

**Optical Imaging of Ion Concentration Dynamics in Synaptic  
Terminals and Dendrites in Brain Slices**

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## I. INTRODUCTION

Fluorescent indicators of ion concentration --molecules that change their spectroscopic properties when they bind an ion--together with optical imaging methods now find widespread application in neuroscience. It is an exciting time, when new indicator molecules are being rapidly developed and cameras and detector arrays are being improved. The modern era in this field began with the development of fura-2, a sensitive fluorescent indicator for intracellular free calcium ion concentration (1). There are now a half dozen related calcium indicators commercially available (2,3) and similar probes for magnesium, sodium, potassium, proton (pH) and chloride ions. There is an increasing use of imaging methods in mammalian brain slice and other semi-intact nervous system preparations. It is possible to image ion concentration dynamics in cell somas, dendrites (4-6), synaptic spines (7,8) and presynaptic terminals (9). It is also possible to monitor large populations of neurons simultaneously (10). Here we discuss technical aspects involved with loading the molecules into brain slice cells and the imaging systems that are appropriate to perform measurements. We do not provide a comprehensive review of ion concentration imaging in brain slice but rather a guide based primarily on our own experience.

A conventional epi-fluorescence microscope is the heart of most optical systems for ion concentration dynamics imaging. Some of the indicator molecules have excitation in the near ultraviolet, so it is important that the microscopes that are used have good transmission characteristics in this wavelength range (to a lower limit of ~300nm). The microscope is fitted with an imaging device (discussed in more detail below) like a CCD camera, confocal microscope attachment, or photodiode array. Unless a laser scanning confocal camera is used, a device for providing pulses of appropriately filtered excitation light during image exposures is also required.

For brain slice experiments, the microscope stage is fitted with a temperature-regulated slice perfusion chamber. When an inverted epi-fluorescence microscope is used, the bottom of the chamber is a thin coverslip (#1 or #0) so that microscope objectives with high numerical aperture (which usually means short-working distance) can be used. Only cells within about 100 microns of the bottom surface of the slice can be successfully imaged. Deeper cells are not clearly resolved because of light scattering. The slice can be held down using small weights around its perimeter, for example gold electron microscope grids. "Harp" shaped structures consisting of a grid of fine gold wires glued on 400 micron spacing to a

U-shaped support wire and placed on top of the brain slice have also been successfully used. One advantage of the submerged slice chamber and inverted microscope system is that oil or glycerin immersion objectives with high numerical aperture can be used against the coverslip bottom. Another is that stimulating electrodes and field-potential electrodes can easily be placed in selected areas from micromanipulators mounted on the microscope stage with no mechanical interference from the microscope objective. A disadvantage is that conventional intracellular recording during imaging must be done using electrodes that have penetrated the thickness of the slice, in order that the imaged cells be near the chamber bottom.

For simultaneous imaging and intracellular electrophysiology, systems based on upright microscopes have advantages for thick brain slice preparations, since electrodes need not pass through opaque tissue before reaching a region of cells that can be imaged. Their major disadvantage is that they require the use of water immersion microscope objectives and microelectrode access is restricted because of the objective size and shape. Air objectives have been successfully used by using caps made of coverslips or saran wrap to prevent water damage. Many metal objectives, even those produced for water-immersion use, electrochemically produce toxic substances when immersed in slice saline and care must be taken to prevent this by coating them with clear nail polish or sylgard. They are also a thermal sink and temperature regulation near the slice may be compromised. If necessary, the objective can be fitted with a heating coil.

For thin slice experiments used together with patch electrode techniques, both upright or inverted microscopes are appropriate. We know of no cases where conventional slice interface chambers have been used for imaging applications. The air/water interface greatly reduces optical stability of the image; in addition, it is difficult to keep moisture from condensing on the objective surface.

## II. LOADING CELLS WITH INDICATOR MOLECULES

Four major methods are presently being used to load neurons in mammalian brain slice with optical ion concentration indicators. They are: (1) intracellular iontophoretic microinjection through conventional (pointy) glass capillary microelectrodes, (2) introduction of the indicator by internal dialysis using whole-cell patch pipettes, (3) bath application of the membrane permeant acetoxymethyl ester (AM) derivatives, and (4) localized perfusion of AM esters. Each method has its own advantages and disadvantages.

