

Intracellular Cytokine Staining Protocol

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). It is critical to include a protein transport inhibitor such as brefeldin A (Cat. No. 420601) or monensin (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.
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 cytokines. 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.

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 (Cat. No. 420801) in the dark for 20 minutes at room temperature.
2. Centrifuge at 350 x *g* 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). 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 Permeabilization Wash Buffer (Cat. No. 421001) to 1X in DI water.
5. Resuspend fixed cells in Permeabilization Wash Buffer and centrifuge at 350 x *g* for 5-10 minutes.
6. Repeat step 5 twice.

Intracellular Staining:

7. Resuspend fixed/permeabilized cells in residual Permeabilization Wash Buffer and add a predetermined optimum concentration of fluorochrome conjugated antibody of interest (e.g. anti-IFN- γ -PE) or an appropriate negative control for 20 minutes in the dark at room temperature.
8. Wash 2x with 2 ml of Permeabilization Wash Buffer and centrifuge at 350 x *g* for 5 minutes.
9. If primary intracellular antibody is biotinylated, it will be necessary to perform fluorochrome conjugated Streptavidin incubations and subsequent washes in Permeabilization Wash Buffer.
10. Resuspend fixed and intracellularly labeled cells in 0.5 ml Cell Staining Buffer and analyze with appropriate controls.

Note: 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 fluorochrome-conjugated anti-cytokine antibody before its addition to the cells.

Activation and Intracellular Staining of Whole Blood:

1. Dilute heparinized whole blood 1:1 with sterile appropriate tissue culture medium.
2. At this stage, *in vitro* cellular stimulation by either antigen or mitogen can be performed. If intending to stain intracellular antigens (e.g. IFN- γ or IL-4), addition of an efficient protein transport inhibitor such as brefeldin A (Cat. No. 420601) or monensin (Cat. No. 420701) is critical. After addition of a suitable cellular activator, aliquot 200 μ l of the whole blood cell suspension into 12 x 75 mm plastic tubes and incubate for 4-6 hours in 5% CO₂ at 37°C.
3. Add 2 ml of 1X Red Blood Cell Lysis Buffer (Cat. No. 420301) and incubate for 5-10 minutes at room temperature.
4. Centrifuge at 350 x *g* for 5 minutes and discard the supernatant.
5. Wash cells 1X with Cell Staining Buffer and perform cell surface immunofluorescent staining.
6. Fix, permeabilize and stain intracellular antigens as described above.

Flow Cytometric Analysis:

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 co-express certain levels of cell surface antigen and intracellular cytokine proteins.

**Related Information:**

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

Reagent List:

1. Cell Staining Buffer (Cat. No. 420201)
2. Monensin (Cat. No. 420701)
3. RBC Lysis Buffer (Cat. No. 420301)
4. Brefeldin A (Cat. No. 420601)
5. Fixation Buffer (Cat. No. 420801)
6. Permeabilization Wash Buffer (Cat. No. 421001)