Brief Communication

Optical Recording of Action Potentials with Second-Harmonic Generation Microscopy

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Nonlinear microscopy has proven to be essential for neuroscience investigations of thick tissue preparations. However, the optical recording of fast (~1 msec) cellular electrical activity has never until now been successfully combined with this imaging modality. Through the use of second-harmonic generation microscopy of primary *Aplysia* neurons in culture labeled with 4-[4-(dihexylamino)phenyl][ethynyl]-1-(4-sulfobutyl)pyridinium (inner salt), we optically recorded action potentials with 0.833 msec temporal and 0.6 μm spatial resolution on soma and neurite membranes. Second-harmonic generation response as a function of change in membrane potential was found to be linear with a signal change of ~6%/100 mV. The signal-to-noise ratio was ~1 for single-trace action potential recordings but was readily increased to ~6–7 with temporal averaging of ~50 scans. Photodamage was determined to be negligible by observing action potential characteristics, cellular resting potential, and gross cellular morphology during and after laser illumination. High-resolution (micrometer scale) optical recording of membrane potential activity by previous techniques has been limited to imaging depths an order of magnitude less than nonlinear methods. Because second-harmonic generation is capable of imaging up to ~400 μm deep into intact tissue with submicron resolution and little out-of-focus photodamage or bleaching, its ability to record fast electrical activity should prove valuable to future electrophysiology studies.

Key words: imaging; action potential; membrane potential; second-harmonic generation; microscopy; nonlinear; optical; voltage-sensitive dye

Introduction

The investigation of the electrical signaling properties of excitable cells such as neurons is predominately accomplished through the use of intracellular microelectrodes. Although these studies are useful for obtaining temporal electrical activity from a few locations on a single neuron (Stuart and Sakmann, 1994), they reveal little about the spatiotemporal modulations of membrane potential (V_m) over the entire cell or the activity of a population of neurons. Optical methods for monitoring V_m enable a greater understanding of the mechanisms underlying single neuron firing properties and cooperative electrical signaling in groups of neurons.

It is currently possible to follow cellular V_m activity optically through the use of linear one-photon methods (i.e., fluorescence, absorption, scattering, and birefringence). Slow transmembrane redistribution of dyes allows for V_m imaging with a high signal-to-noise ratio (S/N) but cannot provide the 1 msec temporal

resolution needed to record fast V_m signals (Rink et al., 1980). Elegant green fluorescent protein (GFP) constructs (Knopfel et al., 2003) and fluorescence resonance energy transfer pairs (Gonzalez and Tsien, 1997) have been used to record V_m but can be limited either in their response time or in their ability to stain intact tissue (Zochowski et al., 2000), respectively. Methods using intrinsic changes in linear scattering or birefringence have been used to record action potentials (APs) in thin specimens (Cohen et al., 1968; Stepnoski et al., 1991), but recent attention has focused on fluorescent probes. These fast probes can respond to a V_m change of 100 mV with up to 10–20% changes in fluorescence emission (Grinvald et al., 1982; Loew et al., 1992; Rohr and Salzberg, 1994), although the response is typically limited to ~1% in practice (Zochowski et al., 2000).

An innovative combination of high dye concentration, large illumination intensities, large collection areas, and/or very sensitive light detectors has allowed researchers to overcome these small signal changes and to image APs with an S/N of ~10 and subthreshold events by temporal averaging at a spatiotemporal resolution of ~10 μm and <1 msec (Zochowski et al., 2000). However, in thick preparations, high-resolution (micrometer scale) one-photon techniques are limited to imaging depths of approximately <50 μm by light scattering, making the poor spatial resolution deep in scattering tissues (such as neural tissue) the most severe limitation.

Nonlinear microscopy [such as two-photon fluorescence, second-harmonic (SH) generation (SHG), etc.] has the potential

