Imaging synaptic vesicle exocytosis and endocytosis with FM dyes

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FM dyes have been used to label and then monitor synaptic vesicles, secretory granules and other endocytic structures in a variety of preparations. Here, we describe the general procedure for using FM dyes to study endosomal trafficking in general, and synaptic vesicle recycling in particular. The dye, dissolved in normal saline solution, is added to a chamber containing the preparation to be labeled. Stimulation evokes exocytosis, and compensatory endocytosis that follows traps FM dye inside the retrieved vesicles. The extracellular dye is then washed from the chamber, and labeled endocytic structures are examined with a fluorescence microscope. Fluorescence intensity provides a direct measure of the labeled vesicle number, a good measure of the amount of exocytosis. If the preparation is stimulated again, without dye in the chamber, dimming of the preparation provides a measure of exocytosis of labeled vesicles. With a synaptic preparation on hand, this protocol requires 1 day.

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INTRODUCTION

FM dyes are widely used to image synaptic vesicle exocytosis and endocytosis. These dyes were originally synthesized by Fei Mao (hence, FM) at Molecular Probes in collaboration with our laboratory, with the specific goal of imaging synaptic vesicles in living preparations[1,2]. Before the introduction of FM dyes, only sulforhodamine had successfully labeled these subdiffraction-limited structures[3]. Unfortunately, sulforhodamine does not work in non-reptilian systems. FM dyes are modified styryl dyes, which have been used as fluorescent probes of membrane potential for many years (reviewed in ref. 4). Once FM dyes were shown to work well in several nerve–muscle preparations[1], the dye was adapted for use in many other synaptic preparations. These included rat hippocampal culture[5], *Drosophila* larval neuromuscular junction[6], rat cerebellar granule cell culture[7], goldfish bipolar cell[8], crayfish neuromuscular junction[9], snake neuromuscular junction[10] and brain slice[11,12]. Additionally, FM dyes have been used to label chromaffin cells[13], anterior pituitary cells[14,15], pancreatic cells[16,17], yeast[18], lung epithelia[19], intestine epithelia[20], fat adipocytes[21] and plant cells[22].

The utility of FM dyes relies on several key structural features of the molecule (**Fig. 1**). First, the tail region is lipophilic and causes the dye to partition into lipids and other hydrophobic domains. Second, the middle region contains two aromatic rings that create the fluorophore. The number of double bonds in the bridge connecting the two rings determines the fluorescence spectrum of the dye; bridges with more double bonds shift the emission spectrum to the red. The excited-state charge transfer across the conjugated region of the molecule is solvent polarity-dependent. In polar solvents such as water, quantum yield is reduced by more than two orders of magnitude compared to non-polar solvents, such as membranes. Thus, virtually the entire signal is emitted by dye molecules in membranes (or other hydrophobic environments; some FM dyes have been shown to bind to lipophilic proteins such as prolactin in rat pituitary lactotrophs[23]). Third, the FM dye's positively charged head group prevents the dye from flipping across the membrane. Thus, once dye is trapped inside a vesicle, it can escape from the cell only by exocytosis of the vesicle.

FM dyes are relatively easy to photoconvert, making it possible to identify ultrastructurally the location of the dye in cells. A discussion of the photoconversion process is outside of the scope of this protocol and the methods may be found elsewhere[24–26].

Labeled synaptic vesicles typically contain a few hundred dye molecules, nearly all of them in the membrane, and only a few at most in solution[24]. During exocytosis of a labeled vesicle, dye molecules rapidly diffuse into the surrounding plasma membrane and then, over a period of a few seconds, departition into the extracellular solution, losing fluorescence[27,28]. The loss in fluorescence intensity is used to quantify the amount and rate of exocytosis.

The FM dyes are stable, non-toxic and relatively easy to use. Even 24 h after staining, labeled synaptic vesicles can be unloaded by nerve stimulation[29]. The dyes are not inert, however, but can bind to and block both nicotinic[30] and muscarinic acetylcholine receptors[31], and pass through and block mechanotransduction channels in hair cells of the cochlea (refs. 32,33 but see ref. 34).

