inhibitor[2,3,4] and is widely used as an anti-microbial agent in laboratory medias. Camptothecin is an anti-cancer drug that is known to disrupt both DNA and RNA synthesis, frequently resulting in cellular apoptosis induction.[5]

The Orflo Moxi GO system is a simple, rapid, and effective flow cytometric platform that can be applied to a wide range of cellular analysis, including mitochondrial membrane potential studies. The system combines the Coulter Principle, the recognized gold standard for precise cell sizing and counts, with simultaneous fluorescent measurements using a green (532nm) laser and 561nm/LP-filtered PMT detection. The fluorescence configuration is ideal for many of the most common fluorophores including phycoerythrin (PE fir immunolabeling), prodium iodide (PI for viability), and RFP (tdTomato/dsRed,for transfection efficiency measurement). The Moxi GO utilizes a disposable flow-cell architecture, does not require warming-up, runs test in under 10 seconds, and does not require cleaning/shutdown protocols. These attributes make the Moxi GO uniquely suited towards rapid testing and analysis of kinetics, such as the mitochondrial kinetics data described below. The result is an affordable flow cytometer that delivers "Assays on Demand," including mitochondrial potential measurements.

Figure 1 – Orflo's Moxi GO – Next Generation Flow Cytometer. Image shows user loading a sample into the two-test disposable flow cell.

Example Data – Results and Discussion

In this application note, the Moxi GO was applied to measurement of the kinetics of mitochondrial depolarization in response to two cellular toxins, sodium azide and camptothecin.

Figure 2 presents data using the Moxi GO to analyze the effect of sodium azide on the TMRE-measured membrane potential of Jurkat cells as a function of time. Cells were loaded with 400nM TMRE and subsequently diluted into PBS solutions that were azide-free (control) or that were supplemented with 0.045% (w/v) sodium azide. Figure 2a and 2b show user-exported, direct screenshots from the Moxi GO presenting data for the azide treated sample at time=0min (post exposure, Figures 2a and 2b, gray) and for time=20min. The shift in TRME fluorescence is clearly resolved and easily represented on-unit by using the built-in test compare/overlay function. The TMRE median fluorescence intensity (MFI) levels for both the control sample and azide-treated sample for all time points were also plot (Figure 2c). The stability of the control sample is contrasted by the rapid depolarization (decline in TMRE fluorescence) reflected in the azide-treated sample. This is consistent with the literature reports of azide as a potent disrupter of mitochondrial function, with associated depolarization of mitochondria membrane and organelle swelling.[2,3,4] Once a lower-level asymptote in fluorescence appeared to have been established in the testing, the azide was washed from the cells with a 2x wash in azide-free PBS. Following the washes, a slight recovery was noted in the mitochondrial potential, demonstrating that mitochondrial function could be partially recovered. A similar approach was applied to monitor the mitochondrial potential shifts associated with Jurkat exposure to 30µM camptothecin. Camptothecin is a toxicological agent known to induce cellular apoptosis.[5] In the "intrinsic" or "mitochondrial" pathway of apoptosis, a key early hallmark is the depolarization of the mitochondria, associated with the opening of the mitochondrial transition pore and subsequent cytochrome c release to the cytosol.[6,7,8] Direct Moxi GO measurement (and associated screenshots in Figures 3a and 3b) show the dramatic effect of Camptothecin-treatment on mitochondrial function by comparing TMRE-reflected membrane potentials of Jurkats at the time=0hr (post drug exposure) and time=25hr post-exposure. Notable in the scatter plot data (Figure 3a) are both the dramatic shift in both TMRE fluorescence (y-axis, associated with loss of mitochondrial polarization) and the corresponding volume decrease in the cell size (x-axis), a morphological hallmark of apoptosis. This is very precisely captured by the precise Coulter-Principle-based size measurements of the

