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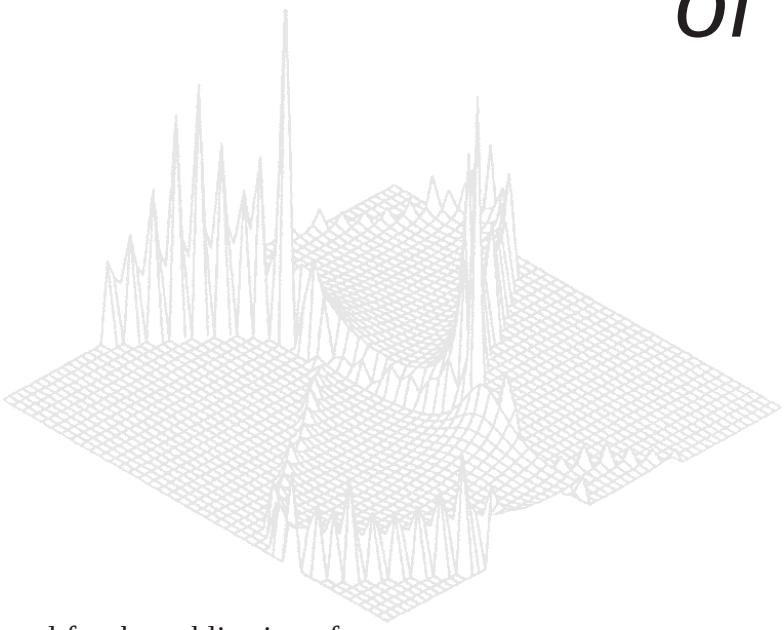

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New Developments in Optical Microscopy for Biological Applications*

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Abstract

In the past ten years a quiet revolution has been taking place in optical microscopy. So quiet indeed that if one has not been working in life sciences research one may have seen little or no evidence of it. Yet what has been going on is an excellent example of how developments in physical instrumentation drive research and research in turn drives further developments in instrumentation. In this paper I will briefly review these developments in optical microscopy and, in particular, show how processes originally proposed 65 years ago as esoteric theoretical solutions to Dirac's equation are now used in practice for some of the most adventurous biological microscopy yet attempted.

1. What do Biologists require from Optical Microscopy?

If one gets down to essentials biologists have three particular requirements of any microscopy technique. It should interfere as little as possible with the sample, it should have specificity, and it should have good resolution. In the case of biological samples these are in fact very demanding requirements. A cell comprises many thousands of different components. How is one to visualise only those of relevance to a particular problem? As regards the requirement of minimal sample interference one must note that in biological microscopy it is required to image not only the surface of cells and tissue but at some depth into what is usually either watery or fibrous material. And in the most demanding applications it will be required to study samples that are in a living state.

From our present perspective we can say with some confidence that optical microscopy offers the best combination of minimal sample interference, specificity and resolution. Biological material is optically transparent over the wavelength range from ∼350 nm to ∼1050 nm (Bhawalkar *et al*. 1996) and thus holds out reasonable hope of being able to image inside cells and tissue. Specificity in optical microscopy is achieved through the technique of fluorescence labelling. This is most commonly done by first attaching the fluorescent probe ('fluorochrome') to an antibody which selectively binds to a particular component of the cell. In other cases probes which bind directly to DNA are used. More recently a technique based on genetic engineering has been developed. In this technique an

* Dedicated to Professor Erich Weigold on the occasion of his sixtieth birthday.

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extra gene (originally isolated from the jellyfish *Aequoria Victoria*) is incorporated in the cell or organism of interest such that a green fluorescent protein (GFP) is produced as a reporter whenever the gene of interest is active (Chalfie 1995). This is a very important development for the study of living cells. In all these ways the daunting structural and biochemical complexity of the cell is tamed and the structures and processes of interest in any study can be visualised with exceptional specificity.