Molecular Probes (an Invitrogen company) offers several different FM dyes designed for different applications (see **Table 1**). FM 1-43 is the most widely used dye and works with fluorescein optics. FM 4-64 was designed to use different optics (rhodamine), which makes it useful for dual labeling with green fluorescent protein[35]. The emission spectra of FM dyes are broad compared to many other fluorescent labels; thus, controls for bleedthrough into other emission channels are always necessary when using an FM dye with another dye. The Molecular Probes website provides a spectrum viewing tool for comparing spectra of a wide variety of fluorescent probes including FM 1-43 and FM 4-64 (http://www.probes.invitrogen.com/resources/spectraviewer). Emission color shifts are

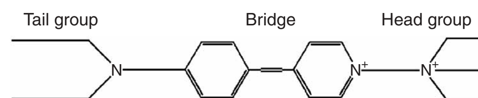

Figure 1 | FM 1-43 structure. Three regions of FM 1-43 structure (see text).

also common with FM dyes. That is, a chemically pure FM dye may appear as different colors in different structures, evidently reflecting differences in the microscopic environments into which FM dye molecules penetrate[1] (Johnson, J.M. & Betz, W.J. Biophysical Society Meeting, abstract **90**, (2006)). In addition to the FM dyes available from Molecular Probes, several "generic" versions of FM dyes are available from Biotium including Synaptogreen (1-43) and Synaptored (4-64). Biotium sells fixable dyes (AM 1-43 and AM 2-10) that are designed to prevent dye loss during fixation and permeabilization of labeled preparations. The fixable dyes have been used successfully with low detergent concentrations (0.01% (vol/vol) Triton-X[15,36]). Molecular Probes recently started offering fixable versions of FM 1-43 and 4-64 as well.

In some preparations, a several minute wash eliminates most background fluorescence. Brain slices, on the other hand, are a special problem. The high density of surface membranes causes high background fluorescence, which can be difficult to wash out. This background can be reduced by adding a quencher (e.g., sulforhodamine 101 (see ref. 12)), or by using a dye scavenger to bind to and remove background dye (e.g., ADVASEP-7, a β-cyclodextran derivative[11]). More recently, Winterer *et al.*[37] claimed improvements over both of the above methods by using two-photon laser scanning microscopy to reduce out-of-focus fluorescence.

Currently, FM dye is still probably the easiest way to label synaptic vesicles, but other methods have been developed and offer new and different opportunities. Fluorescently labeled antibodies have been used to measure uptake and movement of vesicles[38], but cannot show unloading or exocytic rates. Synaptophluorin (spH, a pH-sensitive version of green fluorescent protein connected to the vesicle snare protein synaptobrevin[39]) has become more widely used, but requires either a system for transfection of the spH or a genetically modified organism with spH knocked-in. spH and other phluorin-tagged proteins, have revealed new aspects of synaptic vesicle endocytosis[40–43].

The protocol listed below describes the basic FM dye loading and unloading protocols. Although these steps have changed little since they were first introduced, small refinements in the loading and unloading stimulation times, or adjusting when and for how long FM dye is present have created numerous opportunities to study the synaptic vesicle cycle. Measurement of endocytosis is achieved by quantifying the amount of dye uptake during stimulation. Additionally, spontaneous vesicle cycling can be observed by applying the dye to resting terminals for several to tens of minutes[44–46]. The rate of uptake can be measured by washing away the dye at variable time points after the end of stimulation[29]. Less sticky dyes that wash out faster (see **Table 1**) can facilitate this experiment. Once vesicles have been endocytosed, vesicle locations inside the cell can be measured in resting terminals simply by imaging or by using fluorescence recovery after photobleaching[47–49] or fluorescence correlation spectroscopy experiments (FCS[50,51]). Exocytosis of labeled vesicles is monitored by measuring the decrease in fluorescence during stimulation. Many of the methods utilizing FM dye labeling can be found in reviews on the topic[52–54].

