Detection of Intracellular Cytokines by Flow Cytometry

Yuzhi Yin,[1] Alyssa Mitson-Salazar,[1,2] and Calman Prussin[1]

[1]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland
[2]Yale School of Medicine, New Haven, Connecticut

Intracellular cytokine staining (ICCS), employing fluorescently labeled MAbs detected by flow cytometry, has emerged as the premier technique for studying cytokine expression at the single-cell level. Advances in polychromatic flow cytometry have dramatically enhanced the sophistication of ICCS investigations. ICCS can simultaneously measure multiple cytokines within a single cell, allowing the detection of complex cytokine phenotypes. Additionally, cytokines can be measured with a variety of other analytes, including transcription factors, proliferation dilution dyes, activation markers, and viability dyes. This capability, combined with the high throughput inherent in the instrumentation, gives ICCS an enormous advantage over other single-cell techniques such as ELISPOT, limiting dilution, and T cell cloning. The unit describes intracellular staining of cells that have already been stimulated in vitro and fixed. Methods for in vitro activation by PMA and ionomycin or antigens, fixation of cell suspensions, and cell surface staining are also described. © 2015 by John Wiley & Sons, Inc.

Keywords: cytokines • CD4-positive T lymphocytes • CD8-positive T lymphocytes • immune system • epitopes • T lymphocyte • cytokine staining

How to cite this article:
Yin, Y., Mitson-Salazar, A., and Prussin, C. 2015. Detection of intracellular cytokines by flow cytometry. *Curr. Protoc. Immunol.* 110:6.24.1-6.24.18.
doi: 10.1002/0471142735.im0624s110

INTRODUCTION

Intracellular cytokine detection by flow cytometry has emerged as the premier technique for studying cytokine production at the single-cell level. This fusion of intracellular staining methods with p*olychromatic* flow cytometry results in an immensely powerful technique. P*olychromatic* flow cytometry permits the simultaneous detection of multiple cytokines and other functional attributes within a single cell, allowing the detection of complex cytokine phenotypes. This capability, combined with the high throughput inherent in the instrumentation, gives ICCS an enormous advantage over other single-cell techniques such as ELISPOT, limiting dilution, and T cell cloning. Indeed, ICCS has emerged as a major platform to for multicenter vaccine and viral immunology studies (Maecker et al., 2005; Lamoreaux et al., 2006).

Both commercially available proprietary ICCS kits and literature-described "home-made" ICCS methods generally involve: (1) in vitro activation, (2) immobilization of the cytokine within the cell by fixation, and (3) cell permeabilization to gain access to the intracellular cytokine, followed by (4) immunostaining with anti-cytokine monoclonal antibodies.

Current Protocols in Immunology 6.24.1-6.24.18, August 2015
Published online August 2015 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/0471142735.im0624s110

Multiple reagent suppliers now provide proprietary fixation and permeabilization kits. The value of these kits is that they provide quality-controlled reagents and straightforward time-saving protocols. A limitation of such commercial kits is that they provide a "one size fits all" approach, which may not be optimal for all cytokines or experimental conditions. For the detection of rare events (e.g., antigen specific responses) and cytokines expressed in low abundance (e.g., T_H2 cytokines), the paraformaldehyde-based fixation system included in this unit generally provides superior signal to noise when compared to commercially available kits. The detection of dim, low-frequency, or newly characterized cytokines by flow cytometry can be technically demanding, and often requires optimization by each individual researcher.

The unit first describes the techniques required to perform intracellular staining of cells that have already been stimulated in vitro and fixed (see Basic Protocol). The methods necessary for in vitro activation by PMA and ionomycin (see Support Protocol 1) or antigens (see Support Protocol 2), and fixation of cell suspensions (see Support Protocol 3), are also described. To optimize workflow, typically, cell surface markers are stained concurrently with intracellular cytokines. Alternatively, Support Protocol 4 provides methods for staining cell surface markers on fresh cells prior to fixation and ICCS. Because of the greater level of nonspecific binding inherent in fixed, permeabilized cells, care must be taken in designing specificity controls. Methods to rigorously demonstrate specificity of cytokine staining are addressed in Support Protocol 5.

Cell stimulation and processing will often increase cell death. Dead cells demonstrate greater nonspecific binding, thereby increasing noise and decreasing assay sensitivity. The use of amine reactive viability dyes allow the investigator to exclude dead cells, thereby increasing assay sensitivity.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: Because of the availability of quality anti-cytokine MAbs from many vendors, we have not noted specific MAb sources (see Critical Parameters).

BASIC PROTOCOL

INTRACELLULAR STAINING FOR CYTOKINES

This protocol assumes the use of cells that have been stimulated, fixed, and frozen (see Support Protocols 1 through 4). Here, six-color staining for CD3, CD4, CD8, CD154, IFN-γ, and IL-13, resulting in enumeration of T_H1 versus T_H2 cells, is described.

A major stumbling block in ICCS is the added noise caused by increased nonspecific binding of MAbs, which results from fixation and permeabilization. Careful titration of antibody concentrations and the blocking of nonspecific binding with bland proteins such as BSA, serum, or nonfat dry milk decrease, but do not eliminate, this problem. To optimize the cytokine staining index, all anti-cytokine MAbs should be carefully titrated (Stewart and Stewart, 1997; Hulspas, 2010). Almost all ICCS MAbs yield an optimal staining index (or signal-to-noise ratio) within the range from 0.5 to 5 μg/ml.

Every sample should be analyzed with both a specifically stained tube (i.e., anti-cytokine MAbs) and a control tube (e.g., isotype-matched or fluorescence minus one control; Roederer, 2002). A detailed discussion of specificity controls is described in Support Protocol 5.

NOTE: Saponin is a reversible permeabilization agent; for intracellular staining it must be present in all staining and washing buffers. The optimal concentration of anti-cytokine antibodies is a critical variable that must be determined by each investigator (see Troubleshooting).

Materials

Cells: stimulated, Amine reactive viability dye stained, fixed, and frozen (see Support Protocols 1 to 3)
PBS-S (see recipe)
PBS-S/milk solution (see recipe)
Directly labeled anti-cytokine MAbs (e.g., anti-IFN-γ Alexa Fluor 700 and anti-IL-13 PE-Cy7) and anti-surface marker antibodies (e.g., anti-CD3 Qdot 605; anti-CD4 PerCP-Cy5.5; CD8 PE-TexRed, and anti-CD154 FITC)
PBS/BSA (see recipe)

4-ml V-bottom tubes for staining (Sarstedt)
Tabletop centrifuge

Additional reagents and equipment for flow cytometric analysis (*UNIT 5.4*; Holmes et al., 2002)

Simultaneous surface antigen and intracellular cytokines staining

1. Thaw previously stimulated, fixed, frozen cells in a 37°C water bath.
2. Add 1 ml of PBS-S to each sample and transfer 2-4 × 10^6 cells to a 4-ml V-bottom tube.
3. Centrifuge 5 to 10 min at 1400 × *g*, room temperature. Aspirate supernatant.
4. Add 50 μl of PBS-S/milk solution per tube and resuspend the cell pellet by pipetting up and down or vortexing. Incubate 30 min at room temperature or overnight at 4°C.

   The use of bland proteins such as BSA, serum, or (as in this step) nonfat dry milk decreases nonspecific antibody binding.

5. Dispense proper aliquots of blocked cells into the appropriate number of staining tubes. Centrifuge 5 to 10 min at 1400 × *g*, room temperature. Aspirate supernatant.

