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FM 1–43 Labeling of Synaptic Vesicle Pools at the *Drosophila* Neuromuscular Junction

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Summary

To maintain transmitter release during intense stimulation, neurons need to efficiently recycle vesicles at the synapse. Following membrane fusion, vesicles are reshaped and formed from the plasma membrane by bulk or clathrin-mediated endocytosis. Most synapses, including the *Drosophila* neuromuscular junction (NMJ), can also recycle synaptic vesicles directly by closing the fusion pore, a process referred to as “kiss and run.” While the process of clathrin-mediated vesicle retrieval is under intense investigation, the kiss-and-run phenomenon remains much less accepted. To gain better insight into the mechanisms of synaptic vesicle recycling, it is therefore critical not only to identify and characterize novel players involved in the process, but also to develop novel methods to study vesicle recycling. Although in recent years numerous techniques to study vesicle traffic have been developed (see also this volume), in this chapter we outline established procedures that use the fluorescent dye FM 1–43 or related compounds to study vesicle cycling. We describe how FM 1–43 can be used to study and visualize clathrin-mediated or bulk endocytosis from the presynaptic membrane as well as exocytosis of labeled vesicles at the *Drosophila* NMJ, one of the best-characterized model synapses to study synaptic function in a genetic model system.

Keywords

Bouton; clathrin-mediated endocytosis; exocytosis; exo-endo cycling pool; kiss and run; live imaging; readily releasable pool; reserve pool; vesicle cycling

1 Introduction

In *Drosophila*, genes that affect the synaptic vesicle cycle are often essential for life, and mutant animals often die during development (e.g., Ref. 1). Several mutations in genes critical for neurotransmitter release lead to embryonic or early larval lethality, and their small size hampers functional analyses of motor neuron function. However, most mutants affecting the vesicle cycle, without abolishing it, survive to the third-instar larval stage, allowing the study of loss of gene function at mutant third-instar neuromuscular junctions (NMJs). While the molecular mechanisms of vesicle recycling have been studied at several types of synapses, the NMJ of third-instar *Drosophila* larvae is unique in that it allows testing synaptic structure and function by combining elaborate fly genetics with the ability to conduct numerous in vivo functional assays, including electrophysiology, live imaging, immunohistochemistry, electron microscopy, and more. In this chapter, we focus on the use of FM 1–43 (N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide) to follow vesicle trafficking; excellent chapters on methods for some of these other techniques appear in Ref. 2.

FM dyes are fluorescent molecules widely used to follow endocytosis, vesicle trafficking, and vesicle fusion/exocytosis in various systems. FM 1–43 and its derivatives (*see* Fig. 1 and <http://probes.invitrogen.com/handbook/sections/1601.html>) are styryl dyes that harbor a water-soluble polar head group and a lipid-soluble hydrophobic tail, separated by a central region that determines their fluorescent properties (reviewed in Ref. 3). FM 1–43 is virtually nonfluorescent in aqueous solution but increases its quantum yield more than 40-fold when bound to liposomes, which have an environment very similar to synaptic vesicle membranes (4). FM dyes bind the outer membrane leaflet (or on endocytosis, the inside of a synaptic vesicle), and this membrane association is reversible, allowing the dye to be used to study vesicle fusion and exocytosis.

The affinity of an FM dye for membranes is determined by its tail region. FM variants with a long tail, such as FM 1–84, bind membranes more tightly than those with short tails, such as FM 2–10 (5). In contrast, dyes commonly used to follow vesicle endo- and exocytosis, including FM 1–43, FM 5–95, and FM 4–64 (Fig. 1A), show very similar membrane-binding properties, although FM 4–64 is somewhat more hydrophobic, slightly increasing its affinity for membranes. Finally, different types of FM molecules harbor different spectral properties depending on the linker between the head and the tail region (Fig. 1A). For example when excited with 488-nm blue light, FM 1–43 emits maximally at 598 nm, while FM 4–64 emits maximally at 750 nm, enabling double-labeling experiments with other dyes and probes (Fig. 1B,C).

We and others have used FM 1–43 and FM 4–64 in *Drosophila* to study not only the molecular mechanisms of endocytosis but also vesicle trafficking and exocytosis (6–13). FM 1–43 binds membranes and on endocytosis is internalized and trapped in synaptic vesicles, providing a measure of endocytic efficiency. Noteworthy, during kiss-and-run recycling, the vesicle lumen is only accessible to the extracellular space by a fusion pore too small to allow entry of FM 1–43 (4,14). Hence, whereas vesicles internalized by bulk endocytosis or clathrin-mediated endocytosis are readily tagged in the presence of FM 1–43, kiss-and-run vesicles seem to escape labeling by the dye (4,5,7,14–17) (but see Refs. 18 and 19).

Most synapses contain functionally different types of synaptic vesicles, and depending on stimulation conditions, these vesicles participate in release (Fig. 2). *Drosophila* NMJs, like several other types of synapses, harbor an exo–endo cycling pool of vesicles (ECP; also referred to as “cycling pool” in Ref. 20), participating in release during mild as well as intense stimulation, and a reserve pool (RP) of vesicles that only participates in release during intense stimulation (21,22). While ECP vesicles endocytose during stimulation, work from several laboratories has shown that RP vesicles are mostly derived from internalized membrane following stimulation (reviewed in Refs. 20 and 23). Hence, by using specific stimulation paradigms, it is possible to specifically mobilize ECP or RP vesicles and test their function in vivo at the *Drosophila* NMJ (9,22,24) (Fig. 2A,B). In Table 1, we outline some stimulation conditions that allow for mobilization of specific vesicle pools. Depending on your experimental need, these stimulation protocols can be combined or adapted using the protocols described below (for an example, see Ref. 9).

