Pullen Lab Protocol for Intracellular Flow Cytometry

This protocol was optimized for the detection of intracellular IL-6 in mouse mast cells (both MC/9 and primary BMMC) from *in vitro* culture, using a PE-conjugated antibody. It is based on the protocol developed by BioLegend.

Different cells, different tissues, different proteins, and different antibodies should be optimized carefully. For example, you will need ACK Lysis Buffer if analyzing PBMCs from blood.

Materials:

- FC buffer (0.1% BSA in 1X PBS, or purchased from BioLegend #420201, note that their formulation includes azide!)
- Monensin
- Fixation buffer (4% paraformaldehyde; or BioLegend #420801)
- Permeabilization Buffer (this is basically FC buffer with 0.1% saponin; BioLegend #421002)
- Antibody with directly conjugated fluorophore.
- 2mL microcentrifuge tubes
- Standard range of micropipettes with tips

Things people mess up that are easily prevented (so pay close attention!):

- Double check your dilutions. For example, the monensin and permeabilization buffers are both concentrated stock solutions.
- Watch your pipette tips – be gentle when aspirating! You should use 2mL tubes (not 1.7mL!) with the conical bottom because the cells will pellet on the hinge side where the straight part of the tube meets the cone. Therefore, you can more easily aspirate liquid from the bottom of the tube. IF YOU DISRUPT THE CELL PELLET, spin again.
- Timing is important. Plan your day.
- Protect samples from light throughout this protocol.
- Don't centrifuge your cells too hard!
- Remember controls! You should *at least* have an "unstained" control. You can just split a portion of one of your samples, treat it the same, except you won't give it antibody for your target (STEP 10). Ideally you will have isotype controls.

Protocol:

1. Activate the cells. This protocol is optimized to IgE-XL mast cells by doing the following:
    a. Incubate mast cells with TNP-specific IgE at 0.5μg/mL overnight. (1:1000 dilution)
    b. Add TNP-HSA to a final concentration of 0.3μg/mL. (1:1000 dilution)
    c. 90-minutes after TNP addition, add monensin to prevent exocytosis. This is a 1:1000 dilution from the BioLegend stock solution.
    d. Incubate 4-6 hours before proceeding with IFC. DO NOT go longer than this, or your cells will die from monensin (it's a poison!). YOU MUST proceed through fixation after this, but ideally you would finish the rest of this protocol.
2. Centrifuge cells for 5min at 300-350g
3. Discard supernatant and then resuspend each condition in 0.5mL of fixation buffer. Transfer these suspensions to 2mL tubes.
    a. Incubate 20min at room temperature in the dark.
4. Centrifuge cells, 5min, 300-350g.
5. Discard supernatant. At this point there are a variety of ways to store your cells for later analysis, but they will not be detailed here, because ideally you should finish the rest of this protocol.
6. Dilute the stock permeabilization buffer from 10X to 1X in diH_2O. (for example, 1mL stock in 9mL water)
7. Resuspend cells in 600μL 1X permeabilization buffer, then immediately centrifuge at 300-350g for 5min.
8. Discard supernatant and **repeat step 7 twice**.
9. Remove and discard 500μL of supernatant from each tube (leaving behind 100μL).
10. To each tube add appropriate volume of conjugated antibody for your target. For example, for PE-conjugated IL-6 (BioLegend # 504503), we use a 1:50 dilution (2μL) per million cells in 100μL permebilization buffer.
11. Mix cell suspensions gently with antibody and incubate at room temperature in the dark for 20-30min.
12. Add 500μL fresh 1X permeabilization buffer and immediately centrifuge for 5min at 300-350g.
13. Discard supernatant and resuspend in 600μL 1X permeabilization buffer.
14. Centrifuge for 5min at 300-350g.
15. Repeat steps 13 and 14 once.
16. After the last permeabilization wash, resuspend cells in at least 500μL of FC buffer.
17. Analyze on a flow cytometer with appropriate fluorescence channel(s).