

Chapter 23

Flow Cytometry-Based Monitoring of Mast Cell Activation

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Abstract

Mast cell activation is a central process in the initiation of allergic disorders. As described elsewhere in this volume, this process can be readily monitored by biochemical, antibody-based, and enzyme-based formats when the cell population examined is homogenous. When dealing with mixed and transfected cell populations however, such approaches may not be appropriate. Hence alternative methods are required. Here we describe flow-cytometry-based assays that can be utilized to examine signaling processes and degranulation in both pure mast cell populations and, following appropriate selection, in populations where the mast cells of interest may only represent a fraction of the total cell population.

Key words Mast cell, Signaling, Protein phosphorylation, Calcium signal, Actin polymerization/depolymerization, Degranulation, Flow cytometry

Abbreviations

FcεRI	High-affinity IgE receptor
IP ₃	Inositol trisphosphate
F actin	Filamentous actin
GFP	Green fluorescent protein
rhSCF	Recombinant human stem cell factor
dH ₂ O	Distilled or deionized H ₂ O
BSA	Bovine serum albumin
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
DNP	Dinitrophenol
HSA	Human serum albumin
APC	Allophycocyanin
DMSO	Dimethylsulfoxide
BMMC	Bone marrow-derived mast cell
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin

1 Introduction

Receptor-mediated mast cell activation occurs through a complex cascade of intracellular signaling processes culminating in degranulation and the generation and release of eicosanoids, cytokines, chemokines, and growth factors [1]. With regard to antigen/IgE-induced mediator release, the initiating response is aggregation of the high-affinity receptors for IgE (FcεRI) with resulting phosphorylation of critical tyrosine residues contained within the immunoreceptor-based tyrosine activation motifs (ITAMs) of the FcεRI β and γ chains [2]. These events result in the recruitment and subsequent activation of the ZAP-70-related tyrosine kinase Syk which, in turn, phosphorylates a series of adaptor molecules and other signaling proteins critical for propagation of the signals required for the release of the various classes of inflammatory mediators [3]. Thus, phosphorylation of key signaling molecules within the mast cell activation cascade has been used as an index of their activation. The advent of the generation of antibodies which uniquely recognize the phosphorylated, but not non-phosphorylated, forms of these signaling proteins has allowed monitoring of specific signaling events by immunoblot analysis. The efficacy of this approach is however dependent on the affinity and selectivity of the antibody, the abundance of the protein, and the purity of the cell populations. Nevertheless, this approach has proved to be a relatively sensitive and effective technique for assessing critical intracellular signaling events.

Downstream of these events, an increase in the cytosolic calcium concentration is essential for subsequent events leading to the release of the inflammatory mediators [4]. This calcium signal is primarily produced as a consequence of liberation of calcium from the endoplasmic reticulum in response to inositol trisphosphate (IP₃) liberated from phosphatidylinositol 4,5 bisphosphate by the action of phospholipase C. As a consequence of emptying of these intracellular stores, a secondary, more prolonged elevation of cytosolic calcium is produced via store-operated calcium entry. The calcium signal can be either monitored in cell populations or in single cells using calcium-chelating fluorescence probes such as Indo-1, Fura-2, Fluo-3, and Fluo-4.

For exocytosis of the cytoplasmic granules to proceed, rearrangement of the cytoskeleton through actin depolymerization/repolymerization is required [5, 6]. The toxin phalloidin, isolated from the death cap mushroom, *Amanita phalloides*, has the ability to bind to polymerized filamentous (F)-actin allowing monitoring of changes in the polymerized state of actin, once the phalloidin is conjugated to a suitable fluorescent moiety. This can be done by both fluorescent/confocal microscopy and flow cytometry.

As discussed above, and elsewhere in this volume, the aforementioned signal transduction processes and events leading

to, and including, degranulation can readily be assessed in pure mast cell populations by immunoblot analysis and by a variety of biochemical approaches. However there are specific circumstances where these approaches need to be refined to increase sensitivity or selectivity of the assays, for example, where mast cells may represent only a fraction of the total cell population or where efficiency of transfection is such that only a fraction of cells are accordingly genetically modified. Examples of the former would be the mixed cell population in peritoneal lavage which normally would require purification and/or further culture prior to conducting appropriate studies, and of the latter, where a green fluorescent protein (GFP)-tagged signaling molecule is overexpressed, but only in a proportion of the total cells. In the aforementioned cases the mast cell population can be selected by flow cytometry based on KIT (CD117) or FcεRI expression or by expression of GFP, and then signaling processes or degranulation in these cells assessed.

