Immunofluorescent Staining of Intracellular Cytokines for Flow Cytometric Analysis

I. Introduction
Improved methods have continually been sought to analyze single-cell cytokine production by human and rodent leukocytes and cell lines. Single-cell cytokine analysis techniques include ELISPOT, in situ hybridization, immunohistochemistry, limiting dilution analysis and single cell PCR. These latter techniques have significant drawbacks requiring either high technical proficiency or tedious data collection and analysis. Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters, including size and granularity, as well as the expression of surface and intracellular markers defined by fluorescent antibodies.

Recently, fluorescent anti-cytokine and anti-chemokine monoclonal antibodies have become very useful for the intracellular staining and multiparameter flow cytometric analysis of individual cytokine-producing cells within mixed cell populations. Multicolor immunofluorescent staining with antibodies against intracellular cytokines and cell surface markers provides a high resolution method to identify the nature and frequency of cells which express a particular cytokine(s). For example, multicolor immunofluorescent staining of an individual cell surface antigen and two cytoplasmic cytokines has been used to identify and enumerate cell types which express cytokines in either a restricted (e.g., Th1- versus Th2-like cells) or unrestricted (e.g., Th0-like cells) pattern. In addition to enabling highly specific and sensitive measurements of several parameters for individual cells simultaneously, this method has the capacity for rapid analysis of large numbers of cells which are required for making statistically significant measurements.

Staining of intracellular cytokines depends on the identification of cytokine-specific monoclonal antibodies which are compatible with a fixation-permeabilization procedure. Optimal intracellular cytokine staining has been reported using a combination of fixation with paraformaldehyde and subsequent permeabilization of cell membranes with the detergent saponin. Paraformaldehyde fixation allows preservation of cell morphology and intracellular antigenicity, while also enabling the cells to withstand permeabilization by detergent. Membrane permeabilization by saponin allows the cytokine-specific monoclonal antibody to penetrate the cell membrane, cytosol, and membranes of the endoplasmic reticulum and Golgi apparatus.

Critical parameters for cytokine staining include the following: cell type and activation protocol; the time of cell harvest following activation; the inclusion of a protein transport inhibitor during cell activation; and the choice of anti-cytokine antibody.

II. General Methods

A. Stimulation of Cells
Various in vitro methods have been reported for stimulating cytokine producing cells. Polyclonal activators have been particularly useful for inducing and characterizing cytokine-producing cells. These activa-tors include: phorbol esters plus calcium ionophore; phytohemaglutinin; Staphylococcus enterotoxin B; and monoclonal antibodies directed against subunits of the TCR/CD3 complex (with or without antibodies directed against costimulatory receptors such as CD28).

NOTE: It has been reported that cell activation with PMA alone causes a transient loss of CD4 expression from the surface of mouse T cells. Cell activation with PMA and calcium ionophore together has been reported to cause a greater and more sustained decrease in CD4 expression as well as a decrease in CD8 expression in mouse thymocytes and mouse and human peripheral T lymphocytes.

BDB-Pharmingen recommends the use of an intracellular protein transport inhibitor during in vitro cell activation for cytokine staining. Use of GolgiStop™ (Cat. No. 2092KZ; containing monensin) or GolgiPlug™ (Cat. No. 2301KZ; containing brefeldin A) block intracellular transport processes and results in the accumulation of most cytokine proteins in the rough endoplasmic reticulum or Golgi complex. This leads to an enhanced ability to detect cytokine-producing cells. Since these agents have a dose- and time-dependent cytotoxic effect, exposure must be limited.
NOTE: Investigators should be aware of possible effects of transport inhibitors on the expression levels of cell surface markers. Brefeldin A has been found to cause decreased levels of CD14 staining.

