In situ Neuron Ablation with Laser Beam: Instrumentation and Application

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(*With 3 Text-figures*)

Introduction

The use of fluorescent dyes for staining a single neuron has been widely employed in morphological analysis of vertebrate as well as invertebrate nervous systems. This technique, especially Lucifer Yellow staining (Stewart, 1978), has been used in a variety of neurophysiological approaches: *in vitro* visualization (Stewart, 1978); *in vivo* visualization (e.g. Reichert and Krenz, 1986); killing of neurons or a part of neurons (e.g. Miller and Selverston, 1979; Jacobs and Miller, 1985).

Cell killing by photodynamic action of fluorescent dye was first made practical by Miller and Selverston (1979). Using this technique of photoinactivation, they judged the role and the relative importance of several functional components of the lobster pyloric system (Selverston and Miller, 1980). Since then there have been several other studies using the photoinactivation in neurophysiological and behavioral analysis: cricket cercal sensory system (Jacobs *et al.*, 1986); cricket auditory system and phonotaxis (Selverston *et al.*, 1985; Atkins *et al.*, 1984); cockroach escape behavior (Libersat *et al.*, 1989). All these experiments utilized a combination of the intracellular Lucifer injection and the illumination with intense short wavelength (blue) light.

Several ideas of constructing the illumination apparatus for *in situ* dye visualization have been reported recently (Kater *et al.*, 1986; Reichert and Krenz, 1986; Heitler and Fraser, 1989). Here we report a new epi-illumination microscope system which enables us to perform a quick observation of cell structure and subsequent identification *in situ* of crayfish neuron. The system is also convertible into a cell killing microbeam irradiator by a simple adjustment of its optics.

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Materials and Methods

We adopted a Helium-Cadmium laser (Omnichrom Co. Ltd., model 456-30 MS, California, U.S.A.) for the dye stimulation. Schematic arrangement of the system is shown in Fig. 1. Laser beam was focused on the input end of a quartz fiber light guide (Fujikura Densen Co. Ltd., model GC 50/125, Tokyo, Japan) through a focusing lens. A neutral density filter mount and an electrical shutter were placed between the laser and the lens. The output end of the fiber was mounted on the epi-illuminator of the stereoscopic dissecting microscope of a long working-distance (200 mm) (Olympus Co. Ltd., model MTX, Tokyo, Japan). The conventional illuminator originally provided was modified so as to attach the fiber and suit to pass the laser light. The illuminator focused the laser beam onto the preparation.

A convex lens was inserted in the optical pathway of the laser beam. This lens, which is readily removable from the optical pathway, brings the beam of laser light out of focus on the preparation giving a broader and more diffuse illumination suitable for observation of fluorescent image of the stained cell.

All trial experiments were carried out on adult crayfish *Procambarus clarkii* Girard of both sexes. The abdominal nerve cord was isolated and pinned dorsal side up on a Sylgard-lined dish filled with crayfish saline (van Harreveld, 1936).

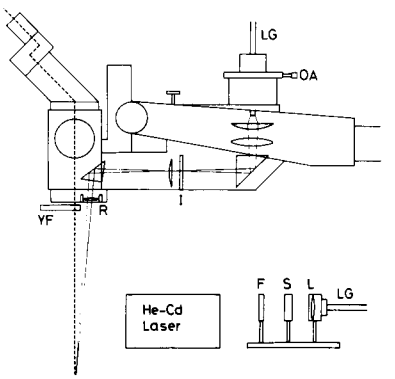

Fig. 1. Schematic diagram of the laser light epiillumination apparatus. Laser light from the generator was focused by a lens (L) and guided to the dissecting microscope through a quartz fiber light guide (LG). An electrical shutter (S) and a neutral density filter (F) for light intensity control were interposed between the generator and the input end of the light guide. An iris (I) and a ring (R) to prevent and absorb internal flare were also inserted in the optical pathway. OA: X-Y stage adjuster holding output end of the light guide. YF: yellow filter for eye protection. This microscope was used for both laser epiillumination and observation of preparation.

Suction electrodes were used for extracellular recording of motoneuron activity and nerve stimulation. Tips of the microelectrodes were filled with Lucifer Yellow CH (5% in 1 mol l^{-1} $LiCl_2$; Stewart, 1978). Electrode resistances were 30-60 M Ohm. The dye was injected with pulsed hyperpolarizing current of 5 nA, for 20 minutes.