MATERIALS

REAGENTS

- Deionized, distilled water
- FM dye (Molecular Probes, http://probes.invitrogen.com)
- Appropriate saline solution for your preparation
- High potassium solution
- Hypertonic sucrose solution
- Optional: ADVASEP-7 (Sigma or Biotium)

EQUIPMENT

- Fluorescent microscope with standard fluorescein or FITC optics (excitation 480, dichroic 505, emission 535 long pass for FM 1-43, 2-10 and 1-84) or standard rhodamine optics (excitation 535, dichroic 565, emission 610 long pass for FM 4-64 and 5-95)
- Electrical stimulator
- Timer or clock

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PROCEDURE

Making up FM dye stock solution

1| Choose an FM dye for your particular needs (see **Table 1**). Dissolve FM dye in deionized, distilled water to make stock solutions (typically 1 mg ml^{-1}). Although Molecular Probes suggests using DMSO as a solvent, it is not necessary or recommended.

■ **PAUSE POINT** This stock solution can be stored at 2–8 °C for several months.

Preparing the synapse for labeling

2| Prepare most neuronal cultures 2–3 weeks before labeling. Brain slices are prepared the day of labeling and should be allowed to recover from the dissection for about 1 h before adding FM dye. Neuromuscular junction preparations are dissected and prepared the same day as labeling. Special care should be used to clean the preparation before adding FM dye because any

TABLE 1 | Commercially available FM dyes.

Dye	Ex (nm)	Em (nm)	Conc (μM)	Notes
FM 1-43	510	626	2–10	Most widely used FM dye, well characterized in many synapses
FM 2-10	506	620	25–40	Shorter tail, departitions faster—better dye unloading
FM 1-84	510	626	2–10	Longer tail, departitions slower—better detects fast endocytosis kinetics
FM 4-64	558	734	2–10	Spectrum allows for dual imaging with GFP
FM 5-95	560	734	25–40	Less lipophilic than 4-64, departitions faster—better dye unloading

Ex: peak excitation wavelength, Em: peak emission wavelength, Conc: typical concentration used.

debris or damaged tissue will be strongly labeled by FM dye. A clean and healthy preparation is essential for a successful experiment. In culture preparations, recurrent activity should be blocked for all following steps with specific antagonists such as CNQX.

Dye loading

3| Add working concentration of dye to buffer or saline solution used with particular neuronal preparation (see **Table 1** for typical working concentrations for each dye). Addition of the dye will label exposed membranes immediately (**Fig. 2b**).

4| Stimulate synaptic vesicle exocytosis using nerve stimulation, field stimulation (using parallel platinum electrodes placed near the synapse), hypertonic sucrose (typically 500 mM) or a depolarizing solution (high potassium–typically 50–100 mM). **Table 2** provides some basic information on choosing a stimulation protocol. Any vesicles exposed to the extracellular space will be labeled by FM dye partitioning into the vesicular membrane (**Fig. 2c**). The time required for stimulation varies depending on the preparation used and is typically 1–5 min.

