

Membrane-Cytoskeleton Dynamics in the Course of Mast Cell Activation

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Abstract

Aggregation of the high-affinity IgE receptor (FcεRI) on the plasma membrane of mast cells and basophils initiates signaling events leading to a rapid release of preformed inflammatory mediators from secretory granules, and overall changes in cell morphology. Mast cell activation also causes reorganization of cytoskeletal components associated with membrane ruffling, spreading, and migration. Here we describe methods used for visualization of mast cell cytoskeleton, focusing on its two major components, microfilaments and microtubules, and their changes after cell triggering.

Key words Mast cells, Plasma membrane, Actin, Tubulin, Microfilaments, Microtubules

1 Introduction

Engagement of immunoreceptors, such as the B cell receptor (BCR), T cell receptor (TCR), and Fc receptors including the high-affinity IgE receptor (FcεRI) on mast cells and basophils, leads to dynamic remodeling of the plasma membrane, facilitating initiation and propagation of intracellular signaling. These early signaling events are followed by other signaling steps resulting in changes in cell morphology, migration, adhesion to various substrates, and, in case of mast cells and basophils, degranulation. Many of these events are dependent on the activity of cytoskeletal components [1–3].

1.1 The Role of Cytoskeletal Components in Mast Cell Physiology

The first indication that cytoskeleton is involved in mast cell signaling was obtained in studies on rat basophilic leukemia (RBL)-2H3 cell line [4]. It was shown that binding of multivalent antigen to IgE-FcεRI complexes induced formation of membrane ridges (lamellipodia). These ridges were observed 30 s after cell triggering, reaching the peak at 5 min, and their formation required continuous presence of antigen. Removal of the antigen resulted in complete reversion of the surface transformation within 5 min.

The extent of lamellipodia formation in IgE-sensitized cells depended on antigen concentration. The lowest dose of antigen was 1 ng/ml and the maximum response was observed at 5 µg/ml. These changes were accompanied by a rapid increase in the amount of filamentous (F)-actin. Enhanced formation of F-actin after cell triggering was reported in several other studies using RBL-2H3 cells [5–7], rat peritoneal mast cells [8], and mouse bone marrow-derived mast cells (BMMCs) [9]. A role of cytoskeleton was also implicated in the formation of signalosomes containing the FcεRI and some key enzymes (kinases and phosphatases) and several adaptor proteins [10–13]. Research has mostly focused on two cytoskeletal systems, namely microfilaments composed of actin, and microtubules formed by αβ-tubulin dimers.

1.2 Microfilaments

Microfilaments, also called actin filaments, are polar and flexible polymers of actin subunits. Actin filaments of about 6 nm in diameter are the thinnest fibers of the cytoskeleton. The two ends of an actin filament polymerize at different rates. The fast-growing end is called the plus (barbed) end, whereas the slow-growing end is called the minus (pointed) end. Although actin filaments are dispersed throughout the cell, they are mostly concentrated in the cortex, beneath the plasma membrane. Cortical actin cytoskeleton is connected to the plasma membrane through an array of closely related cytoplasmic proteins of the ERM (Ezrin, Radixin, Moesin) family. The C-terminal domains of the ERM proteins bind directly to actin filaments, whereas the N-terminal domains bind to transmembrane glycoproteins. Actin filaments are involved in cell movement, changes in cell shape, and organization of cytoplasmic components. They are assembled in two types of structure, bundles and networks.

Most of the studies analyzing the role of microfilaments in mast cell activation have used pharmacological inhibitors, cytochalasin D or latrunculin B. These drugs inhibit actin polymerization by different mechanisms: cytochalasin D by capping the barbed ends of actin filaments and thus preventing their elongation [14], whereas latrunculin B by sequestering the monomeric actin [15]. Previous studies showed that pretreatment of RBL-2H3 cells with inhibitors of actin polymerization enhanced Ca²⁺ mobilization and degranulation [5, 6]. Interestingly, latrunculin B alone initiated some activation pathways, suggesting that actin polymerization is critical for setting the threshold for mast cell triggering [7]. It should be noted, however, that long (1 h) exposure to high concentrations of latrunculin B (40 µg/ml) inhibited degranulation in compound 48/80-activated rat peritoneal mast cells [8]. These data indicate a positive role of F-actin in mast cell degranulation. Pretreatment with latrunculin B completely inhibited the antigen-induced migration [16] or spreading of mast cells on fibronectin ([9] and our unpublished results).

1.3 Microtubules

Microtubules are highly dynamic cytoskeletal polymers that are similarly as microfilaments inherently polar, and contain two structurally distinct ends: minus ends are stably anchored in centrosomes, whereas the plus ends are highly dynamic and switch between phases of growth and shrinkage [17]. Growing microtubules are characterized by accumulation of specialized microtubule-associated proteins, such as the end-binding (EB) family proteins [18]. Tagged versions of these proteins facilitate tracing the growing microtubules in living cells. It is well established that microtubules are involved in mast cell degranulation, since the movement of secretory granules depends on intact microtubules [19, 20]. This is supported by demonstration that agents inhibiting tubulin polymerization (e.g., nocodazole) also suppress degranulation [21, 22]. Importantly, Fc ϵ RI aggregation triggers the reorganization of microtubules at cell periphery [20, 22, 23]. It is of interest that several signal transduction molecules of RBL-2H3 cells and BMMCs form complexes with γ -tubulin, a minor component of the tubulin family, that is essential for microtubule nucleation [23, 24].

2 Materials

2.1 Cells

Several cell types have been used in studies on mast cell cytoskeleton: freshly isolated mast cells, cultured primary mast cells, and cell lines. RBL-2H3 cells and BMMCs predominate.