Results and Discussion

Laser beam illumination, mode of operation

The converted stereoscopic dissecting microscope of a long working-distance kept wide space for electrodes setting between the microscope and the preparation. The system, therefore, allowed electrophysiological recording by micromanipulated intracellular electrode under the microscopic observation with ordinary external light source. Diffused laser light illumination for cell identification and focused irradiation for photoinactivation were done through the epi-illumination installation of the laser light beam.

The optimal stimulating wavelength of Lucifer Yellow CH is 430 nm (Stewart, 1978). The Helium-Cadmium laser has the wavelength of 441.6 nm, which was found to be still appropriate for the dye stimulation and for the observation of fluorescent image of the stained cell. To cut the laser light off from the observing image, a yellow filter of JIS SY-48 was placed before the objective lenses of the microscope. This filter is a lowpass filter and has 50% transmittance wavelength at 480 nm. Since Lucifer Yellow CH has maximum emission wavelength at 540 nm (Stewart, 1978), this filter could effectively cut off the reflection of blue light from the preparation and the saline surface and allow the emission to pass through for observation.

The laser beam was focused into a 40 micrometer in diameter circular spot confocal with the focal plane of the microscope (spot irradiation). This mode of irradiation was used for partial ablation of a neurite of a single neuron. Insertion of a small convex lens into the optical pathway gave a broader illumination suitable for observation of fluorescent image of the cell or whole cell killing (flood illumination). In both cases, the laser light has a incident angle tilted 5.4° off from the optical axis of the microscope. This inclination prevent the direct reflection of laser beam from the saline or preparation surface from coming into the objective lens. An iris and a ring made of a orange colored acrylic were placed in the optical pathway to prevent and absorb the internal flare by spurious reflection of laser beam.

The output end of the optic fiber is held by a X-Y stage attached on the epi-illuminator of the dissecting microscope. Adjusting the stage, the fiber end shifts its position around the optic axis. Since the position determines the target location of the beam in the microscope viewing field, the laser beam can be placed at a certain point (usually at the center) of the viewing field by adjusting the stage in the mode of spot irradiation. Integrated unitary construction of the laser

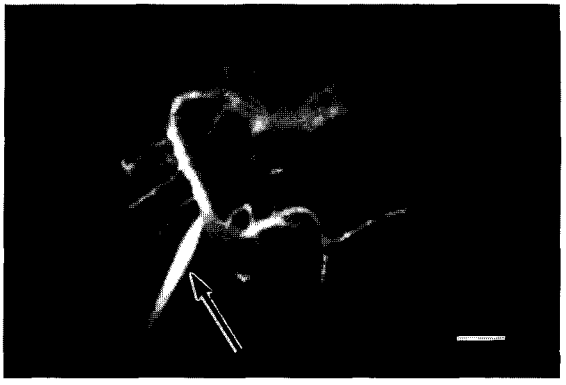

Fig. 2. Photograph taken through the dissecting microscope. This photograph shows the structure of a dye injected neuron in the crayfish sixth abdominal ganglion and a microelectrode penetrating it. The neuron viewed dorsally. An arrow indicates the electrode. Anterior is at the top. Calibration: 100 μm.

optics and the dissecting microscope made the determination of the target location very simple to perform.

Our system thus works both as an epi-illuminating fluorescence microscope and as an intense microbeam irradiation apparatus. An example of *in situ* visualization of a neuron and the electrode penetrating it is shown in Fig. 2. *In situ* visualization of electrode penetration site on a neuron is quite useful to facilitate functional analysis of the local circuit property of the penetrated part of the neuron such as the identification of actual synaptic site of dendro-dendritic connection among motoneurons which has been electrophysiologically postulated by Nagayama *et al.* (1983). Prolonged flood illumination will result in the whole cell killing, which is also useful to determine the role and the relative importance of specific identified neurons in a neural network (Selverston and Miller, 1980).

Spot irradiation was used for the partial destruction of a dye filled neuron (Jacobs and Miller, 1985). This application is to obtain information pertinent with the role of the ablated part of the neuron. An example of this line of study will be given in the following section.

Axonal ablation with laser photoinactivation

Axonal ablation was tried on the slow extensor inhibitory (EI) motoneuron in the fourth abdominal ganglion. The neuron was first identified with dye filling and subsequent *in situ* visualization of its morphology by the flood illumination with the apparatus (Fig. 3A). This neuron could be readily identified by the soma position contralateral to the axonal exit and bilateral dendritic branches of characteristic configuration (Wine and Hagiwara, 1977; Miall and Larimer, 1982).

