# Simultaneous detection of murine antigen-specific intracellular cytokines and CD107a/CD107b by flow cytometry

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**Method Article**

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# Abstract

# Introduction

Assays to detect cytokine production and degranulation are useful measures of T cell function. Multiparametric flow cytometry makes it possible to precisely phenotype and simultaneously detect these intermediate stages of T cell activation.[1,2] The degranulation of cytotoxic cells, an indicator of cytotoxic potential, results in the transient surface expression of CD107a and CD107b \(lysosomal associated membrane proteins).[3] A protocol for the _ex vivo_ assessment of antigen-specific cytokine production by mouse CD4+ and CD8+ T lymphocytes, and the simultaneous presence of CD107a and CD107b, by polychromatic flow cytometry is presented here. In this protocol, C57BL/6 or Balb/c mice were immunized with a live-attenuated strain of _Listeria monocytogenes_ \(actA-Lm). Splenocytes were restimulated _in vitro_ with peptide from listeriolysin \(LLO91-99 for CD8+ \(Balb/c), LLO190-201 for CD4+ \(C57BL/6)) or PMA plus ionomycin. A typical surface staining cocktail consisted of CD4-APC, and CD8-PerCP-CY5.5 and a typical intracellular staining cocktail consisted of IFN-γ-PE and TNF-α-PE-Cy7. To obtain optimal surface staining for CD107a and CD107b \(FITC conjugates) the antibodies were added for the entire activation period. Staining during activation allows access of the antibody to transiently expressed CD107a and CD107b antigens.

# Reagents

1. 15 ml polypropylene centrifuge tube \(BD Falcon™, Cat# 352196) 2. Complete culture media - RPMI 1640 with 10% FBS, 1% L-glutamine and Penicillin-streptomycin \(cRPMI, sterile filtered) 3. Cell culture dish \(60mm x 15mm, polystyrene, Corning, Cat# 430166) 4. Cell dissociation wire screen \(mesh size 50, Sigma, Cat# S0895-5EA) 5. 50 ml polypropylene centrifuge tube \(BD Falcon, Cat# 352070) 6. Dulbecco's phosphate buffered saline \(PBS) without $Ca^{2+}$ and $Mg^{2+}$ \(Hyclone, Cat# SH30028.03) 7. PharmLyse 10X, red blood cell lysing buffer \(BD Biosciences Cat# 555899) 8. Cell strainer \(40µm nylon, BD Falcon Cat# 352340) 9. Phorbol 12-myristate 13-acetate \(PMA, Sigma, Cat# P-8139) 10. Ionomycin \(Sigma, Cat# I-0634) 11. Antigen-specific peptides 12. FITC-anti-mouse CD107a \(BD Biosciences, Cat# 553793) 13. FITC-anti-mouse CD107b \(BD Biosciences, Cat# 558758) 14. Fc Block: rat anti-mouse CD16/CD32 \(BD Biosciences, Cat# 553142) 15. FACS wash buffer – PBS without $Ca^{2+}$ and $Mg^{2+}$ containing 1% bovine serum albumin \(Sigma, Cat# A-7906) + 0.1% Sodium Azide \(NaN3) \(Sigma, Cat# S-80-32), Sterile filtered 16. APC-anti-mouse CD4 \(BD Biosciences, Cat# 553051) 17. PerCPCy5.5-anti-mouse CD8a \(BD Biosciences, Cat# 551162) 18. PE-anti mouse IFN-γ \(BD Biosciences, Cat# 554412) 19. PE-Cy7-anti-mouse TNF-α \(BD Biosciences, Cat# 557644) 20. Perm Wash Buffer 10X solution \(BD Biosciences, Cat# 2091KZ) 21. Round bottom 96-well cell culture plate, sterile \(BD Falcon, Cat# 353077) 22. BD GolgiPlug™ \(Brefeldin A, BD Biosciences, Cat# 555029) 23. BD GolgiStop™ \(monensin, BD Biosciences, Cat# 554724) 24. Cytofix/Cytoperm 1X solution \(BD Biosciences, Cat# 2092KZ)