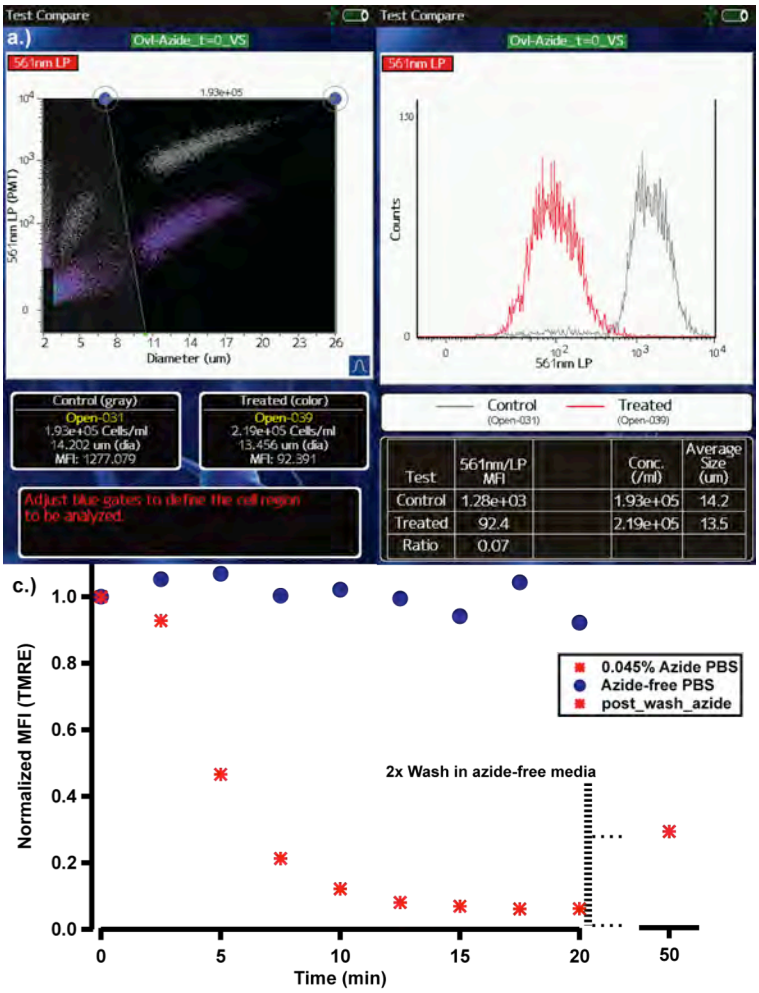

Figure 2 – Time Course of Sodium Azide (0.045% in PBS) alteration of mitochondrial potential in Jurkat E6-1 cells. a.) Moxi GO overlay of fluorescence scatter plots (TMRE Fluorescence vs size) and b.) TMRE fluorescence histograms for t=0min (gray) and t=20min (red/purple). c.) Normalized MFI plots of control (azide-free PBS) and 0.045% azide PBS TMRE fluorescence vs time.

cells. Furthermore, the histogram overlay of the TMRE fluorescence (Figure 3b) provides a clear view of the large shift mitochondrial membrane potential in the sample with time. Finally, as the Moxi GO saves the data in the industry standard FCS 3.1 format (accessible as external drive via USB on-the-go), it can easily be imported into offline flow analysis software for more advanced data visualization and analysis. Figure 3c shows an example of that analysis, using Flowjo X to overlay the TMRE fluorescence of both the control and camptothecin-treated Jurkat samples over a period of 25 hours. Presented in this format, the progression of the drug treatment is easily visualized.

