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Real-time Imaging of Ca^{2+} Mobilization and Degranulation in Mast Cells

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Summary/Abstract

Mast cells play a key role in allergy and inflammation processes as part of the immune response. The activation of mast cells via antigen binding and cross-linking of IgE receptors initiates the onset of dramatic calcium (Ca^{2+}) mobilization dynamics that promote the release of mediators of inflammation and allergy. Ca^{2+} signaling in mast cells has been studied extensively using a variety of research tools and techniques. In these studies, a large number of proteins have been identified to participate in various stages of these processes.

Here we describe single cell imaging as an important approach for examining Ca^{2+} signaling and exocytosis in mast cells. Single cell imaging tools have advanced significantly over the last ten years, in part due to improvements in microscope technology and in part due to the development of a new generation of Ca^{2+} indicators and genetically encoded Ca^{2+} sensors. The single-cell imaging techniques described here provide the spatial and temporal resolution required to decipher the signaling events that are critical for mast cell functions.

Keywords

live cell imaging; calcium (Ca^{2+}) signaling; calcium dynamics; mast cell degranulation

1. Introduction

1.1. Regulation of Ca^{2+} signaling and Ca^{2+} influx in mast cell function

Mast cell signaling is tightly regulated by cytosolic Ca^{2+} dynamics, which are mediated by stimulated Ca^{2+} release from intracellular stores and Ca^{2+} entry via several channels at the plasma membrane (Ma and Beaven 2009). Elevation in cytosolic Ca^{2+} activates a cascade of downstream events that trigger exocytosis of secretory granules. This process, also known as degranulation, is responsible for the release of mediators of allergy and inflammation due to antigen binding to IgE that is associated with its high affinity receptor, $\text{Fc}\epsilon\text{RI}$. For recent reviews on mast cell Ca^{2+} signaling see (Ma and Beaven 2009) and (Holowka et al. 2012). Investigating the spatial and temporal aspects of Ca^{2+} dynamics and identifying the channels

involved in these signaling events are crucial to our understanding of mast cell regulation and function. Cytosolic Ca^{2+} in resting mast cells is maintained in the ~ 100 nM range, whereas activation of these cells results in a rapid rise to μM concentrations. Under physiological conditions, Fc ϵ RI-mediated activation of mast cells induces this rapid elevation via activation of phospholipase C γ (PLC γ) to produce inositol 1,4,5-trisphosphate (IP3), which binds to IP3 receptors at the endoplasmic reticulum (ER) membrane to release a concentrated store of Ca^{2+} . Release of Ca^{2+} from intracellular stores then stimulates store operated Ca^{2+} entry (SOCE), giving rise to a sustained phase of Ca^{2+} elevation. During the past two decades, live cell microscopy has significantly advanced, making single cell imaging possible for a wide variety of applications. In parallel to advances in microscopy, probes to monitor changes in Ca^{2+} concentrations have improved significantly, with fluorescent dyes and genetically encoded proteins providing a panel of specific and versatile tools for the visualization and quantification of Ca^{2+} dynamics. We previously showed that spatial regulation of the initial Ca^{2+} rise in RBL mast cells depends on TRPC1-mediated influx of extracellular Ca^{2+} (Cohen et al., 2009). These events were identified as Ca^{2+} waves that propagate release of Ca^{2+} from the ER, and move directionally through the cytoplasm. Ca^{2+} waves in mast cells are followed by SOCE, which mediates oscillatory changes in cytosolic Ca^{2+} concentration. The oscillations most likely encode temporal information required for the regulation of various cell functions.

Under conditions of weak stimulation by low but physiologically relevant doses of antigen (Ag), we observe spatially restricted Ca^{2+} elevations, termed Ca^{2+} puffs. The nature and function of these puffs in mast cells is not completely understood, but they frequently appear in cellular protrusions, and in many cells they precede full waves and activation of downstream events.

1.2. Ca^{2+} mobilization and entry in mast cells

1.2.1. Real-time imaging—In contrast to single cell imaging, Ca^{2+} measurements can be made on large populations, using either a fluorimeter with suspended cells or a plate reader with adherent cells. A major benefit of single cell imaging experiments is the opportunity for spatial resolution, and this approach should be applied when morphology, motility, or expression pattern of associated proteins are relevant to the scientific question. On the other hand, when information about spatial regulation is not imperative, population experiments provide superior statistical significance with considerably less effort by the researcher.