1. Cultures for Generating Human Cytokine-Producing Cells
   a. IL-3⁺, IL-4⁺, IL-5⁺, IL-13⁺ and GM-CSF⁺ human cells: Human PBMC, purified human CD4⁺ or CD8⁺ cells (especially for IL-5⁺ and IL-13⁺ cells) are stimulated with immobilized anti-human CD3 antibody (clone UCHT1, 10 µg/ml for plate coating, Cat. No. 30100D), soluble anti-human CD28 antibody (clone CD28.2, 2 µg/ml; Cat. No. 33740D), recombinant human IL-2 (10 ng/ml; Cat. No. 19621T) and recombinant human IL-4 (20 ng/ml; Cat. No. 19641V) for 2 days. The cells are washed and subsequently cultured in medium containing rhIL-2 and rhIL-4 for 3 days. Finally, the cells are harvested and restimulated for 4 hr with PMA(5 ng/ml; Sigma, Cat. No. P-8139), calcium ionophore A23187 (250 ng/ml; Sigma, Cat. No. C-9275), or ionomycin (500 ng/ml; Sigma Cat. No. I-0634) in the presence of a protein transport inhibitor.
   b. TNF-α⁺ human cells: Human PBMC are stimulated with immobilized anti-human CD3 antibody (clone UCHT1, 10 µg/ml for plate coating, Cat. No. 30100D) and recombinant human IL-2 (10 ng/ml; Cat. No. 19621T) for 2 days. The cells are washed and subsequently cultured in medium containing rhIL-2 for 3 days. Finally, the cells are harvested and restimulated for 4 hr with PMA (5 ng/ml; Sigma, Cat. No. P-8139), calcium ionophore A23187 (250 ng/ml; Sigma, Cat. No. C-9275), or ionomycin (500 ng/ml; Sigma Cat. No. I-0634) or alternatively the cells can be restimulated with anti-CD3 and anti-CD28. Restimulation should be performed in the presence of a protein transport inhibitor.
   c. IL-2⁺⁺, TNF-α⁺⁺, and IFN-γ⁺⁺ human cells: Human PBMC are stimulated for 6 hr with PMA (5 ng/ml; Sigma, Cat. No. P-8139), calcium ionophore A23187 (500 ng/ml; Sigma, Cat. No. C-9275), or ionomycin (500 ng/ml; Sigma Cat. No. I-0634) in the presence of a protein transport inhibitor.
   d. IL-1α⁺⁺, IL-6⁺⁺, IL-8⁺ and GRO-α⁺ human cells: Human PBMC are stimulated for 4 hr with LPS (1.0 µg/ml; Sigma Cat. No. L-8274) in the presence of a protein transport inhibitor.
   e. IL-10⁺⁺, MCP-1⁺⁺, MIP-1α⁺⁺, MCP-3⁺⁺, and MIG⁺⁺ human cells: Human PBMC are stimulated for 24 hr with LPS (1.0 µg/ml) in the presence of a protein transport inhibitor.
   f. IL-12⁺⁺ human cells: Human PBMC are primed for 2 hr with rhIFN-γ (10 ng/ml; Cat. No. 19751G) and are then activated with IFN-γ (10 ng/ml) and LPS (1.0 µg/ml; Sigma, Cat. No. L-8274) in the presence of a protein transport inhibitor for an additional 22 hr.
   g. RANTES⁺ human cells: Because T cells can make RANTES constitutively (although its expression is upregulated by cell stimulation), human PBMC can simply be cultured for 24 hr in the presence of a protein transport inhibitor (GolgiStop™ is preferred).

