Intracellular – Immunolabeling Protocol

Reagents/Components:

- Moxi GO II system (Orflo Cat #MXG102)
- 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
  - ***525/45nm Filter (PMT1)*** – FITC, BB515, 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) – *Note: Substitute Antibodies that are compatible with 488nm excitation and 561nm/LP emission can also be used instead of PE.*
- *Optional:* 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 II using the "*GO Flow*" app.

   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 II using the "*GO Flow*" app.