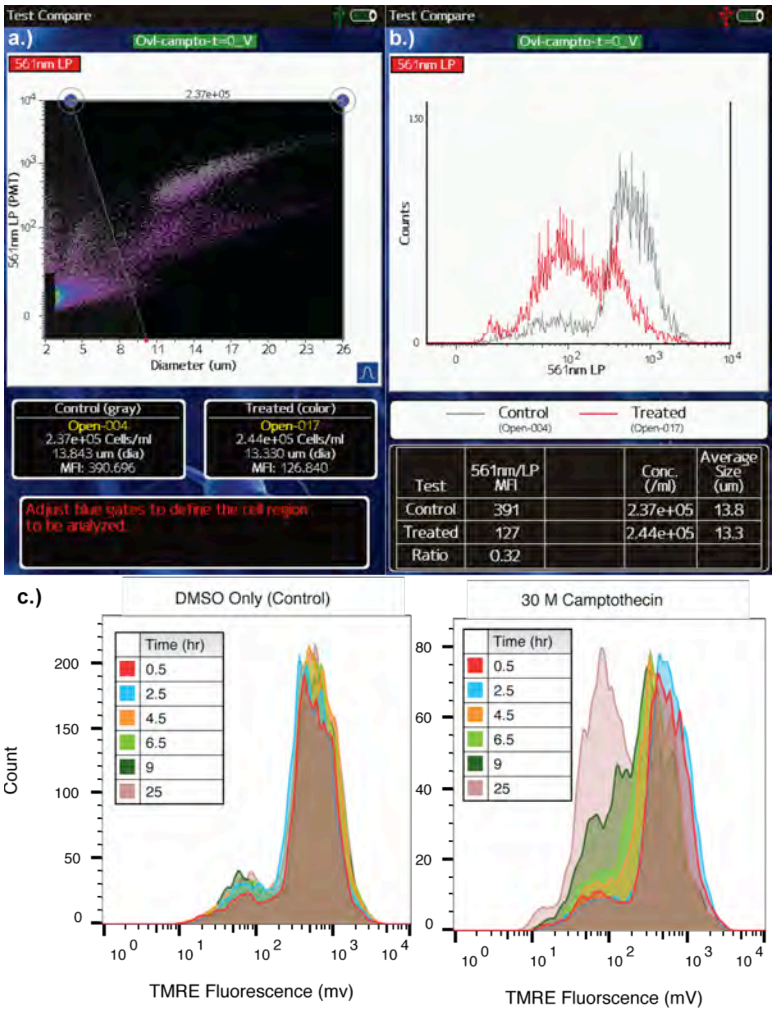

Figure 3 – Time Course of camptothecin(30µM) alteration of mitochondrial potential in Jurkat cells. a.) Moxi GO overlay of fluorescence scatter plots (TMRE Fluorescence vs. size) and b.) TMRE fluorescence histograms for t=0hr(gray) and t=25hr.(red//purple) c.) FlowJo X - generated overlay comparisons of TMRE fluorescence histograms for control (left) and camptothecin-treated (right) samples across all time points.

Summary

In this application note, we provide a protocol and data showing how the Moxi GO can easily be implemented towards the measurement of mitochondrial membrane potential using the TMRE dye. The Moxi GO (Figure 1) is a flow cytometer with a 532nm (green) laser and 561nm/LP PMT collection filter, ideally suited for capturing emission spectra from Phycoerythrin (PE), Propidium Iodide (PI) and RFP (dsRed or tdTomato) cellular labels/stains. One of the most powerful features of the Moxi GO instrument is the ease-of-use and versatility in collection of data. With an ability to run tests with the need for system warm-up, maintenance, or shutdown procedures, uniquely suit it for the implementation of kinetic studies such as the mitochondrial potential kinetic studies described here. In addition, because of it's small footprint and affordable price enable researchers to place the Moxi GO in the culture hood or lab benchtop, allowing for more immediate and frequent flow analysis of their systems over (potentially) long periods of time. Finally the Moxi GO touchscreen GUI is designed to make even complex flow analysis accessible to researchers, regardless of their flow expertise. These features should establish the Moxi GO as a staple in any lab performing mitochondrial potential tracking or any cell-based flow cytometry techniques.

References

1. Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B. & Gelbard, H. A. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques* **50,** 98–115 (2011).
2. Rahn, C. A., Bombick, D. W. & Doolittle, D. J. Assessment of mitochondrial membrane potential as an indicator of cytotoxicity. *Fundam. Appl. Toxicol.* **16,** 435–448 (1991).
3. Johnson, L. V, Walsh, M. L., Bockus, B. J. & Chen, L. B. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. *J. Cell Biol.* **88,** 526 LP-535 (1981).
4. Safiulina, D., Veksler, V., Zharkovsky, A. & Kaasik, A. Loss of mitochondrial membrane potential is associated with increase in mitochondrial volume: Physiological role in neurones. *J. Cell. Physiol.* **206,** 347–353 (2006).
5. Morris, E. J. & Geller, H. M. Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *J. Cell Biol.* **134,** 757–770 (1996).
6. Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35,** 495–516 (2007).
7. Heiskanen, K. M., Bhat, M. B., Wang, H.-W., Ma, J. & Nieminen, A.-L. Mitochondrial Depolarization Accompanies Cytochrome cRelease During Apoptosis in PC6 Cells . *J. Biol. Chem.* **274,** 5654–5658 (1999).
8. Wang, C. & Youle, R. J. The Role of Mitochondria in Apoptosis(). *Annu. Rev. Genet.* **43,** 95–118 (2009).
9. Mitra, K. & Lippincott-Schwartz, J. Analysis of mitochondrial dynamics and functions using imaging approaches. *Curr. Protoc. cell Biol.* **Chapter 4,** Unit 4.25.1-21 (2010).

