Real-time imaging of action potentials in nerves using changes in birefringence

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Abstract: Polarized light can be used to measure the electrical activity associated with action potential propagation in nerves, as manifested in simultaneous dynamic changes in their intrinsic optical birefringence. These signals may serve as a tool for minimally invasive neuroimaging in various types of neuroscience research, including the study of neuronal activation patterns with high spatiotemporal resolution. A fast linear photodiode array was used to image propagating action potentials in an excised portion of the lobster walking leg nerve. We show that the crossed-polarized signal (XPS) can be reliably imaged over a $\geq 2$ cm span in our custom nerve chamber, by averaging multiple-stimulation signals, and also in single-scan real-time “movies”. This demonstration paves the way toward utilizing changes in the optical birefringence to image more complex neuronal activity in nerve fibers and other organized neuronal tissue.

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References and links

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1. Introduction

Fast, noninvasive methods for imaging activity in peripheral nerves and other organized neuronal structures are limited for both research and clinical applications. Direct measures of neural activity, such as electrical recording using microelectrodes, provide a signal with good temporal resolution, but at the expense of irreversible tissue damage and insufficient spatial resolution due to volume conduction effects and the limited number of electrode elements [1,2]. Large-scale imaging methods, such as functional magnetic resonance imaging (fMRI), are limited in both spatial and temporal resolution; moreover, they constitute indirect measures of neuronal activity through the measurement of (typically) local hemodynamic responses, which present with a delay after neuronal impulses propagate [3,4]. Small-scale imaging methods generally use markers such as voltage sensitive dyes (VSDs) and calcium indicators to provide fairly high spatial resolution, but are limited by the dyes’ temporal responsiveness, and are cytotoxic, presenting additional limitations [5,6], although recently dyes are becoming less toxic [7], and genetically encoded voltage indicators have removed some of these barriers for research purposes [8].

Changes in birefringence have been reliably demonstrated to occur simultaneously with action potential (AP) propagation over a variety of scales, from single axonal membranes to complex nerve bundles [9–13]. The change in birefringence is likely a product of induced polarizability by electric-field fluctuations in the vicinity of axonal membranes that occur directly with AP propagation, providing a cleaner method of measuring these activities as compared to other optical methods [11,13–15].

Structurally anisotropic tissues such as nerves inherently exhibit strong structural optical birefringence. The intrinsic anistropy in nerves arises from structures such as microtubules, membrane phospholipids, and surrounding tissues, which include Schwann cells and axon sheaths [14,16]. Dynamic changes in birefringence, measured as the “crossed-polarized signal” (XPS), arise when axonal membranes exhibit a rapid momentary change, typically a decrease, in birefringence as a consequence of the dynamic changes in local electric fields due to propagating APs [9]. This dynamic signal is typically ≤10⁻⁴ compared to the static birefringence. The resulting small XPS is generally recorded as an average of measurements during repeated stimuli, but has been detected successfully in single-stimulus trials in large...
crustacean nerves, as a point measurement with a single photodiode [11]. Limited two-
dimensional spatial resolution of AP activity (over a small length and width of the nerve) has
been obtained using a charge-coupled device (CCD), although a low signal-to-noise ratio
(SNR) allowed only the strongest peak of the signal to be reliably detected after significant
binning and filtering, and the imaging was an average of multiple stimuli, and thus not real-
time [17]. These were, nonetheless, steps in the right direction for real-time imaging of AP
propagation using changes in birefringence.

Here, we provide results of improved instrumentation, which enables reliable tracking of
the propagation of APs in real-time using the XPS measured in the lobster walking leg nerve
(WLN) at various stimulation rates. The rapid onset and gradual recovery times of the XPS
signal that we reported previously for the WLN [13] are also demonstrated here, both in
stimulus-averaged data and for a single stimulus period, depicting real-time imaging. These
results establish a basis upon which this technique could be further developed for
neuroscience research or eventual clinical use.