1.2.2. Ca^{2+} sensors and indicator dyes—Fura2 was the one of the first indicators used to monitor cytosolic changes in Ca^{2+} levels. Since its development by Tsien and colleagues (Grynkiewicz, Poenie et al. 1985), the availability of Ca^{2+} indicators to suit different experimental criteria has expanded widely. In parallel to the development of synthetic Ca^{2+} dyes, the technology for genetically encoding Ca^{2+} sensors within cellular proteins has improved significantly over the last decade. The growing list of synthetic Ca^{2+} dyes provides an excellent toolkit for investigating a range of Ca^{2+} -related phenomena, not only those occurring in the cytosol but also in particular cellular organelles as well as near the plasma membrane (PM). Researchers who employ Ca^{2+} imaging as an integral part of their

experimental investigation should consider the benefits and drawbacks of the two different families of Ca^{2+} indicators: synthetic or genetic.

Synthetic dyes provide superior brightness, ease of delivery (mainly by AM loading, see below), and a panel of dyes with various spectra and affinities for range of Ca^{2+} concentrations have become available. Additionally, some of these dyes provide the possibility of ratiometric measurements of actual Ca^{2+} concentrations (i.e. fura-2 and indo-1; (Grynkiewicz, Poenie et al. 1985)).

Possible limitations associated with synthetic dyes include phototoxicity, leakage from the cells, and concentration in subcellular organelles. Another significant concern stems from relatively fast loading of these dyes into cells and fast diffusion kinetics, which facilitate Ca^{2+} buffering and its unwanted (or unknown) effects on cell physiology. We found this to be a problem in mast cells, and, for example, we previously showed significant differences in Ca^{2+} mobilization dynamics when using Fluo4 and Fluo5 in comparison to genetically encoded GCaMP2 (Cohen et al., 2009).

Genetically encoded Ca^{2+} indicators (GECI) provide an alternative that is often preferable to synthetic dyes for live cell Ca^{2+} imaging. These fluorescent recombinant proteins have improved dramatically over the last decade in terms of their expression levels as well as their sensitivity and rate of response to changes in Ca^{2+} concentrations. The previous generation of FRET-based sensors (i.e. “cameleon” (Miyawaki, Llopis et al. 1997)) has been replaced with permutated GFP constructs that are bright, stable and display fast kinetics. The new generation of GECI, including GCaMP3 and 5 (Tian, Hires et al. 2009) as well as the multi-color sensor GECO proteins (Zhao, Araki et al. 2011), now offer a considerably enhanced toolkit for Ca^{2+} imaging.

Although using these GECIs involves transfection of cells, they benefit from several advantages over synthetic dyes including, gradual expression that maintains the cell's Ca^{2+} homeostasis, as well as very low or no leakage from the cells or into organelles. In addition, GECI can be targeted to specific organelles by means of fusion with targeting peptide sequences for subcellular and local Ca^{2+} measurements. For more information on GECI see McCombs and Palmer (McCombs and Palmer 2008).

1.3. Pharmacological tools for isolation of specific Ca^{2+} pathways in mast cells

Several channel and transporter families play a role in mast cell Ca^{2+} regulation. The major players include transient receptor potential channels (TRPC), calcium-release activated calcium channels (CRAC), inositol trisphosphate receptors (IP3R) and ion transporters (plasma membrane Ca^{2+} ATPase (PMCA), and sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). Numerous pharmacological agents target these various Ca^{2+} channels with variable specificity. Some of the common pharmacological agents include: SERCA Inhibitors: thapsigargin and cyclopiazonic acid. IP3R inhibitors: adenophostin A, xestospongine C, and possibly 2-aminoethoxydiphenyl borate (2-APB). SOCE inhibitors: lanthanides, nickel, 2-APB, and other non-specific Ca^{2+} channel blockers.

In some experiments it is useful to eliminate Ca^{2+} influx from the processes being observed. For this purpose, a Ca^{2+} free buffer (with or without the addition of Ca^{2+} chelators) can be used. Although Ca^{2+} free buffer involves less non-specific effects, various pharmacology agents offer the means to isolate selected channel pathways. However, one must always take into account and control for potential non-specific effects of any reagent used.

