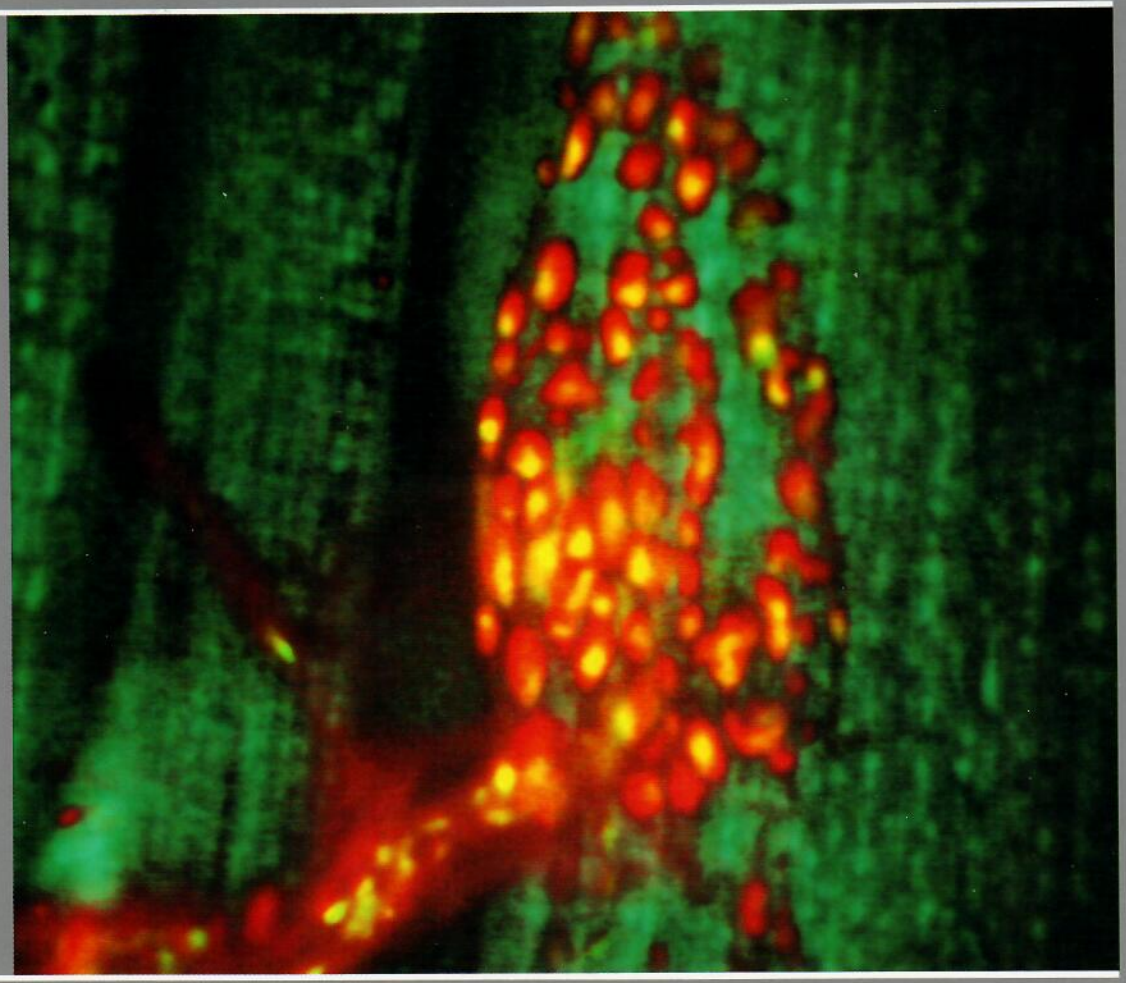
Vol. 1, No. 1 October 1989

THE NEW BIOLOGIST



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High-Resolution Imaging of Synaptic Structure With a Simple Confocal Microscope

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We describe a straightforward modification to a conventional fluorescence microscope that permits confocal fluorescence imaging of living and fixed biological material. The modified microscope has been used to examine the morphology of presynaptic terminals at the snake neuromuscular junction. Among our observations is the presence of two discrete compartments within each terminal bouton.

Received June 7, 1989; revised July 7, 1989.

Studies of the structure and function of synaptic terminals in situ have advanced considerably as a result of the availability of new fluorescent probes that selectively label terminal processes (Tsien, 1989; Yoshikami and Okun, 1984; Magrassi et al., 1987; Lichtman et al., 1985). One can now study the intact nervous system at a much higher level of detail than was previously possible. As a result, previously unanswerable questions such as the relation between structure and function of individual synaptic endings, or the dynamism of living synapses during development and aging, are now approachable. This research trend has depended heavily on microscopic images of structures labeled with fluorescent dyes. However, fluorescence images are subject to a number of optical difficulties that may seriously affect image quality. Over the past decade, computer-enhanced video microscopy has been the principal means of overcoming these difficulties, but certain purely optical methods can also improve image quality (Wilson and Sheppard, 1984). When used in conjunction with digital image processing, such optical methods should provide unparalleled access to the structure of synapses.