2. Methods

A diagram depicting the experimental imaging and electronic setup is shown in Fig. 1. All
optical elements are housed in 30 mm cage mounts (Thorlabs, Inc.). Illumination is provided
by a 625-nm high-power LED (Thorlabs, Inc. M625L3) driven by an LED driver (Thorlabs,
Inc. T-Cube) with variable output current control. Light from the LED is approximately
collimated using a spherical plano-convex lens, and then focused to a line at the nerve by a
cylindrical lens. Light from the nerve is then imaged (using a second spherical lens) onto the
central ~40 pixels of a fast 64-element Si-photodiode (PD) array with a built-in low-noise
amplifier (Hamamatsu S8865-64). The PD array is combined with a dedicated driver circuit
(Hamamatsu C9118). Our custom-designed chamber housing the nerve was described
previously [13]. The chamber is positioned such that a ~2 cm length of the nerve in the
electrically-grounded central well is illuminated. The incident light is polarized by a linear
polarizer (LPi) (Edmund Optics #47-316), and the light transmitted through the nerve is
analyzed by another linear polarizer (LPa), oriented 90° to LPi. The pair of crossed polarizers
are placed with their optic axes at ± 45° relative to the length of the nerve, providing the
maximum birefringence signal [14].

The driver circuit for the PD array is operated by the reset signal (RES), which establishes
the line rate, and a clock signal (CLK), both of which are controlled by a pulse generator (PG)
(Berkeley Nucleonics, BNC 555). The circuit outputs the video signal (VID), as well as two
TTL signals — when each individual pixel is being read out (TRIG), and at the end of each
line scan (EOS). Two National Instruments (NI) data acquisition (DAQ) cards are used. The
first (NI DAQ 1) is a 16-bit, 1 MS/s analog-to-digital converter (National Instruments, PXI-
6120) that uses the TRIG signal as a sample clock, to coincide with a data point recorded for
the analog input for the VID signal. The second (NI DAQ 2) is used to time the electrical
stimulation of the nerve and downstream recording. The electrical signals and data collection
are controlled by LabVIEW (NI).

An algorithm, written in Matlab (Mathworks), is used to parse the VID data into a matrix
containing each pixel’s data in a column, to facilitate data analysis. The static birefringence,
I0, is measured as light transmitted through the crossed polarizers with no stimulation of APs,
and is evaluated as the mean of a short time-span of the pixel readout prior to administering a
stimulus pulse. The optical signal is expressed as the relative dynamic birefringence \( \Delta I/I_0 \),
which is henceforth referred to as the crossed-polarized signal (XPS). To obtain this signal,
we subtract \( I_0 \) from the pixel readout for each stimulus period and divide the resulting
difference by \( I_0 \). Except for the real-time measurements described below, \( \Delta I/I_0 \) is calculated by
first averaging the traces generated by 100 consecutive stimuli, and then applying a 5-point
moving average filter, and a computational bandpass filter between 0.1 Hz and 1000 Hz to the
averaged traces, which do not remove relevant signal information, since an AP spike occurs in around 1-2 ms.

A walking leg nerve (WLN) from one of the four rostral-most walking legs of a lobster, *Homarus americanus*, is removed using the Furusawa pull-out method [18] and placed into the nerve chamber. The nerve is immersed in *Homarus* Ringer’s solution [19,20]. The nerve has ~1000 axons, whose diameters vary from ~0.1 to 100 μm in three groupings of small (0.1-5 μm), medium (20-30 μm) and “giant” (50-100 μm) axons [20–22]. The nerve is stimulated at 2 Hz (unless otherwise noted) using a 1-ms, 1-mA (single-polarity) square pulse from a linear stimulus isolator (WPI, Inc. A395R-C), initiated by another channel of the PG that is gated by a trigger from NI DAQ 2 using a count of the EOS signal. A stimulus current of 1 mA was found to recruit a near-maximum number of axons, while not overstimulating, as discussed previously [13]. The PD array is operated near the maximum rate, ~7000 lines/sec, so for a typical stimulus period, this provides ~3500 frames per stimulus. The electrical recording is amplified by a factor of 1000 using a low-noise bandpass amplifier (Amp) (A-M Systems, 3000) with a bandpass filter between 0.1 Hz and 10 kHz.
3. Results

In Fig. 2, depictions of the average of one hundred stimulus periods of the propagating crossed-polarized signal (XPS) associated with action-potential activity are shown for times of 10, 12, 17, 32, 70 and 200 ms post-stimulus. Each bar represents the XPS for a single pixel, and the signal propagates from left to right, over a ~2 cm length of the nerve. The reduction in peak magnitude, increasing temporal width of the peak, and gradual recovery, reported previously [13], are seen here as well. The traveling signal is reliably tracked, and this is demonstrated in Visualization 1.

