High Resolution Imaging Using Confocal And 2-photon Molecular Excitation Microscopy

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Fluorescence microscopy has proved to be an invaluable tool for biomedical science since it is possible to visualise small quantities of labelled materials (such as intracellular ions and proteins) in both fixed and *living* cells. However, the conventional wide field fluorescence microscope suffers from the disadvantage that objects outside the focal plane also fluoresce (in response to the excitation light) and this leads to a marked loss of contrast for objects in the focal plane. This is especially a problem when the fluorescent probe is distributed throughout the thickness of the cell and the cell is thicker than about 1 µm. The confocal microscope overcomes this problem by illuminating the preparation with a point source of excitation light and limiting the collection of light with a pinhole that is confocal with the illumination source. This converts the microscope from an imaging system to a point detector and images are produced by scanning the illuminating and detecting point over the specimen to build an image (in much the same way that a television set produces an image). The basic idea behind the confocal system is shown in Figure 1, and it should be noted that light from points outside the focal plane is defocused at the pinhole and so does not pass through the pinhole efficiently. This leads to the rejection of out-of-focus fluorescence and an improvement in image sharpness (for further details see Pawley, 1995). For fixed preparations this advantage may be limited since it is possible (at least in principle) to use deconvolution techniques to mathematically correct the in-plane image for out-of-focus fluorescence. With this method, a large number of images at different depths within the specimen are taken and the contribution of out-of-focus fluorescence is calculated and subtracted from data at the image plane. However, such 'deconvolution' techniques are of limited use when images are needed in near real time or when the specimen itself is changing with time.

Since a major problem in fluorescence imaging resides in the existence of fluorescence from outside the image plane, a solution to this problem would be to prevent fluorescence excitation outside the focal plane. This is exactly what has been achieved with 2-photon (or multi-photon) excitation microscopy which was first demonstrated in 1990 (Denk, 1990). In this article, we will describe how application of confocal microscopy to calcium metabolism in cardiac muscle has allowed the discovery of completely unexpected phenomena that has given new insight into how cardiac contraction is regulated (work carried out in collaboration with Jon Lederer's group in the USA) as well as our development of a practical 2-photon microscope system (Soeller and Cannell, 1996).

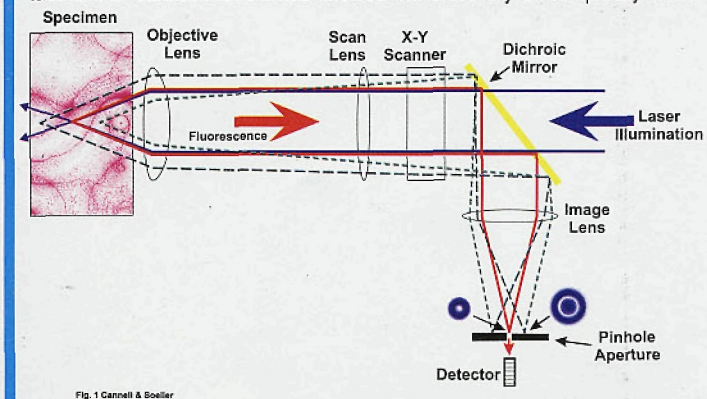

Figure 1: Schematic drawing of the optical path in a confocal microscope. The laser illumination light (blue) is coupled into the optical system through a dichroic mirror. Here a short wave pass mirror appropriate for one-photon excitation. The illuminating beam is scanned across the specimen by an x-y scanner and focused into the specimen by an objective lens. The scan lens ensures that the rear aperture is filled while correcting tube length. Emitted fluorescence (red) is collected by the same objective lens and passes through the x-y scanner ("descanned"). After most of the reflected illumination light has been separated by the dichroic mirror, the fluorescence is imaged onto the pinhole aperture by an image lens. Note how depth discrimination is achieved by the small pinhole aperture which rejects most of the light from locations above and below the plane of focus (dashed green rays).

Detection of 'calcium sparks' with the confocal microscope

Cytoplasmic calcium ([Ca2+]i) levels play an important role in cardiac myocytes by regulating (for example) force production and metabolic rate (for review see Bers, 1991). As shown in Figure 2A, wide field fluorescence imaging of [Ca2+]i (by injecting a fluorescent calcium indicator into the cell) revealed no spatial non-uniformity in quiescent cardiac myocytes. However, application of the confocal microscope to quiescent cells (Figure 2B) showed discrete local increases in calcium ('calcium sparks') -Cheng *et al.*, 1993) whose duration was much less than the image acquisition rate (0.5 s) of the microscope (as shown by the position of these local increases in [Ca2+]i changing between frames). To improve time resolution it is possible to repeatedly scan the same line within the cell which sacrifices spatial information in one dimension for a massive improvement in time resolution (to ~ 2 ms/line). With this method, we have found that the calcium spark spreads from a point source for about 40 ms, eventually encompassing about 2 m (Cheng *et al.*, 1993). The whole cell transient (in response to electrical stimulation) occupies the entire cell volume and appears to be made up of a very large number of calcium sparks (Cannell *et al.*, 1994;1995). Although a calcium spark is of shorter duration than the whole cell calcium transient, this observation can be explained by the calcium associated with the calcium spark diffusing from the site of release, thereby accelerating its decay (Cheng *et al.*,1993). This mechanism would not apply to the whole cell transient since the calcium is elevated nearly uniformly and the dissipation of diffusion gradients cannot make such a large contribution to the decline of [Ca2+]i. The discovery and examination of calcium sparks has led to a significant improvement in our understanding of how cardiac excitation-contraction coupling is achieved (*e.g.*, López-López *et al.*, 1995, Cannell *et al.*, 1994;1995).

Apart from the novelty of such data, it is important to note that the discovery of calcium sparks was the direct result of the application of the confocal microscope to cardiac calcium metabolism. Although the wide field microscope had been applied to calcium imaging since about 1985 (as shown in Figure 2A) cal-

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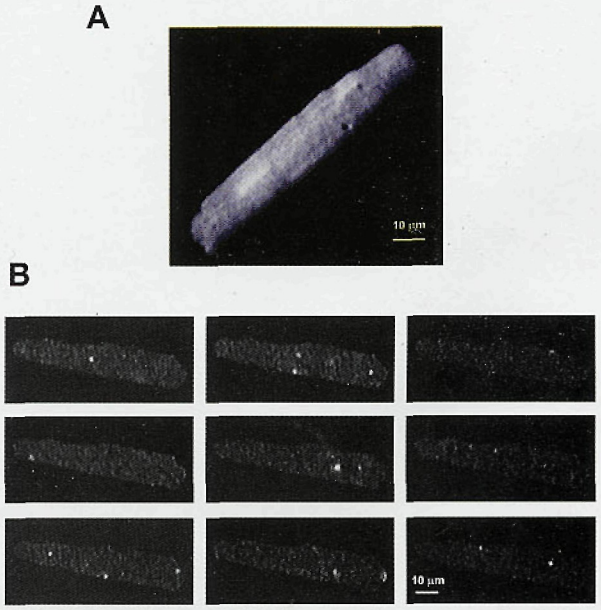

Figure 2: Detection of calcium sparks in quiescent rat heart cells with the confocal microscope. Panel A shows an image of a rat heart cell loaded with the calcium sensitive indicator Fura-2 recorded in conventional wide field mode. There are no pronounced non-uniformities in the fluorescence image. The weak non-uniformities in the fluorescence distribution (e.g., the brighter nuclei) do not change with time (data not shown). Panel B shows 9 successive confocal images of a fluo-3 loaded cardiac cell (images were acquired 0.5 s apart). Note that the fluorescence is generally uniform although there are discrete regions of increased fluorescence ("calcium sparks") at varying positions between images.

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cium sparks had not been observed previously. This is probably because the presence of fluorescence from outside the focal plane results in a marked loss of in-plane contrast. (Fluo-3 was used as the calcium indicator in these experiments as it has low fluorescence in the absence of calcium which also improves image contrast.) The calcium spark illustrates the high sensitivity of optical methods the calcium spark finally occupies about 10 fl (10^{-14} l) and represents calcium binding to only ~10^4 indicator molecules. Until recently, the laser scanning confocal microscope has been the only instrument that could measure fluorescence with a spatial resolution of about 0.4 x 0.4 x 0.8 m (x, y and z respectively) on the millisecond time scale.

2-photon molecular excitation microscopy

As illustrated in Figure 3A, the excited state is normally achieved by the absorption of a short wavelength photon. After radiationless transitions down the vibrational ladder, fluorescence emission occurs with a transition towards the ground state. The difference in energy between the absorbed photon and the emitted photon leads to Stokes Law of Fluorescence which states that the wavelength of emitted light is longer than the exciting light. However, it is also possible to achieve the excited state by the near simultaneous absorption of two longer wavelength photons, as shown in Figure 3B. The probability of absorbing 2-photons will depend on the square of the illumination intensity and for normal light sources this is very low. However, by 'mode-locking' a laser light source it is possible to produce very short, intense light pulses. The short duration of the pulses means that while the average laser power is quite low (~ 1W), the peak pulse powers are very high. For our titanium sapphire (Ti:S) laser system, typical peak powers are about 10 kW in pulses that last about 5 x 10^{-14} seconds with a pulse repetition rate of 80 MHz. The high brightness of the pulses results in a high probability that 2-photons will be able to interact with the fluorochrome and produce the excited state.

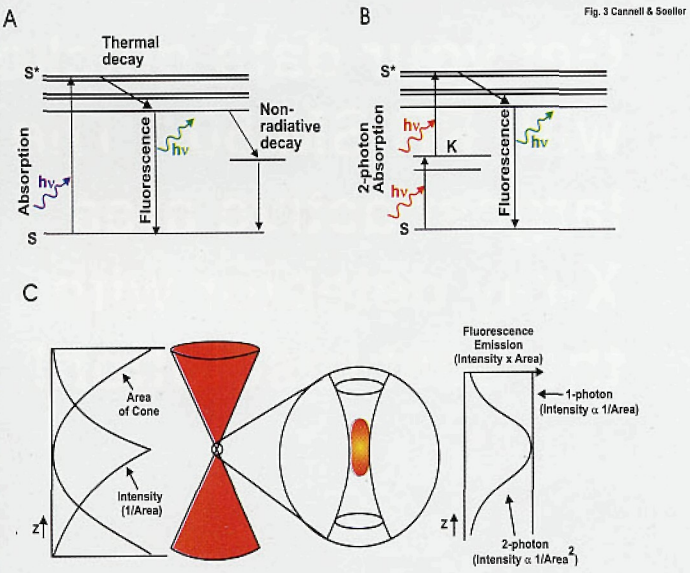

Figure 3: Principles of one- and two-photon excitation microscopy. Panel A shows a simplified Jablonski diagram of the processes involved in one-photon excitation of a generic fluorochrome molecule. Absorption of an excitation photon raises the molecule from the ground state (S) (which is really a quasi-continuum of vibrational sublevels) to an excited state (S*). From there it quickly relaxes to the lowest vibrational sublevel and subsequently decays to the ground state with the emission of a photon of fluorescence. Although most decays lead to the emission of a (red-shifted) photon there is also the possibility of non-radiative decay. The mechanisms of two-photon excitation are similar (panel B). Here, absorption of an exciting (near-infrared) photon raises the molecule to a short-lived "virtual" state from which a second incident photon can raise it to the excited state. The subsequent decay mechanisms are similar to those in one-photon excitation. Photo-bleaching processes, which remove molecules from the pool of fluorochrome molecules, have been omitted for clarity. Panel C illustrates how the different dependence of fluorescence emission on (two photon) excitation intensity results in three-dimensionally localised excitation. In one-photon excitation, the linear dependence of emission on excitation intensity results in any plane having the same contribution to total fluorescence (since excitation intensity area is the same everywhere). For two-photon excitation (with a square dependence on excitation intensity), the total fluorescence from a given plane decays quickly with increasing distance from the focal plane.

