Video Article

Voltage-sensitive Dye Recording from Axons, Dendrites and Dendritic Spines of Individual Neurons in Brain Slices

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Abstract

Understanding the biophysical properties and functional organization of single neurons and how they process information is fundamental for understanding how the brain works. The primary function of any nerve cell is to process electrical signals, usually from multiple sources. Electrical properties of neuronal processes are extraordinarily complex, dynamic, and, in the general case, impossible to predict in the absence of detailed measurements. To obtain such a measurement one would, ideally, like to be able to monitor, at multiple sites, subthreshold events as they travel from the sites of origin on neuronal processes and summate at particular locations to influence action potential initiation. This goal has not been achieved in any neuron due to technical limitations of measurements that employ electrodes. To overcome this drawback, it is highly desirable to complement the patch-electrode approach with imaging techniques that permit extensive parallel recordings from all parts of a neuron. Here, we describe such a technique - optical recording of membrane potential transients with organic voltage-sensitive dyes (V_m-imaging) - characterized by sub-millisecond and sub-micrometer resolution. Our method is based on pioneering work on voltage-sensitive molecular probes [2]. Many aspects of the initial technology have been continuously improved over several decades [3, 5, 11]. Additionally, previous work documented two essential characteristics of V_m-imaging. Firstly, fluorescence signals are linearly proportional to membrane potential over the entire physiological range (-100 mV to +100 mV; [10, 14, 16]). Secondly, loading neurons with the voltage-sensitive dye used here (JPW 3028) does not have detectable pharmacological effects. The recorded broadening of the spike during dye loading is completely reversible [4, 7]. Additionally, experimental evidence shows that it is possible to obtain a significant number (up to hundreds) of recordings prior to any detectable phototoxic effects [4, 6, 12, 13]. At present, we take advantage of the superb brightness and stability of a laser light source at near-optimal wavelength to maximize the sensitivity of the V_m-imaging technique. The current sensitivity permits multiple site optical recordings of V_m transients from all parts of a neuron, including axons and axon collaterals, terminal dendritic branches, and individual dendritic spines. The acquired information on signal interactions can be analyzed quantitatively as well as directly visualized in the form of a movie.

Video Link

The video component of this article can be found at https://www.jove.com/video/4261/

Protocol

1. Equipment Setup

Step 1.1. Imaging setup

The key to recording voltage sensitive dye signals is appropriate setup design. We use an upright microscope (BX51WI Olympus or Zeiss AxioExaminer) equipped with three cameras. The setup is designed for illuminating individual neurons in brain slices by excitation light in epi-fluorescence, wide-field microscopy mode using either Nikon 60X/1.0 NA or Zeiss 63X/1.0 NA water dipping objectives. Our microscopes are bolted to a vibration isolation table and equipped with motorized movable stages. Each microscope is equipped with three camera ports. One camera port has a standard high spatial resolution CCD camera for infrared DIC video-microscopy (IR-1000, Dage MTI, USA). The second camera port has a fast data acquisition camera (up to 20 kHz frame rate) with relatively low spatial resolution (80 x 80 pixels) but outstanding dynamic range (14 bits) and exceptionally low read noise (NeuroCCD-SM, RedShirtImaging LLC, Decatur, GA). The third camera port has a CCD camera with high spatial resolution (PixelFly, 1392x1024 pixels; PCO AG, Germany) mounted on a spinning-disc confocal scanner (CSU-10, Yokogawa, Japan) used to collect z-stacks of confocal images for detailed morphological reconstruction of the stained cell. A frequency-doubled diode-pumped Nd:YVO4 continuous wave laser (400 mW) emitting at 532 nm (MLL-III/400 mW; CNI, Changchun, China) is the source of excitation light. The 2 mm diameter laser beam gated by a shutter (LS6; Vincent Associates) is directed to a light guide coupled to the microscope via a single-port epifluorescence condenser (TILL Photonics GmbH, Gräfelfing, Germany) designed to overfill the back aperture of the objective and provide near uniform illumination of the object plane. The laser light is used in place of a conventional Xenon arc-lamp to maximize the sensitivity of V_m-imaging by: (1) using a monochromatic excitation light at the red wing of the absorption spectrum to maximize V_m sensitivity of the dye [9, 10] and (2) increasing the intensity of the excitation light beyond the level that can be achieved by an arc-lamp. The

