Intracellular Immunolabeling with Orflo's Moxi GO – Next Generation Flow Cytometer

Introduction/Background

Intracellular immunolabeling is a valuable technique applied by researchers to phenotype cells (e.g. verification of stem cell differentiation)[1], to evaluate the functional responses of cells to external stimuli (e.g. pharmaceutical agents or toxins)[2], to identify intracellular markers related to specific diseases and their pathologies[3], and to core research into the progression of and mechanisms underlying signaling cascades in cells.[4] Applying this technique to flow cytometry has correspondingly enabled researchers to rapidly analyze intracellular markers at the cellular level at the rate of thousands of cells per second, ensuring statistical robustness of the resulting data.

Figure 1 – Orflo's Moxi GO – Next Generation Flow Cytometer. Image shows user loading a sample into the two-test disposable flow cell. The Moxi GO is available in 488nm laser and 532nm laser version with user swappable filter sets.

In this application note, we demonstrate how Orflo's newest, "Next Generation Flow Cytometer", the Moxi GO (Figure 1), can be applied towards the measurement of intracellular targets in cell preparations. 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 intracellular immunolabeling. The system combines the Coulter Principle; the recognized gold standard for precise cell sizing and counts; with simultaneous fluorescent measurements using either a green (532nm) or blue (488nm) laser coupled with 561nm/LP-filtered PMT detection (user-swappable to 525/45nm with the 488nm system). The fluorescence configuration is ideal for many of the most common fluorophores including phycoerythrin (PE) for immunolabeling, propidium iodide (PI) for viability, FITC (488nm system immunolabeling) and RFP (tdTomato/dsRed) for transfection efficiency or GFP (488nm system) for transfection efficiency. The Moxi GO utilizes a disposable flow-cell architecture, does not require warm-up, runs a test in under 10 seconds, and does not require cleaning/shutdown procedures. The result is an affordable flow cytometer that delivers "Assays on Demand," including intracellular immunolabeling. Here we provide example data for the application of the Moxi GO to 1.) measuring Neurofilament Heavy (NF-H) and Neurofilament Medium (NF-M) expression in mouse neural progenitor cells and 2.) quantifying ERK1/2 activation in PMA/Ionomycin-stimulated human T-cells and monocytes.

Neurofilaments are intermediate filaments that serve as integral components in the neuronal cytoskeleton.[5] As NF-H and NF-M are mature neuronal markers, intracellular immunolabeling can be applied to help characterize neuronal differentiation from the stem cell precursors.[6] And, pathologies in the function and regulation of neurofilament expression have been linked numerous disease states, adding significance to the detection of NF-H and NF-M.[5,7]

ERK 1/2 are protein kinases in the family of mitogen-activated protein kinases (MAPK). They play a critical role in numerous cell signaling cascades, when activated, through the phosphorylation of amino acid (sernine/threonine) sequences. The central role of ERK 1/2 is reflected in their role in cell growth, motility, differentiation, mitosis, and death.[8,9] Monitoring of ERK 1/2 activation is correspondingly an important intracellular target for immunolabeling studies, including having been identified as a critical

