# Enumeration, Phenotyping, and Identification of Activation Events in Conjugates Between T Cells and Antigen-Presenting Cells by Flow Cytometry

**Kristie M. Grebe and Terry A. Potter***

(Published 10 September 2002)

**INTRODUCTION**

**MATERIALS**

- Mice and Cells
- Antibodies and Detection Reagents
- Chemicals and Reagents
- Other Supplies

**EQUIPMENT**

**RECIPES**

**INSTRUCTIONS**

- Pulsing APCs with Peptide
- Harvesting OT-1 Lymph Node (LN) Cells
- Labeling of APC and LN Populations
- Conjugation of T Cells and APCs
- Intracellular Staining of Conjugates

**NOTES AND REMARKS**

**TROUBLESHOOTING**

**REFERENCES**

Integrated Department of Immunology, National Jewish Medical and Research Center and the University of Colorado Health Sciences Center, 1400 Jackson Street, Denver, CO 80206, USA.

*Corresponding author. E-mail, pottert@njc.org

## Abstract

**Microscopic analysis of T cell–antigen-presenting cell (T cell:APC) interactions at the single cell level has been a powerful, but tedious and subjective, technique. In this paper, we describe a rapid and quantitative method to identify T cell:APC conjugates using succinimidyl ester dyes, which irreversibly label free amine groups on the cell surface. The labeled cell conjugates and subsequent activation events are detected by flow cytometry.**


## Introduction

The biochemical analysis of signal transduction events that occur after cellular activation usually relies on examination of a bulk population, and generally requires a large number of cells. Activation within the population is generally monitored by kinetic activity, such as that resulting in the phosphorylation of endogenous substrates. Alternatively, biochemical activation events can be monitored on a single cell level through microscopic analysis, either by live cell imaging (*1*) or in fixed cells (*2*). However, analysis of single cells provides only a small sample size and is very time-consuming. This is particularly evident in the analysis of interactions between T cells and antigen-presenting cells (APCs). Although single cell microscopy has proved to be a powerful way to view the molecular rearrangements and signaling events that occur after conjugation (*2*), it is still a cumbersome technique that does not lend itself to rapid analysis of cellular interactions in a large sample size. Therefore, a more quantitative and rapid method for characterizing conjugate formation between T cells and APCs is desirable.

Flow cytometry is an alternative approach to the analysis of T cell:APC interactions at the single cell level. We have developed a flow cytometry-based assay that determines the frequency of conjugates formed ex vivo between T cells and APCs. This assay can rapidly and quantitatively identify the conjugating cells in a large sample size. Through the use of antibodies to surface markers on the T cells, the APCs, or both, it is possible to determine the phenotypes of the conjugating cells within a mixed population. Furthermore, the conjugates can be analyzed for activation of various signal transduction cascades after permeabilization and staining with antibodies to cytoplasmic components such as signaling intermediates. The assay as we describe it utilizes two dyes to label the T cell and APC populations. These dyes are succinimidyl esters, which irreversibly label free amine groups on the cell surface. The fluorescent properties of the dyes themselves do not change upon activation of the T cells. Interactions between receptors and ligands on the interacting cells remain unaffected when dyes, rather than antibodies, are used to distinguish the two populations. Although we describe this assay in the context of T cell:APC conjugation, it can certainly be adapted to investigate other cell:cell interactions.

## Materials

### Mice and Cells

OT-1 T cell receptor (TCR) transgenic mice (*3*)

RMA-S (used as an APC cell line) (*4*)

### Antibodies and Detection Reagents

Alexa 488 succinimidyl ester (Molecular Probes, Eugene, OR)

Alexa 633 succinimidyl ester (Molecular Probes)

PerCP-conjugated mouse antibody to CD8 (anti-CD8-PerCP) (Pharmingen, San Diego, CA)

Antibody to phosphotyrosine (4G10) (Upstate, Waltham, MA)

Cy3-conjugated donkey anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA)