1.4. Ca^{2+} signaling dynamics in mast cells: Puffs, waves and oscillations

Mast cell Ca^{2+} dynamics involve a complex ensemble of spatially and temporally restricted changes in Ca^{2+} concentrations. Antigen stimulated signaling mediated by IgE receptors causes local and global changes in Ca^{2+} levels with regulated amplitudes and durations. We recently described Ca^{2+} puffs, waves, and oscillations, which exhibited dynamics that depend on the stimulus and experimental conditions (Cohen et al., 2009). Ca^{2+} oscillations were reported in mast cells more than a decade ago (Kim, Eddlestone et al. 1997). Additional characterization of Ca^{2+} waves and puffs provides a more complete view of regulation of Ca^{2+} signaling in mast cells. We observed a compelling association between mast cell morphology and Ca^{2+} signaling dynamics (Cohen et al., 2009) in which elongated protrusions are sites for initiating spatially restricted changes in Ca^{2+} concentration following IgE crosslinking by Ag. Elongated protrusions are common to mast cells in vivo (Metcalf, Baram et al. 1997; Crivellato, Baldini et al. 1993; Demitsu, Kiyosawa et al. 1999), and these are also manifest in cells grown in culture (see Figure 1). Puffs and waves both demonstrate directional propagation in Ca^{2+} elevation, initiating mostly from the tips of protrusions. However, Ca^{2+} puffs are spatially confined and localized responses, whereas waves propagate through the entire cell. As in the case of waves, Ca^{2+} oscillations radiate throughout the cell and do not display lateral propagation.

Several features of Ca^{2+} waves in mast cells can be quantified using simple analysis. These include the region of origin, wave velocity, and overall Ca^{2+} elevation (see section B9). Other parameters of interest include rise time and decay that can be calculated from wave curve fitting and correlated with Ca^{2+} -induced calcium release (CICR) and Ca^{2+} clearance mechanisms. Features of Ca^{2+} oscillations that are of interest include amplitude and frequency (peak to peak time), as well as oscillation rise and decay times. Here we provide some basic tools for analyzing Ca^{2+} signaling in mast cells. Examination and quantification of other features, not covered here, may also be useful for addressing specific questions about these dynamic responses.

1.5. Imaging mast cell degranulation

1.5.1. Regulation of mast cell exocytosis—As in excitable cells, exocytosis of granules in mast cells depends on Ca^{2+} mobilization. As for other membrane fusion processes, degranulation has been shown to involve specific sets of SNARE proteins (soluble NSF attachment protein receptor proteins) and associated regulators including synaptotagmins (for a recent review see (Blank 2011)). Synaptotagmins trigger membrane-membrane fusion in a process that depends on C2 domain binding to a Ca^{2+} / phosphatidylinositol 4,5-bisphosphate complex (Chapman, 2008).

1.5.2. Mast cell degranulation—Various methods for measuring granule exocytosis in mast cells have been utilized, including measurement of β -hexosaminidase release (Naal et al., 2004), amperometry (Kim et al., 1998), and immunodetection of plasma membrane-associated granule proteins (Gadi et al., 2011). In addition, a number of methods have been developed to visualize individual granule exocytotic events in mast cells (Hohman and Dreskin, 2001). Early studies utilized acridine orange, a fluorescent weak base that accumulates in acidified vesicles (Kawasaki et al., 1991; Williams and Webb, 2000). However, a recent study demonstrated that photosensitization of this dye during imaging limits its utility (Jaiswal et al., 2007). Other strategies have utilized granule-localized serotonin fluorescence imaged using multiphoton microscopy, together with complementary membrane and lysosome-labeling probes (Williams et al., 1999). Although these methods allow detection of individual stimulated granule exocytotic events in mast cells, they are technically demanding and therefore limited in their usage.

1.5.3. Real-time monitoring of mast cell exocytosis—The method we describe here utilizes pre-loading of fluorescein isothiocyanate conjugated dextran (FITC-dextran) into mast cell granules via fluid phase endocytosis, taking advantage of the pH sensitivity of FITC fluorescence. Visualization of granule exocytosis in real time is possible by detection of pH-dependent FITC dequenching that takes place once the granule fuses with the PM and its pH is equilibrated with the extracellular medium (Figure 4). As will be described later, this FITC-dextran method provides a robust and sensitive method for visualizing individual granule exocytosis events, which can be related to other cellular activities.