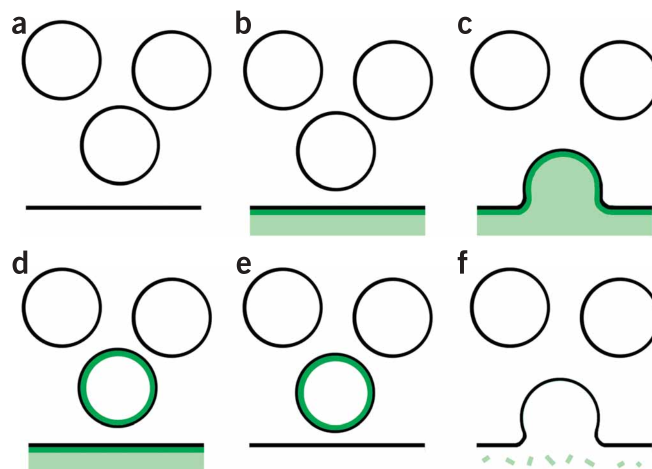

Figure 2 | Typical FM dye experiment. (**a**) Synaptic vesicles near the plasma membrane. (**b**) FM dye is added, binds to the outer membrane, and becomes fluorescent. (**c**) The preparation is stimulated and a vesicle fuses with the plasma membrane exposing the luminal membrane to the FM dye. (**d**) The vesicle is endocytosed with FM dye inside. (**e**) The FM dye is washed out of the bath and a labeled vesicle is imaged. (**f**) The preparation is stimulated again in a dye-free medium and vesicles exocytosis is measured as dye leaves the fusing vesicle.

5| Let the nerve terminal recover in the presence of the dye to allow for complete endocytosis of all released vesicles. Depending on the particular preparation, this may require up to 10–15 min (**Fig. 2d**). Note: spontaneous exocytosis may occur during this step, possibly causing more dye uptake.
▲ CRITICAL STEP In some nerve terminals including both central and peripheral nerve terminals, endocytosis occurs slowly (bulk endocytosis[55,56]) and washing the FM dye out of the sample immediately after stimulation will result in little or no vesicle labeling. In cases where stimulation is applied with high potassium treatment, a switch to normal saline containing FM dye is necessary for about 15–20 min for completion of endocytosis.

6| Wash away the extracellular FM dye by exchanging the solution multiple times with fresh saline solution. This will remove excess dye in exposed membranes (**Fig. 2e**).
▲ CRITICAL STEP Washing should continue for at least 15 min before imaging to minimize background fluorescence and to allow all endocytosed membrane to reform into release-competent vesicles[57]. Spontaneous exocytosis may occur during this step, possibly resulting in some dye loss before imaging begins. Therefore, the length of this step should remain same for all experiments performed. Some laboratories use low- or zero-calcium solutions during the wash step to minimize spontaneous release (*Drosophila* larval neuromuscular junction[58]). Application of ADVASEP-7 (1 mM) may shorten wash times (hours to minutes in some slice preparations[11]) and reduce background fluorescence. Endocytosis should be allowed to complete before addition of ADVASEP-7 (see Step 5). Use of ADVASEP-7 and sulforhodamine to reduce background has seen some use in culture and little use in acute nerve–muscle preparations where just a 15 min wash is usually sufficient.

Imaging

7| Place the sample under a fluorescence microscope and set optics to fluorescein (FM 1-43, 2-10 and 1-84) or rhodamine (FM 4-64 and 5-95). As mentioned in INTRODUCTION, some shifting of the emission spectra may occur depending on the dye's microscopic environment. The signal for the FM 1-43 family of dyes may be stronger in a typical rhodamine emission channel.

TABLE 2 | Stimulation techniques.

Stimulation type	Overview	Comments
Direct stimulation	Electrical stimulation of nerve using an electrode, field stimulation using electrodes placed near the synapse, or stimulation through a patch directly on the terminal	Very controlled amount of vesicle release; may be technically difficult in some preparations
Hypertonic sucrose	500 mM sucrose osmotically stresses the membrane and causes fusion of docked vesicles	Loads selectively, vesicles docked at the plasma membrane
High potassium	Application of higher than normal concentration of potassium to depolarize the neurons (typically 50–100 mM)	Less controlled and non-physiological but easily labels all terminals from many neurons at once