excitation light was reflected to the preparation by a dichroic mirror with a central wavelength of 560 nm and the recorded fluorescence light was passed through a 610 nm barrier filter (a Schott RG610). The combined effect of an increase in light intensity and the use of near optimal monochromatic excitation wavelengths was a dramatic improvement in the sensitivity of voltage imaging by a factor of about 50 compared to previous measurements [6].

Step 1.2. Adjust for uniform illumination

Use a fluorescence slide standard (green excitation/red emission). Insert appropriate neutral density filters in the laser beam path so that the CCD is not saturated. Focus the objective on the surface of the slide. Adjust the position of the receiving end of the quartz light guide in front of the laser launcher objective, and adjust the position of the output end of the light guide attached to the microscope using appropriate actuators on the epi-fluorescence condenser to achieve centered and uniform illumination of the field of view.

Step 1.3. Determine vibrational noise

Place a small black ink mark on the surface of the fluorescence slide. Record light intensity with NeuroCCD in the continuous recording mode. Focus the objective on a dark edge of the black ink mark. Record the light intensity for about 100 msec at 2 kHz frame rate. Display the spatial average of the fractional light intensity traces (ΔF/F) from ~ 20 pixels receiving light from the uniformly illuminated area and from ~20 pixels along the edge of the ink mark. The excess noise in recordings from pixels with high contrast edges reflects the vibrational noise in the system.

Step 1.4. Vibration isolation

It is mandatory to reduce vibrations by using a vibration-isolation table to a level below the shot noise at light intensities comparable to experimental recording conditions. Adjust the vibration isolation table until the vibration noise in the light intensity from pixels covering the sharp edge of the image is negligible. None of the equipment with moving parts (mechanical shutters, fans) can be mounted on the table. The cables from the equipment on the table attached to other components that are not isolated from vibration must be loose so that they do not transmit mechanical vibrations to the microscope.

2. Selection of an Appropriate Neuron for V_m-Imaging

Step 2.1. Selection of neurons

Make brain slices according to standard procedures. Use a mouse line expressing EGFP in individual nerve cells of interest. With a spinning disc confocal system, visualize EGFP labeled neurons in the slice. Select neurons for V_m-imaging with intact dendritic/axonal trees, and with processes running parallel and close to the surface of the slice. This cannot be accomplished in wild-type mice because axons and thin dendrites, for the most part, are not visible in DIC microscopy mode.

3. Loading Neurons with Voltage-sensitive Dye

Step 3.1. Filling of the patch pipette

Fill glass patch pipettes from the tip with dye-free intracellular solution by applying negative pressure for about 15 s up to 2/3 of the electrode taper. The dye free solution in the tip is necessary to prevent the spill of dye onto the slice which increases the background fluorescence and dramatically reduces signal to noise ratio. Back-fill the electrode with the solution containing the membrane impermeant voltage sensitive dye JPW3028 dissolved in intracellular solution (0.8 mM).

JPW3028, the most successful voltage probe for intracellular application, is a doubly positively charged analogue of the ANEP series of lipophilic styryl dyes that is still sufficiently water soluble to be used for microinjection. The di-ethyl analog of this dye has practically identical characteristics (including voltage-sensitivity) and is commercially available as JPW1114 (see **Table 1**). We prepare 20 mM stock solution in distilled H_2O. 50 µl aliquots of the stock solution are kept frozen at -20 °C. For the final dye concentration of 0.8 mM, 2 µl of the stock solution is dissolved in 50 µl of intracellular solution on the day of the experiment. The stock dye solution is stable and can be kept at room temperature for several months. Thus, we keep one 50 µl aliquot at room temperature until it is used up.