The high sensitivity of FITC to pH, together with the robust loading of the dextran into secretory granules, makes this method highly suitable for measurements of degranulation in mast cells with excellent temporal and spatial resolution (Figure 5). In addition, FITC-dextran loading can be used as a tool to measure degranulation in a population of cells (suspended or adherent) using a fluorimeter (as described in section 3F) or a plate reader. This approach provides a means for screening and high throughput assays.

1.5.4. Simultaneous imaging of degranulation and Ca^{2+} mobilization—To allow real time, direct monitoring of the relationship between Ca^{2+} mobilization and granule exocytosis in mast cells, we devised a combined imaging strategy that uses a Ca^{2+} indicator together with FITC-dextran. This strategy provides excellent spatial and temporal resolution of both Ca^{2+} and degranulation dynamics to evaluate how Ca^{2+} waves, puffs and oscillations relate to exocytosis of secretory granules in mast cells. To combine FITC-dextran-based degranulation and Ca^{2+} imaging, one can use a Ca^{2+} indicator dye with red emission (Ca^{2+} Red Asanate or Fura-Red; see Figure 6) or, alternatively, a red-shifted GECI (R-GECO; (Zhao, Araki et al. 2011)) in combination with FITC-dextran.

2. Materials

2.1. Genetically encoded calcium indicators (GECI) cDNA expression plasmids

1. GECI plasmid: 5-8 μg of purified plasmid (e.g., GCaMP2 and GCaMP3) per reaction (5×10^6 mast cells) (Note 1).

2.2. Chemicals and reagents

1. Synthetic Ca^{2+} indicators: A large number of Ca^{2+} -sensitive fluorescent dyes are currently available, and describing all the possibilities is beyond the scope of this chapter. We find that for measurements of Ca^{2+} dynamics in mast cells, the relatively newly developed Fluo-4 family, including Fluo4-AM, Fluo5F-AM and Fluo5N-AM (Invitrogen), is most appropriate. These dyes have a range of affinities for Ca^{2+} , and they are bright, stable, and available as acetoxymethyl esters (AM) for simple and fast loading into cells.
2. Reagents: We use multivalent antigen (DNP-BSA), thapsigargin, Ca^{2+} ionophore (A23187), sulfinpyrazone, hydroxytryptamine (5HT), and FITC-dextran (150kDa) in our studies.

2.3. Tissue culture

1. Mast cells (RBL-2H3 mast cells or bone marrow derived mast cells) (Note 2).
2. Complete medium: Minimal Essential Medium (MEM) containing 20% fetal bovine serum (FBS) and 10 $\mu\text{g}/\text{mL}$ gentamicin sulfate.

2.4. Imaging equipment

1. Glass bottom dishes. (Note 3)
2. Picospritzer. (Note 4):
3. Temperature controlled microscope/objective (Note 5). See Section 2.5

2.4. Solutions and buffers

1. Balanced salt solution (BSS): 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 1 mg/mL glucose, 20 mM HEPES (pH 7.2-7.4).
2. BSS+BSA: BSS with 1 mg/mL bovine serum albumin (BSA).
3. Electroporation buffer: 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 1 mg/mL glucose, 20 mM HEPES (pH 7.4).
4. Stimulating solution: BSS + BSA with antigen (DNP-BSA at 1 ng - 1 $\mu\text{g}/\text{mL}$ final concentration)

¹Several families of GECI are currently available, and newer versions are being developed. Of special interest are the GCaMP indicators, constructed of permutated GFP fused together with calmodulin (CaM) and the M13 peptide. The GCaMP indicators have improved significantly in recent years, and a panel with diverse sensitivity and fluorescent spectra is currently available (e.g., GCaMP2 and GCaMP3, 5 R-GECO, G-GECO, B-GECO, organelle targeted, PM-targeted, etc. Our experience has been mainly with GCaMP2 and GCaMP3, but other GECI could be used with similar procedures.)

²Mast cells: Our experimental system is based on RBL-2H3 mast cells (Barsumian, Isersky et al. 1981). These mast cells are typically sensitized with monoclonal IgE specific for anti-2, 4-dinitrophenyl (DNP) (Posner, Lee et al. 1992))

³We typically use coverslip dishes from MatTek Corp. These dishes combine the convenience of disposable plastic petri dishes with the optical quality of glass. Several other brands are available. The dish diameter and glass bottom thickness should be selected based on the dimensions of the microscope in use.)

