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SHORT COMMUNICATION

IN SITU DYE-VISUALIZATION AND PHOTOINACTIVATION USING AN EPIFLUORESCENT COMPOUND MICROSCOPE

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A very useful technique to emerge in recent years in the field of neural circuit analysis is *in situ* dye-visualization and photoinactivation using Lucifer Yellow (Stewart, 1978). This allows direct anatomical identification of neurones during an experiment, and greatly facilitates placing several microelectrodes within a single neurone (Reichert & Krenz, 1986). If the visualization is kept brief, the neurone is not damaged, but with continued exposure the neurone is specifically killed (Miller & Selverston, 1979). This enables neurones to be selectively deleted from a circuit.

The preferred apparatus for this technique is a helium–cadmium laser, coupled to a flexible fibre-optic light guide. The laser provides high-intensity illumination very close to the excitation frequency of Lucifer Yellow, while the light guide allows the illumination to be applied to the nervous tissue with a minimum of inconvenience and danger (Reichert & Krenz, 1986). Unfortunately, a suitable laser is expensive (currently about £10000 for 50 mW of power), and even the optics required to couple it to the light guide are not cheap. A low-cost system utilizing a mercury arc lamp has recently been described (Büschges *et al.* 1989), but this has no light guide and, instead, relies on simply focusing the beam of the lamp on the tissue. This places severe constraints on the utility of the system in electrophysiological investigation, since an unobstructed light path has to be maintained. Furthermore, the intensity of illumination is not high enough for photoinactivation.

In this report we describe how a standard compound microscope with epifluorescent illuminator can be easily and reversibly coupled to a light guide to provide most of the utility of a laser-based system, at a fraction of the cost. We used a Zeiss Standard 14 microscope, but the method can easily be adapted to other makes and models. In the following text the essential requirements for any dye-visualization and photoinactivation system are listed, followed in each case by a description of how these requirements are met in our system.

High-intensity illumination at the correct wavelength

The first problem is to obtain a suitable source of illumination. Most electro-

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physiological laboratories that use Lucifer Yellow will have access to an epifluorescent compound microscope with the appropriate filter set. Such a microscope is not itself usually suitable for *in situ* experiments, but it provides a very good source of illumination. Our system requires the microscope to be situated relatively close to the electrophysiological set-up (within 1–2 m), but does not significantly disable the microscope for its normal use.

Optical coupling between light source and light guide

The next problem is to couple the light guide to the microscope optically. Here again, the microscope itself contains a good solution. If the substage condenser of the microscope is removed, the tip of a light guide can be inserted into the plane of focus of the objective. The light guide is held in the centre of a collar which fits into the condenser mounting ring (Fig. 1). The objective of the microscope is focused on the flat tip of the light guide by viewing the latter as if it were a normal specimen. The tip should just fill the field of view, and the whole tip should be in focus to ensure that the light guide is exactly orthogonal to the optical axis. The condenser-centring screws can be used for final lateral adjustment of the light guide.

The acceptance cone of the light guide must be matched to the emission cone of the objective lens, or energy will be lost into the light guide cladding. Satisfactory matching is obtained if the numerical aperture of the lens is less than that of the light guide. This restricts the power of the objective lens which can be used, and the consequent diameter of the field of view and light guide. We used a 3 mm diameter liquid light guide (see below) with N.A. 0·47, which fills the field of view