In this chapter, we describe a number of flow cytometry-based assays to monitor mast cell activation that can be used to examine events in relatively pure cell populations or, following selection, relatively impure cell populations. These protocols can be adapted for mast cells from both rodent and human origin.

2 Materials

2.1 Monitoring Signaling in Mast Cells by PhosFlow

1. StemPro-34 complete medium (GIBCO, Grand Island, NY): 13 mL StemPro-34 Nutrient Supplement, L-glutamine (2 mM), penicillin G (100 IU/mL), streptomycin (100 µg/mL) in StemPro-34 serum-free medium.
2. Recombinant human stem cell factor (rhSCF).
3. Biotin-conjugated human myeloma IgE (*see Note 1*).
4. Streptavidin.
5. HEPES buffer: 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄·7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·2H₂O, 1.3 mM Mg²⁺ (as MgCl₂ or MgSO₄) in distilled or deionized H₂O (dH₂O).
 - (a) To make 1 L: Dissolve components *except* the calcium and magnesium salts in 900 mL dH₂O (2.38 g HEPES, 8.00 g NaCl, 0.200 g KCl, 0.103 g Na₂HPO₄·7H₂O, 1.008 g glucose).
 - (b) Adjust pH to 7.4 using concentrated NaOH or HCl.
 - (c) Add 0.265 g of CaCl₂·2H₂O and 1.3 mL of a 1 M MgCl₂ solution.
 - (d) Adjust volume to 1 L with dH₂O.
 - (e) Sterilize by filtration (pore size: 0.22 µm) and store at 4 °C.

6. HEPES buffer with bovine serum albumin (BSA): 0.04 % (w/v) BSA in HEPES buffer (prepare fresh). Add 0.04 g BSA (>96 % purity) to 100 mL HEPES buffer. Warm up to 37 °C and mix well.
7. Fixation solution #1 (prepare fresh): 4 % (w/v) paraformaldehyde (PFA) in PBS. Add 1 mL of a 20 % (w/v) PFA solution (200 mg/mL prepared in dH₂O) to 4 mL PBS.
8. Appropriately labeled phosphorylation-specific antibody (*see Note 2*).
9. 5 mL polystyrene round-bottom tubes.
10. Amaxa Nucleofector cell line Nucleofection kit V (Lonza, Switzerland).
11. Flow cytometer with 488 nm excitation laser and detector for green (filter: 530/30) and orange-red (filter: 585/42) emission.

2.2 Calcium Response Kinetics

1. Mouse anti-dinitrophenyl (DNP) IgE, clone SPE-7.
2. DNP-human serum albumin (HSA).
3. Allophycocyanin (APC) anti-mouse CD117 antibody, 0.2 mg/mL.
4. Fluo-4 solution (50× concentrated): 0.456 mM (0.5 mg/mL) in dimethyl sulfoxide (DMSO). Dissolve 50 µg Fluo-4, AM in 100 µL DMSO. Store in aliquots at -70 °C.
5. 125 mM Probenecid solution (50× concentrated) (pH 7.4): Dissolve 356.7 mg in 9 mL 0.2 M NaOH solution (in dH₂O). Adjust pH to 7.4 using concentrated HCl or NaOH solution. Adjust volume to 10 mL with dH₂O and then sterilize by filtration (0.22 µm pore size). Store at room temperature (RT).
6. Fluo-4 labeling solution: 9.12 µM (10 µg/mL) Fluo-4, 2.5 mM probenecid, and 0.25 µg/mL APC anti-mouse CD117 in HEPES/BSA. Add 20 µL 50× concentrated Fluo-4 solution, 40 µL 50× concentrated probenecid solution, and 1.2 µL APC anti-mouse CD117 to 939 µL HEPES/BSA.
7. Fluo-4 washing solution: 2.5 mM probenecid in HEPES/BSA (prepare fresh). Add 400 µL 50× concentrated probenecid solution to 9.6 mL HEPES/BSA.
8. 5 mL polystyrene round-bottom tubes.
9. Flow cytometer with 488 nm and 635 nm excitation lasers and detectors for green (filter: 530/30) and red (filter: 661/16) emission.

2.3 Actin Polymerization/Depolymerization Kinetics

1. Materials for bone marrow-derived mast cell (BMMC) culture and sensitization (*see Note 3*).
2. Fluorescein isothiocyanate (FITC)-phalloidin.
3. APC anti-mouse CD117 antibody, 0.2 mg/mL.

