

# Using FM1-43 to study neuropeptide granule dynamics and exocytosis

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## Abstract

In the study of neuropeptide secretion and membrane trafficking, the fluorescent dye FM1-43 provides the ability to label selectively those structures that are undergoing exocytosis and endocytosis in living cells in real time. This review describes the unique properties of the FM dyes that make them ideal for studying neuropeptide granule dynamics and discusses various techniques that take advantage of FM dyes.

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## 1. Introduction

Activity-dependent fluorescent dyes [1,2] selectively stain secretory membrane structures that are undergoing exocytosis and endocytosis and have been used extensively to investigate synaptic vesicle recycling. In this review, we will focus on the use of FM1-43 and related dyes to study exocytosis not of synaptic vesicles, but of dense core granules, especially by prolactin-secreting pituitary lactotrophs. Whereas FM dyes typically stain only membranes, in pituitary lactotrophs, but not other neuroendocrine cells, the entire dense core secretory granule, which contains no lipid, stains with FM1-43 [3]. This apparently unique property of lactotroph dense core granules has enabled the monitoring of single exocytic events in living cells in real time with ordinary epifluorescence microscopy [3,4]. We will describe the results of these studies as well as previously unpublished observations using FM dyes in combination with electron microscopy to investigate the post-exocytic fate of prolactin granules. We also consider other applications and caveats for the use of FM dyes.

## 2. FM1-43 and its variants

The family of fluorescent styryl compounds known as FM dyes has been useful for the study of exocytosis, endocytosis, and vesicle trafficking due to three properties: (1) FM dyes reversibly partition into membranes. When dye is applied to a preparation, all surface membranes become stained. When the preparation is washed with dye-free medium, the cell surface “de-stains” as dye molecules leave the plasma membrane. (2) FM dyes do not ordinarily “flip-flop” across membranes and therefore selectively stain only the external leaflet of the lipid bilayer. Because FM dye molecules are permanently charged (valence +2), they cannot diffuse through membranes and are therefore not usually free in the cytoplasm of a healthy cell (an exception will be discussed below). These properties allow selective labeling of endosomes that form in the presence of dye. (3) FM dyes are almost nonfluorescent in water, but their quantum yield increases ~350 times when they partition into a hydrophobic environment. This property allows one to study exocytosis directly: when secretory vesicles fuse with the surface membrane, dye diffuses through the fusion pore and labels the newly exposed membrane. This results in an overall increase in surface fluorescence that is a measure of the cumulative amount of membrane added by exocytosis.

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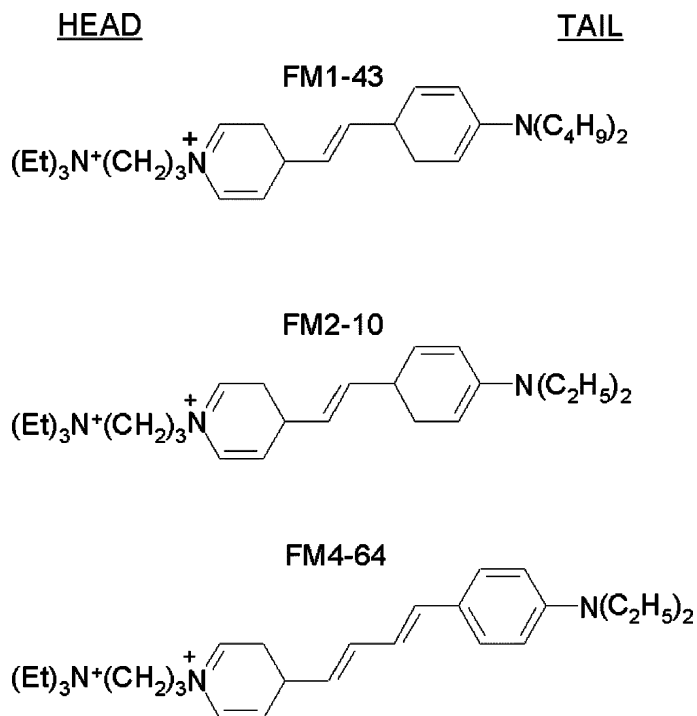


Fig. 1. Structures of commonly used FM dyes. The positively charged head group prevents dye from flip-flopping across membranes while the tail length mediates how “sticky” the dye is in membranes. The spectral properties of the molecule are determined mainly by the number of double bonds linking the head and tail, with more double bonds shifting the fluorescence to longer wavelengths.