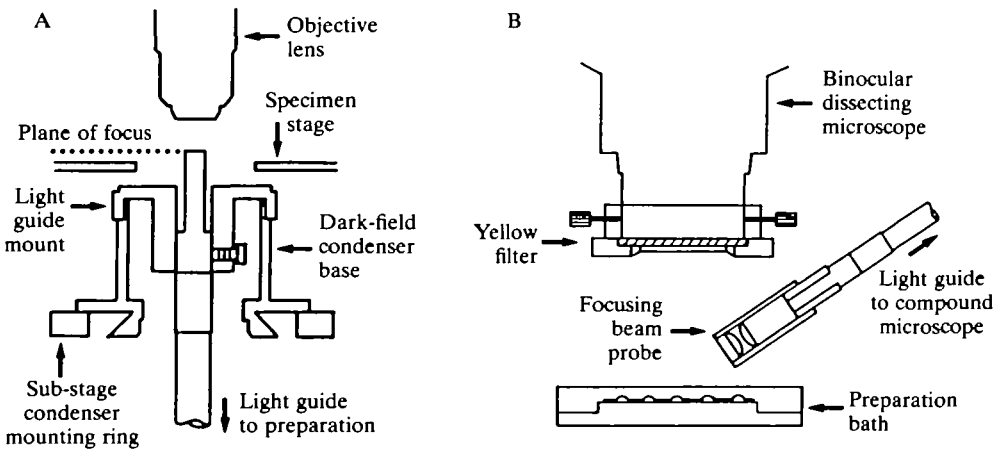


Fig. 1. The optical system. (A) Coupling between light source and light guide. The liquid light guide is held in a home-made collar which screws into a mount consisting of the base of a dark-field sub-stage condenser, located in the normal position. The light guide tip is in the plane of focus of the objective lens of the epifluorescent microscope. (B) Coupling between light guide and preparation. A lens system produces an image of the light guide tip focused on the preparation. A yellow filter is placed between the preparation and the viewing binocular microscope.

of a 6·3× Zeiss Neofluar lens with N.A. 0·2. We thus ensured that virtually all the light emitted by the microscope objective was collected by the light guide.

Transmission through the light guide

Light guides can be obtained with a variety of internal media, including glass, silica and plastic. However, most of these materials have a relatively low transmittance in the deep blue spectral range appropriate for exciting Lucifer Yellow. An exception is the liquid light guide. This consists of a length of tubing filled with a transparent fluid (whose exact composition is, apparently, a trade secret), stopped at both ends with a polished, fused silica plug. This has a transmittance of over 80 % m^{-1} at 420 nm, which is considerably higher than the solid light guide equivalent. We use a 1·5 m length of liquid light guide of 3 mm diameter supplied by Oriel Scientific Ltd (PO Box 31, 1 Mole Business Park, Leatherhead, Surrey KT22 7AU, UK).

Focusing from the light guide onto the preparation

Light diverges from the emitting end of the light guide at approximately 20°. To avoid loss of luminous density it is therefore necessary to focus the light onto the preparation. We used a focusing beam probe (Oriel Scientific) which produces an image of the emitting light guide tip magnified 1·3 times at a working distance of 18 mm. It would obviously be better to have a smaller image (greater luminous density and selectivity of illumination), but there is a trade-off with working distance. The smaller the image, the smaller the working distance.

Filtering the emitted fluorescent light

Lucifer Yellow emits at 530 nm, so a yellow filter placed between the preparation and the viewing binocular microscope both transmits the emitted light and blocks the exciting light. We used a simple long-pass yellow glass filter (Oriel Scientific).

The total cost of our system, excluding the sub-stage light guide mount (which was made in-house with negligible cost in materials) is approximately £300 (provided, of course, an epifluorescent microscope is already available). In our set-up, the microscope is placed on a table adjacent to the electrophysiological rig, and the light guide passed into the Faraday cage. If the metal tip of the emitting end of the light guide is earthed, we have found that it introduces negligible interference into the recording system. The microscope itself remains available for use without change for normal epifluorescent microscopy, and it takes only 1–2 min to remove the light guide mount and replace the normal sub-stage condenser, thus returning the microscope to its original condition.

In experiments on crayfish nervous tissue the illumination system described provides excellent *in situ* dye-visualization (Fig. 2), of a quality which appears equivalent to the pictures published using a laser-based system. Furthermore, photoinactivation takes place within a reasonable time-scale. With a good fill of Lucifer Yellow, the crayfish segmental giant neurone loses its spike within

Fig. 2. Stereo-pair photographs taken through a binocular microscope showing *in situ* dye-visualization. (A) A crayfish abdominal fast flexor motor neurone has been stained with Lucifer Yellow. Faint white-light illumination is also applied, so that the outline of the ganglion can be seen. (B) A bilateral pair of segmental giant neurones have been stained in a crayfish abdominal ganglion. In both pairs of photographs a microelectrode is visible, but is not actually located intracellularly (owing to mechanical difficulties in obtaining the stereo photographs). The neural tissue is alive, and submerged in physiological saline. Scale bar, 1 mm.