⁴This device supplies repeatable pressure pulses for local delivery of a stimulating solution. The system we use requires preparation of pulled capillaries (Parker Hannifin Corp.)

⁵There are many thermal/environmental regulation and control systems available for microscopy. For our purposes we found that a simple objective heater (e.g., from BioOptics Inc) provides sufficient control over temperature.

2.5. Microscope and data analysis software

1. Confocal or wide field microscopy can be used to monitor Ca^{2+} flux and degranulation. The image acquisition rate and detector sensitivity are most important for successful Ca^{2+} imaging. If using an objective heater, an oil immersion objective should be considered for better thermal conductance. To obtain adequate spatial resolution, 40× through 100× objectives with high numerical apertures should be considered.
2. Data acquisition software is usually an integral part of the microscope system being used, and different microscope brands provide different acquisition software with varying control over imaging parameters.
3. Data analysis software. Several commercial and open source options are available. ImageJ (NIH) and Fiji are two open source platforms that enable simple modifications and programming to support specific analysis needs. In addition, ImageJ has numerous readily available plug-ins that are especially useful for live cell imaging and analysis. Some of the most useful plug-ins are discussed in section 4.2 and Note 8.

3. Methods

3.1. Cell preparation

1. Maintain RBL-2H3 mast cells (Barsumian, Isersky et al. 1981) in monolayer culture in MEM with 20% FBS and 10 $\mu\text{g/mL}$ gentamicin sulfate.
2. Transfect cells with GECI plasmid as follows:
 - a. Resuspend 5×10^6 cells in 0.5 mL of cold electroporation buffer containing 5-8 μg of plasmid DNA.
 - b. Electroporate at 280 V and 950 μF (Gene Pulser X (Bio-Rad))
3. Plate cells directly onto MatTek dishes ($\sim 5 \times 10^5$ cells/plate).
4. Sensitize transfected cells with 0.5 $\mu\text{g/mL}$ anti-DNP IgE during overnight cell culture (Posner, Lee et al. 1992).

⁸Troubleshooting Ca^{2+} imaging

- a. Ca^{2+} dynamics and temperature: Suboptimal temperature can dramatically reduce the antigen induced Ca^{2+} response in mast cells and significantly slow its kinetics. To avoid these issues, it is recommended that cells are kept in 37°C at all times and that temperature of the dish is monitored regularly. Buffers and media to be added should be warmed (37°C) in advance.
- b. Optimization of cell density in single cell imaging: for single cell imaging only a few hundred cells per dish are sufficient. We previously showed that protrusions play key functions in mast cell activation (Cohen, Torres et al. 2009), and these need sufficient cell free area to develop and be visualized. In addition, cell-cell interactions might interfere with Ca^{2+} mobilization. Thus, we recommend plating low numbers of cells when preparing for microscopy experiments, as described.
- c. Optimization of Ca^{2+} dye loading: For reasons described in the first section, we recommend using genetically encoded indicators for mast cell Ca^{2+} imaging. However, using synthetic Ca^{2+} indicator dyes can also provide useful information. For these experiments dye-loading conditions must be optimized including: dye concentration, concentration of leakage inhibitor, sulfinpyrazone, duration and temperature of incubation. In our hands, using low affinity Ca^{2+} indicators (such as Fluo5F) improves Ca^{2+} responses in the cells and yet provides sufficient resolution and sensitivity.

5. Under these conditions, the cell transfection efficiency with GCaMP2 is typically 20–30% (Note 6).
6. Load cells with synthetic Ca^{2+} indicators (fluo4-AM family of dyes is recommended). When Ca^{2+} dyes are to be used, harvest cells 3–5 days after passage, and plate overnight (16–24 h) in MatTek coverslip dishes in 2 mL of complete medium (5×10^5 cells/dish).
7. The next day, incubate cells for 30 min at 37°C in BSS containing 0.5 μM of the Ca^{2+} indicator and 2.5 mM sulfinpyrazone.
8. Wash cells into BSS+BSA buffer.
9. Cells can be incubated in BSS+BSA buffer at 37°C for up to 1 h before imaging.

