In situ visualization and photoablation of individual neurons using a low cost fiber optic based system

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Abstract

This paper describes a low-cost system for in situ visualization and photoablation of single neurons. The system includes a fiber optics light source equipped with a filter port, a blue excitation filter and a yellow barrier filter, and a light guide which terminates with a focusing lens. This system is inexpensive, easy to use, and requires minimal maintenance. Given its price, this system is readily accessible and has the potential of becoming standard equipment for in situ visualization and killing of individual neurons.

Keywords: Photoablation; Fluorescent dye; Identified neuron; Fiber optic; In situ visualization; Neuronal circuitry

1. Introduction

Analysis of how neuronal networks control behaviors has benefited greatly from techniques permitting the selective ablation of identified neurons (for review, see Mountcastle Shah and Jay, 1993). A popular method used for deleting an individual neuron while leaving neighboring neurons intact is the fluorescence photo-ablation technique developed by Miller and Selverston (1979). This method takes advantage of the finding that injection of a fluorescent probe inside a neuron, followed by illumination with an intense light of the probe's excitation wavelength, inactivates the neuron. This technique has been used extensively to delete groups of neurons (Garber and Camhi, 1991), single neurons (Miller and Selverston, 1982; Selverston et al., 1985; Fraser and Heitler, 1991; Warzecha et al., 1992; Bodnar, 1993) as well as selected processes of a single neuron (Cohan et al., 1983; Jacobs and Miller, 1985; Libersat et al., 1989; Camhi and Macagno, 1990; Heitler, 1995). A disadvantage of the technique is the requirement of an expensive system consisting of a fluorescent epi-illuminator system and/or a laser system. The present paper describes a low-cost system which can be used for both in situ visualization of dye-filled neurons and photoablation of single neurons. The system, which includes a fiber optic light source and filters, appears to be as effective in visualizing and killing neurons as any of the more expensive systems available on the market.

2. Material and methods

Adult cockroaches were anesthetized with carbon dioxide and pinned dorsal side up on a recording platform after cutting the legs and wings. Then, the ventral nerve cord was exposed from the second abdominal ganglion A_2 to the sixth and most posterior abdominal ganglion A_6, as described previously (Libersat, 1992). Single giant interneurons (GIs) were impaled in the axon within the A5-A6 connectives with glass micro-electrodes filled with 6% carboxyfluorescein (CF) in 0.44 M KOH (resistance 30–50 MΩ). Antidromic spikes were elicited with extracellular hook electrodes placed more anteriorly around the A4-A5 connectives (Fig. 1). In all experiments, carboxyfluorescein was injected by iontophoresis using 20 nA of steady hyperpolarizing current for 20 min.

For in situ visualization and photoablation experiments, the dye-filled neurons were illuminated with blue light emitted by a fiber optic illuminator (Highlight 3000; 150 W light source, Olympus) equipped with a filter port (Fig. 1). A filter holder housed a blue excitation filter (470 nm; blue exciter filter, Olympus) which corresponds roughly to the excitation wavelength of the carboxyfluorescein. The light beam was then brought to the preparation via a
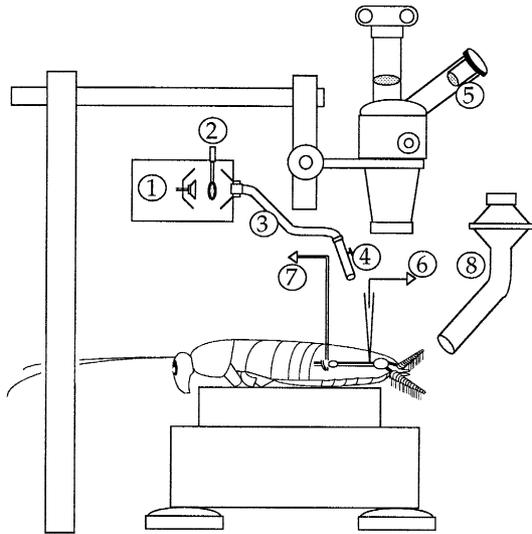
Fig. 1. The experimental setup. The animal is fixed dorsal side up, and the central nervous system is exposed after removing the cuticle and the digestive system. 1, light source; 2, filter holder with blue excitation filter or yellow barrier filter inserted into the filter port. 3, light guide; 4, focussing lens. 5, oculars with yellow filters; 6, intracellular recording of GI's axon; 7, extracellular recording of GIs' axons in the nerve cord; 8, wind stimulator.

