

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 mitochondrial membrane potential on dye loading, TMRE can be used to quantitatively assess changes in the mitochondrial membrane potential¹.

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 systems are simple, rapid, and effective flow cytometric platforms that can be applied to a wide range of cellular analysis, including ROS level measurement. The systems combines the Coulter Principle, the recognized gold standard for precise cell sizing and counts, with simultaneous fluorescent measurements. The systems are available in two configurations. The Moxi GO II has a blue (488nm) laser with two PMT detection channels: PMT1 filtered at 525/45nm and PMT2 filtered at 561nm/LP or 650nm/LP (PMT2 filter is swappable). The Moxi GO 532 has a green (532nm) laser with a single

a.)



b.)



Figure 1 – a.) Orflo's Moxi GO II – Next Generation Flow Cytometer. 488nm Laser, 2 PMT (PMT 1 = 525/45nm, PMT 2 = 561nm/LP or 650nm/LP (swappable filter)). configuration Image shows user loading a sample into the two-test disposable flow cell. b.) 532nm laser (single 580/37nm filtered PMT) configured Moxi GO 532.

PMT, filtered at 580/37nm. The fluorescence configurations are designed for many of the most common fluorophores including:

- 525/45nm filtered PMT: Acridine orange (nucleated counts), Alexa Fluor 488 (immunoprofiling), Calcein (cell health/vitality), CFDA (cell health/vitality), GFP (transfection, CellRox Green (Reactive Oxygen Species Detection), CFSE (proliferation), JC-1 (mitochondrial potential), MitoTracker Green (mitochondrial potential)
- 561nm/LP filtered PMT: Phycoerythrin (PE, immunoprofiling), Proidium iodide (PI, viability), Rhod-2 (cell health, vitality), RFP (tdTomato/dsRed, for transfection efficiency measurement), and TMRE/TMRM (mitochondrial potential)
- 650nm/LP filtered PMT: Proidium iodide (PI, viability) without the need for compensation.

The Moxi GO systems utilize 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 systems 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.

Example Data – Results and Discussion

In this application note, the Moxi GO 532 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

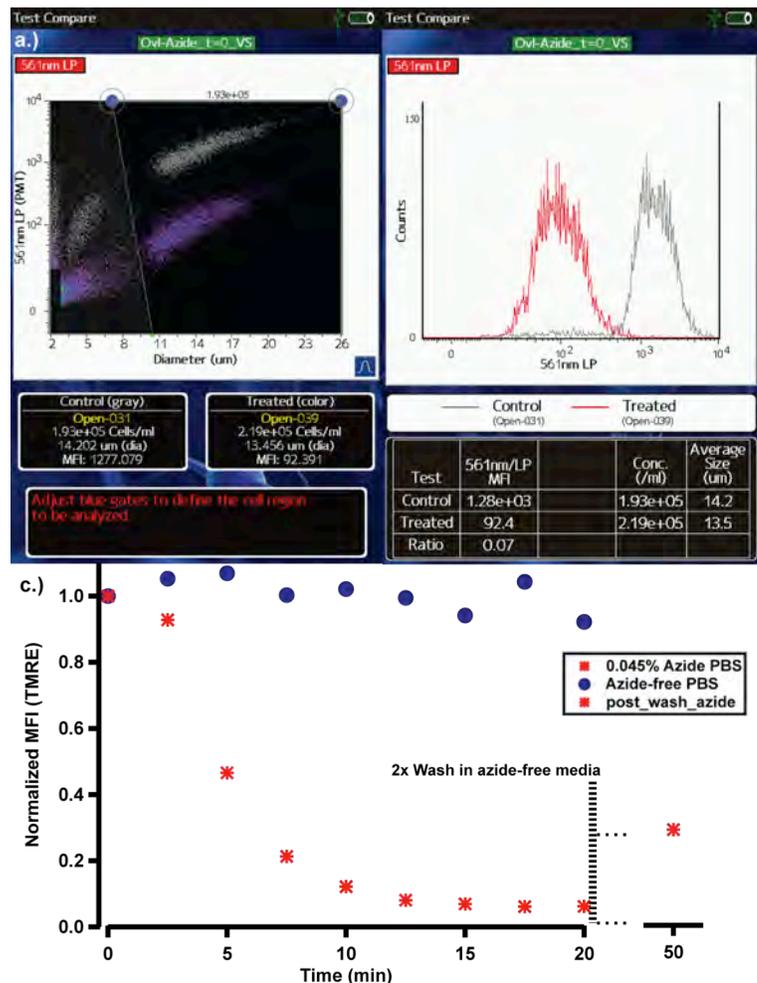


Figure 2 – Time Course of Sodium Azide (0.045% in PBS) alteration of mitochondrial potential in Jurkat E6-1 cells. a.) Moxi GO overlay of fluorescence scatter plots (TMRE Fluorescence vs size) and b.) TMRE fluorescence histograms for t=0min (gray) and t=20min (red/purple). c.) Normalized MFI plots of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

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 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.