4. HEPES/BSA buffer.
5. 0.2 M EGTA (pH 8): Add 7.6 g EGTA and ~6–7 mL 10 N NaOH solution to 90 mL ddH₂O. Adjust pH to 8 with conc. NaOH (or HCl). Once the EGTA is completely dissolved then top up to 100 mL volume.
6. 0.5 M EDTA (pH 8): Add 104.05 g Na₂EDTA · 2H₂O (disodium EDTA dihydrate) to 400 mL ddH₂O. Gradually add 9–10 g NaOH pellets (or ~50 mL 1 N NaOH solution) until the EDTA is dissolved. Adjust pH to 8.0 and top of volume to 500 mL.
7. Fixation solution #2 (prepare fresh): 4 % (w/v) PFA, 5 mM EGTA, 5 mM EDTA in PBS. Add 1 mL 20 % (w/v) PFA solution (prepared in dH₂O), 125 µL 0.2 M EGTA and 50 µL 0.5 M EDTA to 3.83 mL PBS.
8. Incubation solution: 5 mM EGTA, 5 mM EDTA, 2 % (w/v) BSA in PBS (prepare fresh). Dissolve 20 mg BSA in 945 µL PBS. Add 25 µL 0.2 M EGTA, 10 µL 0.5 M EDTA.
9. Saponin solution (50× concentrated): 20 mg saponin in 980 µL dH₂O (prepare fresh).
10. F-actin labeling solution: 1 µg/mL FITC-phalloidin, 0.25 µg/mL APC anti-mouse CD117 antibody, 0.01 % (w/v) saponin in incubation solution. Add 1 µL FITC-phalloidin PBS solution (1 mg/mL), 1.2 µL APC anti-mouse CD117, and 20 µL 50× concentrated saponin solution to 978 µL incubation solution.
11. Flow cytometer with 488 and 635 nm excitation lasers and detectors for green (filter: 530/30) and red (filter: 661/16) emission.

2.4 Degranulation Kinetics

1. Materials for LAD2 culture and sensitization in Subheading 2.1.
2. Materials for BMMC culture and sensitization (*see Note 3*).
3. Phycoerythrin (PE) anti-human LAMP-2 antibody [H4B4].
4. FITC anti-mouse IgE antibody, 0.5 mg/mL.
5. PE anti-mouse CD117 antibody, 0.2 mg/mL.
6. APC anti-mouse CD107b (LAMP-2) antibody, 0.1 mg/mL.
7. HEPES/BSA buffer.
8. Incubation solution: 20 mM EGTA, 20 mM EDTA, 0.2 % (w/v) BSA in PBS (prepare fresh). Add 100 µL 0.2 M EGTA, 40 µL 0.5 M EDTA, and 20 mg BSA to 860 µL PBS.
9. Labeling solution (for BMMCs): 1.25 µg/mL FITC anti-mouse IgE antibody, 0.5 µg/mL PE anti-mouse CD117 antibody, 1 µg/mL APC anti-mouse CD107b (LAMP-2) antibody prepared in incubation solution (prepare fresh).
10. Labeling solution (for LAD2): Dilute PE anti-human CD107b (LAMP-2) antibody 1:100 in incubation solution (prepare fresh).

11. 5 mL polystyrene round-bottom tubes.
12. Flow cytometer with 488 and 635 nm excitation lasers and detectors for green (filter: 530/30), orange (filter: 585/42), and red (filter: 661/16) emission.

3 Methods

3.1 PhosFlow

The analysis of mast cell signaling by flow cytometry can be utilized when transfection of a protein is desired. Mast cells are difficult to transfect and efficiency is generally too low for conventional immunoblots. Thus, flow cytometry can be utilized for the analysis of populations positive for a marker such as GFP. This protocol describes the method for the human mast cell line LAD2, but this method could be adapted for mast cells from other origins.

Transfection of LAD2 Cells with Constructs Labeled with GFP (See Note 4)

1. Remove 2×10^6 LAD2 cells from culture for each transfection and place into a 15 mL centrifuge tube (*see Note 5*).
2. Centrifuge at $90 \times g$ for 10 min at RT with the brake turned off and aspirate the supernatant.
3. Gently resuspend the pellet in 10 mL of pre-warmed (37°C) PBS/0.1 % BSA and centrifuge at $90 \times g$ for 10 min at RT with the brake turned off.
4. Aspirate the supernatant and repeat **step 3**.
5. Aspirate the supernatant, ensuring that as much is removed as possible, and resuspend the cells in 100 μL of pre-warmed (37°C) nucleofection solution V with supplement added.
6. Add 2 μg of high-concentration plasmid DNA to the cells and transfer to a nucleofector cuvette (*see Note 6*).
7. Run the appropriate nucleofector program (*see Note 7*).
8. Using a nucleofector pipette, transfer the cells to 4 mL of pre-warmed (37°C) StemPro-34 medium containing supplement and 100 ng/mL rhSCF in a 6-well plate and transfer to the incubator (**Note 8**).

