Measuring Reactive Oxygen Species with Orflo's Moxi GO Systems– Next Generation Flow Cytometers

Introduction/Background

Reactive oxygen species (ROS) are oxygen containing "free radicals," a group of molecules that are highly reactive due to the unpaired electrons that they contain. While ROS are produced normally in cells by mitochondria as a byproduct of cellular metabolism[1] and have even recently been identified as important messengers in various signaling cascades[2], unregulated build-up of ROS has been implicated in numerous pathologies including neurodegenerative diseases, fibrosis, cardiovascular disease, carcinogenesis, and cochlear damage.[2-6] Because of the critical role of ROS in both cellular function and pathology, tools that facilitate the monitoring of cellular ROS levels are critical to the continued development of our understanding of the progression of ROS levels in various cellular models, including disease states.

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.

In this application note, we demonstrate how Orflo's newest, "Next Generation Flow Cytometers", the Moxi GO systems(Figure 1), can be applied towards the measurement of ROS levels in cell preparations. 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 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 (profileration), JC-1 (mitochondrial potential), MitoTracker Green (mitochondrial potential)
- 561nm/LP filtered PMT: Phycoerythrin (PE, immunoprofiling), Prodium iodide (PI, viability), Rhod-2 (cell health, vitality), RFP (tdTomato/dsRed,for transfection efficiency measurement)., and TMRE/TMRM (mitochondrial potential)
- 650nm/LP filtered PMT: Prodium iodide (PI, viability) without the need for compensation.

Measuring Reactive Oxygen Species with Orflo's Moxi GO Systems– Next Generation Flow Cytometers

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. The result is an affordable flow cytometer that delivers "Assays on Demand," including ROS measurement.

Here we provide example data and a protocol for the application of the Moxi GO to ROS generation. While the measurement of ROS is, in itself, a powerful research tool, the Moxi GO is uniquely suited to ROS studies by allowing for this powerful flow cytometry analysis, to both flow experts and novices, right at their culture hood or lab benchtop. In this regard, the ease-of-use and ready availability of the system will greatly enable researchers' ability to easily monitor ROS progression over periods of time. This will undoubtedly establish the Moxi GO as a required tool for any lab that is involved in ROS studies.

Example Data – Results and Discussion

In the example data provided here, we used Thermo Fishers' CellROX® Orange Kit, and followed their general protocol, for the measurement of ROS levels in both HEK-293 and Jurkat E6-1 cell lines. The CellROX® reagent is a fluorescent probe that converts from a non-fluorescent state when in its reduced form to a strongly fluorescent (545/565nm excitation emission) state in it's oxidized form.[7] As a cell-permeant dye, CellROX® easily loads into cells and will be readily oxidized by intracellular ROS producing a fluorescence signal that is proportional to the level of ROS and easily measured my the Moxi GO.