RBL-2H3 Cells

RBL-2H3 cells [25] grow as adherent to tissue culture-treated plastic or glass surfaces. The cells can be cultured in medium consisting of 1:1 mixture of RPMI-1640 and minimal essential medium (MEM) supplemented with nonessential amino acids, 3 mM L-glutamine, 1 mM sodium pyruvate, antibiotics [penicillin (100 units/ml) and streptomycin (100 μ g/ml)], extra D-glucose (2.5 mg/ml), and 10 % (v/v) heat-inactivated fetal calf serum (FCS). The cultures are maintained at 37 °C in humidified atmosphere of 5 % CO₂ in air. The cell monolayers are dissociated with 0.2 % EDTA in phosphate-buffered saline (PBS), pH 7.4, and subcultured twice a week. RBL-2H3 cells express large amount of Fc ϵ RI and therefore are useful for studies of this receptor. The disadvantage is that they are tumor-derived cells and some of their signaling pathways are therefore changed. They carry a mutation in c-kit [26] and, in contrast to BMMCs, do not thus require stem cell factor (SCF) for continuous growth in culture.

Bone Marrow-Derived Mast Cells

Bone marrow contains mast cell precursors that can be isolated from the femurs and tibias of 6–10-week-old mice. The cells are cultured in RPMI-1640 supplemented with FCS, antibiotics, recombinant interleukin (IL)-3 (PeproTech EC) at concentration 20 ng/ml, and recombinant stem cell factor (SCF) (PeproTech EC)

at a concentration 40 ng/ml for 5–7 weeks. In such cultures almost all cells (>95 %) express the FcεRI and cKIT. Before activation, BMMCs are cultured for 16–20 h in culture medium without SCF, followed by incubation for 3–4 h in SCF- and IL-3-free medium, supplemented with trinitrophenyl (TNP)- or dinitrophenyl (DNP)-specific IgE. The IgE-sensitized cells are then washed in buffered saline solution (BSS; 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 5.6 mM glucose, 1 mM MgCl₂) supplemented with 0.1 % bovine serum albumin (BSA) and challenged with various concentrations of TNP- or DNP-conjugated BSA. Alternatively, cells are cultured for 16 h in culture medium without SCF, followed by incubation in medium deprived for 3–4 h of SCF and IL-3 and activated for various time intervals with H₂O₂, pervanadate (a mixture of H₂O₂ and Na₃VO₄), thapsigargin, or other activators.

Cells of the mouse BMMC line denoted BMMCL [22] have been cultured in medium RPMI-1640, supplemented with 20 mM HEPES, pH 7.5, antibiotics, 100 μM MEM nonessential amino acids, 1 mM sodium pyruvate, 10 % FCS, and 10 % WEHI-3 cell supernatant as a source of IL-3.

2.2 Antibodies, Chemicals, and Kits

1. Primary and secondary antibodies used are summarized in Table 1.
2. Cell-Tak, Cell and tissue adhesive (BD Biosciences, San Jose, CA, USA).

Table 1
Specificity of antibodies and applications

Antigen	Species/clone/supplier, or reference	Conjugation
DNP	Mouse mAb, SPE-7, Sigma	None
TNP	Mouse mAb, IGEL b4 1, [36]	None
FcεRI β subunit	Mouse mAb, JRK, [37]	None
oxPTP	Mouse mAb, 335636, R&D Systems	None
Lyn	Mouse mAb, Lyn-01/Pr [38]	None
Syk	Mouse mAb, Syk-01/Pr [39]	None
α-Tubulin	Rabbit Ab, GeneTex	None
γ-Tubulin	Mouse mAb, TU-30, [40]	None
β-Tubulin	Mouse mAb, TUB 2.1, Sigma	Cy3
Mouse IgG	Goat Ab, Jackson ImmunoResearch Labs	Cy3
Rabbit IgG	Goat Ab, Invitrogen	Alexa Fluor 488
Mouse IgG	Goat Ab, Jackson ImmunoResearch Labs	12 nm gold particles

oxPTP oxidized protein tyrosine (PTP), Cy3 indocarbocyanate

3. DNP conjugated with BSA (Invitrogen, Molecular Probes; OR, USA).
4. Fibronectin from bovine plasma for cell culture (Sigma-Aldrich; Cat. No. F1141).
5. Fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich).
6. Alexa Fluor 488- or Alexa Fluor 555-labeled phalloidin (Invitrogen, Molecular Probes).
7. Mouse macrophage kit for nucleofection (Lonza Cologne AG, Köln, Germany).
8. MOWIOL 4-88 Reagent (Merck, Darmstadt, Germany).
9. Streptavidin conjugated with 5 nm colloidal gold particles (BBInternational; Cardiff, UK).
10. Poly-L-lysine hydrobromide (PLL; Sigma-Aldrich).

2.3 Buffers and Media

1. Buffered saline solution (BSS): 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 5.6 mM glucose, 1 mM MgCl₂.
2. BSS-BSA: 0.1 % BSA (w/v) in BSS.
3. Glutamate/EGTA buffer (GBE): 137 mM K-glutamate, 2 mM MgCl₂, 3 mM EGTA, and 20 mM PIPES, adjusted to pH 6.8 with KOH.
4. HEPES buffer: 25 mM HEPES, adjusted pH to 7.0 with NaOH, 25 mM KCl, 2.5 mM magnesium acetate.
5. Microtubule-stabilizing buffer (MSB): 100 mM MES, adjusted to pH 6.9 with KOH, 2 mM MgCl₂, 2 mM EGTA, 4 % (w/v) polyethylene glycol 6000.
6. RPMI medium for live-cell imaging: RPMI-1640 without phenol red, riboflavin, folic acid, pyridoxal, Fe(NO₃)₃, supplemented with 20 mM HEPES.
7. Osmium tetroxide in cacodylate buffer: 1 ml of 4 % (w/v) osmium tetroxide is mixed with 2.0 ml of 0.2 M sodium cacodylate buffer (pH 7.4) and 1 ml H₂O (*see Note 1*).
8. 1 % aqueous tannic acid: 0.5 g tannic acid is dissolved in 50 ml H₂O. Solution is filtered through 0.2 µm filter disc.
9. 1 % aqueous uranyl acetate: 2 % uranyl acetate (saturated solution) is prepared. Immediately before use, the required amount of the solution is filtered through 0.2 µm filter and mixed with equal volume of H₂O (*see Note 2*).