to solve this problem because of its ability to image at depths of up to ∼400 μm in scattering tissues with submicron resolution (Denk et al., 1990; Denk and Svoboda, 1997; Dombeck et al., 2003). Cells labeled with asymmetric styryl dyes generate an SH signal that is sensitive to V_m (Bouevitch et al., 1993; Ben-Oren et al., 1996; Campagnola et al., 1999). These dyes label only the outer leaflet of the lipid bilayer and therefore satisfy the noninversion symmetry requirement for the generation of an SH signal. Like other forms of nonlinear microscopy, SHG microscopy has imaging-resolution advantages over one-photon techniques deep in tissue and therefore has been the focus of considerable research to prove its capability to record fast (∼1 msec) cellular V_m signals such as APs. However, its sensitivity to V_m has only been demonstrated on fast time scales in model membrane systems such as giant unilamellar vesicles (Moreaux et al., 2003; Pons et al., 2003) or on slow time scales (∼1 sec) in sustained cultured cell lines (Milliard et al., 2003). To date, there has been no demonstration of the ability to record fast V_m activity in living cells with any form of nonlinear microscopy, and therefore ∼1 msec, high spatial resolution optical V_m recording has been limited to thin preparations or superficial regions of thick specimens. Here, we report the response of SHG from labeled *Aplysia* cell membranes during step changes in V_m and demonstrate 0.833 msec and 0.6 μm spatiotemporal resolution of APs with minimal background and photodamage during recording sessions.

Materials and Methods

Primary cell culture. Abdominal and cerebral ganglia from up to 15 different 5–10 gm *Aplysia californica* were dissociated and cultured with like ganglia (i.e., abdominal with abdominal, cerebral with cerebral) according to Goldberg and Schacher (1998). The cultures were grown in glass-bottom dishes (MatTek, Ashland, MA) at 17–18°C. For recordings, 4- to 14-d-old cultures were used.

Staining and imaging. We used 4-[4-(dihexylamino)phenyl][ethynyl]-1-(4-sulfobutyl)pyridinium (DHPESBP) (inner salt) [molecule B from Moreaux et al. (2003)] because of its proven fast (<150 μsec) response to V_m. SHG from giant unilamellar vesicles stained with this molecule was sensitive to V_m on fast time scales through an electrochromic (rather than reorientational) mechanism (Moreaux et al., 2003). Here, staining was accomplished by extracellular perfusion of 8 μM dye in SL-15 extracellular buffer (Goldberg and Schacher, 1998). The dye remained in the bath during the experiments, and the flip-flop time of a similar dye to the inner leaflet of a stained membrane was found previously to be ∼2 hr (Moreaux et al., 2001). The SHG imaging system has been described previously (Dombeck et al., 2003), except here, the transmitted light-condensing objective was replaced by a 0.9 numerical aperture (NA) condenser, modified to switch easily between differential interference contrast wide-field imaging and SHG collection. The focusing objective was also changed to a 20×, 0.75 NA Zeiss (Thornwood, NY) Fluar, making a diffraction-limited ∼0.6 μm radial diameter focal spot. The Ti:Sapphire laser was operated at 940 nm with ∼12 mW of average power at the sample, and a 460/30 bandpass filter was used for SHG detection in front of the bialkali photomultiplier tube. The excitation polarization was linear.

Optical and electrode voltage recording. Electrophysiological recordings of *Aplysia* neuron cultures were made at room temperature with an Axoclamp 2B amplifier and pClamp 8.1 software (Axon Instruments, Union City, CA). Two-electrode voltage clamp was used to clamp V_m at defined values. For stimulation of APs, the neuron was impaled with two microelectrodes (8–15 MΩ, filled with 3 M KCl), one for voltage recording and the other for current injection. AP stimulation was possible with <5 nA, ∼50 msec duration current pulses as used previously (Bedi et al., 1998; Rubakhin et al., 1999); however, shorter more intense pulses (30–100 nA, 1.0–3.5 msec duration) were used here to quickly reach threshold. This shorter stimulation protocol afforded an AP temporal stability of <0.5 msec peak voltage drift over the minutes of signal averaging that was not possible with the longer protocol (>3 msec drift), obviating the

need for postprocessing. The necessary temporal resolution to optically detect APs with SHG was obtained using the line-scanning mode of the scanning system (600–1200 lines/sec). X–Y images were taken by raster scanning the focal spot over the two-dimensional field of view while one line was repeatedly scanned 256 times during line scanning. Each 8-bit image is built of 256 pixels in each dimension. The line scanning and stimulation or voltage-clamping steps were synchronized through trigger pulses coupling the amplifier and image acquisition software. Exact timing of the amplifier signals and line scanning was accomplished by recording the monitor output of the amplifier in one of the line-scan imaging channels. This enabled an increase in the S/N through line-scan averaging. Each recording session consisted of a number (n) of stimulation–line scans in series with pauses, 4 sec for two-electrode voltage clamp and 3 sec for AP stimulation experiments, to save the images and electrode recordings and reset the system for the next line scan. A typical n = 50 session totaled 2–3 min in duration.