Step 3.2. Establish a giga-seal quickly

Patch a previously selected neuron and allow free diffusion of the dye from a somatic patch pipette to the soma in the whole-cell configuration for 20-45 min. It is essential to obtain a seal as fast as possible so that the amount of dye-free solution in the tip can be kept small. Start by practicing fast patching without the dye in the electrode. Next, practice patching and loading neurons with the dye with the goal of using a minimal amount of dye-free solution in the tip without contaminating the surrounding tissue. With practice, it is possible to establish a seal within 1-2 min.

Step 3.3. Monitor the level of staining

During dye diffusion in the whole-cell configuration, monitor the physiological state of the neuron by recording evoked action potentials in the current-clamp mode. Additionally, monitor the amount of staining by recording the resting light intensity (RLI) from the cell soma at a frame rate of 2 kHz and at a fraction of full light intensity adjusted with neutral density filters (we use 0.04% of the laser light intensity from a 400 mW laser). Continue the dye loading process until the action potential starts to broaden, usually after 20-40 minutes, depending on the electrode size and resistance.

The broadening of the spike is completely reversible and most likely due to capacitive load effect of the saturated concentration of the dye in the somatic membrane. The waveform of the spike is fully restored after the dye concentration is equilibrated throughout the neuron.

Step 3.4. Remove the dye electrode

At the end of staining period, carefully pull the patch pipette away from the soma in voltage clamp configuration ensuring that the transition from whole-cell to outside-out patch configuration is attained in the process.

Step 3.5. Wait for dye diffusion

Incubate the slice for an additional 1.5-2 hr at room temperature to allow for the voltage-sensitive dye to spread into neuronal processes. After a significant amount of dye diffuses away from the soma into dendritic and axonal processes, the waveform of the AP is completely restored.

4. Optical Recording

Step 4.1. Select a cellular compartment for imaging

Locate the soma of the stained neuron under low light level fluorescence and re-patch the neuron with a standard (no dye) patch electrode under DIC. Visualize neuronal processes under low light level at a frame rate of 10-40 Hz in the continuous recording mode of the CCD for voltage-imaging. Reduce the light level with neutral density filters to the minimum required to visualize the object of interest. We use 0.01% of the 400 mW laser intensity during positioning of the stained neuron. Using an X-Y stage, position the neuronal process of interest in the middle of the imaging area. Protect the soma from the excitation light using the partially closed field stop iris of the microscope. Shielding the soma from high intensity excitation light will significantly reduce the photodynamic damage during recording.

Step 4.2 Record optical signals related to membrane potential transients

Record optical signals related to backpropagated APs in individual dendritic branches. Record optical signals related to APs in the axon. Record optical signals related to backpropagating APs in dendritic spines. Use frame rates appropriate for accurate reconstruction of signal waveform and keep the recording periods and exposure to high intensity excitation light as short as possible to minimize dye bleaching and photodynamic damage. For example, examining the sequence of initiation and propagation of single action potentials in the axon requires recording periods of 5-10 msec. The excitation light intensity during recording is a compromise between the signal-to-noise ratio on the one hand and the degree of dye bleaching and photodynamic damage on the other hand. We use 100% of intensity of the 400 mW laser in recording from long sections of axons when an area of 300 μm in diameter is illuminated. When the excitation light is focused to a 30 μm diameter area for dendritic spine imaging, we use 10-25% of the laser light intensity. The required duration of recording is an important determinant of the optimal excitation light intensity; shorter recording periods allow higher light intensities. The optimal illumination intensity is best determined empirically for each preparation and measurement settings.