   One cryovial of PMA/ionomycin-activated cells (4 × 10^6 cells) should provide enough cells for staining 4 to 8 tubes, while one vial of antigen-activated cells (4 × 10^6 cells) may only be enough for 1 to 2 tubes. When determining the number of cells to study, the type of activation and expected frequency of positive events should be considered (see Critical Parameters).

6. Prepare a cocktail of MAbs that yields the desired final concentration of each MAb diluted in PBS-S/milk (typically, 0.5 to *5* μg/ml each).
7. Add 50 μl of the MAb cocktail to each tube of cells and resuspend the cells by repeat pipetting or brief vortexing. Incubate for 30 min in the dark at room temperature.
8. Add 1 ml PBS-S to each tube and vortex. Centrifuge 5 to 10 min at 1400 × *g*, room temperature. Aspirate the supernatant, avoiding the pellet.
9. Break up cell pellet by tapping the bottom of the tube.

   Rattling the bottoms of the tubes against a "pincushion" test tube rack can assist in breaking up the pellet.

10. Repeat wash as described in steps 8 and 9.

11. Resuspend cells in 300 to 600 μl PBS/BSA and perform flow cytometric analysis (*UNIT 5.4*; Holmes et al., 2002) within 24 hr.

When determining the number of cells to acquire, the type of activation and expected frequency of positive events should be considered (e.g., antigen versus PMA/ionomycin; see Critical Parameters).

SUPPORT PROTOCOL 1

PMA AND IONOMYCIN ACTIVATION OF T CELLS

T cells obtained from typical sources (e.g., human peripheral blood mononuclear cells, mouse spleen) demonstrate low to undetectable amounts of spontaneous cytokine expression. Thus, cytokine detection typically requires in vitro activation. Typical methods used to activate T cells for both the human and mouse systems can be successfully adapted to this protocol [see *UNIT 7.10* (for human; Muul et al., 2011) and *UNIT 3.12* (for mouse; Kruisbeek et al., 2004)]. Although this protocol is written for human peripheral blood mononuclear cells (PBMC) activated with phorbol myristate acetate (PMA) and ionomycin, identical conditions can be used to stimulate mouse splenocytes.

PMA and ionomycin activation are the most robust stimuli for most T cell cytokines, typically providing the best conditions for observing ICCS (see Basic Protocol). Beginners should master the technique for PMA and ionomycin activation before other, more physiological stimuli are used (see Support Protocol 2). Ionomycin is a Ca^{2+} ionophore and PMA is a protein kinase C activator; together, these two reagents act to pharmacologically activate T cells. Brefeldin A (BFA) is an inhibitor of cytokine secretion and thus increases the amount of cytokine within the cell and, in turn, the signal-to-noise ratio.

Materials

Peripheral blood
Complete RPMI-10 medium (*APPENDIX 2A*), 37°C
10 mM ionomycin (Ca^{2+} salt) in DMSO (add 5 mg Ca^{+2} ionomycin per 0.67 ml DMSO; store at −20°C)
200 μg/ml phorbol myristate acetate (PMA) in 100% ethanol (store at −20°C)
10 mg/ml brefeldin A (BFA) in DMSO (store at −20°C)
3 mg/ml DNase I (60,000 Dornase U/ml; Calbiochem) in PBS, optional
Amine reactive viability dye: LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) or equivalent product from other supplier
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

24-well tissue culture plates
Cell scraper or disposable transfer pipets
Refrigerated centrifuge

Additional reagents and equipment for preparing peripheral blood mononuclear cells [PBMC; *UNIT 7.1* (Fuss et al., 2009)] and counting cells (*APPENDIX 3A*; Strober, 1997)

Prepare and activate PBMC

1. Prepare peripheral blood mononuclear cells (PBMC; *UNIT 7.1*; Fuss et al., 2009).

Mouse splenocytes can also be used; they are prepared using the methods detailed in UNIT 3.1 (Kruisbeek, 2000). Whole-blood techniques have been utilized with similar results. Several manufacturers sell reagents for whole-blood stimulation followed by fixation/lysis, and permeabilization using proprietary kits (Lovelace and Maecker, 2011).

2. Resuspend PBMC in complete RPMI-10 medium, prewarmed to 37°C, at a concentration of 4×10^6 cells/ml (2× cell concentration).

If logistically helpful, cells can be left overnight at 4°C in RPMI-10 medium in a 15-ml V-bottom tube or plated as per step 3 below and left overnight in a tissue culture incubator. If using cryopreserved PBMC, cells should be thawed, plated as per step 3, and rested overnight in a tissue culture incubator.

3. Warm cells to 37°C and plate 4×10^6 cells (1 ml of the suspension prepared in step 2) into each well of a 24-well tissue culture plate.

4. Prewarm RPMI-10 medium to 37°C (requires the same volume as cells). For every 5 ml of medium, add 1 μl each of ionomycin and PMA, and BFA (2× reagent concentration). Mix well.

   All of the chemicals used to activate the cells are lipophilic and will stick to plastic; therefore, once diluted, the activators must be used within 15 to 30 min of preparation. Handle all of these stock solutions with gloves.

5. Add 1 ml of the complete RPMI-10 medium containing PMA, ionomycin, and BFA to each well (final concentrations: 2×10^6 cells/ml, 20 ng/ml PMA, 1 μM ionomycin, and 10 μg/ml BFA) of the 24-well plate containing the cells.

   This can be scaled up or down: it is possible to stimulate 20×10^6 cells/10 ml in a 6-well plate or 4×10^5 cells/200 μl in a 96-well plate.

   Additional medium-control samples should be incubated in the absence of PMA/ionomycin (negative control).

6. Incubate 6 hr in a 37°C, 5% CO_2 incubator.

   This time period is optimal for IL-4 and IL-5, and is a reasonable compromise for IL-2 and IFN-γ. These kinetics are optimized for PMA/ionomycin activation; each investigator should determine the optimal conditions for different stimuli or cytokines of interest (see Critical Parameters).

Eliminate DNA and prepare activated cells for fixation

7. After 6 hr, add 40 μl of 3 mg/ml DNase I to each well and incubate for 5 to 10 min at 37°C (final concentration of 3500 Dornase U/ml).

   The vigorous activation triggered by PMA/ionomycin causes some cell death, with liberation of DNA from dead cells and consequent cell clumping. Cell clumping decreases the number of cells to be analyzed and may clog the flow cytometer. Brief treatment with DNase I abolishes this cell clumping and is not toxic to the cells. Activation with more physiological ligands does not result in as much clumping and does not require DNase I.

8. Add 200 μl of 0.1 M EDTA to each well. Incubate 5 min at room temperature. Pipet cells up and down to remove adherent cells, being careful not to create air bubbles. Transfer the contents of each tube to an individual 4-ml V-bottom tube.

9. Use a cell scraper or disposable transfer pipet to remove adherent cells from the bottom of the well. Transfer contents of each well to a 4-ml V-bottom tube. Pipet cells up and down two to three times to break up cell clumps.

10. Centrifuge 10 min at 300 × *g*, 4°C.

Cell viability staining

11. Prepare violet LIVE/DEAD staining solution by add 1 μl of the stock solution into 4 ml of PBS at 4°C.

12. Centrifuge the cell preparation for 10 min at 300 × *g*, 4°C. Aspirate the supernatant.

13. Resuspend the cells in 500 μl of the violet LIVE/DEAD staining solution.

14. Incubate for 30 min in the dark at 4°C.

15. Wash cells once with 1 ml PBS.

16. Centrifuge 10 min at 300 × *g*, 4°C.

The default protocol is to proceed directly to fixation (Support Protocol 3), after which phenotypic "surface markers" are stained simultaneously with intracellular cytokines. If live cell staining of surface markers is desired, then proceed to Support Protocol 4 prior to fixation.