Dye synthesis. The electroNLOchromic dye DHPESBP was obtained via a multistep synthesis scheme involving a Sonogashira key coupling reaction followed by alkylation with 1,4 butane sultone (T. Mallegol, O. Mongin, M. Blanchard-Desce, unpublished procedure).

Results

Spatiotemporal AP recordings with SHG microscopy

Figure 1, *A* and *B*, shows SHG images of a cultured *Aplysia* neuron stained with DHPESBP. Because of the noninversion symmetry requirement of SHG, a signal is only produced at the labeled membranes; no background from randomly oriented dye is seen. During line scanning of the green line in Figure 1*B*, voltage steps were applied to the cell in voltage clamp. The S/N was ∼1 for single traces, necessitating temporal averaging. This was accomplished by synchronizing the start of the line scan with the amplifier voltage steps. After repeated line-scan averaging (n = 50), the modulations in the SHG emission followed the voltage steps accurately with an S/N of ∼7. This line-scan average is scaled to the dynamic range of the SHG response to visualize the small change in emission (Fig. 1*C*). Negative voltage steps resulted in increased SHG emission, whereas positive voltage steps resulted in decreased emission (Fig. 1*D*). A linear response of the SHG with respect to ΔV_m (linear best fit: $\Delta SHG/SHG = -0.06 \Delta V_m$; slope error = 0.004) was observed by applying a range of voltage steps (Fig. 1*E*).

To prove the ability of SHG to record fast neuronal signals, APs were elicited and optically recorded. Line-scanning positions of two different recording sessions on an *Aplysia* neuron are represented by the green lines in Figure 2*A*, one through the soma and the other through several neurites. During each line scan, two ∼10- to 15-msec-duration APs, consistent with mixed Ca^{2+}–Na^+ APs (Gardner and Kerkut, 1968), were elicited through intracellular electrodes (Fig. 2*B,C*, red traces). Line-scan averaging (n = 50) showed that the optical intensity modulations in the SHG emission follow the fast V_m events with 0.6 μm spatial and 0.833 msec temporal resolution and an S/N of ∼6 (Fig. 2*B,C*, green traces). Note that the somatic recording electrode and SHG optical recording positions are different, so the AP shape and duration differ in some instances, as seen previously (Zecevic, 1996). The temporal response of SHG is easily capable of recording faster events such as the ∼4 msec duration APs, consistent with Na^+ APs (Gardner and Kerkut, 1968), seen in Figure 2*D*. After line-scan averaging (n = 70), the S/N was ∼7 for this recording session.