Pertinent questions are the technical simplicity of application, the specificity of labeling, and the degree to which cellular responses are altered. Comments about each method follow:

#### 1) Iontophoresis Using Intracellular Microelectrodes

The advantages of this method are its simplicity, the excellent signal-to-noise ratios it provides in slice preparations compared to bulk labeling methods, and the minimal alteration of intracellular buffers and biochemistry. Cells are penetrated with fine-tipped microelectrodes that are normally used for conventional intracellular recording. Instead of the high ionic strength solutions used for intracellular recording (for example 4M KAcetate or 3M KCl), electrode filling solutions contain the indicator dissolved in low ionic strength solutions to promote indicator iontophoresis.

In one set of experiments on hippocampal pyramidal cells (5), the electrodes (AM Systems #6010) were pulled on a Flaming-Brown micropipette puller (Sutter Instruments Model P80) and had a DC resistance of 150-250 MOhms. Precautions were taken to prevent chemical degradation of the indicator. A fura-2 stock solution was prepared by dissolving 1mg of the pentapotassium salt in 25 $\mu$ l of 1mM KOH. This was aliquotted and stored frozen in a light-tight container for up to 1 month. Aliquots were diluted 6:4 with a solution containing 50mM KHepes and 300mM of either KAcetate or KCl (pH 7.3) to make a solution with a final concentration of ~20 mM fura-2. This injection solution was prepared fresh each day. To conserve fura-2, only the electrode tips were capillary backfilled with fura-2 solution, and a Ag/AgCl wire was inserted into the rear of the electrode to make electrical contact with the solution maintained along the glass fiber. Individual pyramidal cells were iontophoretically filled with currents of -1 nA for 5 min using a conventional bridge amplifier (Neuro Data model IR-283).

Following injection, an incubation time (30 minutes) was provided to let the indicator diffuse intracellularly to distal dendritic processes. Diffusion along dendrites or axons is 1-dimensional and the average distance ( $x$ ) travelled in a time  $t$  is  $x=\sqrt{2Dt}$ , where  $D$  is diffusion coefficient of the indicator. We have measured the diffusion coefficient of fura-2 in 20 micron diameter crayfish axons to be approximately  $D=1\times 10^{-6}$  cm<sup>2</sup>/sec. For some indicators, active transport is also possible and this could dramatically lower transport times.

When simultaneous imaging and electrophysiological recording are not desired, the iontophoretic microinjection method is particularly attractive. The electrode can be easily removed without injuring the cell, providing good visibility. (When an electrode remains in the vicinity of the cell, its fluorescence can provide a large background, sometimes saturating sensitive cameras.) Intracellular filling and imaging need not be performed on the same setup. For example, a brain slice interface chamber could be used for filling and a submerged brain slice chamber for imaging. When using an inverted microscope for imaging, it can be fitted with a dissecting scope in place of the condenser assembly to allow viewing of the slice from above. Then cells can be micro-injected by observing the slice through the dissecting scope, and filling can be monitored by illuminating the slice from below with the inverted microscope's epi-fluorescence system. Several cells can be filled in one area of the slice and those with the most favorable geometry for imaging selected. One strategy is to first fill several cells within about 50 microns of the upper surface of the slice. For imaging the slice is then turned over using a large-bore pipette so that the cells to be imaged are close to the cover slip bottom of the submerged-slice chamber. Note that the slice should be cut so that the desired dendritic field is parallel to the image plane.

There are two disadvantages to simultaneous imaging and electrophysiological recording with indicator loading through intracellular microelectrodes. The first is that the injection electrodes are generally of high impedance and the quality of the recordings is not optimal. The second is that indicator molecules continue to slowly enter the cell from the electrode during the recording session; therefore, the concentration of intracellular indicator is always increasing and spatially non-uniform.