2.4 Other Materials and Equipment

1. Round glass cover slips, 12 mm in diameter (*see Note 3*).
2. Round glass cover slips, 10 mm in diameter, No. 1.
3. 35 mm glass-bottom culture dishes (MatTek Corp., Ashland, MA, USA).

4. Perfusion insert for 35 mm culture dish (Warner Instruments, Hamden, CT, USA; model RC-37F).
5. 8-Well multitest slides (MP Biomedicals, Irvine, CA, USA).
6. Electron microscopy nickel grids, Athene New 300, 3.05 mm (Ted Pella, Inc., Redding, CA, USA).
7. Thermo-conductive platform (BioCision, Mill Valley, CA, USA).
8. Amaxa Nucleofector II (Lonza Cologne AG, Cologne, Germany).
9. Olympus AX70 Provis microscope equipped with 60x water-immersion and 100x oil-immersion objectives and SensiCam cooled CCD camera (PCO IMAGING, Kelheim, Germany) for recording images.
10. Confocal laser scanning microscope Leica TCS SP5 equipped with 63x/1.4 NA oil-immersion objective.
11. Leica AM TIRF MC (Leica Microsystems): time-lapse sequences are acquired in TIRF mode using HCX PL APO 100x/1.46 NA oil-immersion TIRF objective.
12. Electron microscope Morgagni 268 operating at 80 kV (FEI Czech Republic, Brno, The Czech Republic).
13. Milli-Q water purification system (Millipore S.A., Molsheim, France).

2.5 Software

1. Image J software, version 1.38 (National Institutes of Health, Bethesda, MD, USA) or Olympus Scan^R analysis software (Olympus).
2. Huygens Deconvolution Software (Scientific Volume Imaging, Hilversum, The Netherlands).
3. Ellipse software, version 2.07 (ViDiTo, Systems, Košice, Slovakia).

3 Methods

3.1 Visualization of Microfilaments and Their Interactions with the Plasma Membrane

Immunofluorescence Microscopy

To study the distribution of actin filaments and their changes in the course of mast cell activation, phalloidin labeled with FITC or other fluorescent dyes are used. BMMCs and other nonadherent cells are immobilized to fibronectin-coated surfaces using Cell-Tak and processed as indicated below.

1. To each well of 6 mm multitest slide add 13 μ l of the solution containing 50 mM NaHCO₃, 50 μ g/ml fibronectin, and Cell-Tak (8 μ g/ml) and incubate for 12–16 h at 4 °C in a humidified chamber (*see Note 4*).
2. Wash fibronectin/Cell-Tak-coated wells once with BSS-BSA.
3. Add into each well 1.5×10^4 IgE-sensitized cells in 30 μ l of BSS-BSA.

4. Incubate the cells at 37 °C to allow their attachment.
5. After 1 h wash the cells with BSS-BSA.
6. Activate the cells by adding various concentrations of antigen (1–1,000 ng/ml).
7. After selected time intervals, fix the cells for 20 min at RT (RT) with 3 % paraformaldehyde in GBE buffer supplemented with 4 % polyethylene glycol (MW 3200).
8. For F-actin staining, wash the cells with 50 mM glycine in GBE and then add phalloidin conjugated with FITC, Alexa Fluor 488 or Alexa Fluor 555 diluted in GBE buffer supplemented with mild detergent, L- α -lysophosphatidylcholine (120 μ g/ml).
9. After 30 min, wash the cells four times with GBE and mount cover slips using Mowiol 4-88 containing 2 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) and 6.25 % propyl galate (w/v).
10. Take confocal images (e.g., on Leica TCS SP microscope).
11. Using Image J 1.38 software or Olympus Scan^R analysis software determine the mean cell area (*see* **Note 5**).
12. For each condition, evaluate at least 200 cells.

A typical distribution of actin filaments in nonactivated and activated cells stained by the procedure described above is shown in Fig. 1

Electron Microscopy

To analyze the topography of individual plasma membrane-bound components, plasma membrane sheets are isolated and then analyzed by two-step procedures using antibodies for target structures (antigens) as the first-step reagents and gold nanoparticle-labeled anti-antibodies as the second-step reagents. Alternatively, biotin-labeled antibodies or other ligands such as phalloidin can be used for detection of the target structures and visualized with gold-labeled streptavidin. Both adherent (RBL) and nonadherent (BMMC) cells can be analyzed. Data documenting the applicability of this procedure for analyzing cell signaling events in RBL cells and BMNCs have been presented [10–13, 27–32]. Below is described a protocol for studies using cultured BMNCs which are nonadherent to tissue culture-treated plastic or glass surfaces and require IL-3 and SCF for optimal growth.

1. To sensitize the cells, transfer BMNCs into fresh complete culture medium supplemented with IL-3- and DNP-specific IgE, but without SCF (*see* **Note 6**).
2. After 16–24 h, wash the cells in BSS-BSA, count them, and adjust concentration to 1×10^7 /ml in 1:4 mix of PBS and complete culture medium with FCS, but without SCF, IL-3, and IgE (activation medium).

