Moxi GO II - Whole Blood – Intracellular Staining Protocol

Scope:
This procedure applies to the intracellular antibody labeling of activated white blood cells (WBC's) isolated from a peripheral whole blood sample by 1.) Either performing a buffy coat isolation by gradient or RBC Lysis/Fixation 2.) Stimulating isolated WBC's with an appropriate cellular activation reagent. 3.) Fixing, permeabilizing, and Intracellularly staining cells of interest.

Overview:
As RBC's outnumber the WBC's in a whole blood sample by ~1000x, it is necessary to eliminate the RBC interference prior to running on the Orflo Flow Cytometers. In our experience, RBC lysis buffers vary considerably in their efficacy at removing RBC 's. While the BioLegend RBC Lyse Buffer (used in this protocol) is better than most, we have also found that starting with a buffy coat isolation dramatically reduces the starting RBC concentration, improving the overall results.

Reagents:
- Purified Water (Milli Q or Distilled)
- RBC Lysis/Fixation Solution 10X (Biolegend cat#422401)
- Cell Staining Buffer (BioLegend cat # 420201)
- Intracellular antibody of interest (ex: anti-phospho-Erk ½ -PE)
- Cellular Activation Cocktail (Biolegend cat#423301)
- True-Phos Perm Buffer (Biolegend cat#425401)
- Ficoll Paque Plus (GE cat# 17-1440-02)

1. **Buffy Coat Isolation (option #1):**
   1.1. Obtain a fresh Whole Blood (WB) sample into an anti-coagulant (e.g. Sodium Citrate)-treated tube. Mix 2 mL of WB with 2mL of balanced salt solution (1x PBS). Gently later diluted blood mixture onto of 3mL of Ficoll Paque Plus in a 15mL conical tube.
   1.2. Spin the whole blood at 400 x g for 30 minutes at 18°C with the BRAKE OFF.
   1.3. Remove and discard the majority (all but 2-3mm) of the top (amber/translucent) plasma layer, taking care not to disturb the buffy coat layer (at the interface of the red, RBC layer).
   1.4. Use a Pasteur pipette to extract the leukocyte band (the buffy coat). Notes:
      - The buffy coat is the (cloudy/white) interface layer between the RBC's (red) and the plasma (amber/translucent).
      - To ensure minimal loss of WBC's, it is necessary to include the residual plasma as well as a small portion of the RBC layer in the extracted buffy coat sample.
   1.5. Aliquot buffy coat into a 15ml centrifuge tube, add at least 3 volumes of balance salt solution to the tube.
   1.6. Centrifuge at 100 x g for 10 minutes at 18°C with the Brake ON. Remove supernatant. Repeat.
   1.7. Dilute cells to a concentration of ~5e6 cells/ml. (e.g. for 3ml starting volume of whole blood, add ~2ml of staining buffer to extracted buffy coat sample).
   1.8. Take 100µL of PBMC's, add 100µL of stimulant (ex: Add 100µL of 200ng/mL of IL6 to get a final concentation of 100ng/mL). If using the cell activation cocktail (PMA/Ionomycin) add 2µL to PBMC's. Incubate at 37°C for 15min. (Optional FCR block at this stage).

1.9. Fix cells by adding 2mL of room temperature, 1x RBC lysis buffer on top of stimulated PBMC's. Incubate at 37°C for 15 minutes.

2. <u>RBC Lyse/Fix (option #2):</u>

2.1. Take 100µL of Whole blood, add 100µL of stimulant to Whole blood. (ex: Add 100µL of 200ng/mL of IL6 to get a final concentation of 100ng/mL). If using the cell activation cocktail (PMA/Ionomycin) add 2µL to Whole blood. Incubate at 37°C for 15min. (Optional FCR block at this stage).

2.2. Add 2ml of the room temperature, 1x RBC lysis/Fixation buffer to 100µL of whole blood. Note: Immediately after adding it to the sample, vortex the sample (e.g. level 3) for 1-2 seconds.

2.3. Fix cells by incubating at 37°C for 15 minutes.

3. <u>Perm and Intracellular Staining:</u>

3.1. Spin the sample at 350 x g for 5 minutes at room temperature.

3.2. Add 1ml Staining Buffer, resuspending cells. Spin at 350 g for 5 minutes at room temperature.

3.3. Repeat 2X. Resuspend cells in residual pellet.

3.4. Add 1mL of Chilled True Phos Perm buffer while lightly vortexing.

3.5. Incubate samples at -20°C for 60 minutes.

3.6. Wash cells 2X with Cell staining buffer (centrifuge cells at 1000 x g for 5 min).

3.7. Resuspend cells in 100µL of cell staining buffer.

3.8. Add intracellular detection antibodies and incubate for 30 minutes at room temperature in the dark.

3.9. Wash cells 2X with Cell staining buffer (centrifuge cells at 1000 x g for 5 min).

3.10. Resuspend cells in 500µL of staining buffer, read samples on the Moxi GO II.