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ENHANCING THE LASER SCANNING CONFOCAL MICROSCOPIC VISUALIZATION OF LUCIFER YELLOW FILLED CELLS IN WHOLE-MOUNTED TISSUE

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Abstract

The laser scanning confocal microscope (LSCM) is an extremely useful tool that allows fluorescently labelled cells to be visualized in whole-mount preparations. This is particularly advantageous, for example, in studying the dendritic trees of neurons with respect to their environment.

One of the most popular, and easiest, ways to visualize a cell is to inject it intracellularly with the fluorophore Lucifer Yellow (LY). However, the argon gas lasers of most LSCM's are *not* well matched to the excitation spectrum of aqueous LY. When this largely inappropriate excitation is combined with standard filters, designed for fluorescein fluorescence rather than Lucifer Yellow, the resulting image is poor.

We report that clearing LY-injected neurons in methyl salicylate and mounting them in Entellan, a non-aqueous medium of high refractive index, enhances their visualization on a Bio-Rad LSCM with standard fluorescein (FITC) filters to an unexpected degree. This technique also leads to a substantial reduction in photobleaching.

KEY WORDS: Wholemount, Fluorescence, Photobleaching, 3D-reconstruction, Motoneuron, Retinal ganglion cell, Dendritic tree.

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Introduction

The laser scanning confocal microscope (LSCM) is potentially a powerful tool for analysing three dimensional relationships between cells within a tissue (for review see Fine et al., 1988). Optically sectioning thick specimens (or whole embryos) and reconstructing the images provides access to information on the relationships of a cell and its processes intact within its tissue. This saves a great deal of time and tedium and eliminates the potential for error that physical sectioning and manual reconstruction introduce.

Specimens are scanned systematically by a laser beam, momentarily focused by the objective on a single point in the image plane. Light returning from this point passes through a small aperture to a detector and determines the brightness of the corresponding pixel in a digital image. Potentially image-degrading light from nearby points misses the aperture and fails to reach the detector. In this way the LSCM rejects both out-of-focus blur and scattered light, thus enabling the extraction of thin optical sections of high contrast and resolution from thick biological sections from which it is inherently difficult to obtain clear images. The successive images of the entire three-dimensional dendritic tree of an intracellularly injected neuron, for example, can be captured and compressed into two dimensions with an accuracy otherwise unobtainable.

Structures to be visualized must be either reflective or fluorescent, and not all fluorophores are suitable. Most LSCMs use argon gas lasers that emit light in a few narrow spectral lines with wavelengths in the range 458-514 nm, too long to excite common tracers such as Fast Blue, Diamidino Yellow, DAPI and many of the calcium and pH sensitive dyes. The Bio-Rad MRC-500 that we use seems to be typical in having most of its power in the range 488-514 nm (manufacturers' data). The epifluorescence filter sets currently available for this instrument are designed for use with FITC and TRITC. The FITC one is the most appropriate of the two for imaging LY. This filter set selects the 488 nm laser line for excitation and passes emitted light through a 515 nm long-pass filter.

Lucifer Yellow (LY) is one of the most widely used intracellular dyes in neurobiology and cell communication studies (Buhl and Lübke, 1989). Its emission spectrum in aqueous solution is broadly compatible with the BHS filter set: peak emission occurs at 540 nm, and most of the energy is at wavelengths longer than 515 nm (Stewart, 1978).

However, the excitation spectrum of aqueous LY is much less compatible with this filter set, peaking at 430 nm and falling off rapidly at longer wavelengths so that it is below 10% of the peak value at 488 nm (Stewart, 1978). Cells filled with LY can be visualized on the LSCM in aqueous media if the full power of the laser is used, but the resulting images are less clear than conventional images of the same cells.

We here describe a simple protocol that allows LY-filled neurons to be imaged with increased clarity and detail using the standard BHS filter set on a Bio-Rad MRC-500 and at the same time reduces fading of the dye. Preliminary results obtained using this protocol have been published (Dekkers et al., 1990).