The S/N in SHG recording can be understood by analyzing the noise in the baseline of the optical traces. For example, noise of ∼1% is seen in the baseline of Figure 2, *B* and *C*. Assuming a shot noise-limited system, this equates to a total of ∼10,000 photons collected per membrane during the 50 traces, yielding ∼200 pho-
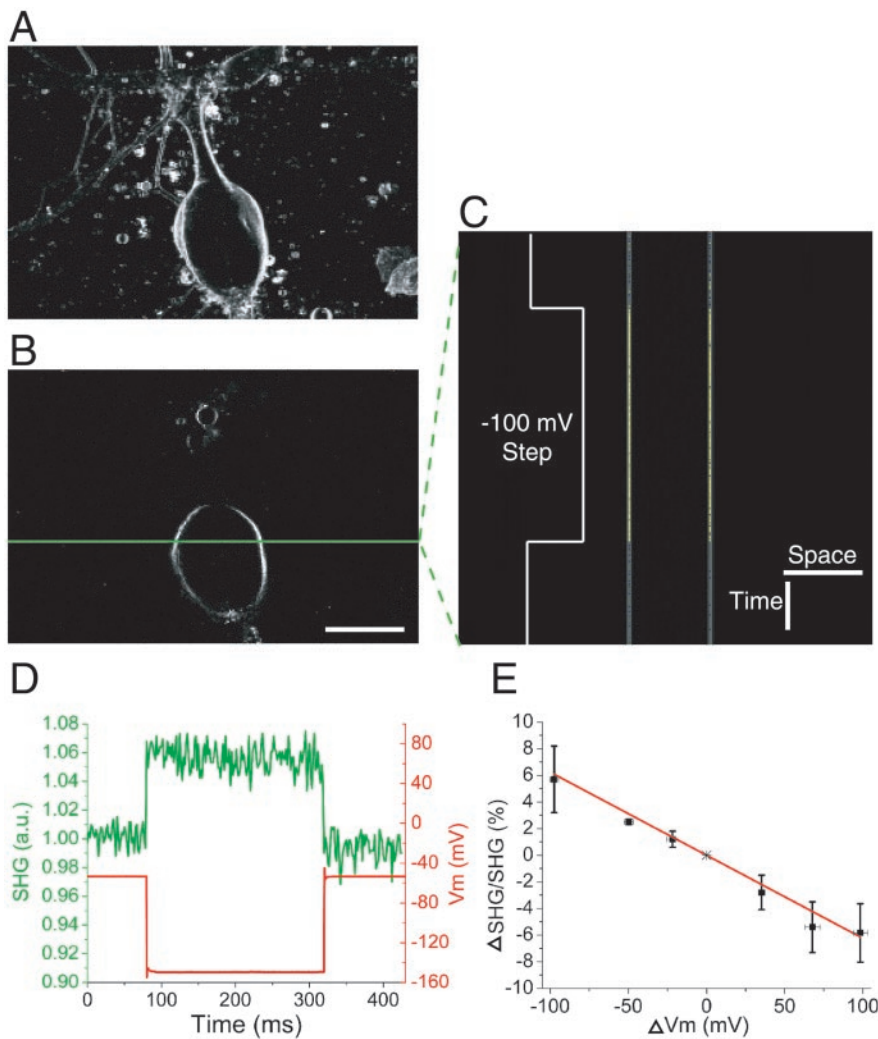
Figure 1. Line-scan recording of V_m with SHG during voltage steps in cultured *Aplysia* neurons. *A*, Projection image superimposing 21 ~1-μm-thick z-sections 2 μm apart. *B*, A single z-section through the neuron in *A* at the plane of line scanning. The green line represents the scanned line where membrane potentials are recorded. *C*, SHG signal changes recorded by line scanning the line denoted in *B* at 600 lines/sec. The voltage-clamped neuron was given a 240 msec duration -100 mV step after 80 msec of scanning during each line scan. $n = 50$ line scans were averaged. The line scan image is scaled to visualize the small change in SHG emission. Calibration: 50 μm, 50 msec. *D*, The green trace, obtained from the left membrane line in *C*, is a normalized intensity plot of SHG emission versus time. The red line represents the measured V_m during the voltage-clamp step. a.u., Arbitrary units. *E*, Plot of ΔSHG/SHG over physiologically relevant ΔV_m. The functional fit in red shows a linear relationship. Error bars represent SDs of three to five different measurements at each $\Delta V_m \pm$ ~4 mV. Note the inverse relationship between ΔSHG/SHG and ΔV_m.