2. Cultures for Generating Mouse Cytokine-Producing Cells
   a. IL-2⁺⁺, TNF-α⁺⁺, and IFN-γ⁺⁺ mouse cells: Mouse splenocytes are stimulated for 4 hr with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the presence of a protein transport inhibitor.
   b. IL-3⁺⁺, IL-4⁺⁺, IL-5⁺⁺, IL-10⁺⁺, GM-CSF⁺⁺ mouse cells: Purified CD4⁺ mouse splenocytes from 6-month old BALB/c mice are stimulated with plate-bound anti-mouse CD3 (clone 145-2C11, 25 µg/ml; Cat. No. 01080D) and soluble anti-mouse CD28 (clone 37.51, 2 µg/ml; Cat. No. 01670D) for 2 days in culture together with rmIL-2 (10 ng/ml; Cat. No. 19231V) and rmIL-4 (50 ng/ml; Cat. No. 01080D) and soluble anti-mouse CD28 (2 µg/ml) in the presence of a protein transport inhibitor. Alternatively, can re-stimulate with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the presence of a protein transport inhibitor.
   c. IL-6⁺⁺, IL-12⁺⁺, TNF-α⁺⁺ mouse cells: 3-day thioglycolate elicited peritoneal cells were harvested and stimulated with 1 µg/ml LPS and GolgiPlug™ for 4 hr.
   d. MCP-1⁺⁺ mouse cells: Thioglycolate-elicited peritoneal macrophages from 6-month old BALB/c mice are stimulated with LPS (1 µg/ml; Sigma Cat. No. L-8274) overnight in the presence of a protein transport inhibitor.

3. Cultures for Generating Rat Cytokine-Producing Cells
   a. IL-4⁺ and IL-10⁺ rat cells: Purified splenic CD4⁺ cells from an adult rat are stimulated with
plate-bound anti-rat CD3 (clone G4.18, 25 µg/ml; Cat. No. 22011D) and soluble anti-rat CD28
(clone JJ319, 2 µg/ml; Cat. No. 22600D) for 2 days in culture together with recombinant
mouse IL-2 (10 ng/ml; Cat. No. 19211T) and rrIL-4 (50 ng/ml; Cat. No. 25011V), followed by a
3 day incubation with only rmIL-2 and rrIL-4. This is followed by a 4-6 hr stimulation with PMA
(5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the
presence of a protein transport inhibitor. Alternatively, can restimulate with plate-bound anti-
rat CD3 and soluble anti-rat CD28 for 4-6 hr in the presence of a protein transport inhibitor.

B. Protocol: Multicolor Staining for Intracellular Cytokines and Cell Surface Antigens

1. Harvest Cells
   Viable activated cell populations can be prepared from in vivo-stimulated tissues or from in vitro-
stimulatory cultures. The cells can be suspended and distributed to plastic tubes or microwell
plates for immunofluorescent staining. Cells should be protected from light throughout staining and
storage.

2. Block Fc Receptors
   Reagents that block Fc receptors may be useful for reducing nonspecific immunofluorescent
staining.\textsuperscript{14}
   a. In the mouse system, purified 2.4G2 antibody directed against Fc\textgamma II/III receptors (Fc Block™;
      Cat. No. 01241D), can be used to block nonspecific staining by fluorochrome conjugated
      antibodies which is mediated by receptors. To block mouse Fc receptors with Fc Block™,
      preincubate cell suspension with 1 µg Fc Block™per 10\textsuperscript{6} cells in 100 µl of Staining Buffer for
      15 min at 4°C. The cells are then washed and stained with a fluorescent antibody which is
      specific for a cell surface antigen of interest.
   b. Fc receptors on human and rat cells can be pre-blocked by incubating cells with an excess of
      irrelevant purified Ig from the same species and with the same isotype as the antibodies used
      for immunofluorescent staining.

3. Stain Cell Surface Antigens
   a. Incubate ~10\textsuperscript{6} cells in 50 µl of Staining Buffer (100 µl for staining in tubes) with a pre-titrated
      optimal concentration (≤0.5 µg) of a fluorochrome-conjugated monoclonal antibody specific
      for a cell surface antigen, such as, CD3, CD4, CD8, CD14, or CD19 (15-30 min, 4°C).
      Multicolor staining of different cell surface antigens can be carried out to provide controls for
      setting proper compensation of the brightest fluorescent signals.
      \textit{NOTE: Some antibodies which recognize native cell surface markers may not bind to
      fixed/denatured antigen. For this reason, it is recommended that the 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 suitable antibody clones be empirically identified.
   b. Wash cells 2X with Staining Buffer (1 ml/wash for staining in tubes), pellet by centrifugation
      (250 X g), and remove supernatant.