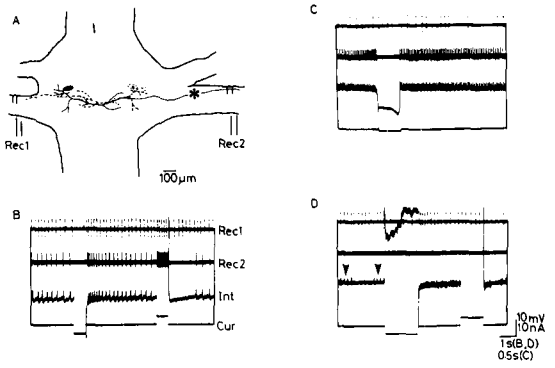

Fig. 3. Effect of axonal ablation on the activity of the slow extensor inhibitory motoneurons in the crayfish fourth abdominal ganglion. A: experimental setting. A solid line drawing shows dye injected and irradiated motoneuron, and a broken line drawing is contralateral homologue of the motoneuron. Asterisk indicates irradiated portion of laser beam. The neurons viewed ventrally. Anterior is at the top. B: spike activity of the motoneurons before irradiation. Intracellular injection of 5 nA current of either polarity into the stained motoneuron (3rd trace) changed its own extracellular spike activity (2nd trace) and that of contralateral homologue (1st trace). C: spike activity of the neurons after for 45 seconds irradiation. Spike activity of the irradiated neuron increased. Injection of -1 nA current into the irradiated neuron effected its homologue. D: the result after the irradiation for 2 minutes. Extracellular spikes of the irradiated neuron disappeared (2nd trace), but motor output to the contralateral homologue (1st trace) and intracellular spikes of the neuron (arrowheads in 3rd trace) still remained.

The EI motoneurons exist as a pair in the ganglion. They are also characterized with spontaneous firing and functional coupling between them. Intracellular current injection into one motoneuron changed spike activity of the contralateral homologue (Fig. 3B).

The laser beam by spot irradiation was aimed at the dye filled axon near the root exit. The irradiated part was located between the intracellular electrode in the ganglion and the extracellular electrode on the root. In the case shown in Fig. 3, the axon was repeatedly irradiated with 15 seconds exposure. The irradiated neuron increased its spontaneous firing rate in the course of irradiation (Fig. 3C). After 1 minute of cumulative irradiation time, orthodromic action potential of the neuron was no more recorded with the extracellular electrode which was distally placed beyond the irradiated part. Spontaneous spike generation, however, was recorded with the intracellular electrode. Fig. 3D shows the result after 2 minutes irradiation. Intracellular injection of hyperpolarizing current still inhibited the spontaneous firing of the contralateral homologue (Fig. 3D). Consequently, the motoneuron has yet kept the ability of its own spike

generation and the functional coupling with its contralateral homologue in the ganglion, after the axotomy by photoinactivation. The continuance of the coupling indicates that the El motoneurons have the sites of local interaction in the central neuropil. The motoneurons could have dendro-dendritic interactions between them as well as to the uropod closer motoneurons in the sixth abdominal ganglion (Nagayama *et al.*, 1983).

We concluded that the inactivation induced by spot irradiation was limited to the irradiated region. The limited inactivation can thus ablate particular dendrites from a crayfish neuron, as shown in cricket cercal interneuron (Jacobs and Miller, 1985; Jacobs *et al.*, 1986). By performing similar experiments in which the laser beam was aimed at different parts of the dendritic tree of a neuron, we will be able to assess the relation between neuronal structure and function. Pearson (1979) proposed that the different arborizing regions of invertebrate neuron are electrically isolated from each other and therefore function more or less independently. If the function of a certain dendrite of a neuron was studied by the dendrite ablation with the current method, Pearson's hypothesis could be practically tested. Also, the response properties of various neurons could be interpreted in terms of synaptic interaction at single dendrites. In addition, *in situ* visualization of neuronal structure enables us to accomplish multiple electrode penetration into a single neuron under visual control (Reichert and Krenz, 1986). The multiple penetration is useful and necessary to study passive properties of a single neuron in the central nervous system.

