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Imaging Synaptic Activity in Intact Brain and Slices with FM1-43 in *C. elegans*, Lamprey, and Rat

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Summary

The fluorescent probe FM1-43 has been used extensively for imaging vesicle recycling; however, high nonspecific adsorption resulting in elevated background levels has precluded its use in certain tissues, notably brain slices. We have found that a sulfobutylated derivative of β-cyclodextrin (ADVASEP-7) has a higher affinity for FM1-43 than the plasma membrane. ADVASEP-7 was used as a carrier to remove FM1-43 nonspecifically bound to the outer leaflet of the plasma membrane or extracellular molecules, significantly reducing background staining. This has enabled us to visualize synaptic vesicle recycling in the nematode *C. elegans*, intact lamprey spinal cord, and rat brain slices.

Introduction

Synaptic terminals by virtue of their small size have proved difficult to study directly. The introduction of the styryl pyridinium dyes such as FM1-43 has made it possible to visualize synaptic vesicles and thus synaptic transmission (Betz and Bewick, 1992; Cochilla et al., 1999). These dyes are particularly powerful in that they allow the time course of both endocytosis and exocytosis to be determined at individually visualized boutons. What makes this possible is that FM dyes are water soluble but partition preferentially into lipid membranes, and once there fluoresce intensely. In the presence of extracellular FM1-43, endocytosed membranes will contain FM1-43 and fluoresce. Perfusing the cells with saline washes off the FM1-43 associated with the external face of the plasma membrane, leaving vesicular fluorescence in place. If the cell is then stimulated so that exocytosis takes place, the FM1-43 will desorb from the membrane to be lost to the extracellular medium and the fluorescence will decline. The fidelity of the procedure is ensured by the fact that FM1-43 bears two positive charges preventing it from traversing membranes and equilibrating between cellular compartments.

Thus far, it has not been possible to image synapses in intact brains or brain slices using FM1-43. The problem appears to be that nonspecific binding of FM1-43 obscures the visualization of synaptic terminals. In this paper, we introduce a method for viewing FM1-43 staining in intact brains and slices of neuronal tissue. The method relies on a carrier molecule, a modified cyclodextrin (Szejtli, 1998), that has a higher affinity for FM1-43 than membranes and efficiently removes FM1-43 from the extracellular space, allowing it to be washed away, greatly reducing background staining.

To demonstrate the utility of the approach, we have shown that it is possible to image synaptic activity in a range of organisms, namely, the nematode *C. elegans*, a powerful model organism for genetic and developmental studies that has proved difficult to probe with electrophysiological methods; the lamprey, an organism with a unique flat spinal cord with particularly large axons; and rat brain slices, which have been used extensively in cellular neurobiology.

Results

Cyclodextrins Have a High Affinity for FM1-43

Cyclodextrins are cyclic oligosaccharides produced by the action of a bacterial enzyme on starch that are widely used as solubilizing agents (Szejtli, 1998). The molecules are toroidal in shape, with a hydrophilic exterior and a hydrophobic interior, into which hydrophobic molecules with the appropriate dimensions can insert. Here, we have used this ability of cyclodextrins to form such "inclusion" complexes as a strategy for removing FM dyes bound to the extracellular membranes and molecules.

Unilamellar liposomes were used to assay the interaction of FM1-43 with a range of cyclodextrins. FM1-43 has distinct spectral characteristics in liposomes compared to that in the sulfobutylated derivative of β-cyclodextrin, ADVASEP-7 (Figure 1a). The spectra in ADVASEP-7 are narrower, and excitation and emission curves are shifted to the right and left, respectively, of FM1-43 in liposomes. Addition of 1 mM ADVASEP-7 to a liposomal solution with FM1-43 results in spectra that are indistinguishable from that of ADVASEP-7-FM1-43, indi-

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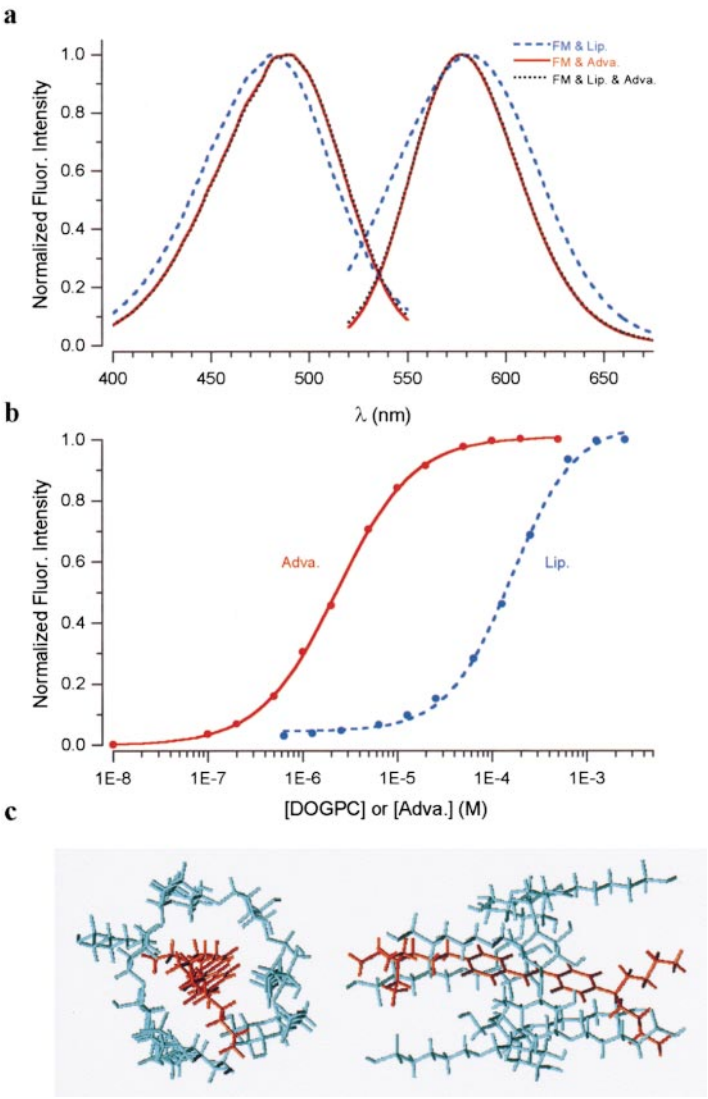