4. Fix and Permeabilize Cells
   a. Thoroughly resuspend cells in 100 µl (250 µl for staining in tubes) of Cytofix/Cytoperm™
      solution for 10-20 min at 4°C.
      \textit{NOTE: Cell aggregation can be avoided by vortexing prior to the addition of the
      Cytofix/Cytoperm™ solution.}
   b. Wash cells two times in 1X Perm/Wash™ solution (1 ml/wash for staining in tubes), pellet,
and remove supernatant.

NOTE: Perm/Wash™ solution is required in washing steps to maintain cells in a permeabilized state.

5. Alternative Fixation and Permeabilization Protocol
Cells can be fixed and stored to continue the intracellular staining at a later time.

a. Fixation and Storage of Cells
1. Resuspend cells in 100 µl (250 µl for tubes or 1 ml/10^7 cells for bulk fixing) of a 4% paraformaldehyde solution at 4°C for 10-20 min.
2. Wash cells 2X in staining buffer
3. Resuspend cells in staining buffer for storing at 4°C or in 90% FCS/10% DMSO for storing at -80°C.

b. Permeabilizing Fixed Cells
1. For frozen cells, wash 2X to remove DMSO.
2. Resuspend cells in Perm/Wash™ for 15 min
3. Pellet by centrifugation.
4. Stain for Intracellular Cytokines
   a. Thoroughly resuspend fixed/permeabilized cells in 50 µl of Perm/Wash™ solution (100 µl for staining in tubes) containing a pre-determined optimal concentration of a fluorochrome-conjugated anti-cytokine antibody or appropriate negative control. Incubate at 4°C for 30 min in the dark.
   b. Wash cells 2 times with 1X Perm/Wash™ solution (1 ml/wash for staining in tubes) and resuspend in Staining Buffer prior to flow cytometric analysis.

C. Alternative Protocol – Activation and Intracellular Staining of Whole Blood Technical Protocols
1. Dilute whole blood 1:1 volume to volume (e.g. 100 µl:100 µl) with RPMI1640 medium and mix well.
2. Add cell activator or mitogen to diluted blood e.g., 50 ng/ml PMA + 1 µg/ml calcium ionophore A23187 or PMA + 1 µM ionomycin (final concentration) in the presence of a protein transport inhibitor such as GolgiPlug™, (Cat. No. 2301KZ) (containing brefeldin A) or GolgiStop™, (Cat. No. 2092KZ) (containing monensin).
3. Vortex briefly to mix. Aliquot 200 µl into 12 x 75 mm plastic tubes. Incubate for 4-6 hr in 5% CO₂ at 37°C.
4. Add 2 ml PharMLyse™ (Cat. No. 35221E), vortex, incubate 10 min at RT in the dark.
5. Spin 5 min, 500 X g.
6. Aspirate supernatant. Wash 1 X in Staining Buffer. Spin 5 min at 500 x g. Aspirate supernatant.
7. Continue with steps 3-5 under part B of the General Methods above.

D. 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.
The frequencies of cytokine-producing cells present in activated human PBMC culture can vary widely due to donor variability. Therefore, cryopreserved cells from a single donor are useful for longitudinal studies.\textsuperscript{5,6}

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. In order to make statistically significant population frequency measurements, sufficiently large sample sizes should be acquired during flow cytometric analysis.\textsuperscript{2} Bivariate dot plots or probability contour plots can be generated upon data reanalysis to display the frequencies of and patterns by which individual cells co-express certain levels of cell surface antigen and intracellular cytokine proteins.\textsuperscript{2}

### E. Staining Controls

1. **Positive Staining Controls**

   The TDS for Pharmingen’s fluorochrome-conjugated anti-cytokine antibodies describe \textit{in vitro} culture systems which can induce detectable frequencies of cytokine-producing cells at specific time-points. Cells stimulated by these methods can be used as positive controls for experimental systems. Published reports of immunofluorescent staining and ELISPOT analysis can also provide useful information regarding different experimental protocols for generating cytokine-producing cells.\textsuperscript{1,12,13}

2. **Negative Staining Controls**

   One of the following three controls can be used to discriminate specific staining from artifactual staining. Investigators should choose which staining controls best meet their research needs. Intracellular cytokine staining techniques and the use of blocking controls are described in detail by C. Prussin and D. Metcalfe.\textsuperscript{5}

   a. **Isotype control:** Stain with an isotype-matched control of irrelevant specificity.