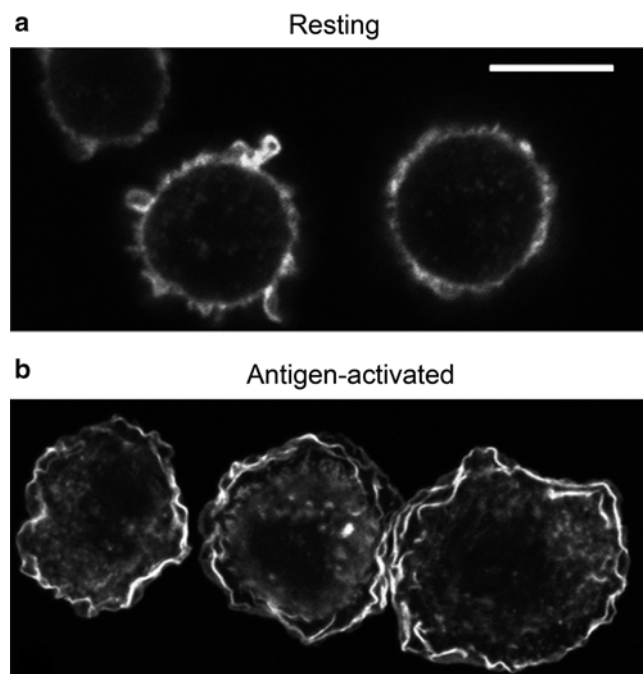
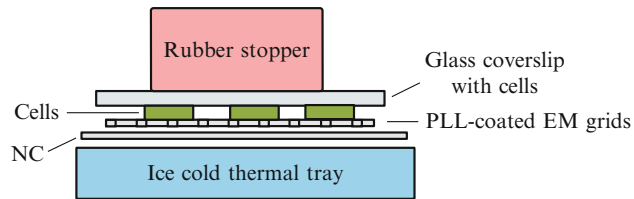


Fig. 1 Topography of actin filaments in nonactivated and activated mast cells visualized by light microscopy. IgE-sensitized BMMCs were attached to fibronectin-coated glass surface and then exposed to BSS alone (**a**; *resting*) or BSS supplemented with antigen (**b**; *antigen activated*). After 20 min at 37 °C, the cells were fixed and stained for F-actin using Alexa Fluor 488-labeled phalloidin. The images are confocal micrographs taken at equatorial planes. Scale bar, 10 μ m. Reproduced by permission from ref. 9. Copyright 2011; John Wiley and Sons

3. Transfer 200 μ l aliquots of the cell suspension into each well of 24-well plate containing ultraclean fibronectin-coated cover slips (*see Note 7*).
4. After 1 h, add 1 μ g/ml of antigen (DNP-BSA conjugate) to activate the cells (*see Note 8*).
5. During activation, prepare a beaker with ice containing a thermal tray. Then transfer wet nitrocellulose on the thermal tray followed by poly-L-lysine hydrobromide (PLL)-covered electron microscopy grids put on the wet nitrocellulose (*see Note 9*).
6. Stop antigen-mediated activation of the cells by immersing the cover slips in ice-cold HEPES buffer.
7. Press cover slips with face-down oriented cells for 10 s onto PLL-covered EM grids placed on wet nitrocellulose filter on ice-cold thermal tray using rubber stopper. This creates a sandwich containing cells bound to both cover slip (via fibronectin) and EM grid (via PLL) (Fig. 2a). Then side-lift the cover slip, exposing the cells to shearing force leading to cell disruption (Fig. 2b).

a A sandwich formed by cells bound to EM grid and glass



b Isolation of EM grids-bound PM sheets

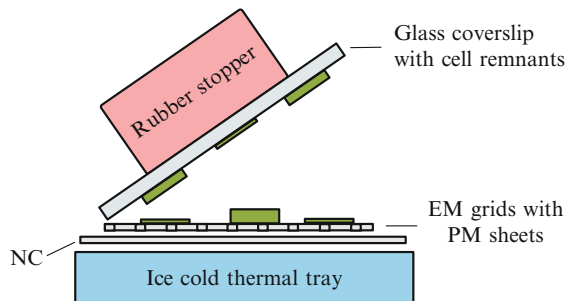


Fig. 2 Schematic outline of the method used for isolation of plasma membrane (PM) sheets bound to electron microscopy grids. **(a)** A sandwich is formed by consecutively adding on thermal tray placed in a beaker with ice (1) NC paper wetted with HEPES buffer, (2) EM grids with the PLL sides up, (3) cover slip with bound/spread cells facing the EM grids, and (4) rubber stopper to facilitate firm pressing of the cells on cover slips towards EM grids. **(b)** Plasma membrane sheets are then isolated by rapid side-lifting of the cover slip exposing the cells to shearing forces and resulting in tearing off the plasma membranes bound to PLL layer on EM grids and glass cover slip

In this way, dorsal plasma membrane is bound to PLL on EM grid and cytoplasmic side is amenable for analysis.

8. Immediately, transfer EM grids with teared PM sheets facing down onto surface of ice-cold HEPES buffer in a beaker.
9. After 5–10 s, transfer floating EM grids into another beaker with ice-cold 2 % paraformaldehyde in HEPES buffer.
10. Fix the cells for 10 min, then transfer sequentially the floating grids into three beakers with PBS, and incubate for 10–20 min in each beaker to remove the fixative.
11. Transfer the grids into another beaker with PBS-0.1 % BSA and incubate for 15 min at 20–25 °C to block the protein-reactive sites.
12. To label the intracellular leaflet proteins, incubate the grids for 30 min on drops of PBS-0.1 % BSA supplemented with selected first-layer antibodies/reagents, followed by four 5-min washes with PBS.