SUPPORT PROTOCOL 2

ANTIGEN ACTIVATION OF T CELLS

Antigen activation of T cells represents a more physiologic stimulus than PMA and ionomycin, and thus may be a desired protocol. In the following protocol, T cells are activated with Staphylococcal enterotoxin B (SEB). However, this protocol can be adapted to other antigens of interest such as tuberculosis, cytomegalovirus, allergens, or superantigens. This same protocol has been used to examine antigen-specific responses in the draining lymph nodes of immunized mice.

Antigen processing and presentation are inhibited by the transport inhibitors brefeldin A and monensin. To allow antigen presentation as well as surface upregulation of costimulatory molecules, cells are first incubated with antigen for 2 hr prior to the addition of these agents. BFA is then added to the cultures for the last 4 hr of the 6-hr incubation.

Materials

Peripheral blood
Complete RPMI-10 medium (*APPENDIX 2A*), 37°C
Anti-CD28
SEB (Toxin Technology)
10 mg/ml brefeldin A (BFA) in DMSO (store at −20°C)
3 mg/ml DNase I (60,000 Dornase U/ml; Calbiochem) in PBS, optional
0.1 M EDTA in PBS (see *APPENDIX 2A* for PBS)

16 × 125-mm round-bottom polystyrene tissue culture tubes (Corning)
Refrigerated centrifuge
4-ml V-bottom tubes

Additional reagents and equipment for preparing peripheral blood mononuclear cells [(PBMC; *UNIT 7.1* (Fuss et al., 2009)], counting cells (*APPENDIX 3A*; Strober, 1997), and cell viability staining (Support Protocol 3)

NOTE: SEB is a Select Agent; distribution, storage and handling are tightly regulated (*http://www.selectagents.gov/*).

Prepare and activate PBMC

1. Prepare peripheral blood mononuclear cells (PBMC; *UNIT 7.1*; Fuss et al., 2009).

2. Resuspend cells in complete RPMI-10 medium, prewarmed to 37°C, at a concentration of 2×10^6 cells/ml.

3. Add anti-CD28 to a final concentration of 1 μg/ml. Mix well by pipetting.

Anti-CD49d has also been used extensively as an additional costimulator.

4. Pipet 4×10^6 cells (2 ml) into 16 × 125–mm round-bottom polystyrene tissue culture tubes.

Round-bottom tubes provide greater cell-cell contact and thus facilitate antigen presentation. 24-well tissue culture plates may work as well for some applications.

5. Add SEB to a final concentration of 10 μg/ml. Mix well.

   The optimal final concentration of antigen should be determined by each investigator.

6. Centrifuge cells 5 min at 300 × *g*, room temperature.

7. Place tubes upright with caps removed or loosed cap in a test tube rack and incubate for 2 hr in a 37°C, 5% CO_2 incubator.

8. Prepare a 200 μg/ml solution (1:50 dilution of the 10 mg/ml stock) of BFA in complete RPMI-10 medium. Gently add 100 μl of the 200 μg/ml BFA solution to each tube for a final concentration of 10 μg/ml. Mix gently without disturbing the pellet.

9. Incubate for an additional 4 hr in a 37°C, 5% CO_2 incubator (total incubation time of 6 hr).

   Each investigator (see Critical Parameters) should determine optimal time and conditions. For most cytokines, antigen-specific responses are optimal over a range from 6 to 16 hr.

Eliminate DNA (optional), prepare activated cells for fixation, and perform cell viability staining

10. *Optional:* Add 40 μl of 3 mg/ml DNase I to each well (to a final concentration of 3500 Dornase U/ml). Incubate 5 min at 37°C.

11. Add 200 μl of 0.1 M EDTA to each well. Incubate 5 min at room temperature. Pipet cells up and down to remove adherent cells, being careful not to create air bubbles. Transfer the contents of each tube to an individual 4-ml V-bottom tube.

12. Centrifuge 10 min at 300 × *g*, 4°C.

Perform cell viability staining

13. Perform cell viability staining starting at step 11 of Support Protocol 1.

FIXATION AND FREEZING OF PBMC

SUPPORT PROTOCOL 3

The following protocol describes fixation with 4% paraformaldehyde followed by freezing and storage of the cells in 10% DMSO/PBS. Formaldehyde works by cross-linking proteins, thereby immobilizing soluble cytokines within the cell. Fixation and freezing with subsequent staining of both cytokines and cell surface markers is the suggested workflow noted in the Basic Protocol. Alternatively, surface staining may be performed prior to fixation (see Support Protocol 4).

Archival cryopreservation of activated fixed samples in 10% DMSO/PBS at −80°C prior to staining allows far greater flexibility, since cells can be analyzed at any time in the future. Such samples are stable for at least 3 years (Kagina et al., 2015) and in our experience for many more years than that.

NOTE: Paraformaldehyde is a carcinogen and must be handled with gloves. Users should check with their local safety department for additional safe handling, storage, and waste requirements.

Materials

4% paraformaldehyde, stored in aliquots frozen at −20°C (see recipe)
Activated PBMC (see Support Protocol 1 or 2) or activated surface-stained cells (see Support Protocol 4)
PBS (*APPENDIX 2A*), 4°C
PBS/BSA (see recipe), 4°C
10% (v/v) dimethyl sulfoxide (DMSO) in PBS (10% DMSO/PBS), 4°C. Use standard reagent grade (not cell culture grade) DMSO.

4-ml V-bottomed tubes for staining (Sarstedt)
"Pincushion" test tube rack
2-ml freezing vials

Warm paraformaldehyde

1. Thaw 4% paraformaldehyde in a 37°C water bath for 10 min. Vortex to redissolve any precipitate.

   Do not use PFA more than 7 days after thawing. Store at 4°C after thawing.

Wash cells

2. Transfer one well (4×10^6) of stimulated cells to a 4-ml V-bottomed polystyrene tube and centrifuge for 10 min at 300 × g, 4°C.
3. Remove supernatant by aspiration, being careful not to aspirate the pellet.
4. Break up pellet by gently knocking the tube against a "pincushion" test-tube rack.
5. Add 1 ml of cold PBS (without BSA) and vortex. Centrifuge 10 min at 300 × *g*, 4°C, then aspirate supernatant.

   This step removes residual protein that may increase nonspecific binding.

6. Break up pellet by gently knocking the tube against a "pincushion" test-tube rack.

Fix cells

7. Add 500 µl of 4% paraformaldehyde (prewarmed to 37°C) to each tube and incubate 5 min at room temperature; vortex briefly three to four times during this period.

   This mixing is essential to avoid cell clumping.

8. Add 2 ml ice-cold PBS/BSA and vortex.
9. Centrifuge 5 to 10 min at 1400 × *g*, 4°C.

Cryopreserve sample

10. Remove supernatant by aspiration. Resuspend pellet in 0.5 ml of 10% DMSO/PBS and transfer samples into 2-ml freezing vials. Dispense cells into one freezer vial. Store cells at −80°C.

    Samples are cryopreserved after fixation, but before permeabilization.