recording from distal neurites resulted in blebbing or transection of the processes at the sites of line scanning. More studies are needed to investigate long-term damage effects and to determine the cause of the phototoxicity and possible avoidance procedures. Although little loss of the SHG signal was observed at ~12 mW, it was more apparent at ~22 mW.
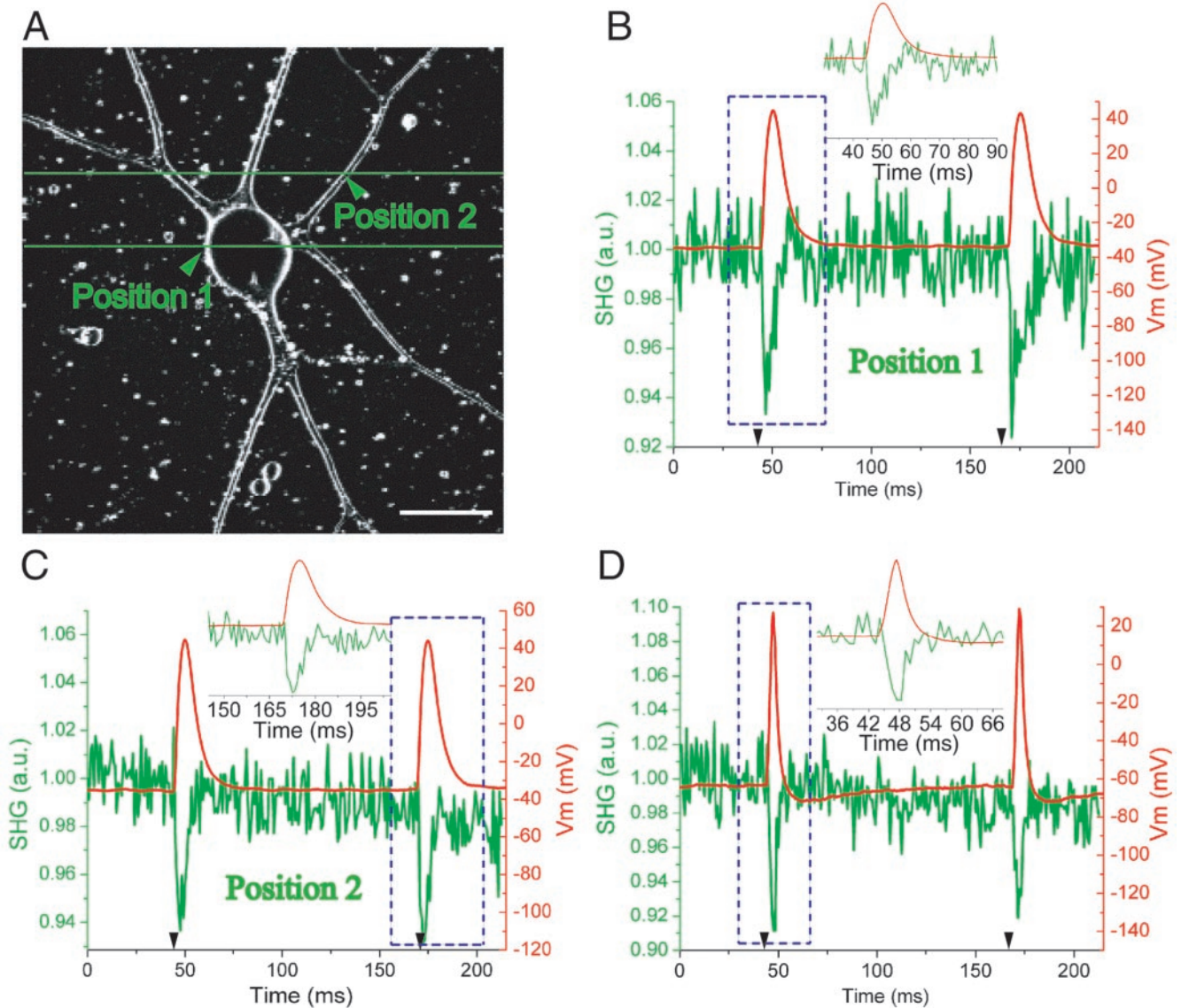
Discussion

An eventual goal of optical V_m recording is to image subthreshold events, without averaging, deep in intact neural tissue with <1 μm and ~1 msec spatiotemporal resolution. To reach this goal, the SHG S/N for V_m recording and imaging frame rate must be improved (i.e., full two-dimensional images must be obtained at the same temporal resolution demonstrated here for line scanning). Commonly used commercial scanning systems have a frame rate for full images of ~1 Hz; thus, to obtain the necessary 1 kHz imaging frame rate, new scanning technologies must be implemented. Resonant galvanometer (Tsien and Bacskai, 1995)- and microlens (Kobayashi et al., 2002)-based scanners have been used for this purpose, but current demonstrations of these devices only achieve a frame rate of ~33 Hz. Newer versions of this technology may show promise for faster scan rates (So, 2003). Another possibility is the use of random access scanning (Bullen et al., 1997; Bullen and Saggau, 1999) to scan over only the membrane region of interest.

Improvements in S/N can come from: (1) increased laser power, (2) higher dye concentrations in the membrane, (3) higher quantum yield detectors, or (4) better SHG molecules, with increases in both hyperpolarizibility and response to V_m. The number of emitted photons is proportional to the square of both the dye concentration and the fundamental laser intensity (Moreaux et al., 2000). Therefore, increases in either should lead to large decreases in the photon shot noise; methods for decreasing photodamage must be investigated concurrently. Rapid progress is being made on option 3. New GaAsP photomultiplier tubes, for example, have greater quantum efficiency than the bialkali model used here. Option 4 will involve the design and screening of many dyes. It is likely that molecules with greater V_m response and increased hyperpolarizibility will be found.

To achieve the goal of V_m imaging with SHG under the guidelines above, many possible variables may be altered. For example, an ~20% ΔSHG/100 mV dye with twice the hyperpolarizibility of DHPESBP, stained and illuminated at twice the concentration and incident power used here, should be capable of recording a 10 mV subthreshold event with $n = 1$ and an S/N of >1.

Recording V_m activity deep in tissue may be possible in the near future, but sample thickness, staining procedures, and tissue

tons during the laser dwell time across the membrane. Therefore, the noise is ~7% and the S/N is ~1 for $n = 1$.

