

Current Protocols in Neuroscience

Loading neurons with dextran conjugated calcium indicators in intact nervous tissue

Kerry R. Delaney
Dept. of Biological Sciences
Simon Fraser University
8888 University Dr., Burnaby
B.C. Canada V5A 1S6

604 291 5619 (voice)
604 291 3496 (fax)
kdelaney@sfu.ca

Keywords: fluorescence imaging, *in vivo* staining, presynaptic terminals, dendrites

Mini Abstract:

Methods are described for filling populations of neurons and their processes, including presynaptic terminals, with dextran conjugated calcium indicators in central nervous tissue of mammals and lower vertebrates. Techniques for filling neurons *in vivo* for subsequent analysis either *in vivo* or in brain slices or *en bloc* preparations are described. These methods are also suitable for staining neurons in acute and organotypic brain slices.

I. Introduction

Dextran conjugated versions of Ca^{2+} indicators were developed to overcome several problems associated with the use of acetoxymethylester or free acid forms. Binding to intracellular proteins can significantly alter the dissociation constant of Ca^{2+} indicators in some cell types, particularly muscle cells. Some cell types, particularly plant cells (Reid *et al.*, 1992) and liver cells rapidly remove free acid forms of Ca^{2+} indicators such as fura-2 or fluo-3 from the cytosol into organelles or to the extracellular space. In neurons sequestration into organelles can result in accumulation of dye that is unresponsive to cytosolic Ca^{2+} changes which is particularly problematic for studies requiring quantitative measurements of fractional fluorescence changes over time. The conjugation of Ca^{2+} indicators to biologically inert dextran molecules of 3000 or 10,000 MW results in an indicator that is not pumped out of cells, does not become sequestered and shows little or no binding to proteins. The superior retention of dextran conjugates in cells permits Ca^{2+} changes to be monitored over periods of many days. Because dextran conjugates are efficiently retained in neurons it is possible to fill populations of neurons by application to the soma/dendrites and subsequently image presynaptic terminals many millimeters away. The main impediment to the use of dextran conjugated Ca^{2+} indicators to monitor Ca^{2+} influx is loading them into cells. However, for reasons which are presently not well understood (see commentary below) dextran conjugates often load into neurons in intact tissue without serious damage when applied locally at high concentration.

Techniques are described for selective loading of groups of neurons by local extracellular application of dextran conjugated Ca^{2+} indicators with particular emphasis on presynaptic terminals. Methods for loading neurons *in vivo* for subsequent quantitative analysis of Ca^{2+} influx *in vitro* or *in vivo* are presented, as well as methods to load groups of neurons and their axonal projections in acute or organotypic brain slices.

II. Protocols

Materials:

Dextran conjugated Ca^{2+} indicator

Glass micropipettes, pulled to a fine point

Microscope slide

Bovine serum albumin (BSA)

Polyvinyl alcohol

Basic Protocol

This protocol is adapted from Gelperin and Flores, 1997, Bohlen-Halbach and Albrecht, 1998, Delaney et al., 2001, Mulligan et al., 2001. It is suitable for loading neuronal structures within a few hundred microns of the surface of a brain, brain slice or

Dextran conjugated calcium indicators in intact nervous tissue

invertebrate ganglion with subsequent intracellular transport of dye to distal structures for imaging. By adjusting the diameter of the glass pipette tip and the extent to which it is coated with dye it is possible to easily restrict dye application to areas as small as 50 μm diameter. It is particularly useful for organotypic slices where localization of dye application with microperfusion or micropressure ejection is difficult.

1. Prepare several sharp glass micropipettes. Pipettes that would be suitable for intracellular recording from neurons with an overall taper length of about 1 cm will work well. Glass micropipettes can be pulled from conventional 1.0 to 1.5 mm electrophysiological pipette glass or other narrow glass tubing or rod using a micropipette puller, or with a little practice, by hand on a small flame.