One of these optical methods, confocal scanning microscopy, improves upon the conventional microscope by means of two modifications. First, only a small region (often a diffraction-limited spot) is illuminated at any one time. Second, the illuminated region is selectively viewed (or electronically detected) through an aperture that masks all but the illuminated area. By virtue of the small area of illumination and the aperture in the return light path, scattered and out-of-focus light resulting from the illumination are minimized (Egger and Petran, 1967; Petran et al., 1968; Wilson and Sheppard, 1984; White et al., 1987; Amos, 1988). An entire image is obtained by scanning the specimen with the illuminated spot. When examined by this technique, images have greater contrast and narrower depth of field, and, with the use of diffraction-limited apertures, potentially greater resolution than otherwise attainable. However, constructing a complete image requires that both the illuminating beam and return light aperture be precisely aligned and that the scan be uniform. As a result, most commercial confocal microscopes are electromechanically complex and expensive.

We describe a device that differs from most previous confocal designs in that a single aperture is used to simultaneously illuminate and view the specimen. This simplification obviates the need for precise alignment of moving apertures, mirrors, or stages and therefore greatly reduces the inherent complexity. Furthermore, this design permits confocal imaging capability to be added to conventional epi-illumination microscopes without special expertise. The required modifications are inexpensive and straightforward. Perhaps most important, the device uses far less illuminating light intensity than laser-driven confocal microscopes, resulting in substantially less bleaching of fluorescent probes. The instrument has allowed us to view living and fixed snake motor nerve terminals at high magnification with high resolution and reduced background, and, by using multiple scans, to construct three-dimensional images of terminals.

RESULTS AND DISCUSSION

The single-aperture microscope was developed as a modification of our fluorescence microscope to improve images of supravitally stained nerve terminals in which

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1043-4674/89/0101-0006$5.00/0

KEY WORDS: confocal microscopy/nerve terminals/neuromuscular junction/synapses

image quality was limited by background fluorescence (Lichtman et al., 1985; Lichtman and Wilkinson, 1987). There are several potential locations in the incident and return light paths where apertures might be inserted to selectively illuminate and view a small area of the specimen. Such "conjugate field planes" (Inoué, 1986) include the field diaphragm, the reticle plane in some eyepieces (ocular field stop), the film or photocathode plane of camera ports, locations designed for the insertion of photo reticles, and the specimen plane. With the exception of the last location, which is inaccessible because it is occupied by the specimen, each such optical plane lies either in the incident (illuminating) path or the return (viewing) path, but not both. However, we noted that a requisite feature of all epi-illumination microscopes is that the incident and return light paths coincide in their final approach to the objective in such a manner that both are focused concomitantly on the specimen.

Our approach makes use of this common path by (i) allowing the original specimen plane to serve as a new conjugate field plane and (ii) extending the combined incident and return paths beyond this plane through an additional objective that now images the specimen (Fig. 1). A single scanning aperture is introduced at the new conjugate plane through which a small region of the specimen is illuminated and viewed simultaneously. Stated another way, an objective at the original objective location illuminates and "views" the aperture, while a second objective and intermediate lens project a real image of the specimen onto the aperture's plane. A detailed description of the modification is contained in the Materials and Methods section.

Although conventional diffraction-limited circular apertures (illuminating 0.2- to 0.5-μm diameter spots of the specimen) may be used for scanning, they require an apparatus for movement in a raster pattern. Furthermore, only a tiny fraction of the specimen is illuminated at any one time. As a result, either very bright illumination (e.g., a laser) or a very slow scanning rate (several minutes per scan with a conventional mercury light source) is required to provide sufficient exposure for image formation. Each alternative has limitations. Slow scanning is tedious, particularly when multiple images

