Immunophenotyping (CD marker labeling) PBMC's with Orflo's Moxi GO Systems – Next Generation Flow Cytometers

Introduction/Background

Peripheral Blood Mononuclear Cell (PBMC) purifications are a critically important cell preparation in a broad range of research and clinical studies including such profound applications as HIV research,[1] cancer immunotherapy,[2] cord blood banking,[3] regenerative medicine,[4] and fundamental studies of cytokine-based immune responses[5].

PBMCs are commonly extracted from peripheral blood, bone marrow and umbilical cord samples through density gradient centrifugation or magnetic bead separations. The approaches are designed to purify the leukocytes (PBMCs) of interest from other cellular components, particularly red blood cells (RBCs). Characterizing the viability and basic composition of the final cell isolates is critical due to the variables associated with:

- The efficiency of the sample extraction, handling, and purification process.
- The diversity of the source samples (i.e. peripheral blood vs. cord blood vs. bone marrow).
- Potential cell trauma/necrosis due to the common need to cryopreserve or "biobank" samples .

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.

Beyond the initial health of the purified samples, researchers subsequently focus on immunophenotyping of the resulting samples to both identify sub-populations of PBMCs for quantification as well as for monitoring the varying expression levels of the characteristic "cluster of differentiation" (CD) markers that uniquely characterize these sub-populations. Specifically, fluorophore-conjugated antibodies are used to target the various CD receptor/markers and flow cytometry is commonly applied to the quantification of the marker expression levels and to the differentiation of sub-populations. Beyond CD marker expression, precise monitoring of counts and cell sizes can be essential to the analysis. One such example is the critical importance of cell size to treatment efficacy in the extraordinarily promising research arena of chimeric antigen receptor T (CAR-T) cell cancer immunotherapy.[6] Sizing, in particular, is an area in which conventional flow cytometry systems have traditional struggled, relying on light-scattering as opposed to more precise sizing techniques, such as Coulter Principle sizing.

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 characterization of PBMC 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 PMBC immunophenotyping, counts, purity checks, viability, and sizing. 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.

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 for PBMC analysis.

Here we provide example data and protocol(s) for the application of the Moxi GO 532 to the characterization of PBMC samples. A unique feature of Moxi GO is that the system allows PBMC analysis, to both flow experts and novices, right in their culture hood or at their lab benchtop. In this regard, the ease-of-use and ready availability of the system will greatly enable researchers' ability to rapidly characterize and study PBMCs. This, in addition to the affordability of the system, will undoubtedly establish the Moxi GO systems as required tools for any lab that is involved in studies that utilize PBMCs.

Example Data – Results and Discussion

In this application note, we applied Orflo's Moxi GO to the study of Ficoll-Paque™ purified PBMCs from human peripheral blood. The system was applied to generating example characterizations of the PBMC sample by demonstrating system counts and sizing, PBMC purity analysis, and basic phenotyping by using phycoerythrin (PE)-conjugated antibody targets to target an array of CD markers. Figure 2 shows a typical PMBC test output on the Moxi GO. After a brief <10 second test, an initial system output of a scatter plot (Figure 2a) of the 561nm/LP fluorescence vs. Coulter-Based sizing is displayed. From this screen, the user can quickly size gate (blue marker/dots, Figure 2a) on sub-populations. The example shows the (predominantly) lymphocyte population in the size gated region (Figure 2a, between blue marker lines) and a fluorescence (Figure 2a, red) gate positioned to distinguish the CD4+ ("upper") vs. CD4- ("lower") lymphocytes. The black boxes underneath the scatter plot provide the precise, Coulter- Principle-based, concentrations and sizes of the lymphocyte sub-populations as well as the associated median fluorescence intensity (MFI) of each population. To the right of the size-gated region, the (predominantly) monocytes population can be uniquely size-identified from the lymphocyte population. As these are not included in the size-gated region, the monocyte statistics are not included in the black boxes below (Figure 2a and Figure 2b only). Figure 2b shows the fluorescence (PE) histogram for the lymphocyte-gated region, clearly showing the fluorescence separation in the CD4+ vs. CD4- populations as boxes below (Figure 2a and Figure 2b only). Figure 2b shows the fluorescence (PE) histogram for the lymphocyte-gated region, clearly showing the fluorescence separation in the CD4+ vs. CD4- populations as well as the relevant populations statistics. Using the touch-screen, the analyzed regions can be quickly adjusted by touching and dragging the gate markers (similar to a smart phone interface). In Figure 2c, the right size marker was moved to the

