

## Real-Time Imaging of $\text{Ca}^{2+}$ Mobilization and Degranulation in Mast Cells

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### 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 ( $\text{Ca}^{2+}$ ) mobilization dynamics that promote the release of mediators of inflammation and allergy.  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signaling and exocytosis in mast cells. Single-cell imaging tools have advanced significantly over the last 10 years, in part due to improvements in microscope technology and in part due to the development of a new generation of  $\text{Ca}^{2+}$  indicators and genetically encoded  $\text{Ca}^{2+}$  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.

**Key words** Live-cell imaging, Calcium ( $\text{Ca}^{2+}$ ) signaling, Calcium dynamics, Mast cell degranulation

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## 1 Introduction

### 1.1 Regulation of $\text{Ca}^{2+}$ Signaling and $\text{Ca}^{2+}$ Influx in Mast Cell Function

Mast cell signaling is tightly regulated by cytosolic  $\text{Ca}^{2+}$  dynamics, which are mediated by stimulated  $\text{Ca}^{2+}$  release from intracellular stores and  $\text{Ca}^{2+}$  entry via several channels at the plasma membrane [1]. Elevation in cytosolic  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signaling, *see* [1, 2]. Investigating the spatial and temporal aspects of  $\text{Ca}^{2+}$  dynamics and identifying the channels involved in these signaling events are crucial to our understanding of mast cell regulation and function. Cytosolic  $\text{Ca}^{2+}$  in resting mast cells is maintained in the  $\sim 100$  nM range, whereas activation of these cells results in a rapid rise to  $\mu\text{M}$  concentrations.

Under physiological conditions, FcεRI-mediated activation of mast cells induces this rapid elevation via activation of phospholipase Cγ (PLCγ) to produce inositol 1,4,5-trisphosphate (IP<sub>3</sub>), which binds to IP<sub>3</sub> receptors at the endoplasmic reticulum (ER) membrane to release a concentrated store of Ca<sup>2+</sup>. Release of Ca<sup>2+</sup> from intracellular stores then stimulates store-operated Ca<sup>2+</sup> entry (SOCE), giving rise to a sustained phase of Ca<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> dynamics. We previously showed that spatial regulation of the initial Ca<sup>2+</sup> rise in RBL mast cells depends on TRPC1-mediated influx of extracellular Ca<sup>2+</sup> [3]. These events were identified as Ca<sup>2+</sup> waves that propagate release of Ca<sup>2+</sup> from the ER and move directionally through the cytoplasm. Ca<sup>2+</sup> waves in mast cells are followed by SOCE, which mediates oscillatory changes in cytosolic Ca<sup>2+</sup> 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<sup>2+</sup> elevations, termed Ca<sup>2+</sup> 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<sup>2+</sup> Mobilization and Entry in Mast Cells**

### *Real-Time Imaging*

In contrast to single-cell imaging, Ca<sup>2+</sup> 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.

### *Ca<sup>2+</sup> Sensors and Indicator Dyes*

Fura2 was the one of the first indicators used to monitor cytosolic changes in Ca<sup>2+</sup> levels. Since its development by Tsien and colleagues [4], the availability of Ca<sup>2+</sup> indicators to suit different experimental criteria has expanded widely. In parallel to the development of synthetic Ca<sup>2+</sup> dyes, the technology for genetically encoding Ca<sup>2+</sup> sensors within cellular proteins has improved significantly over the last decade.

The growing list of synthetic Ca<sup>2+</sup> dyes provides an excellent toolkit for investigating a range of Ca<sup>2+</sup>-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  $\text{Ca}^{2+}$  imaging as an integral part of their experimental investigation should consider the benefits and drawbacks of the two different families of  $\text{Ca}^{2+}$  indicators: synthetic or genetic.

Synthetic dyes provide superior brightness and ease of delivery (mainly by AM loading, *see* below), and a panel of dyes with various spectra and affinities for range of  $\text{Ca}^{2+}$  concentrations have become available. Additionally, some of these dyes provide the possibility of ratiometric measurements of actual  $\text{Ca}^{2+}$  concentrations (i.e., fura-2 and indo-1 [4]).

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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  mobilization dynamics when using fluo4 and fluo5 in comparison to genetically encoded GCaMP2 [3].

Genetically encoded  $\text{Ca}^{2+}$  indicators (GECI) provide an alternative that is often preferable to synthetic dyes for live-cell  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentrations. The previous generation of FRET-based sensors (i.e., “cameleon” [5]) has been replaced with permuted GFP constructs that are bright and stable and display fast kinetics. The new generation of GECI, including GCaMP3 and 5 [6] as well as the multicolor sensor GECO proteins [7], now offers a considerably enhanced toolkit for  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  measurements. For more information on GECI, *see* McCombs and Palmer [8].