Figure 1. Spectral Characteristics of FM1-43 in Different Chemical Environments and a Model of Its Inclusion Complex with ADVASEP-7

(a) Normalized fluorescence of FM1-43 (1 μM) in liposomes (1.27 mM), in ADVASEP-7 (1 mM), and in liposomes and ADVASEP-7. (excitation 481 nm, emission 582 nm).

(b) Normalized fluorescence of FM1-43 as a function of ADVASEP-7 and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOGPC) concentration. The data were fitted with logistic functions and were consistent with the formation of 1:1 complexes (Hill coefficients, 1.07 ADVASEP-7, 1.3 DOGPC).

(c) 3D molecular structure of the FM1-43-ADVASEP-7 complex (FM1-43, red; ADVASEP-7, blue). The degree of substitution and position of sulfobutyl groups in ADVASEP-7 is random. Only one of the possible configurations has been shown here, and no attempt has been made to optimize the structure.

cating that the movement of FM1-43 from liposomes to ADVASEP-7 is almost complete. The movement is also rapid, being completed within the mixing time of the apparatus (<2 s; data not shown). The attraction between the charged groups on FM1-43 (2+) and ADVASEP-7 (average of 7−) may speed the formation of an inclusion complex.

It is worth noting that the increase in fluorescence of dyes such as FM1-43 in hydrophobic environments results from three effects on the fluorophore: the sequestration from quenchers like oxygen, a decrease in the intramolecular rotation, and exposure to a less polar environment (Lakowicz, 1983). All serve to diminish the probability of a nonradiative transition from the excited state.

Of the cyclodextrins tested, ADVASEP-7 (Tait et al., 1992) proved to be the most effective carrier of FM1-43 as judged by the intensity of fluorescence of the inclusion complex. The peak amplitudes of the excitation spectrum as a percentage of that of FM1-43 (2 μM) in ADVASEP-7 were (peak excitation wavelength in brackets, all cyclodextrins 2 mM): 20.3% (490.6 nm) for liposomes (6 mM), 12.4% (488.6 nm) for γ-cyclodextrin, 6.4% (492.6 nm) for hydroxybutyl cyclodextrin, 1.8% (485.6 nm) for β-cyclodextrin.

The effective affinity of ADVASEP-7 for FM1-43 was measured by titrating 1 μM FM1-43 with different concentrations of the cyclodextrin or liposomes. The titration was performed with the lipid rather than the probe to prevent errors resulting from self-quenching. The data was well fitted by a single binding site, with a dissociation constant of 1.8 ± 0.1 μM (n = 3) for ADVASEP-7 and 100 ± 9 μM (n = 3) for 1,2-dioleoyl-sn-glycero-3-phosphocholine (Figure 1b). In calculating the latter dissociation constant, we assumed that FM1-43 could only bind to the outer lipid monolayer (assumed to be 60%), which is consistent with our demonstration below that FM1-43 does not flip-flop across membranes. Our calculated dissociation constant was close to that measured for FM1-43 and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine by high-sensitivity isothermal titration calorimetry: 91 μM at 23°C (Schote and Seelig, 1998). The dissociation constant for γ-cyclodextrin was found by titrating ADVASEP-7 in the presence of 1 mM γ-cyclodextrin, and was ~2 mM.

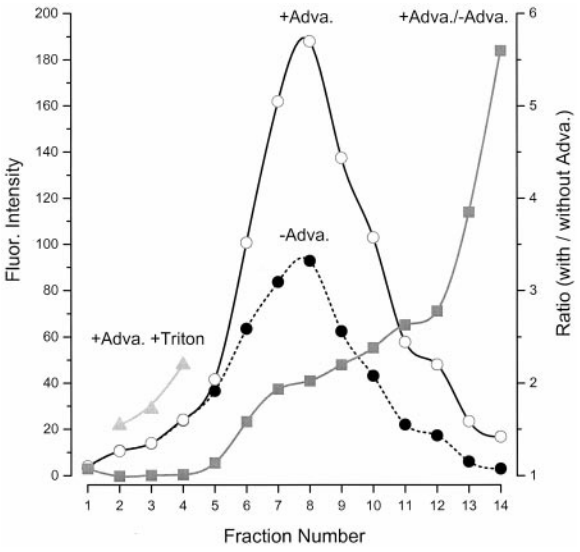

Figure 2. FM1-43 Does Not Flip-Flop across Lipid Membranes

Liposomes were prepared with 10 μM FM1-43 in LS saline. The liposomal solution was then passed through a sephacryl S-1000 column, and fractions were collected at 15 min intervals. The fluorescent intensity (excitation 481 nm, emission 582 nm) of the fraction (closed circle), fraction plus ADVASEP-7 (1 mM) (open circles), and fraction plus ADVASEP-7 and Triton X-100 (0.2%) (shaded triangles) are plotted as a function of fraction number, as is the ratio of the fluorescent intensity of the fraction plus ADVASEP-7 to that of the fraction without the cyclodextrin (shaded squares).

ADVASEP-7 (Tait et al., 1992) is a sulfobutylated derivative of β-cyclodextrin (seven glucose units) with an average of seven sulfobutyl groups randomly attached primarily to positions 2 and 6 of the glucopyranose units. The butyl groups extend the hydrophobic pore, allowing longer molecules to be accommodated. The excellent match between the dimensions of the cyclodextrin's pore and FM1-43 is illustrated in the molecular model in Figure 1c.

An added advantage of ADVASEP-7 is that it was specifically designed to reduce the tendency of cyclodextrins to abstract cholesterol from membranes and thus minimize its possible cytotoxicity (Tait et al., 1992). Moreover, the seven negative charges on ADVASEP-7 makes it unlikely that it will cross the plasma membrane.

To determine if ADVASEP-7 or FM1-43 can move across membranes we used a sephacryl column to remove FM1-43 from the extraliposomal medium. Liposomes were formed in the presence of FM1-43 and then passed through a sephacryl S-1000 column to separate free FM1-43 from liposomes with trapped dye. The first four fractions that came off the column had little FM1-43 in the outer leaflet of the lipid, as judged by the fact that application of ADVASEP-7 led to no detectable increase in fluorescence (Figure 2). These fractions were left for a further 7 hr in the presence of ADVASEP-7, with no increase in fluorescence. Addition of Triton X-100, which breaks the liposomes, led to the doubling of the fluorescent intensity, demonstrating that FM1-43 was trapped in the liposomes (Figure 2). Furthermore, these experiments show that both FM1-43 and ADVASEP-7 are not able to diffuse across pure lipid membranes. Fractions 5–14 contain mixtures of liposomes and extraliposomal FM1-43 with increasing proportions of the latter, as is evidenced by the increase in the ratio of fluorescence in the presence of ADVASEP-7 to that in its absence.

ADVASEP-7 Reduces Background Staining in Rat Brain Slices

The high affinity of ADVASEP-7 suggested to us that it might be able to reduce background staining by FM1-43 by removing the fluorophore from the extracellular surface of the membrane and carrying it out of the tissue. We initially tested this hypothesis on rat brain slices and found that ADVASEP-7 was indeed an effective "clearing" agent. If saline was used to wash FM1-43 from the tissue, high levels of fluorescence remained after 1 hr of washing (Figure 3a). The slice was not stimulated; nevertheless, FM1-43 application led to the staining of numerous structures. This is what one might term "adventitious" staining, in that it is probably not related to synaptic uptake, but to extracellular molecules that have a high affinity for FM1-43. Clearing the slice with ADVASEP-7 led to the almost complete loss of adventitious staining (Figure 3b). The only structures now evident were blood vessels and myelinated axons in mature slices. On applying ADVASEP-7 to the slice, there was an immediate "blooming" of fluorescence resulting from the higher quantum yield of the FM1-43-ADVASEP-7 complex (data not shown).

The affinity of ADVASEP-7 for FM1-43 was further confirmed by the observation that the application of premixed ADVASEP-7 and FM1-43 prevented adventitious staining, labeling of myelin and blood vessels, and stimulus-induced loading.

Application of ADVASEP-7 to hippocampal slices had no significant effect on the slope of the field excitatory postsynaptic potentional (EPSP) in area CA1 elicited by stimulation of the Schaffer collaterals, when applied at a concentration of 1 mM for 10 min (Figure 4a). Similarly, ADVASEP-7 had no effect on action potentials elicited by intracellular current injection in giant axons of the lamprey spinal cord (Figure 4b).

Myelinated axons and blood vessels were intensely labeled with FM1-43, and ADVASEP-7 was ineffective in removing the dye, suggesting that FM1-43 is somehow incorporated into the membranes. This contrasts with labeling of frog myelinated axons in which the dye washes out, albeit slowly (Betz et al., 1992).

In what follows, we demonstrate the activity-dependent staining by FM1-43, in conjunction with our clearing agent, in a range of preparations that have not heretofore been amenable to the FM technique. We will discuss the preparations proceeding from the slices to intact nervous systems, rather than in their phylogenetic order.

Calyces of Held in Slices of Rat Auditory Brainstem

Projections from the contralateral cochlear nucleus form some of the largest synapses in the mammalian brain on the principal cells of the medial nucleus of the trapezoid body (MNTB). These so-called calyces of Held are large presynaptic elements that envelop the cell bodies of the postsynaptic neurons (Forsythe, 1994; Borst et al.,

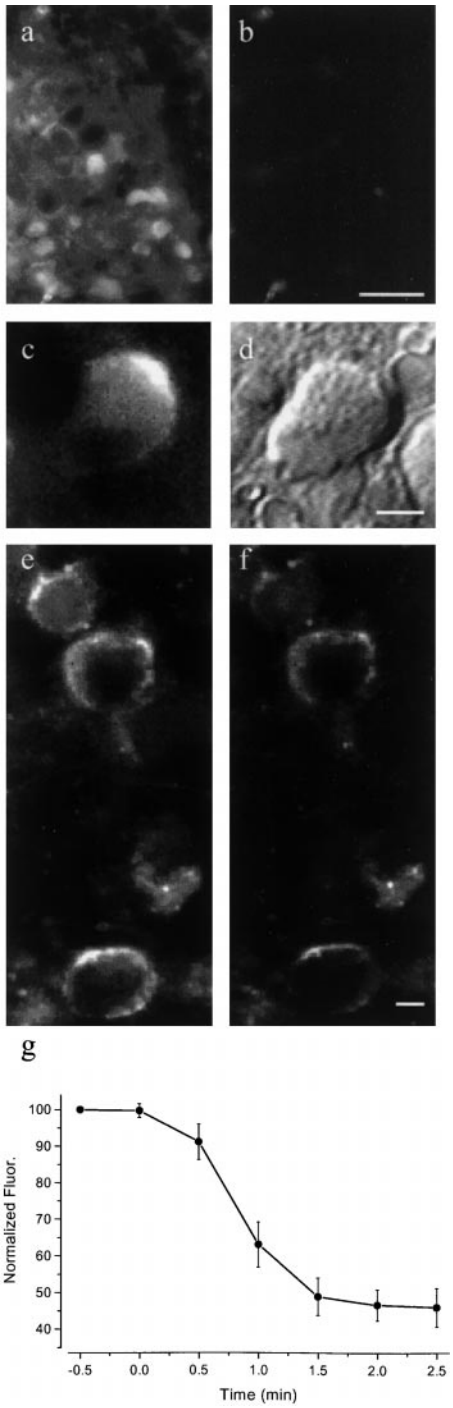

Figure 3. Calyces of Held in the Rat Auditory Brain Stem

(a) Brainstem slice exposed to 8 μM FM1-43 for 1.5 min in RA1 saline and then washed for 30 min with saline.

(b) The same slice as in (a) after washing with 1 mM ADVASEP-7. Scale bar, 30 μm.

(c) Fluorescent image of a single calyx of Held loaded by stimulation with high potassium.

(d) Infrared DIC image of the same. Scale bar, 10 μm.

(e) FM1-43-loaded calyces of Held.

(f) The same after stimulation with high potassium for 1.5 min Scale bar, 10 μm.

(g) Rate of decline of fluorescence in calyces of Held after high-potassium stimulation, commenced at time zero. Average (± SEM) of ten terminals in four different slices.

1995). The distinctive morphology of the synaptic element also allows easy identification of the structure in contrast to the rather nondescript puncta of the rest of the nervous system.

After ADVASEP-7 clearing of MNTB slices that had been stimulated with high potassium in the presence of FM1-43, crescent-shaped fluorescent profiles were evident, consistent with the known morphology of the calyces of Held. Fluorescent synaptic profiles were not seen in the absence of potassium stimulation. When viewed with Nomarski optics, the crescents were found to be in register with the somata of the principal cells, and in some cases the terminals themselves could be discerned (Figures 3c and 3d).

Stimulation of the preloaded calyces with high-potassium saline (90 mM) led to a progressive loss of fluorescence intensity. The average fluorescence intensity from a calyx decreased by 20%–73% (mean ± SEM = 47% ± 5% from ten calyces in four slices) of the control, in 1–1.5 min after application of the high potassium solution (Figures 3e, 3f, and 3g). In control experiments where the fluorescence was imaged at the same rate as during the experiments, the intensity declined by <10% over 2 min (data not shown), suggesting that bleaching or spontaneous activity did not occasion the decline in fluorescence.

C. elegans

The small number of neurons (302) in the adult *C. elegans* nervous system, for which the complete connectivity is known (White, 1986), the availability of mutants in an array of neuronal proteins, and the knowledge of its complete genomic sequence make it a powerful model system to analyze the function of individual neurons and the gene products they express (Bargmann, 1998; Chalfie and Jorgensen, 1998). Electrophysiological analysis of the individual neurons has been difficult as a result of their small size; however, recent advances have opened *C. elegans* to electrophysiological techniques (Raizen and Avery, 1994; Goodman et al., 1998; Richmond and Jorgensen, 1999). One major difficulty with the study of membrane recycling in *C. elegans* is the tough, impermeant cuticle, which keeps the animal under hydrostatic pressure and prevents dyes from permeating into the nervous system. To gain access to the body cavity of *C. elegans*, we have sliced nematodes embedded in agar. Neurons survive this assault, as we will show below, with their synaptic release apparatus intact.

The disposition of the *C. elegans* "brain," the circumpharyngeal nerve ring, is diagramed in Figure 5a. Chemical synapses are made en passant between adjacent processes. The presynaptic elements are relatively simple, and they can be identified because they are filled with synaptic vesicles (White, 1986).

Application of FM1-43 in the presence of high potassium to sliced nematodes led to the development of fluorescence in areas known to contain nerve endings, such as the nerve ring (Figure 5c). Washing with ADVASEP-7 reduced the background by 5- to 7-fold and was used in all subsequent experiments (data not shown). Potassium stimulation promoted an 11-fold increase in fluorescence (62.6 ± 4.3, n = 15) relative to control preparations (5.6 ± 1.8, n = 10; data not shown). Furthermore,

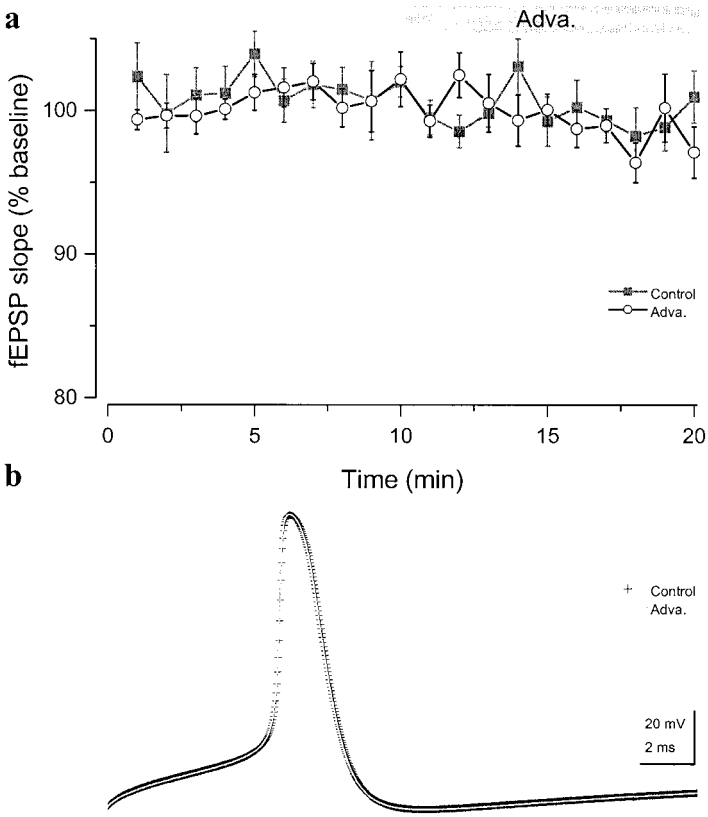

Figure 4. ADVASEP-7 Has Neither an Effect on Synaptic Potentials in Hippocampal Slices nor on Action Potentials in Giant Axons of the Lamprey Spinal Cord

(a) Field EPSP slope (percentage of baseline) in hippocampal slices as a function of time. Field potentials were evoked at a rate of 0.06 Hz, and the slope was measured between 20% and 80% of maximum amplitude. ADVASEP-7 (Adva) (1 mM) was applied for the period indicated by the gray bar (mean ± SEM, n = 9 slices); RA2 solution (Control) was perfused throughout (n = 7 slices).

(b) Spikes evoked by current injection in a giant axon of the lamprey spinal cord in control saline and 3 min after the application of ADVASEP-7 (1 mM).

the omission of calcium from the high-potassium medium prevented FM1-43 loading (data not shown).

In order to confirm that stimulation leads to the accumulation of FM1-43 in presynaptic elements, we have used a transgenic *C. elegans* expressing an UNC-11::GFP fusion protein. UNC-11 is an AP180 homolog that is enriched at the presynaptic terminals, binds clathrin, and participates in the assembly of clathrin coats and recycling and sorting of synaptic vesicle components (Nonet et al., 1999). In these experiments, the styryl dye FM4-64, which has spectral characteristics that do not interfere with that of GFP, was used as the endocytotic marker. Potassium-induced loading of FM4-64 led to a pattern of labeling overlapping that of presynaptic terminals identified by UNC-11::GFP (data not shown).

Application of high potassium to cut nematodes prelabeled with FM1-43 induced the decline of fluorescence, consistent with synaptic release (Figures 5b–5e). The time course of the decline elicited by high-potassium stimulation is shown in Figure 5e (HiK). In sliced nematodes not subjected to high-potassium stimulation after FM1-43 loading, there was little decline in fluorescence (Figure 5e, control).

Lamprey Spinal Cord

The spinal cord of lamprey ammocete is only 150 μm thick, with unmyelinated axons, making it ideal for imaging studies. Moreover, the length of the structure is traversed by giant axons, with diameters of up to 50 μm, that are unusual in having no outward morphological signs of the presynaptic element but rather clusters of synaptic vesicles adjacent to an undeviated axonal membrane (Wickelgren et al., 1985).

In initial experiments, we found that FM1-43 applied via the bathing solution only stained the outside of the spinal cord. To obviate this problem, we pressure ejected FM1-43 into the spinal cord, close to the giant axons (Figure 6a). Extracellular stimulation of the giant fiber was initiated at the same time as FM1-43 infusion, at a rate of 10 Hz for 2000 stimuli. Injection of FM1-43 into the tissue led to a region of strong fluorescence broken by the passage of large axons, indicating that the dye did not penetrate into the axon. At this stage, the staining of tissue was uniform, and no clear puncta were evident. Immediately after stimulation, the application of FM1-43 was stopped and ADVASEP-7 (1 mM) was added to the superfusate. A significant increase in fluorescence was observed (see above), followed by the elimination of all but localized puncta with diameters ranging from 1 to 5 μm (Figures 6b and 6c). The washout of ADVASEP-7 reduced considerably the background fluorescence, and numerous puncta were clearly visible. Stimulation of the giant axon led to the progressive destaining of the puncta in a stimulus-dependent fashion (Figures 6c and 6d). The synaptic origins of the puncta were further confirmed by the fact that superfusing 30 μM Ni^{2+}, which in lamprey blocks presynaptic Ca^{2+} currents and synaptic transmission (R. Freed and S. A., unpublished data) during the loading procedure, blocked the appearance of puncta (data not shown).

To position the stimulus-evoked puncta with respect to their parent axons, a single axon was stimulated intracellularly. The microelectrode was filled with rhodamine