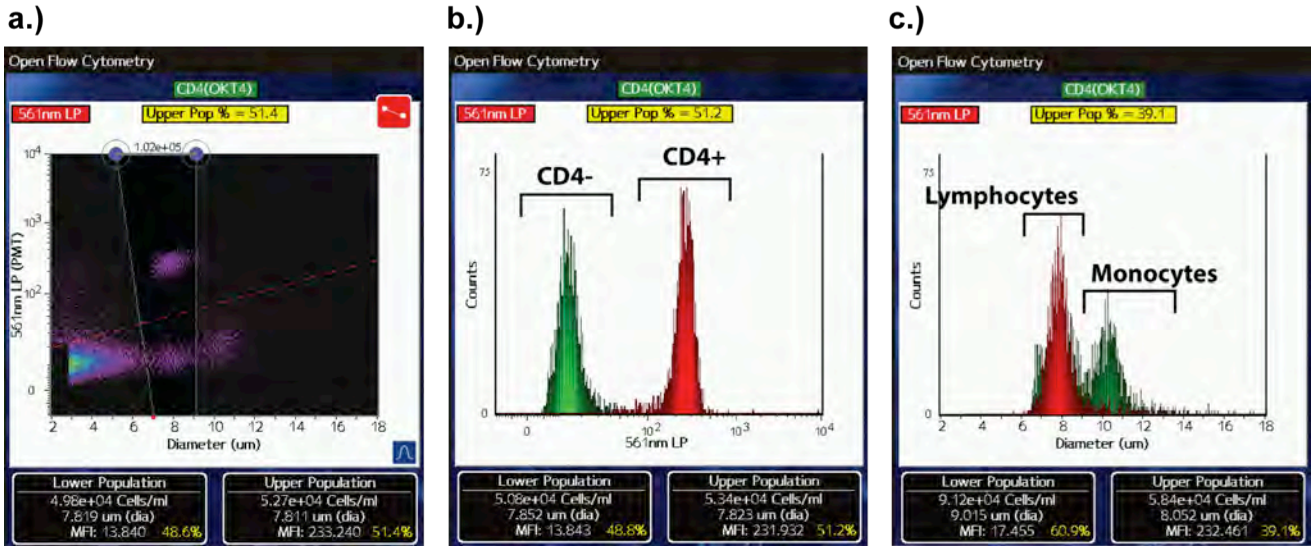

Figure 2 – User-generated screenshots (on-unit) showing the standard Moxi GO output for purified PBMCs. ***a.)*** Scatter plot of fluorescence (example shows PE-CD4) vs. size. User can gate by size (blue markers) to define region for statistical analysis (concentration, size, MFI), fluorescence (red line) for fluorescence threshold, and noise (not shown) to reduce file sizes ***b.)*** Fluorescence histogram view shows the PE fluorescence for easy visualization of + vs. – populations (PE-CD4 example shown, gated on lymphocytes). ***c.)*** Size histogram view highlights the precise Coulter Principle sizing of lymphocytes (left peak) and monocytes (right peak).

far right of the size range and the display was changed to show the size histogram for that region. With that view, the fine size-discrimination of the impedance-based detection approach is clearly highlighted in the resolution of the (labeled) lymphocyte vs. monocyte populations.