Fetal calf serum (FCS) (Sigma-Aldrich, http://www.sigma-aldrich.com)

Normal donkey serum (Jackson Immunoresearch Laboratories)

## Chemicals and Reagents

Dimethyl sulfoxide (DMSO)

Dulbecco's modified Eagle's medium (DMEM) (Gibco, http://www.lifetech.com)

Formaldehyde (Sigma-Aldrich)

NaCl (Sigma-Aldrich)

$NaH_2PO_4$ (anhydrous) (Sigma-Aldrich)

$Na_2HPO_4$ (anhydrous) (Sigma-Aldrich)

Ovalbumin peptide (sequence SIINFEKL)

Paraformaldehyde (Sigma-Aldrich)

Saponin (Sigma-Aldrich)

Sodium azide (Sigma-Aldrich)

Sucrose

## Other Supplies

1-ml syringes

15-ml conical, screw-cap, polypropylene centrifuge tubes (Fisher Scientific)

1.2-ml polypropylene cluster tubes (Fisher Scientific)

25 $cm^2$ tissue culture flasks (Fisher Scientific)

60 mm × 15 mm tissue culture dishes

96-well U-bottom culture plates (Fisher Scientific)

0.22-µM size syringe or bottle top filter (Costar, Cambridge, MA)

Nylon mesh (100 µm) (Fisher Scientific, http://www3.fishersci.com)

## Equipment

FACScaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ)

Hemocytometer (Fisher Scientific)

## Recipes

### Recipe 1: Complete Medium

Prepare a 10% FCS solution in DMEM.

### Recipe 2: SIINFEKL Stock Solution

1 ml of a 1-mM solution of this ovalbumin peptide in DMEM. Sterile filter with a syringe top filter and store at 4°C.

### Recipe 3: Phosphate-Buffered Saline (PBS)

| | |
|---|---|
| NaCl | 145 mM |
| $NaH_2PO_4$ (anhydrous) | 7.46 mM |
| $Na_2HPO_4$ (anhydrous) | 14.5 mM |

Prepare 500 ml in $ddH_2O$.

### Recipe 4: Alexa Dye Stocks

Prepare 1-mg/ml stock solutions of Alexa 633 and Alexa 488 in DMSO. Store at –20°C in 50-µl aliquots.

### Recipe 5: Fixation Solution

| | |
|---|---|
| Sucrose | 3% |
| Paraformaldehyde | 3% |

Prepare 100 ml in PBS (Recipe 3).

### Recipe 6: Fluorescence-Activated Cell Sorting (FACS) Buffer

| | |
|---|---|
| FCS | 2% |
| Sodium azide | 0.1% |

Prepare 500 ml in PBS (Recipe 3).

### Recipe 7: Fc Block

| | |
|---|---|
| Normal donkey serum | 2% |

Prepare 1 ml in FACS Buffer (Recipe 6).

### Recipe 8: Permeabilization Buffer

| | |
|---|---|
| Saponin | 0.5% |

Prepare in 500 ml of FACS Buffer (Recipe 6).

### Recipe 9: Intracellular Staining Buffer

| | |
|---|---|
| FCS | 25% |
| Normal donkey serum | 2% |

Prepare in 50 ml in Permeabilization Buffer (Recipe 8)

## Instructions

### Pulsing APCs with Peptide

In this procedure, the major histocompatibility (MHC) H-2K$^b$ molecules on one population of RMA-S cells (an APC cell line) are loaded with the ovalbumin peptide SIINFEKL. A parallel culture of RMA-S receives no peptide and serves as a negative control.

1. Add RMA-S cells Complete Medium (Recipe 1) to each of two 25-$cm^2$ tissue culture flasks, yielding a density of 0.5 to 1 × $10^6$ cells/ml each.
2. Add 1 μM SIINFEKL (Recipe 2) to one flask.
3. Culture overnight at 27°C, 10% $CO_2$.