### **1.3 Pharmacological Tools for Isolation of Specific $\text{Ca}^{2+}$ Pathways in Mast Cells**

Several channel and transporter families play a role in mast cell  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  ATPase (PMCA), and sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA). Numerous pharmacological agents target these various  $\text{Ca}^{2+}$  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)), and SOCE inhibitors (lanthanides, nickel, 2-APB, and other nonspecific  $\text{Ca}^{2+}$  channel blockers).

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

#### **1.4 $\text{Ca}^{2+}$ Signaling Dynamics in Mast Cells: Puffs, Waves, and Oscillations**

Mast cell  $\text{Ca}^{2+}$  dynamics involve a complex ensemble of spatially and temporally restricted changes in  $\text{Ca}^{2+}$  concentrations. Antigen-stimulated signaling mediated by IgE receptors causes local and global changes in  $\text{Ca}^{2+}$  levels with regulated amplitudes and durations. We recently described  $\text{Ca}^{2+}$  puffs, waves, and oscillations, which exhibited dynamics that depend on the stimulus and experimental conditions [3].  $\text{Ca}^{2+}$  oscillations were reported in mast cells more than a decade ago [9]. Additional characterization of  $\text{Ca}^{2+}$  waves and puffs provides a more complete view of regulation of  $\text{Ca}^{2+}$  signaling in mast cells. We observed a compelling association between mast cell morphology and  $\text{Ca}^{2+}$  signaling dynamics [3] in which elongated protrusions are sites for initiating spatially restricted changes in  $\text{Ca}^{2+}$  concentration following IgE cross-linking by Ag. Elongated protrusions are common to mast cells in vivo [10–12], and these are also manifested in cells grown in culture (*see* Fig. 1). Puffs and waves both demonstrate directional propagation in  $\text{Ca}^{2+}$  elevation, initiating mostly from the tips of protrusions. However,  $\text{Ca}^{2+}$  puffs are spatially confined and localized responses, whereas waves propagate through the entire cell. As in the case of waves,  $\text{Ca}^{2+}$  oscillations radiate throughout the cell and do not display lateral propagation.

Several features of  $\text{Ca}^{2+}$  waves in mast cells can be quantified using simple analysis. These include the region of origin, wave velocity, and overall  $\text{Ca}^{2+}$  elevation (*see* section 3.4). Other parameters of interest include rise time and decay that can be calculated from wave curve fitting and correlated with  $\text{Ca}^{2+}$ -induced calcium release (CICR) and  $\text{Ca}^{2+}$  clearance mechanisms. Features of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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.

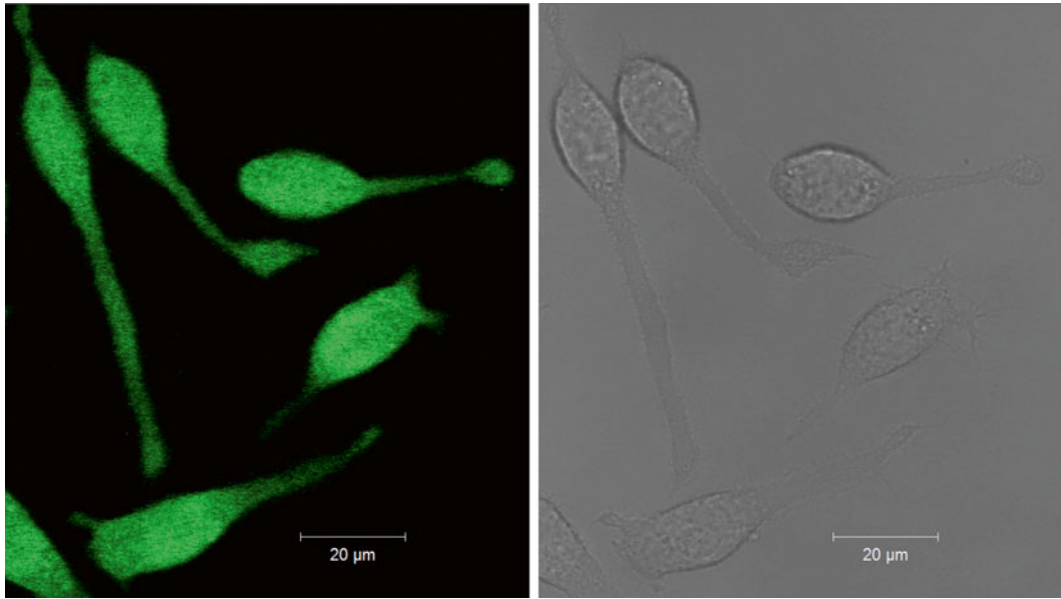


**Fig. 1** Elongated morphology of mast cells. Image of RBL-2H3 mast cells in culture demonstrates their elongated morphology. Typically, cells will have one or two protrusions extending to various lengths away from the cell body. Morphological features of mast cells (length, angle, volume, etc.) can be quantified using various overlay tools in ImageJ or other image processing software. Fluorescent label is fluo4

