Published in final edited form as:

Methods Mol Biol. 2014 ; 1174: 407–421. doi:10.1007/978-1-4939-0944-5_28.

Probing the role of the actin cytoskeleton during regulated exocytosis by intravital microscopy

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Summary

The actin cytoskeleton plays a fundamental role in controlling several steps during regulated exocytosis. Here we describe a combination of procedures that are aimed at studying the dynamics and the mechanism of the actin cytoskeleton in the salivary glands of live rodents, a model for exocrine secretion. Our approach relies on intravital microscopy, an imaging technique that enables imaging biological events in live animals at a subcellular resolution, and it is complemented by the use of pharmacological agents and indirect immunofluorescence in the salivary tissue.

Keywords

Intravital microscopy; Exocytosis; Actin cytoskeleton; Membrane trafficking; Salivary glands

1. Introduction

Exocytosis is the process by which proteins are delivered by membranous carriers to the surface of cells and released into the extracellular space [1]. Proteins can be sorted to undergo either constitutive exocytosis, which occurs in all cell types, or regulated exocytosis, which occurs only in specialized secretory cells following the stimulation of specific cell surface receptor [2]. Activation of those receptors elicits a series of signaling pathways that leads to docking and fusion of the membranous carriers into the plasma membrane [1]. This multistep process is controlled by several molecules including the actin cytoskeleton and its regulators, which have been shown to play multiple roles by either promoting or inhibiting the various exocytic steps [3, 4].

Our lab has developed a methodology to study how regulated exocytosis occurs in the exocrine organs in live rodents by using intravital microscopy (IVM) [5, 6]. Specifically, we focused on studying the dynamics of the large secretory granules in the acinar cells of the submandibular salivary glands (SSGs) that in resting conditions are localized at the apical plasma membranes (APM) [7]. We showed that upon stimulation of the β -adrenergic receptors, secretory granules fuse with the APM and recruit an actomyosin complex that plays three major functions: 1) to provide a scaffold to protect the granules from the hydrostatic pressure generated by fluid secretion, 2) to regulate the process of membrane integration of the secretory granule into the APM, and 3) to act as a barrier to prevent compound exocytosis from occurring *in vivo* [7, 8].

Uncovering the molecular machinery regulating the assembly of the actin cytoskeleton during regulated exocytosis *in vivo* is our ultimate goal. To this end, we employ a series of strategies based on *in vivo* transient transfection of fluorescently tagged probes for the actin cytoskeleton, transgenic animal models, and selective delivery of pharmacological inhibitors. Specifically, we use rats for non-viral based delivery of plasmid DNA and selected transgenic mouse models expressing 1) the F-actin probe Lifeact tagged with either GFP or RFP [9, 10], 2) the membrane-targeted peptide mTomato [7], and 3) cytoplasmic GFP [7]. In addition, we complement data obtained using intravital microscopy with indirect immuno-fluorescence of cryosections of the SSGs, to gain a larger perspective of the molecules involved in the exocytic process. The goal of this chapter is to provide investigators interested in studying the actin cytoskeleton with a basic set of modular protocols that will enable investigating F-actin dynamics and its regulation *in vivo*. Although the procedures described in here are tailored towards regulated exocytosis, they can be easily adapted to other areas of cell biology.

2. Materials

2.1 Animals and Surgical Tools

1. Sprague–Dawley rats, 150–250 g body weight (Harlan Laboratories, Frederick, MD).
2. RFP- and GFP-Lifeact [9] and GFP mice [7] 20–25 g body weight. (see Note 1).

2.2 In Vivo Transient Transfection

1. SZX7 Stereo microscope mounted on an adjustable arm (Olympus America, Center Valley, PA).
2. Custom-made stereotactic device for salivary duct cannulation [11].
3. Polyethylene PE-5 cannula (0.008”I.D. $\times$ 0.020”O.D.) (Strategic Applications Incorporated, Libertyville, IL).
4. Histoacryl tissue adhesive (TissueSeal, Ann Arbor, MI).

¹We use both males and female mice since we did not notice any gender difference in either the extent or the kinetics of the exocytic events.

5. 1 mL Plastic syringes.
6. 30 G 1/2 in. needle (Exelint, Los Angeles, CA).
7. In vivo-jet PEI polyethyleneimine (PEI) (Polyplus Transfection, New York, NY).
8. Plasmid DNA purified from maxi-prep kit (Qiagen, Valencia, CA) (see Note 2).

