Action Potential-Induced Fluorescence Changes Resolved with an Optical Fiber Carrying Excitation Light

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With the use of a single, implantable, optical fiber, to excite fluorescence and detect changes from voltage-sensitive dyes, transmembrane potential changes were measured without the need for a clear line-of-sight path between the excitation light, the tissue, and the detector. In a previous study, we were required to use signal averaging and could detect only cardiac action potentials from frog. In the present study we improved this system so that unaveraged cardiac action potentials were resolved with high fidelity, and action potentials from single nerve axons were detected. Endeavors to optimize the signal-to-noise ratio resulted in the selection of a larger core fiber with a rounded tip, styryl dyes, and filters based upon fluorescence spectra of the dyes when bound to membrane (rather than in solution). The frog gave signals nearly comparable in magnitude and signal-to-noise ratio to those seen with systems that use a fluorescence microscope. Action potential-induced signals could be detected in single lobster axons with the intracellular injection of a dye. The improvement in the signal-to-noise ratio allowed the use of a reduced-intensity excitation illumination which produced less bleaching of the dye.

INTRODUCTION

Optical recording of electrical activity in excitable cells with voltage-sensitive dyes has been used by neurobiologists for some time [5]. Dyes exist that produce linear changes in absorption of transmitted light or fluorescence from excitation light. Two advantages of optical recording of voltages are the ability to make (i) intracellular recordings simultaneously from many sites that could not be penetrated by microelectrodes [10, 15]

and (ii) recordings that are isolated from stimulus artifacts [4].

Until recently, the use of optical recording has required extensive dissection because of the necessity of a direct line-of-sight path between the living tissue and the recording system, usually utilizing a microscope. Two recent works with optical fibers have only partially obviated this constraint. Dillon [3,4] employed a multiple fiber system in which excitation light was emitted from one fiber and the fluorescence detected by other fibers in the bundle. Kudo *et al.* [11] used two fibers held in a micropipette for exciting and detecting, respectively, the fluorescence from a calcium-sensitive dye. As a result of the use of more than one fiber in both of these systems, spatial resolution is limited, areas directly under the excitation fiber (that receive the most fluorescence excitation) are not closest to the detection fiber, and significant tissue disruption would occur if these fiber systems were implanted. In our previous prelimi-

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nary work [1], we described a technique for using a single, implantable, fiber for both delivering the excitation light and carrying the fluorescence to a detector. The technique suffered from a poor signal-to-noise ratio that required the averaging of multiple events, and the signals could be detected only from cardiac muscle, not neurons. The capability of this system was limited by (i) the high concentration of dye required, (ii) the low intensity of the fluorescence generated by the dye, and (iii) the fluorescence collection capability of the 4-μm-core fiber. The latter two limitations affect the size of the optical signal. In this paper we describe improvements to this system, which now can be used with different dyes at lower concentrations and which permit the clear resolution of single (unaveraged) cardiac action potentials and the detection of action potentials from single lobster axons.

MATERIALS AND METHODS

Animal Preparations

Frogs (*Rana pipiens*) were obtained from Carolina Biological Supply (Burlington, NC), and their hearts were prepared as described by Bowmaster *et al.* [1]. The formulae for the frog saline solutions have been described by Salama [16]. To prevent movement, the hearts were bathed in a calcium-free saline solution. Lobsters (*Homarus americanus*) were obtained from local fish markets, and their circumesophageal connectives were removed according to the method of Dalton [2]. The saline solution used was from Maynard and Walton [12].

