

## 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  $2-5 \times 10^6$  cells/ml in cell staining buffer.
2. Aliquot 100  $\mu$ l of cells ( $2-5 \times 10^5$  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 5 $\mu$ l) of each antibody label to 100 $\mu$ l 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  $\leq 18^\circ\text{C}$ .
8. FOR DIRECTLY CONJUGATED ANTIBODIES:  
Re-suspend pellet in 1ml of cell staining buffer (ideal target concentration of  $1e5 - 5e5$  cells/ml) and add 20 $\mu$ l 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 40-50 $\mu$ l) and add Cell Staining Buffer to 100 $\mu$ l total volume. Proceed to Secondary Antibody Staining.

**Secondary/Indirect Antibody Staining**

9. Add 1 test volume (typically 2-5  $\mu$ l) of 2° Ab (conc. 0.2 $\mu$ g/ml) to 100  $\mu$ l cells ( $\leq 0.5\mu$ g per million cells in 100  $\mu$ l).
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 20 $\mu$ l of Moxi Cyte Flow Reagent. Invert 10x to mix. Analyze with the Moxi GO II using the “GO Flow” app.