Beyond concentration and cell sizing, Figure 3 shows some of the key parameters that the Moxi GO can measure in order to determine the overall purity and characteristics of the PMBC isolation. First, the cells were stained with propidium iodide (PI, first image) to assess the cell viability following the procedure. As a cell impermeant dye that increases fluorescence up to 50x upon binding to DNA, PI is widely used in flow cytometry to determine cell viability. In this case, the 97% viability is validation that the cells tolerated the Ficoll separation procedure well. Additionally, as indicated by the yellow arrow, (Figure 3, first image), the degree of RBC contamination can be qualitatively evaluated. The PE-CD45 label (Figure 3, second image) provides a way to label all leukocytes in the sample to verify that the distinct cell populations are, in fact, leukocytes as intended. In the third image (Figure 3), a granulocyte-specific label (PE-CD66) was applied to verify that the level of granulocyte contamination is low as would expected for a properly performed isolation. Finally, a CD41/61 label (Figure 3, fourth image) was applied to identify the level of platelet contamination (yellow rectangle) as well as the activated platelet binding to the monocyte population (yellow circle). Using stains such as those shown in Figure 2 and 3, the quality of PBMC isolation can be easily and quickly evaluated. Results can then be specifically applied in feedback to aspects of the isolation protocol to optimize the overall purification protocol/process.

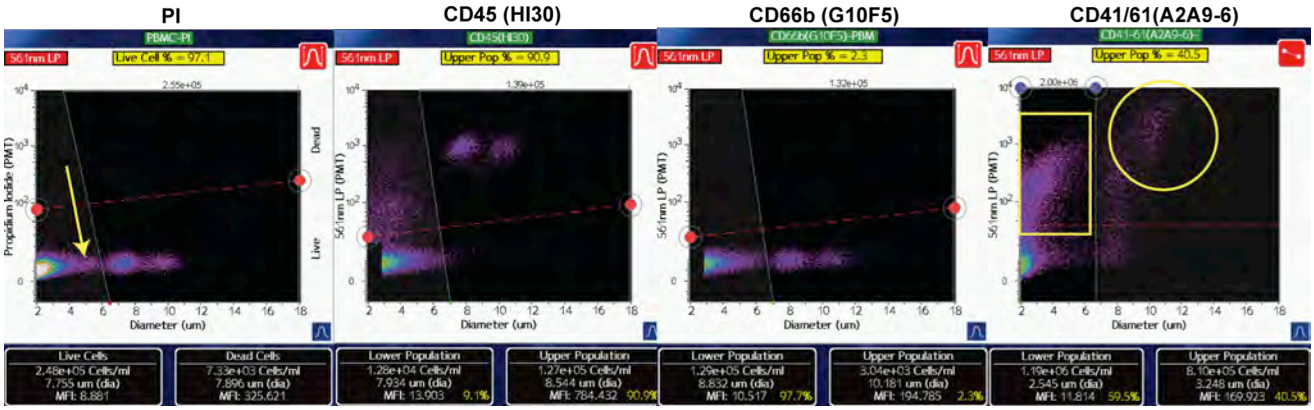

Figure 3 – Basic PBMC purity and characteristic checks. ***(PI)*** Viability stain for PBMC health shows cells tolerated blood draw and Ficoll procedure well (>97% viable). Yellow arrow points to residual RBC population. ***(CD45)*** Leukocyte (WBC) generic label – confirms the gating of predominantly leukocytes (>90%). Note: Lower left cluster "tail" would be residual RBCs, platelet multiplets, and other debris. ***(CD66b)*** Granulocyte label shows very little (~2%) granulocyte contamination. **(CD41/61)** Platelet-specific label. Increased scatter above noise cluster (yellow box) reflects small volume platelet labeling. Fluorescent leukocytes (>6µm, yellow circle) reflect binding of platelets to those cells, with a particular affinity to monocytes.