5. Data Analysis

Step 5.1. Correct the raw data for known errors

The analysis and display of data were carried out using the NeuroPlex program (RedShirtImaging) written in IDL (ITT Visual Information Solutions, Boulder, Colorado) and custom Visual Basic routines. Under the conditions of low light levels, the background fluorescence becomes a significant determinant of the ΔF/F signal size. The raw data were first corrected for this effect by subtracting the average background fluorescence intensity determined from an unstained area on the slice. Subsequently, the signal alignment software was used to correct for temporal jitter in AP initiation as well as for possible small movements of the preparation during averaging. In the temporal domain, AP signals were aligned by cross correlation of the electrically recorded APs in each trial to the reference signal acquired at the start of averaging (**Figure 1B**). In the spatial domain, images were aligned in two dimensions offline by image cross-correlation to compensate for possible small lateral movements of the preparation. The correct focus of the image in the z-dimension was verified before each individual trial; small adjustments were often necessary. The spatially and temporally aligned signals were averaged as shown in **Figure 1B**. Slow changes in light intensity due to bleaching of the dye were corrected by dividing the data by an appropriate dual exponential function derived from the recording trials with no stimulation (**Figure 1B**). The waveform of the AP signal was reconstructed from a set of data points using Cubic Spline Interpolation, a piecewise continuous curve passing through each data point. To confirm that the voltage-sensitive dye signal tracks membrane potential without significant distortion at the millisecond time scale, the electrical AP signal from the soma was compared to the optical AP signal from the adjacent axon hillock as shown in **Figure 3B**. The two signals superimpose very closely, allowing for the shot noise in optical recording.