By feeding such a light source into an objective lens, the intensity of the light in the illumination cone increases as the focal point is approached (see Figure 3C). At the focal point, the intensity of the illumination is at a maximum and there the probability of a 2-photon transition taking place is highest. Outside the focal point, the probability of excitation falls with the fourth power of distance (from the probability of excitation being proportional to the square of intensity which is, in turn, proportional to the square of distance) at a rate that depends on the numerical aperture of the objective lens; being highest for high numerical aperture lenses. Thus while conventional illumination results in excitation throughout the whole thickness of the preparation, 2-photon excitation results in illumination being restricted to the focal plane (Figure 3C). As shown in Figure 4, 2-photon illumination also restricts fluorochrome bleaching to the focal plane which makes obtaining a large number of optical sections for 3D reconstruction easier (see below).

The lower energy of the photons needed for 2-photon excitation implies a longer excitation wavelength for the fluorochrome; typically the 2-photon excitation maximum will be a little less than twice the wavelength of the one-photon excitation maximum. In other words, it is possible to excite fluorochromes, which are normally excited by near UV or visible light (about 340-600 nm), with a tunable infra-red Ti:S laser operating in the range 680-1000 nm. It should also be noted that the use of infra-red light for excitation implies that scattering may be reduced in thick specimens. Since the resolution of the microscope is determined only by the properties of the excitation light cone (rather than the optical behaviour of both the illumination and detection systems) there is no need for any pinhole aperture. The removal of the pinhole (and any associated optics) results in a significant improvement in detection efficiency. This gain can be further increased by avoiding the mirror scanning system by feeding light from the rear aperture of the objective directly to the detector with a suitable dichroic mirror. Despite these advantages, it should be noted that the incident light is very intense and can lead to specimen damage. Where time resolution is not an issue, the damage threshold can be avoided by reducing the excitation light intensity and integrating the output signal for a longer period. While such an approach can be easily applied to fixed specimens, in living specimens we have not yet achieved a higher signal to noise ratio (or time resolution) than can be achieved with one photon excitation in conventional confocal methods.

There are additional problems in developing and using a 2-photon excitation microscope: 1) Accurate alignment of the laser to the microscope is more difficult since the excitation light is invisible (infra-red). 2) The optical path of most scanning microscopes is arranged so that long wave pass dichroic mirrors can be used to split the emission light from the excitation light. Switching to a 2-photon source implies that short wave pass mirrors must be substituted and these mirrors have only limited pass bands. 3) The pulse is distorted by the optical elements in the optical train. This problem arises from the speed of light in

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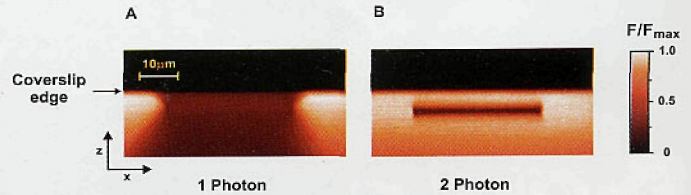
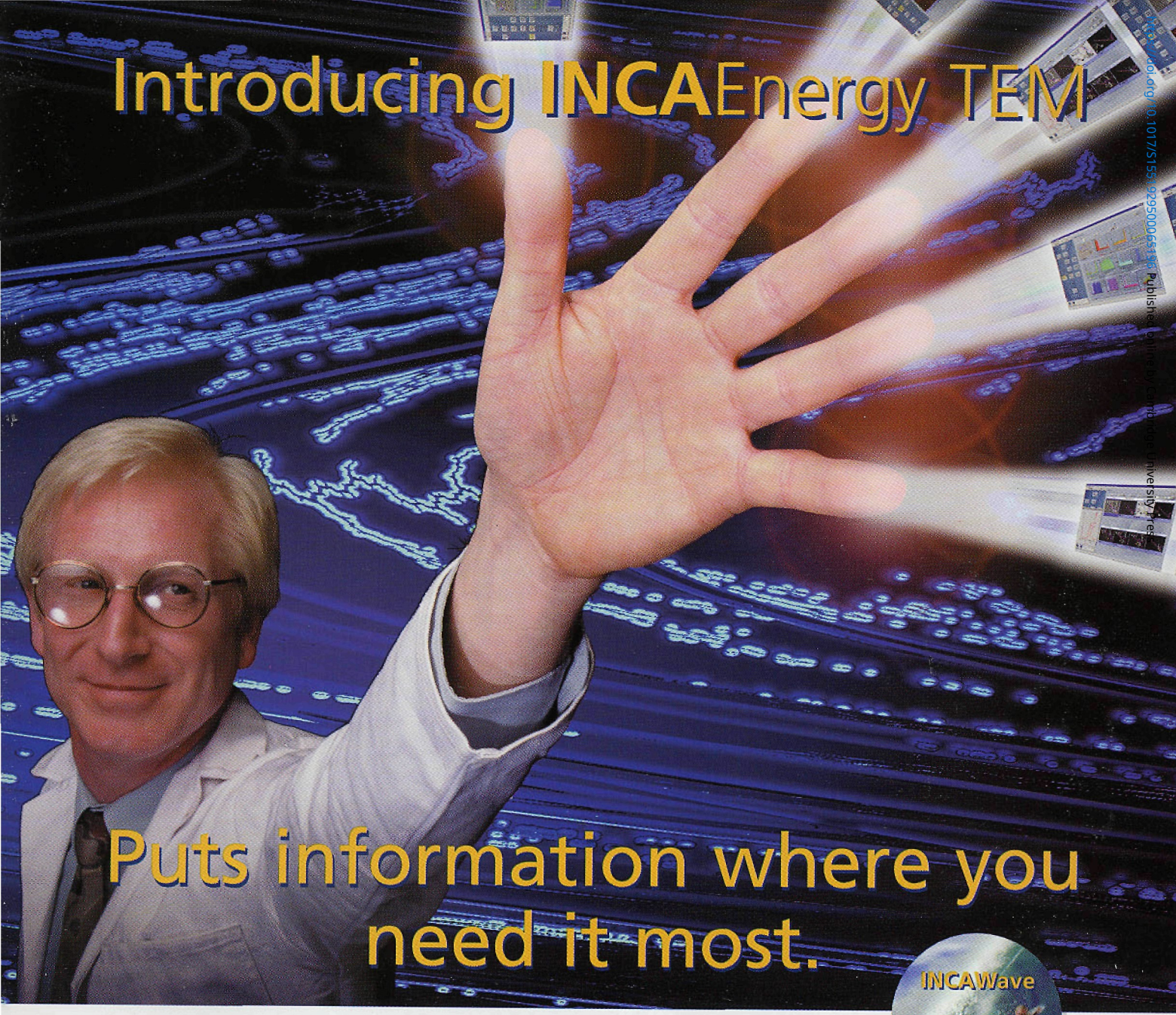
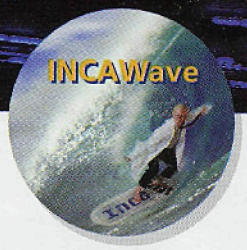


Figure 4: Photo-bleaching in one- versus two-photon excitation microscopy. Panel A shows the photo-bleaching pattern in a thin gel layer stained with immobilised fluorescein. After repeatedly imaging a plane ~5 µm below the coverslip edge, strong bleaching above and below the plane of focus is observed (since the total dosage of excitation light in the double cone of excitation light is independent of distance from the focus). In panel B, the photo-bleaching pattern in an identical sample after repeated imaging in two-photon mode is shown. Here, photo-bleaching is restricted to the immediate vicinity of the focal plane because of the strong axial dependence of the probability of two-photon excitation.





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materials being wave-length dependent, so the relative phase of the wavelength components making up the pulse is changed, resulting in a longer (and lower peak amplitude) pulse. Fortunately, it is possible to use a prism compensator system to pre-distort the pulse in the opposite way to that produced by the lenses in the microscope. Such pre-distortion is then removed by the microscope elements so the pulse shape is restored at the focal point of the objective (Soeller and Cannell, 1996). Such 'pre-chirping' requires an extension of the optical system outside the microscope which increases the overall complexity of the optical design. 4) The mode-locked laser is complicated and requires regular maintenance. 5) Mode-locked laser sources are very expensive and generally require a powerful laser as an energy source (in our system we use a 20W argon-ion laser as a 'pump' for the Ti:S laser).

Despite the above problems associated with 2-photon microscopy, the restriction of photo-damage to the focal plane is an attractive benefit. This also means that it should be possible to photo-release active molecules within cells from their 'caged' precursors. In this technique, the active molecule is modified by the addition of a photolabile side group. When this group is activated by light, it breaks off leaving the active molecule within the cell. Since 2-photon excitation is restricted to the focal plane it is possible to release the caged compound within a defined volume inside a living cell. The 2-photon microscope is the only instrument that makes such '3-dimensionally resolved' photolysis experiments possible. It is also likely that the development of new fluorescent probes that are more readily excited by 2-photon absorption, will further increase the signal to noise ratio obtained in experiments. Although we are currently using probes that were developed for normal fluorescence excitation and have not yet improved on normal confocal microscopy in terms of resolution and signal to noise, such new probes should allow the benefits of 2-photon microscopy to be realised more fully.

Figure 5 illustrates the imaging performance of our microscope in confocal and 2-photon modes. The resolution of a microscope is defined by the 'point-spread' function that can be measured by imaging a sub-resolution fluorescent bead. This point spread function describes how light from a point source is smeared and distorted by the optical train and can never be smaller than the limit set by the wave properties of light (the 'diffraction limit'). As shown in Figure 5A, the image of a 200 nm fluorescent sphere in our confocal microscope using a 63x 1.25 NA objective is an oval spheroid. This 'lozenge' shape results from the relatively poorer axial (than lateral) performance of the confocal microscope. It should be noted that the axial resolution is mainly provided by the pinhole and, if this element is removed, the image degenerates into a series of rings above and below the plane of the object. This distortion in the image of the object is expected from optical theory and is close to the absolute diffraction limit. The image of the same object using 2-photon excitation is shown in Figure 5B and although the pinhole has been removed, the point spread function is comparable to that obtained when using the microscope in confocal mode, despite the fact that the illuminating wavelength was 850 nm instead of 488 nm.

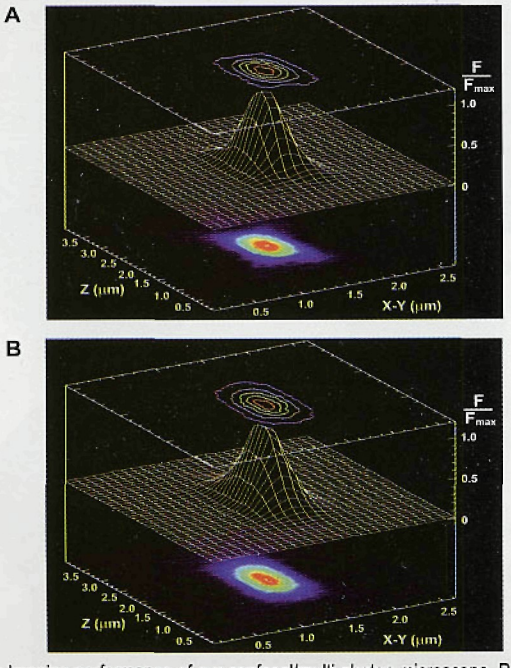

Figure 5: Imaging performance of our confocal/multi-photon microscope. Panels A and B show the point spread functions measured with sub-resolution fluorescent beads (0.2 μm diameter spheres) in conventional confocal and two-photon mode, respectively. The beads were imaged with a Zeiss Plan Neofluar 63x1.25 NA objective. The contour lines show the isophotes from 10% to 90% of peak response in steps of 20%. In conventional confocal mode, x-y and axial full width at half maximum resolutions (FWHM) were 0.3 and 0.75μm, respectively, with the pinhole set to 1 Airy unit. In two-photon mode (without any pinhole) an x-y FWHM resolution of 0.4μm and an axial resolution of 0.8μm were measured.