universal light guide which terminated with a goose neck (Inspection Light Guide; 1800 mm, fiber bundle $d = 5.5$ mm; Volpi). This goose neck was clamped on a magnetic base, permitting orientation of the light spot. A lens focussed the emerging blue light to a spot diameter of roughly 1 cm at a focal distance of 5 cm (focussing lens for fiber bundle, Olympus). The preparation was viewed through a zoom stereo dissecting trinocular microscope (Olympus; SZ6045TR). Both eyepieces were opened by twisting the mounting rings to remove them from the eyepieces; a yellow barrier filter was mounted in each eyepiece (filter supplementary 20EO515-W22; 515 nm). An empty filter holder was also fitted with a yellow barrier filter to illuminate the preparation without exciting the carboxyfluorescein. Photomicrographs were taken with a 35 mm SLR camera (Olympus SC 35) through the trinocular microscope body equipped with a phototube (SZ-PT) and a photoeyepiece (NFK 2.5X). The phototube was also fitted with a yellow barrier filter. This entire fiber optic system was assembled for roughly $1500.

At the end of the experiment, the ganglion was removed, placed on a slide and the structure of the selected neuron was visualized in a solution of 50% glycerol-50% saline using a BH-2 Olympus epifluorescence microscope. The neuron was identified based on the morphology of its dendritic tree and the location of the cell body within the ganglion (Daley et al., 1981). In virtually every case, the identification of the neuron made previously under the trinocular microscope illuminated with the highlight source with the blue filter was confirmed under the compound microscope.

3. Results

3.1. Visualization of neurons

All impaled giant interneurons could be identified in situ providing that the dye was injected in the axon close enough to the ganglion which houses the cell body and dendrites. Fig. 2 shows the structure of a neuron which was injected in its axon within the A5-A6 connectives. In situ, the fine structure of the neuron's dendritic arborization cannot be observed in great detail (Fig. 2B). This is due, in part, to the partial opacity of the tissue, which at this point has not been processed for histology. However, the location of the cell body, the axon and the general outline of the dendritic area of the neuron are seen clearly enough to permit identification of the neuron. Also, these anatomical features provide potential targets for additional intracellular impalement. For instance, this cell was identified as GI2 based on the location and morphology of its dendritic tree and the location of the cell body within the ganglion (Daley et al., 1981). This was later confirmed after treating the tissue in a glycerol-saline solution and observing the whole mount of the ganglion under an epifluorescence compound microscope (Fig. 2C).

3.2. The photoablation procedure

After injection, the dye-filled neuron was illuminated with the blue light emitted by the fiber optic system. The filled neuron exhibited the characteristic signs of ablation within roughly 0.5–6.5 min depending on the intensity of the fill. For instance, Fig. 3A shows the synaptic evoked response of a GI6 to a brief calibrated wind stimulus. Thereafter, the neuron started to produce spontaneous bursts of action potentials (Fig. 3A,B), and always gave a long-lasting high-frequency injury response before losing its resting membrane potential (Fig. 3A,C). After death, it was not possible to evoke any active membrane response with the wind stimulus. Fig. 4A shows in greater detail the broadening of the action potential in a different GI during the photoinactivation. The action potential evoked by antidromic stimulation first broadened, developing a longer repolarizing phase. Then, the membrane potential failed to repolarize rapidly during the action potential and began to show small amplitude oscillations until finally it remained at zero potential. As a control experiment, a GI was filled with carboxyfluorescein and illuminated with maximum light intensity after inserting the yellow barrier filter. Neither the dye nor the high-intensity illumination with yellow light had any effect on the cell membrane potential, the action potential or the wind-evoked response of the GI ($n = 4$, illumination time > 10 min, data not shown). To calibrate the photoablation fiber optic system, the time of death was measured from the beginning of the blue illumi-

nation until the loss of resting membrane potential in 31 neurons (Fig. 4B). The time of death was 140 ± 86 s (mean $\pm$ SD). The maximum time of illumination necessary to photoablate a GI was 6.5 min.