Analysis of Signaling in Transfected Cells

1. Sensitize the LAD2 cells with 100 ng/mL biotinylated human myeloma IgE overnight (16 h) in StemPro-34 medium plus supplement, but without rhSCF (*see Note 8*).
2. Following the incubation, wash the cells by centrifugation ($250 \times g$ for 5 min) with 2 mL of HEPES/BSA buffer pre-warmed to 37°C and aspirate the supernatant.
3. Repeat **step 2**.
4. After the final wash, resuspend the cells in pre-warmed HEPES/BSA buffer to give a final concentration of $2\text{--}5 \times 10^5$ cells in 450 μL (**Note 9**).

5. Transfer 450 μL of cell suspension to separate 5 mL polystyrene round-bottom tube and place in a heating block or water bath set to 37 °C.
6. Allow the cells to equilibrate by incubating in the heating block for 5 min.
7. Add 50 μL of 10 $\times$ pre-warmed (37 °C) activation solution (streptavidin, rhSCF, etc.), start a timer, and quickly swirl the tube before placing it back into the heating block.
8. Incubate for the desired time.
9. Stop the reaction by removing the tube and plunging it directly into ice.
10. Immediately add 1 volume (500 μL) of ice-cold fixative solution.
11. Incubate on ice for 40 min (*see* **Note 10**).
12. After fixation, centrifuge the cells at 300 $\times g$ for 8 min at 4 °C and aspirate the supernatant.
13. Wash the cells with 2 mL of PBS/0.1 % BSA and centrifuge at 300 $\times g$ for 5 min at 4 °C.
14. Aspirate the supernatant and repeat **step 13**.
15. Aspirate the supernatant and resuspend the cells in 1 mL of very cold 100 % methanol (stored at -20 °C and added directly).
16. Cap the tubes and place in the freezer (-20 °C) for >4 h.
17. Centrifuge the tubes at 300 $\times g$ for 5 min at 4 °C and discard the supernatant.
18. Wash the cells with 2 mL of ice-cold PBS/0.5 % BSA and centrifuge at 300 $\times g$ for 5 min at 4 °C.
19. Discard the supernatant and repeat **step 18**.
20. Discard the supernatant and resuspend the cells in 195 μL of ice-cold PBS/0.5 % BSA.
21. Add 5 μL of PhosFlow PE-conjugated antibody and incubate on ice for >1 h.
22. After the incubation, add 1.8 mL of PBS/0.5 % BSA to each tube and centrifuge at 300 $\times g$ for 5 min at 4 °C.
23. Discard the supernatant and repeat **step 22** twice.
24. Discard the supernatant and resuspend the cells in 200 μL of PBS/0.1 % BSA.
25. Run samples on the flow cytometer.

The analysis of phosphorylation by flow cytometry has been shown to correlate well with immunoblots [7]. For the analysis of results, a tight gate should be set on the mast cell population to

eliminate cell debris, and then a secondary gate should be set on the subpopulation of GFP-positive cells (FL1). The FL2 fluorescence can be plotted in the GFP-positive cell population. As an additional control, the FL2 fluorescence can be plotted in the GFP-negative cell population.

A typical example of the phosphorylation of the critical signaling molecules Akt and ERK responses to SCF challenge is shown in Fig. 1.

3.2 Calcium Response Kinetics

The flow cytometry analysis of the calcium response in antigen-stimulated mast cells can be utilized when the mast cell population represents only a fraction of the analyzed cells, or a population of mast cells transfected with a fluorogenic marker, such as GFP. This protocol describes the method for monitoring the calcium response of KIT⁺ (CD117⁺) population in IgE-sensitized and antigen-stimulated mouse peritoneal cells (*see Note 11*).