### 1.5 Imaging Mast Cell Degranulation

#### *Regulation of Mast Cell Exocytosis*

As in excitable cells, exocytosis of granules in mast cells depends on  $\text{Ca}^{2+}$  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* [13]). Synaptotagmins trigger membrane-membrane fusion in a process that depends on C2 domain binding to a  $\text{Ca}^{2+}$ /phosphatidylinositol 4,5-bisphosphate complex [14].

#### *Mast Cell Degranulation*

Various methods for measuring granule exocytosis in mast cells have been utilized, including measurement of  $\beta$ -hexosaminidase release [15], amperometry [9], and immunodetection of plasma membrane-associated granule proteins [16]. In addition, a number of methods have been developed to visualize individual granule exocytotic events in mast cells [17]. Early studies utilized acridine orange, a fluorescent weak base that accumulates in acidified vesicles [18, 19]. However, a recent study demonstrated that photosensitization of this dye during imaging limits its utility [20]. Other strategies have utilized granule-localized serotonin fluorescence imaged using multiphoton microscopy, together with complementary membrane and lysosome-labeling probes [21]. Although these methods allow detection of individual stimulated granule exocytotic events in mast cells, they are technically demanding and therefore limited in their usage.

*Real-Time Monitoring  
of Mast  
Cell Exocytosis*

The method we describe here utilizes preloading 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 (Fig. 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 (Fig. 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 3.5) or a plate reader. This approach provides a means for screening and high-throughput assays.

*Simultaneous Imaging  
of Degranulation and  $\text{Ca}^{2+}$   
Mobilization*

To allow real time, direct monitoring of the relationship between  $\text{Ca}^{2+}$  mobilization and granule exocytosis in mast cells, we devised a combined imaging strategy that uses a  $\text{Ca}^{2+}$  indicator together with FITC-dextran. This strategy provides excellent spatial and temporal resolution of both  $\text{Ca}^{2+}$  and degranulation dynamics to evaluate how  $\text{Ca}^{2+}$  waves, puffs, and oscillations relate to exocytosis of secretory granules in mast cells. To combine FITC-dextran-based degranulation and  $\text{Ca}^{2+}$  imaging, one can use a  $\text{Ca}^{2+}$  indicator dye with red emission ( $\text{Ca}^{2+}$  Red Asanate or Fura Red; *see* Fig. 6) or, alternatively, a red-shifted GECI (R-GECO; [7]) in combination with FITC-dextran.

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## 2 Materials

### 2.1 Genetically Encoded Calcium Indicators (GECI) cDNA Expression Plasmids

1. GECI plasmid: 5–8  $\mu\text{g}$  of purified plasmid (e.g., GCaMP2 and GCaMP3) per reaction ( $5 \times 10^6$  mast cells) (*see* **Note 1**).

### 2.2 Chemicals and Reagents

1. Synthetic  $\text{Ca}^{2+}$  indicators: A large number of  $\text{Ca}^{2+}$ -sensitive fluorescent dyes are currently available, and describing all the possibilities is beyond the scope of this chapter. We find that for measurements of  $\text{Ca}^{2+}$  dynamics in mast cells, the relatively newly developed fluo4 family, including fluo4-AM, fluo5F-AM, and fluo5N-AM (Invitrogen), is most appropriate. These dyes have a range of affinities for  $\text{Ca}^{2+}$ , 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,  $\text{Ca}^{2+}$  ionophore (A23187), sulfinpyrazone, hydroxytryptamine (5HT), and FITC-dextran (150 kDa) in our studies.

### **2.3 Tissue Culture**

1. Mast cells (RBL-2H3 mast cells or bone marrow-derived mast cells) (*see* **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 (*see* **Note 3**).
2. Picospritzer (*see* **Note 4**).
3. Temperature-controlled microscope/objective (*see* **Note 5** and Subheading 2.6).

### **2.5 Solutions and Buffers**

1. Balanced salt solution (BSS): 135 mM NaCl, 5 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 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  $\text{MgCl}_2$ , 1 mg/mL glucose, 20 mM HEPES (pH 7.4).
4. Stimulating solution: BSS + BSA with antigen (DNP-BSA at 1 ng to 1  $\mu\text{g}/\text{mL}$  final concentration).