3.3. Using the fiber optic system for studying cell-cell interactions

The photoablation technique using lasers and epifluorescent attachment has been used to study cell-cell interactions in small neuronal networks (Miller and Selverston, 1982; Selverston et al., 1985; Bodnar, 1993). To study network connectivity, we examined cell-cell interactions in the well-known cockroach escape neuronal network using our fiber optic system. In cockroaches, a group of giant interneurons controls the initiation of the cockroach escape response, a highly directional behavior (Camhi, 1984). Each GI shows a specific wind-receptive field and thus, specifies a given wind angle (Kolton and Camhi, 1995). In this experiment, we tested whether the GIs' directional response is strictly due to the specific pattern of synaptic inputs from the wind-sensitive receptors or also due to specific interactions among the GIs themselves. Because the dendritic arborizations of these neurons overlap consid-
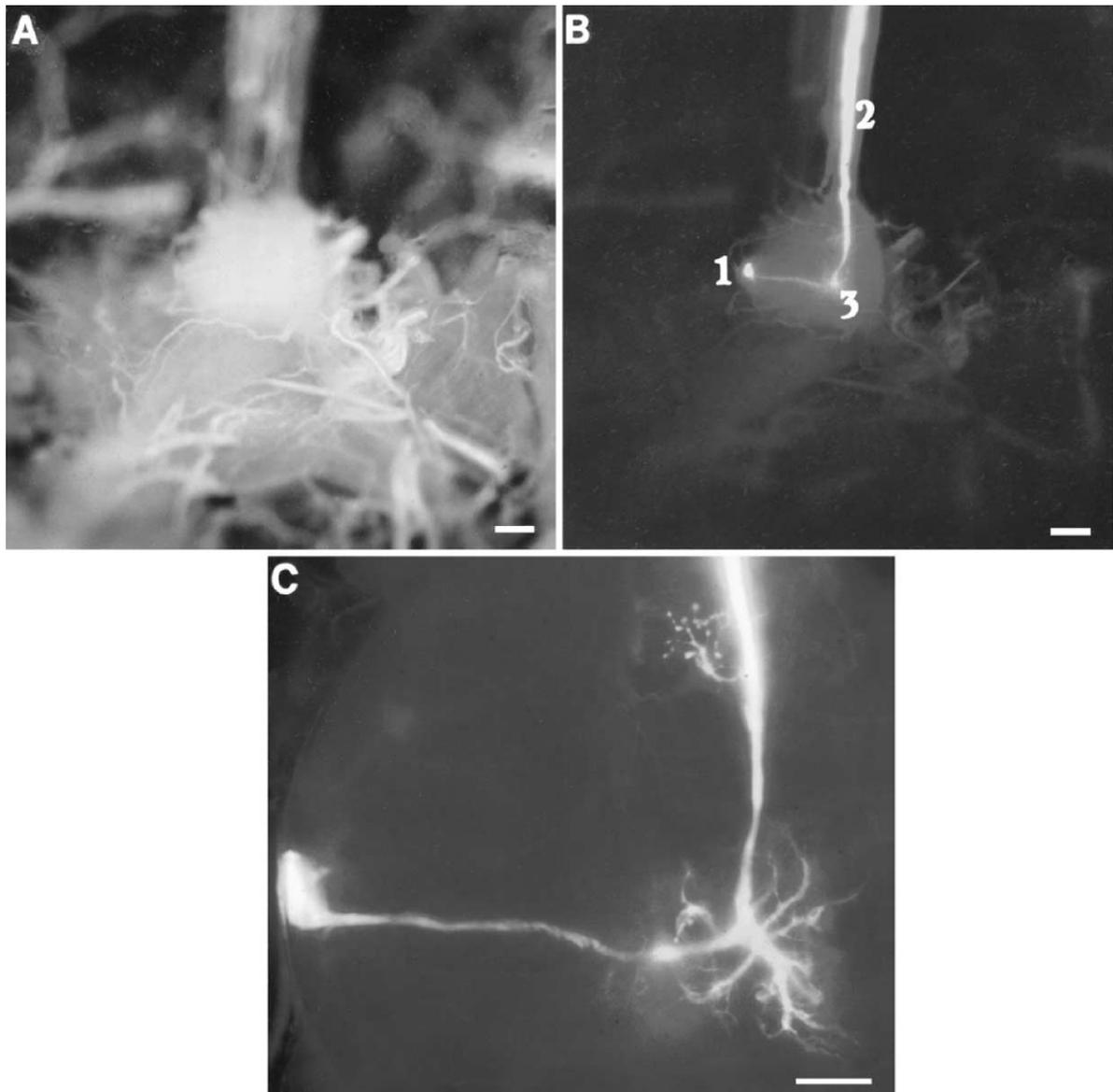