3.2. Microscope set up

1. Before preparing cell samples, ensure that microscope is operating and ready for image acquisition (e.g., excitation light source and filters should be ready).
2. Set temperature of objective or chamber to maintain at 37°C.
3. If using the picospritzer, prepare capillary by loading with stimulating solution.
 - a. Adjust micromanipulator and attach pipette.
 - b. Calibrate picospritzer (required for initial experiments) using fluorescently labeled solution (e.g., FITC-dextran) to monitor stimulating puff intensity and spreading.
 - c. Fabricate glass capillaries using a puller device so that the tip has a final diameter of $\sim 5 \mu\text{m}$.
4. If a picospritzer or an equivalent instrument is not available, add stimulant-containing solution to the dish by pipetting. Remember to adjust the concentration of stimulant according to the desired final concentration and dilution factor.

3.3. Imaging

1. 24 h after GECI transfection (or after loading cells with Ca^{2+} indicator dye), wash cells with pre-warmed (37°C) BSS+BSA, and mount on the microscope.
2. Select appropriate illumination wavelength and filter lines. For GCaMP2, Fluo-4, or Fluo-5F use 488-nm excitation and view with a 505–530 nm band-pass filter.
3. Locate cells in the dish that express GECI (or are loaded with Fluo dyes).
4. Zoom in as much as possible for best resolution (see Figure 1 for common morphology of RBL-2H3 cells in culture).

⁶Several methods of transfection are available, and Ca^{2+} phosphate is probably the simplest and commonly used. However, for the experiments with GCaMP2 as the Ca^{2+} indicator we typically use electroporation because of its relatively high efficiency with RBL-2H3 cells.

5. If using the picospritzer, approach cells with a $\sim 5\text{-}\mu\text{m}$ -diameter pulled-glass capillary, until positioned within $\sim 100\text{ }\mu\text{m}$ distance from the cell.
6. Image cells at approximately 10–30 Hz. Ca^{2+} dynamics in mast cells should be easily detectable when recorded at 5–20 Hz. (Note 7).
7. Care should be taken to optimize conditions for Ca^{2+} imaging. These include dye loading, temperature, and cell density (Note 8)

3.4. Image processing and analysis

Here we describe some simple and basic tools for image analysis that we use for evaluating fundamental Ca^{2+} dynamics in RBL mast cells. Researchers are encouraged to try these and various other analysis approaches and tools. More information about image processing, analysis, and quantification methods is available. See ImageJ documentation (<http://rsbweb.nih.gov/ij/docs/index.html>) or reference (Collins 2007) for examples. In addition, ImageJ has numerous readily available plug-ins that are especially useful for live cell imaging and analysis. Some of the most useful plug-ins are described in Note 9.

1. Ca^{2+} waves (see Figures 2 and 3):
 - a. Spatial origin of the waves can be determined visually, based on the site of the initial Ca^{2+} elevation.
 - b. Wave velocity can be calculated from the number of frames required for a wave to propagate along the cell.
2. Ca^{2+} puffs (see Figures 2C and 2D): Spatial and temporal dynamics of puffs can be determined as described for waves.
3. Ca^{2+} oscillations (see Figure 3): Features of the oscillations can be calculated from a plot of fluorescence intensity over time and integrating the fluorescence signal over a specified region of interest (ROI) (e.g., over the cell body).

3.5. Fluorimetry-based Ca^{2+} measurements

1. Harvest cells 3–5 days after passage.
2. Resuspend 10^6 cells in 1 mL BSS supplemented with 2.5 mM sulfinpyrazone (to reduce dye leakage).
3. Add 0.5 mM Fluo4-AM (or other fluorescent indicating dyes; set emission and excitation filters accordingly) and immediately mix. Incubate at 37°C for 1 min.

⁷Slower imaging rate might impair the temporal resolution of these events. Faster imaging could provide better resolution, but at the cost of increased photobleaching. Pharmacological reagents can be added to the dish just before initiating data collection or earlier as required. It is not recommended to add any solution during acquisition as it may move the dish and impair imaging.