Optical Recording Apparatus

The arrangement for the optical recording is illustrated in Fig. 1. The light source was either a red (633-nm) helium–neon laser (Spectra-Physics, Mountain View, CA; Model 105-1, 5 mW) or a green (543-nm) helium–neon laser (Siemens, Iselin, NJ; Model LGK-7770, 0.5 mW), depending upon whether a red-excited oxonal dye (WW781) or a green-excited styryl dye (RH237, RH414, or RH461) was used. The laser light was attenuated by a neutral density filter (by a factor ranging from 10^1 to 10^5), a procedure necessary to reduce the extent of dye bleaching. The light next passed through an angled, short-pass, interference filter (Oriel, Stratford, CT; Model SP675nm for red excitation and Model SP575nm for green excitation). This filter served as a dichroic beam-splitter, where the longer-wavelength fluorescence was reflected to the photodetector. The light was next fo-
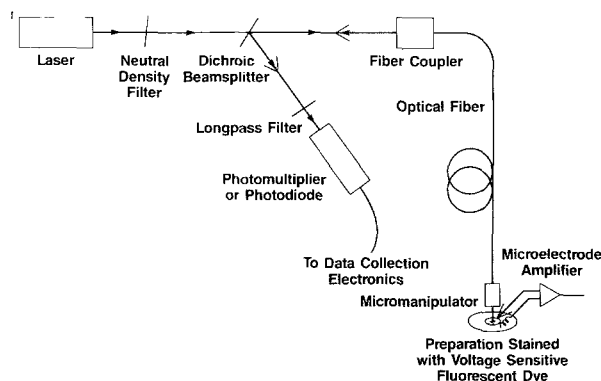

Fig. 1. Diagram of optical recording system (adapted from Ref. 1 with permission).

cused into the fiber through a $\times$20, 0.40-numerical aperture microscope objective held in a fiber coupler (Newport, Fountain Valley, CA; Model F-1015). The three fibers used (Newport Models SW, MSD, and MLD) had core diameters of 4, 50, and 100 μm and cladding diameters of 125, 125, and 140 μm, respectively. For some experiments, the tip of the 100-μm core fiber was rounded (into a hemispherical shape) by melting it with a hydrogen torch, in order to form a crude lens. The fiber end was held by a micromanipulator and placed in the stained tissue. Returning fluorescence was focused by the microscope objective and reflected by the dichroic beam-splitter (angled short-pass filter). A long-pass barrier filter (Schott, Duryea, PA; Model RG665nm for red excitation and Model OG570nm for green excitation) was used in front of the detector to prevent reflected laser light from reaching the detector. The fluorescence detector was usually a photomultiplier tube (Hamamatsu, Bridgewater, NJ; Model R1333) operated at -1.5 kV and terminated with a 10-kΩ resistor as a current-to-voltage converter. A photodiode (Hamamatsu Model S2386-44K) was employed for detection of high light intensities. Without neutral density filters, the light transmission from the laser to the end of the 100-μm core fiber was 40%. Moreover, with the system configured for green excitation, 95% of the light (at 633 nm) that entered the fiber was detected by the photomultiplier tube.

Voltage-Sensitive Dyes

Initial experiments with frog heart [1] used the oxonol dye WW781 (available from Molecular Probes, Eugene, OR) as has been used in a number of previous studies (see Ref. 16). The dye was applied at a concen-

tration of 1 mg/ml (1.32 mM) for 30 min and gave a fractional change in fluorescence of 1/1000 for a cardiac action potential. We also chose to try certain styryl dyes that reportedly can be used at micromolar concentrations with fractional fluorescence changes approximately 10 times greater in magnitude than oxonol dyes [6,7].

In the frog heart the styryl dye RH237 (Molecular Probes) was used. It was dissolved in 100% ethanol to a concentration of 3.8 mM. This dye–ethanol solution was diluted 1/100 with saline solution to obtain a final concentration of 38 μM (20 μg/ml) and applied to excised frog heart or lobster nerve for 30 min.

In the lobster axon the only dye that produced a signal was the intracellularly injected styryl dye RH461. The technique of Grinvald et al. [7] was followed, in which microelectrodes were filled with a 100 mM solution of the dye in distilled water and positive current was used to iontophorese the dye into the cells. None of the extracellular dyes tied, including WW781, RH237, and RH414 (1 mg/ml in saline [6]), produced any detectable signal.

Electrophysiological Recording

Standard techniques were employed for electrophysiological stimulation and recording. The instruments used were a Grass SD9 stimulator (Quincy, MA) for stimulation, a Grass P15 preamplifier for extracellular (electrocardiographic) recording, and a Getting 5A microelectrode system (Iowa City, IA) for intracellular recording. Microelectrodes were pulled from 1.0-mm thin-walled tubing (0.75-mm I.D.) and had resistances of 20 MΩ when filled with 4 M potassium acetate and 70 MΩ when filled with 100 mM WW781. Extracellular stimulation of lobster nerve fibers was accomplished with a suction electrode fabricated from polyethylene tubing.

RESULTS

Fiber Types

Larger multimode fibers have the advantage of better light collection efficiency. This was tested with some fluorescence measurements. Table I compares the amount of green laser light emitted from various core-diameter fibers with the amount of fluorescence returned from a dye (1 mg/ml WW781). Light intensities were measured with a photodiode and the values were normalized to those of the 4-μm fiber. The main finding was that the 100-μm-core fiber allowed a return of 80 times as much

Table I. Comparison of Fibers[a]

Fiber core diameter (μm)	Emitted light	Returned fluorescence	Returned fluorescence/ emitted light
4	1.00	1.0	1.0
50	2.55	82.1	32.2
100	2.84	227.9	80.2

[a]Intensity measurements were made of light emitted from tips of the fibers and returned fluorescence from a solution of voltage-sensitive dye (WW781, 1 mg/ml) for the three fiber diameters.

fluorescence per unit of excitation illumination as the 4-μm-core fiber.

A second advantage of the larger multimode fibers is their greater ability to collect light at a distance. This is illustrated in Fig. 2, which shows plots of percentage maximal fluorescence intensity as a function of fiber distance from a flat fluorescence source. The principal finding to emerge from these graphed relationships was that attenuation was decreased with multimode fibers (half-intensity at 100 μm) compared with the single-mode fiber (half-intensity at 25 μm).

Styryl Dyes

The styryl dyes, compared with the oxonol dyes, have the advantage of generating stronger signals and of being employable at low concentrations [6,7]. Figure 3 illustrates fluorescence spectra from one of these dyes (RH237) in solution when bound to mouse neuroblastoma cells. The mouse cells were selected because they remain viable in suspension during the measurement of their spectra. The dye is about 10 times as fluorescent when bound to cells as it is free in solution, and the fluorescence emission spectrum is shifted to the left. The peak excitation of the dye bound to cells is at 498 nm when emission is measured at 664 nm. We found that, by using 10-nm-band-pass filters between 590 and 700 nm, the action potential-related signal was broad-banded, and the long-pass filter allowed us to detect the entire signal.
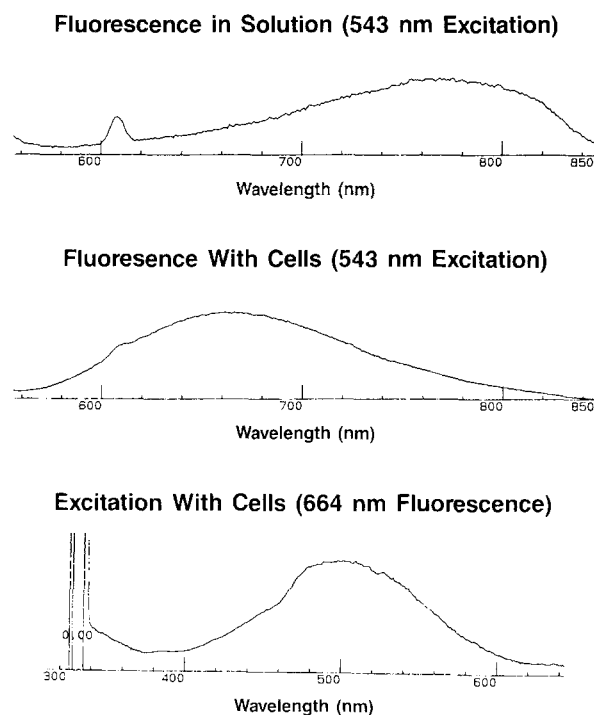
Recordings from Heart