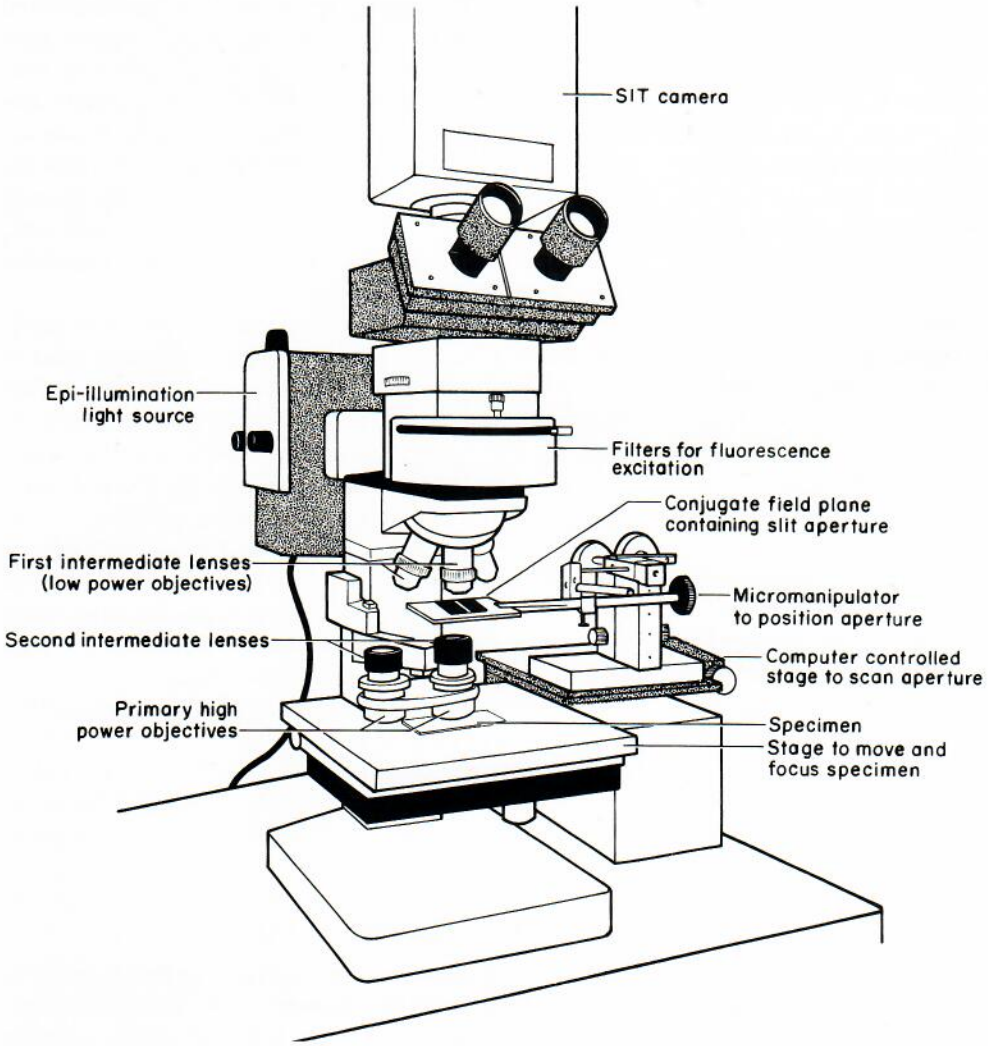

Figure 1. Illustration of single-aperture confocal microscope.

An upright epi-illumination fluorescence microscope (Leitz Laborlux D) was modified to permit confocal imaging. The two modifications are (i) lowering the focusing stage approximately 10 cm and reattaching it to the focusing mechanism, and (ii) adding a swiveling attachment to the microscope to hold the primary high-power objective(s). Next to the microscope is a computer-controlled stage upon which a micromanipulator has been placed to hold the aperture. A 75-Watt xenon bulb provided fluorescence illumination using Leitz cubes (I_2 and N) for excitation and barrier filtration. See Materials and Methods for further details.

for three-dimensional reconstruction are to be obtained, or in living preparations where movement might occur. On the other hand, bright illumination induces a disproportionate increase in the rate of bleaching (or tissue damage) (White et al., 1987) so that multiple scans are often impossible to obtain. As an alternative, we have found that slit apertures provide faster scans with simpler mechanical drives. In addition, because the slit's area of illumination is much larger than that of a circular aperture of similar width, we found that scans could be completed rapidly (within a few seconds) at the moderate light levels produced by the microscope's standard light source (75 Watt xenon).

We wished to see if scanning with slits of different widths affects the resulting image in a manner qualitatively similar to the effect of scanning with circular apertures of different diameters (Wilson and Carlini, 1988). That is, does optical sectioning and reduction of scatter improve as the aperture is narrowed? To do this we scanned the same fluorescent specimen with several different slits. Figure 2a shows an unscanned conventional image of synaptic boutons labeled by activity-dependent internalization of the fluorescent probe sulforhodamine 101 (Lichtman et al., 1985; see below). The image suffers from background light that reduces the contrast of the labeled boutons. Furthermore, the boutons are somewhat blurred because of the contribution of in-focus and slightly out-of-focus light from these roughly spherical objects. Figure 2b shows the same boutons scanned with a slit of 100 μm width. Because the lenses below the slit magnify the image an additional 20 times (see Materials and Methods), the slit's projected width of illumination on the specimen is reduced (20-fold) to only 5 μm. Although this width is 10 to 20 times greater than a diffraction-limited spot, there is noticeable improvement in the image. The background is reduced and the contribution of out-of-focus areas of the boutons is reduced. Figure 2c shows the same synapses scanned with a 25-μm slit (1.25 μm on the specimen). The image is noticeably improved over both the unscanned (Fig. 2a) and wider-slit (Fig. 2b) scanned images. Now the background is minimal and subsynaptic areas of bright and dim staining are easier to define. Use of a 5-μm slit allows scanning with a diffraction-limited (perpendicular to the orientation of the slit) aperture (0.25 μm on the specimen). This image (Fig. 2d) is appreciably better than any of the previous images. Out-of-focus light is at a minimum and areas of boutons that are unstained now appear black. Thus, scanning with slits can dramatically improve image quality in a manner analogous to pinhole apertures. The optical section obtained is progressively thinner as the aperture is reduced. In fact, even when the aperture is not diffraction limited, the quality of the image is still noticeably improved (Wilson and Carlini, 1988).