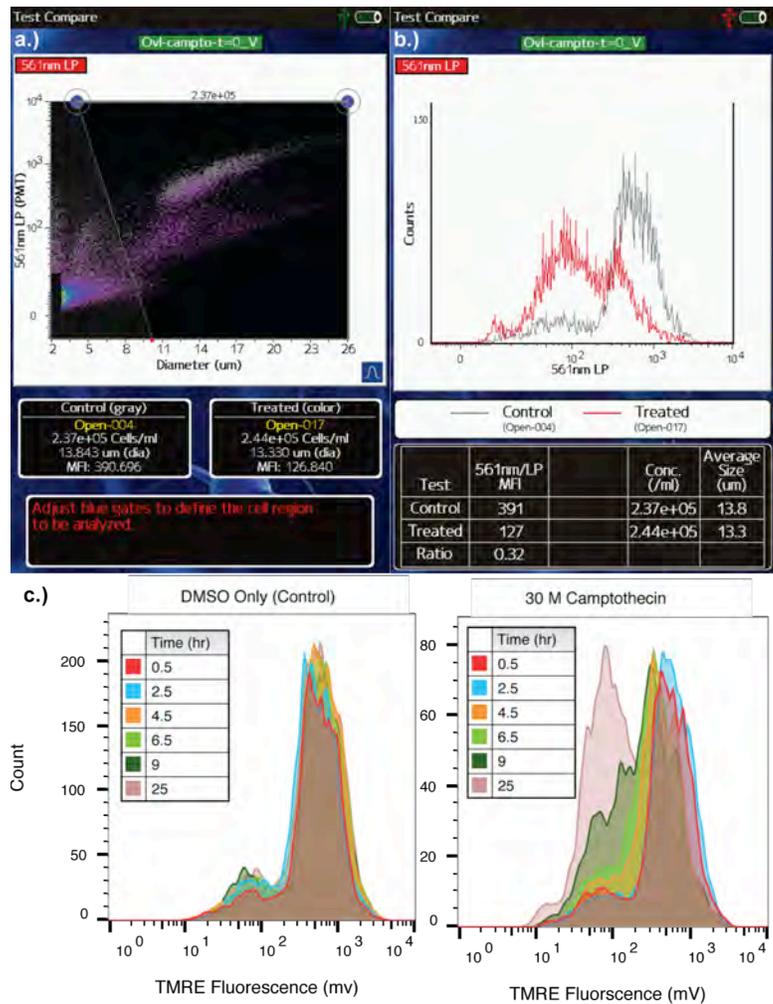


Figure 3 – Time Course of camptothecin(30µM) alteration of mitochondrial potential in Jurkat cells. a.) Moxi GO overlay of fluorescence scatter plots (TMRE Fluorescence vs. size) and b.) TMRE fluorescence histograms for t=0hr(gray) and t=25hr.(red//purple) c.) Flowjo X - generated overlay comparisons 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 system (532nm configuration) can easily be implemented towards the measurement of mitochondrial membrane potential using the TMRE dye. The Moxi GO systems are available in two configurations. The Moxi GO II has a blue (488nm) laser with two PMT detection channels: PMT1 filtered at 525/45nm and PMT2 filtered at 561nm/LP or 650nm/LP (PMT2 filter is swappable). The Moxi GO 532 has a green (532nm) laser with a single PMT, filtered at 580/37nm. The fluorescence configurations are designed for many of the most common fluorophores. One of the most powerful features of the Moxi GO instruments 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 its small footprint and affordable price enable researchers to place the Moxi GO systems 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 systems as a staple in any lab performing mitochondrial potential tracking or any cell-based flow cytometry techniques.

References

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Methods

Cell Culture – Sample Prep

Jurkat E6-1 (ATCC, TIB-152) were cultured (37°C, 5% CO₂) 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 532 system (Orflo Technologies #ZF002) 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 532 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% CO₂) 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 532 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. 10 μ M stock TMRE: 10 μ L of 10mM TMRE stock in 990 μ L DMSO
2. Dilute cells to a concentration of $\sim 2e5$ - $3e5$ 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 500 μ L of cell suspensions (it is useful to generate an FCCP-treated (i.e. 100 μ M), 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 2.5 μ L of 10 μ M 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 20 μ L Orflo Flow Reagent per ml of cells and inversion mix sample.*
8. Following dilution, immediately run the samples on the Moxi GO system using the “Open Flow Cytometry” (Select PMT2: 561nm/LP on the GO II – 488nm system) assay with the “Medium” gain setting.

TMRE Protocol - Core Product/Reagent List

- Either:
 - Orflo Moxi GO 532nm Next Generation Flow Cytometer ([Orflo Cat #ZF002](#))
OR
 - Orflo Moxi GO II 488nm Next Generation Flow Cytometer ([Orflo Cat #MXG002](#)) - with the 561nm/LP filter installed for PMT2
- Type S+ Cassettes ([Orflo Cat #MXC030](#))
- 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)