A Practical Guide: Imaging Action Potentials with Calcium Indicators

Jason N. MacLean and Rafael Yuste

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

The understanding of neuronal circuits has been, and will continue to be, greatly advanced by the simultaneous imaging of action potentials in neuronal ensembles. This chapter describes "bulk" loading of brain slices with acetoxymethyl (AM) ester calcium indicators in order to monitor action potential activity in large populations of neurons simultaneously. The imaging of calcium influx into neurons provides an indirect, but accurate, measure of action potential generation in individual neurons. Single-cell resolution and thus the easy identification of every active cell is the key advantage of the technique.

Starting with Yuste and Katz (1991), this method has been successfully applied throughout the central nervous system including neocortex (e.g., Mao et al. 2001; Cossart et al. 2003), hippocampus (e.g., Tanaka et al. 2002), the cerebellum (e.g., Ghozland et al. 2002), striatum (e.g., Mao and Wang 2003), and spinal cord (e.g., Voitenko et al. 1999), utilizing either acute slices, cultured slices, or cultured dissociated neurons. Recently, the technique has also been applied in vivo to mouse neocortex (Stosiek et al. 2003).

MATERIALS

Cell-permeant Calcium Indicators

Different AM ester calcium indicators under similar experimental conditions have different loading efficacies. Ranked from best loading to worst: mag-fura-2>fura-2>indo-1>Calcium Green-1>fluo-4>Oregon-Green BAPTA-1>Calcium Orange>Calcium Crimson. Several of the indicators are also ranked below by their efficacy as detectors of action potentials: fluo-4>fura-2>mag-fura-2. The majority of our experimental procedures use fura-2. However, the methods described below can be used for any of the listed indicators.

Microscopy

Both single-photon and two-photon excitation of calcium indicators can be utilized to monitor calcium changes in large populations of neurons.

PROCEDURE A

Loading Embryonic and Neonatal Acute Cortical Slices

1. Dissolve 50 μg of fura-2AM (Molecular Probes) in 48 μl of DMSO<!> and 2 μl of Pluronic F-127 (Molecular Probes) for a final concentration of 1 mM.

   Note: Because the solubility of calcium AM indicators is rather poor, vortex the solution for 10–15 min prior to use.

2. Load the slices in oxygenated artificial cerebral spinal fluid (ACSF) and add enough fura-2AM solution for a final concentration of 10 μM fura-2AM, for 30–60 min.
3. Remove slices from loading chamber and place into incubation chamber containing oxygenated ACSF. Imaging may begin after a 30-min recovery period.

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

PROCEDURE B

Loading Juvenile and Adult Acute Cortical Slices

1. Prepare 1 mM fura-2 AM as indicated above (A1).
2. Place slices in loading chamber containing 2.5 ml of oxygenated ACSF.
3. Pipette 5–10 μl of fura-2AM solution on top of each slice.

   Note: This results in a high initial concentration of fura-2AM. The concentration decreases as the fura-2AM diffuses away from the site of application, resulting in a final concentration in the entire chamber of ~10–20 μM.

4. Load slices in the dark for 20–30 min at 35–37°C with 95% O_2/5% CO_2 lightly ventilated into the chamber.

   Note: As a rule of thumb, the loading time should be 10 min, plus as many minutes as the age of the animal in postnatal days.

5. Remove slices from the loading chamber and place into incubation chamber containing oxygenated ACSF for wash. The wash occurs through simple diffusion. Imaging may begin after a 30-min recovery period.

PROCEDURE C

Focal Loading-Slice Painting

Although local neural connections are maintained in the procedure described above (see Kozloski et al. 2001), long axonal projections, such as thalamocortical axons in thalamocortical slice preparations (Agmon and Connors 1991), may be compromised. A modification of Procedure A can be used to circumvent this problem and can also be used in vivo (Stosiek et al. 2003).

1. Dissolve 50 μg of fura-2AM (Molecular Probes) in 13 μl of DMSO<!> and 2 μl of Pluronic F-127<!> (Molecular Probes), achieving a final concentration of 3.3 mM. Vortex the solution for 10–15 min.
2. Deposit the slices into the first loading chamber containing 2 ml of ACSF, ventilated with 95% O_2/5% CO_2, and place onto microscope stage.
3. Fill a fire-polished pipette (tip diameter ~30 μm) with 7.5 μl of fura-2AM.
4. Insert the filled pipette into a standard patch-clamp electrode holder, with tubing attached, and, using a micromanipulator, place the pipette tip 100–200 μm above the surface of the slice. Apply 5–10 psi positive pressure to the pipette. Slowly (1–2 μl in 1 min) move the pipette across the surface of the slice using the manipulator, covering the area of interest with the dissolved fura-2AM.