Because images of very thin optical sections contain virtually no light from out-of-focus areas (Fig. 2d), they can be "stacked" to create three-dimensional images (e.g., Brakenhoff et al., 1986, 1988). There are two advantages of such reconstructed images (usually stereo-pairs). First, the depth of field is restored, but without sacrifice in resolution as occurs if depth of field is increased by using lower numerical aperture lenses. Second, the depth information provided by thin optical sections is still completely accessible and can be visualized by stereo-pairs. For example, even in the thin transversus abdominis muscle there are nerve terminals lying in different focal planes. To visualize the location of all the synapses in one region of this muscle we stacked 30 confocal scans (Fig. 3). The composite image in stereo permits visualization of all the endplates in their appropriate three-dimensional position.

This same approach can be used at very high magnifications to provide structural detail at the submicrometer level (Fig. 4). We have used this method to reconstruct individual synaptic boutons. Boutons were labeled by stimulating them via their axon to cause transmitter release and vesicle recycling, which permits internalization of extracellular markers (Heuser and Reese, 1973). We have induced the internalization of several fluorescent probes, including sulforhodamine, into snake junctions (Lichtman et al., 1985; Lichtman and Wilkinson, 1987). When we scanned at high magnification the terminals labeled in this way we were surprised to find that the boutons were not uniformly labeled; instead, areas of bright and dim (or absent) staining were found in each bouton. Generally, the sulforhodamine-labeled areas were at the periphery of boutons. Conversely, areas without staining were often in the centers.

Examination of electron micrographs of snake synaptic boutons often showed a peripheral area containing vesicles and a central area devoid of them (Fig. 5c). The area lacking vesicles contained mitochondria. However, because of the lability of vesicles within synaptic terminals, it was unclear if the larger areas without vesicles were depleted during fixation. Therefore, to further examine this potential compartmentalization of boutons we stained live boutons in nerve-muscle preparations with the fluorescent probe 4-Di-2-ASP (Magrassi et al., 1987; Lichtman et al., 1987). This probe was selected because it is a cationic styryl pyridinium dye of a class known to label mitochondria in various cell types (Bereiter-Hahn, 1976). Because we wished to see if the compartments unstained by the activity-dependent probe were indeed stained by the mitochondrial probe, we labeled living terminals with both dyes, which have different excitation and emission wavelengths.

Double confocal scans showed that the two stains generally occupied different regions of the same boutons (Fig. 5; see also cover photo).