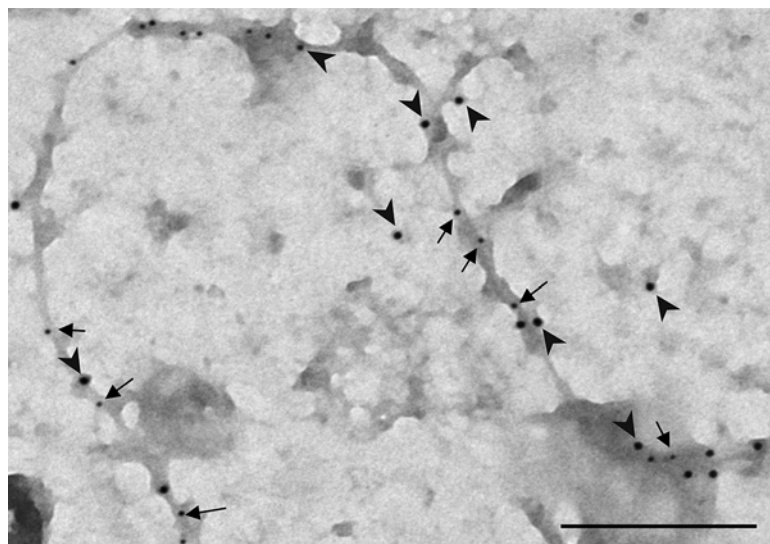


Fig. 3 Topography of actin filaments and oxidized phosphatases visualized by electron microscopy on isolated plasma membrane sheets. Plasma membrane sheets were isolated from BMMCs activated with 0.2 mM pervanadate for 5 min. The sheets were labeled from the cytoplasmic side for oxidized PTPs with oxPTP mAb followed by goat antibody anti-mouse IgG conjugated to 12 nm gold particles (*arrowheads*), and for F-actin with biotin-labeled phalloidin, followed by streptavidin conjugated with 5 nm gold particles (*arrows*). Scale bar, 200 nm. Reproduced from ref. 32. Copyright 2010; the American Society for Biochemistry and Molecular Biology

13. Then incubate the grids with gold-labeled second-layer antibodies and again wash with PBS as above.
14. Postfix the specimens with 2.5 % glutaraldehyde in PBS for 10 min and again wash with PBS for 30 min.
15. Stain the samples with 1 % OsO₄ in cacodylate buffer for 10 min, followed by three 5-min washes in water.
16. Incubate the specimens with 1 % aqueous tannic acid and again wash three times with water.
17. Stain the membranes with 1 % aqueous uranyl acetate and wash again with water.
18. Air-dry the samples and observe them in an electron microscope such as FEI Morgagni 268 operating at 80 kV.
19. Analyze the distribution of gold nanoparticles using proper software (*see Note 10*).

Figure 3 shows the distribution of actin filaments and oxidized phosphatases on cytoplasmic side of the plasma membrane in BMMCs as detected by electron microscopy on isolated plasma sheets from BMMCs prepared by a method as described in this section.

3.2 Visualization of Microtubules and Their Dynamic Changes

Immunofluorescence Microscopy

To study the distribution of microtubules and their changes in the course of mast cell activation, direct or indirect immunofluorescence microscopy with antibodies to tubulin is usually applied. Microtubules are labile structures and therefore proper fixation/extraction conditions have to be selected for their detection with antibodies [33]. When BMMCs are used, they are immobilized on fibronectin-coated surfaces and processed as indicated below.

1. Lay down cover slips (10 mm in diameter) on 30 μ l droplets of fibronectin solution (50 μ g/ml in 50 mM NaHCO₃) on Parafilm and incubate for 12–16 h at 4 °C in a humidified chamber. Rinse, thereafter, cover slips in PBS. Coating of cover slips with fibronectin can be speeded up by incubation at 37 °C for 60 min in a humidified chamber.
2. Transfer cover slips to Petri dishes (diameter 3.5 cm, fibronectin side up) and overlay them with suspension of control or IgE-sensitized cells at a concentration 1.5–2.0 $\times 10^6$ cells/ml (*see Note 11*).
3. Incubate cells for 1 h at 37 °C to allow their attachment.
4. Wash cells, then activate at 37 °C by adding various concentrations of antigen (10–1,000 ng/ml), and incubate for selected time intervals (1–10 min). Alternatively, activate cells by per-vanadate or thapsigargin [22].
5. Fix samples for 20 min at 37 °C with 3 % (w/v) paraformaldehyde in MSB, wash three times with MSB at 37 °C (5 min each wash), extract for 4 min at 37 °C with 0.5 % (v/v) Triton X-100 in MSB, and finally wash again three times with MSB at 37 °C (5 min each wash) (*see Note 12*).
6. Label the cells for 60 min at RT with anti-tubulin antibody. Dilute monoclonal antibody to β -tubulin TUB 2.1 conjugated with Cy3 1:600 in 2 % (w/v) BSA in PBS (*see Note 13*).
7. Wash the cells three times with PBS at RT (5 min each wash).
8. Mount cover slips in MOWIOL 4-88 reagent containing 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI).
9. Observe samples in laser scanning confocal microscope such as Leica TCS SP microscope equipped with 63 $\times$ /1.4 NA oil-immersion objective. For Cy3 visualization use excitation and emission wavelengths 561 nm and 566–633 nm, respectively (diode-pumped solid-state laser). Acquire optical sections in 125 nm steps, and make z series from 70 sections. Perform deconvolution and rotation using Huygens Deconvolution Software.
10. Perform three independent immunofluorescence experiments to estimate the number of cells that respond to activation events by generation of protrusions containing microtubules [22]. In each experiment examine approximately 500 cells.

