FLUORESCENCE MONITORING OF ELECTRICAL RESPONSES FROM SMALL NEURONS AND THEIR PROCESSES

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ABSTRACT To improve the sensitivity of fluorescence measurements of electrical responses from small cells and their processes, we have optimized the optical measuring system. The fluorescence intensity from a stained cell was increased 40-fold relative to our previous apparatus. The increased fluorescence intensity permits the use of an inexpensive photodiode (or a photodiode array) that has a ~10-fold higher quantum efficiency relative to a photomultiplier. Utilizing the improved apparatus, we optically recorded an action potential of a 2 μm wide neuronal process with a signal-to-noise ratio of ~50 (root mean square noise) without averaging. We also report the design of an improved fluorescence voltage-sensitive probe; the fractional change of the fluorescence signal under optimal conditions was 21%/100 mV.

INTRODUCTION

The measurement of the active or passive electrical properties of small neurons and their processes with microelectrodes is often technically difficult or impossible. Fluorescence signals of voltage-sensitive dyes have provided a new and powerful method for measuring changes in membrane potential in a wide variety of preparations (Cohen and Salzberg, 1978; Cohen et al., 1978; Waggoner, 1979). Progress has been achieved in the development of such optical probes for the measurement of rapid changes in membrane potential (Cohen et al., 1974, Ross et al, 1977, Gupta et al., 1981).

Recently we have considerably improved the fluorescence methods, especially for measurements from dissociated cells in tissue culture, and thus were able to record from large nerve processes or growth cones (Grinvald et al., 1981b; Grinvald and Farber, 1981). The synthesis of better fluorescence voltage-sensitive dyes has made the technique more practical (Grinvald et al., 1982a) and has also permitted the recording of electrical activity and synaptic responses from the site of the synapses within a neuropile (Grinvald et al., 1982b).

In this communication we focus on the optimization of the apparatus that led to ~20-fold improvement in sensitivity and that permits the use of a photodiode array to detect the fluorescence signals. We demonstrate here that the predictions made in the preceding paper (Grinvald et al.,

This paper is dedicated by R. Hildesheim to Professor E. Lederer on the occasion of his 75th birthday.

1982a) have now been realized, and report the design and synthesis of a better probe. For a critical discussion of the difficulties associated with the measurements, see the preceding paper.

RESULTS

Our experiments were carried out on mouse neuroblastoma cells, NIE-115, cultured as described by Kimhi et al. (1976). More details were presented in the preceding paper.

Fig. 1 illustrates the improvements in fluorescence recording of changes in membrane potential. Fig. 1 A depicts a single-sweep optical recording from a 70-μm diameter neuroblastoma cell body stained with the new probe, RH-421 (the dipentyl analog of RH-160 [see preceding paper]). This dye is the most sensitive fluorescent dye thus far tested, generating a fractional change in fluorescence of 21%/100 mV potential change. (An average value of 22 ± 3% [SD] was obtained in four cell-body experiments, when the fractional change was corrected for background light.) The experiment was done on the old apparatus, but the signal-to-noise ratio is improved because a better dye was used and a photodiode was employed in place of a photomultiplier. (The previous best dye for neuroblastoma cells, RH-237, generated a fractional change of 12 ± 4% [six experiments, under similar conditions].)