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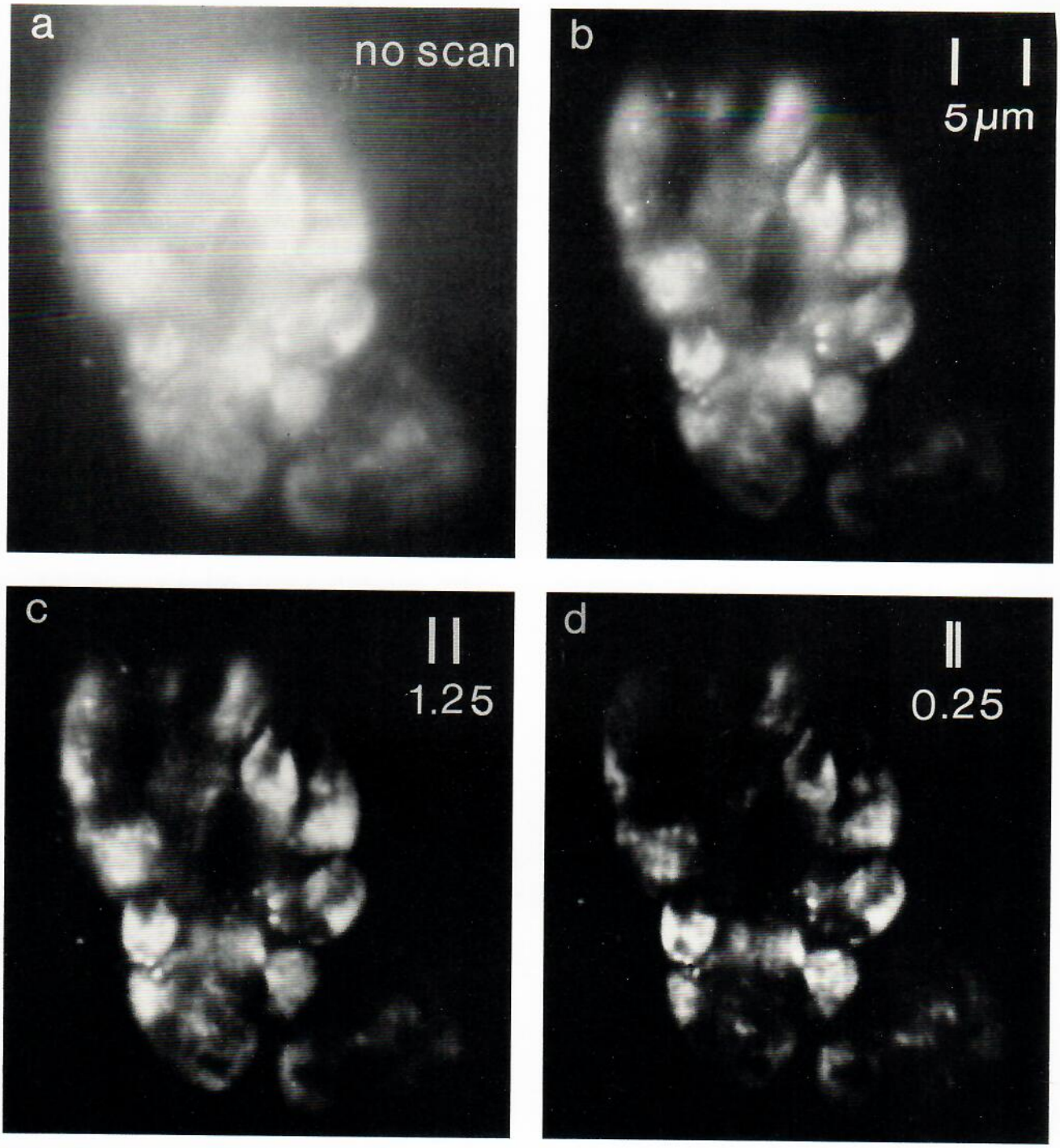

Figure 2. Influence of slit width on confocal images.

Four images of one motor nerve terminal (snake transversus abdominis muscle) were stained with the fluorescent probe sulforhodamine 101. (**a**) Averaged conventional image (32 frames) digitized from a video camera through identical optics and magnification as other panels except that the scanning slit was removed. Note background and out-of-focus fluorescence that obscure details of individual terminal boutons. (**b–d**) Confocal images scanned by slits of three different widths (slit width is shown to scale at upper right of each panel). (**b**) Significant improvement in background was obtained by using the widest slit (100 μm, which illuminated 5 μm in the specimen plane). (**c**) Reduction of slit width to 25 μm (1.25 μm at the specimen) further reduced the background. (**d**) Diffraction-limited slit (5 μm, 0.25 μm at the specimen) provided maximum optical sectioning capability (minimal depth of field). Note that some regions of the terminal are out of the plane of focus and therefore vanish from the image. Images **b** to **d** were obtained by scanning at progressively slower rates proportional to the slit width, such that the total integrated light presented to the video camera was held constant. Slit widths serve as calibration bars.

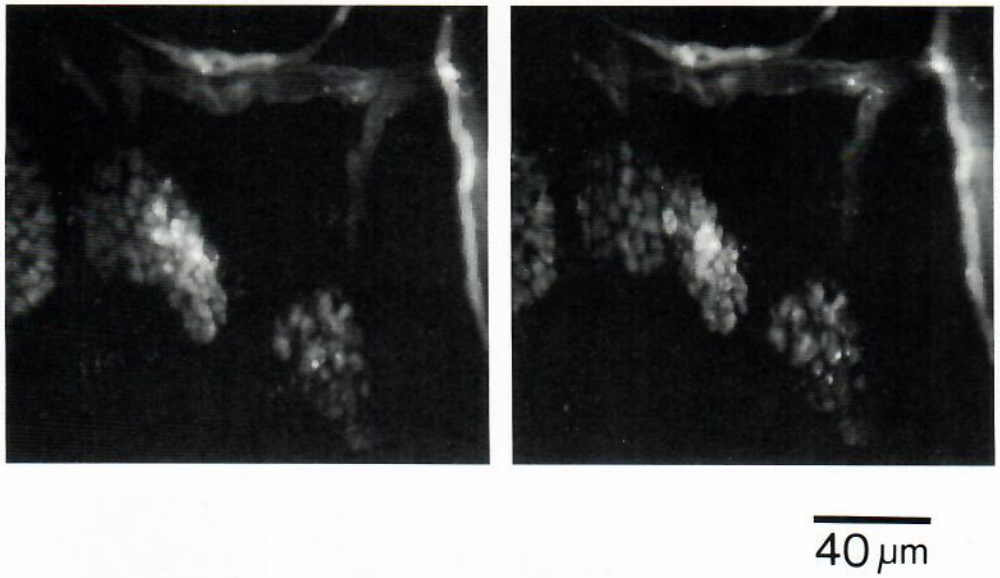

Figure 3. Three-dimensional reconstruction of motor nerve terminals.