Final tip diameters on the order of one to a few μm are desired. The specific shape of the pipette near the tip should be adapted to the tissue and amount of dye that is to be applied. If the pipette is too flexible it will not penetrate the tissue well. If it is too thick it will damage the tissue and deposit more dye than desired.

2. Dissolve approximately 0.5 mg dextran conjugated Ca^{2+} indicator into 1 μL of 1.5% BSA and place this droplet onto a glass microscope slide. Allow the water to evaporate to form a flat “crystal” of dye.

Dextran conjugated calcium indicators in intact nervous tissue

To prepare submilligram quantities of dextran conjugated dyes it is convenient to dissolve several milligrams of the conjugate (or the entire 5 mg jar) in 20 μ L of distilled water, then aliquot this into 10-20 400 μ L plastic microcentrifuge vials.

Place the tubes uncovered in the dark for approximately 30 mins to evaporate the water to yield 0.25-0.5 mg/ vial. Aliquots can be stored at -20° C for many months.

3. Place a 1-2 μ L drop of distilled water at the edge of the dried dye on the glass slide.

The dye will begin to dissolve into the droplet providing an interface at the edge of the droplet where the dye is highly viscous. The BSA helps to increase the viscosity of the dye solution to facilitate coating of the micropipettes.

4. Using a stereomicroscope at a magnification of about 40X hold the pipette at shallow angle to the slide surface and dip the tip into the viscous dye solution to coat its surface with a thin film of dye. Air dry for a few minutes.

Hold the pipette at about 45° to the surface of the slide and rotate it slightly while withdrawing it from the dye solution. Dye should coat the pipette to the tip but not hang off the end. If the dye is too viscous it will tend to extend from the tip when the

Dextran conjugated calcium indicators in intact nervous tissue

pipette is removed forming a long “hair” of dye. If this happens reinsert the pipette into a slightly less viscous region of the dye crystal-water droplet interface. It is important to not break the tip of the pipette to avoid sucking dye solution into the tube by capillary action. Although illumination with normal room light for many minutes will not cause significant dye-fading a green filter in front of the microscope illuminator is recommended. Unused dye can be left on the microslide and stored for months in the freezer.

5. Dry the surface of the brain or slice by wicking away excess physiological ringer or extracellular fluids with a fragment of twisted paper tissue. Insert the micropipette tip into the tissue using a rapid motion. Hold in place until the dye dissolves off the pipette. With a thin coating this will take a few seconds. Depending on the size of the pipette and the amount of dye deposited areas containing somata and neuronal processes within 50-200 microns will be directly filled. Insert more coated pipettes with a spacing of a few hundred micrometers to fill neurons over larger areas. Rinse excess dye from the surface of the tissue.

While some filling of presynaptic terminals is be obtained by application to axonal tracts more efficient staining results from application to dendrites and somata with subsequent transport to the terminals. Insertion of the pipettes with a steady hand is

Dextran conjugated calcium indicators in intact nervous tissue

possible or use a micromanipulator with a coarse drive advancing the pipette into the tissue rapidly (< 1 sec) so that the dye doesn't dissolve only on the surface.

6. Retrograde and anterograde transport will fill dendrites as well as axons and presynaptic terminals with indicator. Allow 1-3 or more days for uptake and transport *in vivo* for mammals or lower vertebrates. With *in vivo* transport presynaptic terminals 2-3 millimeters from the site of dye application will fill thoroughly in as little as 1.5 days. Perfusion with physiological ringers *in vitro* for 3-6 hours after dye application is sufficient to fill dendritic processes in acute or organotypic brain slices or isolated turtle and frog brain located 500 to 1000 μm from the dye application.

Alternative Protocol I

Application of dye by dissolving it off the surface of dye-coated pipettes inserted into nervous tissue achieves highly localized application of concentrated dye solution very simply. The thin diameter of the pipettes that can be used reduces tissue damage and provides for application of very small amounts of dye. With this technique it is difficult to apply dye to structures more than 0.5 mm deep since the dye dissolves off the pipette while it is being inserted. An alternative strategy is to inject concentrated dye solutions into tissue or to mix the dye with a slowly dissolving polyvinyl alcohol.

Dextran conjugated calcium indicators in intact nervous tissue

The following is based on techniques described by Kreitzer et al., 2000 for rodent brain and Mulligan et al., 2001 for frog brain.

Solutions for injection:

- 1 Final concentration of injected dextran conjugated indicator should be 10-20% w/v in distilled water. For Fluo-4 dextran resting fluorescence intensity is usually very low so co-injection of Texas red dextran conjugate is advised to aid in locating dye filled structures. This can be achieved by a 1:1 dilution of a 20-25% Fluo-4 dextran solution with a similar solution of Texas Red dextran.

Coapplication of a fixable lysine derivative of Texas Red also allows histological preparation after physiological measurements are completed.

- 2 Pull fine-tipped micropipettes and then break the tip to a diameter of 30-50 μm . If an oil filled microsyringe (e.g. Hamilton model 7100) or microinjector (e.g. Nanoject II, Drummond Scientific, Broomall PA) is used to eject the dye then fill the body of the pipette with mineral oil and then tip-fill the pipette with 0.5 μL of dye by applying negative pressure to the back of the pipette.

Dextran conjugated calcium indicators in intact nervous tissue

- 3 Insert the tip of the pipette in the desired location in the tissue using a micromanipulator. Eject a volume of 0.1-0.3 μL over the course of 5-20 minutes with a series of small ejections of 20-30 nL each. Leave the pipette in place for a few more minutes to reduce dye leakage from the micropipette track.

Retracting the pipette 50 μm prior to starting ejection can help reduce clogging of the pipette tip with tissue. A simple manual technique for delivering ultra small injection volumes is described by Kreitzer et al., 2000.

- 4 Allow 1-5 days for recovery before preparing slices (mammalian CNS) or *en bloc* brain tissue (amphibians, reptiles) for *in vitro* or *in vivo* measurements.

Alternative Protocol II

Polyvinyl alcohol can be used to slow the rate of dissolution of the dye from the surface of a coated pipette allowing a crystal of dye to be inserted deep into tissue.

- 1 Prepare a crystal of dye as described in step 2 of Basic Protocol 1 substituting a 5% solution of polyvinyl alcohol (Sigma) for 1.5% BSA.

Dextran conjugated calcium indicators in intact nervous tissue

- 2 Coat the pipette tip by inserting it into the dye solution, withdrawing it and allowing the solution on the pipette to harden. Repeatedly insert the pipette into the dye solution drying it for a minute or two between insertions if a thicker coating is desired.
- 3 Insert the pipette into the tissue to the desired depth and hold in place for a few minutes until the dye coating dissolves off the surface of the pipette.

To determine the time needed for dye to dissolve place a test pipette in a droplet of water and observe with a stereomicroscope

Commentary

Background:

Although direct physical cellular damage increases uptake, significant loading of neurons can occur without cell death or severing of processes. Evidence that loading does not require irreversible traumatic injury includes the fact that filled terminals are present even after survival times of many days when neurodegeneration would be expected if somata were killed during loading. Furthermore, we are routinely able to elicit Ca^{2+} transients in

dendrites and nerve terminals of neurons filled 5 days previously by stimulating afferents that are presynaptic to the filled cells indicating that the somata and dendrites of filled neurons are functional (Figure 1). The protocols presented above do not include modifications that might increase loading such as addition of detergents or local acidification. The use of detergents *in vivo* brain tissue is probably not desirable although for loading cut stumps of nerves external to the brain it significantly improves uptake into myelinated axons (O'Donovan et al., 1993). A 10% w/v of 10,000 MW dextran conjugate in distilled water is hyposmotic to vertebrate extracellular fluid (about 10 mOsM) but we have not noticed significant differences in loading efficiency when dye is applied dissolved in isotonic saline. We have not tried injecting dye dissolved in hyperosmotic solutions but to some degree this is the situation when crystalized dye is allowed to dissolve from the surface of a pipette into the extracellular fluid.

Critical parameters

Unfortunately the selection of dextran conjugated Ca^{2+} indicators is limited compared to the free acid derivatives. Dextran conjugates of Calcium Green-1, Oregon Green-1 488 BAPTA, Fura-2, Indo-1 and Fluo-4 are presently available from Molecular Probes (Eugene, OR) in 5 mg packages. Calcium Green is available as conjugates to 3000, 10,000, 70,000 and 500,000 MW dextran. Oregon Green is available conjugated to

Dextran conjugated calcium indicators in intact nervous tissue

10,000 and 70,000 MW dextran. Fura-2, Indo-1 and Fluo-4 are available conjugated to 10,000 MW dextran. 10,000 MW conjugates are efficiently transported and retained intracellularly for many days (at least 7 days in frog brain) so the use of larger conjugates is not required. Conjugation of the indicator to the dextran molecule has so far resulted in decreased Ca^{2+} affinity compared to the free acid forms of the dye. Ca^{2+} affinities vary significantly between production lots and are indicated on the package. The decrease in affinity upon conjugation is typically about a factor of 2, except for Fluo-4 dextran which has an affinity in the range of 2-4 μM in 100 mM KCl compared to approximately 350 nM for the free acid form. The presently available dyes all emit in the blue or green part of the spectrum. A red emitting dye excited in the green or yellow part of the spectrum would be very useful for reducing tissue autofluorescence. Calcium Crimson dextran has been discontinued but may be available by custom synthesis through Molecular Probes.

Fluo-4 dextran partially fulfills the need for a low affinity indicator that has fast reverse binding kinetics that are required to follow rapidly recovering transients in small structures like terminals and sensitivity to higher $[\text{Ca}^{2+}]$ that is required to discriminate individual transients during repetitive firing when $[\text{Ca}^{2+}]$ builds up (Kreitzer et al., 2000). However, this comes at the cost of lower resting fluorescence levels and small fractional fluorescence changes for $[\text{Ca}^{2+}]$ changes on the order of 100 nM. The low resting fluorescence can present problems for finding filled structures and for quantitative

estimates of fractional changes in fluorescence changes since background tissue fluorescence can contribute more than half the total fluorescence from regions containing filled terminals or dendrites when conventional wide-field microscopy is used (Mulligan et al., 2001). Co-application of Texas Red dextran can solve the problem of finding filled structures.

C. Trouble shooting

A shortcoming of the described techniques is that it is difficult to control the amount of dye that is loaded into the neurons. With pressure injection of dye solutions some control is possible by varying the concentration of dye in solution and the total amount of dye solution introduced into the tissue. With dye crystals on micropipettes more staining is achieved if multiple closely spaced applications are made using more than one coated micropipette. As with any technique involving fluorescent Ca^{2+} indicators, the experimenter needs to determine whether the level of loading is sufficient to visualize the $[\text{Ca}^{2+}]$ changes of interest without overly altering the Ca^{2+} handling properties of the filled structures (e.g. for example reducing the peak of transients and slowing recovery to resting $[\text{Ca}^{2+}]$ if these are the variables of interest). In this regard, knowing how to recognize overbuffering, understanding when it is likely to be a problem and when it can be tolerated in any particular system, are essential. Fluo-4 dextran may be an advantage

in some instances because its lower Ca^{2+} affinity means it is less likely to disturb normal Ca^{2+} homeostasis.

Numerous factors need to be considered in order to perform quantitative or semi-quantitative measurements of Ca^{2+} changes associated with neuronal activity using fluorescent dyes. This discussion will focus mainly on problems associated with measuring fluorescence changes in blocks of tissue (slice or whole brain) since general issues pertaining to dye calibration, background subtraction, phototoxicity etc. have been covered in many other publications (reference).

If fluorescence from populations of filled presynaptic terminals is measured then standard methods for equating fractional fluorescence changes with single wavelength excitation (F/F_0 ; Fluo, Ca^{2+} -Green, Oregon Green) or ratiometric (Fura-2 and Indo-1) are usually inappropriate since the change in fluorescence is proportional to the product of the fraction of terminals stimulated and the Ca^{2+} influx into each stimulated terminal. Consequently unless all the filled structures are excited the fractional fluorescent change observed will underestimate the change produced by Ca^{2+} influx into stimulated terminals. Feller et al., 1996 developed a strategy to deal with this difficulty under conditions where loading by bath application of acetomethylester derivatives resulted in significant loading of non-target structures (e.g. glia and terminals other than those of

interest). This method estimates the change in $[Ca^{2+}]$ in presynaptic terminals due to an action potential by calculation of the ratio of the fluorescence changes resulting from a pair of closely spaced stimuli (Figure 2). This method uses the fact that partial saturation of high affinity indicators such as Ca^{2+} Green or Fura-2 occurs with the Ca^{2+} influx that results from one action potential. This approach is particularly useful because even though it is based on a single wavelength measurement it is relatively insensitive to background fluorescence, the number of stimulated terminals or dye fading so it is unaffected by differences in position of the stimulating electrode or the proportion of filled terminals between experiments or changes in excitability of afferent fibers during an experiment. An underlying assumption of the Feller et al., 1996 methodology is that the concentration change evoked by the second of a pair of stimuli is the same as the first, i.e. that the reason the fractional fluorescence change evoked by the second of a closely spaced pair of stimuli is smaller is because influx from the first stimulus partially saturates the indicator. This assumption can be checked, and if it does not hold then corrected for, by observing the fractional fluorescence changes that result from a pair of stimuli applied to terminals filled with a low affinity indicator that has little or no attenuation of the second of a pair of stimuli due to saturation (Figure 2). Fluo-4 dextran is the only dextran conjugated indicator presently available with a low affinity ($3.5 \mu M$) and an example of its use as a control for changes in Ca^{2+} influx can be found in Mulligan et al., 2001.

With widefield fluorescence imaging of dye-filled structures in intact tissue such as invertebrate ganglia, vertebrate brain slice, isolated brains *in vitro* or intact brain *in vivo*, tissue autofluorescence can be a significant problem. Since the dextran conjugated Ca^{2+} indicators presently available are excited by blue or UV light, green or blue-green autofluorescence from metabolites including FAD/FADH and NAD/NADH is prominent. It is common to see an increase in autofluorescence from *in vitro* brain slices over time. Tissue autofluorescence is both activity dependent and subject to fading that can lead to background fluorescence artifacts. This requires that updated background fluorescence measurements must be made if this is being subtracted prior to calculation of fractional fluorescence changes. We find that autofluorescence is more easily faded than the Ca^{2+} sensitive fluorophores we use, even when exciting at the absorption maximum of the dye. This causes a reduction in total fluorescence during illumination that recovers when illumination is stopped as faded metabolites are replaced by fluorescent molecules. With Oregon Green-1 filled neurons resting dye fluorescence in terminals or dendrites is usually several times greater than tissue autofluorescence so the non-stationary background fluorescence is a small problem but with Fluo-4 tissue autofluorescence can be equal to or greater than dye fluorescence. Pre-fading the tissue fluorescence by exposing it for several seconds to light that does not efficiently excite the Ca^{2+} indicator dye prior to measuring dye fluorescence can be helpful since this both reduces

background fluorescence and stabilizes it at a lower level where the rate of fading equals the rate of regeneration of fluorescent metabolites. 410-440 nm light can be used to fade autofluorescence when imaging either the 480 nm excited dyes or Fura-2 (340/380 nm excitation) provided an appropriate dichroic filter is in place. Standard dichroic mirrors for the 480 nm excited dyes will work in this range but standard Fura-2 dichroic mirrors typically pass beyond 400 nm so are not suitable. Finally bath application of drugs, e.g. glutamate agonists can have large effects on tissue autofluorescence that may require adjusting background subtraction values throughout the experiment.

When working with thick tissue the importance of limiting the illuminated area with a field aperture in the excitation path cannot be overemphasized when attempting to image dye-filled structures in intact neural tissue using wide-field microscopy. The problem of autofluorescence, as well as out of focus fluorescence from filled structures that are not of interest, can be greatly reduced by closing the aperture down to illuminate a small portion of the field of view. Likewise, when trying to find small filled structures the increase in contrast that results when the illumination area is restricted can be crucial to success. Ideally an adjustable rectangular slit should be used if linear structures like dendrites are of interest (Applied Scientific Instruments, Eugene OR). Some fluorescence microscopes are delivered so about $1/3^{\text{rd}}$ of the field of view is illuminated when the aperture is in the fully closed position but the aperture can be modified to close

down to 1/10th or 1/8th of full aperture by a trained technician or a patient, adventurous researcher.

D. Anticipated Results:

Figure 1 shows images and activity dependent fluorescence changes from frog olfactory mitral cells filled with Ca²⁺-Green-1 or Oregon Green-1 BAPTA 488 dextran conjugates.

E. Timing:

- Preparation of dried dye aliquots, 1hr
- Preparation of dye coated pipettes, 10 minutes for 5 pipettes
- Loading and transport times, vary from a few hours to several days. Because the dextran
- conjugates are efficiently retained in the neurons after loading, the length of time that elapses between loading and observation is generally not critical and we routinely wait 3-7 days before imaging.

References

Read, ND, Allan, WGT, Knight, MR, Mahlo, R, Russell, A, Shacklock,, P and Trewavas, A.J. 1992 Imaging and measurement of cytosolic calcium in plant and fungal cells. *J. Microscop.*, 166, 57-86.

Delaney KR, Davison I & Denk, W. 2001 Odour-evoked $[Ca^{2+}]$ transients in mitral cell dendrites of frog olfactory glomeruli. *Eur. J. Neurosci. in revision*

Feller MB, Delaney KR & Tank DW 1996 Presynaptic calcium dynamics at the frog retino-tectal synapse *J. Neurophysiol.* 76(1) 381-400

Gelperin A & Flores J 1997 Vital staining from dye-coated microprobes identifies new olfactory interneurons for optical & electrical recording. *J. Neurosci. Meth.* 72, 97-108.

Kreitzer AC, Gee KR, Archer EA, Regehr WG 2000 Monitoring presynaptic calcium dynamics in projection fibers by *in vivo* loading of a novel calcium indicator. *Neuron* 27(1) 25-32

Mulligan SJ & Delaney KR 2001 Mitral cell presynaptic Ca^{2+} influx and synaptic transmission in frog amygdala. *Neurosci. in press*

Dextran conjugated calcium indicators in intact nervous tissue

O'Donovan MJ, Ho S, Sholomenko G, Yee W 1993 Real-time imaging of neurons retrogradely and anterogradely labeled with calcium-sensitive dyes. J Neurosci. Meth. 46(2):91-106

von Bohlen und Halbach O, Albrecht D 1998 Tracing of axonal connectivities in a combined slice preparation of rat brains--a study by rhodamine-dextran-amine-application in the lateral nucleus of the amygdala. J Neurosci. Meth. 81(1-2) 169-75.

Figure 1

A. Dendrites of frog (*Rana pipiens*) olfactory bulb mitral cells filled with Ca^{2+} -Green-1 dextran conjugate by local application of dye dried onto a micropipette to the secondary dendrites. Dendrites were imaged 2-3 hours after application of the dye to an intact olfactory bulb maintained *in vitro*. Panel *i* is a widefield CCD image focusing on the glomerular layer showing many dendritic tufts in different glomeruli. The left edge of the image is 700 μm from the dye application site. Panel *ii* is a CCD image of one glomerulus with more than one apical dendrite projecting to it. Panel *iii* is a maximum intensity projection of 20 optical sections obtained at 1 μm intervals using a 2-photon laser scanning microscope. Scale bar in panel *i* corresponds to 50 μm for panel *i*, 20 μm for panel *ii* and 40 μm for *iii*. B. Physiological responses recorded from an olfactory bulb stained as described for A. The upper pair of overlapped traces are fluorescence changes measured in dendrites of a single glomerulus while the lower vertically offset traces are the corresponding local field potential responses. Thick traces are responses evoked by a brief puff of odorant applied to the nose in an isolated nose brain preparation maintained *in vitro*. Note the odor evoked oscillations in the local field potential and the large long-lasting elevation of $[\text{Ca}^{2+}]$ in the dendrites. Thin traces are responses evoked by a single electrical stimulus of the olfactory nerve for the same tuft and local field potential recording site. Odor and nerve stimulation responses have been aligned along the

time axis so that the onset of the local field potentials are coincident and the local field potential traces are offset vertically for comparison purposes. Scale bars 30% F/F, 400 μ V and 0.5 seconds. C. Neurons and presynaptic terminals stained by injection of Oregon Green-1 BAPTA 488 dextran conjugate into frog the accessory olfactory bulb (AOB). Panel *i* is a CCD image of fluorescence from the AOB located in the lower left, part of the image, the diagonally projecting accessory olfactory tract containing the axons of mitral cells and the mitral cell presynaptic terminals in the amygdala, upper right. A stimulating electrode is positioned on the AO tract. Panel *ii* shows mitral cell somata 150 μ m below the surface of a live AOB imaged using 2-photon laser scanning microscopy presented as a maximum intensity projection of 15 optical sections obtained at 1 μ m intervals. Panel *iii* is a maximum intensity projection of 3 confocal images at intervals of 3 μ m each. Scale bars are 500 μ m for *i*, 25 μ m for *ii* and 20 μ m for *iii*.

Figure 2

Fluorescence changes in presynaptic terminals of mitral cells in frog amygdala resulting from electrical stimulation of the axonal tract. The high affinity indicator Oregon Green-1 (K_d for this dye-lot approximately 250 nM) shows significant attenuation of the fluorescence change resulting from the second of a pair of closely spaced action potentials. In the same terminals in another preparation, the

Dextran conjugated calcium indicators in intact nervous tissue

fluorescence change of Fluo-4 dextran conjugate with lower affinity (K_d approximately 3.5 μM) shows virtually no attenuation to the second of a pair of action potentials indicating the attenuation seen with Oregon Green is the result of partial saturation of the high affinity indicator. The saturation can be used to obtain an estimate of the change in calcium concentration resulting from a single action potential using the method of Feller *et al.*, 1996. $[\text{Ca}] = ([\text{Ca}]_{\text{rest}} + K_d)(1 - \gamma)/2$ where γ is the ratio of the fluorescence transient for two closely spaced Ca influxes of equal magnitude. Strictly speaking the second Ca^{2+} influx should occur prior to any recovery of the $[\text{Ca}^{2+}]$ but in practice some decay is inevitable leading to a slight underestimation of γ and thus Ca^{2+} . Assuming a resting $[\text{Ca}]$ of 50 nM and a K_d of 250 nM the above example with $\gamma = 0.57$ corresponds to $[\text{Ca}]$ of 113 nM.