### **2.6 Microscope and Data Analysis Software**

1. Confocal or wide-field microscopy can be used to monitor  $\text{Ca}^{2+}$  flux and degranulation. The image acquisition rate and detector sensitivity are most important for successful  $\text{Ca}^{2+}$  imaging. If using an objective heater, an oil immersion objective should be considered for better thermal conductance. To obtain adequate spatial resolution, 40 $\times$  through 100 $\times$  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 **Note 9**.

### 3 Methods

#### 3.1 Cell Preparation

1. Maintain RBL-2H3 mast cells [22] in monolayer culture in MEM with 20 % FBS and 10  $\mu\text{g}/\text{mL}$  gentamicin sulfate.
2. Transfect cells with GECI plasmid as follows:
  - (a) Resuspend  $5 \times 10^6$  cells in 0.5 mL of cold electroporation buffer containing 5–8  $\mu\text{g}$  of plasmid DNA.
  - (b) Electroporate at 280 V and 950  $\mu\text{F}$  (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  $\mu\text{g}/\text{mL}$  anti-DNP IgE during overnight cell culture [23].
5. Under these conditions, the cell transfection efficiency with GCaMP2 is typically 20–30 % (*see Note 6*).
6. Load cells with synthetic  $\text{Ca}^{2+}$  indicators (fluo4-AM family of dyes is recommended). When  $\text{Ca}^{2+}$  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 ( $5 \times 10^5$  cells/dish).
7. The next day, incubate cells for 30 min at 37 °C in BSS containing 0.5  $\mu\text{M}$  of the  $\text{Ca}^{2+}$  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 Setup

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  $\text{Ca}^{2+}$  indicator dye), wash cells with pre-warmed ( $37^\circ\text{C}$ ) BSS + BSA, and mount on the microscope.
2. Select appropriate illumination wavelength and filter lines. For GCaMP2, fluo4, or fluo5F 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* Fig. 1 for common morphology of RBL-2H3 cells in culture).
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.  $\text{Ca}^{2+}$  dynamics in mast cells should be easily detectable when recorded at 5–20 Hz (*see* Note 7).
7. Care should be taken to optimize conditions for  $\text{Ca}^{2+}$  imaging. These include dye loading, temperature, and cell density (*see* 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  $\text{Ca}^{2+}$  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 [24], 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.  $\text{Ca}^{2+}$  waves (*see* Figs. 2 and 3):
  - (a) Spatial origin of the waves can be determined visually, based on the site of the initial  $\text{Ca}^{2+}$  elevation.
  - (b) Wave velocity can be calculated from the number of frames required for a wave to propagate along the cell.
2.  $\text{Ca}^{2+}$  puffs (*see* Fig. 2c, d): Spatial and temporal dynamics of puffs can be determined as described for waves.
3.  $\text{Ca}^{2+}$  oscillations (*see* Fig. 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 $\text{Ca}^{2+}$ Measurements

1. Harvest cells 3–5 days after passage.
2. Resuspend  $10^6$  cells in 1 mL BSS supplemented with 2.5 mM sulfapyrazone (to reduce dye leakage).

