

Imaging calcium dynamics in the nervous system by means of ballistic delivery of indicators

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Abstract

The use of fluorescence-based calcium indicators has, over the years, unraveled important calcium-dependent mechanisms underlying neuronal function and development. However, difficulties associated with the loading of calcium indicators have limited their widespread use, particularly for the study of neuronal processing in the adult nervous system. Here, we show that in the central and peripheral nervous systems, populations of neurons and their processes, including dendritic spines and filopodia, can be labeled rapidly and efficiently by delivering calcium indicator-coated particles using a 'gene gun'. Importantly, neuronal labeling occurred both *in vitro* and *in vivo*, and across a wide range of ages and preparations. The labeled cells demonstrate spontaneous and evoked calcium transients, indicating that particle-mediated delivery is not deleterious to neuronal function. Furthermore, unlike loading with patch pipettes, cytoplasmic content is preserved following ballistic loading. This enables the study of calcium-dependent second messenger pathways without loss of signaling components. The ballistic delivery of calcium indicators thus opens up many new avenues for further exploration of the structure and function of the nervous system from single spines to neuronal networks. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Changes in intracellular calcium levels are essential to the development and function of neurons (Berridge et al., 2000). The development of fluorescent calcium indicators has provided a powerful approach for monitoring these calcium fluctuations in both single cells and in populations of cells (Tsien, 1989). However, calcium imaging has been restricted to preparations where loading with the indicator is straightforward with existing methods.

To date, the two primary techniques for loading calcium indicators into cells are incubation with mem-

brane-permeant forms of the indicators, acetoxymethyl (AM)-esters (Takahashi et al., 1999; Tsien, 1981; Regehr and Tank, 1991; Yuste, 1999), and injection of indicators with micropipettes (Takahashi et al., 1999; Neher and Almers, 1986; Eilers and Konnerth, 1999). AM-ester loading is often used to simultaneously label populations of cells, and although efficient in immature tissue or cells in culture, this method is rarely effective in adult preparations. In mature neural tissue, the dense neuropil and numerous glial cells presumably prevent the diffusion of calcium indicators into neurons (Yuste and Katz, 1991; Yuste, 1999). Furthermore, even at ages when AM loading is effective, the high density of labeling prevents the easy discrimination of individual neuronal processes; this makes the study of calcium dynamics in dendrites and axons challenging. Imaging calcium transients in dendrites has therefore typically required loading of indicators into single cells by

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microinjection using intracellular or patch pipettes. However, these approaches are technically demanding and time consuming, restricting the number of cells that can be labeled at any one time (Eilers and Konnerth, 1999). Microinjection is particularly difficult for loading cells *in vivo* where tissue access and movement are problematic. Additionally, loading dyes via patch pipettes dialyses cellular contents including molecules essential to the function of second messenger pathways (Takahashi et al., 1999).

Green Fluorescent Protein-based calcium indicators, such as cameleons (Miyawaki et al., 1997) and pericams (Nagai et al., 2001) that are synthesized by cells, circumvent the problems associated with loading. However, these genetically encoded indicators are generally pH sensitive and show smaller calcium-dependent responses than most synthetic fluorescent indicators. Furthermore, labor-intensive methods are required to stably express the indicators in the cells of interest, and not all neuronal preparations, such as acute or mature brain slices, are suitable for transient gene transfection.

Here, we report a ballistic technique that enables loading of fluorescent calcium indicators into neurons and their processes, in a variety of preparations at different ages. This approach allows imaging of calcium dynamics *in vitro* and *in vivo*, at levels ranging from individual processes to populations of cells.

2. Methods

2.1. Coating particles with calcium indicators

To coat the particles, a small amount (25–50 mg) of tungsten particles (1.3 or 1.7 μm diameter, Bio-Rad) was spread onto a clean glass slide. Between 1 and 2 mg of calcium indicator (Calcium Green-1, Oregon Green 488 BAPTA-1, or Fura conjugated to 10,000 MW dextran; Molecular Probes, Eugene, OR) was dissolved in 12–25 μl of distilled water and thoroughly mixed with the particles on the glass slide. The indicator-coated particles were spread uniformly across the glass slide, air-dried, and then scraped off onto a piece of weighing paper. The particles were then poured into a 15 cm long piece of plastic tubing (Bio-Rad, Hercules, CA, #165-2441) that had its interior pre-coated with polyvinylpyrrolidone (Bio-Rad, #165-2440, 10 mg/ml in distilled water). The tubing was shaken gently for several minutes until the particles stuck onto the inner wall. The particle-coated tube was then cut into 13 mm lengths and stored in the dark at 4 °C in a container with desiccant.