      1. Resuspend cell pellet in 50 µl of Perm/Wash\textsuperscript{™} solution (100 µl for staining in tubes) containing the isotype control antibody at the same concentration for the anti-cytokine antibody (< 0.5 µg/10\textsuperscript{6} cells).
      2. Incubate 15-30 min at 4°C.
      3. Wash cells using the aforementioned procedure for intracellular staining.

   b. **Ligand blocking control:** Pre-block anti-cytokine antibody with recombinant cytokine

      1. Preincubate fluorochrome-conjugated antibodies with appropriately-diluted cytokine in a volume ≥ 50 µl of Perm/Wash\textsuperscript{™} solution at 4°C for 30 min.
      2. Resuspend fixed/permeabilized cells in 50 µl (100 µl for staining in tubes) of pre-blocked labeled anti-cytokine antibody (in Perm/Wash\textsuperscript{™} solution) and incubate 30 min at 4°C.

---

### Intracellular Cytokine Positive control cells

To serve as positive controls for intracellular cytokine staining, PharMingen offers sets of activated and fixed leukocyte populations which have been screened for cytokine production.

<table>
<thead>
<tr>
<th>Cell Set</th>
<th>Cat. No.</th>
<th>Cytokines Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiCK-1</td>
<td>23261Z</td>
<td>IL-2, IFN-γ, TNF-α</td>
</tr>
<tr>
<td>HiCK-2</td>
<td>23262Z</td>
<td>IL-3, IL-4, IL-10, IL-13, GM-CSF</td>
</tr>
<tr>
<td>HiCK-3</td>
<td>23263Z</td>
<td>IL-1α, IL-1b, IL-6, IL-12, TNF-α</td>
</tr>
<tr>
<td>HiCK-4</td>
<td>23264Z</td>
<td>IL-8, GRO, IP-10, MCP-1, MCP-3, MIG, MIP-1α, RANTES</td>
</tr>
<tr>
<td>MiCK-1</td>
<td>20131Z</td>
<td>IL-2, IFN-γ, TNF-α</td>
</tr>
<tr>
<td>MiCK-2</td>
<td>20132Z</td>
<td>IL-3, IL-4, IL-10, GM-CSF</td>
</tr>
<tr>
<td>MiCK-3</td>
<td>20133Z</td>
<td>IL-6, IL-12, MCP-1, TNF-α</td>
</tr>
<tr>
<td>RiCK-2</td>
<td>24142Z</td>
<td>IL-4, IL-10, GM-CSF</td>
</tr>
</tbody>
</table>
3. Wash cells using the aforementioned procedure for intracellular staining.

c. Antibody blocking control: Pre-incubate cells with unconjugated antibody.
1. Resuspend fixed/permeabilized cells in 25 µl Perm/Wash™ solution (50 µl for staining in tubes) containing unconjugated anti-cytokine antibody (same clone as conjugated antibody) diluted to the appropriate concentration (>5 µg/10^6 cells), and incubate 30 min at 4°C.
2. After incubation, add fluorochrome labeled anti-cytokine antibody at an optimal concentration in 25 µl Perm/Wash™ buffer (50 µl for staining in tubes) for a final volume of 50 µl for staining in microwell plates or 100 µl for staining in tubes, and incubate 30 min at 4°C.
3. Wash cells using the aforementioned procedure for intracellular staining.

**SOLUTIONS**

**Staining Buffer**
- Dulbecco's PBS (DPBS)
- 3% heat-inactivated FCS
- 0.09% (w/v) sodium azide
- Adjust buffer pH to 7.4 - 7.6, filter (0.2 µm pore membrane), and store at 4°C

References: