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Intravital imaging of green fluorescent protein using two-photon laser-scanning microscopy *

(Confocal; GFP; 2-photon; **Drosophila** R-cell; rat; hippocampal neuron)

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SUMMARY

Imaging a fluorophore in a living tissue presents several unique problems. The fluorescence from the labeled cell(s) may be weak, the labeled cells may be buried deep within tissue and the presence of a fluorophore may render the cells photo-sensitive. Two-photon laser-scanning microscopy (TPLSM) offers several advantages in meeting these challenges. We show that TPLSM provides greater sensitivity, better resolution and less photo-bleaching, as compared to confocal laser-scanning microscopy. The dramatically reduced photo-bleaching makes it possible to image cells continuously for long periods of time. Therefore, TPLSM allows a safer and higher-resolution means of imaging living cells labeled with a variety of fluorophores, including green fluorescent protein.

INTRODUCTION

Compared to wide-field fluorescence imaging, confocal laser-scanning microscopy (CLSM) provides greatly enhanced resolution, especially in thick specimens, allowing dramatic 3-dimensional renderings to be constructed (Pawley, 1990; Sandison et al., 1994). However, for most applications, especially prolonged intravital imaging, concerns about photodamage (phototoxicity and photobleaching of the label) compel the CLSM user to operate using a fraction of the available laser power. Reducing the total dose of light to which the specimen is exposed has been the only reliable way to deal with photodamage (Laurent et al., 1994; Wells and Johnson, 1994; Rohr and Salzberg, 1994).

Advances in laser technology have made possible a new type of laser-scanning microscopy that offers many of the advantages of CLSM, but with greatly reduced photodamage. Two-photon laser-scanning microscopy (TPLSM), pioneered by Watt Webb and his co-workers (Denk et al., 1990; Williams et al., 1994), make;, use of pulsed infrared (IR) laser light to induce fluorescence in fluorophores normally excited by visible light. Unlike CLSM, there is virtually no fluorescence excitation (and thus no bleaching or phototoxicity) above or below the focal plane. There is no need for a confocal aperture, greatly enhancing the sensitivity of the technique. Furthermore, the increased penetration of IR light through most specimens allows imaging deeper into thick specimens than with CLSM. These advantages suggest

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Abbreviations: BODIPY ceramide, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosine; CLSM, confocal laser-scanning microscopy; DiA, 4-(4-dihexadecylaminostyryl)-*N*-methylpyridinium iodide; DiI, 1-1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; *gfp*, gene encoding GFP; IR, infrared; PBS, phosphate-buffered saline (0.1 M sodium phosphate/0.9% NaCl, pH 7.2); TPLSM, two-photon laser-scanning microscopy.

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that TPLSM would be an ideal technique for imaging thick and living specimens, such as tissues and organisms labeled with green fluorescent protein (GFP, reviewed in Prasher, 1995, and subsequent articles).

We have assembled a TPLSM apparatus, through minor modifications of a standard CLSM, in which the ability to use the microscope as a CLSM has been retained for easy comparison of one-photon confocal and two-photon imaging modes. We demonstrate the usefulness of the TPLSM in a variety of specimens with a number of fluorescent tracers, including GFP.

RESULTS AND DISCUSSION

(a) Construction of the TPLSM instrumentation

Our microscope consists of three main components (Fig. I): A Sarastro 2000 CLSM (Molecular Dynamics), a mode-locked titanium/sapphire laser (Coherent Mira 900), and an 8 W argon-ion pump laser (Coherent Innova 310). The CLSM is controlled by Molecular Dynamics ImageSpace software running on an Indigo workstation (Silicon Graphics). The 8 W pump laser is hundreds of times more powerful than the argon lasers typically used for CLSM, and the output or the Ti/sapphire laser is invisible. Therefore, all laser beams are covered to allow safe operation of the TPLSM by non-physicists.

The Ti/sapphire laser is used for operation in the TPLSM mode. The pulsed IR output of the laser can be tuned across a range from 710–970 nm, with a bandwidth of about 10 nm, allowing excitation of a variety of fluorophores normally excited by visible and UV light of approx. 350–520 nm. No excitation filter is needed. The two-photon process requires a high instantaneous photon flux to cause detectable fluorescence. To accomplish this, the laser produces very short pulses (2×10^{-13} s) of high amplitude (50 kW), with a low duty cycle (1:66 000) allowing a low mean power (a few tens of milliwatts) that is not harmful to living specimens. The repetition rate, 76 MHz, is well matched to the fluorescence lifetime of common fluorophores (a few ns). This minimizes illumination of already-excited fluorophore molecules, which would result in no additional signal emitted but might contribute to photodamage.

In CLSM mode, the on-board 25 mW continuous-wave argon-ion laser of the Sarastro 2000 is used, and the aperture wheel is rotated to either the 200, 100, or 50 μm pinhole. In TPLSM mode, a large, open (6 mm) hole is used.

For standard fluorescence microscopy (wide-field and CLSM), the choice of filters is crucial, since the excitation and emission bands of most fluorophores overlap. One must chose the primary beamsplitter and emission filters carefully to maximize the throughput of emitted fluorescence, while minimizing the detection of reflected excitation light. In contrast, with two-photon microscopy, there is a large separation between the excitation wavelength (in the IR) and the emission wavelength (in the visible). Thus, it is straightforward to collect the entire emission

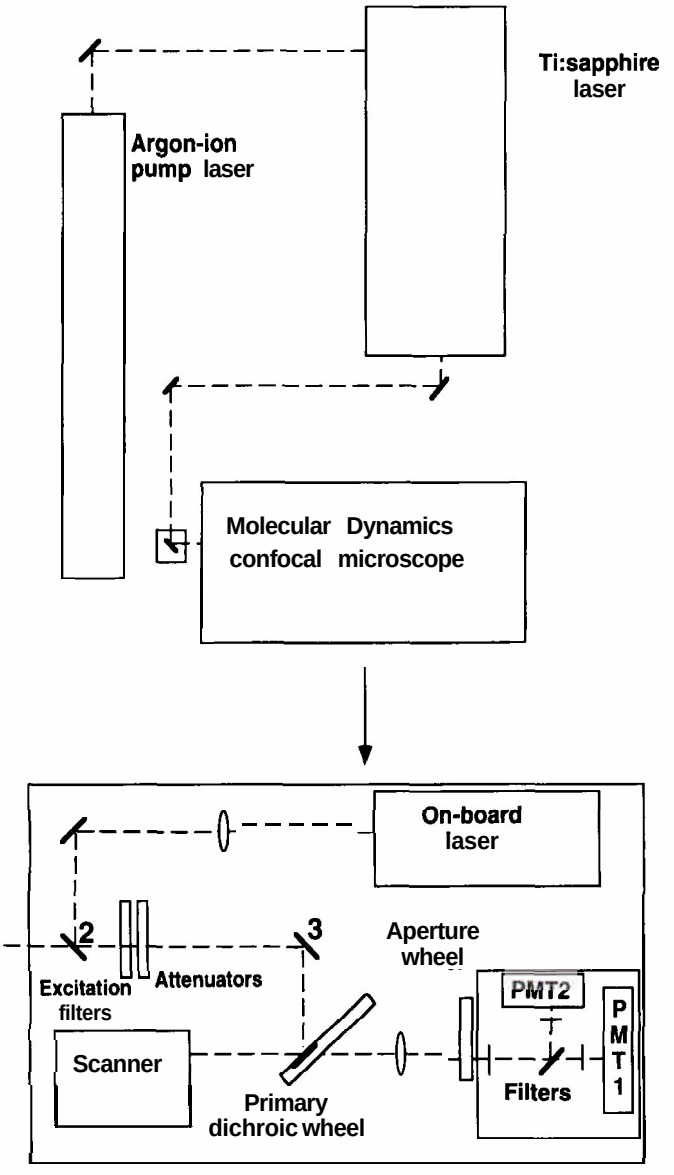

Fig. 1. Layout of the TPLSM/CLSM. Viewed from above, the laser light path is shown, from the pump laser, to the pulsed IR laser and into the scanning microscope, shown enlarged at the bottom. To allow either the IR laser or the on-board visible laser to be used, mirror 2 was replaced with a 680 nm long-pass dichroic beamsplitter (Chroma). Mirror 3 and the two scanning mirrors were replaced with broad-band mirrors (Newport; Ventura Optical Industries) for better IR throughput. A beam-blocker was installed at the auxiliary entrance port to automatically block the IR beam whenever the lid covering the optics is opened. No other modifications were necessary. To switch between CLSM and TPLSM modes, one simply turns off the on-board laser, opens a shutter on the IR laser, rotates the primary dichroic wheel to the short-pass mirror, rotates the aperture wheel to the 'open' position and places the IR blocking filter in the PMT box.

band without interference from excitation light; one beamsplitter and one emission filter can be used for all fluorophores. We use a 690 nm cutoff short-pass dichroic mirror (Chroma) in the beamsplitter wheel, and an IR-blocking emission filter (BG39, Omega). No modifications were made to the Nikon Optiphot II microscope supplied as part of the CLSM.

(b) Comparison of photo-bleaching and resolution in TPSLM and CLSM modes

In order to quantify the two-photon effect on our TPLSM, a homogeneously fluorescent sample of dye mixed in a protein carrier was imaged (fluorescein isothiocyanate in chicken albumin, fixed with glutaraldehyde). Excitation laser intensity was attenuated using neutral-density filters, and the magnitude of fluorescence in CLSM and TPLSM modes was compared. Because 2-photon fluorescence requires the simultaneous absorption of two IR photons by the fluorophore, the probability of exciting a fluorophore depends on the square of the laser intensity. When fluorescence of the fluorescein sample was plotted against excitation on a log-log plot (not shown), the slopes of the one-photon and two-photon curves were 1.0 and 2.0, respectively. This verifies that the two-photon effect has the expected 2nd-order dependence on excitation, and is responsible for the observed signal in TPLSM mode. The intensity of the light in a volume element (voxel) falls off with the square of the distance from the focal plane. This, and the second-order dependence of the 2-photon effect on intensity, means the probability of exciting the fluorophore falls off by the fourth power of distance from the focal plane. This limits two-photon fluorescence excitation to a small voxel at the focus of the beam within the specimen (Nakamura, 1993; Stelzer et al., 1994), allowing the enhanced vertical sectioning capability and reduced photodamage of TPLSM, demonstrated below.

The same sample was used to compare photo-bleaching in CLSM and TPLSM modes. A single x-y plane of focus was repeatedly scanned using CLSM or TPLSM laser intensities that initially gave the same fluorescence signal. Then the TPLSM was used to create an *x-z* cross-section of the bleached region, as shown in Fig. 2. An hour-glass shaped region of the sample scanned in CLSM mode was almost completely bleached after 630 scans, while the TPLSM mode produced only a thin line of bleaching after 630 scans, restricted to the plane of focus (Fig. 2A). On a new sample, two different planes were bleached using different laser intensities in TPLSM mode. As shown in Fig. 2B and C, by decreasing the laser

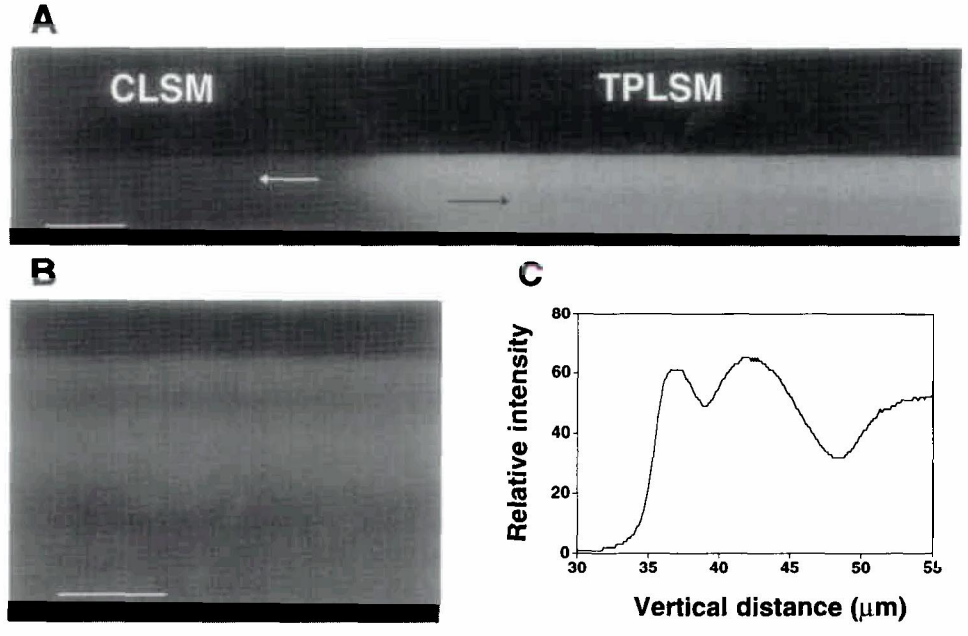

Fig. 2. Comparison of photo-bleaching in CLSM and TPLSM. **(A)** The originally homogenous fluorescent specimen was scanned continuously 630 times in a single horizontal focal plane using the on-board argon laser (left) and the titanium:sapphire laser (right). Then a vertical cross-section image was taken through the thickness of the sample using TPLSM. The CLSM bleached most of the fluorophore on the left side of the sample, resulting in a pair of dark cone-shaped regions expanding above and below the focal plane (left arrow). In contrast, the TPLSM bleached only a thin line at the focal plane (right arrow), leaving the fluorophore above and below unharmed. Scale bar, 5 μm. **(B)** Vertical cross-section of bleaching in two different horizontal planes. The two planes were scanned for the same amount of time, but the upper photo-bleaching was produced using 50% laser intensity and the lower, using full power. Scale bar, 5 prn. **(C)** Graphical representation of the vertical intensity distribution in panel B. The left dip corresponds to the *50%* excitation photo-bleaching and the right dip to the full-power photo-bleaching. The photo-bleaching thicknesses (full width at half maximum) are 1.6 μm and 6.2 μm, respectively.

intensity in TPLSM mode, one can decrease the thickness of the bleached region, and enhance resolution of resulting image, as long as the signal-to-noise ratio is high.

In addition to decreased photo-bleaching, our TPLSM is superior to CLSM in terms of its ability to image and resolve signals from deep within a specimen. To demonstrate this, we imaged a 30-μm spherically-symmetrical pollen grain covered with regularly-spaced sharp points (Carolina Biological prepared slide). In the top two images of Fig. 3, it is clear that even using the smallest available pinhole in CLSM mode, there is more out-of-focus signal than in the TPLSM image taken with no pinhole. In the bottom panels, made by taking a vertical '2-slice' of the pollen through a stack of *x*-*y* images, there is no signal from the lower portion of the pollen grain in CLSM mode, while the points on the bottom of the grain are clearly visible in TPLSM mode. Therefore, TPLSM gives superior results in both resolution and depth in thicker specimens.

(c) TPLSM of fluorescently-labeled rat hippocampal neurons

A number of membrane dyes suitable for neuronal tracing have enabled the study of neural development and synaptic plasticity in living organisms or in living slices of neural tissue (O'Rourke and Fraser, 1990; Dailey et al., 1994; Yu and Brown, 1994). These dyes offer great advantages for imaging in living tissues concerning intensity of signal and ease of application, but many of them cannot be applied due to severe phototoxicity. The characteristics of the dyes, normally excited by blue or green light, would seem to make them ideal candidates for TPLSM. We found that DiI, DiO, DiA and BODIPY ceramide (Molecular Probes) all provide sizable fluorescence signals in stained rat hippocampal neurons in culture, with pulsed illumination at 900 nm (Fig. 4). It was difficult to observe any photo-bleaching during imaging of serial sections in TPLSM mode. No phototoxic effects were observed even from extended periods of imaging (up to 2 h), either acutely or one day after imaging.

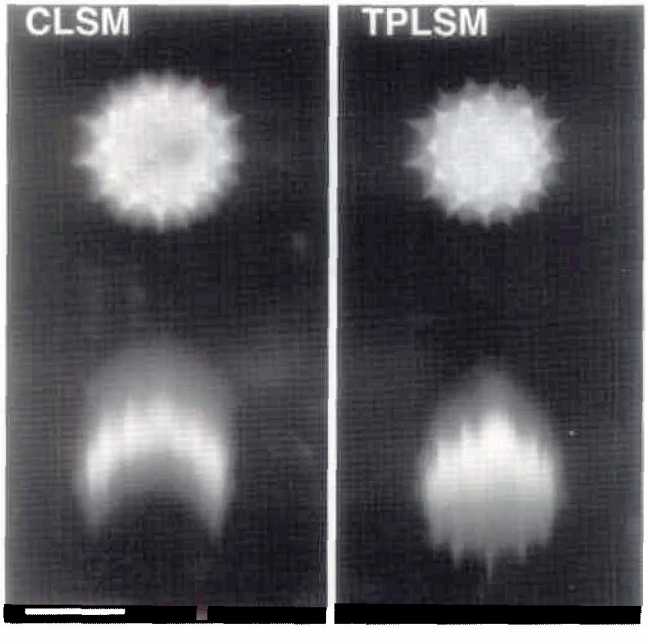

Fig. 3. Three-dimensional imaging of pollen: CLSM vs. TPLSM. A single grain of pollen was imaged using CLSM and TPLSM modes without moving the specimen, using a 40×10.75 numerical aperture Nikon Neofluar objective, 50 r-sections, 1.5 μm *z*-step size, 0.127 μm pixel size. Top images are single *x*-*y* sections. at the same focal plane, near the top of the pollen grain. Bottom images are maximum-intensity projections through a 40-pixel-wide *x*-*z* slice through the center of the pollen grain, reconstructed from the :-series. There was no significant photo-bleaching during the CLSM scans or the subsequent TPLSM scans.

(d) TPLSM of GFP-labeled Drosophila optical disk

The GFP has recently generated a great deal of excitement because it produces visible fluorescence in intact cells in a number of eukaryotic systems (Chalfie et al., 1994; Wang and Hazelrigg, 1994; Marshall et al., 1995). Directed expression of ***gfp*** in a cell type of interest can be used to visualize cells without dye injection, antibodies or histochemical stains. Furthermore, it can facilitate the observation of processes such as axonal outgrowth and targeting in intact living specimens over time (Chalfie et al., 1994).

One promising system for the molecular and genetic study of axonal outgrowth and targeting is the visual system of the fruit fly ***Drosophila melanogaster*** (Martin et al., 1995). The adult ***Drosophila*** compound eye is made up of approx. 750 repeated units. Each unit contains eight uniquely identifiable photoreceptor neurons (called R-cells) numbered R1 through R8. Each R-cell type projects into the developing optic lobe in a stereotyped pattern. The R1 through R6 axons stop at the superficial laminar layer of the brain, while the R7 and R8 axons project through the lamina and terminate in separate layers in the underlying medulla. Each R-cell class forms a topographic map in its target region. R-cell development has been extensively studied and a large number of molecular and genetic tools are available for use in this system (Zipursky and Rubin, 1994). The ability to visualize these axons in unfixed tissue over time, would provide another useful tool for studying the formation of this projection pattern in wild-type animals, as well as in animals containing mutations that disrupt this projection pattern (Martin et al., 1995).

Transgenic ***Drosophila*** were created that express ***gfp*** in all developing R-cells and their axons. We examined R-cell projections into the optic ganglia in third instar larvae, the stage at which R-cell development occurs and R-cell axons innervate their target region. The eye/brain

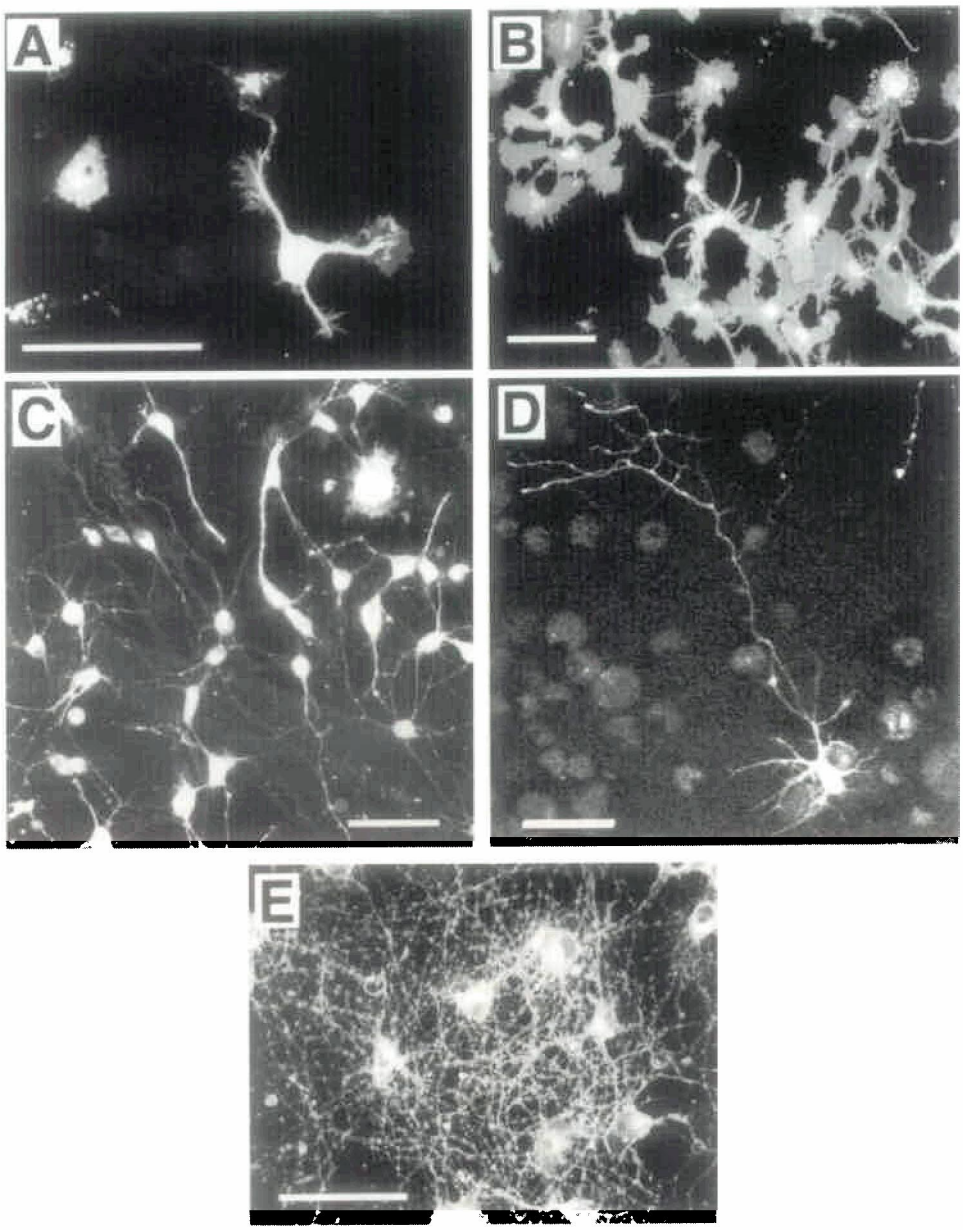

Fig. 4. Two-photon micrographs of cultured rat hippocampal neurons stained with membrane dyes. Hippocampi from embryonic day-17 rats were incubated in 0.25% trypsin for 15 min at 37 C and dissociated by trituration through a Pasteur pipette. Suspensions were filtered through a 70 μm nylon mesh to remove large debris and centrifuged for 2 min at 150 x *g* to remove small debris. Stained cells were washed twice by centrifugation for 6 min at 150 × *g* into a 300 μl layer of 5% bovine serum albumin dissolved in PBS. (**A**) DiA-stained neurons. stained in suspension for 1 h at 37 C with 10 μg nil DiA and imaged 4 h after plating on a polylysine laminin-coated plastic Petri dish. (**B**) DiO-stained neurons, stained in suspension as above. plated on a polylysine-coated Petri dish and imaged after one day in culture. (C) Dil-stained neurons, stained in suspension for 15 min at 37 C with 40 μg Dil mL. imaged 2 days after plating on a polylysine laminin-coated Petri dish. (**D**) Dil-stained neuron, prepared as in (**C**), but plated onto a cultured hippocampal slice (Stoppini. 1991) and imaged 3 days after plating. (**E**) BODIPY ceramide-stained neurons. An unstained dissociated hippocampal culture was prepared as above and stained after 8 days in culture for 1 h at 37 C with 20 μM BODIPY ceramide and washed with three changes of culture medium (Neurobasal B27, Gibco). All images were produced using 900 nm excitation and an open (6 mm) aperture. Scale bars. 50 μm.

complexes were dissected from larvae and then observed immediately without fixation. GFP-filled R-cell axons were imaged using either CLSM or TPLSM mode (Fig. 5). The bundles of incoming photoreceptor axons are visible. as is the lamina which contains many closely packed R1–R6 terminals. The axons from the remaining R-cells project into the medulla as individual fibers; these single axons and terminals are better resolved in the TPLSM image (C) than in the CLSM images (A and B). Any CLSM is seldom operated in a configuration that allows maximum resolution. Even when using intense laser illumination and a sensitive photomultiplier, the fluorescent signal detected by CLSM is relatively weak, due to the rejection of much of the light emitted by the