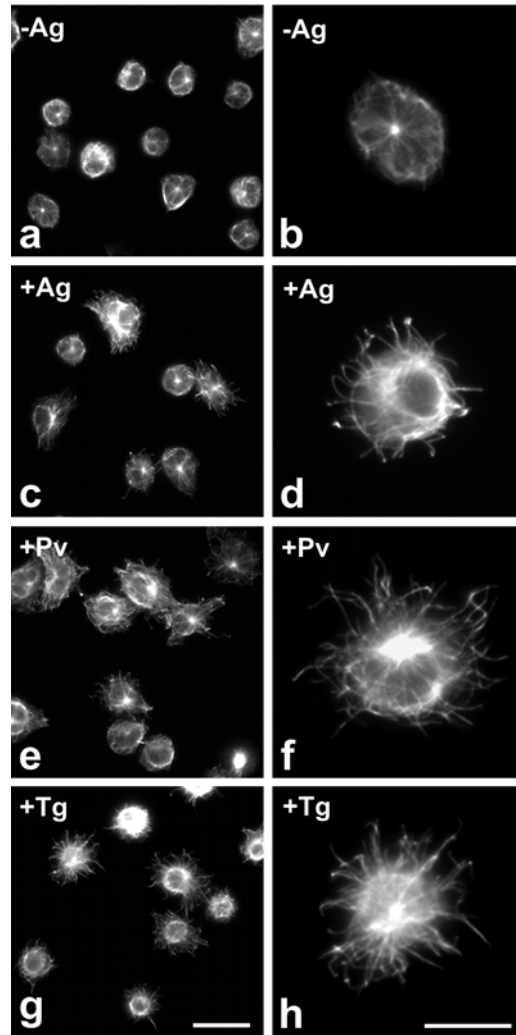


Fig. 4 Organization of microtubules in resting and activated mast cells. Resting BMMCs (**a, b**; $-Ag$), cells activated by Fc ϵ RI aggregation (**c, d**; $+Ag$), pervanadate (**e, f**; $+Pv$), or thapsigargin (**g, h**; $+Tg$) were fixed in formaldehyde and extracted in Triton X-100. Staining for β -tubulin. Scale bars, 20 μ m (**g**) and 10 μ m (**h**). Comparable magnifications are in **a, c, e, g** and in **b, d, f, h**. Reproduced by permission from ref. 22. Copyright 2011; The American Association of Immunologists, Inc.

A typical distribution of microtubules in non-activated and activated BMMCs stained by the procedure described above is shown in Fig. 4. Deconvoluted 3-D images from laser scanning confocal microscopy, shown in Fig. 5, demonstrate that microtubule protrusions in BMMCs do not reflect merely the spreading of cells during activation events, since they are also found on the dorsal side. A typical location of γ -tubulin in resting RBL-2H3 cells is demonstrated in Fig. 6. γ -Tubulin concentrates on centrosomes of interphase and mitotic cells from which microtubules are nucleated.

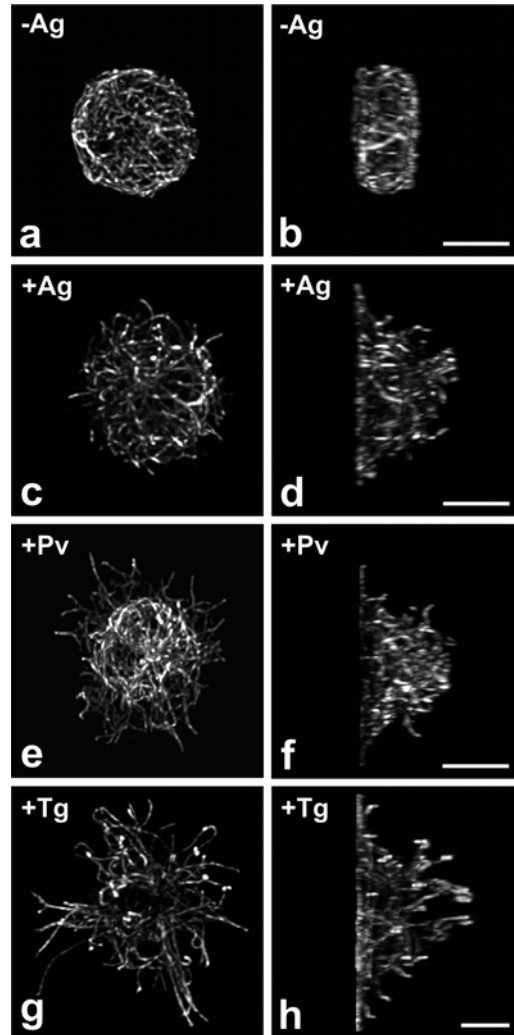


Fig. 5 Laser scanning confocal microscopy of microtubules in resting and activated BMMCs. Resting cells (**a, b**; $-Ag$), cells activated by Fc ϵ R1 aggregation (**c, d**; $+Ag$), pervanadate (**e, f**; $+Pv$), or thapsigargin (**g, h**; $+Tg$) were fixed in formaldehyde and extracted in Triton X-100. Staining for β -tubulin. The stacks of confocal sections were deconvoluted and subjected to three-dimensional reconstruction. Resulting 3-D images viewed from top of the cells (**a, c, e, g**) and from the plane perpendicular to the plane of cell adhesion (**b, d, f, h**). Each pair, **a/b**, **c/d**, **e/f**, and **g/h** represents the same cells. Scale bars, 5 μ m. Reproduced by permission from ref. 22. Copyright 2011; The American Association of Immunologists, Inc.

*Time-Lapse Imaging by
Total Internal Reflection
Fluorescence Microscopy
(TIRFM)*

To study the dynamics of microtubules on their plus ends in the course of mast cell activation, TIRFM on living cells, expressing tagged EB1 protein, is performed. Expression plasmid coding mouse EB1 fused with GFP (pEB1-GFP) was used in the following protocol [34].