1. Sensitize mouse by peritoneal injection with 0.5 mL mouse anti-DNP IgE solution in PBS (2 µg/mL) at days 4 and 2 before peritoneal lavage.
2. Isolate mouse peritoneal cells by peritoneal lavage using 3–5 mL HEPES/BSA buffer and transfer the cell suspension into 5 mL polystyrene round-bottom tubes (*see Note 12*).
3. Centrifuge the cells (200–300 × *g* for 5 min at RT) and aspirate the supernatant.
4. Gently resuspend the cells in 0.5 mL of Fluo-4-labeling solution and incubate at RT for 30 min in the dark.
5. Centrifuge the cells (200–300 × *g* for 5 min at RT), aspirate the supernatant, and gently resuspend in 1–4 mL Fluo-4 washing solution (1 mL per sample).
6. Divide the suspension into 1–4 samples and keep at RT in the dark until further processed.
7. Spin the individual sample (200–300 × *g*, 5 min, RT), aspirate the supernatant, and resuspend in 250 µL HEPES/BSA buffer; temperate the cells at 37 °C for 2–5 min.
8. Record FL1 fluorescence of the gated (CD117⁺) population on flow cytometer at 37 °C for 1 min, then quickly add 200 µL 2× activator (DNP-HSA) in HEPES/BSA buffer (pre-warmed to 37 °C), and continue recording the fluorescence at 37 °C for an additional 5 min (*see Note 13*).
9. Repeat **steps 7 and 8** for the remaining samples.

A typical example of calcium responses is shown in Fig. 2.