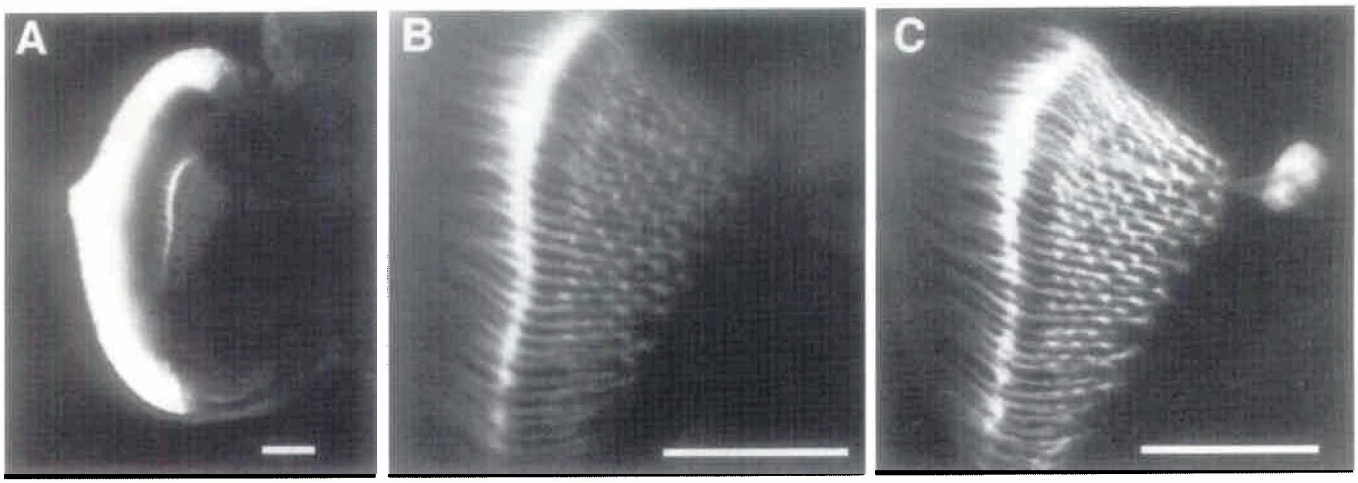

Fig. 5. *Drosophila* photoreceptors filled with GFP innervating the developing optic ganglia (the lamina and medulla) in a third instar larval brain. **(A)** Optic disk. showing photoreceptors and their projecting axons. (B) Higher-magnification projection of the central region of **(A),** imaged using a Bio-Rad MRC600 confocal microscope. Photoreceptor axons enter the target region from the left in this image; cell bodies are not shown. **(C)** Same region as in panel **B**, imaged using the TPLSM. Axons from the R1-R6 cells terminate in the lamina, viewed from the side in this image as a plexus of terminals. Axons from the R7 and R8 cells go through the lamina and terminate in the underlying medulla, which is perpendicular to the lamina and shown en face. The precise array of terminals reflects the topographic nature of photoreceptor axon projections. The large structure at right, too deep to resolve with the MRC600, is the termination site of Bolwig's nerve, which contains axons from the larval photosensitive organ. **Methods:** Standard molecular biology methods (Ausubel et al., 1994) were used to place the *gfp* coding sequence from plasmid TU#64 (Chalfie et al., 1994) under the control of the eye-specific promoter from plasmid pGMR (Hay et al., 1994). This promoter contains synthetic binding sites for the zinc-finger transcription factor glass. Transgenic flies were created using standard methods (Rubin and Spradling, 1982). Eye/brain complexes were dissected from wandering third instar larvae in PBS and immediately mounted in PBS for imaging. Confocal and TPLSM images were made from lookthrough projections through 20 sections of one μm each. Scale bars, 50 pm.

fluorophore by the confocal aperture. Thus, the user is forced to make a compromise in spatial resolution by using a larger confocal aperture so that the signal is not dominated by noise produced by the statistics of light or the electronic noise of the detector (Pawley, 1994; Sandison et al., 1994). With TPLSM, no confocal aperture is used, since the resolution is determined solely by the size of the volume of excitation. None of the emitted light need be rejected and thus, a better signal to noise ratio is possible than with CLSM.

The TPLSM also does a better job of imaging deep structures, such as the termination site of Bolwig's nerve (C, right side), due to the enhanced penetration of IR into tissues, compared to visible excitation light. Thus, the TPLSM is a valuable tool to visualize individual axons filled with GFP in unfixed tissue.

(e) Conclusions

(1) We successfully constructed a two-photon microscope from simple modifications of a conventional confocal microscope, with the ability to switch between TPLSM mode and CLSM mode in seconds by changing the active light source, aperture and filters.

(2) We verified that the observed signal from the TPLSM was due to two-photon excitation, since there was a squared dependence on the laser intensity. This two-photon effect offers excellent selective excitation of only the focal plane and results in dramatically less photo-bleaching than with CLSM imaging.

(3) The TPLSM was shown to have greater ability to image details deep in the specimen than CLSM.

(4) Several commonly used membrane dyes normally excited by blue or green light gave good fluorescence signals under pulsed illumination at 900 nm, producing images comparable to those produced by CLSM, but with dramatically less photo-bleaching and no phototoxicity.

(5) Tracing of neurons expressing ***gfp*** in living *Drosophila* is possible using TPLSM with illumination at 900 nm. Three-dimensional reconstructions of serial sections taken in TPLSM mode surpass those taken using a CLSM.

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