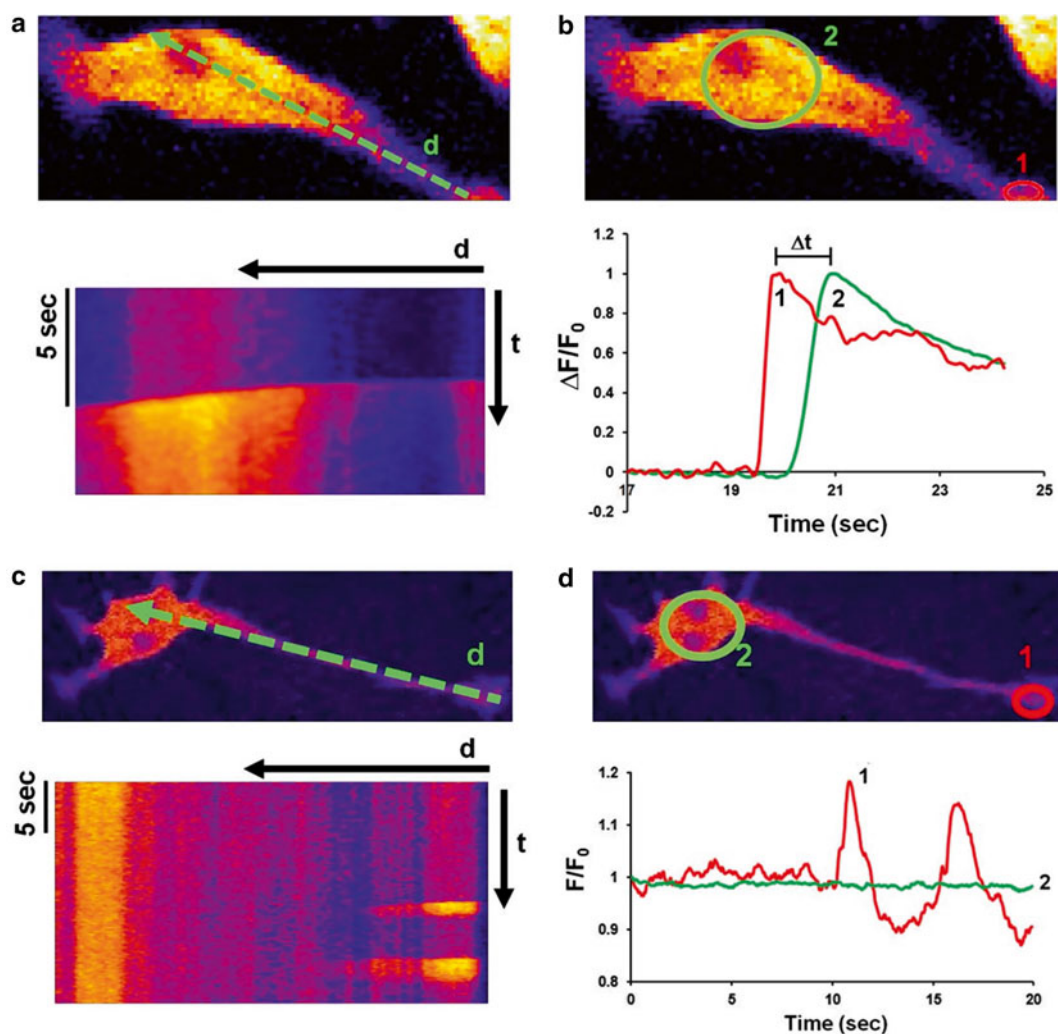


**Fig. 2** Imaging and quantifying  $\text{Ca}^{2+}$  waves and puffs. In these examples, RBL-2H3 cells expressing the  $\text{Ca}^{2+}$  sensor GCaMP2 were monitored for fluorescence intensity as a function of time before and after stimulation by antigen, and changes in  $\text{Ca}^{2+}$  concentration are presented in pseudo colors (*warmer colors* represent higher  $\text{Ca}^{2+}$  levels). (a) *Upper*, image of cell, with direction of  $\text{Ca}^{2+}$  wave indicated; *lower*, timeline analysis (“virtual linescan” plug-in for ImageJ) provides both spatial and temporal information about changes in  $\text{Ca}^{2+}$  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 ( $\Delta t$ ) as a function of distance (in  $\mu\text{m}$ ; “Z Profiler” plug-in for ImageJ). The ROI quantification can be used to determine wave velocity. (c) As described for waves in a, timeline analysis can be used to detect and visualize the spatial attributes of  $\text{Ca}^{2+}$  puffs. In this example, the puffs are localized and confined to the cell’s protrusion. (d) Temporal dynamics of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  elevation during puffs