2 Materials

2.1 Larval Dissection

1. Preparation of Sylgard dissection plates (Dow Corning, Midland, MI, USA, 184 Silicone Elastomer kit, Fisher cat. no NC9644388): Mix 1 volume of silicone elastomer curing agent in 10 volumes silicone elastomer in a plastic disposable cup and place under vacuum to remove air bubbles (takes about 30 min). Pour the solution into small Petri dishes that will fit your imaging station (confocal microscope or

charge-coupled device [CCD] camera); we use 35 × 10 mm plates (Corning New York, NY, USA cat. no. 430165). Place the plates horizontally in a 60 °C oven overnight for the Sylgard to polymerize.

2. Insect dissection pins (Fine Science Tools, Foster City, CA, USA Vancouver, cat. no. 260002–10): Cut the top two-thirds of the pins with scissors and use the bottom (pointy) part. You need six pins per larva.
3. Fine dissection forceps (World Precision Instruments [WPI] Sarasota, FL, USA Dumont no. 55, cat. no. 14099) and dissection scissors (Vannas fine scissors, WPI cat. no. 500086).
4. Dissection microscope (Olympus Tokyo, Japan SZ40). Muscles and motor neurons will be easier to discern with a dissection microscope with diascope (bottom) lighting.
5. HL-3 solution without calcium: 110 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 5 mM HEPES, 30 mM sucrose, 5 mM threolose, 10 mM MgCl₂, pH 7.2. Prepare fresh; do not use this solution for longer than 2 d, and store at 4 °C.

2.2 FM 1–43 Labeling and Unloading

1. FM 1–43 stock solution (1000X): Dissolve FM 1–43 powder (Invitrogen, Carlsbad, CA, USA T-35356 or T-3163) in water to a final concentration of 4mM. Store the FM 1–43 stock solution in 20-μL aliquots at –20 °C. FM 1–43 is light sensitive; keep in the dark. In case you wish to fix the samples after labeling, substitute FM 1–43FX (Invitrogen, F-35355; *see* Subheading 3.6.).
2. HL-3 + 90 mM KCl solution: 25 mM NaCl, 90 mM KCl, 10 mM NaHCO₃, 5 mM HEPES, 30 mM sucrose, 5 mM threolose, 10 mM MgCl₂, pH to 7.2. Prepare fresh; do not use this solution for longer than 2 d, and store at 4 °C. Add CaCl₂ (1 M standard solution) to obtain a final concentration of 1.5 mM CaCl₂ (or another concentration you wish to use in your experiment).

2.3 Electrical Nerve Stimulation

1. HL-3 solution with calcium: 110 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 5 mM HEPES, 30 mM sucrose, 5 mM threolose, 10 mM MgCl₂, pH to 7.2. Prepare fresh; do not use this solution for longer than 2 d, and store at 4 °C. Add CaCl₂ (1 M standard solution) to obtain a final concentration of 1.5 mM CaCl₂ (or another concentration you wish to use in your experiment).
2. Personal computer (PC) with Clampex 8.2 software (Axon Laboratories Union City, CA, USA). Create a stimulation protocol in Clampex to control the Digidata and stimulus isolation unit. For an example of how to create a 10-Hz, 10-min stimulation program, see Note 1.
3. Digidata (Axon Instruments Union City, CA, USA 1322A) connected to the PC.
4. Stimulus isolation unit (WPI stimulus isolator A365); connect the “Digital out” port you configured in the stimulation protocol of the Digidata to the input slot on the stimulus isolation unit (“input”). If you configured the 10-Hz, 10-min protocol as described in Note 1, this will be port 0.
5. Stimulation electrode: Pull a sharp, long, tapered glass electrode (we use 10-cm borosilicate electrodes with filament and outer diameter 1.5 mm, inner diameter 1.1 mm, Sutter Instrument, Novato, CA, USA cat. no. BF150-110-10; and a Sutter Instrument Flaming Brown micropipet puller, model P97). Carefully break the very tip of the electrode (Fig. 3A) and fire polish it using a heated filament (we use a

Narishige East Meadow, NY, USA Microforge MF830) such that the edge becomes smooth (Fig. 3B) and the opening of the electrode will fit the motor neuron snugly (Fig. 3D; see also Ref. 2).

6. Stimulation electrode holder (A-M Systems Sequim, WA, USA suction electrode, cat. no. 573000) mounted on a Narishige micromanipulator (cat. no. M152) with magnetic stand (cat. no. GJ-8) with a fire-polished stimulation electrode (*see* Fig. 3C) backfilled with HL-3 solution with calcium. We use a WPI Microfil tip attached to a syringe for backfilling of electrodes (WPI cat. no. MF28G-5). Connect one of the two poles of the stimulus isolation unit output to the stimulation electrode, wrap a silver wire connected to the other pole of the stimulus isolation unit around the stimulation electrode such that the wire is positioned close to the tip of the electrode (*see* Fig. 3C).

2.4 Image Acquisition and Analysis

1. Water immersion lens (Zeiss Thornwood, NY, USA 40X, NA 0.75).
2. We use a Zeiss 510 confocal microscope or a Zeiss digital camera (AxioCam MRm) mounted on a Zeiss Axioskop microscope for image acquisition. Here, we describe image acquisition with a confocal microscope, but the procedure can very easily be adapted to other image acquisition systems.
3. Amira 2.2. software package (Konrad-Zuse-Zentrum Berlin [ZIB], Indeed Visual Concepts GmbH, Template Graphics Software Inc. Berlin, Germany) for data quantification and image processing.