2.3 Cardiac Perfusion Fixation

1. 1M HEPES Buffer pH 7.3 (Quality Biological, Inc., Gaithersburg, MD).
2. 4% Formaldehyde (Macron Chemicals, Center Valley, PA).
3. 0.05% Gluteraldehyde (Polysciences, Inc., Warrington, PA).
4. Ultrapure water.
5. Monoject 60mL syringes, 2 syringes (Covidien, Mansfield, MA), Surflo winged infusion set 25G $\times$ 3/4" (Terumo, Somerset, NJ), 3-way valve (see Note 3).
6. 70% EtOH.
7. Surgical instruments: Operating scissors (11.5 cm straight); #7 curved tip tweezers (one with blunt and one with sharper tips), 25cm curved tip forceps, microscissors (World Precision Instruments, Sarasota, FL) (see Note 4).

2.4 Cryosectioning and Immunostaining

1. O.C.T compound (Tissue-Tek, Torrance, CA).
2. Disposable Base Molds (Electron Microscopy Sciences, Hatfield, PA).
3. Liquid nitrogen and isopentane.
4. Cryostat (Leica CM1900, Buffalo Grove, IL).
5. Cryostat blade.
6. Histobond Silane coated microscope slides (VWR, Radnor, PA).
7. #1.5 cover slips (Corning, Corning, NY).

2.5 Microscope and Imaging setup

1. Inverted confocal microscope (Olympus IX-81 with a Fluoview1000 scanner, Olympus America, Center Valley, PA) equipped with a motorized stage [12].
2. Custom-made immobilization stage [12].
3. Stage insert for 35 mm dishes to accommodate a 40 mm glass coverslip (Olympus America, Center Valley, PA) [11].

²We noticed that cDNAs cloned in Clontech vectors give the best transfection yield. Alternatively, cDNA can be delivered in rats by using adenoviral vectors. PEI targets the plasmid DNA primarily to acinar cells, whereas adenovirus drives the transgene expression to both acinar and ductal cells.

³Connect the 3-way valve with the two 60mL syringes and winged infusion set.

⁴Instruments should be cleaned and sterilized before use.

4. Glass 40 mm round coverslips, # 1.5 (Bioprotechs, Butler, PA).
5. Objective heater (Bioprotechs, Butler, PA).
6. Gauze sponges 4" × 4" (Tyco Healthcare, Gosport, UK) to be used as blankets.
7. Disposable Foot Warmers (Heat Factory, Vista, CA).
8. IX81 inverted confocal microscope, equipped with a Fluoview-1000 scanning unit (Olympus America, Center Valley, PA).
9. Plan-Apo 60x, 1.2 NA, water-immersion objective. Plan-Apo 60x, 1.42 NA, oil objective (Olympus America, Center Valley, PA) UPLSAPO 10X.

2.6 Imaging Software

1. For processing time-lapse imaging: Metamorph (Molecular Devices, Sunnyvale, CA), ImageJ (NIH, Bethesda, MD). For 3D reconstructions: Imaris (Bitplane, South Windsor, CT).

3. Methods

The procedures described here have been developed to: 1) investigate the dynamics of F-actin recruitment at the apical plasma membrane in the SSGs upon stimulation of exocytosis using IVM, and 2) to study the machinery regulating the recruitment of actin using a complementary pharmacological approach and indirect immunofluorescence. IVM relies on the use of fluorescently tagged proteins that can be either expressed in adult animals or introduced into mouse germ lines. In adult animals, genes can be delivered to selected organs using different approaches: One example being the administration of transgenes to the rat submandibular salivary glands through the salivary duct (Wharton's duct) by using either non-viral or viral methods [13, 14]. Focusing specifically on non-viral delivery, we express fluorescently-tagged Lifeact in the acinar cells by mixing plasmid DNA with polyethyleneimine (PEI), a molecule extensively used for in vivo siRNA delivery [15]. Here, we described the details for the in vivo transfection of fluorescently-tagged Lifeact, but the procedure can be easily adapted to transfect any other construct.

In mice, due to the reduced size of the Wharton's duct, the same approach has been more difficult to implement without damaging the organ. However, this issue has been overcome by the generation of mice expressing either GFP- or RFP-Lifeact [9]. With respect to the rats, transgenic mice offer the possibility to image F-actin dynamics in every cell population in the tissue and ensure homogeneous expression levels of the transgene. On the other hand, in rats, fluorescent Lifeact can be co-transfected with other markers or with constructs that may affect regulated exocytosis, thus providing information on the molecular machinery underlying this process.

