Reagents:

- Cell Staining Buffer (BioLegend cat # 420201 or PBS with 0.5% BSA, 0.1% Azide)
- 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 CD4 - BioLegend cat # 300507)
- *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 II 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 II using the "*Open Flow Cytometry*" or "GOFlow" apps.