A Practical Guide: Two-Photon Calcium Imaging of Spines and Dendrites

Jesse H. Goldberg and Rafael Yuste

Department of Biological Sciences, Columbia University, New York, New York 10027

This chapter describes an approach to two-photon calcium imaging in dendrites and spines of living neurons. The technique can be applied to acute slices from hippocampus, cerebellum, and neocortex, as well as to slice and neuronal cultures (Yuste and Denk 1995; Holthoff et al. 2002; Sabatini et al. 2002). It uses two fluorescence detection channels to provide quantitative estimates of calcium concentration and to minimize the required concentrations of calcium indicator. A method for estimating calcium concentrations from fractional changes in fluorescent light intensity ($\Delta F/F$) is presented, along with two methods for loading neurons with calcium indicator. First, individual cells can be loaded via patch pipette with calcium indicator and Alexa 594 in a recording chamber (see Chapter 33). Alternatively, if the dialysis that occurs during sustained whole-cell recording is a concern (e.g., during spine motility experiments), calcium indicator can be loaded using the bolus injection technique (Majewska et al. 2000).

MATERIALS

General

This protocol requires equipment and reagents for whole-cell patch-clamping or bolus injection (see Chapter 33).

Calcium Indicators

The potassium salt form of a calcium indicator in the green emission range—fluo-4, Calcium Green-1 (CG-1), or Oregon Green BAPTA (OGB) and Alexa Fluor 59 (Molecular Probes)

Microscopy

Two-photon microscope (See Chapter 10) equipped with a recording chamber and a dichroic mirror, which separates red and green light, (565 DCXR from Chroma Technology). At an angle of 45° the dichroic mirror transmits ~80% of light between ~580 nm and 880 nm (Red Channel—IR from laser to sample and red Alexa 594 fluorescence back to internal PMT) and reflects light below 530 nm (Green Channel—emission from calcium indicator) to external PMT. We place an additional bandpass 510/40 (transmits between 490 and 530) filter in front of the external PMT to reduce scattered red light.

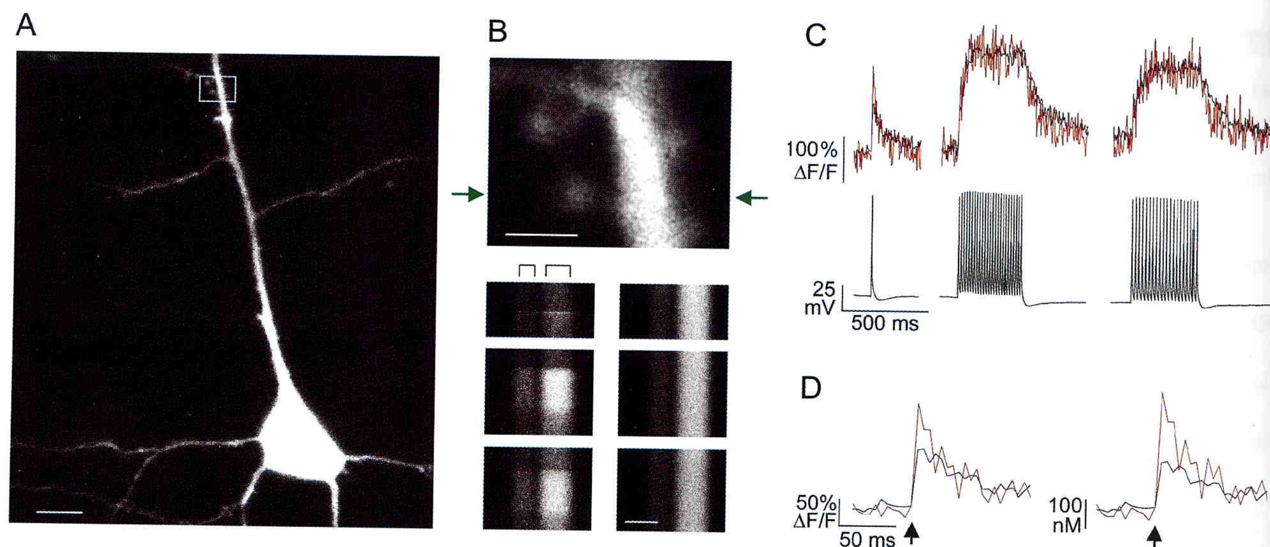

FIGURE 1. See text for details.

