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PR07: Pro5® MHC Pentamer Staining Protocol for Co-Staining with Intracellular Proteins

Introduction

Co-staining $CD8^+$ T cells with Pro5® MHC Pentamers and antibodies against intracellular cytokines has now become a routine procedure in many laboratories. This technique not only enables the frequency of antigen-specific T cells to be determined but also their effector function.

Materials Required

- Pro5® Recombinant MHC Pentamer conjugated to your fluorescent label of choice (e.g. R-PE). **Ensure that the stock Pentamer is stored consistently at 4°C in the dark, with the lid tightly closed.**
- Anti-cytokine antibody conjugated to a different fluorescent label (e.g. anti-Human IFNγ-FITC; ProImmune)
- Anti-CD8 antibody
- Peptide (stock at 10mM in DMSO - may be aliquotted and stored at -20ºC)
- Leukocyte activation cocktail (LAC; optional) for use as a control for IFNγ and TNFα production (BD Biosciences #550583 - may be aliquotted and stored at -80ºC)
- Staphylococcal Enterotoxin B (SEB; optional) for use as a control for IL-2, TNFα and Granzyme B production (Sigma S4881 – a 10mg/ml stock made up in 1x sterile PBS may be aliquotted and stored at –20ºC)
- PBS Wash (1% BSA, 0.1% sodium azide in PBS)
- Permeabilization buffer (0.1% saponin, 1% BSA, 0.1% sodium azide in PBS)
- Fix 1 solution (4% Paraformaldehyde in PBS)
- Fix 2 solution (1% BSA, 2.5% formaldehyde in PBS)
- Complete RPMI medium (500 ml RPMI-1640 supplemented with 5 ml Penicillin/Streptomycin/L-Glutamine (100x) and 37.5 ml fetal calf serum)
- Brefeldin A (Sigma #15870)

Protocol for intracellular staining of IFNγ, TNFα, Granzyme B or IL-2

All procedures up until step 10 should be carried out using sterile buffers and aseptic conditions.

1. Centrifuge Pro5® Pentamer in a chilled microcentrifuge at 14,000 *x g* for 5 minutes.
2. Prepare peripheral blood cells in PBS Wash at a cell concentration of 2×10^7 cells/ml.
3. Transfer the cell suspension to individual tubes in 50 µl aliquots.
4. Add relevant titrated fluorescently-labeled Pentamers to the desired tubes, and incubate for 10 min at 22ºC (non-stimulated single-color controls should not be stained at this stage). Add 10 µl PBS Wash to remaining tubes.
5. Add 500 µl PBS Wash to each tube. Centrifuge at $450 \times g$ for 5 minutes at 10°C.
6. Aspirate supernatant. Agitate to disrupt cell pellets and resuspend in 200 µl complete RPMI.
7. Dilute peptide stock in complete RPMI. Add sufficient to give a 5µM final* concentration to each tube. If using LAC as a control, rapidly thaw this at 37°C in a water bath and add 0.33 µl of this to each desired tube. If using SEB, defrost and prepare a 1mg/ml stock by addition of sterile PBS, and add 0.2 µl of this to each desired tube.

   Note: After use, discard the unused thawed controls.
8. Place the tubes at 37°C in a humidified CO_2 incubator for 15 minutes to 1 hour.
9. Add Brefeldin A (10 µg/ml final) to the desired tubes (n.b. LAC contains Brefeldin A, so LAC-stimulated samples do not require the addition of Brefeldin A) and return to the incubator. Incubate for 15 hours†.

10. Remove tubes from the incubator. Centrifuge at 400 × *g* for 5 minutes at 10°C.
11. Aspirate supernatant. Resuspend desired cell pellets in 50 µl PBS Wash containing an optimally titrated amount of anti-CD8 antibody. Add 50 µl PBS Wash to remaining tubes.

Note: Single-color controls should be stained at this stage. If additional phenotyping of samples is desired, antibodies to other cell surface receptors may also be added at this time.

12. Incubate for 20 minutes on ice.
13. Add 500 µl PBS Wash to each tube. Centrifuge at 450 × *g* for 5 minutes at 10°C.
14. Aspirate supernatant. Agitate to disrupt cell pellets.
15. Add 200 µl Fix 1 solution to each sample tube. Vortex tubes. Incubate for 20 minutes on ice. This step will fix the cell morphology of the activated cells.

Note: The procedure can be stopped at this point. Repeat steps 13 and 14. Resuspend the cells in 100 µl/tube PBS Wash. Cover and store the cells at 4°C for up to 3 days. To proceed, repeat steps 13 and 14. Resuspend the cells in 200 µl/tube permeabilization buffer and proceed to step 17.