An estimate of the concentration of indicator was calculated as follows. Tungsten particles (density = 19.3 g/cm^3) were assumed to be spherical with a diameter of 1.3 μm . Assuming that 50 mg of tungsten particles were

uniformly coated with 2 mg of 10,000 MW dextran-conjugated indicator, each particle would carry 8.9×10^{-17} mol of indicator. For spherical somata with 20 μm diameters and cell volumes that are twice the somatic volume, the concentration of fluorescent indicators in a cell labeled by a single particle would be $\sim 10 \mu\text{M}$.

2.2. Delivery of dye-coated particles

Indicator delivery to the tissue was accomplished using a 'gene gun' (Bio-Rad, Helios Gene Gun System, # 165-2431), essentially using the same protocol described for the 'DiOlistic' approach for delivery of carbocyanine dyes (Gan et al., 2000). The particle-filled tubes were inserted into the cartridge of the 'gene gun' which was held 1–2 cm above the exposed tissue. Brain slices and retinal preparations were placed on nitrocellulose filter papers (Millipore, Bedford, MA, #9004-70-0), transferred to 35 mm culture dishes (Corning, Acton, MA), and superfused with artificial cerebrospinal fluid (ACSF) or Ringer's solution prior to labeling. Solution on top of the tissue was removed just prior to the time of labeling. Dye-coated particles were propelled into the tissue at gas pressures of 80–200 psi. To protect the tissue from clusters of particles and the shock wave that accompanies each shot of the gene gun, an isopore membrane filter (3 μm pore size and 8.0×10^5 pores/ cm^2 density; Millipore, #TSTP04700) was placed between the gun and the preparation. Immediately after shooting, the tissue was washed in Ringer's or ACSF to minimize background labeling due to particles that had landed in the extracellular space.

2.3. Tissue preparation

Tissue was obtained from a variety of species at a range of ages. Rats and mice of ages ranging from 12 days (P12) to 6 months after birth were studied; we considered mice and rats to be mature by 2 months of age, upon sexual maturity. Chick tissue was obtained prior to hatching, at embryonic day 13 (E13) or embryonic day 14 (E14).

Mature mice (Harlan/Sprague–Dawley) and rats (Wistar) were euthanized (> 60 mg/kg sodium pentobarbital) prior to decapitation. Mouse brains were quickly removed and put in cooled, oxygenated ACSF containing (in mM) NaCl (119), KCl (2.5), MgCl_2 (1.3), CaCl_2 (2.5), NaH_2PO_4 (1), glucose (11), and HEPES (20). Transverse sections of the hippocampus were obtained using a vibratome (TPI Inc., St. Louis, MO, Vibratome 1000). Coronal sections of rat brain were obtained by sectioning in chilled HBSS (pH 7.2, Gibco, #14060-057) containing 1 mM kynurenate using a Vibroslicer (EMS, Fort Washington, PA). To prepare organotypic cultures, the slices were then placed on a

cell culture insert (Millipore, Millicell-CM), and cultured in 1 ml of DMEM (Gibco, Carlsbad, CA, #1300-021) supplemented with B27 (Gibco, #17504-010) and 1.2 g/l NaHCO₃ at 37 °C in 5% CO₂/95% air. Explants were in culture 48 h before ballistic indicator loading.

Retinal wholemounts from E13 chick and 2–3-week-old mice were prepared as previously described (Lukasiewicz and Wong, 1997) and maintained in Chick Ringer's composed of (in mM): NaCl (125), KCl (5), MgCl₂ (2), CaCl₂ (2), NaH₂PO₄ (1.25), glucose (20), HEPES (23) or mouse ACSF (as above).