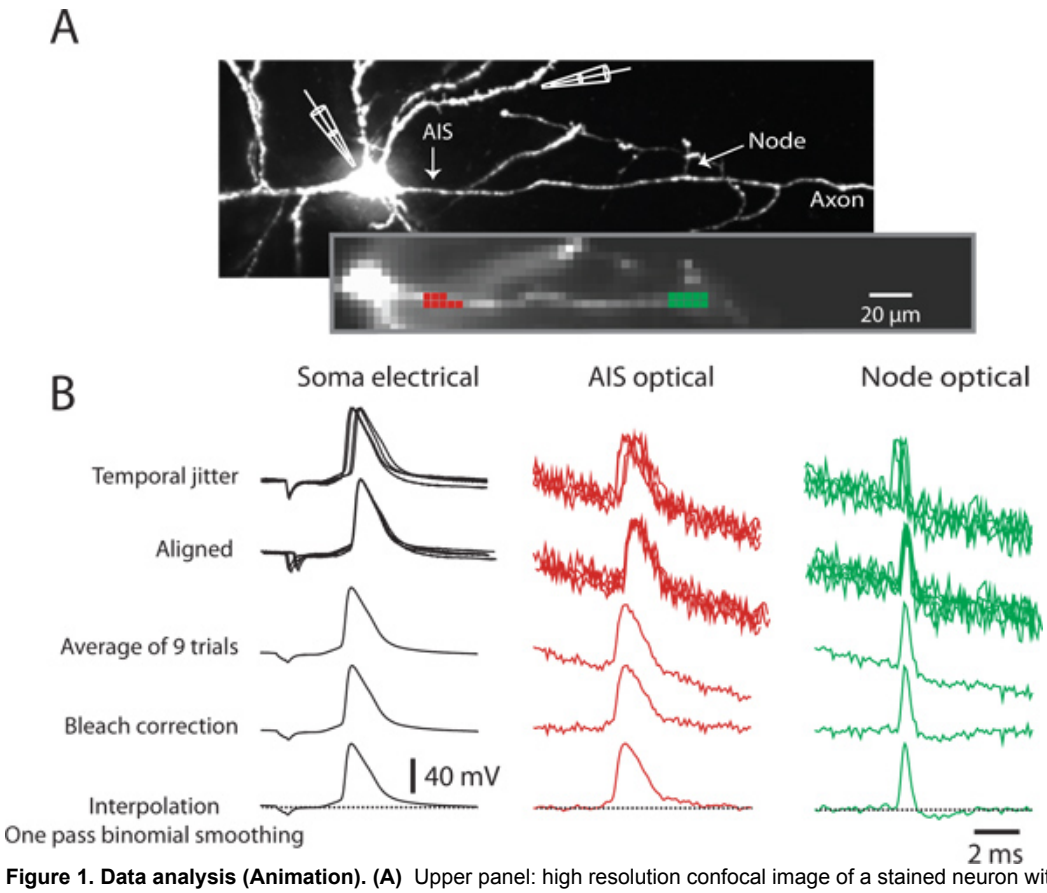

Figure 1. Data analysis (Animation). (A) Upper panel: high resolution confocal image of a stained neuron with axon in recording position. Recording electrode attached to soma and extracellular stimulating electrode next to basal dendrite shown schematically. Action potentials evoked by extracellular, synaptic stimulation. Lower panel: low spatial resolution fluorescence image of the axon obtained by CCD used for V_m imaging. **(B)** Electrode recordings from soma (black traces), optical recordings from AIS (red traces), and from node of Ranvier (green traces). Top traces: raw data from 9 trials showing temporal jitter in AP initiation. Second row of traces: temporally aligned signals. Third row of traces: averaged signal. Fourth row of traces: bleach correction. Bottom traces: cubic spline interpolation with one pass of temporal smoothing.

Representative Results

Successful confocal microscopy should allow clear identification of intact neuronal processes which are close to the surface of the slice and located in one plane of focus. The selection of nerve cells which are appropriate for voltage imaging prior to voltage-sensitive dye loading is critical. An example of confocal images of L5 pyramidal neurons expressing EGFP in a cortical slice (Crym transgenic mouse line) is shown in **Figure 2**. Axons of individual neurons are clearly visible. Cells with long intact axons (white arrows) in one plane of focus close to the surface of the slice were selected.

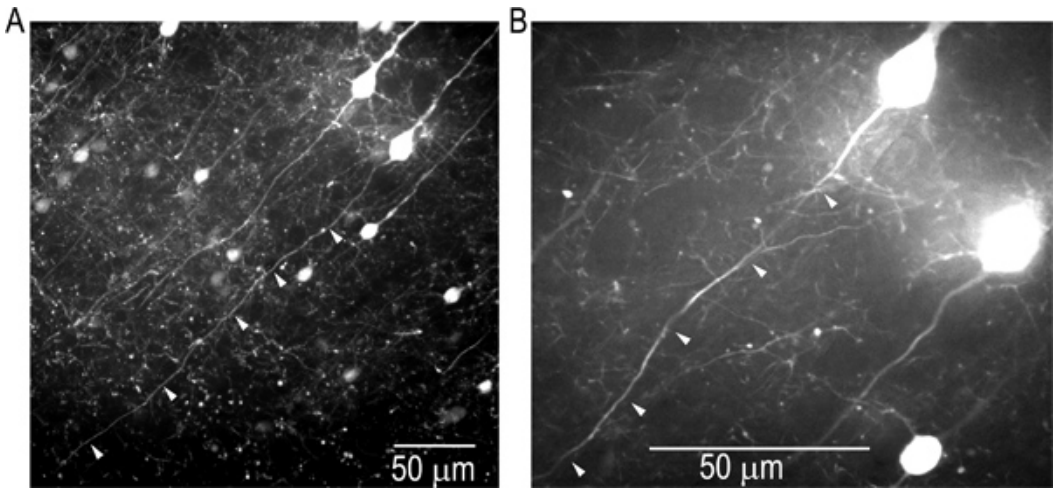

Figure 2. Selection of L5 cortical neurons for V_m-imaging of AP signals from axons. Low (left) and high (right) magnification images of the same slice region; 488 nm excitation using Yokogawa spinning disc scanner.

The spatial pattern of Na-channel clustering in the axon initial segment (AIS) plays a critical role in tuning neuronal computations and changes in Na-channel distribution have been shown to mediate novel forms of neuronal plasticity in the axon. However, immunocytochemical data on channel distribution may not directly predict spatiotemporal characteristics of action potential initiation, and prior electrophysiological measures are either indirect (extracellular) or lack sufficient spatial resolution (intracellular) to directly characterize the spike trigger zone (TZ). A critical methodological improvement in the sensitivity of membrane potential imaging technique described here allows direct determination of the location and length of the spike TZ as defined in functional terms. An example of recording AP signals at high spatial and temporal resolution is shown in **Figure 3**. **Figure 3B** illustrates that the available sensitivity of V_m-imaging was sufficient for accurate monitoring of the subthreshold depolarization preceding the regenerative AP signal. Additionally, the comparison of the optical AP signals from the soma/axon hillock and from a more distal node of Ranvier confirmed that APs have markedly different dynamics at these two locations [8, 15]; both the upstroke and the downstroke of the AP was faster in the node of Ranvier. For details about spatiotemporal resolution limits in the measurements of the size and position of the spike TZ see **Figures 3 and 5** in Popovic *et al.* (2011).