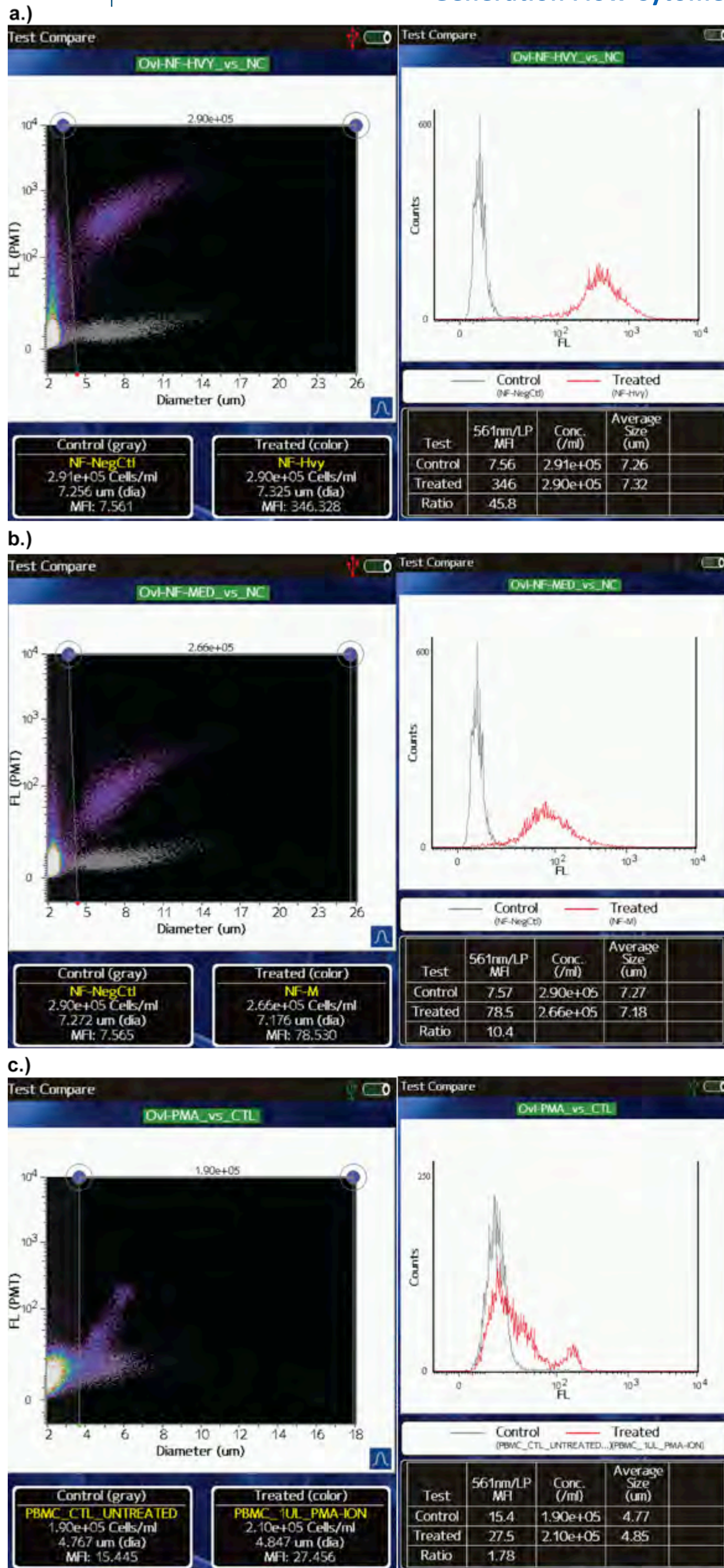


Figure 2 – User-generated screenshots from the Moxi GO showing scatter plot (fluorescence vs. size, left column images) and fluorescence histogram (right column) comparisons/overlays of positive samples vs. controls. a.) Neurofilament-heavy (color scatter/red histogram) vs. unstained control (gray) mNPCs. b.) Neurofilament-medium (color scatter/red histogram) vs. unstained control (gray) mNPCs. c.) PMA/Ionomycin stimulated PBMC's (color scatter/red histogram) vs. untreated sample (gray, neg. control).

target in cancer research and treatment[10].