Figure 4 shows several example results for the application of the Moxi GO to phenotyping of the PBMC sample through PE conjugated antibody CD-marker staining. For each CD marker label, the Moxi GO scatter plot output is presented as the top image. A fluorescence histogram overlay of the CD-marker output vs. the output of the associated isotype control sample is shown below the scatter plot image. All the images were easily generated on unit by the user. The overlay histogram, in particular, highlights the Moxi GO's ability to easily and powerfully provide direct data comparisons on-unit. The examples in Figure 4 show identification of the following sub-populations: All Lymphocytes (CD2), T Lymphocytes (CD3), CD4+ T Cells (CD4, size gated), CD8+ T-Cells (CD8, size-gated), monocytes (CD11b), and B-Cells (CD19).

Immunophenotyping (CD marker labeling) PBMC's with Orflo's Moxi GO Systems – Next Generation Flow Cytometers

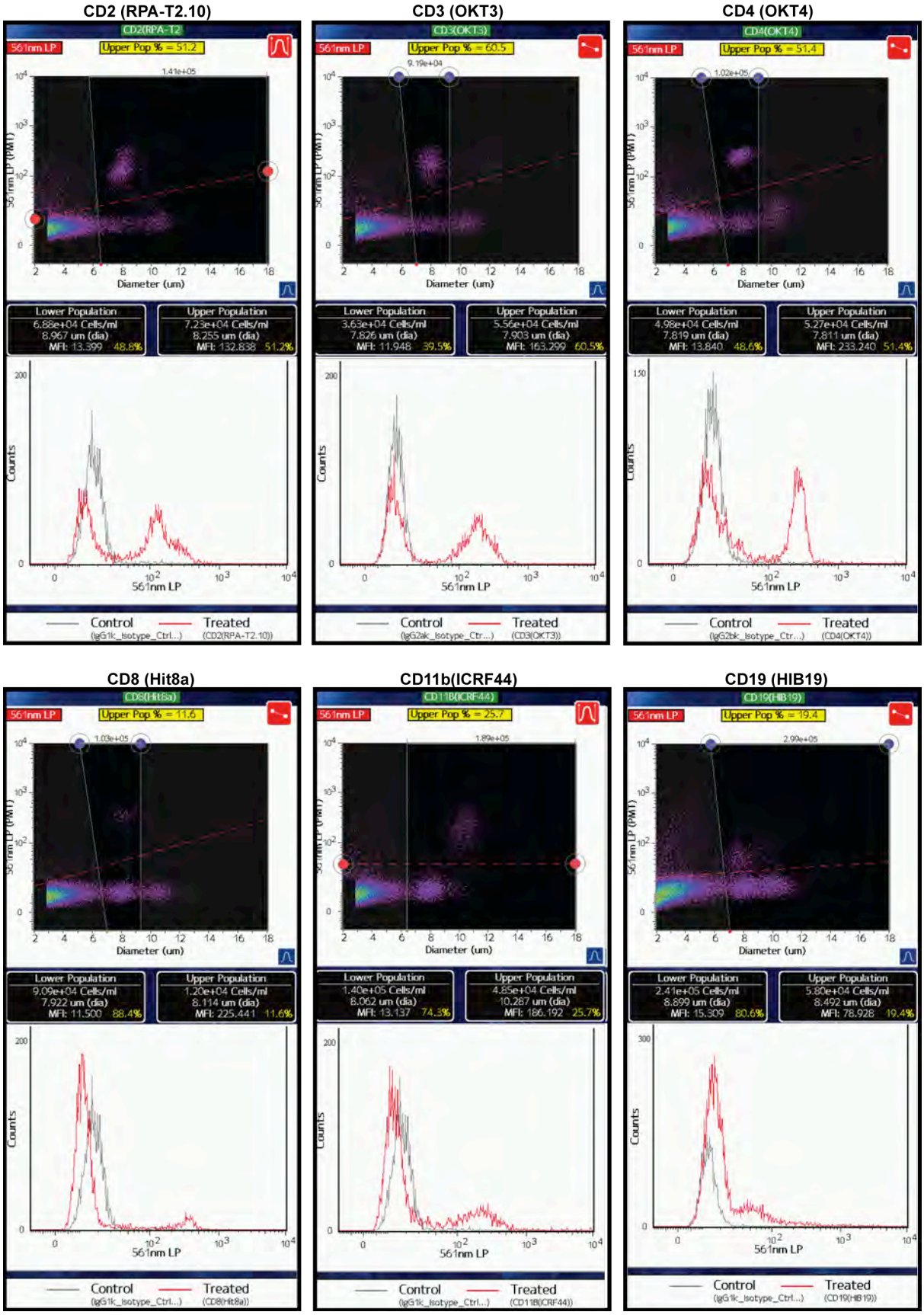

Figure 4– Examples of CD marker labeling of PBMC's. Labeled to identify CD Receptor (clone). For each marker, a scatter plot of the fluorescence output vs. Coulter-based sizing is shown (top) and an on-unit histogram overlay of the marker output (red) vs. the isotype control output (gray) is shown (below).

laser

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Summary

In this application note, we provide a protocol and data showing how the Moxi GO can easily be applied to the characterization of PBMC samples. 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, it is uniquely suited for quick immunophenotyping of samples including design of experiment (DOE) and kinetic-analysis studies. In addition, 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. For PBMC analysis, this easy access allows for great power and flexibility in performing purity checks and CD marker phenotyping. 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 systesm as a staple in any lab performing PMBC studies 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, 17-5442-02) following the manufacturer's protocol. Briefly, 2ml blood was diluted 1:1 with HBSS (Sigma, 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 #420201).

