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FM Dyes in Vesicle Recycling

URL: https://www.jove.com/science-education/5648

Abstract

FM dyes are a class of fluorescent molecules that has found important use in studying the vesicle recycling process. By virtue of a chemical structure, these molecules can insert themselves into the outer leaflet of phospholipid bilayer membranes. After membrane insertion, they are internalized into the cell via endocytosed vesicles, and released when these vesicles recycle back to the membrane. Since, these dyes fluoresce strongly in the hydrophobic environment within membranes and weakly in the extracellular compartment, FM fluorescence levels can be used to track vesicular activity throughout the recycling process.

This video provides an introduction to the use of FM dyes in experiments aimed to examine vesicle recycling. We first review the biochemistry of FM dyes and how their properties permit their use in these experiments. We then go through a general protocol for using FM dyes in such studies, and finally, discuss some recent research that makes use of these unique molecules.

Transcript

FM dyes are membrane dyes that are widely used to image vesicle recycling. This is the process by which a cell forms vesicles from its own membrane to preserve cell size, reuse expensive proteins, and consecutively transport molecules to the extracellular space. This process is most commonly studied at neuronal synapses, where it's involved in release of neurotransmitters. In addition to examining vesicle recycling, FM dyes are used to study several other phenomena, such as secretion in chromaffin cells and cell membrane repair following injury.

This video will focus on the use of FM dyes in an experiment studying vesicle recycling. We'll go over a step-by-step protocol that uses FM dyes to quantify the recycling process in stimulated neurons. Lastly, we will review some example experiments, which utilize this unique molecule in different ways.

Before jumping into the procedure, let's first review the biochemical properties of FM dyes, which will help us understand their function in vesicle recycling experiments.

Structurally, FM dyes are styryl molecules that derive their name from the scientist who synthesized them—Fei Mao. These dyes have three main structural features: head, tail, and bridge. The bridge consists of two aromatic rings with a variable double bond region between them. This entire section creates the fluorophore.

It is the nature of any fluorophore that when struck by incoming light at a certain excitation wavelength, its atoms absorb that energy and raise its electrons to an excited state. These electrons return to the ground state by emitting energy vibrationally, and finally as light of an emission wavelength. The hydrophobic tail allows the FM molecule to partition into the lipid bilayers of the cell membrane, and its hydrophilic head has a charge that restricts its localization to the outer membrane leaflet.

Therefore, the only way it can go into the cell is via endocytosis. FM dyes are acutely environment sensitive, and show weak fluorescence in polar solvents, but strong fluorescence in hydrophobic environments like membranes. This creates high contrast membrane labeling that can be visualized and quantified using a fluorescence microscope.

These properties make FM dyes specifically suited to assess vesicle recycling at synapses. Briefly, the process involves incubating the cultures with FM dye. Some of the FM molecules get attached to the membranes, which significantly increases their fluorescence intensities. Next, a stimulus initiates the membrane internalization process. Therefore, some FM molecules are trapped in the vesicle membrane, and the rest of the outer localized dye molecules are washed away.

On second stimulation, internalized stained vesicles fuse with the cell membrane to release their contents, and the dye quickly departitions, resulting in a rapid decrease in fluorescence intensity. This destaining can be quantified with the help of a fluorescence microscope.

Now that you're familiar with the biochemical principles of FM dyes, let's review a procedure on how to perform an experiment examining synaptic vesicle recycling using these dyes.

Clean, healthy samples of neuronal cultures on coverslips are prepared in advance. These cells are moved to a FM dye solution, causing the membranes to be labeled. The sample coverslip is then mounted onto the imaging chamber of the microscope.

To allow fluid manipulations like washes, fluid input and output lines are attached to form a sealed perfusion chamber. Mount the chamber onto the stage of a fluorescence microscope. Attach wires to the chamber and connect them to the electrical stimulator. Once attached, the excitation and emission filters are set according to the FM dye used.

Dye loading into vesicles via endocytosis is induced using an electrical stimulation. After stimulation, nerve terminals are allowed to recover. Then, extracellular dye is washed off with buffer; this also minimizes the background fluorescence. It is important to keep the excitation intensity minimum because FM dyes are prone to photobleaching, which is weakening of fluorescence intensity due to prolonged excitation. Following the short incubation, initial images are obtained. Next, exocytosis is induced with a second electric signal.

After measuring fluorescence at different time points following stimulation, at the end, nerve terminals can be exhaustively stimulated to unload all releasable dye. The fluorescence remaining after that is considered as the background fluorescence intensity. Then, intrinsic fluorescence of each image is measured using the “Region of Interest” tool in a non-synaptic area of the image. The background fluorescence and the intrinsic fluorescence is then subtracted from the fluorescence intensity of each time point to obtain normalized fluorescence intensity, which is then plotted versus time. The decrease in intensity over time represents destaining, which is an indirect measure of vesicle recycling.

Now that we’ve reviewed the methodology, let’s look at how FM dyes are used in specific experiments.

We discussed the protocol using FM dyes in stimulated neurons. Here, scientists were interested in studying spontaneous vesicle recycling. They did this using the established protocol in combination with an imaging system sensitive enough to detect small changes in fluorescence intensity. The results represent a decrease in fluorescence intensity that is directly due to spontaneous synaptic release.

Within the presynaptic neuron, vesicles are divided into two pools: a reserve pool or RP, and a readily releasable pool or RRP. Here, scientists used FM dyes to analyze the fraction of each type in the vesicle population. By sequential application of electrical stimuli of increasing intensities, these scientists were able to quantify the relative percentages of each type of vesicle pool.

Lastly, cell biologist also use FM dyes to dissect factors involved in the exocytic repair of wounded plasma membranes. In this experiment, researchers were particularly focused on the role of sphingomyelinase, a lipid-modifying enzyme, in the membrane resealing process. First, they generated cells deficient in the enzyme sphingomyelinase with the help of silencing RNA treatment. Next, they treated these cells and a control group with exogenous FM dye and a toxin that creates plasma membrane lesions. Finally, they viewed all the samples using a confocal microscope. The results showed that sphingomyelinase deficient cells showed robust accumulation of intracellular FM molecules. On the other hand, control cells showed minimal FM influx, suggesting a role of sphingomyelinase in plasma membrane repair.

You’ve just watched JoVE’s video on FM dyes in vesicle recycling. The video described the biochemistry of FM dyes, detailed a protocol to stain and quantify synaptic vesicle recycling, and finally outlined current experiments utilizing these dyes in different ways. As always, thanks for watching!