## 2) Internal Dialysis Using Whole Cell Patch Pipettes

This method is described elsewhere in the Syllabus and a detailed description is not provided here. Whole-cell patch methods adapted for use in brain slice are utilized (11-13). The freely diffusible components of the intracellular fluid are replaced by the contents of the patch microelectrode. The patch electrode contains the ionic solution to be used in the cell, including pH and calcium buffer, and the indicator molecule at the final concentration desired. One advantage of whole-cell methods is that the concentration of the indicator molecule can be accurately controlled and excellent quality electrophysiological recordings are possible. Although dialysis of the cell soma is rapid following rupture of the membrane patch, an equilibration time (for example, 30 minutes with hippocampal pyramidal cells) similar to that used with the iontophoresis method described above, is required to

equilibrate indicator with distal dendrites. Although providing excellent indicator loading and high quality electrophysiological recording, internal dialysis methods have one major limitation: important chemical constituents of the cytoplasm may dialyze out of the cell. The intracellular calcium buffering characteristics are altered significantly and loss of ionic currents may be observed (for example "washout" of calcium currents). Other biochemical processes, like those underlying synaptic plasticity, may also be significantly altered.

### 3) Bath Application of Membrane Permeant Derivatives

Most indicator molecules are available in an acetoxymethyl ester (AM) form that, when properly solubilized and accessible, can pass through cell membranes. The AM form is dispersed in saline using an organic solvent/detergent mixture such as DMSO/pluronic acid and this solution is bath applied to the cell preparation. A typical fura-2 labelling solution consists of indicator dissolved in a 10:1 DMSO/pluronic acid solution (the pluronic acid is dissolved in the DMSO by heating). The dissolved indicator is then diluted into the appropriate saline so that the final concentration of DMSO is .1-1%. The nominal indicator concentration is typically 1-100  $\mu\text{M}$ . (The actual indicator concentration is dependent on the critical micelle concentration and is almost always much less.) The preparation is then incubated with this labeling solution in a microwell. The pH and oxygen tension of the labeling solution are controlled by equilibration with bubbled  $\text{O}_2/\text{CO}_2$  gas, as in a conventional slice chamber. After passing through the cell membrane, the indicator molecule is trapped in the intracellular space when de-esterification occurs by intracellular esterases.

Bulk loading of membrane permeant acetoxymethyl ester forms of indicator molecules is simple in concept and has been applied widely to cultured cells and acute preparations of isolated cells from the CNS. Bulk loading of glial cells in cultured brain slice with the calcium indicator fluo-3 has been demonstrated (14). It has also been successfully used in neonatal and early postnatal rat brain slices to label large populations of neurons (10).

Although simple in concept, this method has been difficult to apply to neurons in adult mammalian brain slices. Although good intracellular loading of cortical pyramidal cells with fura-2AM occurs in neonatal and early postnatal rat, there is very little labeling of neuronal cell bodies and dendrites after P7 (10). The inability of solvent dispersed fura-2 AM to load brain slice preparations has been widely observed. The biophysical basis for this problem has not been determined. One idea, however, is that because the molecules are not truly

solubilized on the molecular scale but exist largely as micelles and aggregates, they have limited access to the neurons through the labyrinth of extracellular space, especially when glial cells are prevalent. Evidence for this is the observation that in rat brain cerebral cortex glial proliferation occurs at about the same developmental time that fura-2 AM labeling is lost. The loading problem may yet be solved through the synthesis of indicator molecules with different solubility properties. In addition, many new indicator molecules have not been adequately tested for their ability to label adult brain slices.

Even if access problems can be overcome, significant differences remain between bulk loading and the single-cell labeling methods described above. Bulk labeled brain slice preparations are a tangled meshwork of neuronal somas, dendrites, spines, axons, and presynaptic terminals, as well as glial cells. In conventional epi-fluorescence microscopy, it is difficult to image anything clearly because very little contrast is present. Even when using confocal microscopes, it is difficult to attribute an observed change in indicator fluorescence to a specific cell whose soma may be a great distance away. Independent tests of where a signal is coming from are necessary, for example pharmacological block of postsynaptic currents to examine if an optical signal is presynaptic or postsynaptic in origin.