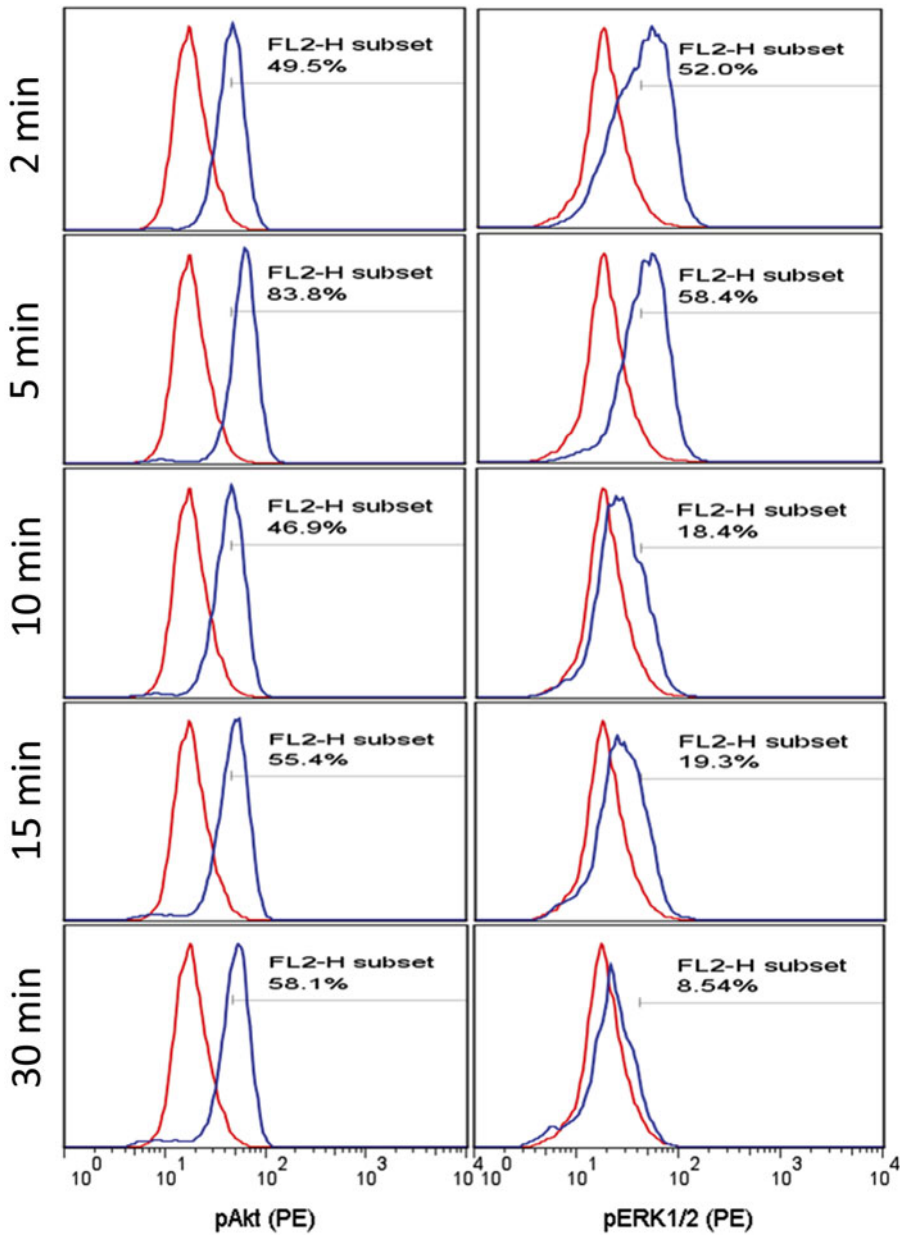


Fig. 1 Phosphorylation of Akt and ERK in LAD2 cells. A time course of phosphorylation in LAD2 cells following stimulation with SCF using phosphorylation flow cytometry demonstrating that Akt phosphorylation was sustained over 30 min, but ERK phosphorylation was more transient. Both Akt and ERK phosphorylation peaked at 5 min. The *red line* demonstrates staining with non-stimulated cells. The *blue line* overlays demonstrate staining at the indicated time point following SCF (100 ng/mL) stimulation

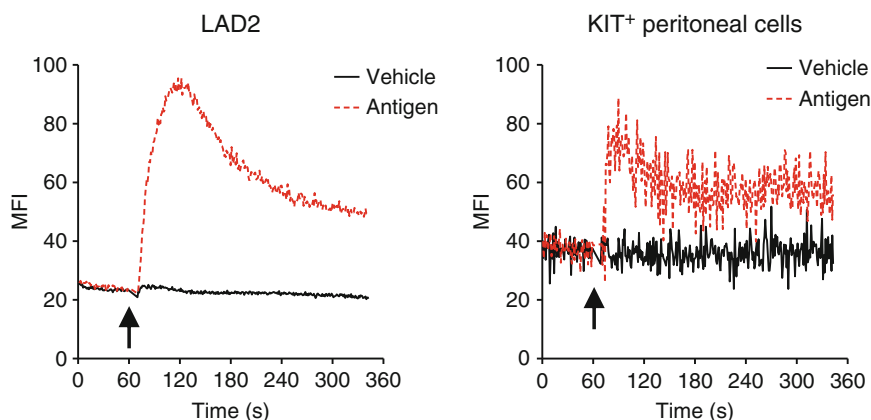


Fig. 2 Calcium response of activated mast cells. A calcium response of IgE-sensitized LAD2 mast cell line or KIT⁺ population of peritoneal cells to IgE cross-linkers (Antigen), respectively, streptavidin (100 ng/mL) or DNP-HSA (100 ng/mL). The cells were labeled by Fluo-4-AM and fluorescence intensity (MFI geometric mean fluorescence intensity) was monitored by flow cytometry before and after cell activation. The arrow indicates time of cell challenge

3.3 Actin Polymerization/ Depolymerization Kinetics

The flow cytometry analysis of actin polymerization/depolymerization in antigen-stimulated mast cells can be utilized both in a homogeneous cell population and in mast cell population that represents only a fraction of the analyzed cells or a population of transfected mast cells with a fluorogenic marker. This protocol describes the method for monitoring actin polymerization/depolymerization in homogeneous cell populations of IgE-sensitized and antigen-stimulated BMMCs (*see Note 14*).

1. Centrifuge at least 1×10^5 IgE-sensitized cells ($200\text{--}300 \times g$, 5 min, RT) and remove the media by aspiration.
2. Gently resuspend the cells in 1–5 mL HEPES/BSA buffer.
3. Centrifuge the cells as in **step 1** and remove the buffer by aspiration.
4. Repeat **steps 2** and **3**.
5. Gently resuspend the cells in HEPES/BSA buffer to a concentration of $0.5\text{--}1.0 \times 10^6/\text{mL}$.
6. Transfer the cell suspension (defined as volume γ) to 5 mL polystyrene round-bottom tubes and temperate the cells at 37 °C for 2–10 min.
7. Add an equal volume (volume γ) of $2 \times$ activator (e.g., antigen) in HEPES/BSA buffer (pre-warmed to 37 °C) and activate at 37 °C for different period of times.
8. Stop activation by addition of $10 \times \gamma$ volume of the fixation solution and incubate at RT for 20 min. Gently agitate the cell suspension every 5 min to avoid formation of aggregates.

9. Centrifuge the cells ($400\text{--}600\times g$, 5 min, RT).
10. Discard the supernatant, resuspend the cells in the incubation solution, and centrifuge ($400\text{--}600\times g$, 5 min, RT).
11. Discard the supernatant, resuspend the cells in the F-actin-labeling solution, and incubate at RT for 1 h in the dark.
12. Centrifuge the cells ($400\text{--}600\times g$, 5 min, RT).
13. Discard the supernatant and resuspend in the $10\times$ diluted incubation solution in PBS.
14. Repeat **steps 12 and 13**.
15. Analyze the cells in the suspension by flow cytometry within 60 min.