Fig. 2. In vivo visualization of a neuron. (A) Photomicrograph of the abdominal posterior portion of the body cavity viewed through the stereomicroscope under yellow light illumination with the fiber optic system. This shows the outline of the last abdominal ganglion housing the carboxyfluorescein filled neuron (calibration bar: 0.5 mm). (B) Photomicrograph of the same area as A but after inserting the blue excitation filter. The blue light excites the intracellular carboxyfluorescein and reveals the structure of the neuron including the soma (1), the axon (2) and the dendritic area (3). Based on these three features of the neuron, it can be identified as a putative GI2. (C) Photomicrograph of the same preparation as viewed through the compound microscope which confirms the in situ identification of GI2 (calibration: 50 μm).

erably in the abdominal ganglion A6 (Daley et al., 1981), it is reasonable to suspect that they may interact here. However, because of this overlap, a double penetration of the two neurons is rather difficult. As an alternative, one could kill one GI of a given pair while simultaneously measuring the changes in the wind sensitivity of the remaining GI.

Fig. 5 shows one such experiment. The right GI5 was impaled with a glass micro-electrode and injected with carboxyfluorescein. Then, using the same procedure, the right GI7 was impaled. The wind-evoked response of GI7 was quantified by delivering a calibrated wind stimulus of fixed velocity every 20 s (Fig. 5A). Roughly after the tenth trial, GI5 was photoablated while continuously monitoring the wind-evoked response of the impaled GI7. The timing of GI5's death was monitored on the extracellular recording, as a dying neuron produces a long-lasting burst of action potentials during the photoablation process (Fig. 5B). The measurement of the wind response of GI7 continued after removing GI5 (Fig. 5C). This suggests that GI5

and GI7 of the same side are not interacting in A6. When sampling other GI pairs (GI5-GI6; GI5-GI7; GI6-GI7), we found no detectable supra-threshold connections (Fig. 5D; $p > 0.05$; two-way ANOVA).

4. Discussion

Fluorescent markers have been used not only to visualize individual neurons, but also to remove these from a network. Visualization and/or photoablation of neurons in situ typically involves expensive equipment. The simplest system consists of a laser connected to a fiber light guide (Reichert and Krenz, 1986). Another more complete system includes an epifluorescent attachment allowing the operator to illuminate the tissue with low light levels using a mercury arc lamp. Once the target is identified, the laser then performs the photoablation (Jacobs and Miller, 1985). Unfortunately, the epifluorescent attachment is no longer commercially available. Lasers generally are expensive,
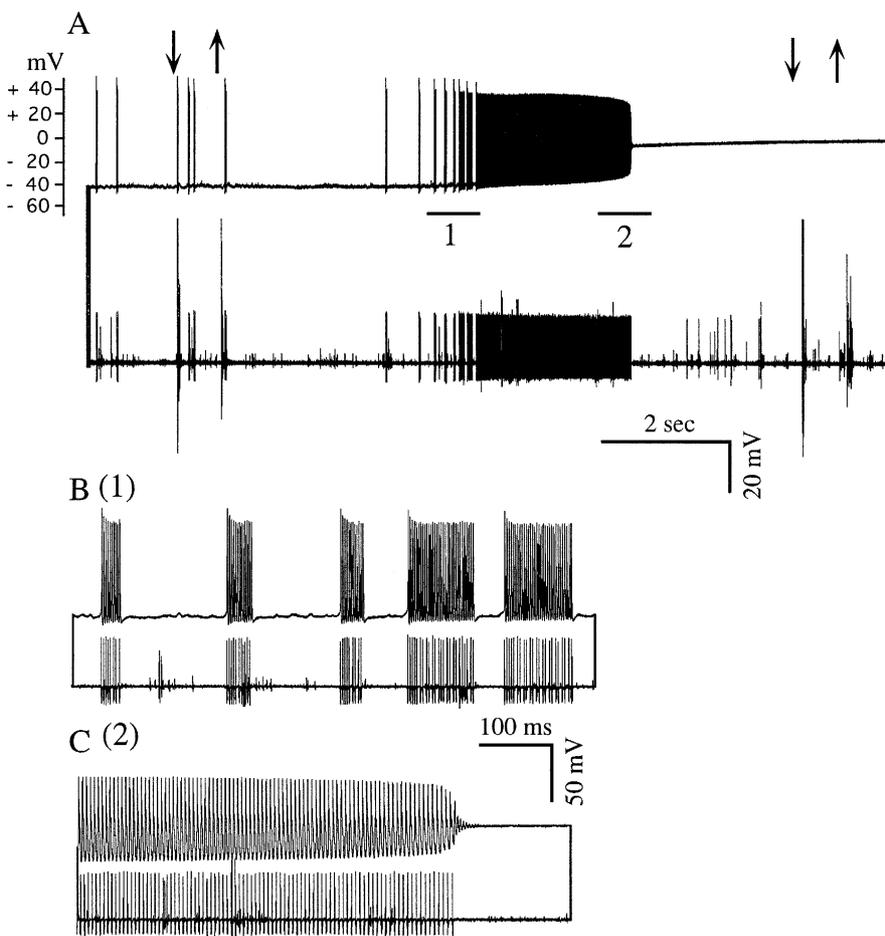