#### 4) Local Perfusion of Membrane Permeant Derivatives

This method is a modification of bulk loading and has been described in detail (15). It combines the simplicity of bulk labelling methods with aspects of the optical specificity previously obtained only by single cell microinjection. A pair of pipettes positioned close to the surface of the brain slice is used to provide a local perfusion of a solution containing the membrane permeant form of the indicator molecule. The fluid stream flows from the orifice of the delivery pipette, hitting a small region of the slice surface, and then flows into the orifice of the a larger suction pipette positioned nearby. A pressure head of about 15 cm is used with a delivery pipette having a 10 micron tip diameter. The stream of solution can be visualized by the fluorescence of the indicator or in bright field illumination by adding fast green to the labelling solution. The streaming seems to be more effective than bulk loading in exposing the membrane permeant form of the indicator to the cell membrane. It is possible that the solution stream cleans the cell membrane surfaces in the same way as that observed in brain slice patch-recording techniques.

Local perfusion labeling has been used to load mossy fiber presynaptic terminals in region CA3 of the guinea pig hippocampus (9,15). It has also been used to load presynaptic terminals of the parallel fiber system in the cerebellum. When applied to a well defined fiber tract, such as the mossy fiber system, the indicator is trapped intracellularly and travels within the axons (anterograde and retrograde). Distant presynaptic terminals are filled. At these distant sites, the signal to noise ratio produced by the labelling is as good as what would be obtained from intracellular microelectrode filling of the presynaptic neuron. There is little background staining of the slice. Local perfusion labeling has also been used to fill dendrites. For example, perfusion labeling in layer 5/6 of rat cerebral cortical slices fills pyramidal cell apical dendrites that can be imaged in more superficial cortical layers.

Many factors determine which of the four labeling methods described above is appropriate for a specific experiment. For example, it would not be sensible to try to bulk label a whole brain slice and image individual presynaptic terminals, because the background fluorescence would be large. Similarly, although single cell injection or dialysis methods have been used to load 3 or 4 neurons that were then imaged as a group, the application of these methods to larger numbers of neurons is impractical and bulk labeling methods must be used. In the future, we hope to see the development of bulk labeling methods that have cellular specificity. For example, it may be possible to use retrograde tracer techniques, such as indicator-conjugated latex beads, to selectively label projection neurons in specific areas of cerebral cortex.

### III. IMAGING SYSTEMS

There are a multitude of different imaging systems currently used in ion concentration imaging. However, they can be largely grouped into four categories: image-intensified video cameras, high-performance cooled charge-coupled device (HCCD) cameras, scanning confocal microscopes, and photodiode arrays. The choice of imaging system depends on the temporal resolution required, that is, how quickly you want independent images acquired. It also depends on what spatial resolution is desired, that is, how many independent picture elements (pixels) are necessary to represent the structure of interest. Most importantly, it depends on how much excitation light can be tolerated by the preparation before phototoxicity and bleaching occur, and how much emitted light is produced by the indicator (See Section III). Some general comments on the four categories of imaging devices used in ion concentration imaging follow:

1) Discrete photodiode or photomultiplier systems are good for low-spatial resolution, high temporal resolution measurements. To measure the spatially averaged calcium concentration with msec time resolution from a single cell soma or a branch point on a dendrite, a single photodiode or photomultiplier is an excellent choice. Many single detector measurement systems (also known as "spot" systems) are commercially available. Small (4 detectors) to large (100-400 detectors) arrays of photodiodes have been successfully used to image ion concentration dynamics in brain slice neurons. For example, a 4-element array was used to simultaneously measure calcium concentrations from cell soma, proximal and distal apical dendrites and basal dendrites of hippocampal CA1 pyramidal cells (5). Small photodiode array imaging systems for use in ion-concentration dynamics applications are not commercially available and are home-built, based on systems that have previously been developed for use in voltage-sensitive dye experiments. A system consists of a photodiode array mounted on a camera port in the microscope, a set of current-to-voltage converters and filtering amplifiers for each detector and a microcomputer based multiplexer and A/D converter for digitization and data storage. When only a few detectors are used, commercial current-to-voltage amplifiers (for example the Ithaco 1211) and commercial data acquisition systems (Axotape, Axon Instruments; Superscope; GW Instruments; etc) can be used effectively, although as always, systems designed specifically for these applications can be less cumbersome. Large photodiode array systems require custom hardware. A large (>400 element) photodiode-array/amplifier/computer system that has been developed by L. Cohen, D. Senseman and colleagues for voltage-sensitive dye applications but that could be used in ion-concentration imaging (6) can be purchased through the Yale instrument shop.