For *in vivo* labeling of submandibular and superior cervical ganglia, 4-month-old NSA mice were anesthetized with a single intraperitoneal injection containing ketamine and xylazine (Gan et al., 1999). The anesthetized mice were put on a metal plate and a midline incision in the skin from the apex of the mandible to the sternal notch was made (Gan et al., 1999). The submandibular and sublingual salivary glands were retracted to expose the ganglia that were distributed over the submandibular ducts (Purves et al., 1987). The superior cervical ganglia were exposed as described previously (Purves et al., 1986).

For labeling of cortical neurons in living animals, 4-month-old NSA mice were anesthetized with ketamine and xylazine. A craniotomy was performed to expose a small window (approximately 3 mm in diameter) over the parietal cortex with a small dental drill and the dura in this region was removed with a pair of sharp forceps. Thirty minutes after ballistic delivery of indicators through the small window, the animal was euthanized with an overdose of sodium pentobarbital (> 60 mg/kg), and cells loaded with indicators were imaged.

2.4. Imaging of preparations

Images were obtained using a Bio-Rad confocal microscope (MRC-1024 or Radiance 2000, 488 nm excitation line, 522±35 nm barrier filter) or with a cooled CCD (Wong, 1999a; Wong et al., 2000). Retinal and slice preparations were imaged in a superfusion chamber within minutes of dye loading. Long-working distance water-immersion objectives (Olympus, Melville, NY; 20×, 40× and 60×) were used. Stacks of confocal images at 0.6–1.0 μm steps were acquired for three-dimensional reconstruction of neurons. Figures were generated by projecting the stack of images onto a single plane (maximum projection). Laser intensity was kept to a minimum to reduce bleaching and phototoxicity. For uncaging glutamate in the retina, 200 μM Nmoc-glutamate (Dr J. Kao, University of Maryland School of Medicine) was continuously superfused in a recirculating superfusion system. Glutamate was locally released upon UV-laser (Spectra Physics) illumination (20 and 30 ms duration flashes).

For *in vivo* imaging, the anesthetized animal was placed on the microscope stage of a Bio-Rad Radiance 2000 confocal microscope. A long-working distance water-immersion objective (60×, 0.9 N.A.) was used for imaging the submandibular and superior cervical ganglia. A high numerical aperture water-immersion objective (Olympus; 60×, 1.2 N.A.) was used for imaging cortical neurons.

3. Results

3.1. Calcium indicators are rapidly loaded by ballistic delivery

We found many cells in mature brain slices labeled within a minute following particle delivery of calcium indicators. Cells could be labeled successfully using a variety of indicators, including Oregon Green BAPTA-1 488, Calcium Green-1 and Fura dextrans. Fig. 1A and B