2.5 Weakly Labeled Samples

1. Solutions and tools described in **Subheading 2.2.** with FM 1–43FX replacing FM 1–43.
2. Formaldehyde fix solution: Prepare a fresh 3.7% (v/v) solution by diluting a 37% stock (Sigma St. Louis, MO, USA cat. no. 2106-01) ten times in HL-3 without calcium.
3. Block solution: 5% (v/v) normal goat serum (Invitrogen cat. no. PCN5000) in HL-3 without calcium, prepared fresh.
4. Rabbit anti-horseradish peroxidase (HRP) antibody (Jackson ImmunoResearch West Grove, PA, USA cat. no. 323-005-021).
5. Goat antirabbit Cy 5-conjugated immunoglobulin G (IgG) secondary antibody (Jackson ImmunoResearch cat. no. 112-175-003) or antirabbit Alexa > 647-nm conjugated antibodies (Invitrogen).
6. Mounting medium: Vectashield (Vector Burlingame, CA, USA cat. no. PK-6102), microscope slides, and coverslips.

3 Methods

We describe a set of protocols to load *Drosophila* NMJ synapses with FM 1–43 by stimulating synapses either using potassium stimulation or using electrical nerve stimulation (*see* Table 1). We subsequently describe a protocol for unloading FM 1–43, allowing not only testing for mobilization of synaptic vesicle pools but also testing the efficiency of vesicle exocytosis. We also outline a procedure to capture FM 1–43 images using a confocal microscope and a method to quantify labeling intensity following loading or unloading. Finally, we deal with weakly labeled synapses such as those in severe endocytic mutants or embryonic NMJs (7,13).

In general, when studying a mutant in *Drosophila*, animals with the same genetic background not harboring the mutation serve as wild-type positive controls. We believe that when performing FM 1–43 dye uptake/release experiments such as those described in Subheadings 3.2–3.4., it is wise also to perform a negative control in which dye uptake is inhibited. One possibility is to perform a mock uptake experiment by incubating the wild-type control preparation in 4 μ M FM 1–43 in HL-3 without calcium for the same duration as the positive control. However, in such a situation, larvae are not stimulated, muscle membranes are not depolarized, and hence these negative controls are not treated exactly as the experimental samples. A better negative control is to repeat the FM 1–43 labeling protocol on *shibire^{ts1}* animals at high temperature and compare these results to controls and experimental samples that were also tested at high (and low) temperature (*see* Subheading 3.6). Hence, negative controls, positive controls, and mutant animals are treated identically, and background labeling caused by muscle contractions or stimulation protocols are similar and can be directly compared (7,8,10).

3.1 Dissection of L3 *Drosophila* Larvae

1. Grow wild-type, mutant larvae, or both under similar conditions and pick L3 larvae of identical size for the experiments.
2. Transfer one larva to a Sylgard plate containing dissection pins and HL-3 solution without calcium. Turn the larva dorsal side up and gently push an insect dissection pin through the anterior portion of the larva, directly posterior to the mouth hooks.
3. Gently push another pin through the posterior end of the larva, in between the two trachea that line the dorsal side, and stretch the larva longitudinally (Fig. 4A).
4. Very gently poke a *small* hole in the dorsal center of the larva by rubbing the sharp end of an insect pin over the cuticle (Fig. 4B).
5. Carefully, and without touching the ventral musculature, insert dissection scissors in the hole and cut the larva longitudinally open in between the two dorsally located trachea (Fig. 4C).
6. Slowly tease out the intestine and fat bodies without disrupting the musculature, central brain, and motor nerves (Fig. 4D).
7. Spread the larva open by placing an additional dissection pin in each corner of the larva, exposing the ventral body musculature. Carefully clean out any remaining intestine, trachea, and fat bodies and inspect the integrity of the muscles and motor neurons (Fig. 4E,F).
8. Wash the preparation with fresh HL-3 solution without calcium. Do not add the solution on top of the larva as this may damage the muscles; instead, gently pipet the solution on the side of the Sylgard plate.
9. Finally, remove the anterior dissection pin to relax the motor neurons that emerge from the ventral nerve cord (Fig. 3G) and cut the motor nerves by inserting the dissection scissors underneath the nerves and cutting them without touching the underlying musculature (Fig. 4H). If the motor nerves are not cut, the central nervous system (CNS) may continue innervating the NMJ, and you do not control the amount of stimulation the NMJ receives during your experiment, confounding comparison between wild-type and mutant samples.

3.2 Labeling of Synaptic Vesicles with FM 1–43 Using KCl Stimulation

1. FM 1–43 labeling solution: Add 1 μ L FM 1–43 stock solution to 1 mL HL-3 plus 90 mM KCl solution with calcium (final concentration of FM 1–43 is 4 μ M).