Materials and Methods

To demonstrate the principles of this method we chose two different types of specimen: motoneurons in hemisected spinal cord which show the problems of reconstruction of a three-dimensional dendritic tree in deep tissue, and retinal ganglion cells, the dendritic tree of which is largely two dimensional and so receives even illumination during studies of dye fading.

Tissue preparation protocol

Tissue specimens containing neurons that have been injected with LY are fixed for 2-24 hours (depending on size) at 4°C in a 4% solution of fresh paraformaldehyde buffered with phosphate to pH 7.4. They are then dehydrated through alcohols, cleared for 2-3 hr in two changes of methyl salicylate (Aldrich), and mounted in Entellan (Merck).

Specimens must be held in an appropriate shape for mounting throughout these steps because clearing can make them brittle and unmalleable. Some tissues also become so transparent that they can be easily lost or damaged by handling. Pinning specimens to blocks of cured Sylgard 184 (Dow Corning) solves both problems: unlike some thermoplastics Sylgard does not soften in methyl salicylate.

Application to motoneurons

To demonstrate the effects of this protocol on image clarity in thick tissue, we iontophoretically injected lumbar motoneurons in sagittally hemisected early postnatal rat spinal cords (Fulton and Walton, 1986; Becker and Navarrete, 1990) with a 3% solution of LY. Three types of LY were tested; Lucifer Yellow CH lithium salt (L-453, Molecular Probes), a Lucifer Yellow derivative that is not fixed by aldehydes (8-methoxypyrene-1,3,6-trisulphonic acid trisodium salt; M-395, Molecular Probes Inc.) and Lucifer Yellow CH dipotassium salt (L-0144, Sigma). All three gave similar results. Individual injected cells and groups of cells in these hemicords were analysed twice, first after temporary mounting in glycerol and then again after dehydration, clearing in methyl salicylate and mounting in Entellan. Observations were made on both a Zeiss Standard epifluorescence microscope using filter set 48 77 05 (exciter, 395-440 BP; reflector, 460 LP; barrier, 470 LP), and a Bio-Rad MRC-500 LSCM, using filter set BHS (exciter, 488 BP; reflector, 510 LP; barrier, 515 LP). Conventional epifluorescence images were photographed on Kodak Ektachrome 200; digital grey-scale images obtained by the LSCM were printed using a Sony UP 930 video printer.