An unexpected difference between 2-photon and normal fluorescence excitation is that the 2-photon excitation spectra are much wider than their 1-photon counterparts (Xu and Webb, 1996). This means that it is possible to excite several fluorochromes simultaneously with the same (infra-red) source tuned to a single wavelength. This completely avoids the problem of longitudinal chromatic aberration which could be a complication when trying to ascertain the relative position of labelled structures within the cell. This point is illustrated in Figure 6 which shows a 3-dimensional representation of the position of two types of cell surface protein in a cultured cell (MDCK epithelial cell line). In this figure, desmoplakin (a protein present in desmosomes) is antibody-labelled with a rhodamine-like compound (TRITC-red). ZO-1 (a protein present tight junctions) is simultaneously labelled with a fluorescein-like compound (FITC -green). Light from the two labels was separated with filters and acquired at the two emission wavelengths simultaneously. The 3-dimensional structure was obtained by taking serial optical sections, a procedure that was helped by the restriction of fluorochrome destruction (bleaching) to the focal plane. In normal fluorescence microscopy, bleaching occurs at the same rate throughout the specimen so it can be difficult to obtain a large enough number of sections for 3-D reconstruction as the later sections may be completely bleached by the prior imaging of the other sections. This would obviously be a serious problem for *quantitative* measurement of the amount of label present at different heights within the specimen. This

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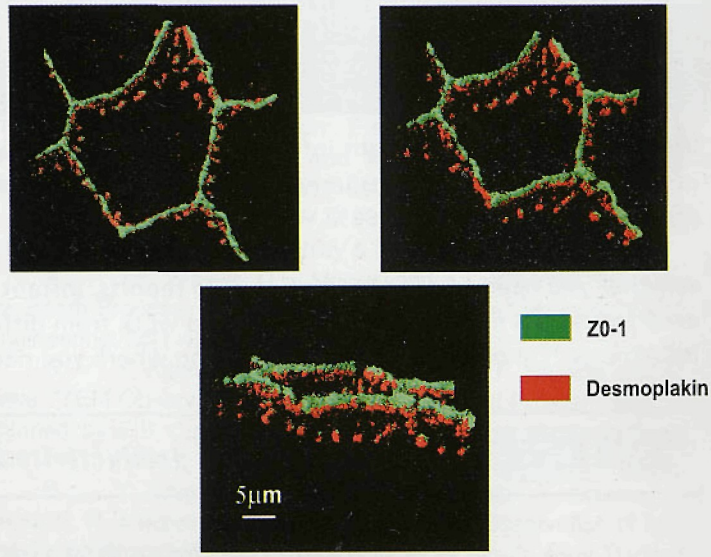

Figure 6: Volume visualisation of desmoplakin and ZO1 distribution in a monolayer of confluent MDCK cells generated from a closely spaced stack of two-photon images. The images show three different views of the three-dimensional distribution of adhesion proteins that have been visualised by dual labelling with TRITS and FITS linked secondary antibodies. Signals from both fluorochromes were excited by mode-locked light from a Ti:S laser at ~840nm and simultaneously recorded with two photomultiplier tubes. This ensured absence of any longitudinal chromatic aberration while the three-dimensionally resolved two-photon excitation avoided problems of bleaching.

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problem does not exist in the 2-photon microscope because photodamage is restricted to each focal plane as it is scanned.

To further illustrate the 3-D resolution available from our microscope we have imaged the transverse tubular structures (see Sommer & Waugh, 1976) within a living cardiac cell. The transverse tubules, which are typically 100-300 nm in diameter, carry electrical excitation into the interior of the ventricular cell to allow rapid activation throughout the cell (Cheng *et al.*, 1994) and are continuous with the cell exterior. By placing a living cardiac cell in a medium that contained dextran-linked fluorescein, the tubules fill with fluorescein which can be imaged—if the massive fluorescence from the fluorescein outside the cell can be rejected or avoided. For such a specimen, the wide field microscope would show almost no detail as the small signal from the fine tubules would be swamped by fluorescence from outside the cell. However, the 2-photon microscope limits excitation to the focal plane and this reduces contamination of the image enormously. As shown in Figure 7, fine structures within the cell are clearly visible and in Figure 8 a 3-dimensional reconstruction of the tubular system is presented. From such reconstruction, we see that the term 'transverse tubular' system is somewhat a misnomer since the tubules actually form a very complicated reticulum. It should be noted that the tubular system images were also enhanced by computer-based image 'deconvolution' (see above) to correct for the slightly poorer axial response of the microscope. To our knowledge, these images represent the first visualisation of the entire transverse tubular system in a living cell. Unfortunately, the 3-dimensional complexity of these structures can only be fully appreciated if the data is rotated by a computer in real time, but Figure 8B shows two stereo pairs of images (red-green anaglyph) to allow some appreciation of the 3-dimensional complexity of the tubular system (in a small part of the cell).

In summary, the confocal microscope has allowed us to image subcellular signal transduction in living cells with unprecedented temporal and spatial resolution. We have also described the performance of a 2-photon molecular excitation microscope. This instrument indicates that there are some real benefits to this technology which may provide a greater understanding of the 3-dimensional inter-relationship of structures within the cell.

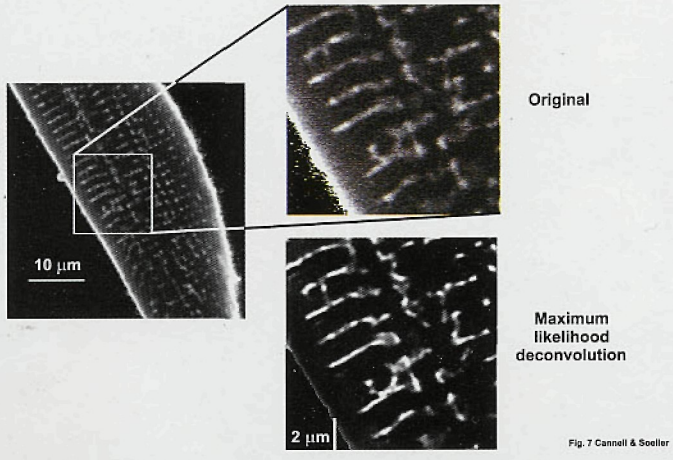

Fig. 7: Imaging of the transverse tubular (t-)system in living rat cardiac myocytes by two-photon microscopy. The left panel shows an "optical slice" of the t-system (visualised by immersing a rat cardiac cell into a bath containing dextran linked fluorescein). The t-system is a network of microscopic tubules (typically 100- 300nm in diameter) . The enlarged view at the upper right shows that the t-system structure can be clearly visualised while fluorescence from the surrounding bathing solution is effectively rejected. To correct for the slightly reduced axial resolution, the three-dimensional image stack formed by sections 0.2µm apart was enhanced by digital deconvolution techniques. The panel on the lower right shows the same detail after application of a maximum likelihood deconvolution procedure which has not only improved the resolution but also enhanced the signal to noise ratio.

In addition, the 2-photon microscope may allow new types of biophysical experiments (on living cells) that require 3-dimensionally resolved fluorescence excitation. With increasing demand, the cost and complexity of mode-locked laser sources should be reduced and the 2-photon microscope should become a powerful new research tool for microscopists and biologists. ■

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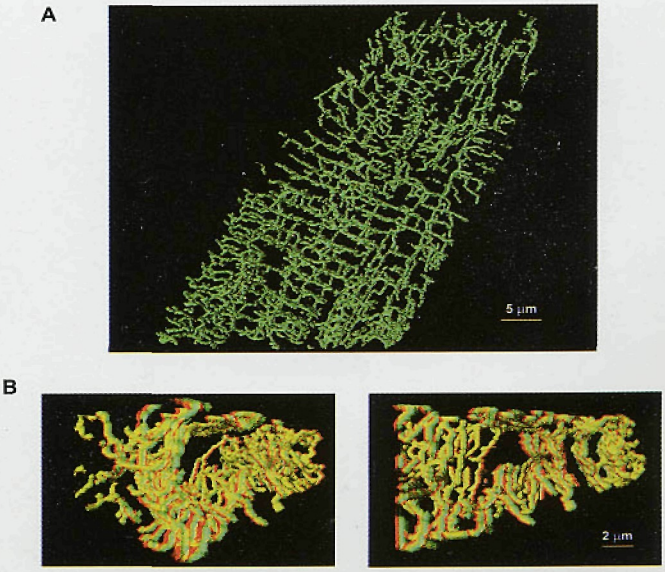

Figure 8: Volume visualisation of the transverse tubular system in living rat cardiac myocytes. Panel A shows a view of the complicated three-dimensional reticulum forming the transverse system that has been generated from the digitally enhanced image stack (one section of which is shown in fig. 7). Since it is difficult to appreciate the complicated architecture from a two-dimensional view, panel B shows two stereo-pairs (red-green anaglyph) of an enlarged part of the structure.