



Intracellular Flow Cytometry Staining Protocol V.3

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Guidelines

Application Notes:

1. Activated cell populations can be prepared from in vivo-stimulated tissues or from in vitro-stimulated cultures (e.g., antigen-specific activation or mitogen-induced). For cytokine and chemokine detection, it is critical to include a protein transport inhibitor such as brefeldin A (BioLegend Cat. No. [420601](#)) or monensin (BioLegend Cat. No. [420701](#)) in the last 4-6 hours of cell culture activation. The cells can be suspended and distributed to 12 x 75 mm plastic tubes or microwell plates for immunofluorescent staining. For details on stimulation methods, please see our [stimulation guide](#) for cytokines/chemokines.
2. Different cytokines/chemokines have different production peaks. In order to obtain optimal staining signals, the stimulation conditions for each stimulant need to be optimized.
3. Some antibodies recognizing native cell surface markers may not bind to fixed/denatured antigens. For this reason, it is recommended that staining of cell surface antigens be done with live, unfixed cells PRIOR to fixation/permeabilization and staining of intracellular targets. Altering the procedure such that cells are fixed prior to staining of cell surface antigens requires that paraformaldehyde-denatured antigen reactive antibody clones be empirically identified. You can also take a look at our [Fixation Webpage](#) to get an idea of how epitopes stain after fixation with 4% PFA. Please note that the ability to stain post-fixation depends on the fluor, antigen expression, and several other factors.

Note: For intracellular, cytoplasmic staining we recommend using our improved Cyto-Fast™ Fix/Perm Buffer Set (BioLegend Cat. No. [426803](#)). Follow the procedure listed under the "Recommended Usage" section on the product webpage or technical data sheet. Procedures outlined below utilize the Fixation (BioLegend Cat. No. [420801](#)) and Intracellular Staining Permeabilization Wash Buffer (10X) (BioLegend Cat. No. [421002](#)).

Related information:

1. Assenmacher, M., et al. 1994. Eur. J. Immunol. 24:1097.
2. Elson, L.H., et al. 1995. J. Immunol. 1995. 154:4294.
3. Jung T, et al. 1993. J. Immunol. Methods 159:197.
4. Prussin C., et al. 1995. J. Immunol. Methods 188:117.
5. Vikingsson A., et al. 1994. J. Immunol. Methods 173:219.



Materials

MATERIALS

⊗ RBC Lysis Buffer **BioLegend Catalog #420301**

⊗ Brefeldin A **BioLegend Catalog #420601**

⊗ Monensin Solution **BioLegend Catalog #420701**

⊗ Cell Staining Buffer **BioLegend Catalog #420201**

⊗ Fixation Buffer **BioLegend Catalog #420801**

⊗ Intracellular Staining Perm Wash Buffer **BioLegend Catalog #421002**

⊗ Cyto-Last™ Buffer **BioLegend Catalog #422501**

⊗ Cyto-Fast™ Fix/Perm Buffer Set **BioLegend Catalog #426803**

Troubleshooting



Fixation

- 1 If staining intracellular antigens (e.g. IFN- γ or IL-4), first perform cell surface antigen staining as described in BioLegend's Cell Surface Immunofluorescence Staining Protocol, then fix cells in 0.5 ml/tube Fixation Buffer in the dark for 20 minutes at room temperature. Tip: For gentler fixation (particularly with tandem fluors), FluoroFix™ Buffer (BioLegend Cat. No. [422101](#)) can be used.
- 2 Centrifuge at 350xg for 5 minutes, discard supernatant.
- 3 To put the experiment "on hold" at this point for future staining and analysis, wash cells 1x with Cell Staining Buffer. Resuspend cells in Cell Staining Buffer and store cells at 4°C (short term) or in 90% FCS/10% DMSO for storage at -80°C (long term, for fixed cells without surface antigen staining). Alternatively, cells can be kept in Cyto-Last™ Buffer for the storage of cytokine-producing cells for up to two weeks. The frequencies of cytokine-producing cells present in activated human PBMC cultures can vary widely due to donor variability. Therefore, cryopreserved cells from a single donor are useful for longitudinal studies.

Permeabilization

- 4 Dilute 10X Intracellular Staining Perm Wash Buffer to 1X in DI water.
- 5 Resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 350xg for 5-10 minutes.
- 6 Repeat step 5 twice.

Intracellular Staining

- 7 Resuspend fixed/permeabilized cells in residual Intracellular Staining Perm Wash Buffer and add a predetermined optimum concentration of fluorophore-conjugated antibody of interest (e.g. PE anti-IFN- γ) or an appropriate negative control for 20 minutes in the dark at room temperature.
- 8 Wash 2x with 2 ml of Intracellular Staining Perm Wash Buffer and centrifuge at 350xg for 5 minutes.
- 9 If primary intracellular antibody is biotinylated, it will be necessary to perform fluorophore conjugated Streptavidin incubations and subsequent washes in Intracellular Staining Perm Wash Buffer.



- 10 Resuspend fixed and intracellularly labeled cells in 0.5 ml Cell Staining Buffer and analyze with appropriate controls.
Tip: To confirm specific anti-cytokine staining, a blocking experiment is recommended in which cells are fixed/permeabilized then preincubated with an excess amount of unlabeled anti-cytokine antibody and/or the recombinant cytokine of interest is preincubated with fluorophore-conjugated anti-cytokine antibody before its addition to the cells.

Activation and Intracellular Staining of Whole Blood

- 11 Dilute heparinized whole blood 1:1 with sterile appropriate tissue culture medium.
Tip: For details on stimulation methods, please see our [stimulation guide](#) for cytokines/chemokines.
- 12 At this stage, in vitro cellular stimulation by either antigen or mitogen can be performed. If intending to stain intracellular cytokines or chemokines (e.g. IFN- γ or IL-4), addition of an efficient protein transport inhibitor such as brefeldin A or monensin is critical. After addition of a suitable cellular activator, aliquot 200 μ l of the whole blood cell suspension into 12 \times 75mm plastic tubes and incubate for 4-6 hours in 5% CO₂ at 37°C.
- 13 Add 2ml of 1X Red Blood Cell Lysis Buffer and incubate for 5-10 minutes at room temperature.
- 14 Centrifuge at 350xg for 5 minutes and discard the supernatant.
- 15 Wash cells 1X with Cell Staining Buffer and perform cell surface immunofluorescent staining as described above.
- 16 Fix, permeabilize, and stain intracellular antigens as described above.

Flow Cytometric Analysis

- 17 Set PMT voltage and compensation using cell surface staining controls. Set quadrant markers based on blocking controls, isotype controls, or unstained cells. For proper flow cytometric analysis, cells stained by this method should be inspected by light microscopy and/or flow light scatter pattern to confirm that they are well dispersed. Bivariate dot plots or probability contour plots can be generated upon data analysis to display the frequencies of and patterns by which individual cells coexpress certain levels of cell surface antigen and intracellular cytokine proteins.