Styryl dyes like FM1-43 are amphiphilic molecules in which a lipophilic tail is linked to a cationically charged head via a double-bond bridge (Fig. 1). The positively charged head group prevents the dye from permeating membranes, trapping the dye within endosomes. The length of the lipophilic tail determines how “sticky” the dye is in membranes: the longer the tail, the longer the washout time. (With a four-carbon tail, FM1-43 is more hydrophobic than FM2-10, which has a two-carbon tail.) Finally, the number of double bonds linking the head and tail mediates the fluorescent properties of the dye molecule. For example, FM1-43 has one double bond and can be excited with standard fluorescein optics whereas FM4-64 has three double bonds and its excitation and emission are both red-shifted [5]. Because its spectra are sufficiently red-shifted, FM4-64 can be used simultaneously with GFP-tagged proteins with little bleedthrough.

### 3. Monitoring exocytosis

FM dyes have been used to study membrane dynamics in several peptide-secreting cells. Adrenal chromaffin cells [6] and pituitary somatotrophs [8], when stimulated in the presence of FM dye, exhibit diffuse plasma membrane brightening (Fig. 2). This increase in surface membrane fluorescence is proportional to the

amount of vesicular membrane added during exocytosis. Thus, although resolution of individual granule fusion events in many endocrine cells is not achieved, monitoring FM fluorescence allows for the examination of the kinetics of membrane fusion in the whole cell in real time [6–8].

In contrast to the diffuse fluorescence observed in somatotrophs and chromaffin cells, pituitary *lactotrophs* exhibit discrete punctate spots, each spot marking exocytosis of a single granule ([3,4]; Fig. 2). These spots can be co-stained with anti-prolactin antibodies and are consistent with the dense core granules fused to the membrane that are seen at the ultrastructural level [3].

It is not clear why individual dense core granules can be visualized in lactotrophs with FM1-43 but not in somatotrophs or chromaffin cells. Perhaps, lactotroph dense cores present a uniquely hydrophobic and FM-accessible structure that the others lack.

In lactotrophs, the fluorescent spots persist for as long as FM1-43 is present in the extracellular solution. However, if dye is washed from the chamber during the first 10 min following exocytosis, the spots destain. If cells are reexposed to dye, the cores can be restained, indicating that FM staining is reversible and that the fusion pores remain open for several minutes after exocytosis. However, when the cells are exposed to FM1-43 for longer periods of time (20 min) before wash out, the plasma membrane destains while the dense