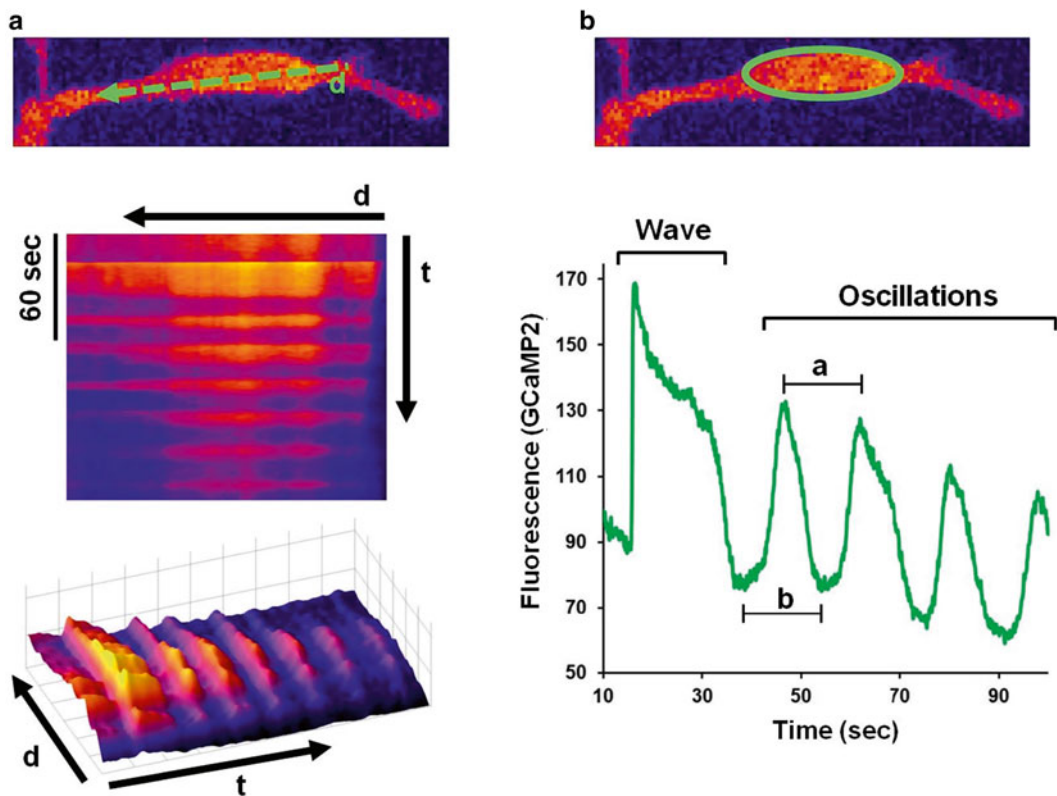


**Fig. 3** Visualizing and quantifying  $\text{Ca}^{2+}$  oscillations. RBL-2H3 cell expressing GCaMP2 was stimulated with Ag and imaged for  $\sim 100$  s. **(a)** Timeline analysis along the cell body (*upper panel*) reveals repetitive  $\text{Ca}^{2+}$  oscillations (*middle panel*; warmer colors represent higher  $\text{Ca}^{2+}$ ). *Lower panel* illustrates a “3D” representation of the kymograph (“Interactive 3D Surface Plot” plug-in for ImageJ), 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  $\text{Ca}^{2+}$  influx), down phase (representing  $\text{Ca}^{2+}$  clearance), and the integrated  $\text{Ca}^{2+}$  elevation during various time points

3. Add  $0.5 \mu\text{M}$  fluo4-AM (or other fluorescent indicating dyes; set emission and excitation filters accordingly) and immediately mix. Incubate at  $37^\circ\text{C}$  for 1 min.
4. Add 9 mL of BSS + BSA, and incubate cells in  $37^\circ\text{C}$  for 30 min; during this incubation sensitize cells with IgE ( $2 \mu\text{g}/\text{mL}$ ).
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  $\text{Ca}^{2+}$  concentration upon addition of various stimulants to stirred cells at  $37^\circ\text{C}$ .
7. Add Triton X-100 (0.1 %) at the end of the experiment to determine maximal indicator response to  $2 \text{ mM}$   $\text{Ca}^{2+}$ , followed by excess EGTA ( $10 \text{ mM}$ ) to assess background signal when no  $\text{Ca}^{2+}$  is bound.

### 3.6 Monitoring Degranulation with Fluorescence

1. Harvest cells 3–5 days after passage and plate in MatTek dishes.
2. Culture  $1 \times 10^6$  cells in 2 mL of full medium in the presence of 2 mg/mL FITC-dextran and anti-DNP IgE (0.5  $\mu\text{g}/\text{mL}$ ) (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 [25].
4. After 24 h incubation (37 °C) with FITC-dextran ( $\pm$ IgE), wash cells once with PBS, and resuspend in fresh buffer (BSS + BSA) at  $1 \times 10^6$  cells/mL.
5. Incubate washed cells for 1 h at 37 °C.
6. Add 0.5 mL of the resuspended cells (approx.  $2.5 \times 10^5$  cells) to 1.5 mL BSS in a stirred acrylic cuvette.
7. Monitor FITC fluorescence (excitation ( $\lambda = 490$  nm), emission ( $\lambda = 520$  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* Figs. 4, 5 and 6.
10. Optional co-imaging of degranulation and  $\text{Ca}^{2+}$  changes.
  - (a) If using dye for  $\text{Ca}^{2+}$  imaging, load dye following the wash step as indicated in  $\text{Ca}^{2+}$  imaging section (i.e. Fura Red; *see* Fig. 6).
  - (b) Image acquisition: As described in the  $\text{Ca}^{2+}$  imaging section.
11. Care should be taken to optimize conditions for imaging degranulation. These include temperature and dye and dextran loading (*see* **Note 10**).

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## 4 Notes

1. Several families of GECI are currently available, and newer versions are being developed. Of special interest are the GCaMP indicators, constructed of permuted 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).