Fig. 3. Disappearance of the action potential and resting potential during cell death. (A) A wind stimulus (arrows) elicits a response of a GI6 (top trace) and a short burst of spikes on the extracellular recording of the nerve cord (bottom trace). The process of cell death starts after the wind stimulus and consists at the beginning, of short bursts of action potentials recorded both intracellularly (top trace) and extracellularly (bottom trace). Then, before depolarizing to zero, the neuron fires a high-frequency burst of action potentials. After cell death, the wind stimulus (arrows) does not evoke a response in GI6 but still produces a burst of spikes recorded on the nerve cord. 1 and 2 indicate the portions of the recording which have been expanded in time in B and C.

requiring maintenance, and often provide only a single wavelength. The system described in this report permits in situ visualization and photoablation of individual neurons with considerable advantages: (1) the system is inexpensive enough to become a standard piece of equipment in most laboratories; (2) it requires minimal maintenance; (3) it provides fluorescence filters for blue or green excitation at 470 and 530 nm, respectively, for different fluorescent markers. Despite its simplicity, this system can be used effectively for photoablating neurons in less than 4 min, i.e., within the same time range for photoablating neurons
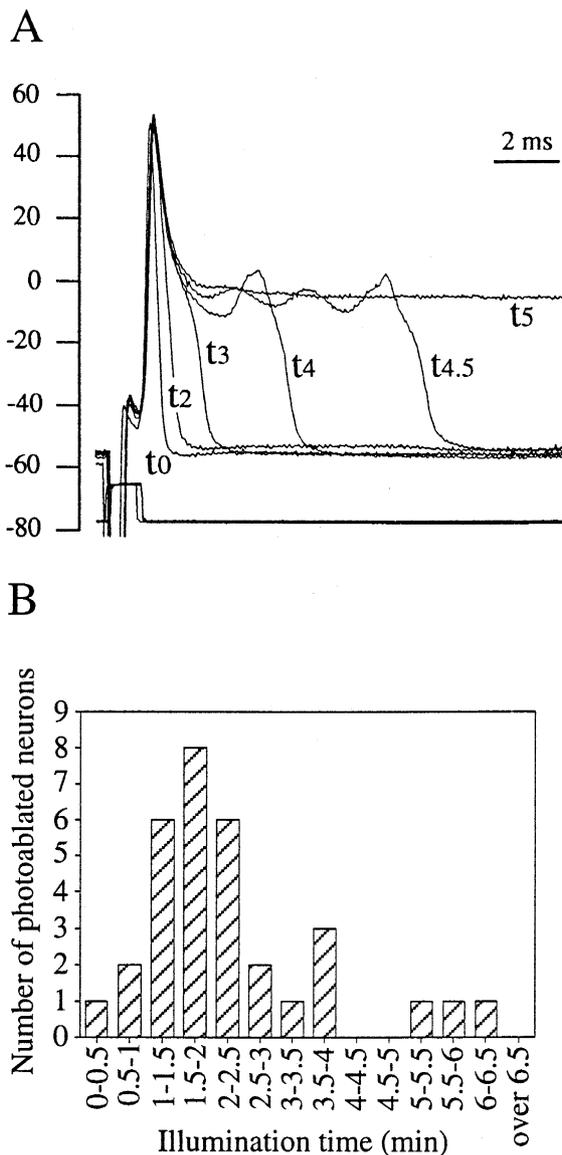
A
B

