

***In vitro* Assessment of Immunological Synapse Formation by Flow Cytometry**

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[Abstract] In adaptive immune system, formation of immunological synapse between T cells and antigen presenting cells (dendritic cells, B cells, and macrophages) or target cells (tumor cells and viral-infected cells) is critical for the execution of T cell immune responses via cytokine secretion or direct killing activity. Here, we describe the practical methods that directly measure the number of conjugates as a result of immunological synapse formation between T cells and superantigen-loaded B cells or between cytotoxic T cells and antigen-loaded target cells by dual-color flow cytometry.

Materials and Reagents

1. 15 ml and 50 ml conical tube
2. 12 well cell culture plate
3. Falcon™ Cell strainer (40 µm Nylon) (Corning, catalog number: 352340)
4. 1 ml syringe
5. Falcon™ 5 ml Polystyrene Round-Bottom tube (Corning, catalog number: 352052)
6. MACS MS column (Miltenyi Biotec, catalog number: 130-042-201) and MACS separator (Miltenyi Biotec)
7. Jurkat T cell (ATCC, catalog number: TIB-152)
8. Raji B cell (ATCC, catalog number: CCL-86)
9. EL4 cell (ATCC, catalog number: TIB-39)
10. C57BL/6 mice (8-10 weeks)
11. OTI mice (8-10 weeks) (THE JACKSON LABORATORY, catalog number: 021773)
Note: These mice have the transgenic T cell receptor designed to recognize ovalbumin residues 257-264 in the context of H2Kb.
12. *Staphylococcus Enterotoxin E* (SEE) (Toxin Technology, catalog number: ET404)
13. *Staphylococcus Enterotoxin B* (SEB) (Toxin Technology, catalog number: BT202)
14. OVA 257–264 peptide (InvivoGen, catalog number: vac-sin)
15. CellTracker Green CMFDA (Life Technologies, Molecular Probes® , catalog number: C7025)
Note: Currently, it is "Thermo Fisher Scientific, Molecular Probes™ , catalog number: C7025".
16. CellTracker Orange CMRA (Life Technologies, Molecular Probes® , catalog number:

C34551)

Note: Currently, it is "Thermo Fisher Scientific, Molecular Probes™", catalog number: C34551".

17. RBC lysis buffer (BioLegend, catalog number: 42030)
18. Mouse CD3⁺ T cell enrichment column kit (R&D Systems, catalog number: MTCC535)
19. Mouse B cell enrichment kit (STEMCELL Technologies, catalog number: 19754A)
20. CD8a (Ly-2) MicroBeads (Miltenyi Biotec, catalog number: 130-049-401)
21. Recombinant human IL-2 (rhIL-2) (NIH AIDS Reagent Program, catalog number: 11697)
22. RPMI1640 (Thermo Fisher Scientific, Gibco™, catalog number: 31800)
23. 10% heat-inactivated fetal bovine serum (FBS) (Siyaku, Wako Pure Chemical Industries, AusGeneX, catalog number: FBSUS500)
24. Penicillin/streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15140)
25. Non-essential amino acids (Thermo Fisher Scientific, Gibco™, catalog number: 11140)
26. Sodium pyruvate (Thermo Fisher Scientific, Gibco™, catalog number: 11360)
27. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M-7522)
28. Complete RPMI1640 medium (see Recipes)
29. Mouse T cell medium (see Recipes)

Equipment

1. Water Jacketed CO₂ incubator (37 °C, 5% CO₂) (Thermo Fisher Scientific)
2. FACSCanto instrument (BD Bioscience)

Procedure

A. Conjugation assay using cell lines

1. Incubate Jurkat T cells (2 x 10⁵/sample) with 1 μM of CellTracker Green CMFDA and Raji B cells (2 x 10⁵/sample) with 3 μM CellTracker Orange CMRA in 0.5-1 ml of complete RPMI1640 medium in 15 ml conical tubes for 30 min at 37 °C in a CO₂ incubator.

Note: Perform all steps in the dark to prevent bleaching of fluorescence.

2. For washing, centrifuge the cells at 1,300 rpm for 3 min, discard the supernatant, and resuspend cell pellet using complete RPMI1640 medium. Repeat twice. After washing, resuspend the cells in 200 μl/sample of complete RPMI1640 medium.
3. Treat CMRA-labelled Raji B cells in 200 μl/sample of complete RPMI1640 medium with 5 μg/ml SEE for 30 min at 37 °C.

Notes:

- a. *SEE and SEB (B.5) act as the superantigens. Prepare SEE and SEB stock*

solution of 1 mg/ml in Sterile Distilled Water (SDW) and store in -80 °C.