Besides the neurophysiological application of the current method, principle of the photoinactivation and *in situ* visualization will be utilizable in other fields of biology. Some possible applications are: tracing of a cell-lineage by fluorescent dye injection into a cell and subsequent laser killing of the cells belonging to the line to know the developmental fate and the role of the cells in an embryo; assessment of the functional role of a specific cell group in development by selective laser killing; visualization of morphological change of living cells during pre- and postembryonic development. This laser apparatus thus may serve as a new versatile tool in biology.

Summary

Cell killing apparatus was devised by combining a Helium-Cadmium laser with a stereoscopic dissecting microscope. The system can operate as both an epi-illuminating fluorescence microscope and an intense microbeam irradiation apparatus, during electrophysiological recording. A retractable convex lens in the light path can convert the mode of operation of the system. Broader beam illumination by out of focus (flood illumination) was used for *in situ* observation of fluorescent image and whole cell killing. Intense microbeam irradiation (spot irradiation) was used for partial destruction of a cell.

Spot irradiation to the motoneuron axon could sever it by the photodynamic

action. As an evidence of the severance, no orthodromic action potential was recorded from the portion peripheral to the irradiated spot, while the rest of the neuron central to it could function normally. The result indicates a possibility that a particular dendritic branch of a neuron could be ablated with this system.

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References

Atkins, G., Ligman, S., Burghardt, F. and J.F. Stout 1984. Changes in phonotaxis by the female cricket *Acheta domesticus* L. after killing identified acoustic interneurons. J. comp. Physiol. A. **154**: 795-804.

Heitler, W.J. and K. Fraser 1989. *In situ* dye-visualization and photoinactivation using an epifluorescent compound microscope. J. exp. Biol. **145**: 477-481.

Jacobs, G.A. and J.P. Miller 1985. Functional properties of individual neuronal branches isolated in situ by laser photoinactivation. Science. **228**: 344-346.

————, ————, and R.K. Murphey 1986. Integrative mechanisms controlling directional sensitivity of an identified sensory interneuron. J. Neurosci. **6**(8): 2298-2311.

Kater, S.B., Cohan, C.S., Jacobs, G.A. and J.P. Miller 1986. Image intensification of stained, functioning, and growing neurons. In: Optical methods in cell physiology, Vol. 40. (P. DeWeer and B. Salzburg eds), John Wiley & Sons, New York. pp. 31-50.

Libersat, F., Selverston, A., Camhi, J.M. and R.S. Goldstein 1989. Photoinactivation of a portion of a neuron for long-term studies of its role in behaviour. J. exp. Biol. **142**: 453-459.

Miall, R.C. and J.L. Larimer 1982. Central organization of crustacean abdominal posture motoneurons: connectivity and command fiber inputs. J. exp. Zool. **224**: 45-56.

Miller, J.P. and A.I. Selverston 1979. Rapid killing of single neurons by irradiation of intracellularly injected dye. Science. **206**: 702-704.

Nagayama, T., Takahata, M. and M. Hisada 1983. Local spikeless interaction of motoneuron dendrites in the crayfish *Procambarus clarkii* Girard. J. comp. Physiol. A. **152**: 335-345.

Pearson, K.G. 1979. Local neurons and local interactions in the nervous systems of invertebrates. In: The neuroscience, fourth study program. (F.O. Schmitt and F. G. Worden eds), The MIT press, Cambridge, MA. pp. 145-157.

Reichert, H. and W.D. Krenz 1986. In vivo visualization of individual neurons in arthropod ganglia facilitates intracellular neuropil recording. J. comp. Physiol. A. **158**: 625-637.

Selverston, A.I., Kleindienst, H-U. and F. Huber 1985. Synaptic connectivity between cricket auditory interneurons as studied by selective photoinactivation. J. Neurosci. **5**(5): 1283-1292.

————, and J.P. Miller 1980. Mechanisms underlying pattern generation in lobster stomatogastric ganglion as determined by selective inactivation of identified

neurons. I. Pyloric system. J. Neurophysiol. **44**: 1102-1121.
Stewart, W.W. 1978. Functional connections between cells as revealed by dye-coupling with a highly fluorescent Naphthalimide tracer. Cell. **14**: 741-759.
Van Harreveld, A. 1936. A physiological solution for freshwater crustaceans. Proc. Soc. exp. Biol. Med. **34**: 428-432.
Wine, J.J. and G. Hagiwara 1977. Crayfish escape behavior. I. The structure of efferent and afferent neurons involved in abdominal extension. J. comp. Physiol. A. **121**: 145-172.