PROCEDURE

Loading Neurons with Calcium Indicator and Alexa 594

1. For whole-cell recording: Patch the neuron of choice with a pipette filled with 20–100 μM of green-emission calcium indicator and 20–100 μM Alexa Fluor 594<!> in standard internal recording solution, such as: 130 mM $KMeO_4$, 5 mM KCl <!> , 5 mM NaCl, 10 mM HEPES, 2.5 mM Mg-ATP, 0.3 mM GTP, titrated to pH 7.3. (See Chapter 33.)
2. After 20–30 min of recording, dendrites are well visualized on the red channel, and the green channel can be used to image calcium changes during stimulation (see Fig. 1).

   Note: Resting indicator fluorescence is necessary to visualize dendrites if calcium imaging is going to be performed with only one (green) channel. In this case, a higher concentration of indicator (> 100 μM) must be used.

   CAUTION: *See Appendix 3 for appropriate handling of materials marked with <!>.*

Alternatively, for the bolus injection technique, prepare millimolar concentrations (1–5 mM) of calcium indicator in internal recording solution and hold the cell in whole-cell mode for ~3 min. When patching the cell, target a region of the cell body that does not overlie the nucleus; this will ensure cell viability after electrode withdrawal. Twenty minutes after withdrawal, the dendritic tree should be loaded satisfactorily and will be ready for calcium imaging.

Estimating Changes in Calcium Concentration from ΔF/F

Maravall et al. (2000) developed a method for estimating changes in calcium concentrations that is ideal for use during two-photon excitation of calcium indicators with large dynamic ranges (such as fluo-4 and OGB). Briefly, the approach takes advantage of the ability to estimate $\Delta F/F_{max}$ during experiments by achieving near-indicator saturation with strong stimulation, such as during high-frequency action potential (AP) trains. An alternative method was pioneered by Feller and coworkers (Feller et al. 1996). By comparing the ΔF/F plateau at two frequencies, $\nu_2 > \nu_1$, the percentage, x, of true indicator saturation ($\Delta F/F_{max}$) can be estimated using the following equation:

$$x = 100 \cdot \frac{1 - Q^{\nu_1/\nu_2}}{1 - \nu_1/\nu_2}$$

where Q equals the plateau ΔF/F reached during v_2 divided by that reached during v_1 (see Fig. 1). Equipped with a derived value of $\Delta F/F_{max}$, $\Delta[Ca^{2+}]$ can be solved by

$$\Delta[\mathrm{Ca}] = K_d\left[\left((\Delta F/F)_{max}+1\right)(1-R^{-1})\left(\frac{\Delta F/F}{\left((\Delta F/F)_{max}-\Delta F/F\right)(\Delta F/F)_{max}}\right)\right]$$

where R is the dynamic range of the indicator, and K its dissociation constant (Maravall et al. 2000; Sabatini et al. 2002).

SHORT EXAMPLE OF APPLICATION

Figure 1 shows a L2/3 pyramidal neuron from a slice from p11 mouse visual cortex filled with 25 µM Alexa Fluor 594 and 50 µM fluo-4. The morphology of the cell and region of interest, indicated in Figure 1A and B, were obtained from the calcium-independent Alexa 594 fluorescence recorded on the red channel. Line scans through spine and dendrite, at positions indicated by the green arrows in Figure 1B, were performed using the calcium-dependent green channel, (B, bottom left), during 1 AP, and during 66- and 50-Hz trains (B, from top to bottom). Note the that the Alexa Fluor 594 signal does not change in fluorescence, reflecting its insensitivity to calcium (B, bottom right). In Figure 1C, calcium fluorescence signals acquired from the green channel over spine and dendrite, at positions indicated by red and black brackets, respectively, in Figure 1B, are plotted above time-locked APs. $\Delta F/F_{max}$ was estimated from the $\Delta F/F_{plateau}$ reached during the 66-Hz (v_2) and 50-Hz (v_1) AP trains as indicated above. In Figure 1D, the calcium signal during a single AP is plotted as %ΔF/F, left, and as $\Delta[Ca^{2+}]$, right. Arrowheads indicate timing of AP generation. Note how the spine $\Delta[Ca^{2+}]$ signal appears "stretched" compared to the %ΔF/F signal, reflecting how ΔF/F sublinearly reflects $\Delta[Ca^{2+}]$ as indicator saturation is approached.