5. Move the slices to the second loading chamber containing 2.5 ml of ACSF and the remaining 7.5 μl of fura-2AM.
6. Load the slices in the dark for 20–30 min (as a rule of thumb, 10 min plus the age of the animal in days) at 35–37°C with 95% O_2/5% CO_2 lightly ventilated into the chamber.
7. Remove the slices from the loading chamber and transfer them to an incubation chamber containing oxygenated ACSF for wash. Imaging may begin after a 30-min recovery period.

PROCEDURE D

Imaging of Calcium AM Indicators

Two-photon imaging of calcium indicators allows highly sensitive detection of changes in neuronal calcium concentration with relatively little bleaching and photodamage. However, the simultaneous imaging of large populations of neurons is necessarily slow. Epifluorescent imaging of calcium indicators is sufficient to detect changes in neuronal calcium concentration and has the advantage that, using a fast camera, the detection of changes can be of higher temporal resolution than in the two-photon system. However, fluorescent imaging of bulk-loaded slices is subject to rapid bleaching and is also prone to high background fluorescence. Finally, in our experience, spinning disk confocals, together with fast cameras, can be used to image thousands of neurons simultaneously, without significant photobleaching and with good signal-to-noise ratios, over long periods of time.

EXAMPLE OF APPLICATION

Figure 1 illustrates the two-photon imaging of fura-2 and the utilization of fura-2 as an indicator of neuronal activity. Figure 1A illustrates thousands of loaded neurons in a neocortical slice imaged using a two-photon microscope. Figure 1B reveals the correlation between neuronal activity and a change in fluorescence as detected by epifluorescence. The fluorescence of fura-2 decreases in the

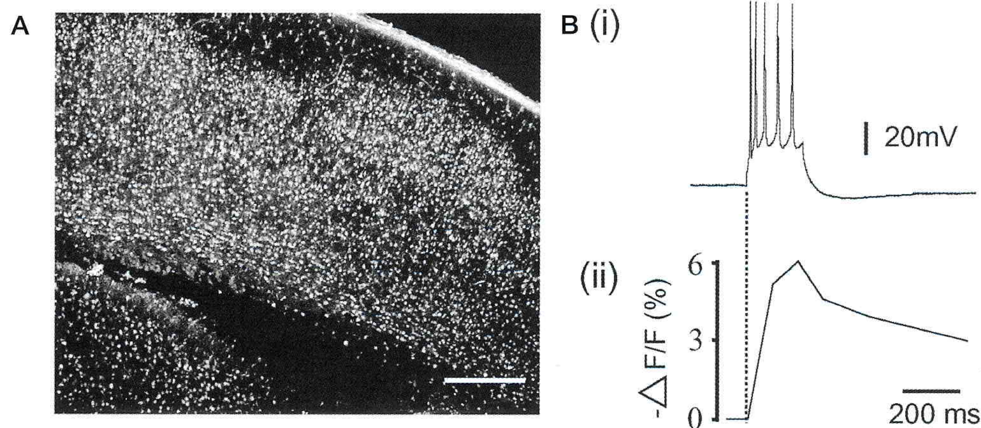

FIGURE 1. High-resolution calcium imaging of neuronal activity. (*A*) Two-photon image of transverse section of mouse neocortex loaded with fura-2AM. Bar, 250 μm. Note how thousands of neurons can be adequately visualized. Courtesy of Dr. Hajime Hirase. (*B*) Correspondence between action potentials and somatic fluorescence change. (i) Whole-cell recording of a burst of action potentials in response to a single intracellular depolarizing current step (150 pA, 200 msec). (ii) Normalized fluorescence change for the recorded neuron imaged with a cooled CCD camera (Micromax, Princeton Instruments). Recording pipette contained 50 μm of fura pentapotassium salt (comparable to the intracellular concentration of fura following bulk loading). Although individual action potentials cannot be resolved, onset of neuronal activity is accurately imaged.

presence of calcium, thus the negative fluorescent change in Figure 1B(ii). As illustrated, even one-photon imaging of fura-2 is sufficient to detect and resolve action potential generation in a single neuron within a field of hundreds or thousands of loaded cells. Because of the rapid time course for the onset of its response, fura-2 lends itself to the elucidation of network dynamics when simultaneously imaging large populations of neurons.