2) High-performance cooled CCD cameras represent the state of the art systems for moderate spatial resolution, moderate temporal resolution systems and have been used in many brain slice imaging experiments. Sometimes called "slow scan" cameras or HCCD (for High-performance), their advantages include geometric stability, linearity of response, excellent quantum efficiency, large dynamic range and low noise. They are built for accurate digital readout with low-noise and are not to be confused with CCD cameras designed for video standards. Cameras are usually very flexible and there is a tradeoff possible between spatial resolution and temporal resolution: at lower spatial resolution faster imaging is possible (16). For example, the low-noise, 12-bit, 500K pixel per second HCCD camera produced by Photometrics Inc. (Tucson, AZ), operating in frame-transfer mode, can take 100 x 100 pixel images at 15 frames per second and 200 x 200 pixel images at 6 frames per second. When not operated in frame transfer mode, the frame rate at a given spatial resolution is reduced.

In mammalian brain slice, the ratio of indicator signal intensity in the cell soma to that in dendrites can easily be 100:1. HCCD cameras with 12 or 14 bit A/D conversion and low readout noise can easily handle this dynamic range; many video-based cameras cannot without post-processing, so that the effective frame rate of these latter cameras is lowered. Because of their low-noise, geometric stability and flexibility, cooled scientific grade HCCD cameras appear to be the system of choice for quantitative imaging applications under conditions of moderate temporal and spatial resolution.

Despite the flexibility to trade off spatial resolution for increased speed, HCCD cameras currently used for quantitative fluorescence imaging applications could benefit from faster readout electronics. The manufacturers of these systems are aware of this and we can expect to see an order of magnitude increase in readout rate in the very near future. In fact, as prototype systems, 12 bit cameras are now available that can take 100x100 pixel images at greater than 100 frames per second while still maintaining excellent low noise characteristics similar to that of present cameras.

3) Many lower-performance CCD-based video cameras are available that operate at a fixed frame rate, usually 30 frames per second, having a format in compliance with RS-170A NTSC standards. They have a limited dynamic range of about 200:1 and are considered by many to be, at best, a qualitative measuring tool (See the discussion in ref. 17). When used in commercial microfluorescence imaging systems, they are used in conjunction with an image intensifier. They are popular for qualitative imaging applications because they are self-scanning--you can simply hook the output up to a video monitor to see it-- and there exists a diverse set of reliable, easy-to-use devices for digitization, storage and display. They are also somewhat less expensive than the slow-scan high-performance HCCD cameras. Some commercial imaging workstations now offer the option of using either the lower-performance video output CCD or the more expensive HCCD cameras.

4) Both photodiode arrays and cooled HCCD cameras are typically used with conventional epi-fluorescence microscopy. As such there is always a problem with light scattering and fluorescence from sources outside of the image plane. Software deconvolution schemes can be used to enhance optical sectioning (18), but they require multiple images at different planes and are thus slow to perform. They have not yet been used extensively in imaging ion concentration dynamics in neurons. Confocal microscopes are specifically designed to

provide excellent spatial resolution and reduce out-of-plane fluorescence and are becoming increasingly more popular in ion concentration imaging. There are excellent reviews of these instruments available (19,20) and we will not discuss their technical features here. Many people have become discouraged with using confocal microscopes for quantitative ion concentration imaging because strong phototoxicity problems have been encountered. However, some of these problems have been traced to poor signal conditioning in early microscopes and have been eliminated by using better scanning and sampling procedures. Confocal microscopy has been successfully used with fluo-3 with no apparent phototoxicity in cultured brain slice experiments (14). When attempting to compare scanning confocal microscopes to HCCD based systems under comparable image signal-to-noise ratios, a fundamental difference exists: excitation light for HCCD systems illuminate a pixel area at a low intensity for the entire frame time whereas in the confocal microscope, a pixel location on the sample is illuminated with a spot having an intensity orders of magnitude higher but for orders of magnitude briefer time. For the probes used with ion concentration imaging, it is unknown if brief high-intensity exposures cause more, or less, photodamage than constant low level exposures having the same time-average incident light power.