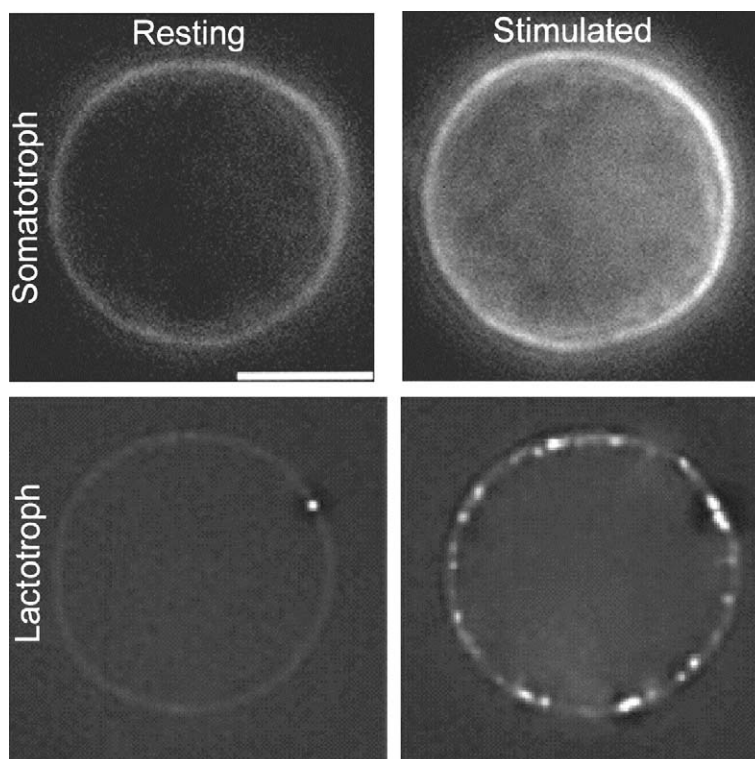


Fig. 2. Lactotroph secretory granules stain with FM1-43 upon cell stimulation. The top panels show a somatotroph in the presence of FM1-43 before (left) and after stimulation with Growth Hormone Releasing Hormone. The bottom panels show a lactotroph in the presence of FM1-43 before (left) and after stimulation with high potassium. Whereas the somatotroph membrane diffusely brightens after stimulation, the lactotroph membrane displays punctate staining. Images reproduced from Kilic et al. [8] and Angleson and coworkers [4]. Scale bar = 5  $\mu$ m.

cores retain their dye, suggesting that the cores are retrieved by endocytosis and do not fully release their granule content [3].

The fluorescence intensity of FM1-43-stained spots is quantal in nature. This property revealed prevalent multi-granular or compound exocytosis in living lactotrophs and was used to determine that granule-to-granule and granule-to-plasma membrane fusion events are subjected to distinct regulation [4]. While at present it is not clear what property of dense cores in lactotrophs accounts for the apparently unique, intense staining with FM1-43, this property has turned out to be useful for monitoring single exocytic events in living cells with conventional epifluorescence microscopy.

#### 4. FM1-43 in combination with other techniques

##### 4.1. GFP-tagged proteins

Green Fluorescent Protein (GFP)-tagged proteins have been used extensively to monitor secretory vesicle biogenesis, trafficking, and secretion [11]. If cells are transfected with a releasable GFP-tagged secretory granule protein and also stained with FM4-64, it is possible to monitor individual secretory granules before,

during, and after membrane fusion. As a granule undergoes exocytosis, the GFP-tagged protein is released into the extracellular space and the green fluorescence of the granule dims [9,10]. Concurrently, the red fluorescence signal increases as the granule core takes up the FM dye. Thus, one can monitor an individual granule's approach, fusion, endocytic retrieval, and subsequent path. By introducing different sizes of fluorescent cargo molecules into the secretory granules, one can examine the size and dynamics of the fusion pore that forms during exocytosis [12].

##### 4.2. Capacitance measurements

Whole cell capacitance recordings measure the net change in cell membrane surface area. When the amount of membrane being added to the surface via exocytosis is greater than the amount of membrane being retrieved by endocytosis, the cell capacitance increases; conversely, when the amount of endocytosis is greater than exocytosis, cell capacitance decreases. The combination of whole cell capacitance recording with FM1-43 imaging allows simultaneous, independent measurements of exocytosis and endocytosis [3,6–8]. When a cell undergoes exocytosis in the presence of FM1-43, the plasma membrane fluorescence increases as vesicles fuse with

the surface of the cell. As vesicles subsequently undergo endocytosis, the cell fluorescence remains constant because the same amount of dye is present in the vesicles whether they are fused to the surface membrane or internalized via endocytosis. By combining these two techniques one obtains simultaneous and independent measures of exocytosis (fluorescence) and endocytosis (capacitance minus fluorescence). In cells such as pituitary somatotrophs [8] and adrenal chromaffin cells [6] in which only the membrane stains with FM dye, there is a

tight correlation between the increases in capacitance and fluorescence upon stimulation (Fig. 3).

In contrast, in pituitary lactotrophs the dense cores themselves are fluorescently labeled with FM dye. As shown in Fig. 3, when lactotrophs were stimulated to undergo exocytosis, the total fluorescence increase of the cell perimeter greatly exceeded the rise in whole cell capacitance even at times when significant endocytosis of the cores would not yet have occurred [3]. However, the total capacitance increase could be predicted when