SUPPORT PROTOCOL 4

CELL-SURFACE STAINING OF PBMC

The Basic Protocol describes staining for both cell-surface markers and intracellular cytokines simultaneously, after the fixation and permeabilization steps. However, 4% paraformaldehyde may, in rare cases, alter or destroy antigenic determinants, rendering them undetectable by specific MAb clones. Typically, an alternative MAb, recognizing a different antigenic determinant, will work in such cases. If no alternative MAb is available or if MAb staining of live cells is superior (typical for chemokine receptors), it is best to stain for phenotypic surface markers after activation (see Support Protocols 1 or 2), but before fixation (see Support Protocol 3). To be consistent with the reagents used in the Basic Protocol, CD4 is used as an example in this protocol.

Materials

Stimulated cells to be stained (see Support Protocols 1 and/or 2)
PerCP/Cy5.5-conjugated anti-human CD4 MAb
PBS/BSA (see recipe), 4°C
PBS (*APPENDIX 2A*), 4°C

4-ml V-bottomed tubes for staining (Sarstedt)
Refrigerated centrifuge

Additional reagents and equipment for counting cells (*APPENDIX 3A*; Strober, 1997) and fixation of cells (see Support Protocol 3)

NOTE: It is important that all washes and incubation be done on ice to decrease secretion of intracellular cytokine.

1. Transfer stimulated cells from Support Protocols 1 or 2 to a 4-ml V-bottomed polystyrene tube and centrifuge for 10 min at 300 × *g*, 4°C.
2. Aspirate supernatant and break up the cell pellet by knocking the bottom of the tube.

   Rattling the bottoms of the tubes against a white "pincushion" test-tube rack can assist in breaking up the pellet.

3. Resuspend the cells in 1 ml of ice-cold PBS/BSA. Count cells with a hemacytometer (*APPENDIX 3A*; Strober, 1997) or other cell counting device. Dispense 4×10^6 cells into the appropriate number of 4-ml V-bottomed tubes.
4. Centrifuge cells for 10 min 300 × *g*, 4°C. Aspirate supernatant.
5. Prepare a solution of PerCP/Cy5.5-conjugated anti-human CD4 MAb in 50 μl of PBS/BSA at the desired final MAb concentration. Keep antibody solutions ice-cold.
6. Add 50 μl of MAb solution to the cell pellet and resuspend cells by repeat pipetting. Incubate 20 min in the dark on ice.
7. Add 1 ml ice-cold PBS (no BSA) to each tube and gently vortex. Centrifuge 10 min at 300 × *g*, 4°C.
8. Complete the wash and fixation steps as described in Support Protocol 3, steps 6 through 10.

DEMONSTRATING SPECIFICITY OF CYTOKINE STAINING

SUPPORT PROTOCOL 5

Fixation increases the hydrophobicity of cellular proteins, thereby increasing their nonspecific binding. Furthermore, permeabilization increases the amount of cellular protein accessible for nonspecific antibody binding by several orders of magnitude. The use of bland proteins such as BSA, serum, and nonfat dry milk decreases but does not eliminate this problem. ICCS requires only one antibody to recognize a given cytokine. Thus, the intrinsic specificity of this method is less than that of a sandwich ELISA or western blot, in which additional factors (e.g., second MAb, molecular weight) confer greater specificity. For all of these reasons, a greater level of scrutiny must be placed upon the results obtained from intracellular staining relative to those obtained from surface staining. This is particularly true for new antigens or MAbs.

Specificity of staining can be demonstrated by blocking with recombinant cytokine, in which a 10- to 1000-fold molar excess of recombinant cytokine is added to the anti-cytokine MAb prior to staining. Because recombinant cytokine blocking can be technically challenging, and requires large quantities of expensive cytokine, a second, more versatile and cost-effective method using unlabeled MAb is suggested. With this technique, the negative control sample is preincubated with an excess of unlabeled anti-cytokine antibody. The specifically stained samples are similarly treated with an identical amount of irrelevant isotype-matched control antibody. Both tubes are then stained with identical amounts of directly conjugated anti-cytokine antibody. This approach provides a negative control in which specific binding is blocked by an excess of unlabeled

anti-cytokine antibody, but in which nonspecific staining remains unaffected relative to the control-blocked sample (Prussin and Metcalfe, 1995).

This protocol uses unlabeled anti-IFN-γ, anti-IL-4, and anti-IL-2 MAbs as an example, to be consistent with the reagents used in the Basic Protocol.

Additional Materials *(also see Basic Protocol)*

Unlabeled and FITC-labeled anti-IFN-γMAb
Unlabeled and PE-labeled anti-IL-4 MAb
Unlabeled and APC-labeled anti-IL-2 MAb
Unlabeled irrelevant isotype-control MAbs matching each of the anti-cytokine Ig isotypes

1. Prepare and block cells with PBS-S/milk (see Basic Protocol, steps 1 to 4), then divide cell suspension into two 25-μl aliquots in two separate 4-ml tubes.
2. Centrifuge 5 to 10 min at 1400 × *g*, room temperature. Aspirate supernatant.
3. Resuspend the pellet in one tube with 50 μl PBS-S/milk containing 100 μg/ml each of unlabeled specific anti-IFN-γ, anti-IL-4, and anti-IL-2 MAbs. In the second tube, resuspend the pellet with 50 μl PBS-S/milk containing 100 μg/ml unlabeled isotype-control MAbs matching each of the anti-cytokine Ig MAbs. Incubate 1 hr at room temperature.

   Isotype controls should match each of the anti-cytokine Ig isotypes.

   Dilution of saponin (present in the PBS-S) to <0.06% by adding large volumes of MAb should be avoided, as this reduces permeabilization. If significant dilution of saponin cannot be avoided, add 10× saponin at 1/10 the volume of the added MAbs.
4. Add FITC-labeled anti-IFN-γ MAb, PE-labeled anti-IL-4 MAb, and APC-labeled anti-IL-2 MAb at optimal concentration to all tubes and incubate 30 min at room temperature.

   Do not wash out unlabeled antibody prior to the addition of labeled MAbs.
5. Wash cells twice and perform flow cytometric analysis (see Basic Protocol, steps 8 to 11).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****.*

Brefeldin A (BFA)

Dissolve the BFA powder in DMSO to a final concentration of 10 mg/ml. Store up to 5 years at −80ºC.

Paraformaldehyde (PFA), 4%

Mix 100 ml of 32% paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences; *http://www.emsdiasum.com*) with 700 ml PBS (*APPENDIX 2A*) to make 4% PFA. Prepare 12-ml aliquots in 15-ml tubes.

PFA tubes should be affixed with the following label:

"4% PFA—carcinogen

Dispose of 7 days after thawing

Date of thaw _____

4% PFA must be stored frozen at –20°C"

After thawing, label tube with date and store at 4°C for up to 1 week.

CAUTION: Paraformaldehyde is a carcinogen and must be handled with gloves. Users should check with their local safety department for additional safe handling, storage, and waste requirements.

PBS/BSA

PBS (*APPENDIX 2A*) containing:
0.1% (w/v) BSA
0.05% (w/v) NaN_3
Store up to 6 months at 4°C

CAUTION: NaN_3 can potentially form explosive azides in plumbing. After treatment with bleach, all NaN_3-containing buffers should be disposed of by pouring down the sink with an excess of running tap water.

PBS-S

PBS (*APPENDIX 2A*) containing:
0.1% (w/v) saponin (add from 10× stock, see recipe)
1 mM $CaCl_2$
1 mM $MgSO_4$
0.05% (w/v) NaN_3
0.1% (w/v) BSA
10 mM HEPES
Adjust pH to 7.4 using 1 M NaOH
Store up to 6 months at 4°C

Saponin is a very light powder; handle so as to avoid spillage, aerosol formation, or inhalation.