# Equipment

BD LSRII or equivalent multicolor flow cytometer

# Procedure

**A. Cell Preparation** 1. Sacrifice mouse and harvest spleen. 2. Place spleen in a 15ml polypropylene tube containing 5 ml of cRPMI. Keep on ice until ready to use. 3. Pour the spleen and media into a cell culture dish containing a wire mesh screen. 4. Prepare a single cell suspension of the splenocytes by gently pushing the spleen tissue through the wire screen with the rubber plunger end of a 5 ml syringe. Use as little force as possible. Most connective tissue should remain on screen. 5. Transfer cell suspension from the culture dish into a 15 ml polypropylene tube. 6. Place the 15 ml tube, containing the cell suspension, upright on ice for 3-5 minutes \(until clumps settle to bottom). 7. Transfer the cell suspension to a 50 ml tube with a pipet. Leave the clumps behind. 8. Dilute the cell suspension to 50 ml by adding 1X PBS. 9. Centrifuge cells @ 400 x g for 5 minutes at room temperature. Discard supernatant. 10. Add 3–5 ml of 1X PharmLyse to cell pellet and mix by gently pipetting up and down. 11. Let cell suspension stand at room temperature for 7 minutes. 12. Add 10 ml of cRPMI to the cells and mix by gently pipetting up and down. 13. Pass cell suspension through a 40 µm cell strainer into a fresh 50 ml tube. 14. Dilute the cell suspension to 50 ml by adding 1X PBS. 15. Centrifuge cells @ 400 x g for 5 minutes at room temperature. Discard supernatant. 16. Wash with 1X PBS two more times \(repeat steps 14 and 15). 17. Resuspend cells in 5 ml of cRPMI. 18. Count the splenocytes and bring the sample to a concentration of $10x10^6$ cells/ml in cRPMI. **B. Preparation of Activation and Staining Reagents** 1. PMA: Prepare a stock solution of 1 mg/ml in DMSO, aliquot and store at -20°C. This solution is stable at -20°C for at least six months. On day of experiment, serially dilute an aliquot of PMA stock from freezer 1:1000 in PBS to make 1 µg/ml concentration. 2. Ionomycin: Prepare a stock solution of 1 mg/ml in DMSO, aliquot and store at -20°C. This solution is stable at -20°C for at least six months. On day of experiment, dilute an aliquot of frozen ionomycin stock 1:10 in PBS to make 100 µg/ml concentration. 3. Peptide: Peptides representing optimal antigenic epitopes are superior to whole proteins for _in vitro_ restimulation. Prepare a sterile peptide stock solution of 2 mM in DMSO, aliquot and store at -80°C. This solution is stable at -80°C for at least six months. On day of experiment, serially dilute the frozen peptide aliquot 1:1000 in cRPMI to make 2 µM concentration. 4. CD107a/CD107b-FITC Cocktail: on day of experiment combine 0.1 µg CD107a-FITC with 0.5 µg CD107b-FITC in 1X PBS. Prepare enough for all required wells such that 10 µl of the cocktail is added to each well. 5. Fc Block: Add Fc block to FACS wash buffer at a 1:50 ratio. Make enough to add 100 µl per well \(98 µl FACS wash buffer + 2 µl Fc block per well). 6. Prepare cell surface staining mAbs at optimal \(previously determined) concentrations by diluting in FACS wash buffer. Mix all surface mAbs in the same tube so that the total volume added per well is 50 µl. 7. Prepare intracellular staining mAbs \(i.e., cytokines) at optimal \(previously determined) concentrations by diluting in Perm Wash buffer. Mix all intracellular staining mAbs in the same tube so that the total volume added per well is 50 µl. **C. Activation and Staining** 1. Prepare working stock of PMA+Ionomycin activating reagent. 100 µl of working stock is needed for every well. To prepare 1 ml, add 2 µl of GolgiPlug, 2 µl of GolgiStop, 40 µl of the diluted PMA \(prepared in Step 1 of previous section), and 40 µl of the diluted ionomycin \(prepared in Step 2 of previous section) to 916 µl cRPMI. Store on ice