Methods

Cell Culture – Sample Prep

Jurkat E6-1 (ATCC, TIB-152) were cultured (37°C, 5% CO_2) in RPMI-1640 supplemented with 10% FBS, 1mM Sodium Pyruvate, and 10mM HEPES (all Thermo Fisher). Cell counts and viability during culture were verified using the Moxi GO system (Orflo Technologies #MXG001) with the MoxiCyte Viability reagent (Orflo Tech, #MXA055) and the "Viability Assay" application

Azide Treatment

Jurkat cells were stained with TMRE following the labeling protocol (listed below). Following incubation (Step 4) the cells were diluted (Step 5) in azide-free PBS (control, Gibco, Cat #10010023) or 0.045% sodium azide (BDH, #BDHVBDH7465-2) supplemented PBS. Tests were run every 2.5minutes, following PBS dilution, on the Moxi GO using the "Open Flow Cytometry" assay with the default fluorescence gain. After 20 minutes of TMRE measurements, the cells were washed (250xg, 4min) and re-suspended in azide-free PBS. An additional ~13minute recovery period was applied and a final TMRE measurement was made. Time course plots were generated in IGOR Pro (Wavemetrics).

Camptothecin Treatment

30µL of 10mM camptothecin (Tocris, #1100) stock (in DMSO, Sigma #D8418) was added to 4.97ml of Jurkat culture media. 5mL of the Jurkat culture/suspension was then added (30µM final camptothecin). For the negative control, DMSO was substituted for camptothecin. Cells were continually incubated (37°C, 5% CO_2) during testing. Measurements were made at several post-incubation time points (0, 2.5, 4.5, 6.5, 7.5, 9, 25 hours) to monitor the effect of camptothecin toxicity on the mitochondria polarization. An aliquot of each cell sample was sampled and stained with TMRE following the labeling protocol (listed below) and run at each of the specified, post-incubation, time points on the Moxi GO using the "Open Flow Cytometry" assay with the default fluorescence gain. Overlay data was subsequently analyzed in FlowJo v10.0.8 (Treestar, Mac OSX El Capitan).

Mitochondria - Tetramethylrhodamine ethyl ester perchlorate (TMRE) – Labeling Protocol

1. Make initial stock solutions of TMRE by sequential dilution of TMRE (Sigma Cat #87917) as follows:
    a. 10mM stock TMRE: 25mg dissolved TMRE in 4.85ml DMSO
    b. 10µM stock TMRE: 10µL of 10mM TMRE stock in 990µL DSMO
2. Dilute cells to a concentration of ~2e5-3e5 cells/ml with Cell Staining Buffer. *Notes:*
    a. Cells can be labeled directly in in culture media if they are already at the correct concentration (or are under-concentration).
    b. Cells need to be in a single cell suspension for testing. Detachment with Accutase/Accumax is recommended with pipette trituration to break apart clusters
3. Aliquot 500µL of cell suspensions (it is useful to generate an FCCP-treated (i.e. 100µM), Sigma Cat #C2920, sample as a negative control) into separate polypropylene micro-centrifuge tubes (Santa Cruz, Cat #sc-200271) – *Note: do not use polystyrene (PS) as TMRE can bind significantly to PS.*
4. Add 2.5µL of 10µM TMRE stock solution to each vial to achieve a 50nM final TMRE concentration and gently vortex to disperse. *NOTE: At high overly high concentrations, TMRE has a quenching effect. Consequently, optimal TMRE concentration is dependent on cell type and sample prep and can vary from 20nM –200nM. Initial titration tests should be performed for optimal concentrations. As an initial guess, start with 50nM TMRE.*
5. Incubate TMRE/Cell media at 37°C for 15-30 minutes in the dark.
6. Post-incubation, incubate cells for an additional 15min (RT/Dark).
7. *Optional/Recommended: Add 20µL Orflo Flow Reagent per ml of cells and inversion mix sample.*
8. Following dilution, immediately run the samples on the Moxi GO system (561nm/LP filter) using the "Open Flow Cytometry" assay with the "Medium" gain setting.

TMRE Protocol - Core Product/Reagent List

- Cat #MXG001 – Orflo Moxi GO Next Generation Flow Cytometer
- Cat #MXC030 – MF-S+ Cassettes
- Cat #MXA080 – Orflo Flow Reagent
- Tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma Cat #87917)
- PBS (Gibco, Cat #10010023)
- DMSO (Sigma #D8418)

Monitoring Mitochondrial Membrane Potential with Orflo's Moxi GO – Next Generation Flow Cytometer

http://www.orflo.com