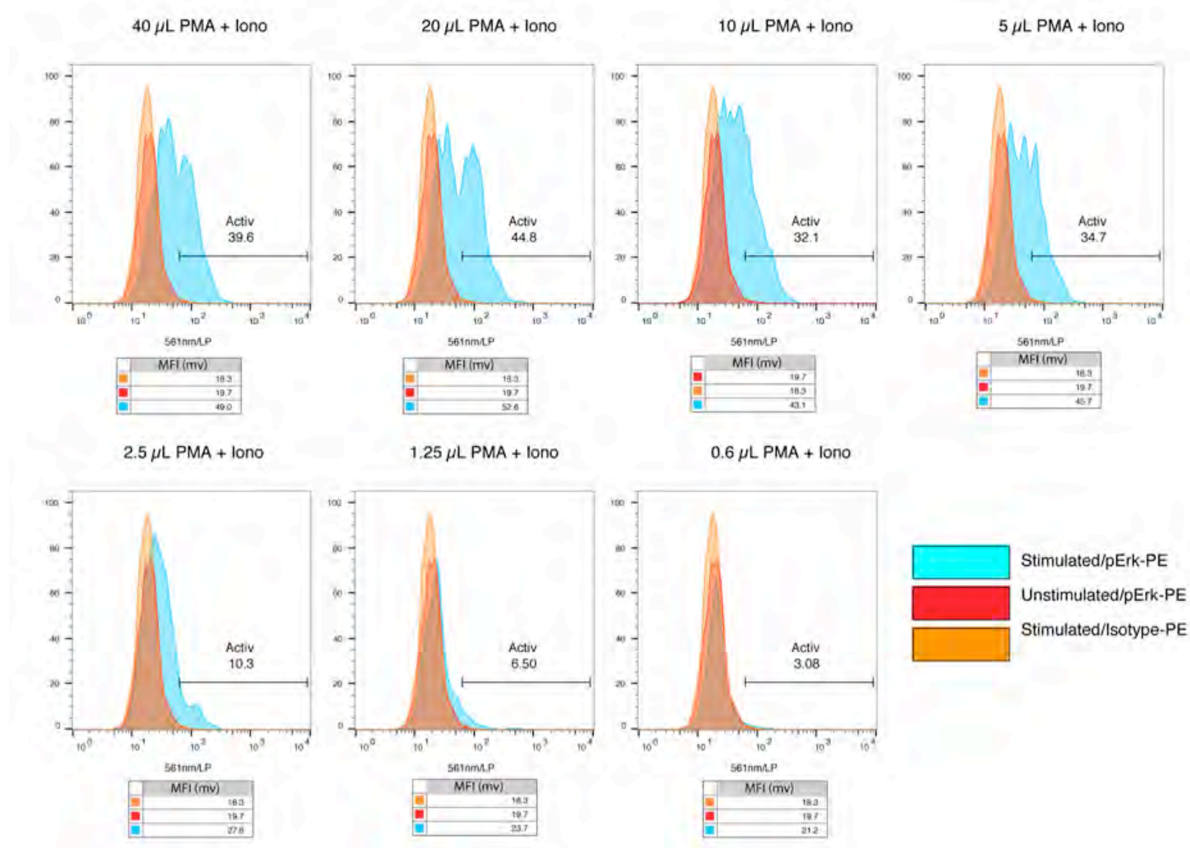
The Moxi GO is uniquely suited to intracellular immunolabeling studies by enabling flow cytometry analysis of intracellular targets by both flow experts and novices, right at their culture hood or lab benchtop. In this regard, the ease-of-use and ready availability of the system can greatly enable researchers' ability to easily monitor the progression of most intracellular antigen expression levels over periods of time, short or long. This will undoubtedly establish the Moxi GO as a required tool for any lab that is involved in studies that study intracellular targets.

