Flow Cytometry for Intracellular Staining (Conjugated Antibodies Only)

This method may be used for both extracellular and intracellular targets. Some targets like FoxP3 or other nuclear factors may require specific staining buffers. Always consult the product manufacturer's recommendations when working with specialized stains.

Adaptations may be necessary for specific protein targets within the cells. Please consult the references given at the end of the document for additional help.

Reagents:

- PBS
- 4% Paraformaldehyde (dilute 16% stock 4x with PBS).
- Staining Buffer (PBS + 3% FBS + 0.01% Sodium Azide)
- Perm buffer (PBS + 1% FBS + 0.01% Sodium Azide + 0.2% Saponin)

Stain for Surface Markers:

- Collect cells and transfer approximately 1 x 10^5 - 10^7 cells/mL into a 5mL (12 x 75 mm tube
- Wash 2x with ice cold PBS, by centrifuging at 1000 rpm, 10 mins, 4°C in a centrifuge with a swinging bucket rotor.
- Remove supernatant (do not aspirate to dryness, so as not to lose cells)
- Block surface Fc receptors for 10 minutes at room temperature (or 15-20 minutes at 4 °C) – DO NOT WASH
- Stain cells with directly conjugated antibodies at optimized concentrations (see antibody titration) for 15 – 20 minutes at RT or 30 minutes at 4°C, in the dark.
- Wash cells 2x with PBS and resuspend in staining buffer.
- Add a dead cell marker, incubate 10 -15 minutes at room temperature (do not wash)
    - Live/dead fixable amine reactive dyes are recommended for fixed panels.
    - See protocol for adding live/dead markers before staining extracellular markers (best staining is achieved in protein free buffer).

******* See Direct Immunofluorescent staining protocol for more details

Fixation and Permeabilization:

- Collect cells by centrifugation and aspirate supernatant
- Resuspend cells in 300 µL staining buffer
- Add an equal volume of 4% paraformaldehyde (final concentration should be 1-2%)
- Fix cells for 30 - 60 minutes at 4 °C.
- Wash Cells 2x with staining buffer
- After last wash, remove supernatant and resuspend in 100 µL PBS containing 0.2% Saponin or 0.2% Digitonin (Perm Buffer)
- Incubate for 30 minutes on ice or 30 minutes at 4 °C
- Wash 3x with Perm buffer

Alternate Method: Use a pre-made company supplied perm/fix buffer for a single step perm and fix.

- BD Cytofix/Cytoperm (BD Biosciences - 554714)
- Fix & Perm Cell Permeabilization Kit (ThermoFisher Scientific: Invitrogen – GAS-003)

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Intracellular Target Staining:

- Block non-specific intracellular binding sites with 5% serum or BSA for 15 – 30 minutes
- Wash 3x with Perm buffer
- Resuspend cells in 100 µL Perm Buffer
- Stain cells with Conjugated antibodies and prepare controls as follows (fluorophores mentioned are for example only)
    - Fixed, unstained sample
    - Fixed, compensation controls
    - Fixed, FMO gating controls
- Incubate 30-60 minutes in the dark at room temperature
- Wash Cells with Perm Buffer
- Resuspend cells in PBS + 2% FCS with 0.01% Sodium Azide
- Analyze cells by flow cytometry.

Peer Reviewed Reference Articles:

Turaç G, Hindley CJ, Thomas R, Davis JA, Deleidi M, et al. (2013) Combined Flow Cytometric Analysis of Surface and Intracellular Antigens Reveals Surface Molecule Markers of Human Neuropoiesis. PLoS ONE 8(6): e68519. doi: 10.1371/journal.pone.0068519

Bingham, K. N., Lee, M. D., & Rawlings, J. S. (2015). The Use of Flow Cytometry to Assess the State of Chromatin in T Cells. *Journal of Visualized Experiments : JoVE*, (106), 53533. Advance online publication. http://doi.org/10.3791/53533

Ehx, G., Hannon, M., Beguin, Y., Humblet-Baron, S., & Baron, F. (2015). Validation of a multicolor staining to monitor $_{phospho}$STAT5 levels in regulatory T-cell subsets. *Oncotarget, 6*(41), 43255-43266. Retrieved from http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path%5B%5D=6486

Online Intracellular Staining Protocols:

- Proteintech: Flow Cytometry Intracellular Staining Protocol
- eBioscience: Staining Intracellular Antigens for Flow Cytometry
- BD Biosciences: Intracellular Flow Cytometry. Multiparameter analysis of cytokine, transcription factor, and phosphorprotein expression by flow cytometry.
- Biolegend - Intracellular Cytokine Staining Protocol
- R&D Systems - Flow Cytometry Protocol for Staining Intracellular Molecules using Alcohol to Permeabilize the Cell Membrane
- Nature.com Protocol Exchange - Simultaneous detection of murine antigen-specific intracellular cytokines and CD107a/CD107b by flow cytometry