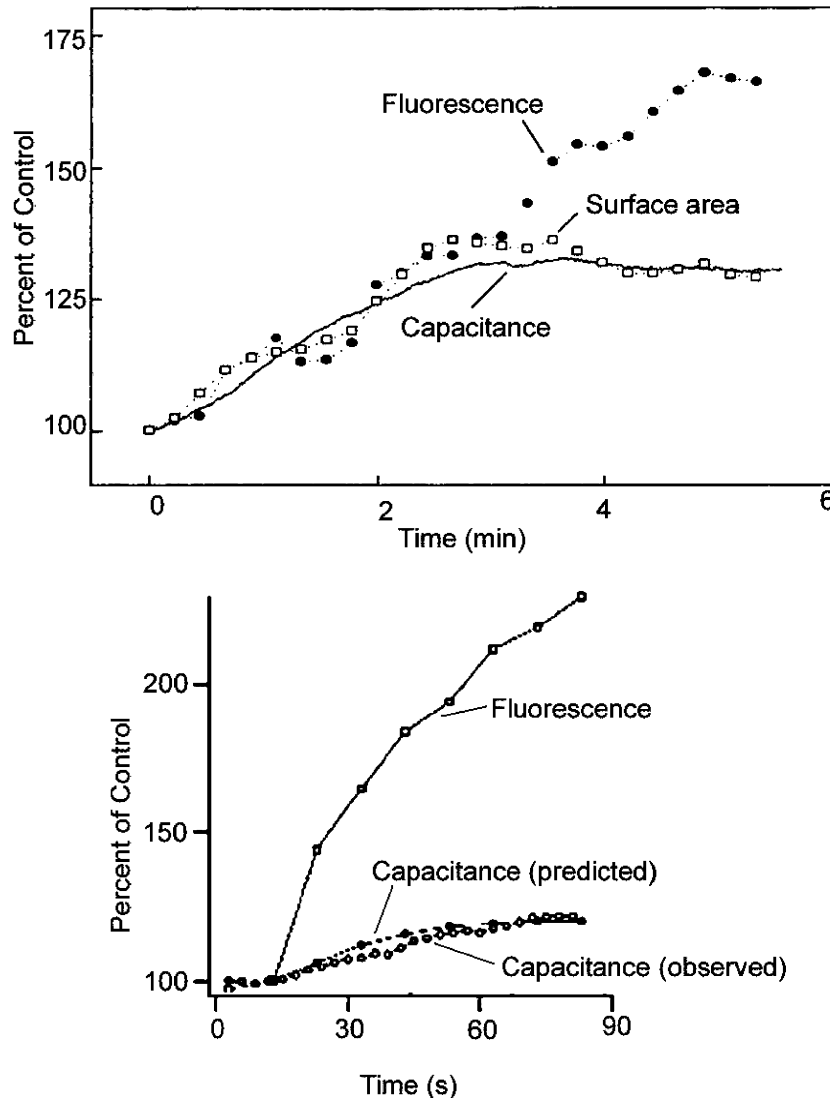


Fig. 3. The change in FM1-43 fluorescence reflects the amount of exocytosis in adrenal chromaffin cells and pituitary lactotrophs. (Top) The graph plots the plasma membrane fluorescence (circles), whole cell capacitance (solid line), and calculated surface area (squares) of an adrenal chromaffin cell. The patch pipette contained  $\text{Ca}^{2+}$  buffered to  $50 \mu\text{M}$ . For the first 3 min of stimulation, the capacitance increase closely mimicked the change in fluorescence, suggesting that only exocytosis occurred during that time. After 3 min, the capacitance trace leveled off while the fluorescence trace continued to increase. This divergence suggests that compensatory endocytosis began after 3 min of stimulation. During the entire experiment, the surface area of the cell, estimated from its diameter, closely mirrored the capacitance increase. (Bottom) A similar experiment in lactotrophs gave different results. The graph shows the changes in fluorescence (squares) and capacitance (circles) of a patch-clamped pituitary lactotroph internally dialyzed with  $10 \mu\text{M}$   $\text{Ca}^{2+}$ . The increase in fluorescence was  $\sim 5.5$  fold greater than the increase in capacitance. However, the amount of exocytosed membrane calculated from the number of fluorescent spots predicted the observed capacitance trace. Figures reproduced from Smith and Betz [6] and Angleson et al. [3].

individual FM1-43-stained exocytic events were counted and used to predict the increase in capacitance that would occur from the addition of membrane of the corresponding number of granules [3]. Thus for lactotrophs, the discrepancy between the FM1-43 fluorescence increase and capacitance increase reflected the intense staining of the dense core and not the high rate of endocytosis. In addition, the good agreement between predicted and observed capacitance increases demonstrated that the majority of, if not all, fusion events retained dense cores under these stimulation conditions.