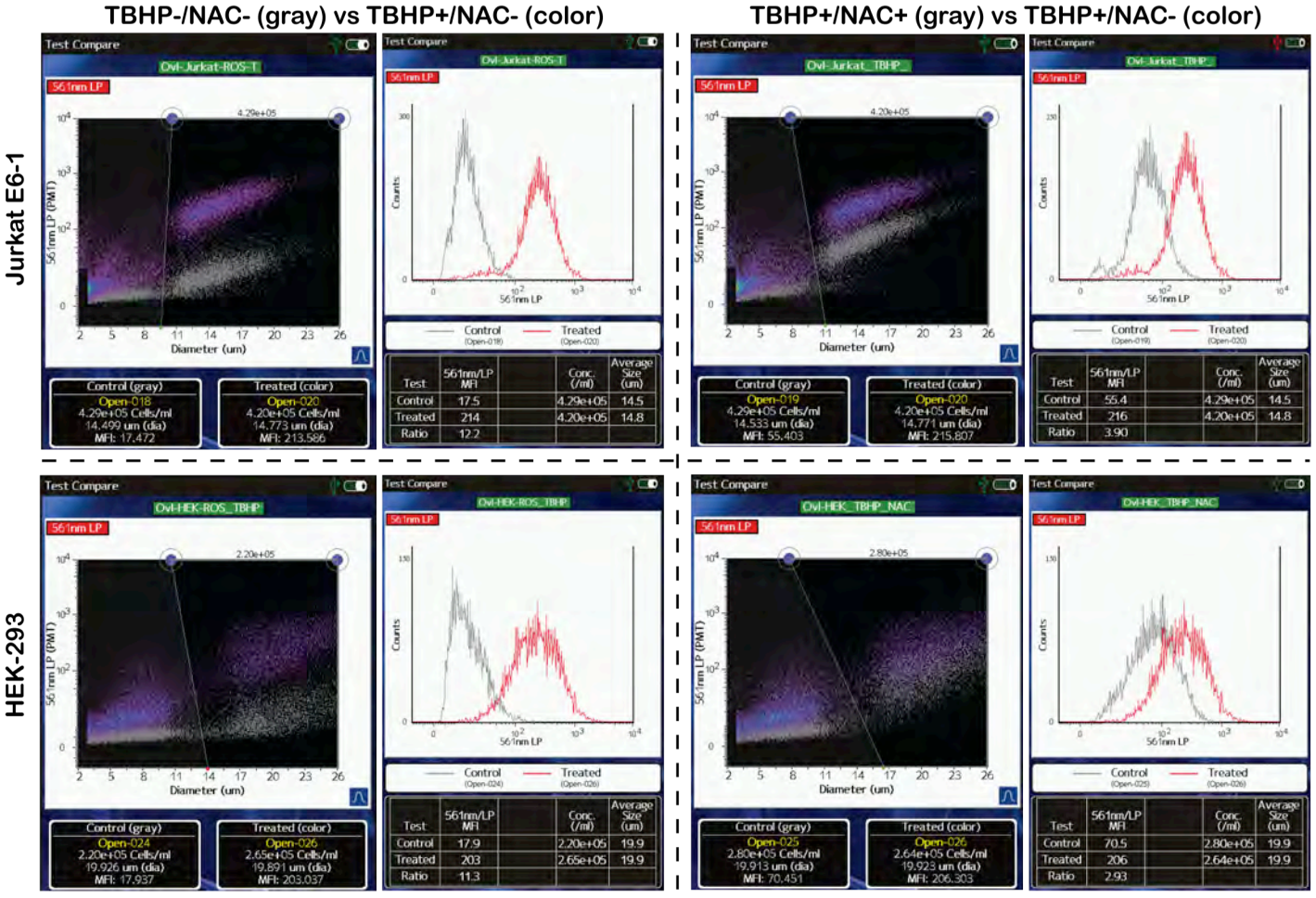

Figure 2 – Direct user-generated screenshots from the Moxi GO showing comparison/overlays of CellROX® Orange fluorescence measurement of ROS levels in various culture states for Jurkat E6-1 cells (row 1) and HEK -293 cells (row2). The first column provides scatter plot and histogram comparisons of the untreated (TBHP-/NAC-, gray) cells vs. the TBHP-only treated cells (TBHP+/NAC-, red/purple). The second column highlights the comparison of the ROS levels of an anti-oxidant (NAC) pre-treated sample (TBHP+/NAC+ in gray) with that of the TBHP –only treated (TBHP+/NAC- red/purple) .

Following the Thermo CellROX® Assay Kit protocol, three ROS conditions were prepared for each cell line: (HEK-293 and Jurkat E6-1): 1.) Negative Control (untreated cells), 2.) ter-Butyl Hydroperoxide(TBHP) treated cells, and 3.) N-Acetlycysteine (NAC) and TBHP – treated cells. Using this approach, three representative states of ROS levels in the cell preparations were realized. With normal, healthy cultures (untreated), the expected ROS levels would be minimal due to cellular homeostatic regulation. For cells treated with TBHP (and organic peroxide that is known to induce ROS production), higher cellular ROS levels would be generated. However, in CellROX® the third condition, the (pre-) application of NAC, a known anti-oxidant would serve to counteract the effects of TBHP, protecting against ROS generation and driving the levels towards the basal (untreated) range.

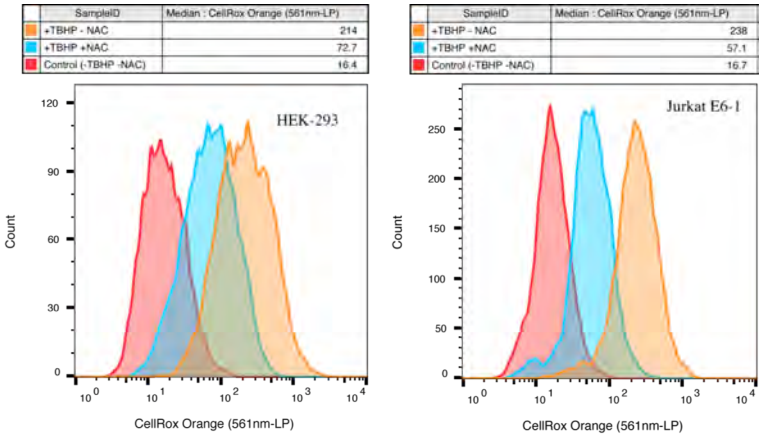

Figure 3 – FlowJo overlays of the CellROX® Orange fluorescence for the three ROS states (red – TBHP-/NAC-, blue-TBHP+/NAC+, and orange – TBHP+/NAC-) for both the HEK-293 (left) and the Jurkat E6-1 (right) cell lines.