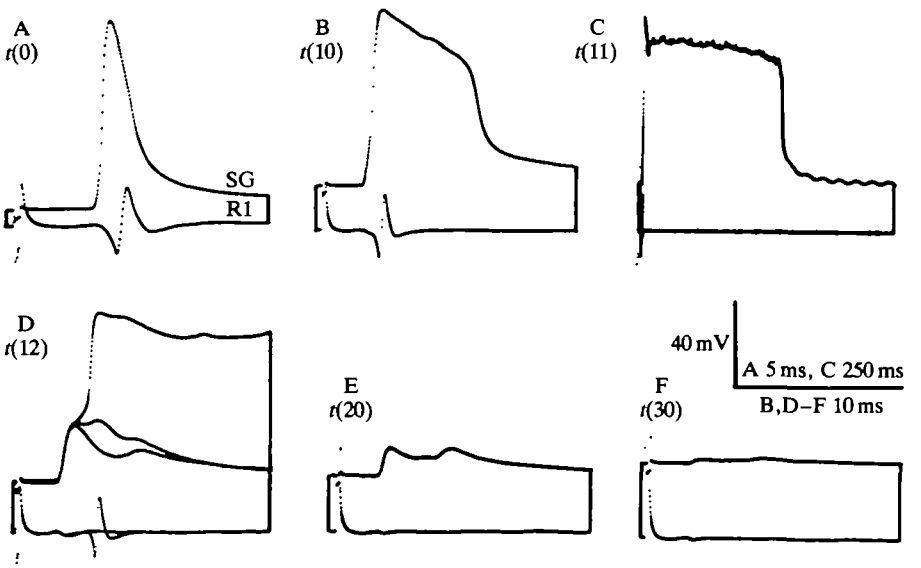
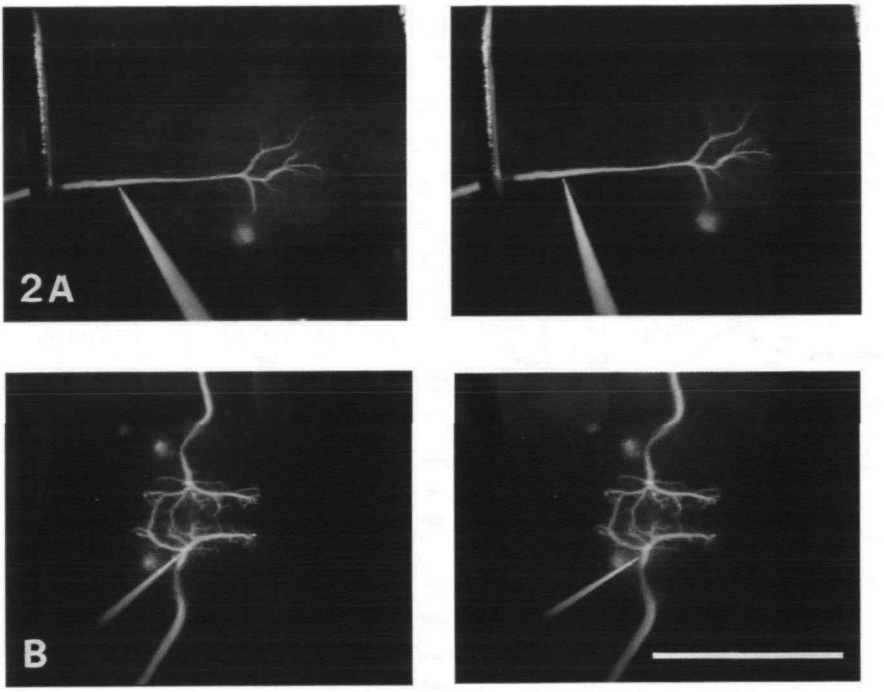


Fig. 3. Dye photoinactivation. A segmental giant (SG) neurone was stained with Lucifer Yellow in a crayfish abdominal ganglion, and then inactivated. A giant fibre (GF) was stimulated extracellularly, while recording intracellularly from the SG (upper trace), and extracellularly from the first root (R1) containing the SG axon (lower trace). (A) Prior to illumination a large, short-duration spike is recorded in the SG (time 0). (B) After 10 min of illumination the SG spike starts to broaden. (C) At 11 min a massive depolarized plateau develops on the falling phase of the SG spike. (D) At 12 min, SG spikes start to fail, revealing the underlying electrical EPSP from the GF. Three sweeps are superimposed. (E) At 20 min, SG spikes have been totally abolished. (F) At 30 min all synaptic input has been abolished. The resting potential of the intact SG is typically 90–100 mV, and fell to less than 10 mV at the termination of this experiment.

5–10 min, and suffers total loss of membrane potential and all signs of input within 20–30 min (Fig. 3). The main disadvantage of our system compared to one based on a laser is the relatively large spot-size of the illumination. This means that highly selective illumination of only parts of a neurone is not feasible. However, this requirement is only found in rather specialized experimental circumstances. For many purposes the system we describe will be perfectly adequate, and provide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the same utility as a laser-based system at a cost which is at least an order of magnitude lower.

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