Activation and Intracellular Staining of Whole Blood: For the Detection of Intracellular Cytokines and Other Intracellular Targets

BioLegend, Inc.

Abstract

This is part of BioLegend's, "Intracellular Flow Cytometry Staining Protocol: For the Detection of Intracellular Cytokines and Other Intracellular Targets".

Citation: BioLegend, Inc. Activation and Intracellular Staining of Whole Blood: For the Detection of Intracellular Cytokines and Other Intracellular Targets. **protocols.io**
dx.doi.org/10.17504/protocols.io.ez3bf8n
Published: 31 May 2016

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.

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.

Note

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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.

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.

Reagent List
1. Cell Staining Buffer (BioLegend Cat. No. 420201)
2. Monensin (BioLegend Cat. No. 420701)
3. RBC Lysis Buffer (BioLegend Cat. No. 420301)
4. Brefeldin A (BioLegend Cat. No. 420601)
5. Fixation Buffer (BioLegend Cat. No. 420801)
6. Intracellular Staining Perm Wash Buffer (BioLegend Cat. No. 421002)
7. Cyto-Last™ Buffer (BioLegend Cat. No. 422501)

Materials

Cell Staining Buffer 420201 by BioLegend

RBC Lysis Buffer 420301 by BioLegend

Fixation Buffer 420801 by BioLegend

Monensin 420701 by BioLegend

Brefeldin 1 420601 by BioLegend

Intracellular Staining Perm Wash Buffer 421002 by BioLegend

Cyto-Last Buffer 422501 by BioLegend

Protocol

Activation

Step 1.
Dilute heparinized whole blood 1:1 with sterile appropriate tissue culture medium.

Activation

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Step 2.
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 (BioLegend Cat. No. 420601) or monensin (BioLegend Cat. No. 420701) is critical.

Activation

Step 3.

After addition of a suitable cellular activator, aliquot 200 µl 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.

DURATION
04:00:00

Activation

Step 4.
Add 2 ml of 1X Red Blood Cell Lysis Buffer (Cat. No. 420301) and incubate for 5-10 minutes at room temperature.

DURATION
00:05:00

Activation

Step 5.
Centrifuge at 350 x g for 5 minutes and discard the supernatant.

DURATION
00:05:00

Activation

Step 6.

Wash cells 1X with Cell Staining Buffer.

Fixation

Step 7.

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 (BioLegend Cat. No. 420801) in the dark for 20 minutes at room temperature.

DURATION
00:20:00

Fixation

Step 8.
Centrifuge at 350 x g for 5 minutes, discard supernatant.

DURATION
00:05:00

Fixation

Step 9.

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To put the experiment “on hold” at this point for future staining and analysis, wash cells 1x with Cell StainingBuffer (BioLegend Cat. No. 420201). 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).

NOTES

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Alternatively, cells can be kept in Cyto-Last™ Buffer (BioLegend Cat. No. 422501) for the storage of cytokineproducingcells for up to two weeks.

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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

Step 10.

Dilute 10X Intracellular Staining Perm Wash Buffer (Cat. No. 421002) to 1X in DI water.

Permeabilization

Step 11.

Resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 350 x g for 5-10 minutes. (1/3)

DURATION

00:05:00

Permeabilization

Step 12.

Resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 350 x g for 5-10 minutes. (2/3)

DURATION

00:05:00

Permeabilization

Step 13.

Resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 350 x g for 5-10 minutes. (3/3)

DURATION

00:05:00

Intracellular Staining

Step 14.

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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.

DURATION
00:20:00

Intracellular Staining

Step 15.

Wash with 2 ml of Intracellular Staining Perm Wash Buffer and

centrifuge at 350 x g for 5 minutes. (1/2)

DURATION
00:05:00

Intracellular Staining

Step 16.

Wash with 2 ml of Intracellular Staining Perm Wash Buffer and

centrifuge at 350 x g for 5 minutes. (2/2)

DURATION
00:05:00

Intracellular Staining

Step 17.

If primary intracellular antibody is biotinylated, it will be necessary to perform fluorophore conjugatedStreptavidin incubations and subsequent washes in Intracellular Staining Perm Wash Buffer.

Intracellular Staining

Step 18.

Resuspend fixed and intracellularly labeled cells in 0.5 ml Cell Staining Buffer and analyze with appropriate controls.

NOTES
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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 fluorophore-conjugated anti-cytokine antibody before its addition to the cells.

Flow Cytometric Analysis

Step 19.

Set PMT voltage and compensation using cell surface staining controls. Set quadrant markers based

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on blocking controls, isotype controls, or unstained cells.

NOTES

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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.

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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.

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