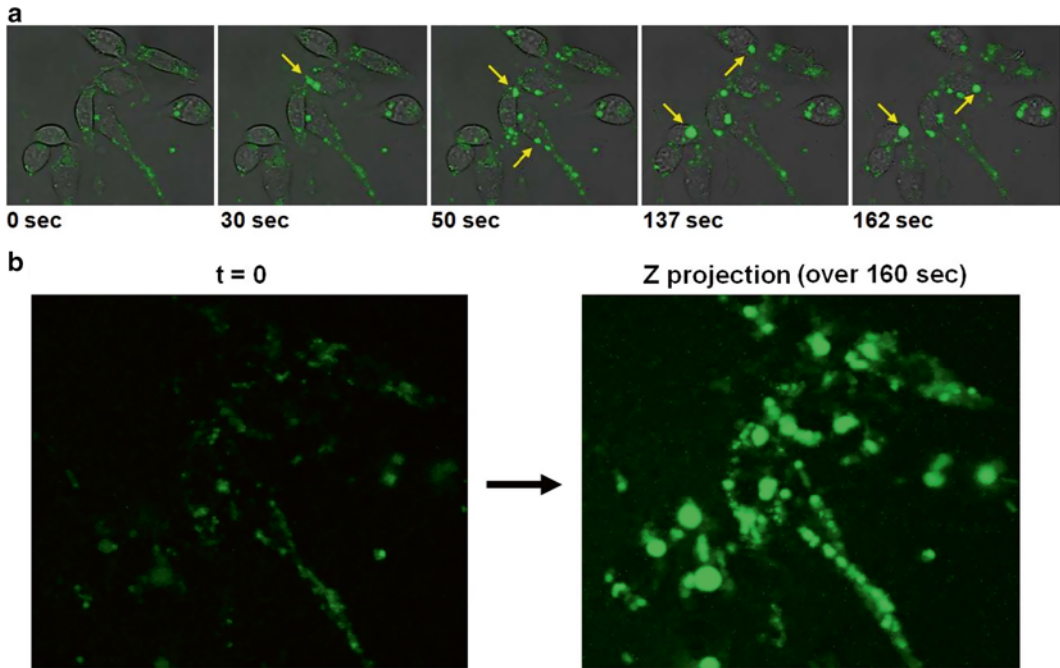


**Fig. 4** Imaging of single-cell degranulation events using FITC-dextran. **(a)** Individual frames taken from ~3 min of imaging RBL-2H3 cells loaded with FITC-dextran and stimulated with Ag. *Arrows* point to some of the degranulation events taking place in each frame. **(b)** For initial evaluation of the total degranulation magnitude from cells, a Z projection (integrating 160 s) can be used which shows the sum of all or a range of frames

2. Mast cells: Our experimental system is based on RBL-2H3 mast cells [22]. These mast cells are typically sensitized with monoclonal IgE specific for anti-2, 4-dinitrophenyl (DNP) [23].
3. We 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.
4. This device supplies repeatable pressure pulses for local delivery of a stimulating solution. The system we use requires preparation of pulled capillaries (Parker Hannifin Corp.).
5. There 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.
6. Several methods of transfection are available, and  $\text{Ca}^{2+}$  phosphate is probably the simplest and commonly used. However, for the experiments with GCaMP2 as the  $\text{Ca}^{2+}$  indicator, we typically use electroporation because of its relatively high efficiency with RBL-2H3 cells.

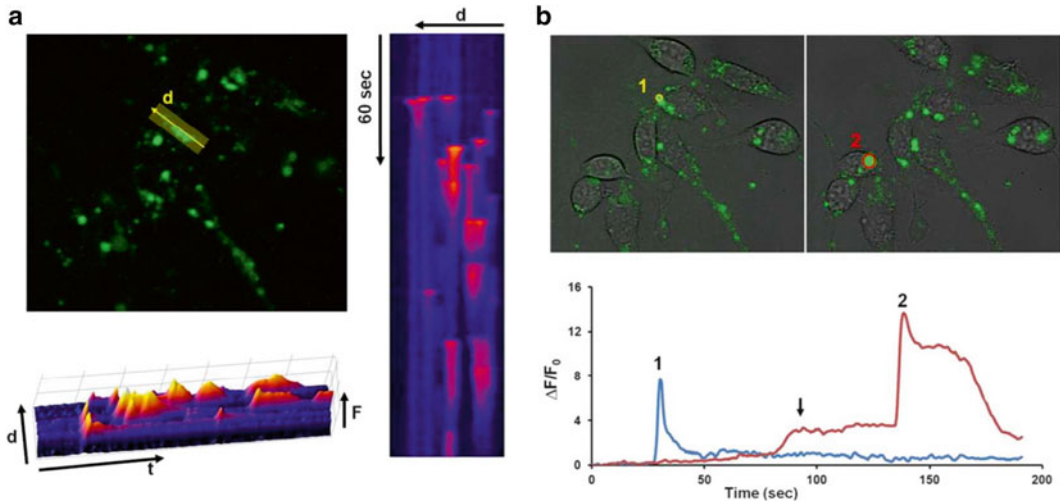


**Fig. 5** Quantification of single-cell degranulation events using FITC-dextran. As for  $\text{Ca}^{2+}$  waves and oscillations, integration of fluorescence intensity over ROI or timeline analysis can be used for quantification of degranulation visualized with FITC-dextran. (a) Timeline analysis (*middle 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 [26]