ADVANTAGES AND LIMITATIONS

The use of calcium AM ester indicators provides a very robust method of imaging activity in large populations of neurons simultaneously. In our experience, voltage-sensitive dyes are insufficient for the detection of activity in an individual neuron within a population of neurons such as a cortical slice. Their nonspecific staining pattern and poor signal strength currently make optical recording with single-cell resolution unfeasible in cortical slices (Yuste et al. 1997). Calcium indicators (Tsien 1989) that can be bulk-loaded into brain slices using their AM ester derivatives (Yuste and Katz 1991) act as very good, albeit indirect, measures of action potential generation (Smetters et al. 1999). These still provide the best means of imaging activity in large populations of neurons when single-cell resolution is desirable.

The limitations of the technique are due to the properties of the dyes themselves. Calcium indicators, being charged molecules, do not easily cross the cell membrane and need, therefore, to be microinjected. To circumvent this problem, AM ester derivatives of the indicators were synthesized (Tsien 1981). The AM esters mask negative charges, making the indicator molecules more lipophilic and membrane-permeant, thus allowing them to enter the cell. Once inside the cell, cytoplasmic esterases hydrolyze the acetyl ester linkage, releasing formaldehyde and free indicator, which then accumulates intracellularly as it is once again charged. However, the dependence on intracellular enzymatic cleavage makes this process cell-dependent. This can result in differential loading efficiency in different neurons. In addition, the increased hydrophobicity of the AM ester derivatives of the indicators can cause problems in delivering sufficient amounts to their targets. This problem becomes significant in adult preparations, where the slice painting method appears to be the best loading strategy. Finally, although the time constant for the onset of quenching indicator fluorescence in neurons is rapid, the offset is proportionally slow due to saturation of the dye. Thus, although the calcium indicators are excellent measures for the onset of activity, they do not provide adequate temporal resolution to detect single action potentials during a burst of action potentials.

REFERENCES

Agmon A. and Connors B.W. 1991. Thalamocortical responses of mouse somatosensory (barrel) cortex in vitro. *Neuroscience* **41:** 365–379.

Cossart R., Aronov D., and Yuste R. 2003. Attractor dynamics of network UP states in the neocortex. *Nature* **423:** 283–288.

Ghozland S., Aguado F., Espinosa-Parrilla J.F., Soriano E., and Maldonado R. 2002. Spontaneous network activity of cerebellar granule neurons: Impairment by in vivo chronic cannabinoid administration. *Eur. J. Neurosci.* **16:** 641–651.

Kozloski J., Hamzei-Sichani F., and Yuste R. 2001. Stereotyped position of local synaptic targets in neocortex. *Science* **293:** 868–872.

Mao L. and Wang J.Q. 2003. Group I metabotropic glutamate receptor-mediated calcium signalling and immediate early gene expression in cultured rat striatal neurons. *Eur. J. Neurosci.* **17:** 741–750.

Mao B.Q., Hamzei-Sichani F., Aronov D., Froemke R.C., and Yuste R. 2001. Dynamics of spontaneous activity in neocortical slices. *Neuron* **32:** 883–898.

Smetters D.K., Majewska A., and Yuste R. 1999. Detecting action potentials in neuronal populations with calcium imaging. *Methods* **18:** 215–221.

Stosiek C., Garaschuk O., Holthoff K., and Konnerth A. 2003. In vivo two-photon calcium imaging of neuronal networks. *Proc. Natl. Acad. Sci.* **100:** 7319–7324.

Tanaka E., Uchikado H., Niiyama S., Uematsu K., and Higashi H. 2002. Extrusion of intracellular calcium ion after in vitro ischemia in the rat hippocampal CA1 region. *J. Neurophysiol.* **88:** 879–887.

Tsien R.Y. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **290:** 527–528.

———. 1989. Fluorescent probes of cell signaling. *Annu. Rev. Neurosci.* **12:** 227–253.

Voitenko N.V., Kostyuk E.P., Kruglikov I.A., and Kostyuk P.G. 1999. Changes in calcium signalling in dorsal horn neurons in rats with streptozotocin-induced diabetes. *Neuroscience* **94:** 887–890.

Yuste R. and Katz L.C. 1991. Control of postsynaptic Ca^{2+} influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron* **6:** 333–344.

Yuste R., Kleinfeld D., and Tank D.W. 1997. Functional characterization of the cortical microcircuit with voltage-sensitive dye imaging of neocortical slices. *Cereb. Cortex* **7:** 546–558.