PBS-S/milk solution

Add nonfat dry milk to PBS-S (see recipe) to make a 5% (w/v) solution and mix 15 min with a magnetic stirrer. Centrifuge 30 min at 15,000 × *g*, room temperature, to sediment milk solids, and retain supernatant, which should be clear or slightly opalescent. Store up to 6 months at 4°C.

RPMI-10 medium

RPMI 1640 medium (e.g., Invitrogen) supplemented with:
10% fetal bovine serum (FBS; *APPENDIX 2A*)
100 U/ml penicillin
100 μg/ml streptomycin
1 mM L-glutamine
1× nonessential amino acids
1 mM Na-pyruvate
Store up to 1 month at 4ºC

Saponin, 10×

Prepare 1% (w/v) saponin (Sigma) in PBS (*APPENDIX 2A*). Store up to 6 months at 4°C.

COMMENTARY

Background Information

ICCS originated with work done by Ulf and Jan Andersson at Stockholm University in the late 1980s. The Anderssons developed the PFA/saponin system, which had the advantage of leaving cellular antigens intact for immunodetection with minimal disruption of cell morphology or scatter. Subsequently, other investigators combined that method with flow cytometry, yielding the techniques in use today.

Advantages and limitations of ICCS

Flow cytometry is a single-cell technique: individual cells are analyzed as they pass through the laser beam in single file, with data typically expressed as the frequency of cytokine-expressing cells. Analogous single-cell cytokine techniques include ELISPOT (*UNIT 6.19*; Klinman, 2008), in situ hybridization, limiting dilution, and single-cell PCR. These other techniques can have significant drawbacks, such as requiring high technical proficiency, laborious manual scoring of data, or sophisticated image analysis. By taking advantage of the sophisticated flow cytometry instrumentation that is generally available to immunologists and coupling it with relatively straightforward immunostaining, one is able to overcome many of the limitations of these other techniques.

Advantages of ICCS

1. *Single-cell analysis.* Cytokine expression is analyzed at the single-cell level, giving an indication of both the frequency and intensity of production.

2. *Multiparameter analysis* allows simultaneous detection of multiple cytokines in a single cell and the detection of cytokine production within a rare or specific cell population.

3. As a *high-throughput technique*, ICCS increases the number of samples or subjects that can be studied.

4. *Minimal in vitro culture* is required. Unlike T cell cloning, cells can be studied with little or no in vitro culture, decreasing the potential for artifacts arising from prolonged in vitro culture.

Limitations of ICCS

1. Not all cells make a given cytokine synchronously and not all cytokines have the same expression kinetics.

2. Cytokine is often undetectable in cells examined without in vitro stimulation.

3. Anatomical information is lost in single-cell suspensions.

4. Seemingly minor populations of cells may dominate the outcome of complex biological responses. A dense population of cells making a specific cytokine should not be taken to indicate that the disease state will be determined by these cells.

5. The presence of a cytokine within cells does not necessarily mean that it would have been released, or that it would have exhibited biological effects in vivo.

Use of ICCS to examine T_H1/T_H2 differentiation

ICCS has become the method of choice to examine T_H1/T_H2 differentiation because it allows simple and direct measurement of multiple cytokines within a single cell. IFN-γ has been used as the signature T_H1 cytokine almost without exception. IL-4, IL-5 and IL-13 can all be used as T_H2 cytokines; IL-13 is the most technically straightforward T_H2 cytokine to measure and is recommended if a singleT_H2 signature cytokine is studied.

Use of ICCS with lineages other than T cells

Because of their diversity and central role in immunity, T cells are the cell type most often studied by ICCS. Other cell lineages can be similarly studied, although these may require different activation conditions as well as strategies to address autofluorescence and Fc-mediated binding. Monocytes and dendritic cells (Bueno et al., 2001; Jansen et al., 2008), natural killer cells (Mendes et al., 2000), neutrophils (Wang et al., 2008), and basophils (Devouassoux et al., 1999) have been studied using these methods.

Critical Parameters

ICCS is a reagent-driven technique. Detection of dim and low-frequency cytokines such as IL-4, IL-5, and IL-13 can only be optimized by using antibody probes with the highest signal-to-noise (S/N) ratio. Phycoerythrin (PE) and allophycocyanin (APC) conjugates provide superior S/N ratios and should be used for such cytokines. That said, for the most part, quality ICCS MAbs detecting T_H1 and T_H2 cytokines are available from many vendors. As such, we have not noted specific sources for each anti-cytokine MAb. Users are

encouraged to examine recent papers in their area of research to identify sources of MAbs. We have noted that one anti-human IL-4 MAb clone, 8D4, exhibits substantial nonspecific staining, and thus have avoided using it.

The use of phenotypic markers to isolate cytokine production within a specific cell population often improves the S/N ratio. For example, gating on CXCR3+ or CRTH2+ CD4 cells will dramatically increase the frequency of T_H1 and T_H2 cells, respectively, within that gate. Similarly, gating on CD154 as an early activation marker will enhance the detection of low-frequency antigen-specific T cells (Meier et al., 2008; Prussin et al., 2009). Dot plots are often more revealing than histograms for displaying rare events.

Many cytokines are not expressed in a sufficiently large enough number of cells to allow straightforward compensation using the anti-cytokine antibody itself. Although lysine compensation beads are increasingly preferred as a solution to this problem, bright cell surface marker MAbs (e.g., CD3, CD8) conjugated with the same fluorochrome as the anti-cytokine MAb can be used to compensate in place of the anti-cytokine antibody. All FITC, PE, PerCP, and APC conjugates require the same compensation as others with the same fluorochrome, since the compensation is a function of the dye and not the conjugate. Tandem dyes, such as PE/Cy5 and Brilliant Violet, have spectral characteristics that vary from lot to lot, and cannot be used in this way. To preserve valuable samples, compensations can be performed on fixed unstimulated PBMC rather than experimental samples. For this purpose, large numbers of human PBMC or murine splenocytes can be fixed in bulk, dispensed into aliquots, and cryopreserved. In other aspects, compensation is carried out as per Roederer (2002).

Investigators should acquire an appropriate number of cells on the flow cytometer to obtain statistically significant numbers of cytokine-positive events. Data comprising 100 positive events will have a coefficient of variation of 10%, which should be sufficient for most uses (Hedley and Keeney, 2013). PMA and ionomycin activation yields approximately 15% IFN-γ-positive and 4% IL-13-positive $CD4^+$ cells in healthy donors; therefore, a relatively low number of PBMC (e.g., 20,000 total events) yields a sufficient number of cytokine-positive events. In contrast, antigen-specific activation typically yields frequencies from 0.001% to 0.10%, and therefore requires correspondingly greater numbers of acquired events.

Virtually all published protocols for ICCS use an aldehyde-based fixative such as formaldehyde or paraformaldehyde (PFA). Aldehyde fixatives act to immobilize the cytokine within the cell, preventing their loss after permeabilization. Twenty years after the initial reports, PFA/saponin-based ICCS remains the system of choice.

The paraformaldehyde/saponin fixation/permeabilization detailed in this chapter efficiently detects cytoplasmic antigens, but not nuclear antigens, such as transcription factors. Commercially available kits for transcription factor (e.g., Foxp3) or nuclear staining will allow for simultaneous staining of transcription factors and cytokines. However, these nuclear staining buffers do not provide as high a quality of cytokine staining as the paraformaldehyde/saponin system detailed within.