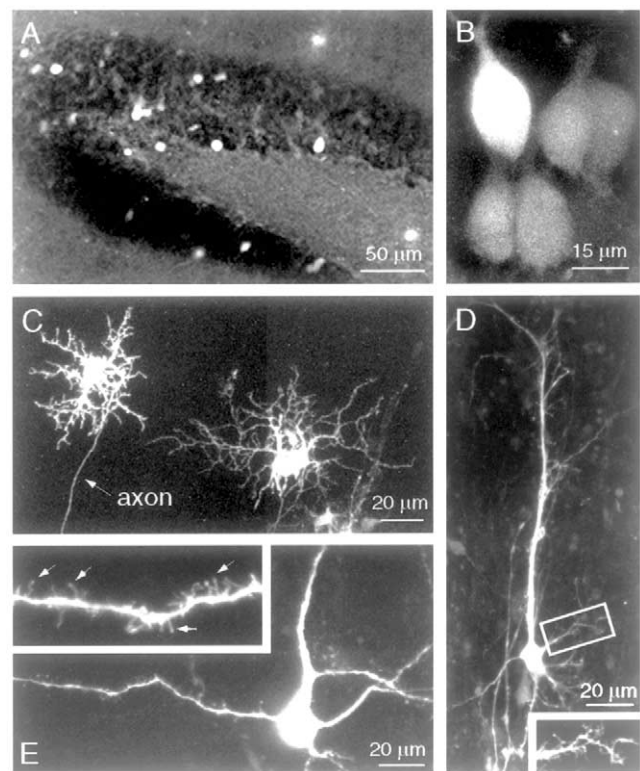


Fig. 1. Ballistic delivery of calcium indicators labels a diversity of neuronal preparations. Examples of neurons labeled with Calcium Green-1 (A–B) or Oregon Green 488 BAPTA-1 dextran (C–E). (A) Labeling of many cells in a 300 μm thick hippocampal slice from a 6-month-old mouse. (B) Higher magnification view of several cells labeled in the dentate gyrus of a hippocampal acute slice from a 4-month-old mouse. (C) Retinal ganglion cells from an E13 chick retina showing complete labeling of dendritic arbors and axonal labeling. (D) Pyramidal cell and fine filopodia (inset) in an organotypic slice culture from the neocortex of a P12 rat. (E) Pyramidal cell and spines (arrows; inset) from an acute slice preparation of a P12 rat.

shows random labeling of many cell somata in mature (6- and 4-month-old, respectively) hippocampal slice preparations. The labeled cells were generally located at a depth of 10–60 μm from the surface of the slices. Typically, we found only one particle within each cell body, similar to biolistic transfection with DNA-coated particles (Lo et al., 1994; Wong et al., 2000). The intensity of labeling varied from cell to cell, most likely because particles carried different amounts of indicator. Depending on cell sizes, we estimated that the average concentration of fluorescent indicators such as Calcium Green-1 dextran loaded through individual particles was in the range of 1–10 μM (see Section 2), consistent with estimated concentrations of indicators loaded with other methods (Takahashi et al., 1999; Peterlin et al., 2001). In order to obtain a density of 15 labeled cells/ mm^2 in the hippocampal granular cell layer, delivery of approximately 100–200 particles/ mm^2 was required. In the retina, 15% of cells in the ganglion cell layer were routinely labeled (Fig. 2A). Labeling density could be increased either by producing bullets with a denser coating of particles or by shooting the preparation multiple times.

In addition to labeling cell somata, we found that many axons and dendrites, including dendritic spines and filopodia, were labeled (Fig. 1C and E). For example, neocortical pyramidal neurons showed labeling of filopodia in an organotypic slice culture from a P12 rat after 2 days *in vitro* (Fig. 1D), as did E13 chick retinal ganglion cells (Fig. 2H). Dendritic spines were also visualized in a cortical pyramidal cell from an acute brain slice from a P12 rat (Fig. 1E). The time required for complete filling of dendritic arbors depended on the cell size and the amount of indicator on the particle, but generally occurred within minutes. We also found that cells and their arbors can be labeled by particles penetrating axons or dendrites. Presumably dye loading occurred by anterograde or retrograde transport of the dextran conjugated indicator from the loaded processes.

3.2. Calcium signals in labeled neurons *in vitro*

To determine whether the method of delivery can faithfully report neuronal function, we monitored calcium levels in various preparations loaded with the indicators. Populations of ganglion cells in a 2-week-old mouse retina ballistically labeled with Oregon Green 488 BAPTA-1 dextran (Fig. 2A and B), or the ratiometric indicator Fura dextran, exhibited synchronous spontaneous elevations in intracellular calcium concentration that resembled activity patterns previously shown by AM-loading of Fura-2 (Wong, 1999b) and by multi-electrode recordings (Meister et al., 1991). Patch clamp recordings from retinal ganglion cells labeled via ballistic delivery of calcium indicators demonstrated that increases in intracellular calcium within the soma are