Minimal phototoxicity during recordings

Little cellular photodamage was detected during the recording sessions with an average power of ~12 mW, as in those examples presented here. To evaluate this, we first noted that the variation in resting potential was <4 mV during individual sessions, equivalent to nonillumination controls. Second, the elicited APs remained constant in amplitude and duration throughout recording sessions compared with nonillumination controls. Finally, no gross morphological changes were detectable in the soma or processes after individual sessions. Repeated optical recording from single neurons was possible over the course of ~0.5 hr. When the power was increased to ~22 mW, damage was observable. Recording from the soma was still possible with little damage, but

Figure 2. Fast SHG line-scan recording of APs at multiple sites in cultured *Aplysia* neurons. *A,* An SHG projection image of six ∼1-μm-thick z-sections 3 μm apart. Green lines represent the scanned lines. Scale bar, 50 μm. *B, C,* The green traces, obtained from the averaged line scans, are normalized intensity plots of SHG emission versus time at membrane position 1 and position 2, respectively, in *A* at 1200 lines/sec (0.833 msec/line). Two APs were elicited by current injection (timing of current pulses given by black arrowheads), one at 45 msec and the other at 170 msec, during each line scan. $n = 50$ line scans were averaged. The red trace is the V_m from the recording electrode at the soma. The inset is an expanded time base around the AP in the blue dashed box. *C* reveals the usefulness of high-resolution optical V_m recording by showing the clear difference in AP duration that can occur between the soma and neurites of individual neurons. *D,* Example of shorter APs from a different neuron than in *A*. $n = 70$ line scans were averaged.

type must first be carefully evaluated. Intrinsic SHG imaging at depths of up to 300–400 μm with submicron resolution has been demonstrated previously in hippocampal brain slices (Dombeck et al., 2003). Because SHG is collected in the transmitted light direction, sample thickness is limited to approximately <500 μm. Staining thick turbid media, such as intact ganglia or mammalian neural tissue, has been accomplished by bath-applying dye (Delaney et al., 1994), but other techniques such as pressure injection of dye (Stosiek et al., 2003), intracellular labeling (Antic et al., 1999), novel GFP constructs (Knopfel et al., 2003), or the addition of dye crystals into tissue (Gan et al., 2000; Moreaux et al., 2001) have been needed previously to stain at these depths. Additionally, varying preparations have led previously to different sensitivities of the same V_m dyes (Zochowski et al., 2000), but a greater issue for SHG dyes may be their effects on more delicate mammalian cells.

With these issues in mind, numerous applications should be possible with SHG V_m recording. The current capabilities demonstrated here are most immediately applicable to reproducibly stimulated systems, as are often used for current optical V_m recording techniques (Zecevic, 1996). It should be feasible to investigate spike initiation zones (Zecevic, 1996) and AP propagation properties (Fromherz and Muller, 1994; Meyer et al., 1997) deep in intact ganglia, where one-photon methods are not appropriate. Repeated line scans at varying spatial positions over processes will allow for the generation of high-resolution time series movies of AP propagation, leading to the experimental analysis of electrical propagation properties at complex structures, such as axon bifurcations and varicosities. In addition, SHG V_m recording simultaneously with powerful Ca^{2+} imaging techniques (Szmacinski et al., 1993; Yuste and Denk, 1995) should prove to be a useful combination.

Any increase in spatial resolution is valuable for the investigation of V_m properties of small structures (i.e., dendritic spines) or for providing higher-resolution data of AP propagation. The spatial resolution of $\sim$0.6 μm demonstrated here represents the highest-resolution optical recording of fast V_m activity to date. The potential usefulness of this resolution for AP propagation studies in which shape and duration vary depending on position is seen in Figure 2C, where these differences are clear. Theoretically, similar spatial resolution is possible for the fast one-photon techniques while maintaining the same S/N; however, a higher illumination intensity would be needed, likely resulting in greater photodamage. The highest demonstrated resolution of $\sim$2 μm was demonstrated by one-photon random-access scanning, but like other linear methods, it was limited to thin specimens (Bullen et al., 1997; Bullen and Saggau, 1999).

Here, we demonstrated that SHG microscopy is capable of recording APs from excitable neurons with 0.833 msec temporal resolution at the highest spatial resolution yet reported for fast optical V_m recordings. This technique for optically recording V_m holds the following important advantages over current techniques: submicron resolution at depths up to $\sim$400 μm in scattering tissue, little out-of-focus photodamage or bleaching, and no background. The above work represents a key step toward $\sim$1 msec time-scale, high-resolution V_m imaging deep in intact neural tissue. We expect that rapid progress will continue to be made toward this goal in the near future.

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