⁹ImageJ functions and plug-ins for analysis and quantification of Ca^{2+} imaging

- a. Time line analysis (“virtual linescan” plug-in) – this plug-in is used for generation of kymographs (see Figure 2A).
- b. Z profiler plug-in – this plug-in can be used for plotting F (intensity) over time (see Figure 2B).
- c. Interactive 3D Surface Plot – a useful plug-in for generating a more vivid visual representation of fluorescence as a function of time (F/t; see Figure 3A lower panel).
- d. All plug-ins can be downloaded directly from ImageJ website (<http://rsbweb.nih.gov/ij/>)

4. Add 9 mL of BSS+BSA, and incubate cells in 37°C for 30 min; during this incubation sensitize cells with IgE (2 µg/mL).
5. Wash the cells into fresh BSS+BSA, and verify appropriate dye loading by microscopy. It is important to make sure that the dye is loaded into the cytosol and not into organelles.
6. Use steady-state fluorimeter to measure changes in Ca²⁺ concentration upon addition of various stimulants to stirred cells at 37°C.
7. Add Triton X-100 (0.1%) at the end of the experiment to determine maximal indicator response to 2 mM Ca²⁺, followed by excess EGTA (10mM) to assess background signal when no Ca²⁺ is bound.

3.6. Monitoring Degranulation with Fluorescence

1. Harvest cells 3-5 days after passage and plate in MatTek dishes.
2. Culture 1×10⁶ cells in 2 mL of full medium in the presence of 2 mg/mL FITC-dextran and anti-DNP IgE (0.5 µg/mL) (if desired) for 24 h at 37°C.
3. Optional: Add 5-hydroxytryptamine (HT) (0.2 mM final concentration) to induce increased granule diameter for better visualization with microscopy (Bonifacino, Yuan et al. 1989).
4. After 24 h incubation (37°C) with FITC-dextran (± IgE), wash cells once with PBS and resuspend in fresh buffer(BSS+BSA) at 1×10⁶ cells/mL.
5. Incubate washed cells for 1 h at 37°C.
6. Add 0.5 mL of the resuspended cells (approx. 2.5×10⁵ cells) to 1.5 mL BSS in a stirred acrylic cuvette.
7. Monitor FITC fluorescence (excitation (λ=490 nm), emission (λ=520 nm)) at 37°C using an SLM 8100C steady-state fluorimeter (SLM Instruments, Urbana, IL) - or similar instrument -- in a time-based acquisition mode.
8. Add 0.1% Triton X-100 to lyse cells at the end of each experiment to record unquenched FITC fluorescence to normalize the time course.
9. Degranulation analysis: For basic analysis see Figures 4, 5 and 6.
10. Optional co-imaging of degranulation and Ca²⁺ changes.
 - a. If using dye for Ca²⁺ imaging, load dye following the wash step as indicated in Ca²⁺ imaging section (i.e. Fura Red; see Figure 6).
 - b. Image acquisition: As described in the Ca²⁺ imaging section.
11. Care should be taken to optimize conditions for imaging degranulation. These include temperature and dye and dextran loading (Note 10).

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¹⁰Troubleshooting FITC-dextran degranulation imaging

- a. As for Ca²⁺ imaging, degranulation is highly temperature sensitive, and special care should be taken to make sure cells are maintained at 37°C at all times.
- b. Sulfinpyrazone used for loading of Ca²⁺ indicator dyes should be avoided in degranulation experiments as it can inhibit of this cellular response.
- c. Increasing FITC-dextran concentration during loading period could improve signal to noise ratio under conditions where low degranulation may occur. Up to 4mg/mL dextran-FITC can be used.
- d. Combining FITC-dextran with a non pH-sensitive fluorophore conjugated to the dextran (such as TexasRed-dextran) can confirm proper loading into granules. This can also be useful in focusing on the cells (and finding the right field of cells), zooming, or focusing for FITC-dextran imaging. In addition, this combination enables the researcher to follow granule movement in the cells prior to exocytosis.

Zhao Y, Araki S, et al. An expanded palette of genetically encoded Ca(2) indicators. Science. 2011; 333(6051):1888–1891. [PubMed: 21903779]

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Figure 1. Elongated morphology of mast cells

Image of RBL-2H3 mast cells in culture demonstrates their elongated morphology. Typically, cells will have one or two protrusions extending to various lengths away from the cell body. Morphological features of mast cells (length, angle, volume etc.) can be quantified using various overlay tools in Image J or other image processing software. Fluorescent label is Fluo4.