![Fig. 2](image)

Fig. 2. The average of the XPS over 100 stimulus periods as a function of pixel number are shown at post-stimulus times of 10, 12, 17, 32, 70 and 200 ms (A-F, respectively). The onset of the peak resulting from the compound action potential is evident (A-C) and the gradual recovery to baseline (D-F) lasts hundreds of milliseconds. The propagating peak of the XPS takes ~20 ms to travel a distance of ~2 cm. This is demonstrated in Visualization 1.

To demonstrate ‘real-time’ XPS detection, the frames associated with a single stimulus period, from among the hundred were averaged in Fig. 2. One such example is shown in Fig. 3. We found that a computational 4th-order Butterworth filter with cutoff frequencies at 0.3 Hz and 100 Hz provides smooth data without significantly removing relevant information. In this sample, the central ~1.5 cm of the nerve length is depicted. The ‘real-time’ traveling XPS from left to right is observed, and the same temporal characteristics of the onset and recovery of the peak are evident, as were seen in the averaged data. This ‘real-time’ tracking is demonstrated in Visualization 2.
Fig. 3. A random, single stimulus period is extracted from the data, and a smoothing computational filter with cutoff frequencies of 0.3 Hz and 100 Hz is applied. These data provide a demonstration of a ‘real-time’, fast tracking of the XPS signal. Timepoints of 10, 12, 17, 32, 70 and 200 ms are shown (A-D, respectively). The onset and recovery of the peak can be reliably detected without averaging. This real-time tracking is demonstrated in Visualization 2.

Figure 4 shows XPS traces (from the same data presented in Figs. 2 and 3) for specific pixels correlating to two distances from the stimulus site along the length of the nerve. These are shown for both the average of one hundred stimulus periods (A and B) and for a single stimulus (C and D). The delayed onset and broadened width of the peak as a function of distance is evident when comparing data from the location at 18 mm with that from 6 mm, for both averaged and ‘real-time’ data. The noise is on the order of $10^{-4}$ relative to the baseline intensity in a non-averaged, single trial after filtering, and the signal is consistently $>10^{-4}$. With one hundred averages, the noise is reduced by a factor of ~10.

When the repetition rate of stimulation is increased from 2 Hz to 14 Hz, the shape of the XPS peak is altered, as shown in Fig. 5 for post-stimulus times 15, 20, 35 and 65 ms. As can be seen, the temporal width decreases substantially and the recovery is forced to occur at a
much faster rate. Whereas the gradual recovery lasts hundreds of milliseconds under less-frequent stimulation, when stimulated at a faster rate, the recovery lasts only until the next stimulation event. This is demonstrated in Visualization 3.

Fig. 5. XPS data for averaged stimulus periods at a faster stimulation rate of 14Hz are shown for post-stimulus times of 15, 20, 35 and 65 ms. The recovery of the peak occurs at a faster rate and the gradual recovery is forced to return to baseline just before the initiation of the next stimulus pulse. The temporal width of the peak is reduced. This is demonstrated in Visualization 3.

In Fig. 6, a comparison of the electrical recording and XPS measured close to the electrical recording site for both 2-Hz (A) and 14-Hz (B) stimulation rates is shown. As is the case with the XPS, the electrical signal in the faster stimulation rate is forced to return to baseline just before the subsequent stimulus event. For the 2-Hz stimulation rate, the duration of the return to baseline of the electrical signal is in a range 300-450 ms (depending on the sample), and is generally about the same for the XPS. The temporal widths of the peaks of the electrical recordings coincide with the XPS peaks for both the 2-Hz and 14-Hz stimulation rates.