In both experimental systems, mice and rats, we have been able to visualize F-actin dynamics upon stimulation of regulated exocytosis through subcutaneous injection of isoproterenol (ISOP). In addition, we have developed a procedure to extract quantitative information about the kinetics of F-actin assembly in vivo. Finally, we have complemented the imaging in both species with the use of pharmacological agents that perturb the

dynamics of the actin cytoskeleton, and with a procedure to label for several intracellular markers by using indirect-immunofluorescence. Finally, the procedures described below can be used in a modular fashion, as illustrated in the diagram in Figure 1, in order to better suit the specific questions that the investigator seeks to address.

3.1 Animal preparation

The procedures to prepare both mice and rats for IVM, which include pre-anesthesia, anesthesia, surgical procedures, and positioning of the animals on the microscope stage, have been extensively described elsewhere [11].

3.2 Transfection of Fluorescently-tagged Lifeact via Cannulation of the SSGs

1. Place the anesthetized rat on a stereotactic device [11] with mandibles held open and the tongue bent back into the mouth to prevent the obstruction of the airways.
2. Move the stereotactic device under the stereomicroscope and tilt it to approximately a 45 degrees angle. Adjust the focus to visualize the area below the tongue. The two orifices of the Wharton's duct will appear as two small flaps.
3. Grab the PE-5 cannula with a pair of tweezers close to the tip. Gently push the orifice with the tip of the cannula until it gets inserted into the duct (see Note 5). Do the same for both orifices, if transfecting both glands.
4. Apply a small drop of histoacryl tissue glue around the cannulated orifices, and let it dry.
5. Prepare the transfection mixture. Typically, efficient transfection is achieved by using 12–24 µg of plasmid DNA/gland. Mix 50 µl of 10% glucose with the plasmid DNA and adjust the volume to 100 µl (solution 1). Mix 50 µl of 10% glucose with 7.5 µl of Jet PEI and adjust the volume to 100 µl (solution 2). Mix solution 1 and solution 2 and incubate them for 30 min at room temperature.
6. Aspirate the transfection mixture with a syringe (30-gauge needle) and make sure that no air bubbles are released when injecting the fluid. Connect the needle to the cannula without piercing the tubing.
7. Inject the transfection mixture gradually over a 5 min time period by applying gentle pressure on the plunger.
8. Remove the cannula and the syringe from the mouth and allow the animal to recover in the cage. A warm environment should be provided to facilitate the recovery and the animal should be monitored for at least 2 h.
9. After 12–48 h from the injection, perform IVM (Note 6).

⁵Do not apply too much pressure, as the mucosa can be ruptured, damaging the duct, and causing bleeding from the mucosa.

⁶Before performing IVM, determine the yield of transfection on a separate set of rats. Some variability may occur depending on the quality of the plasmid preparation, the batch of PEI, and the operator. After 12–48 h from the transfection expose the salivary glands, as previously described [11], excise, and place them in ice-cold saline. The tissue can be imaged for maximum 30 minutes after the excision. Select 10 random fields of view per gland using a 10x dry objective and acquire the images by confocal microscopy using a large pinhole (5–10 µm optical slice). Score the number of cells per field of view and divide them by the area. Typically, both GFP- and RFP-Lifeact are expressed in 5–10 cells/mm².

3.4 Intravital Microscopy

1. Set up the microscope with a 60x objective (oil or water) connected to an objective heater to maintain the temperature at 37–38 °C.
2. Set up the stage and position the animal with the glands exposed and immobilized as previously described for rats [11] and mice [16].
3. Set imaging parameters to approximately 320 × 320 pixels and 4 microseconds per pixel (resulting in a scanning speed of 0.4 sec/frame) and with a 0.2 µm/pixel spatial scale (field of view of approximately 50 µm × 50 µm). In order to image the assembly of F-actin around the secretory granules, the optical thickness should be set between 0.9–1.2 µm. To excite GFP-Lifeact and the RFP-Lifeact use a wavelength of 488 nm (peak power 0.5 mW) and 561 nm (peak power 1 mW), respectively.
4. Use epifluorescence illumination to locate the cells expressing the transgene.
5. Image the cell by confocal microscopy and select the appropriate focal plane, which should include the apical plasma membrane that is enriched with Lifeact (Figure 2).
6. Acquire an image to be used as a reference to correct for changes in focus and shifts in the xy direction that are due to the motion artifacts.
7. Inject 0.1 mg/Kg of ISOP sub-cutaneously (SC) and image in time lapse mode for 10–15 min.
8. After 1–2 minutes, a Lifeact "ring" appears around the secretory granules (Figure 2). Within 40–60 the ring shrinks in diameter and disappears, designating the completion of the integration of the secretory granule into the APM.
9. Manually correct for any shift in the xyz directions.
10. Save the images in the appropriate format to be used with the available image processing software.