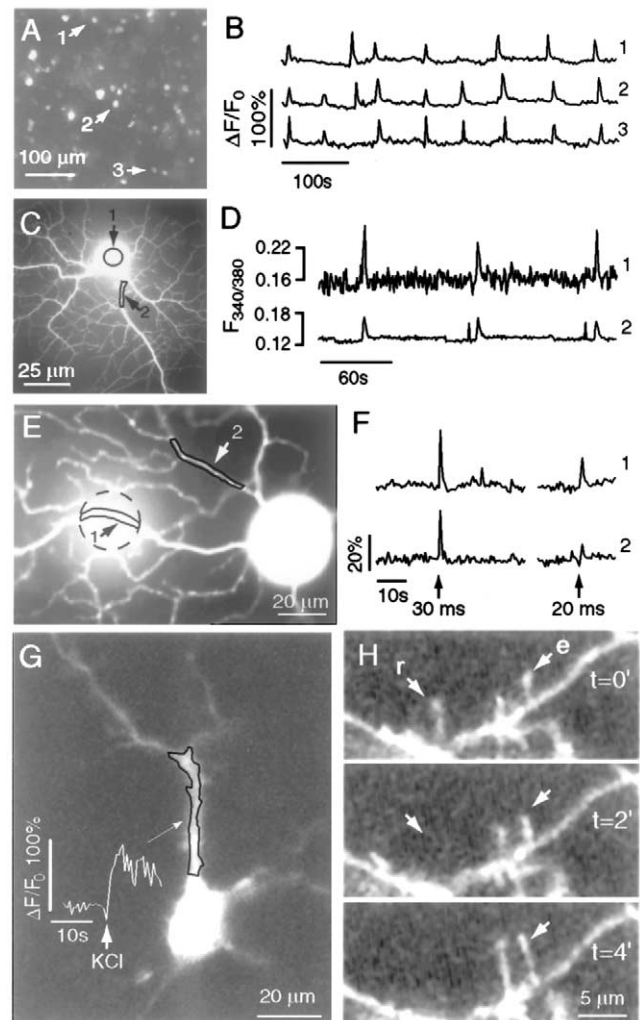


Fig. 2. Cellular function persists after ballistic loading. Cells labeled with Oregon Green 488 BAPTA-1 dextran (A, E, H), Fura dextran (C) or Calcium Green-1 dextran (A). (A, B) Labeled cells in the ganglion cell layer of a 2-week-old mouse retina and traces showing spontaneous elevations in intracellular calcium levels, given as the fractional change in fluorescence intensity levels ($\Delta F/F_0$) for cells 1–3. (C, D) Spontaneous rhythmic activity in an E14 chick ganglion cell indicated by periodic increases in the ratio of fluorescence emission at 340 and 380 nm wavelength excitation ($F_{340/380}$); lines enclose the measured regions 1–2. (E, F) Dendritic calcium signals are observed in a 3-week-old mouse retinal ganglion cell upon uncaging (arrows) of N-methyl-D-glutamate (200 μM). The dashed circle indicates the position of the UV laser spot; measured regions 1–2 are shown in (E). The arrows in (F) indicate when the laser spot was turned on. The duration of the laser flashes, 30 and 20 ms, respectively, is specified below the arrows. (G) Calcium elevation (change in fluorescence given by $\Delta F/F_0$) in the apical dendrite (region enclosed) of a 4-month-old mouse cortical pyramidal cell evoked upon stimulation with KCl (50 mM). (H) A time sequence showing the motility of dendritic filopodia from an E13 chick retinal ganglion cell. ‘e’, extension; ‘r’, retraction.

correlated with spiking activity (Lohmann et al., 2002). Spontaneous calcium elevations could be seen in processes as well, as shown in a recording of an E14 chick retinal ganglion cell using Fura dextran (Fig. 2C and D). In addition, photo-uncaging of caged-glutamate in the

vicinity of labeled ganglion cells from a 3-week-old mouse retina evoked a rise in intracellular calcium concentration within the dendrites; the rise could be spatially restricted to a subset of dendrites by reducing the amount of uncaging (Fig. 2E and F). An increase in intracellular calcium was also seen in mature (4-month-old mouse) hippocampal neurons upon stimulation with KCl (Fig. 2G). Furthermore, dendritic filopodia from labeled E13 chick retinal ganglion cells were highly motile, both extending and retracting on a time scale of minutes (Fig. 2H). Such dendritic filopodial motility observed after ballistic delivery of calcium indicators was similar to that previously documented in cells expressing Green Fluorescent Protein (Wong et al., 2000). Together, these observations indicate that the ballistic method of indicator loading is not deleterious to cell function and allows for the monitoring of calcium dynamics in cells.