Four nerve terminals are shown, each innervating a twitch muscle fiber in the transversus abdominis muscle. The nerve was stimulated with the activity-dependent probe sulforhodamine 101 added to the bath, labeling the terminals. Myelinated axons (right and above) are also labeled by the probe; the preterminal region of each axon is unmyelinated and therefore unstained. Muscle fibers (unstained) course vertically in a single-fiber-thick sheet. All boutons of each terminal are sharply in focus, although the terminals lie at different depths. Note that the two central terminals are separate in depth although they overlap in the plane of the figure. The image was generated from 30 confocal scans (10 μm slit, 0.5 μm on specimen), each obtained after incrementing the microscope's focus 1 μm. Images are arranged for crossed-eye viewing.

CONCLUSIONS

We have found the most useful configuration of our microscope to consist of a slit scanned in one direction only (rather than a raster-scanned circular aperture) and a video camera and frame buffer to permit subsequent digital image processing if desired. Depending on the slit width chosen, a scan can be completed and digitally displayed in 2 to 60 s. Reduction in scattered and out-of-focus light, and optical sectioning capability, were progressively improved as slit width diminished, similar to the effect of aperture diameter in commercial

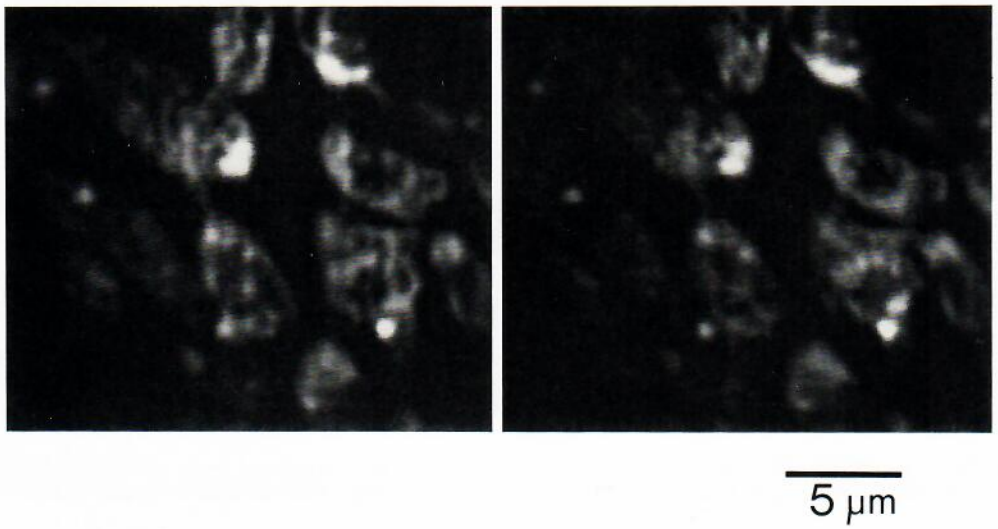

Figure 4. Three-dimensional reconstruction of individual synaptic boutons at an endplate.

A high-magnification reconstruction of 20 scans (0.5 μm steps in depth) of synaptic boutons at a twitch motor nerve terminal is shown. The boutons were stained by the activity-mediated uptake of sulforhodamine 101. Connections between the boutons are visible including one crossing on top of a bouton in the center. Areas with little or no staining within the boutons are evident. In some of the boutons one can see that the areas without stain are located in the center (see Fig. 5). Images are arranged for crossed-eye viewing.