The dye RH237 gave signals with a high signal-to-noise ratio when a 100-μm-core fiber was used (Fig. 4A). The heart that generated the signal was spontaneously rhythmic. The upper trace is an electrocardiogram with a QRS complex at 0 ms and a T-wave at 900 ms; the lower trace is the optical signal which reflects
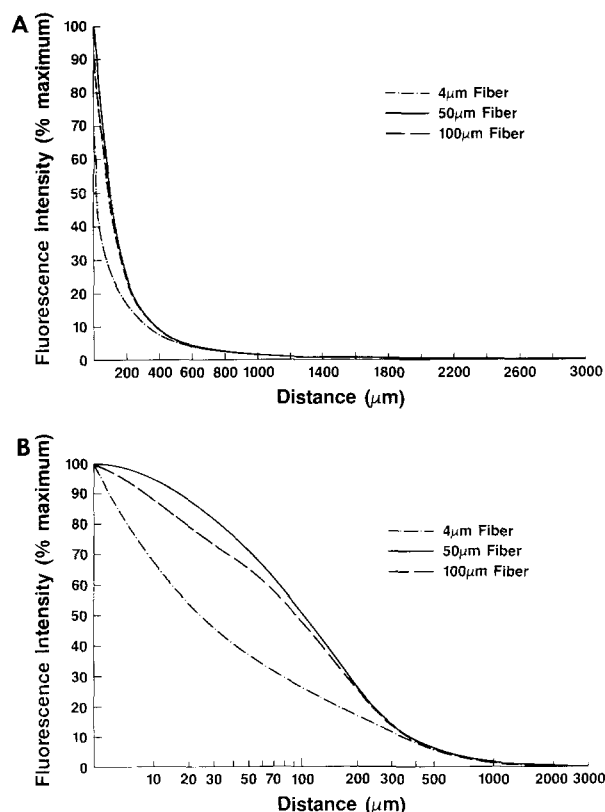
Fig. 2. Attenuation of fluorescence with distance. This is a plot of the percentage of maximum fluorescence intensity with distance from a fluorescent surface in air. The plot is shown for fiber-core diameters of 4, 50, and 100 μm. A is plotted on normal axes, and in B distance is plotted logarithmically. Measurements were made at 10-μm intervals. The fluorescent intensities have been normalized; the actual magnitudes for the 50- and 100-μm fibers were 92 and 450 times the value of the 4-μm fiber. Note that half-intensity is reached at distances of 24 μm for the 4-μm fiber, 103 μm for the 50-μm fiber, and 95 μm for the 100-μm fiber.

Fig. 3. RH237 spectra. The top spectrum is from a 38 μM aqueous solution of RH237 in phosphate-buffered saline (PBS). The middle spectrum is from a mouse C-1300 neuroblastoma cell suspension [9] stained with 38 μmol of the dye (for 10 min) and rinsed with PBS; the fluorescence magnitude is plotted at 1/10 the scale as for the dye in solution (upper spectrum). The bottom spectrum is of the dye excitation bound to the cells measured at the peak fluorescence of 664 nm. These spectra were made with an SLM Instruments (Model SPF 500C) spectrofluorometer (Urbana, IL).

the cardiac action potential. This optical signal is expressed as a change in fluorescence over baseline ($\Delta F/F$). It was possible to average the optical signal (middle trace) because bleaching of the dye was not a significant problem at the excitation intensity used. It took approximately 40 min of continuous illumination for the fluorescence intensity to drop to half-intensity. One interesting feature of the 100-μm-core fiber was that the movement artifact that occurs in beating hearts when calcium was present is less than with the 4-μm-core fiber [1]. The lower trace in Fig. 4B illustrates this. The trace begins with the depolarization within 10 ms of the stimulus (time 0); after 100 ms it is followed by a second deflection, which represents the contraction; the third deflection, at 570 ms, simultaneous with the T-wave, represents

repolarization; relaxation occurs after 700 ms. The size of the part of the signal related to movement varied greatly and depended on the location of the fiber in the heart.