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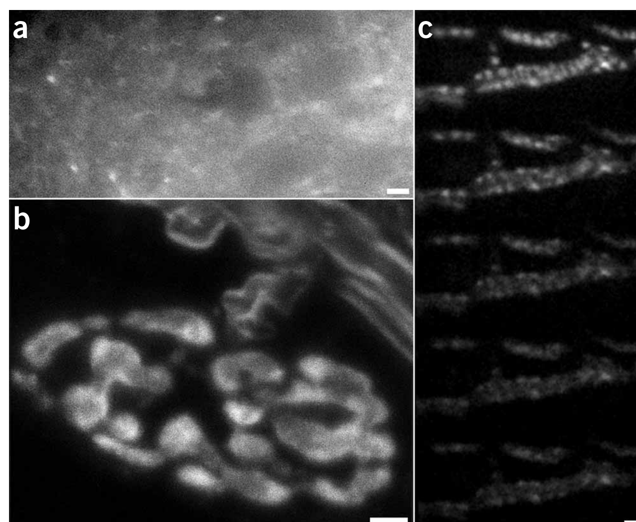

Figure 3 | ** FM dye-labeled synaptic vesicles in nerve terminals. (a**) Mossy fiber boutons are seen as bright spots on the left side of the image in a brain slice preparation after loading with FM 1-43 and washing with ADVASEP-7. Cell bodies are seen as dark ovals on the right side of the image. (**b**) Mouse motor nerve terminals imaged after loading with FM 1-43; note that FM 1-43 also labels the myelinated axon in the upper right. (**c**) From top down, sequential images of a frog motor nerve terminal taken 1 min apart during 30 Hz nerve stimulation and resulting FM 1-43 destain. Scale bars, 10 μm (**a**), 2 μm (**b**,**c**). **a**: courtesy of Dr Sukumar Vijayaraghavan.

Using long-pass emission filters (see EQUIPMENT) will collect all emitted light wavelengths from FM 1-43. However, in cases where dual imaging or choice of filter sets is limiting, use whichever filter set (FITC or rhodamine) produces the strongest fluorescence for the FM 1-43 signal, so that excitation intensity can be minimized (see below).

8| Image total fluorescence levels to quantify the relative number of synaptic vesicles endocytosed during the stimulation and recovery periods (Steps 4 and 5).
▲ CRITICAL STEP It is important to reduce excitation intensity reaching the sample because phototoxicity can be a problem with FM dyes. The light reaching the sample should be reduced to the point where detection of 7–8 bits of dynamic range is achieved (meaning the brightest pixel is 200–300 counts higher than the dimmest pixel). Reducing the light reaching the sample will also minimize any photobleaching of the FM dye resulting from the image collection.
? TROUBLESHOOTING

Dye unloading

9| To image vesicle release, start by collecting several control images. Next stimulate synaptic vesicle exocytosis (as in Step 4) while imaging. As vesicles containing dye exocytose, dye releases into extracellular space and quickly washes away (**Fig. 2f**). Any loss in fluorescence measured during stimulation is indicative of the rate and amount of synaptic vesicle exocytosis. Note: to image exocytosis during stimulation in nerve–muscle preparations, a drug such as curare should be added to prevent muscle contraction.
? TROUBLESHOOTING

10| Exhaustively stimulate the nerve terminal (either prolonged nerve stimulation or repeated high potassium applications) to unload completely all releasable FM dye. Any dye remaining after this step can be used as the terminal background level. This background contribution has been attributed to non-vesicular fluorescence as well as non-releasing vesicles[26].
▲ CRITICAL STEP To quantify observations made on preparations loaded with FM dye, terminal background levels must be measured. This step allows for measurements in Steps 8 and 9 to be attributed to activity-dependent, recycling synaptic vesicles.

Analysis

11| Quantify the amount of fluorescence in each terminal for each time point. Using image analysis software (ImageJ is a commonly used, open source freeware with a large community of contributors; http://rsb.info.nih.gov/ij/), outline the nerve terminal and record the fluorescence intensity inside the region.

12| Measure the fluorescence in the image background or non-synaptic area of the image. The background is attributed to non-terminal FM dye, autofluorescence or image detector settings. Subtract this value from the terminal fluorescence for each time point. The resulting background-subtracted fluorescence reflects the amount of vesicle release.

