Pullen Lab Protocol for Cell Surface Target Flow Cytometry (FC) with Primary Antibodies Directly Conjugated to Fluorophores

This protocol is optimized for FC with primary and immortalized cell cultures. FC on samples harvested from tissues will require modification to prepare appropriate single cell suspensions.

This is ***not*** a protocol to prepare cells for FC analysis of intracellular targets.
This is ***not*** a protocol for cell cycle analysis.

Materials:

- Container with ice
- 2mL microcentrifuge tubes
- refrigerated microcentrifuge (set to 4°C)
- micropipettes with tips
- Incubation Buffer (0.5g BSA in 100mL 1X PBS)
- Blocking antibody solution (anti-CD16/32 diluted 1:50 in Incubation Buffer)
- Primary antibodies. if more than one target is being assessed at the same time you must have fluorophores with minimal fluorescence spectral overlap. Most vendors have tools to help you figure this out.
- Isotype control antibodies. These should be the same isotype(s) and fluorophore(s) for each primary antibody, but they should not recognize any target on your cells. Their purpose is to set the background for non-specific antibody binding, for example through Fc receptors due to incomplete blocking. Most vendors will recommend an isotype for each primary antibody you use.

Procedure:

1. For suspension cells: proceed to step 2.
    a. For adherent cells: gently scrape. An inverted 1mL pipette tip is useful for this. **Do NOT use trypsin** or any other form of enzymatic material to get cells in solution… doing the could destroy the epitopes on your target proteins that your antibodies are raised against.
2. Centrifuge cells 500xg for 5min.
3. Discard supernatant and resuspend cells in 800µL Incubation Buffer.
4. Divide cells into separate tubes for each of the following: (1) unstained control; (2) isotype control; (3) primary targets.
5. Repeat steps 2 & 3.
6. Count cells – all antibody concentrations will be optimized for cell number.
7. Centrifuge cells 500xg for 5min.
8. Add appropriate amount of blocking antibody solution. For example, 100µL per million cells.
    a. Some will add 2µL blocking antibody directly to the pellet and then 98µL Incubation Buffer, per million cells, and then mix.
9. Incubate on ice for 10min., then bring to 800µL with Incubation Buffer.
10. Centrifuge 500xg for 5min.
11. Add antibodies to their respective tubes (isotype and primary). Do NOT add anything but Incubation Buffer to your unstained control! NOTE: every primary antibody will be optimized differently, so concentrations will vary; additionally, some commercial formulations are sold as "tests" which are pre-diluted and dispensed as a volume per cell #.
12. Incubate on ice **IN THE DARK** for at least 20min.
13. Bring volume to 800µL with Incubation Buffer, and centrifuge 500xg for 5min.
14. Discard supernatant.
15. Wash cells two more times with incubation buffer (800µL) and centrifugation (500xg for 5min).
16. Resuspend in 500µL-2mL Incubation Buffer (depends on cell # and your needs), keep on ice, protect from light, and run on the Flow Cytometer!

a. Ideally, before the FC you would also add a viability stain, such as propidium iodide, per manufacturer's protocol.