   *Note: RMA-S cells need to be grown at 27° because of a deficiency in antigen processing. Growth at 27° allows the stabilization of H-2K$^b$ peptide complexes.*
4. Harvest the cells by pipetting and transfer them to 15-ml centrifuge tubes.

## Harvesting OT-1 Lymph Node (LN) Cells

OT-1 TCR transgenic mice produce $CD8^+$ T cells that recognize the peptide SIINFEKL in the context of the H-2K$^b$ molecule.

1. Kill the mouse by $CO_2$ asphyxiation.
2. Remove the lymph nodes and place them in a 60 mm × 15 mm tissue culture dish containing 10 ml of Complete Medium (Recipe 1)
3. Mash the lymph nodes through 100-μm nylon mesh with the plunger from a 1-ml syringe.
4. Transfer the cells to a 15-ml centrifuge tube.

## Labeling of APC and LN Populations

In this procedure, the surface of both RMA-S (APCs) and OT-1 LN cells are labeled with succinimidyl esters (Alexa 488 and Alexa 633, respectively). During the labeling, the peptide-pulsed RMA-S cells are kept separate from the unpulsed RMA-S. After labeling, the cells should be protected from light as much as possible.

1. Wash the cells twice with 5 ml of PBS (Recipe 3), pelleting the cells by centrifugation at 1200 rpm for 5 min after each wash.
2. Count the cells with a hemocytometer.
3. Resuspend the cells in PBS (Recipe 3) to a concentration of 2 × $10^7$ cells/ml.
4. Add an equal volume of 1:100 diluted Alexa 488 (Recipe 4) to the RMA-S cells and an equal volume of 1:50 diluted Alexa 633 (Recipe 4) to the OT-1 LN cells.
5. Incubate at room temperature protected from light for 15 min.
6. Wash cells three times with 5 ml of Complete Medium (Recipe 1), pelleting the cells by centrifugation at 1200 rpm for 5 min after each wash.

## Conjugation of T Cells and APCs

The OT-1 LN cells are conjugated to the peptide-pulsed RMA-S or the unpulsed RMA-S.

1. Pellet by centrifugation and then resuspend both the peptide-pulsed RMA-S and unpulsed RMA-S to a concentration of 1 × $10^8$ cells/ml in Complete Medium (Recipe 1).
2. Pellet by centrifugation and then resuspend the OT-1 LN cells to a concentration of 1 × $10^8$ cells/ml in Complete Medium (Recipe 1).
3. Add 10 μl of OT-1 LN cells to 10 μl of peptide-pulsed RMA-S or to 10 μl of unpulsed RMA-S in the wells of a 96-well U-bottom plate.
4. Incubate at 37°C, 10% $CO_2$ for 5 min or more.

   *Note: To enumerate the percentage of T cells that can form conjugates, an incubation time of 30 min is ideal. To detect transient phosphorylation events, a shorter incubation of 5 min is needed. There should be enough conjugates present at 5 min to perform the analysis.*

5. Add 100 µl of Fixation Solution (Recipe 5) to each well.

   *Note: If only enumeration of conjugates is desired, this step is not necessary, but the samples must be run on the FACS caliber immediately.*

6. Incubate 15 min at room temperature protected from light.

## Intracellular Staining of Conjugates

In this procedure, the fixed conjugates are stained for the T cell marker CD8 and then permeabilized and stained for phosphotyrosine. All steps are performed at room temperature. Protect the cells from light as much as possible during staining. This portion of the protocol has been adapted from Zell and Jenkins (*5*).

1. Wash the cells twice with 100 µl of FACS Buffer (Recipe 6), pelleting the cells by centrifugation at 1200 rpm for 2 min after each wash.
2. Resuspend the cells in 80 µl of Fc Block (Recipe 7) and incubate 15 min.
3. Add 20 µl of 1:40 diluted anti-CD8-PerCP (final dilution in well should be 1:200) and incubate 15 min.
4. Add 100 µl of FACS Buffer (Recipe 6) and centrifuge at 1200 rpm for 2 min.
5. Wash the cells once with 100 µl of FACS Buffer (Recipe 6), pelleting the cells by centrifugation at 1200 rpm for 2 min after wash.