3.5 Optimization of the Administration of the Drugs Affecting the Actin Cytoskeleton

1. Before performing IVM to study the effect on regulated exocytosis of a drug that perturbs the actin cytoskeleton, it is necessary to determine the conditions for its administration (i.e. concentration and incubation time). To this end we use a mouse that expresses cytoplasmic GFP [7] and that enables the visualization of the secretory granules. The rationale for using this mouse is that when the cytoskeleton is impaired the secretory granules do not collapse normally, and instead form large vacuoles due to the stimulation of compound exocytosis. This is a quick assay that can rapidly determine the concentration of the drug necessary to perturb regulated exocytosis (Note 7).

⁷We have previously established that to block the collapse of the secretory granules the optimal conditions are 10 µM cytochalasin D or 10 µM of latrunculin A in saline for 20 min. We will use these drugs as an example but the same approach can be used for any other molecule.

2. Surgically expose both glands as previously described [11, 16].
3. Install on the exposed glands a small circular ring (0.5–0.75 cm thick) made from cutting a 1 mL syringe (Figure 3A). Secure the ring having its edges covered by the skin.
4. Pour the inhibitor into the ring (see Note 8).
5. After 20 minutes stimulate exocytosis by injecting SC 0.1 mg/Kg ISOP.
6. After 20 minutes stop the process by performing intra-cardiac perfusion of the fixative (see section 3.7) (see Note 9).
7. Excise the glands and fix further in 4% formaldehyde for 1 hour at R.T.
8. Image the glands by confocal microscopy to assess any effect on regulated exocytosis (Figure 3B). Drugs disrupting the cytoskeleton will generate large vacuoles [7].
9. Label the actin cytoskeleton to determine the efficacy of the drug (see section 3.8.).

3.6 IVM of Salivary Glands Exposed to Drugs Affecting the Actin Cytoskeleton

1. Place the animal with the exposed glands onto the microscope stage using the bathing chamber (Figure 3C) (see Note 10).
2. Fill the chamber with the drug in saline at the appropriate concentration (see Note 11).
3. After 20 minutes begin imaging and stimulate exocytosis by SC injection of 0.1 mg/Kg ISOP (see Note 12).
4. At the end of the imaging session, intracardiac fixation and immunofluorescence can be performed (see sections 3.7 and 3.8).

3.7 Intra-cardiac Perfusion of the Fixative in the Live Animal

1. Place the fixative and saline in a water bath at 37°C (see Note 13).
2. Place a Styrofoam board inside of a plastic container, which is capable of holding excess fluids and blood.
3. Place the anesthetized animal ventral side up and pin its feet in a stretched out position.

⁸Make sure that the glands are fully bathed in the drug. If the chamber is leaky, keep adding more inhibitor for the length of the experiment. The dose of the drug and the length of the incubation have to be established depending on the goal of the experiment. Finally, keep the animal warm with a heated lamp.

⁹Perfusion of the fixative in mice and rats gives the quickest fixation of the SSGs, which ensures the best structural preservation.

¹⁰The bathing chamber is made of stainless steel to provide a frame for holding a coverslip that enables imaging the exposed glands of the live animal, and to create a reservoir that ensures a constant exposure of the tissue to the drug.

¹¹Make sure that the glands are fully bathed in the drug. The dose and the length of the incubation have to be established depending on the goal of the experiment. It is important to maintain the body temperature of the animal by exposing it to a heated lamp.

However, closely monitor for excessive heating that may provoke the evaporation of the drug. If this occurs, add fresh drug every 5–10 min.

¹²The time to wait before stimulating exocytosis may vary depending on the concentration and penetration rate of the drug.

¹³Fixative is composed of 4% formaldehyde, 0.05% glutaraldehyde, and 200mM HEPES buffer at pH 7.3 to be made fresh right before use.