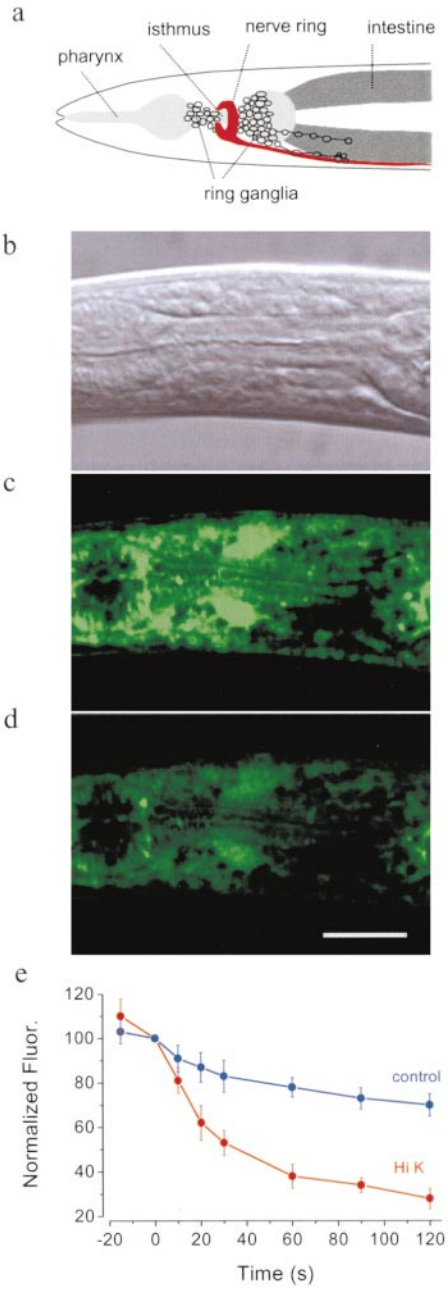

Figure 5. *C. elegans*

(a) Schematic diagram of the *C. elegans* head. The nematodes in all other panels have the same orientation as the schematic.

(b–d) Destaining of sliced nematode after high-potassium stimulation and clearing with ADVASEP-7. DIC image is shown in (b), fluorescence at time zero is shown in (c), and fluorescence 120 s after commencing stimulation is shown in (d). Scale bar, 10 μm.

(e) Time course of FM1-43 release. Sliced nematodes were loaded with 5 μM FM1-43 by subjecting them to high-potassium saline for 5 min. This was followed by a wash with 1 mM ADVASEP-7 in CE-0Ca saline. "HiK" indicates stimulation with high-potassium saline at time zero (n = 10 nematodes). "Control" indicates wash with normal *C. elegans* saline at time zero (n = 5 nematodes). The fluorescence intensity was measured in two areas on the nerve ring, and the background was measured in the isthmus of the pharynx.

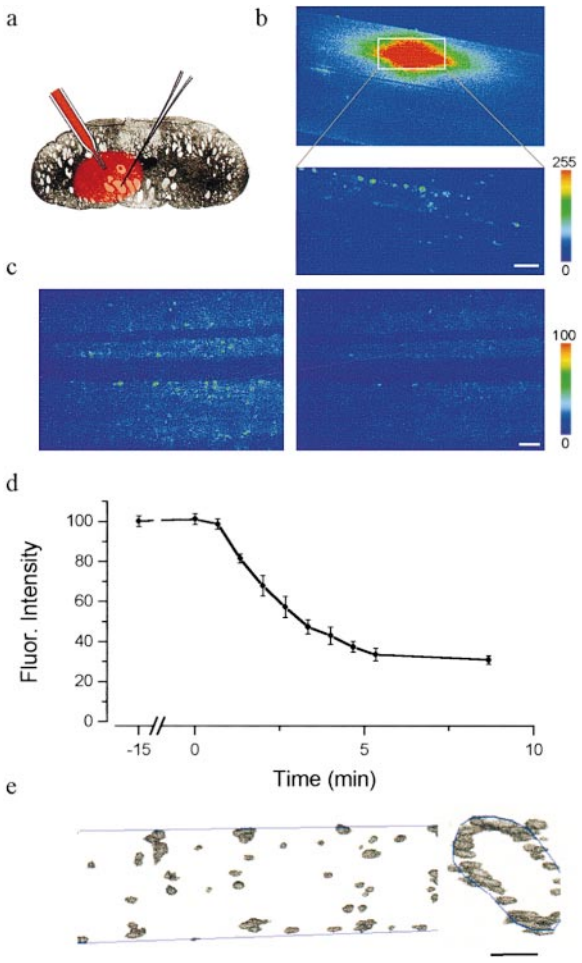

Figure 6. Imaging Synaptic Activity in Intact Lamprey Spinal Cord

(a) Cross section of a lamprey spinal cord fixed in osmium acetate and counterstained with toluidine blue, illustrating the placement of electrodes.

(b, top) Low power confocal image of lamprey spinal cord injected with FM1-43 and electrically stimulated (2000 stimuli) prior to clearing with ADVASEP-7.

(b, bottom) Confocal image of a portion of the field above, after washing with ADVASEP-7 for 3 min. Scale bar, 20 μm.

(c) Stimulus-induced decline of fluorescence.

(left) Confocal image of puncta flanking two giant axons after stimulus-induced loading.

(right) Same field after applying 3200 stimuli. Scale bar, 20 μm.

(d) Rate of decline of fluorescence in the same field as shown in (c). Average (± SEM) of 15 puncta. The axons were stimulated at 10 Hz.

(e) Two orthogonal projections of confocal sections of fluorescent puncta inside a giant axon stimulated while in the presence of FM1-43 and cleared with ADVASEP-7.

(left) Parallel to the axis of the axon.

(right) Cross section of the axon. The outline of the axon was reconstructed from the fluorescent image of the axon. Scale bar, 10 μm.

red (1 mM) to disclose the axonal morphology. Two thousand stimuli (10 Hz) were delivered to the axon during FM1-43 injection. The recorded axon was stimulated in whole-cell mode during the pressure application of dye, and ADVASEP-7 was subsequently applied to the superfusate. Projections of confocal sections into two orthogonal planes show multiple puncta nestling

Table 1. Composition of Saline Solutions

Abbreviations	NaCl	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	HEPES	Glucose	
CE	140	6	2	1		5	10	
CE-0Ca	140	6		1		5	10	0.5mM EGTA
CE-HiK	60	86	2	1		5	10	
LP	100	2.1	2.6	1.8	26		4	
LP-HEPES	112	2.1	2.6	1.8		2	4	
LS	150		2	2		10		
RA1	125	2.5	2	1	25		25	*
RA1-HiK	90	37.5	2	1	25		25	*
RA2	124	2.5	2	2	23		10	

All solutions at pH 7.4.
Asterisks indicate 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 myo-inositol, and 2 sodium pyruvate. Abbreviations: CE, *C. elegans*; LS, liposome; LP, lamprey; and RA, rat.

within the confines of the axon (shown in Figure 6e), and this is consistent with the known ultrastructure of these synapses (Pieribone et al., 1995). Stimulation of the axon led to the progressive destaining of the puncta (data not shown). Similar results were obtained in two other experiments. These results show that FM1-43 can be used to locate the synaptic terminals of active neurons in an intact nervous system.