Application to retinal ganglion cells

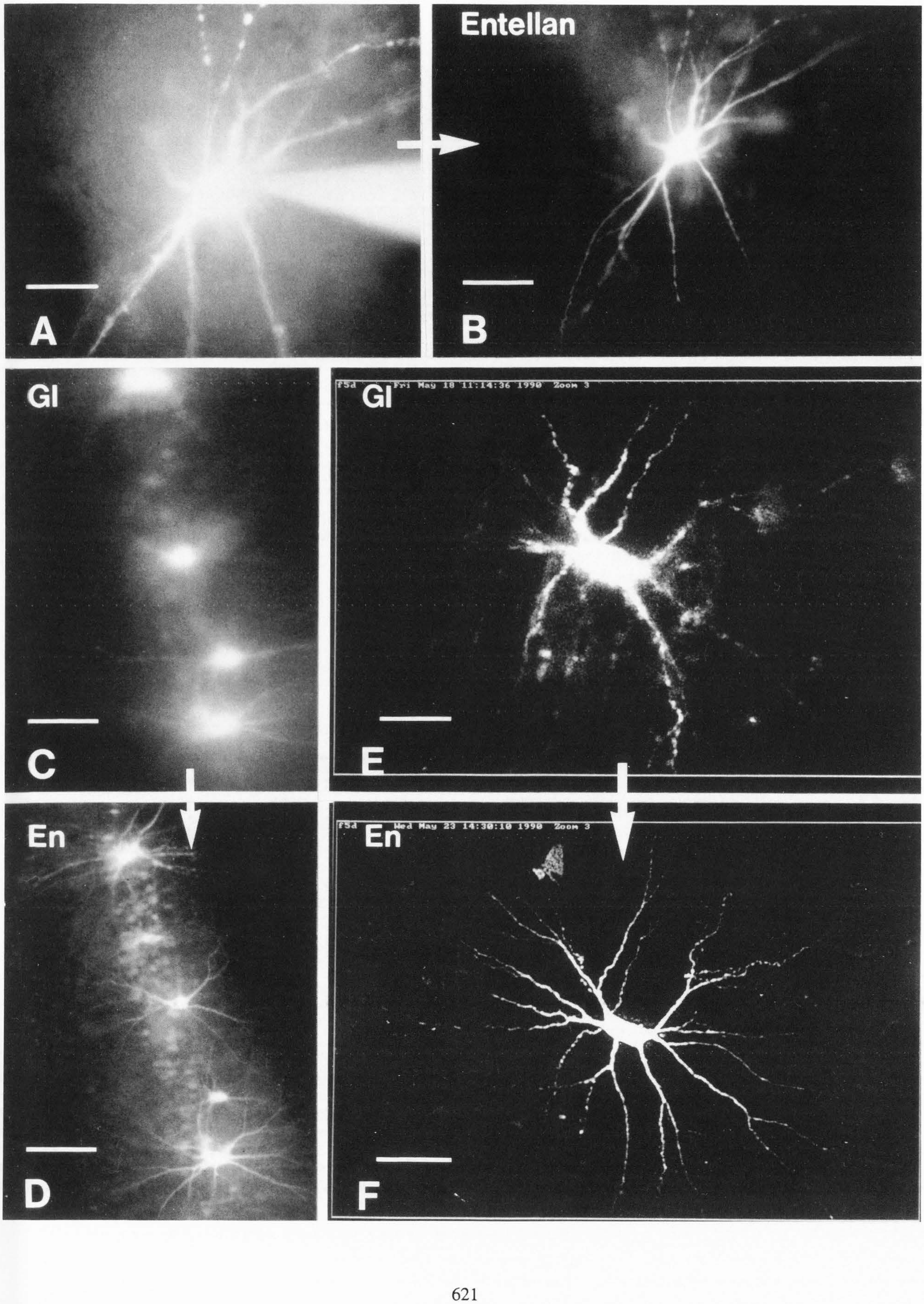
To demonstrate effects on photobleaching we used isolated goldfish retinae. Ganglion cells were injected iontophoretically with 3% LY (Becker and Cook, 1990), and the retinae fixed for up to 1 hr. Some specimens were then mounted in glycerol, while others were dehydrated, cleared and mounted in Entellan. Photobleaching tests were carried out on a microscope fitted with a Zeiss epifluorescence illuminator IV FL (without neutral density filters) and a Wotan HBO 50 W mercury lamp, again using filter set 48 77 05. For each test, two neighbouring LY-filled cells were aligned in such a way that both were in the field of view of a x25/0.8 objective but only one was in the field of a x63/1.2 objective (both Zeiss Plan-Neofluars using water immersion). Photographs were taken, using each objective in turn, at the start of the experiment, after 1 min, and then at 5 min intervals up to 31 min. Between photographs, a circular field including the test cell was illuminated continuously through the x63 objective. Automatic exposure control (Zeiss MC100 camera system) ensured that the overall density of the photographic image remained constant as bleaching proceeded, but exposure times (using the x63/1.2 objective) rose from 0.7 s at t=0 to 15 s at t=31 min.

Results

Conventional microscopy

With thick specimens such as the hemisected spinal cords and retinae used here, the effects of dehydration, clearing and mounting in non-aqueous media could be seen even by conventional epi-fluorescence microscopy. The clarity with which LY-filled cells could be observed was substantially improved. In Figure 1, parts A and B are conventional photomicrographs of the same LY-filled motoneuron, whilst being injected (A) and after clearing and