Example Data – Results and Discussion

In the first part of this work, mouse neuronal progenitor cells (mNPCs) were fixed, permeabilized and labeled with PE-conjugated anti-Neurofilament Heavy (NF-H) and anti-Neurofilament Medium (NF-M) antibodies. Cells were then processed on the Moxi GO using the MFS cassette type with the "Open Flow Cytometry" application. Figure 2a and 2b are on-unit user-generated screenshots comparing the labeled (colored scatter, red histogram) vs. unlabeled (gray) fluorescence signals of the NF-H (Figure 2a) and NF-M (Figure 2b) immunolabeled mNPCs. The strong fluorescence signals (46x higher reported MFI for NF-H and 10x for NF-M) of the labeled cells are confirmation of the differentiation of these cells from their embryonic stem (mES) precursors into the neural progenitor derivatives. Analysis such as these can be used to phenotype not only stem cell differentiation but also to distinguish normal and diseased states in cells. The Moxi GO on-unit overlay feature, shown in Figure 2, correspondingly provides a rapid, but clear, comparison of the target marker/protein content/expression levels within any two samples.

Figure 2c shows a similar data comparison of the ERK 1/2 activation between PMA/ionomycin treated (colored scatter, red

a.)



b.)

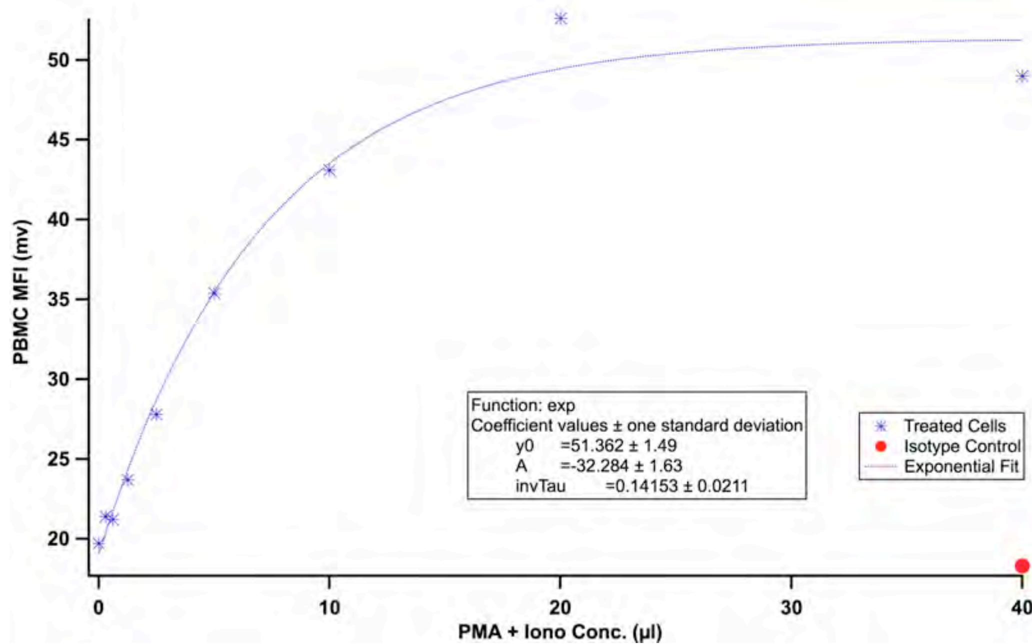


Figure 3 – a.) FlowJo overlays of the dose-response of fixed/lysed peripheral blood (blue) to varying PMA/Ionomycin cocktail levels vs. negative (red) and isotype (orange) controls. Treatment levels are reported in µL of stock activation cocktail (40.5µM PMA/669.3µM ionomycin) per ml of sample. b.) Plot (blue markers) of activated cell region MFI (mv) values vs. stock activation cocktail dose (µl/ml). Exponential fit (blue line) allows for extraction of dose-response parameters.

histogram) peripheral blood mononuclear cells (PBMCs) and untreated PBMCs. Cells were labeled with a PE anti-phospho ERK 1/2 monoclonal antibody to specifically target the activated ERK 1/2 proteins. The clear fluorescence shift, reflecting the activation of ERK1/2 in the PBMCs, is clearly visualized. This result would be expected for PMA/Ionomycin stimulation which as a known activator of both T-cells and Monocytes, the primary constituents of PBMC isolations. In fact, in looking at the activated portion of the Figure 2c scatter plot, the clear emergence of two fluorescence clusters is visible with the dimmer/smaller cluster ostensibly corresponding to the T-cell stain and the brighter/larger fluorescence cluster corresponding to the monocytes.