3.4 Degranulation Kinetics

The flow cytometry analysis of cell degranulation in antigen-stimulated mast cells can be utilized both in homogeneous cell populations and in mast cell populations that represent only a fraction of the analyzed cells, or in a population of transfected mast cells expressing a fluorogenic-tagged protein. This protocol describes the method for monitoring degranulation kinetics in a homogeneous cell population of IgE-sensitized and antigen-stimulated LAD2 cells and BMMCs.

1. Centrifuge at least 3×10^5 IgE-sensitized cells ($200\text{--}300\times g$, 5 min, RT) and remove the media by aspiration.
2. Gently resuspend the cells in 1–5 mL HEPES/BSA buffer.
3. Centrifuge the cells as in **step 1** and remove the buffer by aspiration.
4. Repeat **steps 2 and 3**.
5. Gently resuspend the cells in HEPES/BSA buffer to a concentration of $0.5\text{--}1.0\times 10^6$ cells/mL (the final volume of the cell suspension defined as volume γ) (*see Note 15*).
6. Transfer the cell suspension to 5 mL polystyrene round-bottom tubes and temperate the cells at 37°C for 2–10 min.
7. Add an equal volume (volume γ) of $2\times$ activator (DNP-HSA) in HEPES/BSA buffer (pre-warmed to 37°C) and activate at 37°C for different period of times.
8. Stop activation by addition of equal volume ($2\times \gamma$) of the labeling solution and incubate at RT for 30–60 min.
9. Analyze the cells in the suspension by flow cytometry within 60 min (*see Note 16*).

A typical example of degranulation kinetics is shown in Fig. 3.

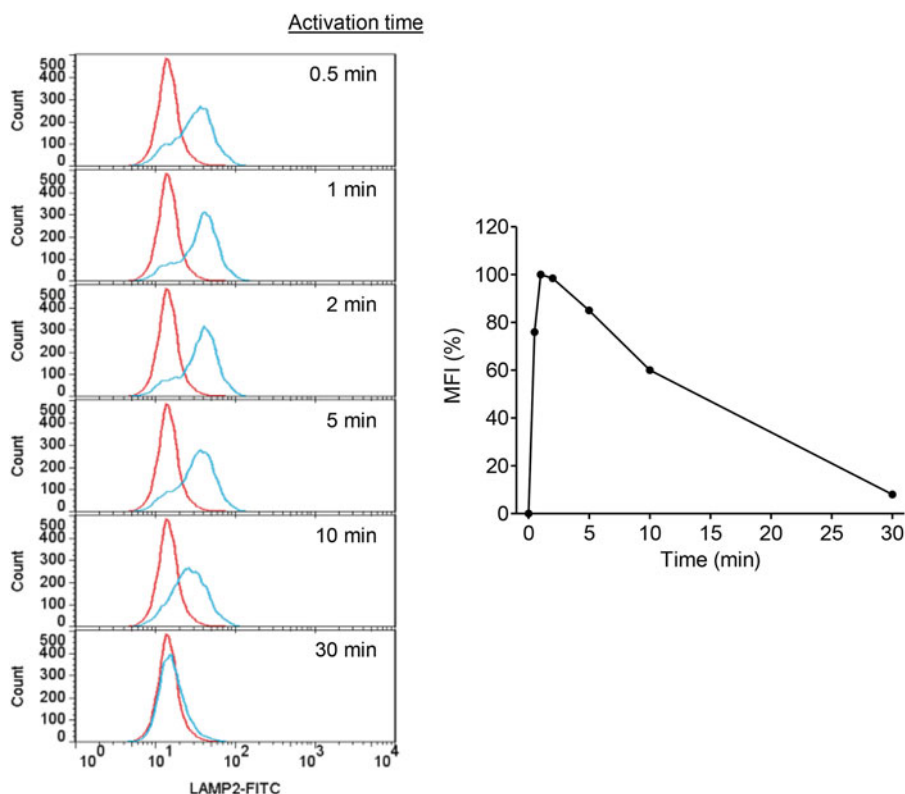


Fig. 3 LAMP-2 externalization kinetics in activated mast cells. A LAMP-2 externalization of gE-sensitized and antigen (streptavidin, 100 ng/mL)-activated LAD2 mast cell line. The externalization was detected by FITC-labeled LAMP-2-specific antibody and fluorescence intensities (*MFI* geometric mean fluorescence intensity) of non-activated (*red*) and activated cells (*blue*) were normalized to maximal response (*right*)