until ready to use. Discard leftovers after activation. 2. Prepare working stock of unstimulated control. 100 µl of working stock is needed for every well. To prepare 1 ml, add 2 µl of GolgiPlug and 2 µl of GolgiStop to 996 µl cRPMI. Store on ice until ready to use. Discard leftovers after activation. 3. Prepare working stock of peptide activating reagent. 100 µl of working stock is needed for every well. To prepare 1 ml, add 2 µl of GolgiPlug, 2 µl of GolgiStop, and 1 µl of the diluted peptide \(prepared in Step 3 of previous section) to 995 µl cRPMI. Store on ice until ready to use. Discard leftovers after activation. 4. Dispense 100 µl of cell suspension \(1x106 cells) into appropriate wells of a 96-well round-bottom cell culture plate. Avoid cell clumps and cells which have settled to the bottom of the tube. 5. Dispense cells into additional wells \(one well per color plus an unstained control) for use as instrument setup and compensation controls. These cells will be stained with a single antibody each \(e.g., CD4-FITC, CD4-PE, CD4-PE-Cy7, CD4-PerCP-Cy5.5, or CD4-APC). 6. Add 100 µl of the working stock of each activator to the appropriate wells. 7. Add 10 µl of CD107a/CD107b-FITC cocktail \(prepared in Step 4 of previous section) to each well as required. 8. Mix wells by pipetting up and down several times. 9. Incubate 5-6 hrs at 37°C, 7% CO2. 10. Optional step – after incubation, the procedure can be interrupted for overnight storage \(not to exceed 20 hours) of cells by transferring the plate to 4°C \(refrigerator). 11. Centrifuge the plate at 400 x g for 5 min at 4°C. 12. Remove supernatant from the wells by quickly “flicking” the plate upside down. 13. Add 100 µl Fc block \(prepared in Step 5 of previous section) to each well and mix by pipetting up and down. 14. Incubate plate on ice for 20 min. 15. Add 100 µl of FACS wash buffer to each well. 16. Centrifuge the plate at 400 x g for 5 min at 4°C. 17. Remove supernatant by flicking the plate. 18. Add 50 µl of cell surface staining mAbs \(prepared in Step 6 of previous section) to the appropriate wells, mix by pipetting up and down. 19. Incubate in the dark, on ice, for 30 min. 20. Add 150 µl of FACS wash buffer to each well. 21. Centrifuge the plate at 400 x g for 5 min at 4°C. 22. Remove supernatant by flicking the plate. 23. Add 200 µl FACS wash buffer to each well, mix by pipetting up and down. 24. Centrifuge the plate at 400 x g for 5 min at 4°C. 25. Remove supernatant by flicking the plate. 26. Add 100 µl Cytofix/Cytoperm to each well and mix by pipetting up and down. 27. Incubate in the dark, on ice, for 30 min. 28. Optional - procedure can be interrupted at this point by freezing the cells. Wrap plate in parafilm and foil. Transfer to a -80°C freezer. To continue the procedure, thaw the plate at 37°C for ~20 min. 29. Add 100 µl of 1X Perm Wash Buffer to each well. 30. Centrifuge the plate at 500 x g for 5 min. Note that the cells are permeabilized and fixed at this point and require stronger centrifugation to pellet. Be sure to centrifuge at 500 x g for this and all following centrifugation steps 31. Remove supernatant by flicking the plate. 32. Add 50 µl of intracellular staining mAb \(prepared in Step 7 of previous section) to appropriate wells, mix by pipetting up and down. 33. Incubate in the dark, on ice, for 30 min. 34. Add 150 µl Perm Wash buffer to each well. 35. Centrifuge the plate at 500 x g for 5 min at 4°C. 36. Remove supernatant by flicking the plate. 37. Add 200 µl Perm Wash buffer to each well, mix by pipetting up and down. 38. Centrifuge the plate at 500 x g for 5 min at 4°C. 39. Remove supernatant by flicking the plate. 40. Add 200 µl of FACS wash buffer to each well, mix by pipetting up and down. 41. Cells are ready for acquisition \(leave cells in the plate for acquisition on an HTS plate reader, or transfer samples to 12x75 ml tubes for single tube acquisition). 42. Acquire samples on a BD LSRII or equivalent multicolor flow cytometer. Acquire at least 30,000 CD4+ or CD8+ T cells.

# Timing

Spleen recovery and preparation – 30-60 minutes Preparation of activation and staining reagents – 30 minutes Activation procedure – 1 hour set up plus 5-6 hours incubation Staining – 2.5-3 hours FACS analysis – dependent on number of samples and acquisition rates

# References

1. Betts, M.R. _et al_. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. _J. Immunol. Methods_ 281, 65-78 \(2003). 2. Suni, M.A. _et al_. _Ex vivo_ analysis of T-cell function. _Curr Opin Immunol_ 17, 434-440 \(2005). 3. Casazza, J.P. _et al_. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. _J. Exp. Med_. 203, 2865-2877 \(2006).