As a basis for comparison with the technique of Bowmaster et al. [1], who employed a 4-μm-core fiber, we repeated the work employing a 100-μm-core fiber. The signal generated by a cardiac action potential recorded with 1/360 the excitation light used by Bowmaster et al. [1] was half the amplitude (relative to the noise). Single, unaveraged, action potentials could be resolved with the same lower excitation light intensity. A photodiode was employed for these recordings because the fluorescence intensity was high enough to obviate the use of the photomultiplier tube.
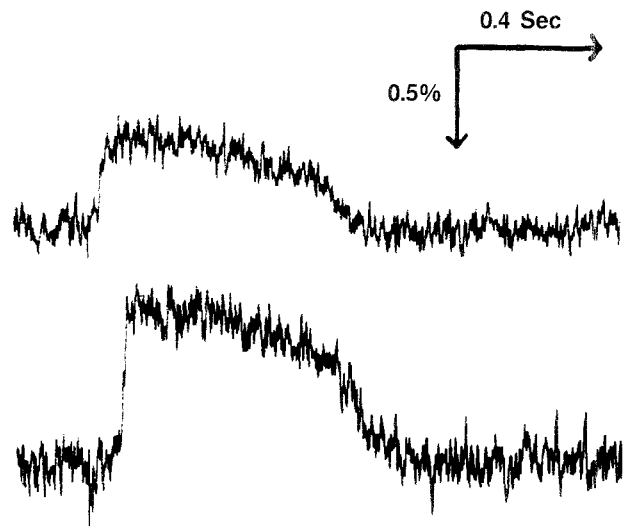
The one additional measure taken to improve the signal-to-noise ratio was rounding the tip of the fiber with a hydrogen torch to create a crude hemispherical lens. The beam diameter of the excitation light was thereby made smaller, and presumably, so was the field of col-
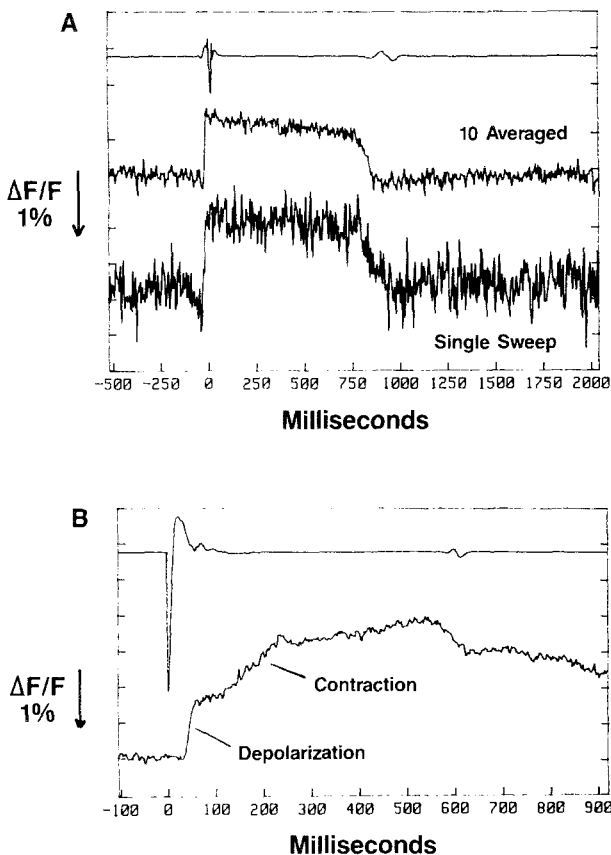
Fig. 4. Cardiac action potential. The frog heart was stained with RH237; green (543-nm) excitation, a 10^{-2} neutral density filter, and a 100-μm-core fiber were used. The signals were processed through a passive high-pass filter ($\tau = 10$ s), which removed the DC component of the signal, and a passive low-pass filter ($\tau = 1.4$ ms), which removed high-frequency noise. (A) Spontaneously generated action potentials from the ventricle in calcium-free saline solution. The upper trace is the electrocardiogram (ECG), the lower trace is a single unaveraged optically recorded action potential, and the middle trace is an average of 10 action potentials that was synchronized from the peak of the QRS-wave of the ECG. (B) Recorded in the same manner as A from a paced heart with 0.1 mM Ca^{2+} present; a movement-related signal (contraction) that occurred 100 ms after the depolarization.