Fig. 1 B illustrates the experimental arrangement for simultaneous electrical recording from the soma and fluorescence recording from a process. Fig. 1 C depicts a
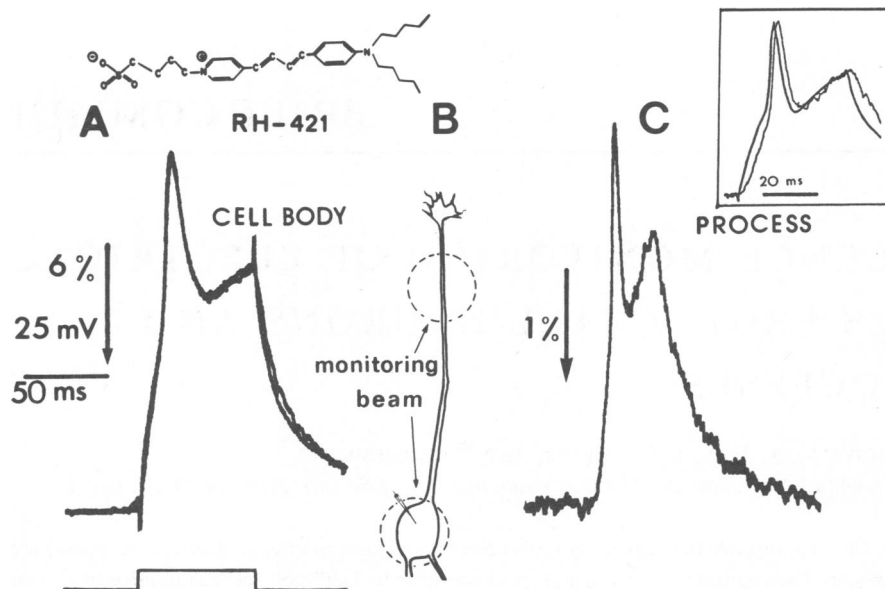
FIGURE 1   Comparison of optical and electrical recordings. (*A*) Comparison of single-sweep fluorescence and intracellular recordings from the soma. The cell was stained for 5 min with a 2-μM solution of the new probe, RH-421, in balanced salt solution. The structure of the dye is shown at the top. The soma was then stimulated with a current pulse (*lower trace*). The two recordings are essentially identical in time course. The arrow shows the normalized fractional change in fluorescence (depolarization results in a fluorescence decrease) that was 21%/100 mV in this experiment (corrected for the 31% background that was measured when the cell was removed from the field of view). The time constant of both the optical and electrical detection systems was 1.5 ms. (*B*) Schematic drawing of the neuron illustrating the experimental arrangements for somatic or processes recordings. The mercury lamp excitation beam (monitoring beam) was positioned over the desired target by moving the xy-positioner under the microscope (see text). Soma recording is shown in *A* and the process in *C*. (*C*) A single-sweep recording from a 2-μM wide process. The cell was stained with 1-μM RH-273. The soma was stimulated with a current pulse and the measurement was made 500-μm away from the soma. The arrow shows the fractional change (uncorrected for background light) in fluorescence. *Insert:* comparison between the electrical somatic recording and the same fluorescence recording on an expanded scale. (In both experiments *A* and *C*, the cells were hyperpolarized to ~ -80 mV with a DC current and the fluorescence trace was corrected for bleaching and light-source noise.)

typical single-sweep fluorescence recording of an action potential from a 2-μm process of a neuroblastoma cell stained with another voltage sensitive probe, RH-237 (Grinvald et al., 1982*a*). The signal-to-noise ratio for this process recording, which was done on the new apparatus, is even better than the best signal previously obtained with the old apparatus for a somatic recording from a 65-μm cell that had ~50 times larger membrane area. The reasons for the strikingly better performance of the new optical apparatus are discussed below.

OPTIMIZATION OF THE APPARATUS

The signal-to-noise ratio (S/N) obtained in fluorescence measurement is given by

$$(S/N) = (\Delta F/F) \cdot (2\tau q)^{1/2} (F)^{1/2} \qquad (1)$$

(see preceding paper and references cited therein). ΔF is the fluorescence signal; F is the total detected light (fluorescence) intensity; τ is the response time of the detection system; and q is the quantum efficiency of the detector. In this equation we assumed that the light-source noise and the dark-noise of the detection system are both negligible relative to the shot noise resulting from random fluctuations in the rate of arrival of photons at the photodetector

surface. This assumption holds only if the fluorescence-light level is relatively high, and the light source is well regulated.

The preceding paper discussed theoretically the optimization of various spectroscopic (i.e., $\Delta F/F$) and instrumental parameters. Here we shall describe their implementation. Since the S/N is proportional to $F^{1/2}$, maximization of the fluorescence-light level is a key to the improvement of apparatus sensitivity. The amount of fluorescence light that reaches the photodetector is given by

$$F \propto I_0 \, (T_{ex} \cdot \beta_{ex} \cdot R_{ex}) \, (T_{em} \cdot \beta_{em} \cdot D_{em}). \qquad (2)$$

I_0 is the intensity of the light source; T_{ex} is the percent transmission of excitation filter; β_{ex} is proportional to the square of the overall numerical aperture (NA) of the epi-illumination system; and R_{ex} is the percent reflectance of the dichroic mirror; T_{em} is the percent transmission of the fluorescence-barrier filter that blocks the excitation wavelengths but passes the fluorescence maximally; β_{em} is proportional to $(NA)^2$ (of the objective) and D_{em} is the percent transmission of the dichroic mirror. (All of the terms except the β's are functions of wavelength.)

It is important that the excitation and barrier filters,

together with the dichroic mirror, allow maximum possible transmission and/or reflection. However, careful attention should also be given in order to transmit and/or reflect exclusively those excitation and emission wavelengths that contribute to the fluorescence signal. Practically, the optimal filters can be determined by testing the signal size as a function of different excitation and emission wavelengths. These are selected with narrow-band (10 nm) excitation and emission filters. Only then can the optimal filter be designed.