- b. Control samples of conjugation assay are samples untreated with SEE, SEB (B), and OVA peptide (C).
 - c. SEE and SEB act as a superantigen that cause non-specific activation of T cells resulting in polyclonal T cell activation and massive cytokine release. Although SEE and SEB can bind both TCR and MHC to induce T cell activation, binding sites are different. SEE is used for formation of conjugation between Jurkat T cell-Raji B cell because Jurkat T cells have the human TCR Vβ recognized by SEE, not SEB. SEE can also apply to form mouse T cell-B cell conjugates, but SEB is more widely used in mouse T cell activation.
4. Mix and incubate equal cell number of CMFDA-T cells (2 x 10⁵/200 μl/sample) and CMRA-B cells (2 x 10⁵/200 μl/sample) in complete RPMI1640 medium in the polystyrene round-bottom tube for 30 min at 37 °C in a CO₂ incubator.
 5. Analyze the T cell-B cell conjugates using dual-color flow cytometry.
 - a. Prepare the samples as the table below.

Unstained		Single-Color		Dual-Color
T cells	B cells/EL4 cells	CMFDA (green) labeled T cells	CMRA (red) labeled B/EL4 cells	CMFDA (green) labeled T cells co-incubated CMRA (orange) labeled B/EL4 cells with or without treatment of SEE/SEB/OVA
Set aside 2 x 10 ⁵ of these cells before starting step A1 for step A5b	Set aside 2 x 10 ⁵ of these cells before starting step A1 for step A5b	Set aside 2 x 10 ⁵ of these cells before step A3 for step A5c	Set aside 2 x 10 ⁵ of these cells before step A3 for step A5c	These samples will be used for step A5d

- b. Analyze unstained control to set voltage and gates (FSC, SSC, fluorescence signals).
- c. Analyze single-color stained controls for compensation.
- d. After compensation, analyze dual-color stained samples.

Notes:

- i. Read the fluorescence of 10,000-20,000 cells in all controls and samples for analysis.
- ii. The percentage of green-orange events indicates the proportion of T cell-B cell conjugates (Figure 1).

B. Conjugation assay using mouse T cells and B cells

1. Isolate splenocytes from the spleen from a C57BL/6 mouse.
 - a. Cut out the spleen.
 - b. Mash the spleen through the cell strainer into 50 ml conical tube using the plunger end of the 1 ml syringe.
 - c. Wash the cell strainer with 5-6 ml mouse T cell medium and discard the strainer.
 - d. Transfer the suspended cells into a 15 ml conical tube, and spin down cells at 1,800 rpm for 3 min.
 - e. Discard supernatant and resuspend cell pellet in 1 ml RBC (Red blood cell) lysis buffer to remove RBCs. After 1 min, add 5 ml mouse T cell media and discard any dead cell mass.
 - f. Spin down at 1,800 rpm for 3 min, and discard supernatant. Resuspend pellet in T cell enrichment buffer provided by the manufacturer or B cell enrichment buffer (2% FBS in PBS).
 2. Isolate CD3⁺ T cells from splenocytes using Mouse CD3⁺ T cell enrichment column kit according to the manufacturer's protocol. Isolate B cells from mouse splenocytes using Mouse B cell enrichment kit according to the manufacturer's protocol. After isolation, resuspend cells in mouse T cell medium.

Note: The yield of T cell and B cell enrichment are above 95%.
 3. Incubate CD3⁺ T cells with 1 μM of CellTracker Green CMFDA (2 x 10⁵/sample) and B cells with 3 μM CellTracker Orange CMRA (2 x 10⁵/sample) in 0.5-1 ml of mouse T cell medium at 37 °C in 15 ml conical tubes as described in step A1.
 4. For washing, centrifuge the cells at 1,800 rpm for 3 min, discard the supernatant, and resuspend cell pellet using mouse T cell medium. Repeat twice. After washing, resuspend the cells in 200 μl/sample of mouse T cell medium.
 5. Treat B cells with 5 μg/ml SEB in 200 μl/sample of mouse T cell medium for 30 min at 37 °C.
 6. Mix and incubate equal cell number in 200 μl/sample of mouse T cell medium of CMFDA-CD3⁺ T cells and CMRA-B cells in the polystyrene round-bottom tube for 30 min at 37 °C in a CO₂ incubator.
 7. Analyze the T cell-B cell conjugates using dual-color flow cytometry as described in step A5.
- C. Conjugation assay using antigen-specific cytotoxic T cells and target cells
1. Isolate splenocytes from an OTI mouse as described in step B1, and resuspend splenocytes in mouse T cell medium.

Note: CD8⁺ T cells purified from an OTI-transgenic TCR mice express Ova-specific TCR that can recognize OVA 257-264 peptide as an antigen.
 2. For differentiation to OVA-specific cytotoxic T cells, stimulate splenocytes (2 x 10⁶ cells/well) with OVA 257-264 peptide in mouse T cell medium with 10 U/ml rhIL-2 in 12 well cell culture plates for 2-3 days. Add rhIL-2 to media daily.

Note: Thaw frozen stock of rhIL-2 (kept at -80 °C) and dilute in mouse T cell medium. Diluted rhIL-2 can be stored at 4 °C for one month.

3. Isolate OVA-specific cytotoxic T cells by MACS separation using CD8a (Ly-2) MicroBeads according to the manufacturer's protocol.
4. Incubate OVA-specific cytotoxic T cells with 1 μM of CellTracker Green CMFDA (2 x 10⁵ - 1 x 10⁶ /sample) and EL4 cells (as target cells; 2 x 10⁵/sample) with 3 μM CellTracker Orange CMRA at 37 °C in mouse T cell medium, wash, and resuspend in mouse T cell medium as described in steps B3-4.