One of the distinguishing features of the Moxi GO is to affordably empower non-flow users the ability to rapidly screen samples right in their lab. This feature particularly suits the instrument and allows: design of experiment (DOE) studies, time-course studies, and/or dose-response studies that might be challenging and/or costly to accomplish otherwise. To highlight this capability, we applied a PMA/Ionomycin cocktail dose titration to a peripheral whole blood sample, followed by fixation, permeabilization and staining of intracellular phosphorylated Erk. Figure 3a shows the FlowJo-analyzed (off-unit) results of this analysis. As the Moxi GO generates Flow Cytometry Standard (FCS) 3.1 files, they can be readily loaded into FlowJo (or other FCS 3.1 compatible analysis software) for more detailed analysis. The FlowJo data in Figure 3a presents fluorescence (PE anti-phospho ERK 1/2) histograms visually demonstrating the effect of increasing concentrations of PMA/Ionomycin treatment on treated cells (blue) vs. untreated cells (red, *Note: the isotype control fluorescence histogram is shown in orange*). Plotting the mean fluorescence intensity (MFI, mv) values of the activated cell regions vs. the amount of stock PMA/Ionomycin activation cocktail used per ml of cells (Figure 3b) provides a clear representation of the drug dose-response and the corresponding ability to curve-fit the data for extraction of critical dosing parameters. As this data highlights, the Moxi GO can provide rapid and powerful quantification of intracellular targets in response to external stimuli (e.g. PMA/ionomycin activation cocktail).

Summary

In this application note, we provide a protocol and data showing how the Moxi GO can easily be implemented towards the measurement of several intracellular targets (NF-M, NF-H, and phosphor-ERK 1/2). The Moxi GO (Figure 1) is a flow cytometer with either a green (532nm) or blue (488nm) lasers coupled with 561nm/LP-filtered PMT detection (user-swappable to 525/45nm with the 488nm system). The fluorescence configuration is ideal for many of the most common fluorophores including PE for immunolabeling, PI for viability, FITC for immunolabeling (488nm system only), and RFP (tdTomato/dsRed) for transfection efficiency or GFP (488nm system only} for transfection efficiency. 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 without the need for system warm-up, maintenance, or shutdown procedures, the Moxi GO is ideally suited for the implementation of immunophenotyping, intracellular protein quantification, and dose-response studies such as with the neurofilament and ERK 1/2 activation data described here. In addition, the Moxi GO'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 over (potentially) long periods of time. Finally, the Moxi GO touchscreen GUI is designed to make even the most 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 intracellular immunolabeling measurements or other cell-based flow cytometry techniques.

References

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Methods

PBMC Isolation From Peripheral Blood

Human peripheral blood samples were collected into sodium citrate or sodium heparin coated vacutainers. The blood was then processed with Ficoll-Paque Premium (GE Healthcare, cat #17-5442-02), within 3 hours of extraction, following the manufacturer's protocol. Briefly, 2ml blood was diluted 1:1 with HBSS (Sigma, cat #H6648) and carefully layered over 3ml of Ficoll medium in 15ml centrifuge tubes. Multiple tubes were prepared and centrifuged at 400xg (40min, 18°C). The PBMC layer was extracted with a pasteur pipette and washed (100g, 10 min 18°C) twice before final re-suspension in staining buffer (BioLegend cat #420201).

PBMC Isolation From Peripheral Blood

Human peripheral blood samples were collected into sodium heparin coated vacutainers. Samples were processed within 3 hours of the initial blood draw.