4. Load two 60 mL syringes with the pre-warmed saline and fixative and connect them to the 3-way valve (see Note 14).
5. Wet the skin on the ventral side of the animal with 70% EtOH.
6. Remove the skin from the ventral side of the animal. Make an incision in the skin starting at the navel and proceed to cut up towards the mouth (be careful not to cut through the membrane underneath). At the shoulder joints, cut laterally to allow the skin to be peeled open and repeat at the lateral incision at the pelvic girdle. Use forceps and fingers to peel back the skin layer and pin it to the board.
7. Remove the abdominal membrane to reveal the internal organs and the lower part of the sternum: At the navel, gently cut the membrane upwards. Then, cut membrane flaps laterally and pin them to the board.
8. Clamp the end tip of the sternum with the curved tip forceps and proceed to cut up the sides of the rib cage while pulling the sternum towards the mouth to reveal the thoracic cavity.
9. Insert a needle into the left ventricle of the heart, cut the right atrium and proceed to fix the animal by first injecting pre-warmed saline at 1–2 mL/min until the blood flowing from the atrium becomes clear. Inject pre-warmed fixative at 1–2 mL/min (see Note 15).

3.8 Whole Mount Staining of the Tissue

1. Once the animal is thoroughly fixed (see Note 16), excise the glands and place them in 4% formaldehyde for 1 hour at room temperature. Then, slice the glands in half and fix them again in 4% formaldehyde for an additional 30 minutes.
2. Place the sliced tissue in block solution (composed of 10% FBS in PBS, 0.02% saponin and 0.02% NaN_3) overnight at 4 °C.
3. Incubate with the primary antibody in the presence of saponin for the required length, wash the primary, and incubate with a secondary antibody mixed with fluorescently labeled phalloidin (see Note 17).
4. Wash 3 times with block solution and store in block at 4 °C until ready to image (see Note 18).

¹⁴Make sure the tubing are primed and that no air bubbles are pushed through the syringe. Also, push a few mL of saline through the needle to clear the line of any fixative prior to starting the procedure.

¹⁵Saline can be injected within 30 seconds to 1 min in mice and about within 1–1.5 min in rats. It is best to start injecting the fixative while the animal is still alive and therefore it is important to rapidly perform the steps between the opening of the chest cavity and injecting into the heart. Finally, avoid inserting the needle too far into the left ventricle, as it could penetrate the wall of the heart between the right atrium and left ventricle, which would reduce the pressure in the vascular system.

¹⁶Over-fixation may cause excessive background due to the glutaraldehyde, and can also interfere with the labeling.

¹⁷Proper conditions for the primary and secondary antibody have to be determined for each case. As for the phalloidin, we recommend to incubate 0.5 U/ml in block solution for 30 min at RT.

¹⁸Samples cannot be stored for more than 1 week.

3.9 Cryosections and Labeling Procedures

1. Dispense the OTC compound into the disposable base molds without creating any bubbles.
2. After intra-cardiac fixation, excise the glands, cut them in half and fully immerse them in the OTC compound.
3. Carefully bring the bottom of the disposable base molds to the surface of liquid nitrogen and hold them there until the OTC compound fully freezes (see Note 19).
4. Place the frozen gland embedded in OTC into the cryostat and cut sections (10–15µm in thickness).
5. Quickly place the sections on the silane coated side of the slide and once the sections have fully melted and adhered to the slide, move them to room temperature. (see Note 20).
6. Proceed to stain and/or mount and image the sections.

3.10 Quantitative Analysis of the Lifeact Recruitment Around the Secretory Granules

1. Import the raw time-lapse series into ImageJ (Figure 4).
2. Use the Stackreg plugin (rigid body option) to stabilize the frames and eliminate shift in the XY plane (Note 21).
3. Use the rectangular region selection tool to crop the image series.
4. Save the image series as a Tiff image sequence, and import it into Metamorph using the quick build stack option.
5. Identify secretory granule fusion profiles on which Lifeact is recruited and highlight the edges of those areas using the region tool (Note 22) (Figure 4).
6. Use the display region measurements tool to view the integrated fluorescence intensity of the ROI's in all the frames of the time-lapse series.
7. Select the parameters to record for data logging including:
 - i. Integrated fluorescence intensity
 - ii. ROI label
 - iii. Frame number
8. Record the raw data in an excel file using the log data tool.