PBMC Viability Stain

PBMC's were stained with Orflo's Moxi Cyte Viability Reagent (Orflo #MXA055) by adding 15µL of the PBMC final suspension to 135µL of viability reagent and incubating the mixture (5min/room temperature/dark). Following incubation, 75µl of the cells were run on the Moxi GO 532 (Orflo #ZF002) using the "Viability Assay."

CD marker Labeling Protocol

All cells were labeled following the "Moxi GO – Cell Surface Immunolabeling Protocol" detailed below. PE-conjugated antibodies used in this application note were sourced as follows:

Target (Clone)	Manufacturer	Cat. #
CD2 (RPA-T2.10)	Tonbo BioSciences	50-0029-T025
CD3 (OKT3)	BioLegend	317308
CD4 (OKT4)	BioLegend	317409
CD8 (Hit8A)	BioLegend	300907
CD11b (ICRF44)	Tonbo BioSciences	50-0118-ST05
CD19(HIB19)	Tonbo BioSciences	50-0199-ST05
CD41/CD61(A2A9/6)	BioLegend	359805
CD66b(G10F5)	BioLegend	305105

PE-conjugated isotype controls were sourced as follows:

Isotype (Clone)	Manufacturer	Cat. #
IgG1, κ (MOPC-21)	Biolegend	400111
IgG2a, κ (MOPC-173)	BioLegend	400213
IgG2b, κ (MPC-11)	BioLegend	400311

Data Analysis and Output

All images for the application note were generated directly from the Moxi GO unit using the "File | Print | Export Screenshot" functionality available for each test. Histogram Overlays (Figure 4) were generated using the Moxi Go's on-unit "Test Compare/Overlay" functionality. Off-unit analysis (not used directly in the application note) was performed by exporting the Moxi GO FCS 3.1 test files into FlowJo X (Mac OSX).

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Reagents:/Components:

- Either:
  - Orflo Moxi GO 532nm Next Generation Flow Cytometer (Orflo Cat #ZF002)
  - Phycoerythrin (PE/R-PE) labeled antibody (e.g. PE anti-human Cell Staining Buffer (BioLegend cat # 420201 or PBS with 0.5% BSA, 0.1% Azide)