ADVANTAGES AND LIMITATIONS

Two-Channel Calcium Imaging

Calcium imaging in small structures such as dendrites and spines is complicated by the fact that the calcium indicators, by binding calcium, effectively perturb calcium efflux, and diffusion. To better approximate physiological conditions, exogenous buffer capacity must be reduced by using low-affinity indicators, such as Oregon Green BAPTA-6F, or, as described here, low indicator concentrations. One problem associated with low indicator concentrations is insufficient resting fluorescence and, hence, poor visualization of the dendritic tree. This problem can be solved by using a separate channel to visualize morphology by using a calcium-insensitive fluorophore with nonoverlapping emission (Fig. 1).

An important consideration when working with low indicator concentrations, however, is indicator saturation. This can be advantageous when examining small calcium influxes such as during single APs, since it allows estimation of $\Delta F/F_{max}$, and, therefore, $\Delta[Ca^{2+}]$ (see above). However, if large calcium signals, such as during N-methyl-D-aspartate (NMDA) receptor activation or release from intracellular stores, are of interest, the use of higher indicator concentrations or low-affinity indicators, such as Oregon Green BAPTA 6-F, is recommended.

Choice of Indicator

CG-1, OGB, and fluo-4 have all been used successfully in single-channel imaging, and all three indicators are excited well by a mode-locked laser at 800 nm. However, each indicator is suited to different conditions. When imaging with one channel (using the indicator's resting fluorescence to visualize the dendritic tree), CG-1 and OGB (150–200 µM) are recommended for their high fluorescence at resting calcium concentration. However, partly because of their high fluorescence, their increase in intensity on binding calcium is compromised. These indicators are, therefore, unsuitable for detecting small

signals. Fluo-4 has a low fluorescence at resting calcium concentration. This means that higher concentrations (200–400 μM) are necessary for one-channel use, but fluo-4 has a large dynamic range and is ideal for imaging small or heavily buffered signals, such as single APs in cortical interneurons (Goldberg et al. 2003). Fluo-4 is also a good indicator to use in tandem with Alexa 594 during two-channel image acquisition (Fig. 1).

REFERENCES

Feller M.B., Delaney K.R., and Tank D.W. 1996. Presynaptic calcium dynamics at the frog retinotectal synapse. *J. Neurophysiol.* **76:** 381–400.

Goldberg J.H., Tamas G., and Yuste R. 2003. Ca^{2+} imaging of mouse neocortical interneurone dendrites: Ia-type K^+ channels control action potential backpropagation. *J. Physiol.* **551:** 49–65.

Holthoff K., Tsay D., and Yuste R. 2002. Calcium dynamics of spines depend on their dendritic location. *Neuron* **33:** 425–437.

Majewska A., Tashiro A., and Yuste R. 2000. Regulation of spine calcium compartmentalization by rapid spine motility. *J. Neurosci.* **20:** 8262–8268.

Maravall M., Mainen Z.F., Sabatini B.L., and Svoboda K. 2000. Estimating intracellular calcium concentrations and buffering without wavelength ratioing. *Biophys. J.* **78:** 2655–2667.

Sabatini B.L., Oertner T.G., and Svoboda K. 2002. The life cycle of $Ca^{(2+)}$ ions in dendritic spines. *Neuron* **33:** 439–452.

Yuste R. and Denk W. 1995. Dendritic spines as basic units of synaptic integration. *Nature* **375:** 682–684.