13| Use the fluorescence in the control images taken before stimulating the preparation to measure any loss of fluorescence as a result of imaging. Any measurable loss is likely due to photobleaching of the terminal. If loss is occurring before

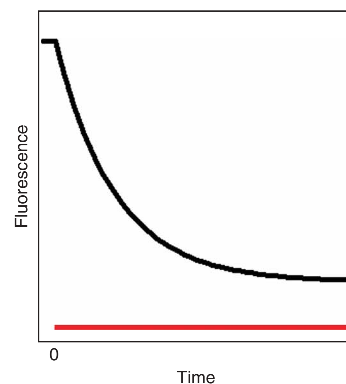

Figure 4 | ** Expected destain rates. Fluorescence plotted versus time. The fluorescence should be mostly flat before stimulation begins. Any reduction in fluorescence before stimulation is likely a result of photobleaching or spontaneous release. See PROCEDURE for ways to reduce these two possibilities. During stimulation (red line), the fluorescence will decay during a timescale *t* depending on the preparation. **Table 4 lists typical timescales (*t*) for various preparations fully loaded with FM dye and stimulated as listed.

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stimulation, it is likely occurring during stimulation as well. Therefore, the decline in fluorescence during destaining needs to be corrected to account for photobleaching.

● TIMING
Step 1: <1 min (only occurs once per vial of FM dye)
Step 2: cell culture 2–3 weeks, all others $<$ half day
Steps 3–5: <30 min depending on stimulation protocol
Step 6: $<$ 1 h (wash times can be adjusted to reduce background fluorescence)
Steps 7 and 8: 1–2 h (a few minutes per synaptic terminal imaged)
Step 9: 1–30 minutes (varies depending on stimulation protocol used)
Step 10: 5–20 min
Steps 11–13: 1–10 minutes per image series

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Problem	Possible reason	Solution
Step 8: no dye visible in terminals	Little or no exocytosis	Terminal must be healthy for vesicles to recycle properly. In many terminals, checking for transmitter release by recording post-synaptically should confirm the health of the terminal. In preparations where the nerve is stimulated to generate exocytosis, the health of the nerve may not be sufficient to transmit the signal. Try using high potassium stimulation to load the vesicles
Step 9: dye does not unload during stimulation	Phototoxicity	If the total amount of loading is not important, try loading with a weaker stimulus. Fewer FM dye molecules in the excitation light will reduce photodamage. Otherwise, take images farther apart in time to reduce the amount of light reaching the sample

ANTICIPATED RESULTS

Imaging (Step 8) should reveal fluorescently labeled terminals (**Fig. 3a**,**b**). Unloading (Step 9) should result in fluorescence loss over time (**Fig. 3c**, **Supplementary Video 1** online). Depending on the particular synapse under study and the stimulation protocol used, the rate of destaining may vary dramatically. **Figure 4** shows a plot of fluorescence decline during time *t*. **Table 4** lists typical timescales for *t* for a variety of synaptic preparations and stimulation protocols. These destain rates are typical for fully loaded terminals undergoing destain. Under these conditions, central neurons typically destain more quickly than motor nerve terminals. Different loading protocols designed to label specific pools of synaptic vesicles (reviewed in ref. 59), such as the readily releasable pool, will result in quicker destain rates. One terminal type not listed in **Table 4** is the ribbon synapse. In these terminals, exocytosis rates may be measured during dye loading[60].

TABLE 4 | FM dye destain times.

Preparation	Stimulation	*t*
Amphibian neuromuscular junction	30 Hz nerve	~5 min[57]
Fly larval neuromuscular junction	30 Hz nerve	~10 min[61]
Rat hippocampal culture	10 Hz field	~1 min[62]
Rat hippocampal slices	10 Hz mossy fiber	~10 min[12]
Rat hippocampal slices	90 mM K^+	~2 min[12]
Rat cerebellar granule cells	20 Hz field	~1 min[63]

Note: Supplementary information is available via the HTML version of this article.

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