   *Note: If only enumeration of conjugates is desired, skip to step 16.*

6. Wash the cells once with 100 µl of Permeabilization Buffer (Recipe 8), pelleting the cells by centrifugation at 1200 rpm for 2 min after wash.
7. Resuspend the cells in 50 µl of antibody to phosphotyrosine [diluted 1:500 in Intracellular Staining Buffer (Recipe 9)].
8. Incubate 30 min.
9. Add 100 µl of Permeabilization Buffer (Recipe 8) and centrifuge at 1200 rpm for 2 min.
10. Wash the cells once with 100 µl of Permeabilization Buffer (Recipe 8), pelleting the cells by centrifugation at 1200 rpm for 2 min after wash.
11. Resuspend the cells in 50 µl of Cy3-conjugated donkey anti-mouse IgG antibody (diluted 1:200) in Intracellular Staining Buffer (Recipe 9).
12. Incubate 30 min.
13. Add 100 µl of Permeabilization Buffer (Recipe 8) and centrifuge at 1200 rpm for 2 min.
14. Wash the cells once with 100 µl of Permeabilization Buffer (Recipe 8), pelleting the cells by centrifugation at 1200 rpm for 2 min after wash.
15. Wash the cells once with 100 µl of FACS Buffer (Recipe 6), pelleting the cells by centrifugation at 1200 rpm for 2 min after wash.
16. Resuspend the cells in 200 µl of FACS Buffer (Recipe 6) and transfer into 1.2-ml polypropylene cluster tubes containing an additional 200 µl of FACS Buffer (Recipe 6) for flow cytometry acquisition.
17. Set a live-cell gate on the forward-by-side scatter plot.
18. Collect data for the fluorescence intensity in the FL-1, FL-2, FL-3, and FL-4 channels for at least 30,000 events that fall within the live cell gate. Use a low or medium flow rate setting.
19. To determine the extent of T cell conjugation, compare the number of $CD8^+$ Alexa $633^+$, Alexa $488^+$ cells to the number of $CD8^+$, Alexa $633^+$, Alexa $488^-$ cells (Fig. 1).
20. To determine whether activation markers (such as phosphotyrosine) are induced on T cells that form conjugates with APCs, compare the fluorescence intensity in the FL-2 channel of the Alexa $633^+$, Alexa $488^+$ conjugates to Alexa $633^+$, Alexa $488^-$ LN cells (Fig. 2).

**Fig. 1.** Enumeration of conjugates. Representative pseudocolor dot plots of OT-1 lymph node (LN) cells labeled with Alexa 633 on the *x* axis and RMA-S cells labeled with Alexa 488 on the *y* axis. The gate identifies cells involved in conjugates. (**A** and **C**) All live cells in the sample. (**B** and **D**) Cells gated on CD8$^{+}$ cells. The numbers above the gates in B and D show the percentage of cells in the conjugate gate.