### 5. AM1-43 fixation combined with immunocytochemistry

To assess the colocalization of various proteins with stained granules, it is now possible to perform immunocytochemistry on fixed and permeabilized cells that have been stained with a modified form of FM1-43. AM1-43 (Biotium, Richmond, CA) is the modified form of FM1-43 in which an aldehyde-reactive amine has been added to the hydrophilic tail [13]. This offers the advantage of making AM1-43 more amenable than FM1-43 to standard aldehyde fixation and detergent permeabilization protocols used for immunocytochemistry [13]. One important consideration when using AM1-43 is to use a lighter permeabilization protocol (such as 0.01% Triton X-100 for 12 min at room temperature) than is typically used for standard immunocytochemistry. Stronger permeabilization washes out the AM1-43 fluorescence. In addition, because AM1-43 has broad excitation and emission spectra, when co-staining for another marker it is useful to use a secondary antibody labeled with a long wavelength fluorophore such as Cy5 that is excited outside the spectrum of AM1-43.

Fig. 4 shows an example of a pituitary lactotroph depolarized in the presence of AM1-43 for 1 min and then washed with normal saline containing AM1-43 for 20 min. After being washed with dye-free extracellular solution, the cell was fixed with 2% paraformaldehyde and permeabilized with 0.01% Triton X-100. The cell was imaged using an Olympus IX70 inverted microscope and a Silicon Graphics O2 computer with DeltaVision deconvolution software (Applied Precision).

### 6. FM1-43 photoconversion

While FM1-43 has been most extensively used to monitor membrane traffic in living preparations at the resolution of fluorescence microscopy, the ability to photoconvert the dye in the presence of diaminobenzidine (DAB) has enabled studies of FM1-43-stained membranes to be extended to the ultrastructural level.

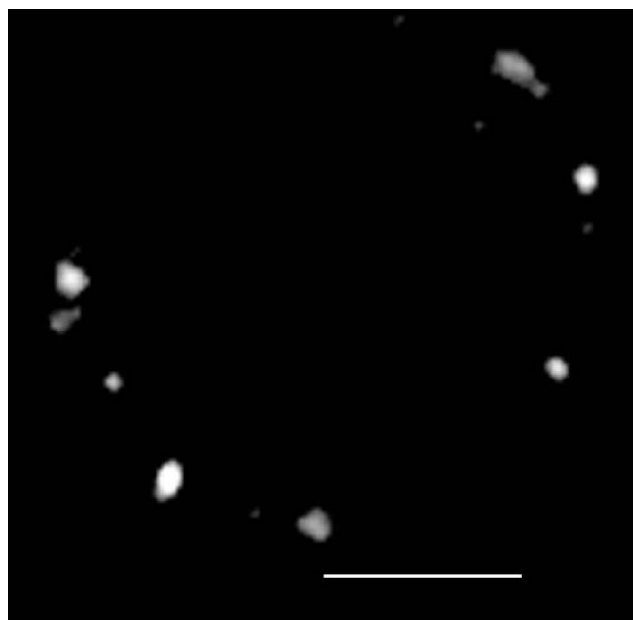


Fig. 4. AM1-43 labeled granules can be visualized in fixed and permeabilized cells. This cell was depolarized in the presence of AM1-43 for 1 min and then transferred to normal saline containing AM1-43 for 20 min. Before imaging, the cell was fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.01% Triton X-100 for 12 min at room temperature. Scale bar = 5  $\mu$ m.

This technique has proven useful for identifying intermediates of the synaptic vesicle cycle [14–17].

We photoconverted FM1-43 (Molecular Probes, Eugene, OR) stained dense cores that had been internalized in pituitary lactotrophs (Fig. 5). Lactotrophs were stimulated for 5 min in the presence of an external solution containing 100 mM KCl, 5 mM CaCl<sub>2</sub>, and 4  $\mu$ M FM1-43. The cells were switched to standard external solution containing 4  $\mu$ M FM1-43 for an additional 30 min before washing with dye-free standard external and then fixation with ice-cold 2% glutaraldehyde, 2% paraformaldehyde in 100 mM phosphate buffer for 20 min at 4 °C. The cells were then incubated in 100 mM NH<sub>4</sub>Cl for 10 min and then switched to a solution containing 1.5 mg/ml DAB in 100 mM phosphate buffer. Photoconversion was achieved by illumination for 45 min with 490 nm light through a Zeiss Plan-Neofluar 20 $\times$  (0.5 n.a.) air objective lens. The sample was then rinsed in phosphate buffer, post-fixed for 1 h in 1.0% OsO<sub>4</sub>, before ethanol dehydration and embedding in Epon, and then sectioned. Thin sections (100 nm) were viewed without post-staining with a Hitachi 7000 transmission electron microscope.