2. Incubate the dissected larva in FM 1–43 labeling solution by replacing the HL-3 solution without calcium. Do not add the solution on top of the larva but gently pipet the solution on the side of the larva. Start a timer; once you add the labeling solution, exocytosis and endocytosis are induced, and FM 1–43 labeling ensues. The incubation time will depend on your experiment (Table 1) but a 1-min incubation leads to robust labeling in wild-type animals (e.g., Fig. 5D). Since some mutants are temperature sensitive, it may be necessary to perform experiments at high temperature (*see* Note 2 if this is the case).
3. Following stimulation, remove FM 1–43 labeling solution and wash five times over a total of 5–10 min with a generous amount of HL-3 solution without calcium to stop stimulating and to remove dye not internalized. Do not add the solution on top of the larva but gently pipet the solution on the side of the larva to avoid muscle damage. Gently perfuse the wash solution by pipeting up and down.
4. Image labeled vesicles on a confocal microscope with an $\times 40$ water immersion lens and quantify intensity of labeling as described in Subheading 3.5.

3.3 Labeling of Synaptic Vesicles with FM 1–43 Using Electrical Nerve Stimulation

The procedure described here is best performed on an electrophysiology rig equipped with a compound or stereomicroscope, a stimulus isolation unit, and a head stage that allows you to attach the suction electrode as described in Materials.

1. FM 1–43 solution: Add 1 μ L FM 1–43 stock solution to 1 mL HL-3 solution with calcium (final concentration of FM 1–43 is 4 μ M).
2. Incubate the dissected larva in FM 1–43 solution by replacing the HL-3 solution without calcium. Do not add the solution on top of the larva but gently pipet the solution on the side of the larva.
3. Turn the stimulus isolation unit on. Then, use the head-stage controllers to bring the suction electrode to a position close to one of the cut nerves innervating the segment you wish to stimulate and suck either the end of the nerve or a loop of the nerve into the polished end of the electrode (Fig. 3D). Make sure the stretching of the nerve does not strain the NMJs excessively. You can relax the strain by repositioning the suction electrode using the head-stage controllers.
4. Set the stimulation strength on the stimulus isolator to two times threshold. Please refer to Note 3 if you need to determine the threshold for stimulation.
5. Open the 10-min, 10-Hz stimulation protocol in Clampex and click the start button. Since some mutants are temperature sensitive, it may be necessary to perform experiments at high temperature. Please refer to Note 2 if this is the case.
6. When stimulation stops, quickly remove FM 1–43 labeling solution with a pipet and wash five times over 5–10 min with a generous amount of HL-3 solution without calcium to remove dye not internalized. Do not add the solution on top of the larva but gently pipet the solution on the side of the larva to avoid muscle damage. Gently perfuse the wash solution by pipeting up and down. While washing, blow the nerve out of the suction electrode and remove electrode.
7. Image labeled vesicles on a confocal microscope with an $\times 40$ water immersion lens and quantify intensity of labeling as described in Subheading 3.5.

3.4 Unloading of FM 1–43-Labeled Vesicles

We describe a protocol to unload synapses that were previously loaded with FM 1–43. The most straightforward approach is using KCl stimulation, the method described here. However, it is also possible to use nerve stimulation to unload vesicles, as is briefly described in Note 4.

1. Label vesicles with FM 1–43 using KCl stimulation or nerve stimulation by following a protocol outlined in Subheading 3.2. or 3.3.
2. Remove the HL-3 solution without calcium wash solution and incubate the labeled larva in HL-3 plus 90 mM KCl solution with calcium (*without* FM 1–43). Do not add the solution on top of the larva but gently pipet the solution on the side of the larva. Start a timer; once you add the HL-3 plus 90 mM KCl solution, exocytosis and endocytosis are induced, and FM 1–43-labeled vesicles will fuse with the membrane and release their FM 1–43 in the bath solution. The incubation time depends on your experiment (Table 1), but 10-min incubation leads to unloading of most of the dye in wild-type animals. Since some mutants are temperature sensitive, it may be necessary to perform experiments at high temperature. Please refer to Note 2 if this is the case.
3. Following unloading, remove the HL-3 plus 90 mM KCl solution and wash five times over 5–10 min with a generous amount of HL-3 solution without calcium to stop stimulation. Do not add the solution on top of the larva but gently pipet the solution on the side of the larva. Perfuse the wash solution by pipeting up and down.
4. Image remaining labeling on a confocal microscope with an $\times 40$ water immersion lens and quantify intensity of labeling as described in Subheading 3.5. Take extra care to image the same boutons that were imaged after loading with FM 1–43 (Subheading 3.2. or 3.3.).

3.5 Imaging and Quantification of FM 1–43 Labeling

3.5.1 FM 1–43 Imaging—We describe a procedure to image FM 1–43-labeled boutons using a confocal microscope, but this strategy can easily be adapted if you wish to use a CCD camera as outlined in Note 5. Historically, most analyses of *Drosophila* NMJs are performed on synapses located on muscles 4, 6, 7, 12, or 13, likely because they are large and easily accessible. We therefore suggest imaging boutons on these muscles. Figures 4H,I and 5A show the L3 larval musculature and the NMJs that innervate them (Fig. 5B–E). NMJs on specific muscles in each segment show a stereotypic innervation pattern (e.g., muscle 4 in Fig. 5D,E), and before imaging boutons, it is useful to familiarize yourself with the muscle and NMJ layout (25,26).