3.3. Indicator loading in living animals

One area in which the ballistic delivery is especially useful is imaging calcium in living animals. The rapid delivery of particles makes it possible to label neurons in vivo despite tissue movement. Fig. 3A shows labeling of multiple cortical neurons in a 4-month-old living mouse brain upon shooting Calcium Green-1 dextran-coated particles through a small window in the cranium. In the

peripheral nervous system, the dendrites and axons of superior cervical ganglion neurons could also be labeled in vivo in mice of the same age (Fig. 3B). In addition, more than 50% of neurons in the submandibular ganglia, again from 4-month-old mice, were successfully loaded with indicators in the living mice (Fig. 3C). These cells demonstrated a rise in intracellular calcium levels upon stimulation with KCl, indicating that they remained viable after labeling (Fig. 3D).

4. Discussion

We present here a new technique that delivers calcium-sensitive indicators into multiple neurons and their processes in the developing and adult nervous systems, both in vivo and in vitro. Both spontaneously occurring and evoked activity could be observed after neurons were loaded with calcium indicators using this ballistic technique. The present loading technique provides several advantages over existing loading methods. First, ballistic delivery makes it possible to label mature neuronal tissue, which has previously been very challenging to achieve. For example, incubation with membrane-permeant AM forms of calcium indicators typically stain populations of cells in developing rat cortical slices up to P7 (Yuste and Katz, 1991). Recent improvements in AM-ester loading has enabled cell loading in acute cortical slices up to P30 (Yuste, 1999), but monitoring neuronal activity beyond this period remained difficult.

Second, the ballistic technique allows extensive dendritic arbors to be labeled rapidly without dialyzing cellular content as seen in patch pipette loading. Prolonged perfusion of the cytoplasm in the whole-cell patch configuration has been found to alter many aspects of cell physiology, for example, resulting in a loss of spine mobility (Dunaevsky et al., 1999; Majewska et al., 2000). Because patch pipettes are not required to fill the cells, it should be possible to record calcium events in neurons that depend crucially on soluble second messenger molecules that remain in the cells after ballistic delivery of the indicators. Indeed, experiments using the ballistic technique recently demonstrated the presence of prolonged local calcium elevations in immature retinal ganglion cell dendrites that result from calcium release from internal stores (Lohmann et al., 2002).

Third, unlike loading cells using sharp micropipettes, ballistic delivery enables simultaneous labeling of many cells, therefore allowing rapid selection of cells for imaging. This is particularly important for preparations in which health can be maintained only within a limited period of time. Furthermore, injection with microelectrodes is extremely difficult in immature cells that are fragile and susceptible to damage. We found that cells in

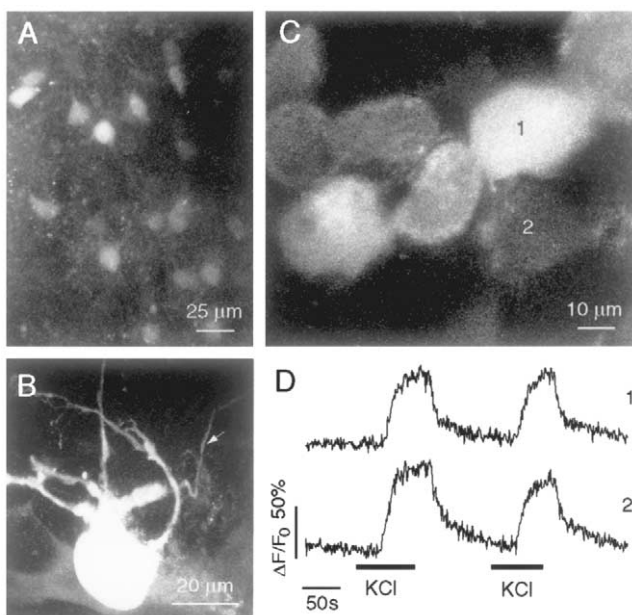


Fig. 3. Ballistic delivery enables in vivo labeling and calcium imaging. Calcium Green-1 dextran in the mature mouse. (A) Neurons in the parietal cortex labeled by shooting through a small window in the cranium of an anesthetized, 4-month-old mouse. (B) Axon (arrow) and dendrites of a superior cervical ganglion neuron were labeled in a living, 4-month-old mouse. (C) Labeling of neurons in the submandibular ganglia of a 4-month-old mouse and their responses (D) to applications of KCl (50 mM).

the embryonic chick retina can be successfully labeled with indicator without perturbation of their structure or function after ballistic delivery of the indicators. In fact, dendritic spines and filopodia were clearly visualized and were motile, enabling structural changes and local calcium levels to be monitored simultaneously. Such a study should help us to better understand how activity regulates morphological changes that take place during synaptic development and plasticity (Wong and Wong, 2000; Yuste and Bonhoeffer, 2001; Lohmann et al., 2002).