4 Notes

1. Biotinylate as described [8] or by use of an appropriate commercially available biotinylation kit.
2. In this chapter we describe the use of PE mouse anti-human phosphorylated ERK1/2 (pT202/Y204) and mouse anti-human phosphorylated Akt (pS473) PhosFlow antibodies. The antibodies used in this chapter represent well-characterized proteins in mast cell signaling. A variety of alternative antibodies are available for other signaling molecules. However, the specificity of the antibody is critical. Thus only well-characterized, highly specific, and validated antibodies should be used and adequate controls should always be included. Validation of the flow cytometry data with western blots should be carried out in control cells to establish the reliability of the antibodies prior to carrying out experiments with transfected populations.

3. Materials for BMMC culture and sensitization have been described previously [9].
4. The transfection of mast cells can be achieved using several approaches. This method describes the use of the Amaxa Nucleofector II. The process is quite harsh on the cells. Thus cells should be fed 24–48 h prior to transfection to ensure a healthy population. LAD2 cells should be cultured as described [10].
5. The optimal cell number for nucleofection may vary depending on the type of mast cells used. However, for LAD2 cells 2×10^6 is optimal.
6. The transfection of appropriate controls should always be included. For example, the empty GFP vector should be included. Extra tubes should be set up for each experiment to allow correct setting of the compensation for the flow cytometer. Unstained and untreated cells should be run as well as unstained tubes for each transfection (GFP positive but not stained for PE). Isotype controls and PE-stained GFP-negative cells should also be included. Each GFP construct will differ in fluorescence depending on the characteristics of the tagged protein. Thus each construct may need to be set up independently if fluorescence varies greatly.
7. The nucleofector program will vary according to the mast cell type used. For human mast cell lines, T-020, T-030, and X-001 have all been used. Optimization should be carried out for each cell type.
8. After transfection, it is recommended to culture the cells in complete medium to allow recovery from the procedure. It is not recommended to perform the cytokine withdrawal directly after nucleofection. Withdrawal of rhSCF is required for these assays since rhSCF induces significant signaling, but the cells should be given a minimum of 6 h in complete medium to recover before withdrawal of rhSCF.
9. The number of cells required for each condition will vary depending upon the efficiency of transfection for the different constructs. Each construct will differ in efficiency (even when using the same plasmid). Thus, to get enough cells in the GFP-positive population with a construct that transfects poorly, it will be necessary to increase the total number of cells. All of the transfections from each set of experiments should contain the same number of cells, so the number of cells per condition should be set from the construct with the lowest transfection efficiency.
10. Methanol will disrupt the conformation of GFP and consequently diminish fluorescence, so it should not be used to fix the cells. It is therefore necessary to adequately fix the cells with PFA (>30 min). This fixation will reduce the loss of fluorescence with methanol treatment. If the fluorescence is very

low, then other forms of permeabilization can be used, such as Triton X-100, which will not diminish fluorescence. However, these alternative permeabilization methods will reduce the signal on flow cytometry and do not work as well as methanol. If the cells are adequately fixed in PFA then loss of fluorescence with methanol is minimized.

11. In this example protocol we use KIT⁺ (CD117⁺) peritoneal cells as our mixed population. This method could also be applied to other various mast cell populations. All studies conducted in our laboratory mice and on mouse BMMCs are done so under a protocol (LAD2) approved by the Institutional Animal Care and Use Committee at NIH.
12. It is crucial to use the polystyrene tubes throughout the procedures as other polymers, such as polypropylene, cause strong attachment and possible activation of the processed cells. This leads to significant or even complete loss of the cells and their quality during the procedures.
13. The cell population of interest must be gated before the measurement and the basal fluorescence of the entire population must be on the scale. The wider the fluorescence peak of the population, the higher the basal level is required. As many flow cytometers are not equipped with a tempering system to keep the tube with cells at 37 °C, use a beaker or a thermo-beaker with 37 °C warm water instead to encase and temperate the tube with cells during the acquisition by flow cytometer.
14. In this example protocol, we use BMMCs, but this protocol could be modified for any mast cell type and can be used to study actin dynamics in mixed populations with the use of appropriate markers and gating.
15. The volume of cell suspension can vary depending on number of cells analyzed and number of cells available. The convenient volume is 50–200 µL.
16. The parameters of cell activation are sufficiently stable in the final suspensions. However, to ensure that there are no alternations in these parameters during the time of sample analysis, it is recommended to always re-measure the first measured sample also at the end of the measurement procedure.

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