Mouse Neural Progenitor Cells (mNPCs)

mNPCs, differentiated from mouse embryonic stem(mES) cells, were kindly provided by Predictive Bio (Carlsbad, CA). Cells were fixed following the Orflo Ethanol Fixation Procedure outlined below. Cells were then labeled following the Orflo Intracellular Immunolabeling Protocol (below) following the secondary labeling protocol. Primary antibodies were kindly provided by Biosensis (Temecula, CA): anti-mouse Neurofilament Heavy (NAP4, Biosensis cat #M-1387-50) and anti-mouse Neurofilament Medium (3H11, Biosensis cat #M-1394-100). A phycoerythrin (PE) anti-goat IgG secondary (BioLegend cat #405307) was applied to generate the fluorescent signal.

Leukocyte PMA/Ionomycin Activation

Seven (7) 2x stock activation cocktail titration levels were generated starting with a 3.24μM PMA/53μM Ionomycin stock (16μL 500x BioLegend activation cocktail (Biolegend cat #423301) into 184μL PBS). The six additional 2x stock serial dilution levels were generated by mixing 100μL of the prior level into 100μL of PBS. For each dilution level, 100μL of the activation cocktail stock was then mixed (vortexed) with 100μL of cells for final PMA/Ionomycin concentrations as shown in the table below:

Dilution Level	PMA (µM)	Iono (µM)	500x activator stock (µL)/1 ml cells
1	1.62	26.50	40
2	0.81	13.25	20
4	0.41	6.63	10
8	0.20	3.31	5
16	0.10	1.66	2.5
32	0.05	0.83	1.25
64	0.03	0.41	0.625

An extra stimulated and non-stimulated sample were also performed for use in isotype control stainings. Cells were stimulated with the activation cocktail for 15min (37°C).

Phospho-Erk1/2 staining

100µL of anti-coagulant treated peripheral blood or Ficoll-Paque purified PBMCs (1e6 – 10e6 cells/ml) were added to microcentifuge tubes (post activation treatment). One test volume (e.g. 5µL) FC receptor block (BioLegend cat #422301) was added to the cells and incubated for 5 minutes at 37°C. 2mL of warm 1X RBC Lyse/Fix buffer (Biolegend cat #422401) was added to cells and cells were gently (3- 4 setting) vortexed. Cells were incubated for 15minutes in the dark at 37° and subsequently pelleted (350xg, 5 minutes) at room temperature. Supernatant was removed and cells were washed cells 2X (350xg, 5min) with Cell Staining Buffer(Biolegend cat #420201). Cells were resuspended in residual volume and 1mL of cold True-Phos Perm Buffer was added to the cells while vortexing. Cells were incubated for 1 hour at -20°C. Following incubation, cells were washed 2X with Cell Staining Buffer by centrifuging at 1000xg for 5 minutes at room temperature. Cells were then labeled following the Orflo Intracellular Immunolabeling Protocol (below) using an anti-human p-Erk-PE monoclonal Antibody (Biolegend cat #369505)

Flow Cytometry Analysis

Flow cytometry data was acquired on the Moxi GO 532nm system (Orflo cat #MXG001) using the "Open Flow Cytometry" application, MFS cassettes (Orflo cat# MXC020), and the default 561nm/LP PMT filter. Scatter plots and histogram overlays were generated on-unit using the built-in "Compare/Overlay" functionality.

Offline Data Analysis

Flowjo 10.2 (TreeStar) on Mac OSX 10.11 was used in the analysis of the PMA/Ionomycin dose-response of fixed/lysed peripheral blood. IGOR 6.37 (Wavemetrics) was used in the display and curve-fitting of the dose-response (MFI vs. dose level) data/curve.