¹⁹Be careful not to crack the tissue or to drop it into the liquid nitrogen while the OTC compound is still freezing. Alternatively, place the base molds on top of frozen isopentane, which is placed in liquid nitrogen, since the freezing is quick, yet more gentle and better preserves the membranes.

²⁰Make sure to move the slides to room temperature within 30 seconds after their first contact with the sections. Avoid leaving the slides too long in the cryostat to prevent distorting the sections.

²¹If necessary, crop the image and repeat the Stackreg alignment on a smaller area to ensure proper XY stabilization. Measurements will be inaccurate without proper image alignment.

²²Fusion profiles of Lifeact are identifiable as rings of intense fluorescence that form and collapse over 40–60 seconds and can reach up to 1 µm in diameter.

9. Normalize the raw data for the fluorescence intensity just before the onset of Lifeact recruitment (Note 23) and plot the data versus time elapsed (Figure 4).

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, National Institute of Dental and Craniofacial Research.

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²³The onset of recruitment can be identified as a sharp increase in integrated fluorescence intensity within the selected region of interest

Figure 1.

General layout of procedures for probing the dynamics and regulation of the actin cytoskeleton in the SSGs of mice and rats, which can be extended to other subcellular structures. Intravital microscopy (IVM) experiments with mice and rats follow similar protocols, differing mainly in that introduction of fluorescent probes into rats is accomplished through transient transfection, while transgenic mice would serve for a similar purpose. The SSGs can further be treated pharmacologically to perturb the regulation of F-actin assembly

and also fixed and immuno-stained to investigate the involvement of molecules that may regulate F-actin dynamics during regulated exocytosis.

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Figure 2.

Lifeact dynamics in transfected rats (A–C) and transgenic mice (D, E). A–C. Transient transfection typically results in one transfected cell per acinus (A, left diagram). Rats were transfected with GFP-Lifeact and individual acinar cells were imaged before (A right panel, B left panel) or after (B right panel, C) SC injection of ISOP. Single slice (A) or volume rendering of 3D stacks (B) show the basolateral (arrow) and the apical (arrowhead) plasma membrane and the secretory granules (asterisk). C. Time-lapse series of the dynamics of Lifeact recruitment around granules. The broken line highlights the cell border, whereas the

arrow points to the apical plasma membrane (green line); asterisks are placed near Lifeact rings around the secretory granules. Scale bars, 5 μm . **D, E.** In transgenic mice, GFP-Lifeact is expressed in all the acinar cells (**D**, left diagram). Single slide (**D**, center) and max projection of a 3D stack (**D**, right) show the basolateral (arrow) and the apical (arrowhead) plasma membrane, and the myoepithelial cell (asterisk) that also express GFP-Lifeact. **E.** Time-lapse series of recruitment and loss of Lifeact (left). Apical plasma membrane (arrow) and secretory granules (asterisks) are shown. Scale bar, 5 μm .

Figure 3.

Set up for bathing the salivary glands in pharmacological inhibitors. **A.** Bathing ring cut from a 1mL syringe, and example of its insertion into the anesthetized animal. **B.** The exposed SSGs of anesthetized GFP mice were bathed in either saline (upper panels) or 10 μ M cytochalasin D (lower panels) for 20 min followed by stimulation of exocytosis for 10 min. SSGs were fixed by perfusion, and labeled for phalloidin. The animals treated with cytochalasin D displayed large vacuoles as a result of swelling and compound exocytosis due to actin depolymerization (as shown, by phalloidin staining). Scale bar, 10 μ m. **C.** Stainless steel chamber used for bathing SSGs in pharmacological drugs during intravital imaging.

Figure 4.

Analysis of the Lifeact recruitment around the secretory granules. **A.** Time series in tiff format are processed with ImageJ. Images in the series are aligned by applying the plug-ins Stackreg. **B.** After processing the stabilized series is cropped to eliminate the discarded areas, saved as tiff series, and opened with Metamorph. The secretory granules coated with Lifeact are highlighted and ROI are created with the region function. **C.** The integrated fluorescence intensity is measured and plotted as a function of time using the “region measurement” function. This allows to identify the beginning of the exocytic events that will be used to normalize the fluorescence intensity. **D.** The data are exported in excel files and processed to generate the curves. A comparison between GFP-Lifeact transfected in rats and transgenic mice expressing GFP- or RFP-Lifeact is shown.