1. Place the Sylgard plate with the labeled preparation bathed in HL-3 without calcium on the stage of the confocal microscope and view FM 1–43-labeled NMJs using a mercury lamp and FITC optics under the $\times 40$ water immersion lens. (While FITC optics allow you to capture some of the FM 1–43 emission, an FITC excitation filter and TRITC emission filter configuration will allow you to capture significantly more fluorescence). Although background labeling in muscles and trachea is typically observed, clear, bright bouton labeling should be obvious in wild-type animals. You should observe labeling in individual boutons (Figs. 2A,5D). You may observe hazy rings around the boutons because of insufficient washing (*see* Note 6). If you are testing uptake in mutants, synaptic vesicle labeling may be reduced, and it may be hard (if not impossible) to identify boutons. If this is the case, please refer to Subheading 3.6.
2. When you find a string of boutons on an identified muscle you wish to image, open the program that controls the confocal microscope and excite the sample using a 488-nm laser line (at 20% laser power) and acquire fluorescence of more than 505nm using

a 505-nm long-pass filter (Fig. 1B). Please refer to Subheading 3.6. if you are using fixed samples labeled with Cy 5-conjugated antibodies as you will need to use a bandpass filter to exclude Cy 5 fluorescence in the FM 1–43 channel (Fig. 1B).

3. Use standard confocal settings and scan the boutons from top to bottom at 0.5- μ m intervals. In 8-bit mode, individual pixels can acquire an intensity between 0 and 255. However, pixel values of 0 or 255 are undefined; they could just as well be less than 0 or more than 255. Hence, to avoid erroneous quantification results, it is therefore important that all bouton pixels have an intensity value of less than 255, and all muscle pixels have a value of more than 0. Finally, scanning your samples with laser light will bleach the FM 1–43; adjust the scan speed accordingly to minimize bleaching.
4. Save the image stack either as a Zeiss database file or a series of TIFF files if you plan to use Amira 2.2 for quantification of labeling intensity.

3.5.2 Quantification of FM 1–43 Labeling Intensity—The basic procedure to quantify labeling intensity includes segmenting your data into a volume that comprises the pixels with intensity that you wish to quantify and then calculating the average pixel intensity within this volume. You will need to select a volume encompassing FM 1–43-labeled pixels (i.e., the boutons) and a volume that contains background pixels. In general, you can manually outline which volume is boutonnic using the FM 1–43 fluorescence as a guide. However, if you are working with weakly labeled samples, you may choose to fix the preparation and label it with antibodies that mark the boutons independent of FM 1–43 labeling, allowing you to use the independent marker as an outline of the bouton volume (Fig. 6). While we describe the basic protocol to quantify FM 1–43 labeling intensity here, a strategy using an independent marker of boutons to quantify FM 1–43 labeling intensity is explained in Subheading 3.6.

1. To quantify FM 1–43 labeling intensity: Open Amira 2.2, load the series of TIFF files.
2. Right click the data field that contains your imaging data and select “compute,” then “label voxel.”
3. Click the label voxel compute module, drag the “exterior-interior” slide bar to its maximum value of 255, and click “Do it.” This will exclude all pixels at this point from the analysis, allowing you now to manually add the pixels you wish to include.
4. To add pixels: Click the green “labels” data field and then the pencil tool to open the image segmentation editor of Amira. The image segmentation editor is a tool that will allow you to add pixels to the volume you wish to quantify. The editor shows the scanned confocal slices to guide you in adding or removing pixels to this volume. You can scan through the different confocal slices using the left and right arrows or using the slide bar in the segmentation editor.
5. Click on “interior” and, using the tools provided in the editor, highlight the boutonnic areas in each section that should be included in the analysis. When done, click 3D and “+” to add the highlighted area to the “interior” field. The selected areas in each slice should be surrounded by a brown line.
6. Select background area for quantification by right clicking below the “interior” field and select “new material.” Again, using the editor tools, select the muscle area you wish to use for background quantification in each section of the confocal stack. Click “+” to add the selected area to “material 1.”
7. To measure average pixel intensities in the selected volumes, right click the green “labels” field, select “measure” and then “tissue statistics.” Next, right click on the very left side of the tissue statistics measure module and connect “field” in the drop-down menu to the original FM 1–43 data field. Click on tissue statistics and click “do

it”; a statistics data field will appear. Finally, click this data field and click “show.” The values you read in the “mean” column for “interior” and “material 1” are the mean boutonnic and background intensities, respectively. The average absolute pixel intensity is the difference between these two values.

3.6 Adapted Protocol for Weakly Labeled Samples

If you are testing FM 1–43 dye uptake in mutants that severely affects membrane uptake or if you are using embryo fillets (e.g., 1,7,8,13), it is difficult to image the boutons and to outline the boutonnic areas for quantification. In such cases, one might be tempted to image those boutons that did take up some dye, ignoring those that did not, erroneously overestimating the average FM 1–43 labeling intensity (e.g., 18). Here, we describe a protocol to label neuronal membranes independently of FM 1–43 dye uptake, allowing one to use the label as an outline of boutons for quantifying FM 1–43 intensity (Fig. 6). However, we would like to note that although this procedure may circumvent problems with weakly labeled samples, fixing of FM 1–43FX slightly reduces its fluorescence, decreasing the signal-to-noise ratio. In addition, since fixing your preparation precludes additional loading or unloading steps, you will not be able to image the same boutons in loading/unloading experiments.