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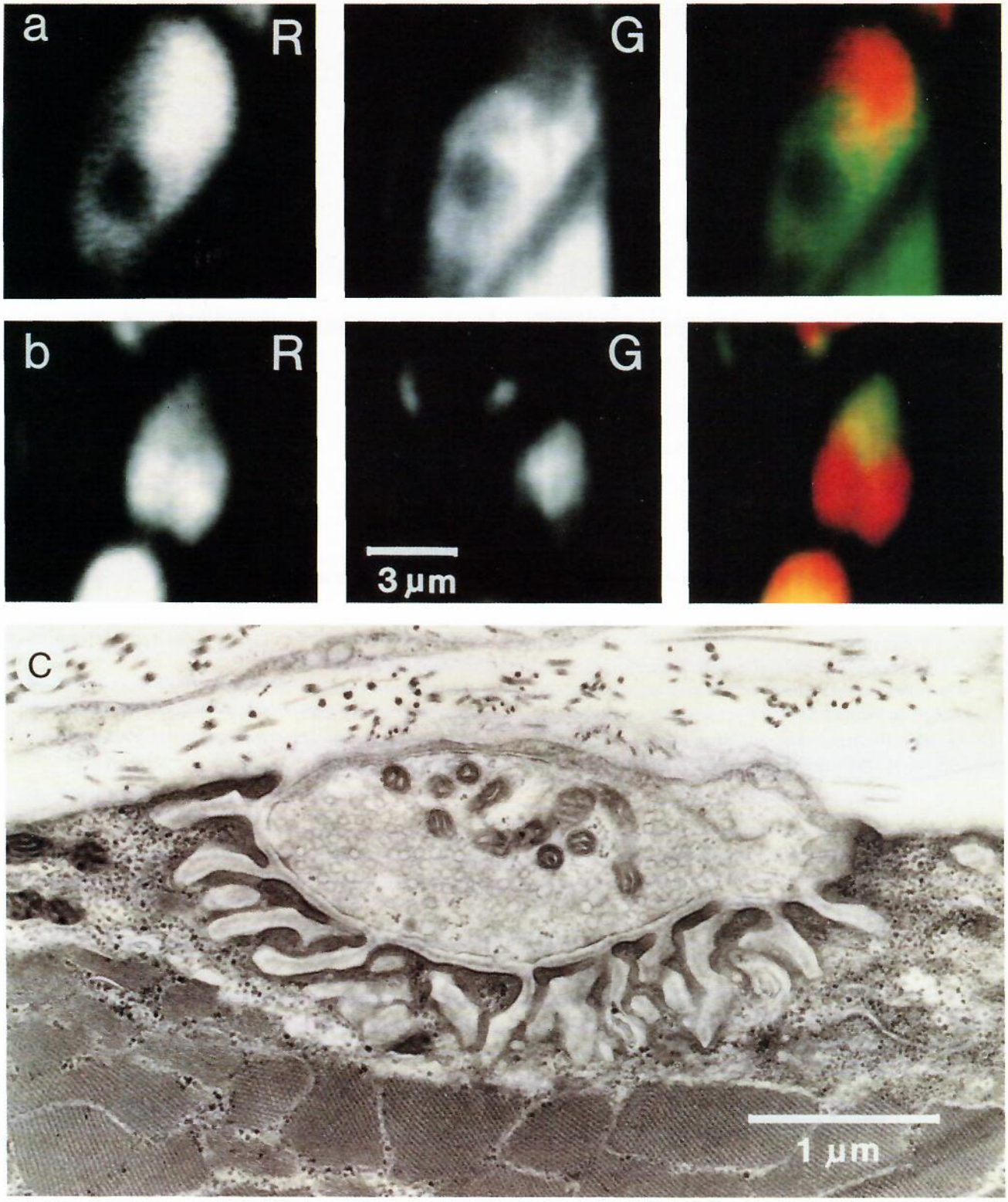

Figure 5. Confocal images of synaptic boutons supravitally stained with two fluorescent probes.

Synaptic boutons at the neuromuscular junction of the transversus abdominis muscle were supravitally stained with the activity-dependent probe sulforhodamine 101 (red fluorescing, R) and the mitochondrial dye 4-Di-2-ASP (green-fluorescing, G). The two probes label different compartments in the boutons. Two examples are shown in (a) and (b); the color images show the stains superimposed. (c) An electron micrograph of a snake synaptic terminal reveals a parcellation of mitochondrial and vesicular compartments, similar to the pattern revealed by the supravital stains.

confocal microscopes. Furthermore, the single-aperture microscope operates at low enough light intensity to prevent photobleaching.

Operation of the microscope at low light intensities also makes it possible to obtain confocal images using supravital probes in living tissue. Such probes often either fade or damage the tissue (possibly because of free radical formation) when exposed to too much light. We plan to exploit this unique feature of the single-aperture microscope in two types of experiments. First, stereo images of individual boutons (e.g., Fig. 4) should improve our measurement of size of physiologically characterized synapses by assigning an approximate volume, rather than area or length, to the presynaptic terminal. Second, it should be possible to document terminal morphology in three dimensions in experiments where the same synapses are repeatedly studied over time.