Dextran conjugated calcium indicators in intact nervous tissue

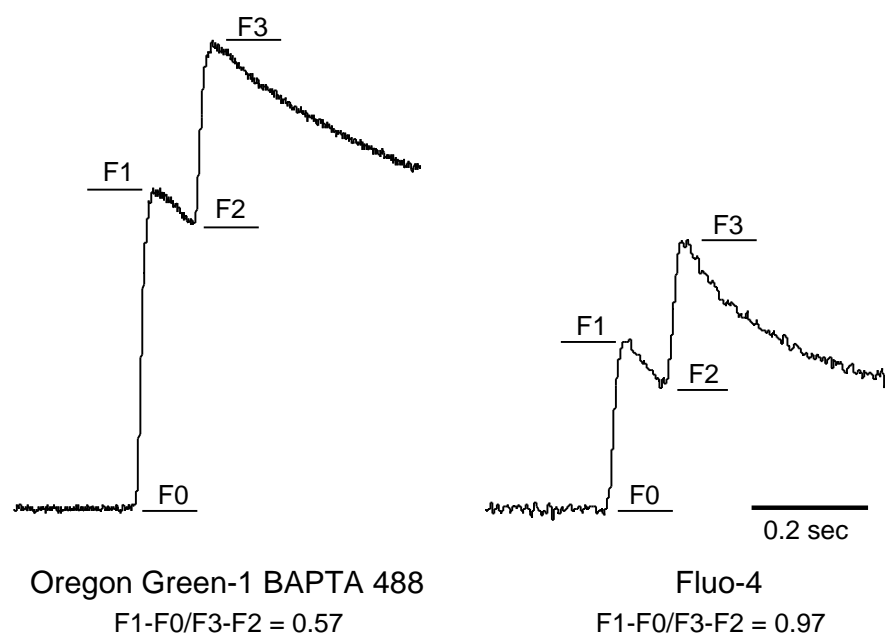


Figure 1

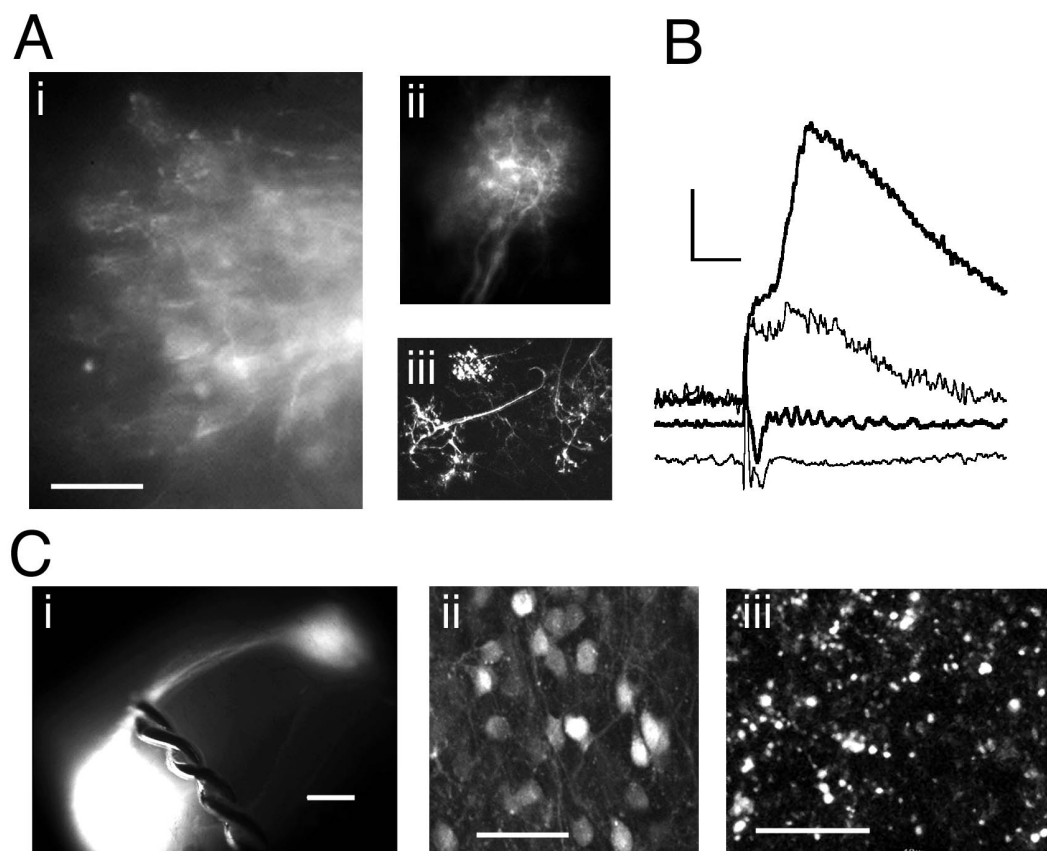


Figure 2