1. Follow loading/unloading protocols described in Subheadings 3.2.–3.4., replacing FM 1–43 with FM 1–43FX.
2. Remove the HL-3 without calcium and gently add formaldehyde fix solution.
3. Fix the sample for 5 min and quickly wash several times with HL-3 without calcium. Carefully remove the insect dissection pins and transfer the fixed sample to a 1.5-mL microfuge tube filled with HL-3 without calcium. Rock the tube gently and replace the HL-3 without calcium several times (> ten times) over a 10-min period.
4. Add 200 μ L block solution and 4 μ L anti-HRP antibody. Gently rock the preparation at room temperature for 30 min. Note that permeabilization with Tween-20 or Triton X-100 is not recommended as this will partly wash out the FM 1–43. However, since the anti-HRP antibody recognizes an extracellular glycoprotein (β -subunit of neuronal $\text{Na}^+\text{K}^+\text{ATPase}$); adding detergent is not necessary.
5. With a 1-mL pipet, remove the antibody (store for later reuse) and wash the preparation several times (> ten times) in HL-3 without calcium over a period of 10 min.
6. Add 200 μ L block solution and 2 μ L Cy 5-conjugated antirabbit antibody. Gently rock the preparation at room temperature for 30 min.
7. With a 1-mL pipet, remove the antibody (and store for later reuse) and wash the preparation several times (> ten times) in HL-3 without calcium over a period of 10 min.
8. Remove the preparation from the microfuge tube and place brain side up on a microscope slide in a drop of Vectashield. Cover with a coverslip and seal with transparent nail polish.
9. Use a confocal microscope to image FM 1–43 (488-nm excitation; emission: bandpass filter 505–600 nm) and Cy 5 (633-nm excitation; emission: long-pass filter > 650 nm). See also Subheading 3.5.1., steps 3 and 4 for additional instructions on how to capture and save images. You should save the FM 1–43 channel and Cy 5 channel as two separate series of TIFF files if you plan to use Amira 2.2 for quantification.
10. To quantify FM 1–43 labeling intensity in Cy 5-labeled samples, open the FM 1–43 channel series of TIFF files and the Cy 5 channel series of TIFF files in Amira 2.2.

Rather than using the FM 1–43 scans to segment the data, you should use the Cy 5 channel by right clicking on the data field and choosing “compute” and then “label voxel.” Now, follow the instructions in Subheading 3.5.2., steps 3–6 to outline the bouton volumes for quantification. Alternatively, you may choose to automatically outline boutons using intensity thresholding, as is briefly discussed in Note 7 (Fig. 6C,F). Finally, right click the Cy 5 “labels” field, choose “measure,” then “tissue statistics.” Next, right click on the very left side of the tissue statistics measure module and connect “field” in the drop-down menu to the FM 1–43 data field. Click on tissue statistics and click “do it”; a statistics data field will appear. Finally, click this data field and click “show.” The values you read in the “mean” column for “interior” and “material 1” are the mean bouton and background FM 1–43 labeling intensities, respectively, as defined in the Cy5 channel. The average absolute FM 1–43 pixel intensity is the difference between these two values.

4 Notes

1. To create a 10-Hz, 10-min stimulation protocol program in Clampex, open the program and select acquire, new protocol. In the mode/rate tab, change runs/trial to 1, sweeps/run to 6000, and samples/sweep/signal to 1000 (one sweep should now be 100 ms). Change the sampling interval per signal to 100 (the sampling rate should read 10 kHz). In the trigger tab make sure “start trial with” shows “immediate” and “trigger source” shows “internal timer.” In the wave 0 tab, uncheck “analog waveform,” check “digital outputs,” and make sure “waveform 0” shows “use holding.” Then, in columns A, B, and C change “type” to “step” and “first duration (samples)” to 100, 3, and 0, respectively. Finally, the “digital bit pattern” for column A should read “0000,” for column B “0001,” and for column C “0000.” These settings run a program that will open Digital Port 0 (the fourth position in the Digital bit pattern) on the Digidata for 0.3 ms every 100 ms (i.e., 10 Hz) for 10 min. When the program runs, the stimulus isolator will stimulate the motor neuron each 100 ms for a duration of 0.3 ms over a total period of 10 min. Save the protocol for future use. Stimulation frequency and duration can be adjusted as required for your experiment.
2. It is sometimes necessary to perform FM 1–43 labeling experiments (such as those in Subheadings 3.2.–3.4.) at high temperature since some mutants show normal or marginally compromised FM 1–43 dye uptake at low temperature that is dramatically exacerbated at high temperature (Fig. 6D–F) (e.g., 10). To heat your samples, you may use a commercially available heating element (Harvard Apparatus Temperature Controller TC-202A), or you can improvise a heated chamber using a water bath. Regardless of the method used, it is absolutely critical that you monitor the temperature of the labeling solution with a thermometer during the entire experiment. If you are, for example, using *shibire^{ts1}*, a temperature-sensitive mutant that blocks all vesicle recycling at high temperature (6,7,27), the temperature should never drop below 30 °C, or some endocytosis will ensue, and synapses will be partly labeled.
3. To determine stimulation threshold of a preparation incubating in HL-3 with calcium (1.5 mM) (see Subheading 3.3.), you can stimulate the motor neuron at 1 Hz (using a 1-Hz stimulation protocol in Clampex) and then slowly increase the stimulation strength on the stimulus isolator until you observe muscle twitching in the segment stimulated (Fig. 3D). Note, however, that if your preparation at this point is incubating in FM 1–43 solution, you are inducing some FM 1–43 dye uptake. To keep dye uptake to a minimum, try to keep the number of stimuli presented during this test period limited or add the FM 1–43 only after determining the stimulation threshold.
4. In Subheading 3.4., we described a protocol for dye unloading using KCl stimulation. However, unloading using nerve stimulation is also possible. Simply incubate the

labeled preparation in HL-3 with calcium (1.5 mM) but no FM 1–43 dye and stimulate the motor neuron as described in Subheading 3.3. Please refer to Table 1 for paradigms that lead to loading or unloading of vesicle pools at the *Drosophila* NMJ.