Fig. 5. Lens on fiber. Rounding the tip of the fiber improved the signal size from the frog heart. The optical recording was performed as in the legend to Fig. 4. The upper trace is a single record from a frog atrium detected with a 100-μm-core nonrounded fiber. The lower trace was made 10 min later with the rounded-tip fiber from the same spot on the atrium.
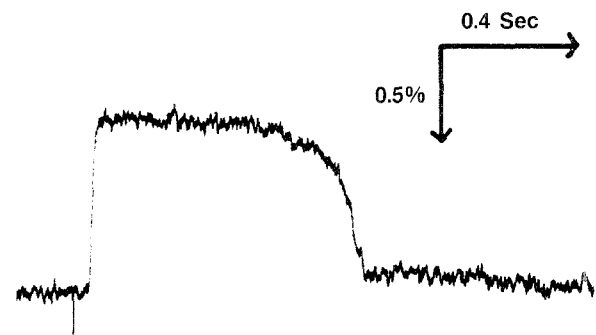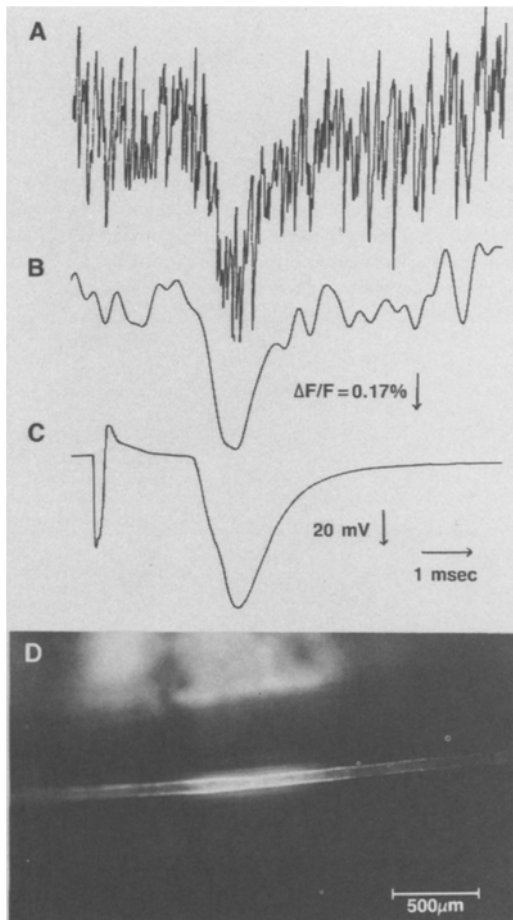

Fig. 6. Optimized recording. A single (unaveraged) action potential from frog atrium. It represents a typical recording employing a 100-μm-core fiber with a rounded end and 10 times the light intensity (10^{-1} neutral density filter) that was used in Fig. 4.

lection. Experiments performed to compare the non-rounded-tip and rounded-tip fibers were performed at the same location in the heart. Figure 5 illustrates the gain in signal size from the rounded-tip fiber. The increased signal is due to reduced baseline fluorescence (F) rather than an increased fluorescence change (ΔF).

With the use of the aforementioned changes to the system, a good-quality cardiac action potential could be recorded without averaging as illustrated in Fig. 6. The heart that generated this signal was stained with RH237, a rounded-tip 100-μm-core fiber was used, and the ex-

citation light intensity was increased by a factor of 10 over the intensity used in Figs. 4 and 5. With the higher light intensity, bleaching of the dye to half-fluorescence intensity occurred in 6 min.

Recordings from Lobster Axons

Numerous attempts were made to record from lobster axons in desheathed circumesophageal connectives

with superfused fluorescent dyes (WW781, RH237, RH414). All of the dyes stained and fluoresced strongly, but no action potential-related signals could be discerned from the noise. Success came with the intracellular injection of the styryl dye RH461. The baseline fluorescence (and noise) was less by a factor of approximately 100, and action potential signals could be detected above the noise. Figures 7A and B illustrate an average of 64 traces from a lobster axon with the dye iontophoretically

applied. The recording in Fig. 7B was digitally filtered [15]. The nerve was stimulated with a suction electrode at one end, and the optical fiber was located about 1 mm from the intracellular (dye-filled) microelectrode with which the electrical recordings were made (Fig. 7C). The signals are inverted because the dye molecules are bound to the inner membrane so they experience an opposite electric field than when the dye is applied to outer membrane. Dye fluorescence was detected up to 3 mm from the injecting electrode. The excitation light intensity was kept low (as in Figs. 4 and 5) because photodynamic damage at greater intensities caused the action potential to decrease in amplitude (probably due to propagation failure beyond the damaged spot). Dye bleaching did not occur at this intensity since the baseline fluorescence did not change over 5 min; however, the signal size was reduced considerably over that 5 min. This may be due to the dye leaving the cell and binding to the outer membrane (Lev-Ram, personal communication). The dye was visible with a fluorescence microscope as shown by Fig. 7D. The dye fluorescence in this micrograph was visible only to 1 mm from the injection site, even though it could be detected to 3 mm.