16. Add 200 µl permeabilization buffer to each tube.
17. Centrifuge at 450 × *g* for 5 minutes at 10°C. Aspirate supernatant.
18. Add 100 µl permeabilization buffer to the sample tubes that are to be stained with anti-cytokine antibody. Add 100 µl PBS Wash to the remaining tubes (i.e. Single-color controls).
19. Incubate for 5 minutes at room temperature.
20. Add an optimally titrated amount[§] of fluorochrome-conjugated anti-cytokine antibody to the desired sample tubes and mix.
21. Incubate for 20 minutes at room temperature.
22. Add 200 µl permeabilization buffer to each tube and centrifuge at 450 × *g* for 5 minutes at 10°C. Aspirate supernatant and agitate tubes to disrupt the cell pellets.
23. Resuspend the cells in 200 µl Fix 2 solution. Vortex tubes. It is important to vortex well when adding this fixative so that cells do not clump.
24. Incubate (protected from light) the intracellular cytokine staining tubes for 5 minutes at room temperature, and the control tubes for at least 1 hour at 4°C before analysis.
25. The samples are now ready for data acquisition and analysis on a flow cytometer but may be stored overnight at 4°C in the dark prior to analysis.

Background

Production of cytokines plays an important role in the immune response. Examples include the induction of many anti-viral proteins by IFNγ, the induction of T cell proliferation by IL-2 and the inhibition of viral gene expression and replication by TNFα. Cytokines are not preformed factors; instead they are rapidly produced upon relevant stimulation. Intracellular cytokine staining relies upon the stimulation of T cells in the presence of an inhibitor of protein transport thus retaining the cytokines inside the cell.

Cellular activation to trigger cytokine production generally results in down-regulation of the T cell receptor. For this reason, Pentamer staining is carried out prior to activation to ensure a good level of staining. The Pentamers may be internalized with the T cell receptor during this period, but can still be detected in permeabilized cells.

To analyze the effector function of antigen-specific T cells, the cells are first stained with Pentamer, then stimulated with antigen. This is followed by staining with antibodies specific for extracellular epitopes (such as CD8), then by membrane permeabilization and intracellular cytokine staining. We outline above a protocol applicable for Pentamer co-staining with anti-IFNγ, TNFα, or IL-2. Please note that certain cytokines such as IL-4 and IL-10 are difficult to detect by intracellular staining methods. In addition to this, frequencies of Pentamer positive cells producing these cytokines may also be extremely low.

Protocol Optimization

The detection of rare, cytokine-producing, antigen-specific T cells by Pro5® Pentamer staining is a powerful and versatile tool for immunological analysis. However, success requires both the design of suitably controlled experiments as well as a well-maintained flow cytometer. The binding affinity of the MHC molecule for the TCR varies depending on the allele/peptide combination. This means that different MHC complexes, and different cytokines will have slightly different characteristics in the way they stain. The following guidelines will assist with protocol optimization for the best possible results:

† ***Incubation period*** The optimal stimulation period for induction of a given cytokine is variable and has to be determined. The incubation period of 16 hours described above was determined to be optimal for human PBMCs stimulated with 10 µg/ml peptide to induce an IFNγ response. The incubation period will vary dependent on the method of stimulation and the cytokine response you wish to determine. For Granzyme B, a 5-hour incubation may be sufficient, and for IL-2 a longer incubation, of 24 hours, may give optimal results. For mouse splenocytes, the best IFNγ response is seen after a 24 hour incubation.

*** *Peptide concentration*** Investigate the effect on cytokine response of titrating your peptide.

Pentamer staining Cellular activation to trigger cytokine production also results in the down-regulation of the T cell receptor, which can make it difficult to subsequently perform Pentamer staining. For this reason, we recommend Pentamer staining prior to activation. However, in some cases where the incubation period is short (< 5 hours), you may prefer to incubate activated cells with Pentamer at stage 11 instead of at stage 3.

§ ***Antibody concentrations*** Investigate the effect of titrating your anti-CD8 and anti-cytokine antibodies. Titration of anti-CD8 will prevent any antibody-mediated blocking of the Pro5® Pentamer-binding site.

Protein Transport Inhibitor Brefeldin A is an inhibitor of intracellular protein transport. Incubation of cells in culture with Brefeldin A leads to blockade of protein transport to the Golgi complex and accumulation of proteins in the endoplasmic reticulum. Brefeldin A is effective for enhanced detection of a majority of intracellular cytokines; however, it is advised that you investigate the use and efficacy of this reagent as well as other protein transport inhibitors such as Monensin in your specific assay system.