__Figure 1__ A,B: Paired images of identified flexor motoneurons in the sagittally hemisected early postnatal rat lumbar spinal cord, filled with Lucifer Yellow. The first (A) was obtained during the injection of the motoneurone on postnatal day 11, the second (B) after dehydration, clearing and mounting in Entellan. Here, the most obvious advantage of dehydration is reduced light scatter in bright areas. Colour film also recorded a colour shift in the emitted light, described in the text. Scale bars: 50 µm.
C,D: Conventional epifluorescence images of a group of motoneurons belonging to a single motor pool, injected on postnatal day 3; before processing (C) and after (D). The greater clarity of D makes it possible to see many fine processes, and also the nuclei of other neurons belonging to the same motor pool, retrogradely labelled from the limb with Diamidino Yellow. Scale bars: 100 µm.
E,F: Laser scanning confocal microscope images of a single identified flexor motoneuron filled on postnatal day 5. In the best image obtainable (E) the detail is poor and the image grainy. Scattered light makes the soma appear larger than it is, and only two orders of dendritic branching are resolved. In F, much more detail can be seen despite some shrinkage, and scattered light is better suppressed. More peripheral dendrites could be observed by manipulating brightness and contrast, and up to 7 orders of branching, showing details such as dendritic spines, could be resolved using objectives of higher power. Scale bars: 50 µm.


Entellan
A
B
Gl
C
f5d Fri May 18 11:14:36 1990 Zoom 3
Gl
E
En
D
f5d Wed May 23 14:30:10 1990 Zoom 3
En
F

permanent mounting in Entellan (B). Parts C and D show, in a similar way but at a lower magnification, a region of hemicord containing several such cells. In both cases, the specimen reveals much more dendritic detail after dehydration and the soma appears smaller and better defined, partly because the tissue has indeed shrunk, but partly because its outline is no longer obscured by a strong halo of scattered light.

One of the most obvious changes was that the light emitted by the LY became distinctly green in the dehydrated and cleared tissue, whereas before dehydration it was more nearly a neutral yellow. We do not have the facilities to measure this spectral shift but it has been noted both directly and in colour transparencies. The intensity of the fluorescence was little changed by dehydration and clearing. In most preparations the level of background autofluorescence was low, and also little changed by dehydration, though a slight green shift was seen here also. It should be noted that fixation with paraformaldehyde that is no longer fresh (or formalin or glutaraldehyde) can increase autofluorescence, and that some tissues, including the retina, require particular care.

Confocal microscopy

When individual LY-filled specimens were examined with the LSCM both before and after dehydration, clearing and permanent mounting, a great improvement in clarity was seen. Better pictures were obtained after processing, despite any fading caused by the earlier exposure to the laser beam. Whereas the best LSCM images of motoneurons obtainable from glycerol mounted specimens only revealed the basic cell shape and primary dendrites, the images of processed specimens yielded significantly more information (see Figure 1, E & F). Not only was the image sharper but much more (if not all) of the dendritic tree could be resolved using even low power objectives. At higher magnifications it became possible to resolve details deep within the tissue, such as dendritic spines (Dekkers et al., 1990), and some dendrites could be traced to the seventh order of branching.

Photobleaching

One well-known disadvantage of fluorescent dyes is their tendency to fade on exposure to light. The severity depends on the objective in use. High numerical apertures that capture as much as possible of the light emitted by the specimen also increase the amount reaching it unless neutral density filters are interposed. High magnifications increase fading by concentrating the exciting light into a small area. Thus, the problem is at its worst with the high power, high aperture immersion objectives that are needed to observe fine detail, in both conventional and confocal microscopes. The objective used in our bleaching tests, a water immersion x63/1.2, fits this category.

Photobleaching of the LY image and the background autofluorescence occurred in both aqueous and non-aqueous media, though not to the same extent. In glycerol the image faded faster than the background, leading to a severe loss of contrast. In Entellan the Lucifer Yellow did not appear to fade as fast as in glycerol but the background faded faster. Figure 2 shows the results of one such test: details are given in the legend.