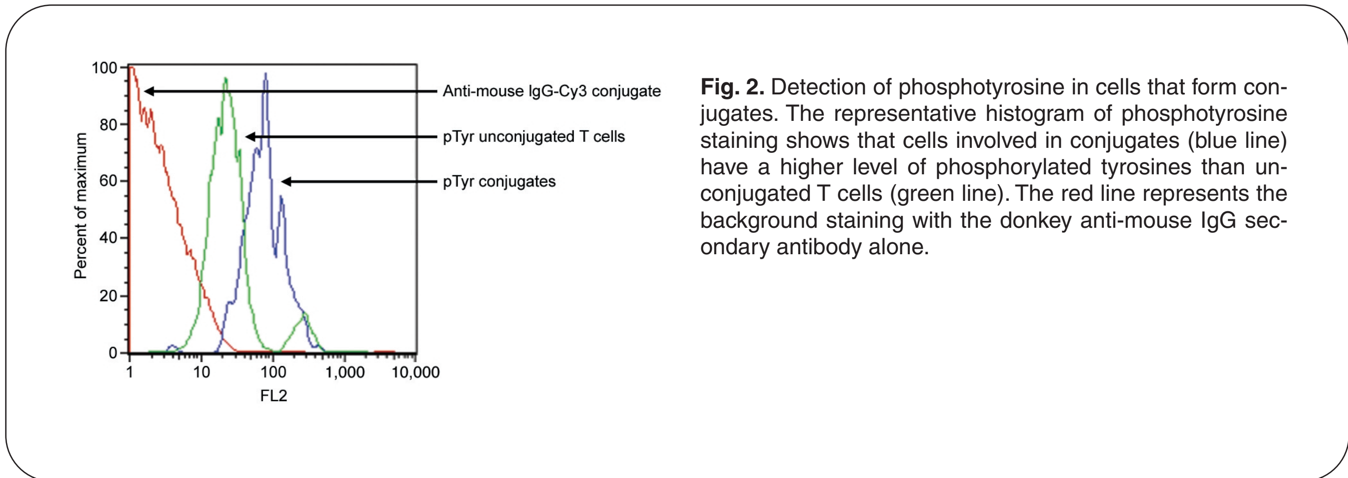


**Fig. 2.** Detection of phosphotyrosine in cells that form conjugates. The representative histogram of phosphotyrosine staining shows that cells involved in conjugates (blue line) have a higher level of phosphorylated tyrosines than unconjugated T cells (green line). The red line represents the background staining with the donkey anti-mouse IgG secondary antibody alone.

## Notes and Remarks

To express sufficient levels of H-2K$^{b}$-SIINFEKL complexes on the cell surface, RMA-S cells need to be cultured at 27°C. These cells have a deficiency in antigen processing, but at 27°C, exogenous peptide can stabilize H-2K$^{b}$. This step is not necessary for cell lines competent in antigen processing. When not required for use as APCs, RMA-S cells are best maintained in culture at 37°C.

This assay has been performed with many different APC cell lines or transgenic T cells. The time point at which the extent of conjugation is maximal may vary and should be determined empirically.

This assay can also be used with different antibodies to analyze cell-specific activation markers. However, the intracellular staining requires fixation of the cells, and the epitopes for some antibodies may be destroyed.

## Troubleshooting

Alexa 488 is a bright dye that bleeds into other channels more than fluorescein isothiocyanate (FITC) does. Increased compensation between the FL-1 channel and the FL-2 is required. It is important to gate out any dead cells in the sample, because they take up large quantities of dye and create compensation problems.

### References


1. K. H. Lee, A. D. Holdorf, M. L. Dustin, A. C. Chan, P. M. Allen, A. S. Shaw, T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539-1542 (2002).
2. C. R. Monks, B. A. Freiberg, H. Kupfer, N. Sciaky, A. Kupfer, Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82-86 (1998).
3. K. A. Hogquist, S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, F. R. Carbone, T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17-27 (1994).
4. M. Attaya, S. Jameson, C. K. Martinez, E. Hermel, C. Aldrich, J. Forman, K. F. Lindahl, M. J. Bevan, J. J. Monaco, Ham-2 corrects the class I antigen-processing defect in RMA-S cells. *Nature* **355**, 647-649 (1992).
5. T. Zell, M. K. Jenkins, Flow cytometric analysis of T cell receptor signal transduction. *Science's STKE* (2002), http://stke.sciencemag.org/cgi/content/full/sigtrans;2002/128/pl5.


6. We would like to thank B. Schaefer and S. Gauld for technical assistance in development of this assay. We would also like to thank B. Townend, S. Sobus, and J. Loomis for flow cytometry assistance.



**Citation:** K. M. Grebe, T. A. Potter, Enumeration, phenotyping, and identification of activation events in conjugates between T cells and antigen-presenting cells by flow cytometry. *Science's STKE* (2002), http://www.stke.org/cgi/content/full/sigtrans;2002/149/pl14.