Fig. 6. A) The electrical recording (black) and XPS (red) at a standard stimulation rate, 2 Hz. The nerve demonstrates an adaptive response to the faster stimulus, and no reversal of polarity, which is evident with a standard stimulation rate. B) The electrical recording (black) and XPS (red) at a faster stimulation rate, 14 Hz. The XPS peak width is reduced with a faster stimulation, which may indicate a reduction in the recruitment of axons to generate action potentials as a result of adaptation to fast stimulation. The peak of the XPS coincides with the peak of the electrical recording for both fast and standard stimulation rates.

4. Discussion

Fast, noninvasive methods for real-time imaging of neuronal signals are lacking. Changes in birefringence of nerve tissues occur as a result of the direct polarization changes induced by local electric fields fluctuating with propagating action potentials (APs) [13–15].
discussed in our previous work [13], other intrinsic tissue phenomena, such as cellular swelling, may marginally contribute to these changes in birefringence, but the effects are largely overshadowed by those caused by changes in the electric field. The measurement of changes in birefringence is adequate for fast, real-time detection in structured neuronal tissues such as peripheral nerves. In this study, we demonstrate real-time imaging based on changes in birefringence along a lobster walking leg nerve (WLN).

In previous work, we described the temporal characteristics of the crossed-polarized signal (XPS) for the WLN in detail, finding that the evolution of the XPS peak and gradual recovery to baseline can be explained by the spreading compound APs, a bulk tissue capacitance, and ephaptic coupling between adjacent axons [13]. Here, using a fast linear photodiode array, we observe the same phenomena in real time, including the temporal broadening of the peak as a function of distance from the stimulus location (and the concomitant loss of peak height), and a gradual recovery to baseline. This serves as a verification that our new setup and detection method are capable of capturing in a “movie” much of the same detail as our previous method, which captured the temporal history at a single point using a single photodiode. Repeated stimulus averaging is helpful to reduce the relative shot noise and increase the SNR, but would be a limitation when attempting to study spontaneous neuronal activity in vivo. In analyzing a single stimulus period, we show that real-time tracking of the XPS is feasible. Visualization 2 demonstrates real-time imaging of the propagating compound XPS in the WLN with adequate SNR.

With the type of instrumentation employed in this set of experiments, the compound AP electrical recording is measured between two electrodes across a ~4 mm length of nerve, through Ringer’s solution. When subjected to a faster stimulation rate, the nerve exhibits an adaptation, which is evident in the electrical recording seen in Fig. 6(B). The shapes of the XPS traces in Fig. 6 further demonstrate this adaptation, as the width and magnitude of the peak decreases with a faster stimulation rate (note the time scales). The XPS appears to recover just before a subsequent stimulation event, which is due in part to a DC drift in the pixel readout caused by the rate of stimulation being higher than the recovery time of the XPS. Despite the rapidity of the fast stimulation rate, the signal is still cleanly tracked and the characteristic phases of the XPS in the WLN are evident (shown in Fig. 5).

Since changes in birefringence in neurons are associated instantaneously with electrophysiological phenomena, they are well-suited for fast imaging of propagating action potentials in neuronal tissue. While these signals have been imaged primarily in a transmission setup, measurement has also been demonstrated in a backscattering geometry, although the SNR was lower than in transmission [23]. Both regimes may be useful toward utilizing these signals to noninvasively image AP activity in nerves. Backscattering geometry could be used to image activity in higher-level neuronal tissues, such as the cortex, but due to their less-oriented structure, the XPS would be more difficult to detect. Polarization-maintaining fibers could be used to direct light to and from a sample in vivo for transmission or reflection geometry. Additionally, circularly polarized light may be advantageous for imaging back-scattered light in thicker tissues, since circularly-polarized light retains its polarization state through more scattering events than linearly polarized light [24]. In summary, imaging based on polarization sensing of changes in birefringence offers promise for an improved noninvasive method of detecting and tracking AP activity.

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