In the present experiments we have used a new apparatus built around a Zeiss IM35 inverted fluorescence microscope (Carl Zeiss, Inc., Federal Republic of Germany), rather than the Universal upright microscope. We have detached the microscope stage from the microscope body and rigidly fixed it to the vibration isolation tabletop. The microscope itself was mounted under its fixed stage on a movable XY-table (Calvet and Calvet, 1981). The use of an inverted microscope has the following advantages. (a) One can use an oil-immersion high-numerical aperture objective with relatively short working distance (the microelectrode insertion being done from the opposite side of the preparation). Using a 1.25 NA objective with a magnification of 63 (Zeiss 461820; Carl Zeiss, Inc.), we obtain nine times more fluorescence than with the 0.75 NA water immersion objective with a magnification of 40 used previously (Zeiss 461702; Carl Zeiss, Inc.). The fluorescence intensity was approximately proportional to $(NA)^4$, i.e., $(\beta_{ex}\beta_{em})$. (b) With the inverted microscope one can make microelectrode penetrations at a nearly vertical angle, using heavy and stable manipulators mounted directly on the modified fixed microscope stage. The field of view can be changed after impalement by moving the microscope on its XY-positioners. By contrast, using the Universal microscope and the water-immersion objective, the maximum electrode angle is only $27°$, and only small manipulators mounted directly on the moving microscope stage are useful (e.g., Narashige M103 hydraulic manipulators; Tokyo, Japan). (c) The IM35 has a different optical path than the Universal. A real magnified image of the preparation is formed not only at the photodetector position but also at an intermediate position where the reticle is located. We have modified the reticle carrier to include a rotating disk that carries variable size pinholes or slits. This arrangement permits convenient visually controlled positioning of slits to block background light from neighboring objects while passing the fluorescence emission of the target(s) under investigation, thus maximizing the signal-to-noise ratio.

In addition, the dichroic-beam splitter (FT-580, 466305; Carl Zeiss, Inc.) and the wide-band excitation filter (467994; Carl Zeiss, Inc.) produce about five times more fluorescence relative to the semisilvered mirror and excitation filter used previously (Grinvald et al., 1982a). However, it is our experience that custom-made excitation filters with the sharpest possible red edge are frequently

superior to the standard filters used in microscopy because they yield a better fluorescence per scattering ratio.

The significant increase in light intensity enabled the use of an inexpensive, relatively low-noise photodiode-amplifier combination (HAV-4000; EG&G Inc., Electro Optic Div., Salem, MA) instead of a photomultiplier (other photodiodes may be even more emittable). With the present light level, the light shot noise was larger than the dark noise of the amplifier even when recording from fine nerve processes (see Discussion in Grinvald et al., 1982a). Evidently the increase in excitation intensity increases photodynamic damage and bleaching. If they become a limiting factor, then one may attempt to find suitable antioxidants or free radical scavengers to reduce them (e.g., Giloh and Sedat, 1982).

Photodiodes have the following advantages. (a) Their quantum efficiency of $\sim70\%$ is ~10 times larger than the quantum efficiency of the photomultiplier over the emission wavelengths range of 610–750 nm. Thus the signal-to-noise ratio is improved by a factor of 3.3. (b) An array of photodiodes can be used to obtain simultaneous fluorescence recordings from multiple sites, as previously done only in transmission measurements (Grinvald et al., 1981a). (c) The use of photodiodes is less worrisome than that of photomultipliers because they cannot be easily damaged by over-exposure to high light intensities.

The considerable reduction of the fractional shot-noise level (Noise/F) by a factor of 20 (that is, the square root of a 40-fold increase in fluorescence light intensity times a 10-fold increase in quantum efficiency) has led to a situation where the lamp noise becomes the dominant noise in the experiment. (The peak-to-peak noise of the mercury lamp, occurring at the line frequency, typically constituted $5–10 \times 10^{-4}$ of the steady light level.)

In our modified apparatus, a second photodiode was used to record the time course of the excitation light. The reference diode was mounted directly on the carrier of the dichroic mirror. Thus the line-frequency noise of the illumination was corrected for by subtracting this (appropriately scaled) excitation signal from the fluorescence signal. A factor of 5–10 improvement in the peak-to-peak noise of the optical recording was thus obtained. Typically the remaining noise in a single trial was $0.5–2 \times 10^{-4}$. Thus with all of these improvements, a signal-to-noise (rms) ratio of 50 was obtained for only 1% change in fluorescence intensity.

CONCLUSIONS

The present results demonstrate that remarkable sensitivity of the fluorescence recording technique has been obtained. Thus it can become a powerful tool for the study of electrical properties of small cells and their processes, both in dissociated-cell culture and in intact central nervous system preparations (when the dye is iontophoretically injected into single cells, Grinvald et al., 1982b). The increase in fluorescence intensity by a factor of ~40

permits the use of photodiodes and often eliminate the need for averaging experiments. (200–700 trials had to be averaged to get a similar signal-to-noise ratio in absorption measurements from barnacle dendrites [Krauthamer and Ross, 1982].) However, under such conditions photodynamic damage limits the duration of the measurements to 1–2 s, allowing averaging of only 2–40 trials depending upon their duration. (The bleaching correction is still effective under these experimental conditions.) The sensitivity already obtained justifies additional efforts in the design and synthesis of even better fluorescence probes that will cause less photochemical damage to the investigated system and thus permit longer experiments.

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