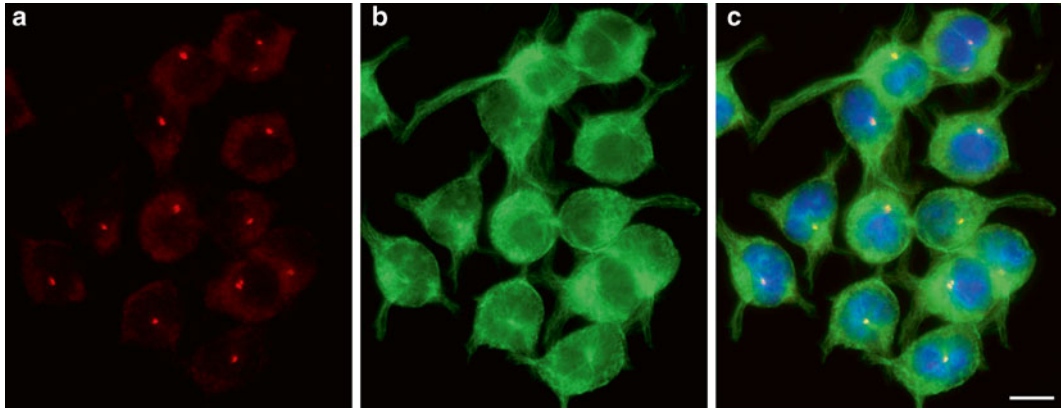


Fig. 6 Distribution of microtubules formed by $\alpha\beta$ -tubulin dimers and γ -tubulin in resting RBL-2H3 cells. Cells were fixed with cold methanol before staining with mouse monoclonal antibody TU-30 to γ -tubulin (**a**, *red*) and rabbit antibody to α -tubulin dimer (**b**, *green*). DNA was stained with DAPI (*blue*). Superposition of $\alpha\beta$ -tubulin and γ -tubulin is shown in **c**. Scale bar, 10 μm . Photography E. Dráberová (Institute of Molecular Genetics AS CR, Prague)

1. Transfect cells with pEB1-GFP by nucleofection using Mouse Macrophage Kit and program Y-001 on Amaxa Nucleofector II according to the manufacturer's instructions. After nucleofection, transfer cells into culture media supplemented with IL-3 and culture for 24 h before analysis.
2. Overlay a 35 mm glass-bottom culture dish (MatTek) pre-coated with fibronectin, as in Subheading "Immunofluorescence Microscopy," **step 1**, with 100 μl sample of cell suspension at a concentration 1.5×10^6 cells/ml. Allow cells to attach for 1 h at 37 °C.
3. Insert perfusion insert for 35 mm culture dish, wash cells, and subsequently incubate in RPMI-1640 medium for live-cell imaging.
4. Observe cells in TIRF microscope such as Leica AM TIRF MC at 37 °C. Acquire time-lapse sequences of EB1-GFP in TIRF mode (GFP cube, laser line 488 nm, Ex: 470/40, Em: 525/50, penetration depth 150 nm) using HCX PL APO 100 $\times$ /1.46 NA oil-immersion TIRF objective. Take images for 3 min at 1-s intervals with 30-40 % laser power and exposure times ranging from 500 to 800 ms. Scan cells before, during, and after adding the activation agents (*see Note 14*).
5. Adjust time-lapse sequences and analyze by particle-tracking program. Detect regions of pixels with distance less than 3 μm from cell boundary. Calculate the speed of particles as the ratio of particle trajectory length and trajectory duration. Calculate the histogram of the particle speed from the trajectory speed weighted by the trajectory duration (*see Note 15*).

6. Compute statistical data from a total of 15 to 21 different cells tracked in three independent experiments.

Activation of mast cells raises the number of growing microtubules in cell periphery as documented in Fig. 7.

4 Notes

1. Osmium tetroxide is extremely toxic heavy metal and, therefore, all work with it should be done carefully. Sodium cacodylate is also highly toxic by ingestion, inhalation, or skin contact. Work with osmium and sodium cacodylate should be done in fume hood.
2. Uranyl acetate is extremely toxic heavy metal and slightly radioactive (0.37–0.51 $\mu\text{Ci/g}$). Therefore, all work with it should be done carefully and in fume hood.
3. Glass cover slips should be thoroughly cleaned with synthetic detergent. After cleaning, the cover slips must be washed several times with ultraclean water (deionized water further purified to resistivity of $\geq 18 \text{ M}\Omega\cdot\text{cm}$ using a Milli-Q water purification system) to remove all traces of detergent, followed by a brief wash with 96 % ethanol, drying and transferring individually into a beaker with 35 % HCl. After 20–24 h the cover slips are washed several times with ultraclean water to remove all HCl. Finally, they are washed with ethanol and stored in ethanol until use.
4. To enhance the adhesiveness of BMMCs on fibronectin-coated glass, the cell adhesive BD Cell-Tak (8 $\mu\text{l/ml}$) is combined with fibronectin; importantly, cell spreading on Cell-Tak alone is not apparent.
5. The areas of activated cells can be normalized to the mean area of appropriate resting cells. The proportion of spread cells is calculated from the total number (100 %) of rounded, spread, and “ambiguous” cells.
6. RBL and some other mast cell lines can be cultured in simplified culture medium lacking IL-3 and SCF.
7. Cover slips are covered with fibronectin by incubation at 4 °C for 18–24 h with fibronectin (50 $\mu\text{g/ml}$ in 0.43 M NaHCO_3), followed by washing with distilled H_2O .
8. The cells can be activated with various concentrations of antigen or with other activators, such as 0.2 mM pervanadate [32].
9. EM grids covered with pioloform and coated with carbon are glow-discharged for 45–60 s by 300 V, incubated for 30 min with 1 mg/ml PLL in H_2O , washed for 5 s in H_2O , and dried. Immediately before isolation of PM sheets, EM grids are placed