Fig. 4. Changes in the shape of the action potential during cell death and time of illumination required to kill cells. (A) The action potential (top trace) is elicited by antidromic stimulation of the nerve cord (pulse on the bottom trace). The subscript 't' is the time (in min) after the onset of the illumination. Six sweeps taken at different time intervals are shown. (B) Most neurons are photoablated after 2.5 min of blue light illumination. The maximal time of illumination required to kill a given neuron is 6.5 min.
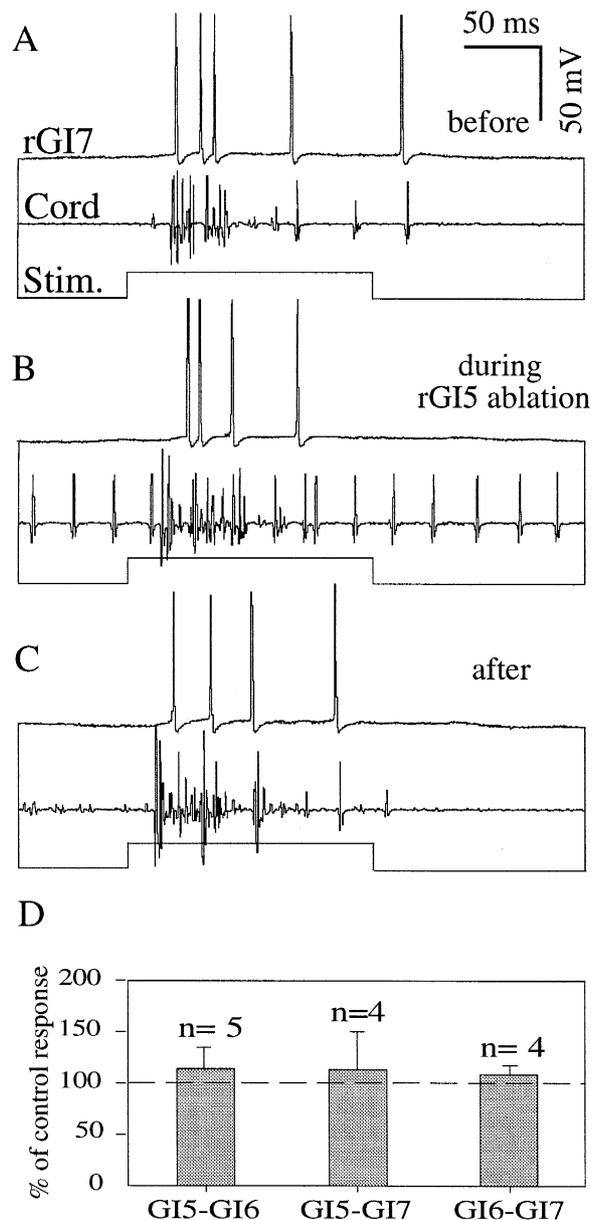
Fig. 5. Interactions between cockroach giant interneurons studied with photoablation. (A) Response of a right GI7 to a wind puff (the pulse indicates the onset of the wind) after right GI5 has been injected with carboxyfluorescein but before it is ablated; CORD: extracellular recording of the cord. (B) Response of this rGI7 to a wind puff during rGI5's ablation (notice the injury tonic firing of GI5 on the nerve cord recording). (C) After GI5's ablation, the number of GI7 action potentials does not change. (D) Histogram summarizing the results of 13 tested pairs of GIs. The number of wind-evoked action potentials before (control) and after photoablating one of a given GI pair is measured. Each bar represents the percentage of the control response of a given GI after ablating another GI.

using an epifluorescence compound microscope (i.e. 4 min; Camhi and Macagno, 1990) or the fluorescent epi-illuminator mounted on a microscope (i.e. 9 min; Libersat et al., 1989). This is longer, however, than the time required for a laser system (10 s to 1.5 min depending on the light intensity; Selverston et al., 1985).

The system also permits direct in-situ visualization of neurons (Fig. 2) and thus, could facilitate, among other uses, in vivo cell identification and multiple intracellular recordings of the same neuron. For instance, many neurons project long distances before reaching their targets. Because the electrical activity recorded in the cell body does not necessarily reflect the activity reaching the terminals, it would be useful to be able to record at these two electrotonically distant locations. Although this has been successfully achieved in several systems without direct visualization of the target neuron (e.g. Coleman and Nusbaum, 1994), in situ visualization of the entire neuron including the soma, dendrites and axon, would greatly facilitate the sampling of electrical activity at different distant sites. Finally, the fiber system has the added advantage of providing regular white light for dissection and in addition, a yellow barrier filter which provides filtered light with which one can search for another target for intracellular impalement without exciting and killing a previously filled neuron.

Acknowledgements

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