Figure 2. Imaging and quantifying Ca^{2+} waves and puffs

In these examples, RBL-2H3 cells expressing the Ca^{2+} sensor GCaMP2 were monitored for fluorescence intensity as a function of time before and after stimulation by antigen, and changes in Ca^{2+} concentration are presented in pseudo colors (warmer colors represent higher Ca^{2+} levels). A) Upper: image of cell, with direction of Ca^{2+} wave indicated; lower: time line analysis (“Virtual Linescan” plugin for Image J) provides both spatial and temporal information about changes in Ca^{2+} concentration. This analysis measures the changes in fluorescence over time along a designated line across the cell. Here the line width is 2 pixels. This visualization can be used to determine the point of origin of the wave. B) Wave propagation can be visualized and quantified by plotting the fluorescence signal from two regions of interest (ROI; 1-protrusion, 2- cell body) over time and measuring the time difference between them (Δt) as a function of distance (in μm ; “Z Profiler” plug-in for Image

J). The ROI quantification can be used to determine wave velocity. C) As described for waves in A, time line analysis can be used to detect and visualize the spatial attributes of Ca^{2+} puffs. In this example, the puffs are localized and confined to the cell's protrusion. D) Temporal dynamics of Ca^{2+} puffs can be quantified from fluorescence (F) vs time plots, as in the case of waves. Here we integrated the fluorescence intensity over the cell body (2) or protrusion (1) to show the transient Ca^{2+} elevation during puffs.

Figure 3. Visualizing and quantifying Ca^{2+} oscillations

RBL-2H3 cell expressing GCaMP2 was stimulated with Ag and imaged for ~100 sec. A) Time line analysis along the cell body (upper panel) reveals repetitive Ca^{2+} oscillations (middle panel; warmer colors represent higher Ca^{2+}). Lower panel illustrates a “3D” representation of the kymograph (“Interactive 3D Surface Plot” plug-in for Image J), for enhanced visualization of spatial and temporal dynamics of the oscillations. B) Integration of the fluorescence signal over the cell body plotted against time can be used to quantify the oscillation's dynamics. With this approach, one can measure the oscillation peak-to-peak interval (a, frequency) and peak width (b). Additional parameters can be extracted including oscillation rise phase (representing Ca^{2+} influx), down phase (representing Ca^{2+} clearance), and the integrated Ca^{2+} elevation during various time points.

Figure 4. Imaging of single cell degranulation events using FITC-dextran

A) Individual frames taken from ~3 minutes of imaging RBL-2H3 cells loaded with FITC-dextran and stimulated with Ag. Arrows point to some of the degranulation events taking place in each frame. B) For initial evaluation of the total degranulation magnitude from cells, a Z projection (integrating 160 sec) can be used which shows the sum of all or a range of frames.

Figure 5. Quantification of single cell degranulation events using FITC-dextran

As for Ca^{2+} waves and oscillations, integration of fluorescence intensity over ROI or time line analysis can be used for quantification of degranulation visualized with FITC-dextran. A) Time line analysis (middle panel) over cell of interest (upper left panel, broader yellow line) reveals the spatial and temporal dynamics of degranulation events as they occur along the cell in a single kymograph. Further enhancement of visualization can be achieved by a 3D representation (lower panel). This analysis can be used, for example, to evaluate the spatial distribution of degranulation events across the cell. B) Integration of fluorescence intensity over individual degranulation events provides a simple tool for quantifying the exocytosis dynamics. Here we show a fast exocytosis event (left panel, 1) and a biphasic degranulation event where the granule undergoes two-step fusion and release of contents (right panel, 2). This method enables detection and distinction between “kiss and run”, “full” and “compound” types of exocytosis common to mast cells (Cohen, Corwith et al. 2012).

Figure 6. Simultaneous imaging of Ca^{2+} mobilization and degranulation in cells using Fura-Red and FITC-dextran

In these experiments a fluorescent Ca^{2+} indicator (synthetic or GECI) is used in combination with FITC-dextran loaded into cells. In this case we used Fura-Red to measure Ca^{2+} changes. A) Time line analysis of Ca^{2+} concentration changes (red) or degranulation (green) channels is shown separately (left and middle lower panels) and combined to create an overlay kymograph (right panel). The overlay kymograph provides temporal and spatial view of the Ca^{2+} changes and degranulation throughout the cell. B) Integration of fluorescence over ROI in red or green channels provides high temporal resolution of correlated Ca^{2+} mobilization and degranulation events over particular regions of the cell (e.g., protrusions or cell body), as can be seen in the plot of $\Delta F/F_0$ vs time (lower panel). Black trace shows Ca^{2+} response, and individual colors show different exocytic events.