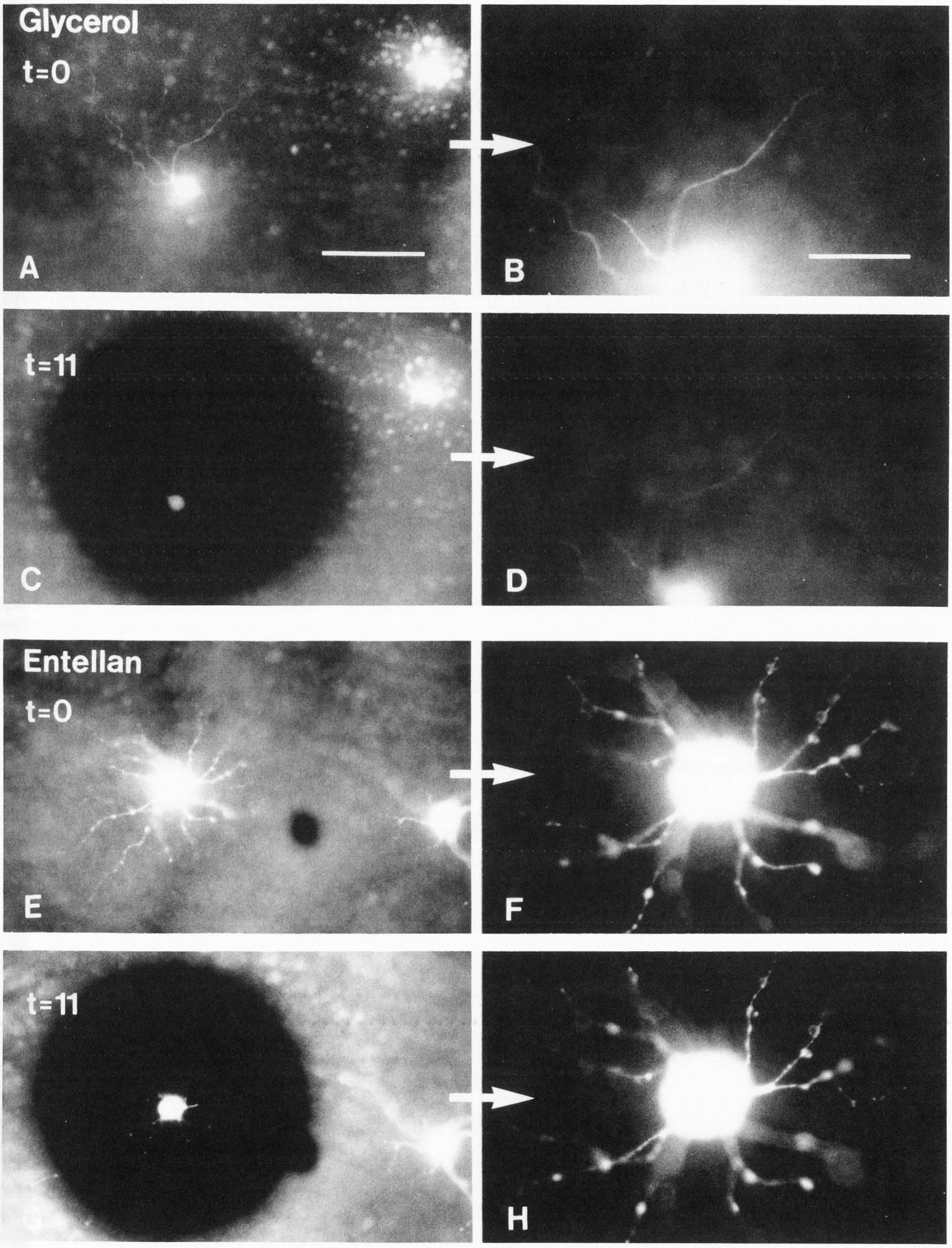
Discussion

Many factors could potentially account for the enhanced visualization of our LY-filled cells after dehydration, clearing and permanent mounting. Mounting in non-aqueous media, though sometimes employed with fluorescent specimens to improve permanence (Furness et al., 1977; Sripanidkulchai and Wyss, 1986), is thought to reduce sensitivity (Stewart, 1978).