Note: The relevant ratio of cytotoxic T cells and EL4 cells is 1:1-5:1.

5. Add 10 μg/ml OVA 257–264 peptide to EL4 cells in 200 μl/sample of mouse T cell medium for 30 min at 37 °C.

Note: OVA peptide loaded on EL4 cells acts as the antigen recognized by OVA-specific cytotoxic T cells.

6. Mix and incubate equal cell number in 200 μl/sample of mouse T cell medium of CMFDA-cytotoxic T cells and CMRA-EL4 cells in the polystyrene round-bottom tube for 30 min-1 h at 37 °C in a CO₂ incubator.
7. Analyze the cytotoxic T cell-target cell conjugates using dual-color flow cytometry as described in step A5.

Representative data

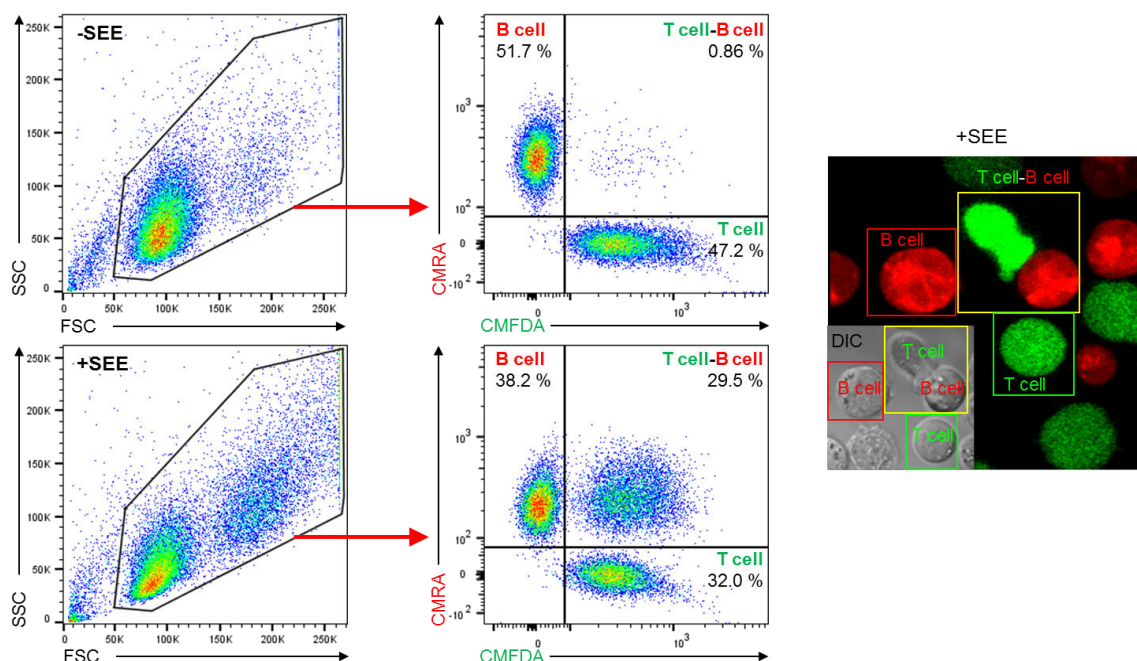


Figure 1. SEE-induced Jurkat T cell-Raji B cell conjugates formation. Conjugates formation using Jurkat T cells and Raji B cells with or without SEE were performed as described in Procedure section A and then analyzed by flow cytometry. Dot plot showing

FSC and SSC is gated to exclude cell debris and select cells for dual-color analysis (Left). Cells inside the gate were analyzed by CMFDA (green; T cell) and CMRA (red; B cell) fluorescence signals (middle dot plots). Quadrants are established using negative control and single-color controls. CMFDA⁺ CMRA⁻ cells (lower right quadrant) indicate T cell only, CMFDA⁻ CMRA⁺ cells (upper left quadrant) indicate B cell only, and CMFDA⁺ CMRA⁺ cells (upper right quadrant) indicate T cell-B cell conjugates. After loading of SEE by Raji B cells (+SEE), T cell-B cell conjugates were increased in compared with SEE-unloaded B cells (-SEE). Numbers in dot plots indicate the percentage of each quadrant. Confocal fluorescence image and differential interference contrast (DIC) image show T cell only (green box), B cell only (red box), and T cell-B cell conjugate (yellow box) after conjugation assay with SEE (Right).

Recipes

1. Complete RPMI1640 medium
 - RPMI1640
 - 10% heat-inactivated fetal bovine serum
 - 100 U/ml Penicillin/streptomycin
2. Mouse T cell medium
 - RPMI1640
 - 10% heat-inactivated fetal bovine serum
 - 100 U/ml Penicillin/streptomycin
 - 1% non-essential amino acids
 - 1 mM Sodium pyruvate
 - 50 μM 2-Mercaptoethanol

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References

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