Although investigators will no doubt wish to study other activation conditions and cytokines than those detailed in this unit, it is strongly advised that novices start with the detection of an abundant cytokine such as IFN-γ or IL-2 using PMA/ionomycin activation.

Detailed conditions for activation with specific antigen and PMA/ionomycin activation are contained in this unit; however, these protocols will work with any number of stimuli including anti-CD3, lectin, antigens (Waldrop et al., 1997; Kagina et al., 2015), peptide antigens (Maecker et al., 2001; Sylwester et al., 2005; McDermott et al., 2014), allergens (Devouassoux et al., 1999; Prussin et al., 2009), and innate immune activators (Bueno et al., 2001; Jansen et al., 2008; Wang et al., 2008). Antigen-induced T cell cytokine is often more easily detected when a costimulation is added in the form of anti-CD28 ± anti-CD49d (Waldrop et al., 1997).

The protocols in this unit are designed for the study of red blood cell–depleted cell suspensions; however, whole-blood techniques have also been published (Lovelace and Maecker, 2011). A number of proprietary whole-blood fix/lyse/perm reagents are commercially available. These kits work well for many applications and have distinct advantages in regard to time requirements and workflow. However, such whole blood kits typically result in lower-fidelity staining than that found using this protocol.

ICCS requires the presence of an inhibitor of intracellular transport, either brefeldin A

(BFA) or monensin. These agents block intracellular trafficking, thereby increasing the amount of intracellular cytokine to be detected. For specific activation conditions and cytokines, one or the other may have an advantage. Most investigators prefer BFA because monensin is a Na^+/H^+ ionophore, which can induce cytokine expression in some cell types.

Both monensin and brefeldin A are toxic to cells and generally should not be left in cultures for >12 to 16 hr. Maximal cytokine accumulation occurs within 2 to 4 hr after the addition of these inhibitors, so there is little need for prolonged culture. Rather, they should be added for 2 to 4 hr at the end of culture. Although 6 hr was used here as an optimal time point to examine IFN-γ, IL-4, IL-5, and IL-13 after PMA/ionomycin activation, longer incubations may be needed for other cytokines, such as IL-10. However, with longer incubations, the amount of cell death increases in the culture, thus adding to the background noise and possibly negating the improvements in signal obtained from prolonged culture.

Both CD69 and CD154 are up-regulated on T cells within 4 hr of activation. Antigen-specific cytokine detection is enhanced by first gating on such activation markers. CD154 is generally favored, as it is more specific for antigen-activated cells and stains brighter (Frentsch et al., 2005; Kirchhoff et al., 2007; Meier et al., 2008; Lovelace and Maecker, 2011). When used in conjunction with ICCS, both CD69 and CD154 are best detected as intracellular antigens.

Alternative methods

Alternatives allowing flow cytometric cytokine detection include the magnetic-assisted cell sorting (MACS) cytokine secretion assay, in which cell surface bound MAb captures secreted cytokine with subsequent detection using fluorochrome-labeled MAbs (see *UNIT 6.27*; Assenmacher et al., 2002). Unlike ICCS, the cytokine secretion assay allows for bead enrichment of cytokine expressing cells and results in viable cells that can be used for cell culture or analysis of mRNA. Recently, several flow based RNA assays have been developed (Affymetrix/eBioscience PrimeFlow, EMD Millipore SmartFlare). These latter techniques are just beginning to be applied to cytokine biology.

Tetramer staining similarly detects antigen-specific T cells by flow cytometry and has the advantage of allowing the analysis of living cells without the requirement for cell activation. ICCS has much greater flexibility than tetramer staining, in allowing studies with complex antigens on any MHC background. ICCS can be used in conjunction with tetramers, to examine the cytokine phenotype of antigen specific cells (Sylwester et al., 2005).

ELISPOT has a similar capacity to determine the frequency of cytokine-producing cells. Because of its multiparameter capacity, ICCS has a greater capability for analysis of complex cytokine phenotypes and greater availability of instrumentation.

Kinetics of cytokine expression

ELISA measures the accumulation of cytokine secreted into the cell culture supernatant over the incubation period. ICCS measures cytokine production at a specific time point and for individual cells, without such summation. Thus, no one time point will detect every cytokine-producing cell. Similarly, when examining more than one cytokine, a single time point may not be optimal for all cytokines examined. Maximal T cell intracellular cytokine accumulation occurs rapidly after cell activation, often within the first 4 to 12 hr, depending on the cytokine examined. A common mistake novices make is to use the 48-hr time point typical for ELISA. With PMA/ionomycin activation, peripheral blood T cells exhibit optimal immunostaining for IL-4 and IL-5 after 4 to 6 hr, and for IL-2 and IFN-γ after 8 to 10 hr. Published studies have generally used a 4- to 6-hr incubation, as it yields optimal results for this combination of cytokines within a workable time frame. It is important to select a culture duration that optimizes detection of the cytokines being studied.

Specificity

For cell-surface staining, specificity is demonstrated using isotype-matched controls, and, indeed, this approach can be used for intracellular staining. However, differences in nonspecific binding between the anti-cytokine MAb and isotype control are magnified by the inherent stickiness of the fixed cells, such that some isotype-matched controls may not yield a useful control. Selected isotype-matched controls that have been shown to exhibit similar levels of nonspecific binding to the anti-cytokine MAb provide a reasonable control, once specificity has been demonstrated using one or both of the more rigorous methods described in Support Protocol 5. However, in critical situations, the veracity of recombinant cytokine and unlabeled antibody-blocked

controls is superior to that of simple isotype-matched controls.

Troubleshooting

Obtaining high S/N ratio results requires an investment in optimizing both signal and noise. Signal can be maximized by optimizing activation conditions, duration of activation, anti-cytokine MAb selection, MAb concentration, and choice of fluorochrome. Because fixation and permeabilization increase noise relative to cell-surface staining, strategies to decrease noise were necessary for the development of the technique, and are incorporated into the Basic Protocol. A thorough understanding of the factors contributing to noise and the ability to evaluate each of them is critical. Autofluorescence may increase after aldehyde fixation, and is best evaluated by running unstained fixed cells through a flow cytometer and comparing the results to either fixed or unfixed cells of known low autofluorescence (e.g., lymphocytes). Both cell death and prolonged fixation should be avoided, as these conditions may increase autofluorescence and nonspecific binding. Effective permeabilization of cells can be verified by staining with an antibody to vimentin or actin.

Nonspecific binding, the tendency for cells to be sticky and bind antibody through low-affinity non-receptor-mediated interactions, can be evaluated using either isotype-matched controls or blocked controls (see Support Protocol 5). Fc-mediated binding generally is not a problem in T cell ICCS. Some investigators concerned about Fc binding add either anti-Fc receptor MAbs or purified IgG prior to the addition of anti-cytokine MAbs. The degree of nonspecific or Fc-mediated binding can be assessed by comparing the staining of fixed, unstained cells to that of isotype-control stained cells or to the negative peak of the cytokine stained cells. The increase in fluorescence resulting from nonspecific or Fc-mediated binding should be no more than two to three-fold greater than that due to autofluorescence—i.e., the mean fluorescence intensity of the isotype control is <2 to 3 times than that of unstained cells. This assumes negligible increases in autofluorescence resulting from fixation (see above).

Because of the increased nonspecific binding associated with intracellular staining, the range of optimal MAb staining concentrations is relatively narrow. Too little MAb yields poor signal, and too much increases nonspecific binding and noise. All MAbs used should be carefully titrated to obtain an optimal concentration, which is typically within the range from 0.5 to 5 μg/ml (Stewart and Stewart, 1997; Hulspas, 2010).