Background staining When performing Pentamer staining, if the background staining is unusually high, it may be necessary to first block the cells with Fc block or 10% mouse serum prior to Pentamer staining. Further suggestions for reduction of background staining can be found on our website.

References

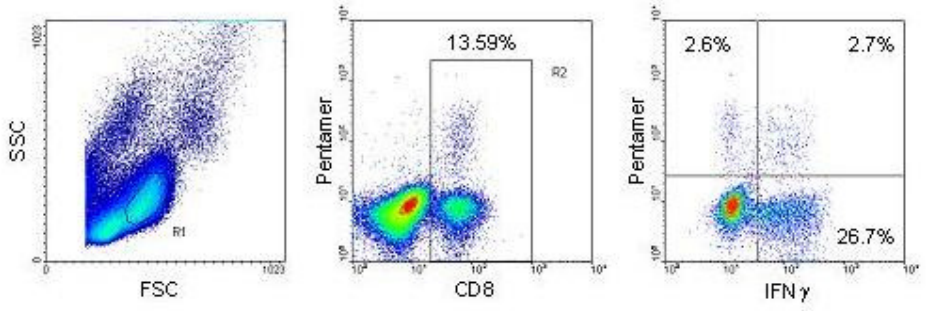
Appay, V. *et al*. 2000. HIV-specific $CD8^+$ T cells produce antiviral cytokines but are impaired in cytolytic function. J Exp. Med. 192: 63-75.

Appay, V. and Rowland-Jones, S. 2002. The assessment of antigen-specific $CD8^+$ T cells through the combination of MHC class I tetramer and intracellular staining. J. Immunol. Methods 268: 9-19.

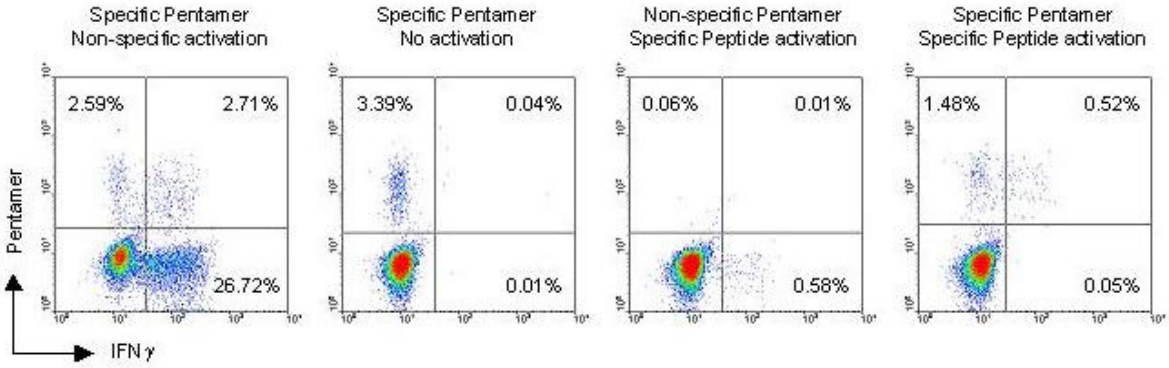
Example staining data

1. Gating strategy

- Create a 2D density plot showing FSC vs. SSC. Draw a tight region around the lymphocyte population (R1).
- Create a second density plot, gated only on events encircled within R1, showing CD8 staining on the x-axis and Pentamer staining on the y-axis. Draw a region around all of the CD8-positive cells (R2).
- Create a third density plot, gated on both R1 and R2, showing cytokine staining on the x-axis and Pentamer staining on the y-axis. Draw a quadrant that clearly separates positive cells from negative cells on both axes, and use quadrant statistics to obtain quantitative information about the frequency of antigen-specific cytokine-positive T lymphocytes.



2. Example figures



The figures illustrate IFNγ versus Pentamer staining of live lymphocytes. PBMCs were incubated with either a negative control (non-specific) Pentamer (A*0201/EBV (GLCTLVAML)) or a Pentamer specific for the cells of interest (B*0801/EBV (RAKFKQLL)), then stimulated with LAC (non-specific activation) or B*0801/EBV peptide (specific peptide activation) for 15 hours in the presence of Brefeldin A. Fixation, permeabilization and staining for IFNγ were carried out exactly as detailed in the protocol.

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