# Acknowledgements

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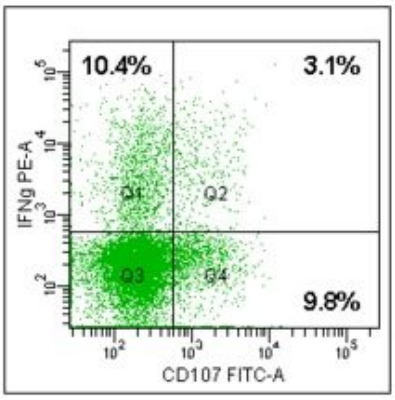
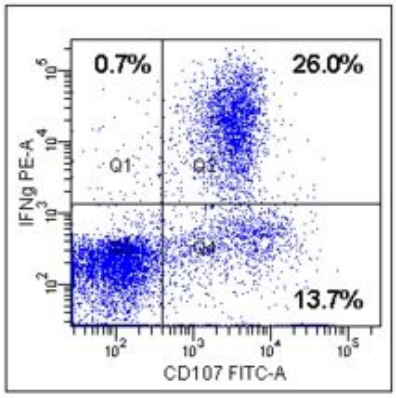
# Figures



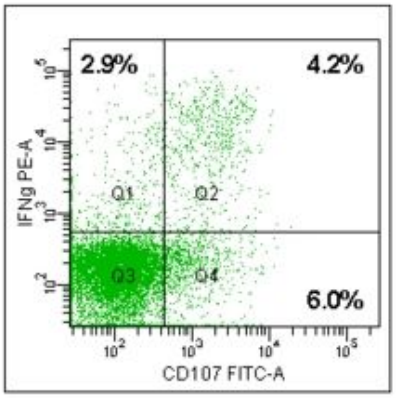

## Figure 1

CD107a+CD107b (CD107), and IFN-γ staining of CD4+ splenocytes from a Balb/c mouse immunized with actA-Lm and restimulated _in vitro_ with PMA+ ionomycin. Quadrant markers were set based on staining of an unstimulated sample. The numbers in the quadrants represent the percentage of CD4+ cells staining positive.



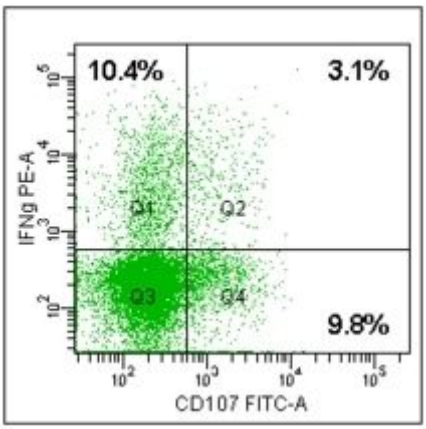

## Figure 2

CD107a+CD107b (CD107), and IFN-γ staining of CD8+ splenocytes from a Balb/c mouse immunized with actA-Lm and restimulated _in vitro_ with LLO91-99 peptide. Quadrant markers were set based on staining of an unstimulated sample. The numbers in the quadrants represent the frequency of CD8+ cells staining positive.

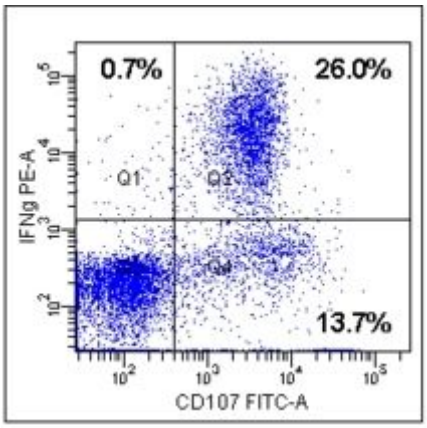



## Figure 3

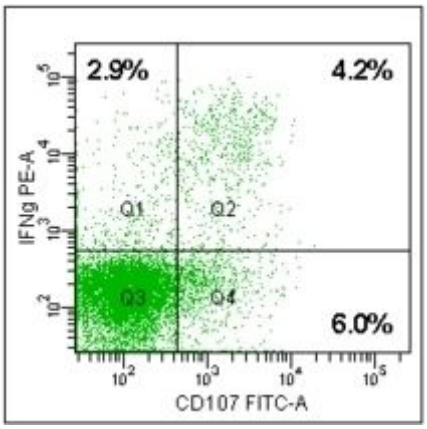
CD107a+CD107b (CD107), and IFN-γ staining of CD4+ splenocytes from a C57BL/6 mouse immunized with actA-Lm and restimulated _in vitro_ with LLO190-201 peptide. Quadrant markers were set based on staining of an unstimulated sample. The numbers in the quadrants represent the percentage of CD4+ cells staining positive.



## Figure 4




## Figure 5




## Figure 6