#### IV. LIMITATIONS AND CONSTRAINTS

A common misconception is that high speed and high spatial resolution movies of ion concentration dynamics in neurons could be produced if only a suitable camera or imaging systems were available. There are physical and physiological limitations that can prevent high-speed imaging. The first is the characteristic response time of the indicator. The binding and unbinding reactions of the ion to the indicator are what ultimately determine the temporal response limit. But even before that limit is reached, physiological constraints are present.

When illuminated with a 100 W Hg arc lamp and imaged with a 10 x (0.5 NA) objective and a scientific grade CCD camera, we find that a 140x140 pixel image of a fura-2 filled (100  $\mu$ M) hippocampal CA1 pyramidal cell (the soma and dendritic field) requires between 200ms and 1 sec exposure time per image to have good signal-to-noise ratio in the dendrites. So, at this spatial resolution we can only achieve, at best, a few frames per second, and typically we end up with something more like 1 calcium measurement (1 pair of images; 340 and 380 nm excitation) per second. To go faster, we would require more light reaching the detector.

One way to potentially increase the amount of fluorescent light is to increase the intensity of the excitation light source. In the hippocampal pyramidal cell experiment, increasing the incident light intensity much higher produces photodamage. One characteristic signature of this photodamage is that the intracellular calcium levels irreversibly rise during a sequence of images. However, photo-induced calcium oscillations with irreversible rises occurring much later have also been observed. A limit is thus imposed on the excitation light level. Photodamage is indicator, cell, and possibly, ion concentration level dependent. It must be evaluated in each specific imaging situation.

A second way to increase light level in order to increase frame rate is to increase the concentration of indicator. However, most of the fluorescent ion indicators currently in use are buffers. All such indicators thus alter the intracellular buffering system of the cell and change ion concentration dynamics. For example, increasing the concentration in fura-2 from 150  $\mu\text{M}$  to 300  $\mu\text{M}$  changes the calcium decay kinetics in hippocampal CA1 pyramidal cells by a factor of  $\sim 2$  and severely attenuates short calcium transients. (The effects of buffering can change dramatically the ion concentration level reached by short stimuli. For long stimulus trains the level reached is not affected; this level is determined by influx=efflux and not by buffering.)

Thus, there are physical limits that are imposed by toxicity and altered cell physiology that constrain rapid imaging experiments. The best one can do is optimize the signal-to-noise ratio while operating within these limits. A basic strategy for optimization would include:

- 1) Use the largest numerical aperture objective possible. This is primarily to maximize the efficiency of collected (emitted) fluorescence. Collected light efficiency goes like the square of the NA ( for example, doubling the NA will increase collected light by a factor of 4). Unfortunately, large NA objectives usually have shorter working distances.
- 2) Increase the incident light as high as possible, but below the level where phototoxicity becomes a problem. Note that increasing NA can also increase the intensity of incident (excitation) light, but the real reason for using high NA is to increase the efficiency of the collected light, since phototoxicity usually limits incident light levels.
- 3) Increase the concentration of intracellular indicator, but only to a level where the changes in cell buffering do not alter significantly the property being studied.

4) Do not use more spatial resolution or temporal resolution than is necessary to answer the experimental question. If possible, average areas (bin pixels if using a CCD) and temporally average image sequences.

Another potentially serious complication in brain slice imaging is changes in background fluorescence. In all imaging experiments, the fluorescence produced by the indicator molecule is measured by subtracting a representative background fluorescence image, usually taken in a nearby area of the slice. Changes in brain slice background fluorescence, produced, for example, by changes in NADH levels in response to neural activation, can produce errors in the optical measurements of ionic levels. It is necessary to ascertain the stability of the background fluorescence and to determine the magnitude of activity dependent changes in background fluorescence. In area CA1 of the guinea pig hippocampus, artifacts produced by changes in background fluorescence are typically quite small. On the other hand, we have observed that background fluorescence changes in rat neocortical slices produced by low concentrations of bath-applied neurotransmitters such as NMDA can be large enough to severely compromise fura-2 measurements of calcium transients in the dendrites of pyramidal neurons (Yuste, Delaney & Tank, unpublished observations).

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