Photoconverted FM1-43 is apparent as a dark precipitate that fills the dense core of labeled granules (Fig. 5). The omission of post-staining with lead or uranyl acetate allowed unlabeled granules to be readily distinguished from FM1-43 containing granules, which contained dense reaction product. This FM1-43 photo-

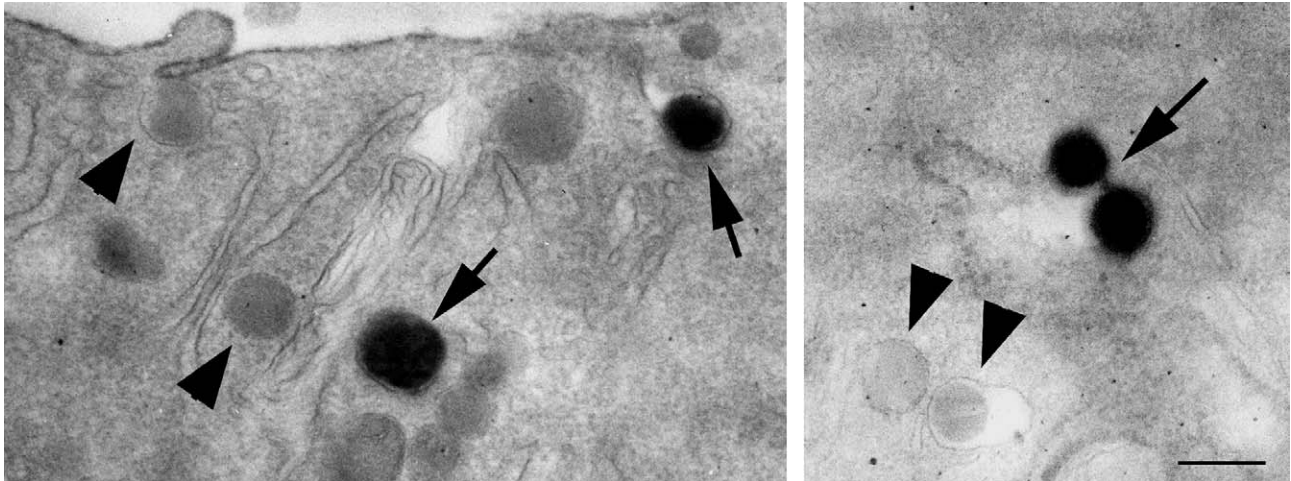


Fig. 5. FM1-43 photoconversion allows ultrastructural dye localization. Pituitary lactotrophs were stimulated with high potassium saline containing FM1-43 and then washed with normal saline containing FM1-43. Cells were then fixed and photoconverted. Full arrows mark secretory granules containing photoconversion product. Arrowheads mark secretory granules that were not stained with FM1-43 and therefore do not contain photoconversion product. Scale bar = 200 nm.

conversion labeling procedure should allow for morphological characterization of the post-exocytic traffic of internalized dense cores.

## 7. Other considerations

Although technical problems such as photobleaching and phototoxicity are encountered during live cell imaging with most fluorescent probes, some additional considerations should be taken into account when using FM dyes.