Rarely, some antigenic epitopes may be altered or destroyed by fixation, rendering them undetectable by certain MAb clones. If, after PFA fixation, a specific MAb does not stain, the simplest approach is to use an alternative clone that recognizes a different (PFA insensitive) epitope. Alternatively, for cell surface phenotypic markers, cell surface staining can be performed before fixation (Support Protocol 4).

After in vitro activation, expression of cell-surface markers may be altered. In particular, after PMA exposure, a large fraction of the CD4 molecules are internalized. As such, staining for intracellular CD4 simultaneously with cytokines yields a greater staining index when compared to cell-surface staining prior to fixation. Detection of human CD4 after PMA activation is highly dependent on the specific clone; clones SK3 and Q4120 consistently yield superior results when compared to other clones that the authors have screened. After physiologic activation, intracellular staining of CD3, CD4, and CD8 is minimally altered.

Anticipated Results

The light scatter of paraformaldehyde-fixed preparations of splenocytes or PBMC appears very close to that of unfixed cells, with good differentiation of lymphocytes and monocytes/macrophages.

Results obtained using ICCS should generally agree with results obtained with other assay systems such as ELISPOT, ELISA, or in situ hybridization. Results that seem counter-intuitive or in disagreement with other assays should be scrutinized using blocking controls (see Support Protocol 2). Fixed, fresh ex vivo T cells from unmanipulated mice or healthy human donors demonstrate few ($<0.002\%$) cytokine-positive cells when stained with antibodies to IL-2, IL-4, IL-5, IL-10, IL-13, or IFN-γ, and can be used as negative controls. When stimulated with PMA/ionomycin, fresh ex vivo T cells demonstrate a defined range of expression of cytokine production, much as frequencies of $CD4^+$ and $CD8^+$ T cell subsets have a normal range (Prussin and Metcalfe, 1995). Results differing greatly from these values should be scrutinized.

Abundantly expressed cytokines such as IFN-γ and IL-2 often stain as a bimodal population, whereas less bright cytokines such as

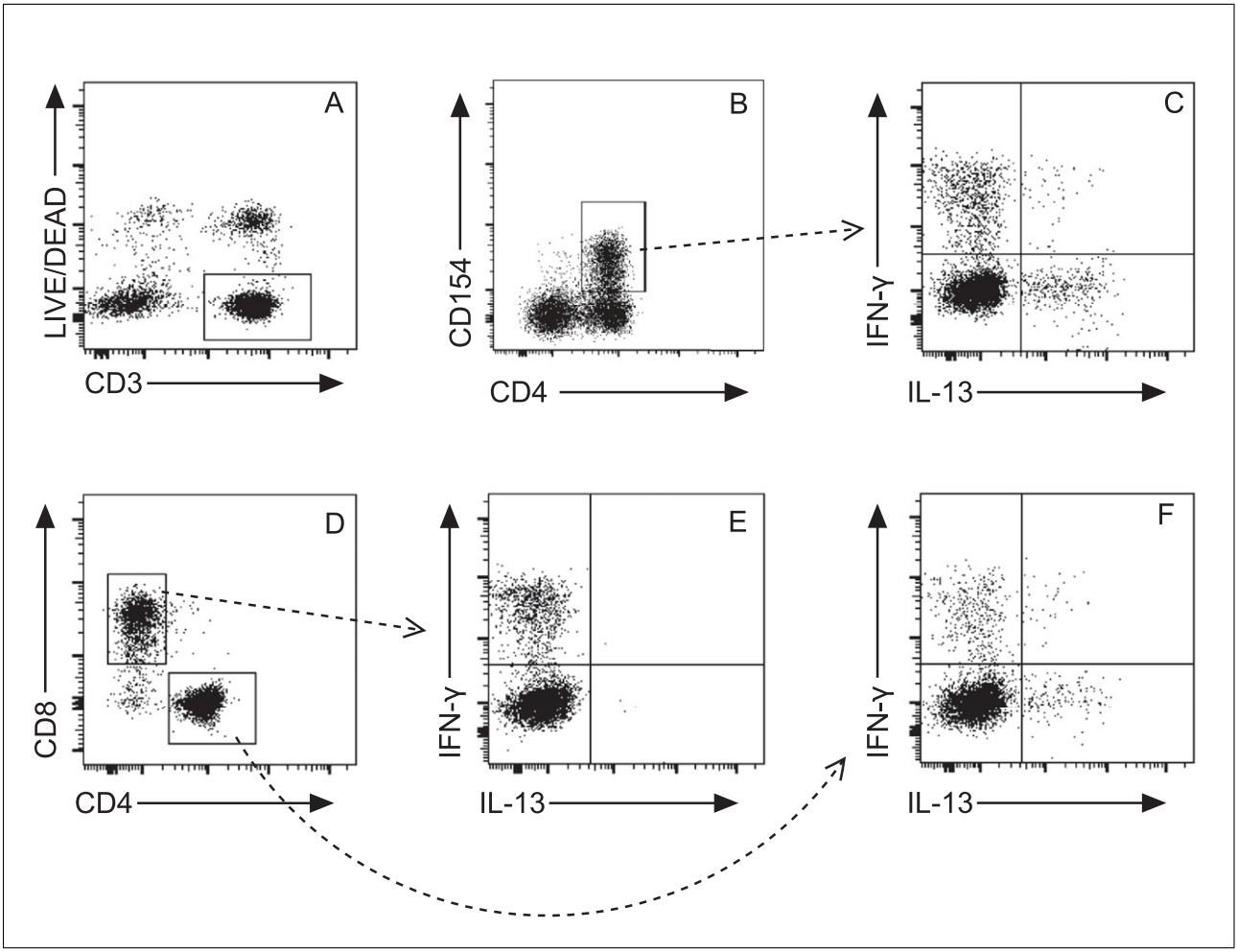

Figure 6.24.1 Intracellular cytokine staining of superantigen-activated PBMC. PBMC were activated, stained with violet LIVE/DEAD, PFA fixed, and stained with MAbs to CD3, CD4, CD8, CD154, IL-13, and IFN-γ. (**A**) After gating on viable CD3+ lymphocytes, the CD4 versus CD8 dot plot in (**D**) was generated. (**E**,**F**) Cytokine staining of CD8 and CD4 T cells, respectively. (**B**) After initially gating on viable CD3+ lymphocytes, a CD4 versus CD154 dot plot was generated. (**C**) Cytokine staining of CD4+, CD154+ cells. Rectangles indicate gated populations; dotted lines indicate cytokine staining of specific gated populations.

IL-4 and IL-13 may stain as a shoulder beyond the negative peak. In both cases, placement of statistical markers using isotype-matched or blocked controls allows the determination of the frequency of cells staining specifically for a given cytokine.

Examples of flow cytometric results obtained with human cells activated with SEB are shown in Figure 6.24.1.

Time Considerations

Stimulation and fixation are typically performed on one 6- to 10-hr day, followed by staining the next day. Alternatively, cryopreservation of stimulated and fixed cells (see Support Protocol 3) allows more flexibility in experimental design and work schedule. Cryopreserved fixed cells can be stored for several years at −80°C and still provide excellent results. Staining typically takes 1 hr longer than for traditional flow cytometry, ∼3 hr total.

Acknowledgements

Support for this work was provided by the NIAID Division of Intramural Research, National Institutes of Health.