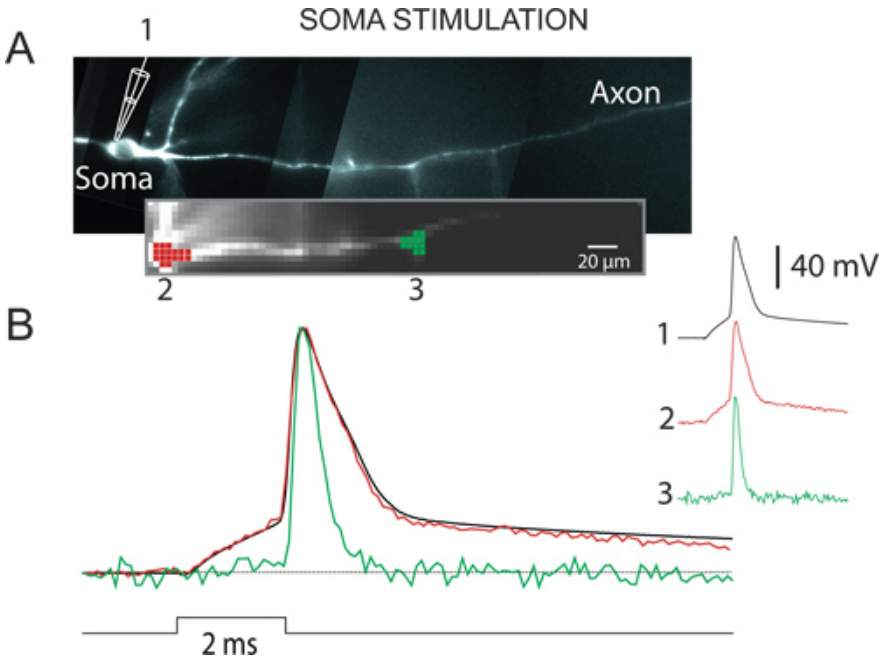

Figure 3. Action potential signals from axon. (A) Upper panel: high resolution confocal image of a L5 cortical neuron loaded with the voltage-sensitive dye with axon in recording position; projection from a z-stack of confocal images. Recording/stimulating patch electrode attached to soma. Lower panel: low spatial resolution fluorescence image of the axon obtained by CCD used for V_m-imaging. (B) AP related signals recorded at a frame rate of 10 kHz. Traces on right: AP transients from three locations: 1-electrode recording from soma; 2-optical recording from axon hillock; 3-optical recording from the first node of Ranvier. Bottom traces: Superimposed signal from the same three locations.

The non-linear interaction between the excitatory postsynaptic potentials (EPSPs) and bAPs in the dendrites which is responsible for the induction of LTP is not fully understood. This interaction depends critically on the amplitude of both signals and must, therefore, be spatially nonuniform. The experimental test of this prediction requires spatially well resolved measurements that have not been carried out because dendritic branches of small diameter are not accessible to electrode measurements. The membrane potential imaging technique described here allows monitoring of electrical signaling from multiple locations in the entire dendritic arbor as shown in **Figure 4**. The pattern of bAP activity

in the dendritic arbor is characterized by sodium current dominated spikes in proximal regions which gradually changed to prolonged calcium current dominated depolarizing events in distal dendrites.