Figure 2 shows the direct, user-generated, Moxi GO screenshots of data comparing the various conditions through the on-unit overlay/test-compare capability. The data in Figure 2 is arranged in a grid to show Jurkat E6-1 (row 1) and HEK-293 (row 2) ROS state comparison/overlays. The first column provides the corresponding scatter plot overlay (CellROX® Orange fluorescence vs size) and CellROX® Orange fluorescence histograms comparing the untreated (TBHP-/NAC-, gray) cells vs the TBHP-stimulated samples (TBHP+/NAC-, purple/red). The ROS-stimulating effect of the TBHP treatment is clearly evident in the large fluorescence shift in the data vs. the control sample. The second column shows the same data layout to demonstrate effect of the anti-oxidant pre-treatment of the sample prior to applying the TBHP. In this case, the NAC treated sample (TBHP+/NAC+, gray) shows a downward shift in fluorescence vs. the TBHP-only sample (TBHP+/NAC-, purple/red), reflecting the expected scavenging effect of the anti-oxidant on the TBHP-induced ROS generation.

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 3 succinctly shows the overlays for the three ROS states (red – TBHP-/NAC-, blue-TBHP+/NAC+, and orange – TBHP+/NAC-) for both the HEK-293 (Figure 3 left) and the Jurkat E6-1 (Figure 3 right) cell lines. Presented in this format, the effect of the ROS-inducer (TBHP) and anti-oxidant application can be clearly visualized simultaneously.

Summary

In this application note, we provide a protocol and data showing how the Moxi GO (532 configuration) can easily be implemented towards the measurement of ROS using ThermoFisher's CellROX® Orange kit. 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 without the need for system warm-up, maintenance, or shutdown procedures, the Moxi GO systems are uniquely suited for the implementation of kinetic studies such as the ROS

measurement described here. In addition, its 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 systems as a staple in any lab performing ROS measurements or other cell-based flow cytometry techniques.

References

1. Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552,** 335–344 (2003).
2. Bazhin, A. V, Philippov, P. P. & Karakhanova, S. Reactive Oxygen Species in Cancer Biology and Anticancer Therapy. *Oxid. Med. Cell. Longev.* **2016,** 4197815 (2016).
3. Waris, G. & Ahsan, H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **5,** 14 (2006).
4. He, F. & Zuo, L. Redox Roles of Reactive Oxygen Species in Cardiovascular Diseases. *International Journal of Molecular Sciences* **16,** (2015).
5. Seidman, M. D. *et al.* Age-related hearing loss and its association with reactive oxygen species and mitochondrial DNA damage. *Acta Otolaryngol.* **124,** 16–24 (2004).
6. Richter, K. & Kietzmann, T. Reactive oxygen species and fibrosis: further evidence of a significant liaison. *Cell Tissue Res.* **365,** 591–605 (2016).
7. CellROX® Green and CellROX® Orange Flow Cytometry Assay Kits – User Manual - https://tools.thermofisher.com/content/sfs/manuals/cellrox_green_orange_flow_cyt_assays_man.pdf

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). HEK-293 cells were cultured (37°C, 5% CO_2) in DMEM supplemented with 10% FBS 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 (data not shown).

ROS Staining and Drug Treatment

Reagents for the ROS fluorescence staining were acquired through the CellROx Orange Flow Cytometry Assay Kit (Thermo #C10493). The data generated in this study followed the protocol outlined in the corresponding kit manual[7]. A brief overview of the specific implementation of the protocol is provided below.

N-acetylcysteine (NAC) Treatment[7]

NAC (10mg, component C) was reconstituted with 245µL of PBS (Gibco, Cat #10010023) to create a 250mM stock solution. For the TBHP+/NAC+ (anti-oxidant treated samples), the Jurkat and HEK cells were pre-treated with 500µM NAC (2µL of 250mM stock per 1ml of cell media) and incubated (37°C, 5% CO_2) for one hour prior to TBHP treatment.

Tert-butyl hydroperoxide (TBHP) Treatment[7]

3.22µL of the 7.78M TBHP stock (Component E) was added to 496.8µL PBS (Gibco, Cat #10010023) to create a 50mM intermediate stock solution. 4µL of the 50mM TBHP stock was added per mL of both the TBHP-only (TBHP+/NAC-) and NAC-treated (TBHP+/NAC+) cell samples to achieve a 200µM final TBHP concentration. Following application of TBHP, cells were incubated (37°C, 5% CO_2) for an additional hour.