Conflict of Interest

The authors have no conflicts of interest.

Literature Cited

Assenmacher, M., Löhning, M., and Radbruch, A. 2002. Detection and isolation of cytokine secreting cells using the cytometric cytokine secretion assay. *Curr. Protoc. Immunol.* 46:6.27.1-6.27.10.

Bueno, C., Almeida, J., Alguero, M.C., Sanchez, M.L., Vaquero, J.M., Laso, F.J., San Miguel,

J.F., Escribano, L., and Orfao, A. 2001. Flow cytometric analysis of cytokine production by normal human peripheral blood dendritic cells and monocytes: Comparative analysis of different stimuli, secretion-blocking agents and incubation periods. *Cytometry* 46:33-40.

Devouassoux, G., Foster, B., Scott, L.M., Metcalfe, D.D., and Prussin, C. 1999. Frequency and characterization of antigen-specific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *J. Allergy Clin. Immunol.* 104:811-819.

Frentsch, M., Arbach, O., Kirchhoff, D., Moewes, B., Worm, M., Rothe, M., Scheffold, A., and Thiel, A. 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat. Med.* 11:1118-1124.

Fuss, I.J., Kanof, M.E., Smith, P.D., and Zola, H. 2009. Isolation of whole mononuclear cells from peripheral blood and cord blood. *Curr. Protoc. Immunol.* 85:7.1.1-7.1.8.

Hedley, B.D. and Keeney, M. 2013. Technical issues: Flow cytometry and rare event analysis. *Int. J. Lab. Hematol.* 35:344-350.

Holmes, K.L., Otten, G. and Yokoyama, W.M. 2002. Flow cytometry analysis using the Becton Dickinson FACS Calibur. *Curr. Protoc. Immunol.* 49:5.4.1-5.4.22.

Hulspas, R. 2010. Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells. *Curr. Protoc. Cytom.* 54:6.29.1-6.29.9.

Jansen, K., Blimkie, D., Furlong, J., Hajjar, A., Rein-Weston, A., Crabtree, J., Reikie, B., Wilson, C., and Kollmann, T. 2008. Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation. *J. Immunol. Methods* 336:183-192.

Kagina, B.M., Mansoor, N., Kpamegan, E.P., Penn-Nicholson, A., Nemes, E., Smit, E., Gelderbloem, S., Soares, A.P., Abel, B., Keyser, A., Sidibana, M., Hughes, J.E., Kaplan, G., Hussey, G.D., Hanekom, W.A., and Scriba, T.J. 2015. Qualification of a whole blood intracellular cytokine staining assay to measure mycobacteria-specific CD4 and CD8 T cell immunity by flow cytometry. *J. Immunol. Methods* 417:22-33.

Kirchhoff, D., Frentsch, M., Leclerk, P., Bumann, D., Rausch, S., Hartmann, S., Thiel, A., and Scheffold, A. 2007. Identification and isolation of murine antigen-reactive T cells according to CD154 expression. *Eur. J. Immunol.* 37:2370-2377.

Klinman, D. 2008. ELISPOT assay to detect cytokine-secreting murine and human cells. *Curr. Protoc. Immunol.* 83:6.19.1-6.19.9.

Kruisbeek, A. M. 2000. Isolation of mouse mononuclear cells. *Curr. Protoc. Immunol.* 39:3.1.1-3.1.5.

Kruisbeek, A.M., Shevach, E., and Thornton, A.M. 2004. Proliferative assays for T cell function. *Curr. Protoc. Immunol.* 60:3.12.1-3.12.20.

Lamoreaux, L., Roederer, M., and Koup, R. 2006. Intracellular cytokine optimization and standard operating procedure. *Nat. Protoc.* 1:1507-1516.

Lovelace, P. and Maecker, H.T. 2011. Multiparameter intracellular cytokine staining. *Methods Mol. Biol.* 699:165-178.

Maecker, H.T., Dunn, H.S., Suni, M.A., Khatamzas, E., Pitcher, C.J., Bunde, T., Persaud, N., Trigona, W., Fu, T.M., Sinclair, E., Bredt, B.M., McCune, J.M., Maino, V.C., Kern, F., and Picker, L.J. 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J. Immunol. Methods* 255:27-40.

Maecker, H.T., Rinfret, A., D'Souza, P., Darden, J., Roig, E., Landry, C., Hayes, P., Birungi, J., Anzala, O., Garcia, M., Harari, A., Frank, I., Baydo, R., Baker, M., Holbrook, J., Ottinger, J., Lamoreaux, L., Epling, C.L., Sinclair, E., Suni, M.A., Punt, K., Calarota, S., El-Bahi, S., Alter, G., Maila, H., Kuta, E., Cox, J., Gray, C., Altfeld, M., Nougarede, N., Boyer, J., Tussey, L., Tobery, T., Bredt, B., Roederer, M., Koup, R., Maino, V.C., Weinhold, K., Pantaleo, G., Gilmour, J., Horton, H., and Sekaly, R.P. 2005. Standardization of cytokine flow cytometry assays. *BMC Immunol.* 6:13.

McDermott, D.S., Knudson, C.J., and Varga, S.M. 2014. Determining the breadth of the respiratory syncytial virus-specific T cell response. *J. Virol.* 88:3135-3143.

Meier, S., Stark, R., Frentsch, M., and Thiel, A. 2008. The influence of different stimulation conditions on the assessment of antigen-induced CD154 expression on CD4+ T cells. *Cytometry A* 73:1035-1042.

Mendes, R., Bromelow, K.V., Westby, M., Galea-Lauri, J., Smith, I.E., O'Brien, M.E., and Souberbielle, B.E. 2000. Flow cytometric visualisation of cytokine production by CD3-CD56+ NK cells and CD3+CD56+ NK-T cells in whole blood. *Cytometry* 39:72-78.

Muul, L.M., Heine, G., Silvin, C., James, S.P., Candotti, F., Radbruch, A. and Worm, M. 2011. Measurement of proliferative responses of cultured lymphocytes. *Curr. Protoc. Immunol.* 94:7.10.1-7.10.26.

Prussin, C. and Metcalfe, D.D. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol. Methods* 188:117-128.

Prussin, C., Lee, J., and Foster, B. 2009. Eosinophilic gastrointestinal disease and peanut allergy are alternatively associated with IL-5+ and IL-5(-) T(H)2 responses. *J. Allergy Clin. Immunol.* 124:1326-1332.

Roederer, M. 2002. Compensation in flow cytometry. *Curr. Protoc. Cytom.* 22:1.14.1-1.14.20.

Stewart, C.C. and Stewart, S.J. 1997. Titering antibodies. *Curr. Protoc. Cytom.* 00:4.1.1-4.1.13.

Strober, W. 1997. Monitoring cell growth. *Curr. Protoc. Immunol.* 21:A.3A.1-A.3A.2.

Sylwester, A.W., Mitchell, B.L., Edgar, J.B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P.R., Grabstein, K.H., Hosken, N.A., Kern, F., Nelson, J.A., and Picker, L.J. 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J. Exp. Med.* 202:673-685.

Waldrop, S.L., Pitcher, C.J., Peterson, D.M., Maino, V.C., and Picker, L.J. 1997. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: Evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J. Clin. Invest.* 99:1739-1750.

Wang, J.P., Bowen, G.N., Padden, C., Cerny, A., Finberg, R.W., Newburger, P.E., and Kurt-Jones, E.A. 2008. Toll-like receptor-mediated activation of neutrophils by influenza A virus. *Blood* 112:2028-2034.