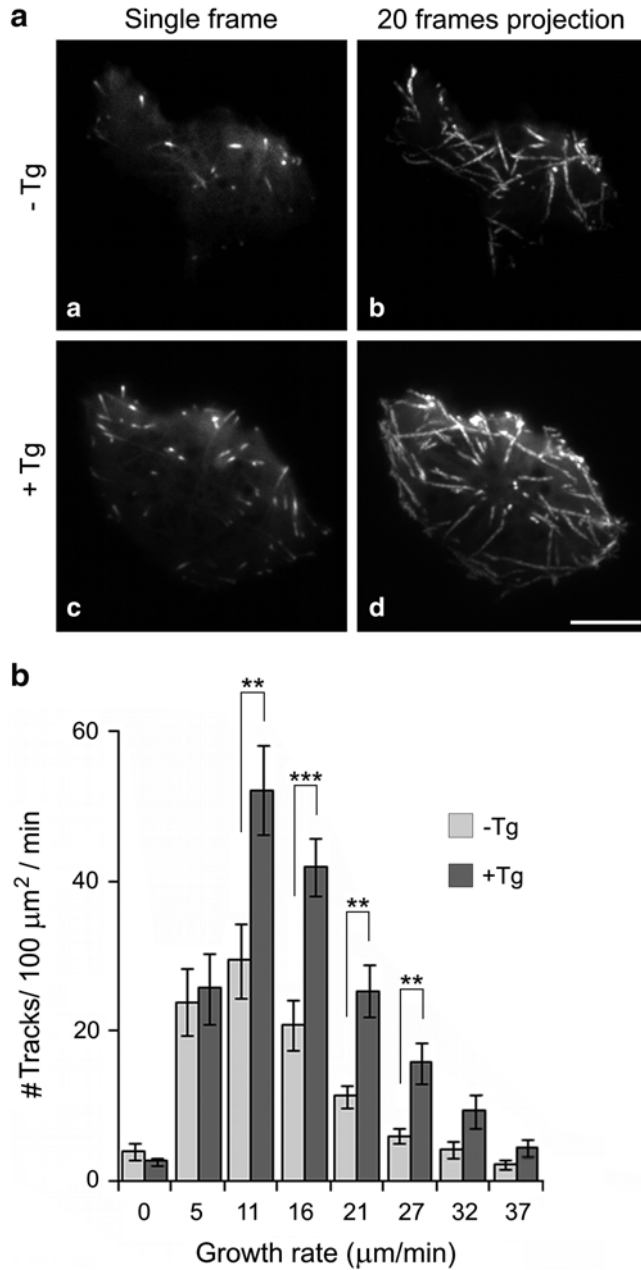


Fig. 7 Changes in the number of growing microtubules in cell periphery during mast cell activation as determined by TIRFM time-lapse imaging in living cells. **(A)** Time-lapse imaging of resting (*a, b*) and thapsigargin-activated (*c, d*) BMMCs expressing EB1-GFP. Still images of EB1 (*a, c*) and tracks of EB1 comets over 20 s created by maximum intensity projection of the 20 consecutive frames (*b, d*). Scale bar, 5 μm . **(B)** Histogram of microtubule growth rates in cell periphery of resting (-Tg) and thapsigargin-activated (+Tg) cells. A total of 15 different cells were tracked in five independent experiments. Values indicate mean $\pm$ SE, $n=15$ (** $p<0.01$; *** $p<0.001$). Reproduced by permission from ref. 22. Copyright 2011; The American Association of Immunologists, Inc.

with the PLL side up onto a HEPES buffer-soaked nitrocellulose membrane filter (Millipore, 0.45 μm pores) kept on ice-cold thermo-conductive platform.

10. Typically, three independent experiments are performed for each condition and 10–20 micrographs covering 22–44 μm^2 of the plasma membrane are obtained from each grid. The coordinates of gold particles are determined using ImageJ software. Statistical evaluation of particle clustering is based on program Gold [35] using the pair correlation function (PCF), which expresses the ratio of the density of gold particles at a given distance from typical particle to the average density of such particles.
11. When cells are activated by pervanadate or thapsigargin, the sensitization step is skipped.
12. Fixation with formaldehyde followed by extraction in Triton X-100 is preferable for direct immunofluorescence staining of microtubules with Cy3-TUB 2.1 conjugate. When γ -tubulin is detected, cold methanol fixation is used. In this case, samples are incubated for 20 min in methanol precooled to -20°C .
13. In double-label experiments intended to detect both microtubules and γ -tubulin, the methanol-fixed cells are washed with PBS at RT (three times, 5 min each wash) and incubated simultaneously with primary antibodies for 60 min at RT. Rabbit antibody to α -tubulin is diluted 1:200 and mouse monoclonal antibody to γ -tubulin (TU-30; spent hybridoma culture medium) to 1:5 in 2 % (w/v) BSA in PBS. After washing with PBS at RT (three times, 5 min each wash), the cover slips are incubated simultaneously for 45 min at RT, with conjugated secondary antibodies. Alexa Fluor 488-conjugated anti-rabbit antibody is diluted 1:200 and Cy3-conjugated anti-mouse antibody 1:1,000.
14. To follow the depolymerization of microtubules by TIRFM, nocodazole can be added at a concentration of 10 μM .
15. For analysis of time-lapse sequences we have been using in-house-written particle-tracking plug-in that is implemented into Ellipse software version 2.07 [22].

Acknowledgements

We thank Dr. M. Hibbs (Ludwig Institute for Cancer Research, Melbourne, Australia) for the gift of BMMCs cell line and Dr. Y. Mimori-Kiyosue (KAN Research Institute, Kyoto, Japan) for EB1-GFP construct. Work on mast cells in the laboratories of authors has been funded, over the years, by several grants including 204/09/1777, P302/14/09807S, P305/14/00703S, and

P302/12/G101 from the Czech Science Foundation; Grants LC545, LD12073, LD 13015, and 1M6837805001 from the Ministry of Education, Youth and Sports of the Czech Republic; Project KAN200520701 and M200520901 from Academy of Sciences of the Czech Republic; and the Institutional Research Support (RVO 68378050).

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