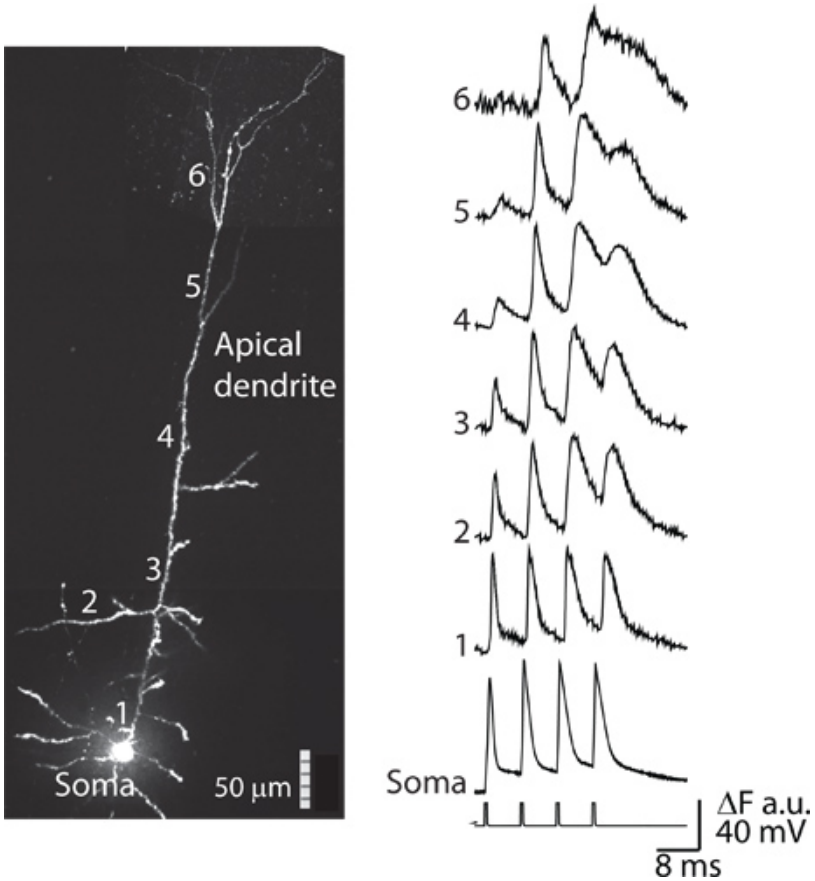

Figure 4. Action potential signals from multiple locations on the dendritic arbor of a L5 cortical neuron. Left panel: high resolution image of a L5 cortical neuron loaded with a voltage-sensitive dye; projection from a z-stack of confocal images. Right panel: a burst of 4 AP initiated at 100 Hz by short depolarizing current pulses (bottom trace) delivered to the soma. Backpropagating action potential signals from six selected locations (1-6) along the apical and oblique dendrites. Traces 1 through 3 were obtained from a single trial recording. Trace 4 is a four trial average, while traces 5 and 6 are sixteen trial averages.

The hypothesis that spines have an electrical role of modifying synaptic efficacy that underlies plasticity and possibly learning and memory mechanisms has recently received considerable attention because of its critical implications for brain function (Yuste, 2010). There is, however, very little direct experimental evidence in favor of or against this hypothesis. The uncertainties in the interpretation of indirect results and the lack of direct evidence about electrical behavior of dendritic spines are due primarily to a methodological limitation - spines are small and not accessible to conventional methods of electrophysiology. Thus, attempts to investigate this question in the absence of experimental data relied on computer simulations with estimates of electrical parameters based on spine morphology and diffusional properties of the spine neck. The voltage-imaging approach described here makes it possible to monitor action potential signals and synaptic potential signals at the spatial scale of individual dendritic spines with high sensitivity. The experiments can now be designed to directly test the fundamental theoretical predictions about electrical behavior of dendritic spines. An example of optical signals related to backpropagating APs in an individual dendritic spine and its parent dendrite is shown in **Figure 5**.