Orflo Ethanol Fixation Protocol

Reagents:

- 70% Ethanol (e.g. Sigma E7023, in PBS or distilled water) - Store at -20°C
- Ice cold Ca2+-free and Mg2+-free PBS (e.g. Phosphate Buffered Saline (PBS, e.g. Life Tech #10010-23)

Protocol:

Fixation:

1. Pellet cells (300xg, 5min) and remove supernatant (Note: if you are detaching adherent cells, pipette triturate in detachment media (e.g. Accutase) to break up clusters. (Note: Put pipettor on a lower setting to avoid excess trauma to cells)
2. Wash the cells twice in PBS (300xg, 5min, 4°C). Count the cells when finished.
    a. *Note 1:* Recommend a total of 5e5-2.5e6 cells so that the re-suspension is 1-5e6/ml (when adding 0.5ml final re-suspension later).
    b. *Note 2:* Put PBS on ice during centrifugations to keep cold.
    c. *Note 3:* Pipette triturate 10-20x when re-suspending pellet to ensure single cell suspension. Preferred approach is add ~1mL PBS to pellet and use a 1mL pipette to triturate. Then add remaining 4ml PBS for wash. Invert 3x.
3. Re-suspend the pellet in approximately 500 ul of ice-cold PBS. Pipet with 1000µL pipette, up and down, 20 times. It is important that this be a good single-cell suspension at this point, or the cells will be fixed as clumps.
4. Aliquot 4.5mL of ice cold 70% ethanol to a 15mL centrifuge tube.
5. Hold ethanol tube and cell tube in a cold pack and vortex gently.
6. Add .5ml cells drop-wise to the 70% ethanol tube (while vortexing) using a 100uL pipette (max size to ensure drop volume is small).
7. Place in Freezer for 1hr to 4days

Post-Fixation Recovery/Use:

1. Centrifuge fixed cells at 800xg, 10°C, 5min with BRAKE OFF. Remove the ethanol.
2. Re-suspend in 1ml ice-cold PBS with 20x pipette trituration (1000uL pipette)
3. Add 4mL ice-cold PBS and invert 3x.
4. Centrifuge at 700xg, 10°C, 5min with BRAKE OFF. Remove the supernatant.
5. Re-suspend in 1ml ice-cold PBS with 20x pipette trituration (1000uL pipette)
6. Add 4mL ice-cold PBS and invert 3x
7. Centrifuge at 600xg, 10°C, 5min with BRAKE OFF. Remove the supernatant.
8. Re-suspend the pelleted cells in in desired solution for staining

Orflo Intracellular Immunolabeling Protocol

Reagents/Components:

- Either:
  - Orflo Moxi GO 532nm Next Generation Flow Cytometer (Orflo Cat #MXG001)
  - 561nm/LP Filter (Orflo Cat #MOG002)

  OR

  - Orflo Moxi GO 488nm Next Generation Flow Cytometer (Orflo Cat #MXG002)
  - 525/45nm Filter (Orflo Cat #MOG001)
  - 561nm/LP Filter (Orflo Cat #MOG002)
- MF-S+ Cassettes (Orflo Cat #MXC030)
- Fixation/ Permeabilization Buffer (discussed below)
- Cell Staining Buffer, E.g.
  - BioLegend Cat #420201

  OR

  - PBS + 0.5% BSA + 0.1% Azide
- Compatible fluorophore conjugated antibody
  - ***561nm/LP Filter (538nm or 488nm GO systems)*** **-** Phycoerythrin (PE/R-PE) labeled antibody (e.g. PE anti-human CD4 - BioLegend cat # 300507) – *Note: Substitute Antibodies that are compatible with 532nm excitation and 561nm/LP emission can also be used instead of PE.*
  - ***525/45nm Filter (488nm GO ONLY)*** – FITC or Alexa Fluor 488 labeled antibody
- *Optional/Recommended:* Orflo Flow Reagent (Orflo Cat #MXA080)

Fix and Permeabilize Cells

Fixation and permeabilization of cells is critical for intracellular labeling of proteins. Depending upon the fixation method, an additional permeabilization step may be required. Some options/approaches are discussed below.