Moxi GO – Oxidative Stress Staining using Thermo Fisher's CellROX™ Orange or CellROX Green ROS Staining Protocol (from Thermo Fisher CellROX™ Orange Manual*)

*CellROX® Green and CellROX® Orange Flow Cytometry Assay Kits – User Manual - https://tools.thermofisher.com/content/sfs/manuals/cellrox_green_orange_flow_cyt_assays_man.pdf

Overview

The following protocol is sourced and adapted from the Thermo Fisher CellRox Orange and CellRox Green Flow Cytometry Kit User Manual. The CellRox family of dyes allows for detection of reactive oxygen species (ROS) in cells. Specifically the dyes are readily oxidized by ROS and convert from a non-fluorescent to fluorescent state upon oxidation. As the dyes are plasma membrane permeant, they can be used to measure oxidative stress in living cells. The CellRox kits include the CellROX Orange Reagent, N-acetyl cysteine (an antioxidant, for negative control), and tert-butyl hydroperoxide solution (TBHP, an inducer of ROS for use as a positive control).

Reagents/Components:

- Either:
  - Orflo Moxi GO 532nm Next Generation Flow Cytometer (Orflo Cat #ZF002)
  - CellRox Orange Flow Cytometry Assay Kit (ThermoFisher, Cat# C10493

  OR

  - Orflo Moxi GO II 488nm Next Generation Flow Cytometer (Orflo Cat #MXG002)
  - CellRox Green Flow Cytometry Assay Kit (ThermoFisher, Cat# C10492)
- Type S+ Cassettes (Orflo Cat #MXC030)
- Cell media (e.g. RPMI or DMEM)
- *Optional:* PBS (any formulation, e.g. Gibco, Cat #10010023)
- *Optional/Recommended:* Orflo Flow Reagent (Orflo Cat #MXA080)

Protocol:

1. Dilute cells to ~1e5 – 5e5 cells/ml in standard cell media (e.g., RPMI or DMEM). If an adherent cell line is used, ensure that the cells are sub-confluent. **Note: Staining of cells in phosphate buffered saline (PBS) is not recommended.**
2. Induce ROS in cells using the desired method (TBHP).
3. Prepare positive (TBHP) and negative controls (no TBHP). You can prepare the negative control by incubating the cells in the absence of the ROS inducing agent or by incubating the cells with the antioxidant (NAC).

   a. Reconstitute one vial containing 10 mg of NAC (Component C) with 245µL of PBS to make a 250mM solution.
   b. Prepare a 50mM intermediate dilution of TBHP (Component E) by adding 3.22µL of the 70% stock (7.78 M) to 496.8µL of PBS or complete media.
   c. *Negative control:* Prepare a negative control by incubating the cells with NAC before treatment with TBHP. Add NAC to the negative control sample and incubate for 1 hour under normal growth conditions (ex: 37°C, 5% CO2). Although the suggested final concentration of NAC for use is 200–5000µM, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.
   d. *Positive control:* Create a positive control by adding 100–400µM of TBHP to a sample of cells. Ensure that the same concentration of TBHP is used in both positive and negative controls (Ex: add

4µL of the 50mM intermediate TBHP solution per mL cells for a final concentration of 200µM TBHP).

e. Following the 1hour incubation with NAC, add TBHP to the negative control cells from step 3c.
f. Incubate the samples from step 3d and 3e for 30–60 minutes under normal growth conditions before staining with the CellROX ROS detection reagent.

4. Briefly centrifuge the vial of CellROX reagent (Component A) before opening the vial. Add the CellROX reagent at a final concentration of 500–1000nM to the samples and/or appropriately induced cells, and incubate for 30–60 minutes at 37°C, protected from light.

   a. It is best to prepare an intermediate dilution of the CellROX reagent in DMSO (Component D). Mix the intermediate dilution well by pipetting up and down, and then add the specified amount of the diluted solution to the cells, so that the final concentration of the reagent incubated with the cells is 500–1000nM (Ex: combine 1µL of CellROX reagent with 9µL of DMSO to make a 250µM solution; use 2µL of this intermediate solution to stain 1 mL of the cell suspension for a final concentration of 500nM).

5. *Optional:* Wash the cells once with 3X the stain volume using PBS or other appropriate buffer (ex: wash 1 mL of stained cells with 3 mL of PBS). However, washing is not required following staining with CellROX reagents.
6. *Optional Recommended: Add 20µL Orflo Flow Reagent per ml of cells and inversion mix sample*
7. Read the samples using the Moxi GO systems with the "Open Flow Cytometry" assay. Make sure you are using of the of the flowing system/filter selections:
   - CellRox Green: 488 system Moxi GO II, select PMT1 (525/45nm filter)

   OR

   - CellRox Orange: 532nm Moxi GO