### 7.1. Background FM1-43 fluorescence

The experiments described above were performed on primary cell monolayer cultures, where background staining is minimal. In thicker tissues, such as brain slices, background fluorescence is much higher. Recently, two methods for reducing background were described. Kay et al. [18] showed that the cyclodextrin ADVASEP-7 dramatically decreased background FM1-43 staining. Cyclodextrins are toroidal molecules with a hydrophilic exterior and hydrophilic interior, into which molecules of the appropriate size can insert. ADVASEP-7 efficiently binds stray FM dye and, since it is water soluble, can be readily washed from the preparation, leaving only the FM dye found inside endosomes. In other studies, sulforhodamine 101 (S-Rhd) has been shown to quench FM1-43 fluorescence [19]. Because the absorption and emission spectra of S-Rhd coincide with the broad emission spectrum of FM1-43, S-Rhd can act as an acceptor for fluorescence resonance energy transfer (FRET). An emission filter with a narrow band-pass at  $540 \pm 20$  nm was then used to collect light in the range

of wavelengths at which FM1-43 emits, but S-Rhd does not. Because FRET only occurs when the donor and acceptor molecules are in close proximity ( $\sim 50$  Å) only the extracellular FM1-43 fluorescence was quenched. Using either of these methods, background staining was reduced enough so that the vast majority of detectable FM1-43 fluorescence came from the endocytic structures of interest. Finally, there is evidence that FM4-64 may also be used in some preparations to quench FM1-43 fluorescence via FRET [20].

### 7.2. Variable emission spectrum

In addition to having a broad emission spectrum, FM1-43 fluoresces with different colors depending on its microscopic environment. For example, when a frog nerve–muscle preparation is stained with FM1-43, Schwann cell vacuoles fluoresce orange–red, myelin appears green, and synaptic vesicles are yellow–orange [2]. This property has not been exploited experimentally, but it could be potentially useful for monitoring changes in membrane composition.

Quantum yield is also specific to the microscopic environment. For example, when Jurkat human leukemic T cells were bathed in FM1-43 and stimulated with ionomycin to induce plasma membrane lipid scrambling, surface membrane fluorescence rapidly increased as much as eightfold [21]. In most healthy cells, the plasma membrane lipid composition is asymmetric, with most of the phosphatidylserine residing on the inner leaflet. However, early in apoptosis, membrane asymmetry is disrupted and phosphatidylserine is detectable on the outer surface of cells. In apoptotic cells, this change in lipid composition and packing density causes a large increase in FM1-43 quantum yield. Therefore,

when used as an optical indicator of membrane recycling, one must be aware that FM1-43 fluorescence also increases as an early indicator of cell death. This caveat is especially important to consider when imaging FM1-43 for prolonged times and/or in combination with UV excitation of dyes such as fura-2, as both can lead to phototoxicity and potentially trigger increases in FM1-43 fluorescence.

### 7.3. Entry through ion channels in sensory neurons

When most cell types are bathed in FM dye, the only structures that are labeled are the plasma membrane and any endocytic structures that form when the dye is present. In most healthy cells, dye is almost never present in the cytoplasm. However, two reports provide evidence that when sensory hair cells are bathed in FM1-43, the cytoplasm becomes labeled within a few seconds by entering through mechanotransduction channels [22,23]. In these studies, dye entry through these channels could be reduced by high extracellular calcium and was also voltage dependent, decreasing at extreme positive and negative potentials. It should be noted that another group recently reported that FM dye uptake by hair cells is only due to membrane recycling and not passage through mechanotransduction channels [24]. If FM dye is able to enter cells through mechanotransduction channels, high cytoplasmic background fluorescence may preclude the use of FM dyes to study secretion in cells that express these channels.

## 8. Summary

By using FM dyes to label the dense core granules of pituitary lactotrophs, we have been able to monitor optically single exocytic events in real time. This technique has allowed us to study the spatial organization of membrane fusion sites, examine signaling pathways involved in the regulation of compound exocytosis, and investigate the time course of exocytosis and endocytosis in these cells.

The utility of being able to stain dense cores with FM dyes has been enhanced by several techniques. First, because it is now possible to image FM-stained cells that have been fixed and permeabilized, we can use immunocytochemistry to dissect the protein network associated with recently endocytosed vesicles. In addition, photoconversion with FM dyes will allow for characterization of the path of endocytosed dense core granules at the ultrastructural level. Finally, because fluorescent proteins can be packaged into dense core granules, we can follow the route of a granule from biogenesis through exocytosis and subsequent recycling. Comparing the release of different sized granule markers to the uptake of FM dye will also help characterize the

pore that forms when a vesicle fuses with the surface membrane. In conclusion, the ability to stain lactotroph dense core granules in an activity-dependent and reversible fashion has provided a powerful tool to study neuropeptide exocytosis and dense core granule recycling.

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