Discussion

The primary impediment to using FM1-43 in slices is the nonspecific adsorption of the probe that is resistant to washing and obscures the visualization of synapses loaded with FM1-43. Application of ADVASEP-7 effectively acts as a scavenger of FM1-43 bound to extracellular structures and, because the inclusion complexes so formed are soluble, serves to clear FM1-43 from the extracellular space, unmasking synapses loaded by stimulation. In the preparations employed in this paper, the need for clearing with ADVASEP-7 varied; in rat and lamprey, it proved essential to observe stimulus-induced loading, while in *C. elegans* and *Xenopus* tadpole optic tectum (K. Haas and H. T. C., unpublished data) ADVASEP-7 was not essential but did improve the signal-to-background ratio. These variations in adventitious staining may be accounted for by differences in the accessibility of the extracellular space or variations in the presence of molecules with a high affinity for FM1-43.

We have in this communication chosen to perform our experiments on rat brain slices on the calyces of Held because of their idiosyncratic morphology that serves as a clear sign of presynaptic labeling. Moreover, functional synapses in these slices are close to the cut edge, making FM1-43 permeation less of a problem than for synapses within the depths of a slice.

We offer a few pointers for using our method in other tissues. First, it may be necessary, as was the case in lamprey spinal cord, to inject FM1-43 into the depths of the slice. FM1-43 appears to be trapped by cells located at the cut edge of slices, because of the high affinity of the dye for membrane and restricted access to the extracellular space. Second, the apparent dynamics of the release process in slices and intact brain may be slowed with respect to that in a more isolated preparation. The local environment of the synapses in slices or intact brain is unstirred, and this should slow the removal of FM1-43 that is released. Moreover, the released FM1-43 has a high probability of encountering membranes into which it is likely to partition, in preference to the extracellular solution. The overall effect of these factors would be to slow the measured rate of release of FM1-43. The provision of a low concentration of ADVASEP-7 (10–100 μM) in the extracellular space may serve to carry the released FM1-43 into the intracellular matrix and thence out of the slice. A more effective solution to this problem might be to conjugate a chemical group to ADVASEP-7 with an absorption spectrum that overlaps that of the emission of FM1-43, to quench its fluorescence by fluorescence resonance energy transfer (Lakowicz, 1983).

A disadvantage of our procedure is that it takes some time for the cyclodextrin-FM1-43 complex to wash out, preventing destaining experiments from being commenced during this period. A quenching cyclodextrin would serve well to obviate this problem, as then it would not be necessary to wait for the scavenger to wash out. While completing this paper, we became aware of the work of Pyle et al. (1999 [this issue of *Neuron*]), who have used sulforhodamine 101 to quench extracellular FM1-43 and thereby extinguish extracellular signals.

FM dyes are unique in that they allow one to study the process of exocytosis in addition to endocytosis. However, the slowness of the washout limits the temporal precision of such investigations. The introduction of ADVASEP-7 greatly speeds the rate of washout and will allow more precise estimates to be made of the dynamics of vesicle recycling (T. A. Ryan, personal communication).

Because of ADVASEP-7's high affinity for FM1-43 and the high quantum yield of the complex, ADVASEP-7 can act as a detector of FM1-43. For example, it can be used to measure the concentration of FM1-43 released into the solution bathing tissue. It could also be used to generate a "positive" signal of synaptic activity rather than the customary destaining. If cells are loaded with FM1-43 and cleared, then provision of ADVASEP-7 in the extracellular medium during stimulation may lead to a transient increase of fluorescence during vesicular fusion.

The strategy introduced here of using natural or synthetic organic carrier molecules to shepherd fluorophores into or out of tissues might be of value with other

indicator molecules (e.g., acetoxy-methyl ester derivatives of ion probes), allowing the ingression or removal of probes that would otherwise get trapped in the interstices of the tissues. Organic chemists have developed an array of synthetic carrier molecules (Lehn, 1995; Rebek, 1999) that are excellent resources for such experiments, but because of artificial disciplinary boundaries have had little exposure in the field of neuroscience.

In semi-intact enteric ganglia, FM dyes have been used as markers of cell body activity by Kirchgessner et al. (1996). Our results with the activity-dependent staining of lamprey spinal cord and *Xenopus* tadpole tectum (K. Haas and H. T. C., unpublished data) suggest that our method may be of general value in imaging the spatiotemporal dynamics of synapses of other intact or semi-intact nervous systems. The 2-deoxy-glucose method has long been used to visualize neuronal activity on the basis of the accumulation of this nonmetabolizable glucose analog. However, it does not provide a distinction between the different cellular compartments of neurons. In contrast, FM1-43 offers the prospect of providing an image whose intensity is related to the extent of activation of the synapses.