The optical benefits of viewing a thick specimen in a medium of uniform refractive index must account for much or all of the improvement in the conventional epifluorescence images, though we were surprised to find that so little of the fluorescence was lost during processing. In the case of the LSCM, however, the improvement was more marked, despite the theoretical advantage that confocal microscopes have over conventional ones with tissues that scatter light. We assume, therefore, that further improvements arose from changes in the fluorescence properties of the dye in the non-aqueous environment. We observed a shortening in the dominant wavelength of light *emitted* by LY, but cannot attribute the improvement to this because the long-pass 515 nm barrier filter should have passed the yellow light from aqueous LY just as readily as its more green non-aqueous equivalent. We had no means of observing any accompanying change in the *excitation* spectrum, but such a change is to be expected when a soluble fluorophore passes out of solution and become bound to a surface (for a useful outline of the molecular basis of fluorescence, see Taylor, 1975). To account for the observed improvement in sensitivity, a red-shift of the excitation band would be needed, bringing the energy levels of the absorbed and emitted photons closer together.

Other potential causes of the improvement after clearing and mounting might be reductions in fluorescence saturation, in the excited state lifetime of the fluorophore, or in the proportion of excited molecules entering the much longer-lived triplet state (Tsien and Waggoner, 1989). All these factors would be expected to affect sensitivity much more in the LSCM than in the conventional microscope because the laser beam and detector of the LSCM dwell on each point only for a few microseconds, exciting the fluorophore intensely for a very short period and then detecting only that energy which is re-emitted before the beam moves on.

__Figure 2__ A demonstration of the effects of photobleaching on goldfish retinal ganglion cells filled with Lucifer Yellow. A-D are from a retina mounted flat in glycerol; E-H from a similar retina dehydrated, cleared and mounted in Entellan. The low power image of each pair (x25 objective; A,C,E,G) shows two similar LY-filled cells, but only the cell shown at higher power (x63 objective; B,D,F,H) was continuously exposed to intense light for 11 minutes between the photographs. Photobleaching of both the Lucifer Yellow and the background autofluorescence occurred in both media. However, in glycerol it led to a severe loss of contrast (B,D), while in Entellan it did not (F,H). Thus, by increasing the exposure time (in this case by a factor of 12) we obtained images of bleached cells that were as clear as those obtained before bleaching. Little loss of contrast was seen even after 31 minutes of continuous exposure to this high level of illumination (not shown).
Scale bars: 100 μm for A,C,E,G and 40 μm for B,D,F,H.


Glycerol
t=0
A
B
t=11
C
D
Entellan
t=0
E
F
t=11
G
H

To our knowledge only one group has published high quality LY images produced on an LSCM (Sarastro LSCM - Wallén et al., 1988). It is of note that this group also cleared their specimens with methyl salicylate, but they did not comment on its benefits for the LSCM or mention any reduction in fading. Under strong, sustained illumination, both the specific fluorescence of LY and the autofluorescence of the surrounding tissue fade and shift towards the red (Stewart, 1978). Photobleaching of some catecholamine derivatives (Furness et al., 1977), Fast Blue (Sripanidkulchai and Wyss, 1986) and the stilbene derivative Fluorogold (Ju et al., 1989) seems to be reduced when they are removed from an aqueous environment, but we are not aware that this had been shown for naphthalimides such as LY. However, evidence that the photobleaching of aqueous LY can be directly coupled to the oxidation of diaminobenzidine (Maranto, 1982) suggests that the availability and mobility of molecular oxygen from which singlet oxygen radicals can be formed within the tissue may have an important regulating influence here, as with many other fluorophores (Tsien and Waggoner, 1989). Reduced photobleaching is a great advantage because it increases the contrast and life of the specimen, and particularly when the specimen is repeatedly exposed to the concentrated power of the laser beam on an LSCM during optical sectioning.

Whatever the causes of these changes in the properties of LY, the simple technique described here allows the LSCM to image LY-filled cells within thick specimens with increased resolution and reduced bleaching. We hope it will help others to exploit the capabilities of LSCMs using argon gas lasers, while retaining established and commonly used protocols for cell injection.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.