1. *Alcohol (Ethanol/Methanol) Based Fixation* – With alcohol fixation, no additional permeabilization step is needed. An example protocol for ethanol fixation is provided with "Orflo Ethanol Fixation Protocol.pdf"
2. *Formalin or Paraformaldehyde (PFA, 3-4%) fixation* (min 10min) followed by permeabilization. Permeabilization can be accomplished through:
   a. Treatment with 0.5 – 1% Triton X-100 (in PBS) for 15 min
      OR
   b. 0.5% Digitonin or Saponin (in PBS) for 15min
3. *Use a commercial "Fix and Permeabilize" kit.* - These are widely available (BioLegend, Life Tech, eBioscience, BD, etc). Most of these are based on the above PFA and Triton X-100 fixation approach.

Notes:

- For "whole" blood samples, it is necessary to lyse the red blood cells in the sample. Several vendors offer combined "Lyse/Fix" buffers for this purpose (e.g. Biolegend cat #422401)
- For phospho-protein labeling, stronger permeabilization buffers are typically required. Commercially available buffers are offered for this purpose (e.g. BioLegend True-Phos Perm, cat #42540)

- FC receptor block steps (add 1 test volume FC Block, e.g. BioLegend cat #422301, vortex and incubate 5min) must be performed BEFORE fixation and permeabilization.

Staining Protocol

1. Harvest and wash the fixed cells (note: for ethanol fixed cells refer to the "Post-Fixation Recovery/Use" section of the "Orflo Ethanol Fixation Protocol.pdf" for maximum cell yield). Re-suspend into the cell staining buffer at a density of 2-5 x 10^6 cells/ml in cell staining buffer.
2. Aliquot 100 µl of cells (2-5 x 10^5 total cells) into a 1.5ml microfuge tube. Optional: Setup additional vials for isotype controls and untreated/negative control samples as necessary.

Primary/Direct Antibody Binding/Staining:

3. Quick spin (e.g. 500 x g, 5 seconds) antibody vials for maximum volume.
4. Add 1 test volume (typically 5µl) of each antibody label to 100µl cell suspension.
5. Vortex gently.
6. Incubate for 20 min at 4°C, protect from light.
7. Wash 2X with 1.5ml of Cell Staining Buffer by centrifugation at 1000 x *g* for 5 minutes at ≤18°C.
8. FOR DIRECTLY CONJUGATED ANTIBODIES:
   Re-suspend pellet in 1ml of cell staining buffer (ideal target concentration of 1e5 – 5e5 cells/ml) and add 20µl of Moxi Cyte Flow Reagent. Invert 10x to mix. Analyze with on the Moxi GO or Moxi Flow using the "*Open Flow Cytometry*" apps. For the Moxi GO 488nm system, verify the proper filter is inserted (i.e. 525/45nm for FITC, 561nm/LP for PE)

   FOR PURIFIED ANTIBODIES:
   Re-suspend pellet in residual buffer (typically 40-50µl) and add Cell Staining Buffer to 100µl total volume. Proceed to Secondary Antibody Staining.

Secondary/Indirect Antibody Staining

9. Add 1 test volume (typically 2-5 µl) of 2° Ab (conc. 0.2µg/ml) to 100 µl cells (≤0.5µg per million cells in 100 µl).
10. Incubate for 20-30 minutes at 4°C, protect from light.
11. Wash 2x with 1.5ml of Cell Staining Buffer.
12. Re-suspend pellet in 1 ml of Cell Staining Buffer (ideal target concentration of 1e5 – 5e5 cells/ml).
13. Add 20µl of Moxi Cyte Flow Reagent. Invert 10x to mix.
    Analyze with the Moxi GO or Moxi Flow using the "*Open Flow Cytometry*" app. For the Moxi GO 488nm system, verify the proper filter is inserted (i.e. 525/45nm for FITC, 561nm/LP for PE)