  OR

  - Orflo Moxi GO II 488nm Next Generation Flow Cytometer (Orflo Cat #MXG002)
    - Compatible fluorophore conjugated antibody
      - ***525/45nm Filter (PMT1)*** – FITC or Alexa Fluor 488 labeled antibody
      - ***561nm/LP Filter (PMT2)*** - Phycoerythrin (PE/R-PE) labeled antibody (e.g. PE anti-human Cell Staining Buffer (BioLegend cat # 420201 or PBS with 0.5% BSA, 0.1% Azide)
- *Optional*) Moxi Cyte Flow Reagent (Orflo MXA079/MXA080)
- (*Optional*) Human TruStain FcX - Fc Receptor Blocking Solution (BioLegend cat # 422301)
- *(Optional)* Accutase (Orflo MXA020) or Accumax (Orflo MXA021) dissociation reagents

Cell Isolation/Preparation

Prepare cells into a single-cell suspension free of large (>30um) particulate. E.g. following Ficoll-Paque PBMC isolation protocol https://www.gelifesciences.com/gehcls_images/GELS/Related Content/Files/1314729545976/litdoc71716700_20161013221551.pdf).

Notes:

- Primary harvests (e.g. blood or tissue extractions):
  - If large extracellular debris is present, pass sample through a 40um cell strainer (e.g. BelArt FlowMI 40µm tip strainer) to remove it.
  - If large amounts of RBCs are present in the sample, an appropriate RBC lyse method must be applied to get proper counts (RBCs will mask the signal of the cell population of interest due to their extremely high concentration levels).
- Cell aggregation/Clustering – Clusters and aggregates can be dissociated through a combination of:
  - Protease treatment – suspend cells in an appropriate dissociation reagent (e.g. Accumax or Accutase) for a minimum of 5 minutes.
  - Pipette trituration (repeatedly, e.g. 10x, pull a substantial fraction of the cell sample in and out of a pipette tip). If a dissociation treatment was used, perform this step while cells are still suspended in the dissociation reagent.

Primary Antibody Staining Protocol

1. Suspend cells at density of 2-5 x 10^6 cells/ml in cell staining. (Note: For PBMC Samples, this would be following the last wash of the PBMC's, re-suspend the pellet directly into the staining buffer). Combine pellets if necessary. Verify counts using the Moxi GO instrument.
2. Aliquot 100 µl of cells (2-5 x 10^5 total cells) per 1.5ml microfuge tube for each sample type. Note prepare as many samples as would be required for proper compensation (e.g. 4 samples: Dual-label, antibody 1 positive control, antibody 2 positive control, negative control/unlabeled)

(Optional) Block Fc-Receptors *(recommended for reducing nonspecific staining of antibodies)*

i. *Add 1 test volume (typically 5 µl) of Fc Receptor Blocking Solution (BioLegend cat#422301) to each 100 µl vial of cells.*
ii. *Incubate for 10 minutes at room temperature.*

3. Quick spin (e.g. 500 x g, 30seconds) antibody vials for maximum volume.
4. Add appropriate antibody to each vial.
5. Vortex each vial gently.
6. Incubate for 15-20 min at 4°C, protect from light.
7. Wash 2X with 2ml of Cell Staining Buffer by centrifugation at 350 x *g* for 5 minutes at ≤18°C.
8. FOR DIRECTLY CONJUGATED ANTIBODIES:
   Re-suspend pellet in 1ml of cell staining buffer (ideally target 2e5 – 5e5 cells/ml)
9. *(Optional) 20µl of Moxi Cyte Flow Reagent.*
10. Invert 10x to mix and analyze with Moxi GO II using the "*Open Flow Cytometry*" or "GOFlow" apps.

    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 Antibody Staining

11. 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).
12. Incubate for 15-20 minutes at 4°C, protect from light.
13. Wash 2x with 2ml of Cell Staining Buffer.
14. Re-suspend pellet in 1 ml of Cell Staining Buffer (ideally target 2e5 – 5e5 cells/ml).
15. *(Optional) 20µl of Moxi Cyte Flow Reagent*.
16. Invert 10x to mix and analyze with Moxi GO using the "*Open Flow Cytometry*" or "*GOFlow*" (Moxi GO II only) apps.