5. Although image acquisition with a confocal microscope has the advantage of capturing fluorescence throughout the 3D volume of the bouton, it is also possible to image FM 1–43-labeled boutons using a CCD camera (*see* Subheadings 2.4. and 3.5.1.). A CCD camera has the advantage that it is faster and quantification of labeling intensity is a bit easier as you do not need to highlight bouton areas in each section, but only in one image (*see* Subheading 3.5.2.). Regardless of the method of imaging, comparative studies on wild-type animals show very similar absolute fluorescence intensities for both acquisition methods (around 100 arbitrary units on a scale of 0 to 255 for CS larvae labeled for 1 min with KCl stimulation; P.V., unpublished results). In summary, imaging with a confocal microscope may yield more “in-focus” images with less background, whereas a CCD camera has the advantage of speed and easier quantification.
6. If you observe hazy rings around the boutons, you may not have washed enough. This is usually membrane labeling in the subsynaptic reticulum of the muscle and does not represent labeled synaptic vesicles; extra washes should clear this labeling.
7. As an alternative to manual thresholding of bouton areas for quantification (as outlined in Subheadings 3.5.2. and 3.6.), it is also possible to semiautomatically determine bouton areas. To do this, you should follow the procedure outlined in Subheading 3.6. and stain FM 1–43-labeled preparations with a neuronal membrane marker such as anti-HRP antibodies. Scan the data and open the data files in Amira 2.2. Right click the Cy 5 channel data field and choose “compute” and then “label voxel.” Rather than excluding all pixels by dragging the “exterior-interior” slide bar to its maximum value of 255, you should place the slide bar at a lower intensity threshold value. All pixels with an intensity above this set value will be automatically included for analysis: When you click “do it” and open the segmentation editor of the labels field, you will notice that some pixels are now included in the “interior” (Fig. 6). These are the pixels with an intensity value equal to or higher than the threshold you set. If needed, in the segmentation editor you may now manually add/remove areas you do not wish to use for quantification, and you can also add another material that contains an area for background quantification as is described in Subheading 3.5.2., step 6. Then, right click the labels data field, choose measure and then tissue statistics. Finally, right click on the very left side of the tissue statistics measure module and connect “field” in the drop-down menu to the FM 1–43 data field. Use the tissue statistics module in “measure” to display the bouton and background FM 1–43 intensities as outlined in Subheading 3.6., step 10.

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Fig. 1.

Properties of FM 1-43 and other styryl dyes. **(A)** Chemical structure of FM 1-43, FM 4-64, and FM 5-95. The “head” region of these dyes is charged and therefore water soluble, while the length of their tail determines their membrane affinity. These dyes each differ in specific spectral properties as described in the text. **(B)** and **(C)** Spectral properties of FM 1-43 and FM 4-64 when bound to lipids. The spectral properties of Cy5 or eGFP (enhanced green fluorescent protein) are sufficiently different from FM 1-43 or FM 4-64, respectively, to allow for double-labeling experiments. The 488-nm laser line excites both FM dyes.

Fig. 2.

Different vesicle pools at the *Drosophila* NMJ. **(A)** Larval fillet incubated for 5 min in HL-3 with 4 μ M FM 1–43, 60 mM KCl, and 1.5 mM calcium, washed, and imaged (see text and Table 1 for details). Note the doughnut-like bouton labeling representing FM 1–43-labeled exo–endo cycling vesicles (ECP). **(B)** Preparation first incubated in HL-3 with 4 μ M FM 1–43 and 5 mM calcium and the motor nerve stimulated for 10 min at 10 Hz. The FM 1–43 was left in the bath an additional 5 min and then washed. This protocol labels both the ECP and the reserve pool (RP) (not shown). The preparation was then incubated in HL-3 with 60 mM KCl and 1.5 mM calcium without FM 1–43 for 5 min, washed, and imaged. This unloading protocol mobilizes (and unloads) the ECP. FM 1–43-labeled vesicles that are retained following KCl unloading are located in the reserve pool (RP). **(C)** Schematic representation of the different vesicle pools in a bouton of the *Drosophila* NMJ. Dark vesicles are RP vesicles; they only participate in release during intense stimulation, and newly endocytosed vesicles *following* stimulation will preferentially refill this vesicle pool. In contrast, ECP vesicles participate in exocytosis during both mild and intense stimulation and are refilled preferentially during stimulation (9,22,23).

Fig. 3.

Preparation of a suction electrode for nerve stimulation. **(A)** A fine, long, tapered glass electrode with a broken end. **(B)** The same electrode as in **(A)** with fire-polished edges. The opening will snugly fit the motor nerve of a wild-type larva. **(C)** A head stage (HS) with an attached stimulation electrode holder. The fire-polished suction electrode (se) was backfilled with bath buffer inserted in the holder and the silver (Ag) ground wire wrapped around it. **(D)** Nomarski image of the ventral musculature of an L3 larva in which one motor nerve loop (mn; marked by arrowheads) is sucked into the suction electrode (se). The brain is located on top. Motor neurons always enter the anterior portion of each hemisegment they innervate. The motor neuron innervates marked muscle fibers 6 and 7. See Figs. 4 and 5 for additional details. The ventral midline is marked by a dashed line.

Fig. 4.