Lastly, the ballistic delivery of calcium indicators opens up many new windows for studying neuronal development and function in living animals. This is underscored by our observation that neurons in living animals can be labeled despite substantial tissue movement due to respiratory and cardiac pulsation. The new technique makes it possible to image, *in vivo*, calcium dynamics from neurons in the peripheral and central nervous system, which had been prohibitively difficult to load using micropipettes. In the central nervous system, *in vivo* imaging of ensemble activity is often obtained using intrinsic signal optical recordings that have resolution limits of, at best, 50 μm (Ts'o et al., 1990). The ballistic technique offers an opportunity to record *in vivo* from populations of cortical neurons with single cell resolution.

A drawback of the ballistic technique is that it is difficult to label cells located at a depth greater than 100 μm from the surface of the tissue, due to the penetration depth of particles using the commercially available Bio-Rad 'gene gun'. However, recently reported modifications of the gun barrel can increase the depth of penetration substantially, up to approximately 300 μm (O'Brien et al., 2001). This modification can be of great use for calcium imaging in neuronal preparations where it is possible to image down to 500 μm with a two-photon microscope. Another possible disadvantage to ballistic delivery is an inability to target a particular cell or cell type of interest. However, using a mask to cover part of the preparation during shooting permits loading of random subsets of cells within the unmasked area.

One possible concern with the ballistic method is that variability in the concentration of indicator delivered per cell complicates direct comparisons of calcium signals across cells. This problem is not exclusive to ballistic loading; AM-ester loading also results in a variable concentration of free indicator across cells. However, even with this variability, ratiometric indicators, such as Fura, enable the comparison of calcium fluctuations between cells within the same preparation, as long as they have similar baseline fluorescence. Additionally, methods to quantify the absolute changes in intracellular calcium of Fura labeled cells by ballistic delivery are subject to the same constraints as with AM-ester loading of the indicator (Helmchen, 1999).

We found that labeling was best using dextran-conjugated indicators; coating with the AM-ester or potassium salt forms of the indicators was less robust. The use of dextran-conjugated indicators is advantageous because dextrans diffuse rapidly, thus quickly labeling the entire cell. Furthermore, dextran-conjugated indicators are not readily compartmentalized or sequestered, allowing the indicator to function for extensive periods of time (Schlatterer et al., 1992). Indeed, we observed that cell labeling persisted in organotypic slice cultures for more than 24 h, without appreciable loss of indicator from the cells. The ability to coat particles with any dextran-conjugated indicator implies that indicators with distinct calcium affinities and spectral properties are compatible with the ballistic method. This enables the investigator to easily choose the appropriate indicator for studying calcium dynamics in their system. For example, a low affinity indicator such as Fluo-4 dextran (Kretitzer et al., 2000), used to study the rapid calcium dynamics associated with neurotransmitter release, can be easily coated and delivered ballistically.

Finally, ballistic delivery is potentially useful for delivering other dyes and indicators, such as fluorescent and biotinylated dextrans, voltage-sensitive dyes, dextran-conjugated pH indicators, and other ion indicators. This is of particular interest because it is possible to simultaneously deliver more than one dye or indicator into a cell, by simply co-coating the particles with both substances. For example, cells could be filled with both Calcium Green-1 dextran and Fluoro-ruby dextran, a photoconvertible fluorescent dye (Schmued and Snavely, 1993), allowing for electron microscopic analysis of the same neuron in which calcium signaling was examined. In combination with rapidly advancing imaging technology (Denk et al., 1991; Helmchen et al., 2001), ballistic delivery of calcium indicators and other dyes or indicators provides exciting new opportunities for probing the structure and function of the nervous system.

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