7. Slower 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.
8. Troubleshooting  $\text{Ca}^{2+}$  imaging.
  - (a)  $\text{Ca}^{2+}$  dynamics and temperature: Suboptimal temperature can dramatically reduce the antigen-induced  $\text{Ca}^{2+}$  response in mast cells and significantly slow its kinetics. To avoid these issues, it is recommended that cells are kept in  $37^\circ\text{C}$  at all times and that temperature of the dish is monitored regularly. Buffers and media to be added should be warmed ( $37^\circ\text{C}$ ) 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 [3], and these need sufficient cell-free area to develop and be visualized. In addition,

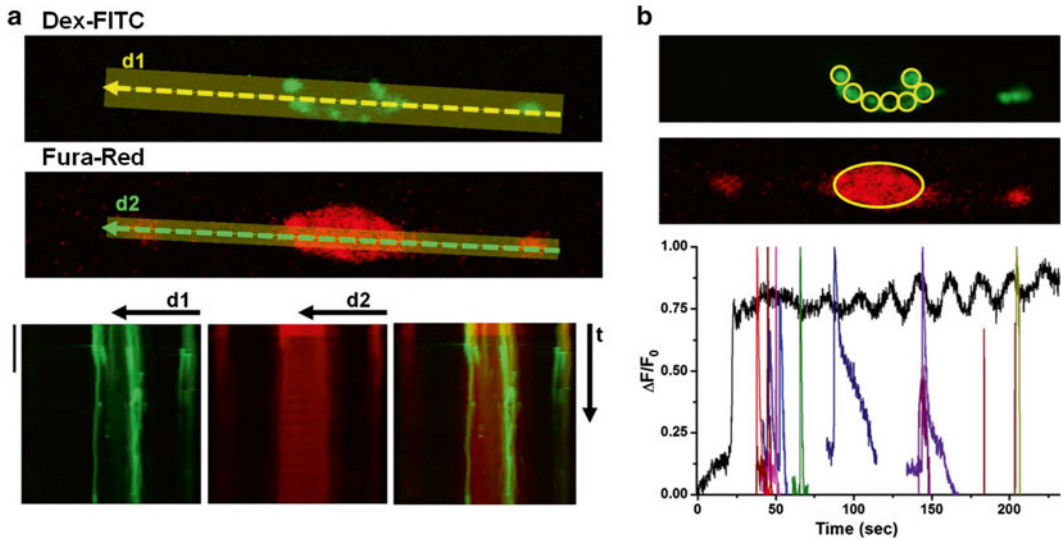


**Fig. 6** Simultaneous imaging of  $\text{Ca}^{2+}$  mobilization and degranulation in cells using Fura Red and FITC-dextran. In these experiments a fluorescent  $\text{Ca}^{2+}$  indicator (synthetic or GECI) is used in combination with FITC-dextran loaded into to cells. In this case we used Fura Red to measure  $\text{Ca}^{2+}$  changes. (a) Timeline analysis of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  changes and degranulation throughout the cell. (b) Integration of fluorescence over ROI in red or green channels provides high temporal resolution of correlated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  response, and individual colors show different exocytotic events

cell-cell interactions might interfere with  $\text{Ca}^{2+}$  mobilization. Thus, we recommend plating low numbers of cells when preparing for microscopy experiments, as described.

- (c) Optimization of  $\text{Ca}^{2+}$  dye loading: For reasons described in the first section, we recommend using genetically encoded indicators for mast cell  $\text{Ca}^{2+}$  imaging. However, using synthetic  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  indicators (such as fluo5F) improves  $\text{Ca}^{2+}$  responses in the cells and yet provides sufficient resolution and sensitivity.
9. ImageJ functions and plug-ins for analysis and quantification of  $\text{Ca}^{2+}$  imaging.
  - (a) Timeline analysis (“virtual linescan” plug-in)—this plug-in is used for generation of kymographs (see Fig. 2a).
  - (b) Z profiler plug-in—this plug-in can be used for plotting  $F$  (intensity) over time (see Fig. 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 Fig. 3a lower panel).
  - (d) All plug-ins can be downloaded directly from ImageJ website (<http://rsbweb.nih.gov/ij/>).
10. Troubleshooting FITC-dextran degranulation imaging.
- (a) As for  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 4 mg/mL dextran-FITC can be used.
  - (d) Combining FITC-dextran with a non-pH-sensitive fluorophore conjugated to the dextran (such as Texas Red-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.

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