MATERIALS AND METHODS

Labeling of Snake Neuromuscular Boutons

Garter snakes (*Thamnophis sirtalis*) were anesthetized by submersion in iced water for 10 min and killed by rapid decapitation. The thin transversus abdominis muscle was dissected from the animal and pinned out in a small chamber containing reptilian saline solution. The muscle nerve was then raised onto a small Ag/AgCl wire electrode for stimulation; a similar wire in the bath served as a reference electrode. The activity-dependent probe sulforhodamine 101 was added to the bath (200 μg/ml), and the nerve supramaximally stimulated (50 Hz) for 5 min. The preparation was rinsed, fixed, and whole-mounted on a standard microscope slide (see Wilkinson and Lichtman, 1985 and Lichtman and Wilkinson, 1987 for details of the dissection and labeling procedure). For the examination of living terminals the preparation was rinsed but not fixed, and viewed with a water immersion objective placed in the bath. When desired, the mitochondrial probe 4-Di-2-ASP (10 μM) was added to the bath before viewing (Magrassi et al., 1987).

Snake neuromuscular junctions were prepared for electron microscopy as described elsewhere (Wilkinson and Nemeth, 1989).

Details of Microscope Construction and Adjustment

To adapt a particular objective for confocal scanning we used the following procedure. After lowering the microscope stage 10 cm, the objective was similarly positioned in a lower location and adjusted laterally and vertically to give a focused and centered image as viewed through the eyepieces (Fig. 1). This established that the objective was aligned with the original optical axis and operating near its original working distance and magnification (a slight error in both is introduced by the increase in tube length). Next, the first intermediate lens (a low-power objective, 2.5 or 10×) was introduced into the light path by rotating the microscope's nosepiece. At this time the required second intermediate lens was placed between the two objectives and positioned vertically so that the image was once again in focus. We selected this lens empirically and for convenience so that the total magnification of the optical path was similar to that of the objective lens used in a conventional microscope. Finally, a movable aperture was positioned within the optical path at the specimen plane of the first intermediate lens. Thus, both the aperture and the specimen were in focus concurrently. Similarly, the epi-illumination was in focus both at the aperture and the specimen plane.

The primary objective in our device was either a Leitz 100× NPL Fluotar (n.a., 1.2; water immersion) or a Leitz 160× Plan Apochromat (n.a., 1.25; water immersion). The first intermediate lens was either a Leitz 2.5× Plan (n.a., 0.08) or a Leitz 10× EF (n.a., 0.25). The second intermediate lens was the upper compound element of a Leitz eyepiece (Periplan GW 8× M). Although maximal resolution and image quality requires a primary objective with a high numerical aperture, the microscope significantly improved the quality of images from lower numerical aperture objectives as well.

Because the same aperture was used simultaneously in the illumination and return light path it was not difficult to experiment with a number of different aperture designs. When our aim was to see a confocal image in real time, we found that a series of parallel slit apertures rapidly oscillated by an inexpensive electromagnet (an audio speaker) gave good results. The total intensity of the return light is, of course, proportional to the fraction of the image that is illuminated at any one time. The greater the number of slits or the wider the slits, the more light returns; however, if the slits are too close together, illuminating light entering one slit may cause scatter of fluorescence exiting an adjacent slit. Therefore, the slits must be spaced at a reasonable distance. We found that slits that were about 10 slit widths apart gave good results. The width of the slit also affects image quality (see Fig. 2), so we typically used diffraction-limited slits.

When our aim was to obtain high-quality video images using a silicon intensified target camera (Dage MTI-66, Michigan City, IN), we scanned more slowly with either single "precision" air slits or pinholes (Melles Griot, Rochester, NY), both consisting of apertures cut into a thin metal foil. The principal advantage of slower scanning comes with video microscopy when one uses cameras in which the light level of the image and resolving power (lines of resolution) are directly related. By scanning slowly, the camera need not be pushed to its maximum gain (lowest resolution). The slow scans were generated by moving the aperture with a computer-driven motorized stage (517 MF, Stahl Research Laboratories, Inc., Port Chester, NY) placed next to the microscope upon which was mounted a micromanipulator holding the aperture (see Fig. 1). The scan speed was adjusted in relation to the size of the aperture so that each part of the specimen was continuously illuminated for several (two to eight) thirtieths of a second (video frames) to provide some signal averaging (see below). A single pass of the slit aperture across the specimen was sufficient to obtain the image. For pinhole apertures the process was much slower because of the need for raster scanning. Video images were always obtained using manual camera gain, setting it such that the image was black in the absence of light. A 16-bit frame buffer in our image processor

(Trapix Image Recognition Concepts, Inc., Incline Village, NV) was used to accumulate and digitize the scanned image. The data were compressed to 8 bits and printed with a video printer (Sony UP-5000). Software to drive the stage and image processor and create three-dimensional images was written locally by us and Ladan Hedayati, using the IMAGR language developed by J. Voyvodic.

Acknowledgments

We thank L. Hedayati for expert help in software development. This work was supported by NIH grants NS 20364, NS 24752, a Javits Center for Excellence Award, and the Muscular Dystrophy Association.

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