DISCUSSION

Our results show that the technique of optical recording with fluorescent voltage-sensitive dyes can be extended to use with single optical fibers with comparable signal-to-noise ratios as with other techniques. Cardiac action potentials were recorded through a fine optical fiber (with an outer diameter of 140 μm) without signal averaging. Action potentials from single axons were discerned. One improvement made was using a 100-μm-core fiber rather than the 4-μm-core fiber used previously [1]. This allowed 80 times as much fluorescence to be collected, which should theoretically improve the signal-to-noise ratio by about a factor of 9 ($\sqrt{80}$). Although the signal-to-noise ratios varied between preparations and locations, we generally saw signal-to-noise ratio improvements of this magnitude.

A second improvement was utilizing green excitation (543 nm) light rather than red (633 nm). The availability and relatively low-cost green helium–neon lasers allowed us to try a variety of styryl dyes that are excitable at this shorter wavelength and can be used at lower concentrations, with less photodynamic damage [6,7]. We also found that the fluorescence spectrum of one of these dyes, when bound to cells, is of a short enough wavelength to be optimally detected by a photomultiplier tube. Additionally, calcium-sensitive fluorescent dyes are

Fig. 7. Axonal recording. This is an averaged (64 traces) fluorescence recording from a lobster axon stained with the intracellularly injected dye RH461. The same optical arrangement as that used for RH237 was employed here with a 10^{-2} neutral density filter in front of the laser and a nonrounded 100-μm-core fiber. (A) The optical data processed through passive RC filters (5 kHz, low pass; 30 Hz, high pass). (B) The same signal digitally filtered at 1 kHz [15]. (C) An intracellular microelectrode recording made 1 mm distal from the optical recording site. The fluorescence micrograph (D) was made of the axon following recording. Note that the dye appears bound to the membrane. The fluorescence recordings in A and B appear inverted because the dye was applied to the inner membrane, as opposed to the outer membrane as in C and D.

excitable at the green wavelength [13] and can theoretically be used with this system. Recently, Tung and colleagues [14] have used an optically equivalent system to achieve recordings of a high fidelity from frog heart with the voltage-sensitive dye di-4-ANEPPS [8].

A third improvement was rounding the fiber tip. This is probably related to the crude lens narrowing the field of detection and limiting the collection of fluorescence from extraneous fluorescing tissue. The rounded tip has the additional advantage of probably being less traumatic when inserted into tissue.

The difficulty in recording from lobster axons and the necessity for intracellular dye injection underscore a limitation intrinsic in using optical fibers. The problem is that fibers have a low collection efficiency as the distance between the fiber and the fluorescing tissue increases (Fig. 2). Most likely, in the case of bath application of dyes to lobster nerve, nonexcitable glial and connective tissue generated most of the background fluorescence (and noise), which obscured the signal from the more distant axon membrane. With intracellular dye application, only the excitable tissue was stained and thus fluoresced. This is in contrast to frog heart, in which a great proportion of the stained tissue is excitable.

This single-fiber technique is potentially useful in applications other than those described here. The chief advantage of the fiber technique is that a line-of-sight path is not needed for fluorescence measurements. The fiber is implantable in tissue with relatively little disruption. Light, once entering a fiber, can be carried many meters without attenuation, therefore intracellular optical recording is possible with all of the hardware located remotely from the tissue preparation. We measured fluorescence changes of less than 1% using excitation illumination that did not cause photodynamic damage, and we detected fluoroscence that was not visible through a fluorescence microscope. Although we have improved this system considerably, our recordings are still from single sites and are noisier than those made through the optics of a microscope by Salama [16]. These disadvantages must be considered before adopting such a system. Similar fluorescence measurements through optical fibers can, in theory, be applied to any other fluorescent indicator, either physiological or anatomical.

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