Brain slices are an important preparation for the study of synaptic transmission and plasticity. The method introduced here extends the powerful methodology of FM1-43 to such semi-intact preparations. The method will allow investigators to address issues of endo- and exocytosis in fully differentiated central neurons, as opposed to cultured neurons, where the state of differentiation and neuronal identity are in question. In addition, FM1-43 may allow one to visualize the distribution of synaptic terminals resulting from the activation of a neuron or group of neurons ("synaptic field"). Measurement of the synaptic field may be of value in finding target postsynaptic neurons in electrophysiological experiments. Moreover, synaptic fields may be reshaped by bouts of neuronal activity—as in, for example, synaptic potentiation—and our method may be of value in rendering such shifts manifest.

Experimental Procedures

The abbreviations and compositions of the salines used in this paper are specified in Table 1. All experiments were carried out within the guidelines of the respective institutions. Results are expressed as mean ± SEM.

Spectrophotometry and Liposomes

Spectra were measured on a Hitachi F4500, with emission and excitation bandwidths set at 5 nm. The spectral characteristics of FM1-43 were measured in LS saline at 26°C.

Liposomes were prepared by ultrasonicating (Laboratory Systems) 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti) at a concentration of 10 mg/ml in LS saline and used at a concentration of 1 mg/ml (1.27 mM).

C. elegans

Nematodes (larval stages 1 and 2) from the Bristol strain N2 were used as the wild-type strain, and a transgenic *C. elegans* strain expressing an UNC-11::GFP fusion protein (Nonet, 1999) was cultured using standard procedures. Nematodes were embedded in 5% agar (Sigma) and cut into 100 μm thick slices (Vibratome). Slices were sandwiched between a glass slide and a cover slip. Solutions were changed by pipetting the solution in at one end and drawing off solution at the opposite end with a paper wick. Sliced nematodes were loaded with dye by incubating in CE-HiK saline in the presence of 5 μM FM1-43 or FM4-64. The wash procedure was as follows: wash for 1 min with CE-0Ca saline, followed by a 2 min wash with 1 mM ADVASEP-7 in CE-0Ca saline. For FM1-43 loading studies, nematodes were selected that were cut approximately in half, making the cut end 100–200 μM from the nerve ring.

Confocal images were collected on a Zeiss LSM510 microscope equipped with the LSM software. Images were taken such that the optical sections were in the longitudinal plane approximately midway through the nematode. The average fluorescence intensity was measured with NIH Image 1.61 software, and the experimental results were plotted as the difference between fluorescence at the nerve ring minus background acquired from the neighboring muscle.

Lamprey

Lamprey ammocetes (*Petromyzon marinus*) were anesthetized with tricaine methyl sulfonic acid, and sections of the spinal cord were removed. The tissue was maintained at 10°C in LP bubbled with 95% O_2/5% CO_2. The integument of the tissue, the mininx primitiva, was removed. For those experiments in which Ni^{2+} was added to the superfusate, LP-HEPES bubbled with 100% O_2 was used.

The spinal cord was placed ventral surface down in the recording chamber of the confocal microscope. A pipette (<1 μm tip) was filled with LP saline and 4 μM FM1-43. The tip of this pipette was then positioned in the tissue adjacent to the giant axons. FM1-43 was pressure ejected into the tissue while the axon was simultaneously stimulated either with an extracellular electrode or with an intracellular electrode. Immediately after stimulation was terminated, the pressure application of FM1-43 was stopped and ADVASEP-7 (1 mM) was added to the superfusate for 1 min, followed by a wash with LP saline, and this procedure was repeated twice.

Images were acquired with a confocal microscope (Biorad MRC600). Analysis of the imaging data was performed on a Macintosh computer using NIH Image software. NIH image was used to calculate the brightness value for each pixel in the field of view. For each individual punctum of interest, the brightness values were measured and background brightness (mode of the entire field of view) was subtracted. The data were then normalized to the baseline fluorescence to give ΔF/F values, where 100 is the baseline value.

Rat Brain Slices

Slices (200 μm thick) were cut (Campden microslicer) from the brainstem of 8- to 10-day-old Wistar rats in RA1 solution, transferred to a recording chamber, and perfused at room temperature (23°C–24°C) with RA1 saline. High-potassium saline, RA1-HiK, was used to load slices with FM1-43 and to stimulate them to destain.

The staining procedure was as follows: slices were exposed to high-potassium saline with 8 μM FM1-43 for 1.5 min, washed with normal saline for 2 min, perfused with 1 mM ADVASEP-7 for 1 min, washed with normal saline for 2 min, perfused with 1 mM ADVASEP-7 again for 1 min, and washed with normal saline for 20–30 min.

The optical recordings were performed on an upright epifluorescence microscope (Axioskop, Achroplan 40×, NA 0.75, Zeiss) equipped with a monochromator (TILL Photonics), a dichroic mirror (510 nm), a long-pass (520 nm) emission filter, and a CCD camera (CH250, Photometrics LTD). Excitation light (470/10 nm) was coupled to the microscope via a light guide. Images were analyzed using the NIH image software.

Transverse slices (400 μm) were cut (Vibratome) from the hippocampi of male Long-Evans rats (4–6 weeks old) in RA2 solution. Extracellular field potentials were recorded in stratum radiatum of CA1 with glass electrodes using an Axoclamp 2A amplifier. Bipolar electrodes were used to stimulate the Schaffer collaterals, and the stimulus strength was adjusted to give approximately half-maximal field EPSP (fEPSP) amplitude. The slope of the fEPSP was calculated between 20% and 80% of the maximal amplitude.

Chemicals

The following chemicals were used in the experiments: FM1-43, FM2-10, and FM4-64 (Molecular Probes); ADVASEP-7 (CyDex, Overland Park, KS); β-cyclodextrin and γ-cyclodextrin (Sigma), and hydroxybutyl cyclodextrin (Tocris).

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