Overview of a third-instar larval fillet preparation. (A)–(F) Different steps involved in filleting a third-instar larva. (A) Pinned-down L3 larva; ventral is down, dorsal is up, anterior is to the top, and posterior is to the bottom. (B) The same larva with a small hole poked in its dorsal side. (C) Dorsally cut open larva (D) cleaned of gut, fat bodies, and large trachea. (E) and (F) Pinned-open larva, exposing the ventral musculature (ventral is medial, and dorsal is lateral). (G) Higher magnification image showing the ventral nerve cord (VNC) and motor nerves that emerge from it. The asterisk marks the same location in the fillet in panels (G)–(I). (H) The same larva with severed motor nerves, preventing direct CNS-mediated stimulation of the NMJs. The dashed line marks the ventral midline in (H) and (I). (I) High-magnification

image of two hemisegments (each marked by an arrow) showing the repeated pattern of muscle fibers. Fibers commonly used for analyses are marked (4, 7, 6, 13, and 12).

Fig. 5.

FM 1–43 labeling of a third-instar larval fillet. **(A)–(E)** Third instar larval fillet incubated for 1 min in HL-3 with 4 μ M FM 1–43, 90 mM KCl, and 1.5 mM calcium, washed, and the FM 1–43 imaged **(D)**. The same preparation was then briefly fixed with 3.7% formaldehyde and labeled with mouse anti-DLG antibodies (28; 4F3 monoclonal antibody; Developmental Studies Hybridoma bank) used at 1:50 **(B)**, **(C)**, and **(E)** to reveal synaptic boutons at the NMJs and Alexa 635-conjugated phalloidin (Invitrogen cat. no. A34054) used at 0.001 unit/ μ L **(A)** and **(C)** to reveal muscles. **(A)–(C)** two hemisegments labeled with phalloidin **(A)** and DLG **(B)**, merged in **(C)**. The dashed line in **(A)** shows the ventral midline, and muscles commonly used in the genetic analysis of synaptic function are marked. **(D)** FM 1–43 labeling (before fixation of the preparation) of the NMJ in the boxed area on muscle 4 shown in **(C)**. **(E)** The same synapse as shown in **(D)**, labeled with DLG (see **B**) following fixation of the preparation.

Fig. 6.

Weakly labeled FM 1-43FX preparations. (A)–(C) A wild-type fillet incubated for 1 min in HL-3 with 4 μ M FM 1-43FX, 90 mM KCl, and 1.5 mM calcium, washed, fixed in formaldehyde, and labeled with anti-HRP antibodies without permeabilization (13). HRP antibodies (B) label boutons that endocytosed FM 1-43FX (A). Using Amira, the HRP channel (B) was segmented; all pixels with intensity 45 or higher are white in (C) and mark the area that is used to quantify the average pixel intensity of the FM 1-43 channel (A). (D)–(F) A *shibire^{ts1}* mutant fillet incubated at 34 °C for 1 min in HL-3 with 4 μ M FM 1-43FX, 90 mM KCl, and 1.5 mM calcium, washed, fixed in formaldehyde, and labeled with anti-HRP antibodies without permeabilization (13). (D) *shibire^{ts1}* shows minimal FM 1-43FX uptake at high temperature, and boutons are not discernible; however, boutons can be revealed using extracellular anti-HRP labeling (E). Using Amira, the HRP channel was then segmented; all pixels with intensity 45 or higher are white in (F) and mark the area that is used to quantify the average pixel intensity of the FM 1-43 channel shown in (D).

Table 1
ECP and RP Loading and Unloading Protocols at the *Drosophila* Neuromuscular Junction

SV pool	No.	Stimulation protocol	Ca concentration (mM)	FM 1-43 loading period	Reference
ECP	1	60-90 mM K ⁺ , 1-10 min	1.5-2	Add FM 1-43 during stimulation	9,21,24
	2	3 Hz, 5-50 min	1.5-2	Add FM 1-43 during stimulation	9,22
ECP + RP	1	30 Hz, 5-15 min	0.2-2	Add FM 1-43 during stimulation	21,29
	2	10 Hz, 10 min	2-5	Add FM 1-43 during stimulation and then incubate in FM 1-43 for 5 min with or without Ca ²⁺ right after stimulation	9,21
RP	3	(1) Pretreatment with cyclosporin A ^a (2) 60 mM K ⁺ , 5 min	2	Add FM 1-43 during stimulation	21,30
	4	(1) 60 mM K ⁺ stimulation, 5 min (2) Wash with HL-3 without Ca ²⁺ (3) 10 Hz, 5 min	2	Add FM 1-43 during K ⁺ and nerve stimulation	24
	5	Veratridine (100 μM), 5 min ^b	2	Add FM 1-43 during stimulation	22,29
	1	30 Hz, 5 min	2	Add FM 1-43 in HL-3 without Ca ²⁺ only right after stimulation, for 5 min	22
	2	(1) ECP + RP 1, 2, or 4 (2) Wash with HL-3 without Ca ²⁺ (3) 60-90 mM K ⁺ (unload), 3-10 min	2	Add FM 1-43 during loading of ECP + RP; do not add FM 1-43 during unloading of the ECP with K ⁺ stimulation	22-24
	3	Veratridine (100 μM), 5 min ^b	0	Add FM 1-43 during stimulation	22,29

^aCyclosporin A: Inhibitor of calcineurin, Ca²⁺/calmodulin-dependent protein phosphatase 2B, which enhances endocytosis: The pretreatment consists of an incubation in 10 μM cyclosporin A for 20 min in the absence of FM 1-43. Subsequent stimulation with K⁺ will label the ECP as well as the RP (21,30).

^bVeratridine: Opens voltage-gated Na⁺ channels and increases Ca²⁺ release from internal stores. Ca²⁺ release from internal stores has been linked to loading of vesicles in the RP (22,29).