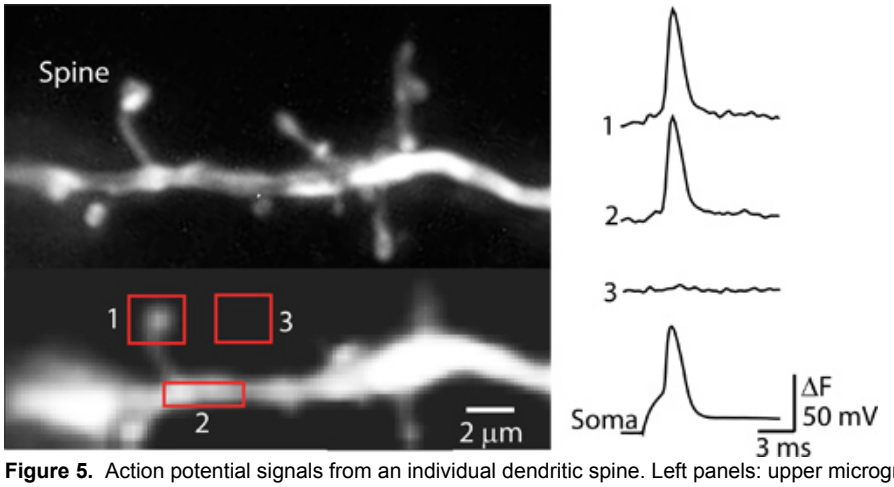

Figure 5. Action potential signals from an individual dendritic spine. Left panels: upper micrographs - anatomical reconstructions obtained from a stack of spinning-disk confocal images. Lower micrographs - fluorescence images of the same region obtained with the CCD camera for V_m-imaging. Right panel: Fluorescence intensity traces corresponding to bAP from locations 1-3 outlined on CCD images. Temporal averages of 9 trials. Bottom trace: electrode recordings from the soma. Note that trace 3 from an area without a spine has no detectible signal indicating low level of light scattering in the superficial layer of the slice.

Discussion

This article describes a voltage-sensitive dye recording method for monitoring electrical activity of individual neurons with sub-micrometer and sub-millisecond spatiotemporal resolution. Laser excitation at near-optimal wavelength (regarding signal size) improved the sensitivity of recording by a factor of ~50 over previous approaches. The current sensitivity enables monitoring electrical signals from all parts of individual neurons, including dendrites, axons, axon collaterals and axon terminals as well as individual dendritic spines. With present sensitivity, recordings of membrane potential transients can be carried out at frame rates of up to 20 kHz. Modest signal averaging (4-25 trials) can readily improve the sensitivity of recording expressed as the signal-to-noise ratio by a factor of 2-5. The major limitation of voltage imaging is the lack of simple calibration of optical signals from multiple locations on an absolute voltage scale. In some preparations this can be resolved by finding a membrane potential signal that has a known amplitude at all locations. AP signals in the axon and in some fully excitable dendrites [6] provide an excellent calibration standard.

Critical steps in the application of this methodology are:

a. Minimizing light scattering effects by restricting the recordings to neurons located near the surface of acute brain slices (< 30 µm). This required optimizing the slicing procedure to obtain a high percentage of healthy neurons in the upper layer of the slice [1].
b. Optimizing the amount of clear solution in the tip of the electrode for delivering the dye to insure quick loading of neurons.
c. Eliminating mechanical vibration of the preparation which can be a source of excess noise in optical recording.
d. Employing low-noise continuous wave (CW) lasers with the RMS noise of amplitude < 0.5% as a source of excitation light.
e. Controlling photodynamic damage by selecting appropriate excitation light intensity relative to the duration of the recording period and by separating successive recordings by dark periods. Longer recording periods require lower light intensities to prevent photodynamic damage.

The recording examples shown above indicate a turning point in spine and axon physiology. These recordings reveal the remarkable power of being able to directly record electrical events which could only be analyzed on theoretical grounds in the past.

Disclosures

Dejan Zecevic declares that he is a co-owner of RedShirtImaging LLC., a company specialized in high-speed, low noise CCD cameras used in voltage-sensitive dye recording. All other authors report no financial interests or potential conflicts of interest related to the current study.

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