2. The Problem with Conventional Optical Microscopy

The big difficulty in optical microscopy of biological samples is to achieve the required resolution. The problem is not so much that the achievable diffraction limited resolution is poor, $0 \cdot 2$ microns lateral resolution is readily achieved, but that so much of the image is out-of-focus. In fluorescence microscopy this introduces 'flare' which obscures or obliterates the significant contrast. The easiest way to overcome this problem, indeed the only way until recently, is to mechanically section the sample into a thin slice, typically 10 microns thick. This drastic procedure hardly meets the requirement of minimal sample interference. More canny scientists, keen to preserve as much of the structural organisation of the cell as possible will squash the sample until it is more nearly two-dimensional. This approach clearly denies any prospect of studying the three-dimensional structure of cells and will not be appreciated by a cell in a living state.

What the biologist ideally requires is an optical microscopy technique which can produce an image of the sample free of out-of-focus flare. Ten years ago this was made possible with the introduction of a new kind of optical microscope which could indeed produce such an image by use of *confocal* collection optics. The principle of confocal optics is shown in Fig. 1. The sample is illuminated with a focussed probe of light which excites fluorescence throughout the full cone of illumination. The emitted fluorescence is re-imaged onto a small aperture before it is detected. The trick is to set the size of this aperture so that the in-focus fluorescence reaches the detector with little or no attenuation but the out-of-focus fluorescence, which forms a diffuse spot of light at the aperture, is strongly attenuated. One then has an imaging system which discriminates against out-of-focus signal.

In confocal microscopy the rule is: 'in-focus bright $\cdots$ out-of-focus black'. Such an image is called an *optical* section image to emphasise that it can be obtained without need for *mechanical* sectioning.

A confocal microscope illuminates the sample with a diffraction limited focussed spot of light and builds up an image by scanning this spot over the field of view. It is the fact that neighbouring points in the image are illuminated sequentially that allows use of confocal optics. The history of confocal microscopy goes back to the work of Marvin Minsky at Harvard University who applied for a patent in 1957 for a microscope that built up an image by scanning the sample beneath a fixed focal spot. Unusually, there are several distinct scanning strategies in confocal microscopy (for a full review see Pawley 1995). However, for the purposes of this short review I will only consider the most common system, which is to use a focussed laser beam as the probe and to build up an image by scanning the probe in a raster pattern over the field of view in a stationary specimen. This is the optical analogue of the scanning electron microscope.

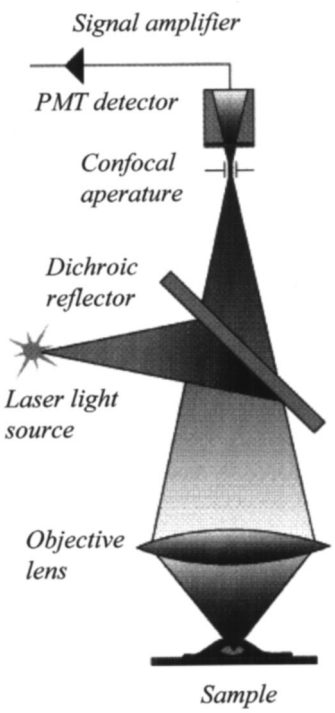

Fig. 1. Schematic diagram showing the principle of confocal optics.

In the 1980s confocal systems were developed with fluorescence imaging capability. A seminal paper by White *et al.* (1987) highlighted the marvellous image quality that was possible. Fig. 2 is an example from this paper showing a direct comparison between conventional wide field fluorescence and confocal fluorescence imaging. So began the quiet revolution to which I referred at the beginning of this review.

Confocal microscopy is now a well characterised technique, widely used in all areas of life science research. There are perhaps 3000 systems in use around the world. Modern instrumentation delivers excellent imaging performance combined with sophisticated software for collection, visualisation and analysis of 3-D data sets. The quality of a confocal fluorescence image far exceeds what is produced by a conventional (i.e. widefield) fluorescence microscope except for the thinnest section samples. Confocal microscopy has opened up new possibilities in biological microscopy and to a great extent meets the three requirements of minimal sample interference, specificity and resolution. However, even confocal microscopy has limitations. It is when one considers these limitations that the motivation for the development of the new technique of multi-photon fluorescence microscopy can be understood.

3. The Problem with Confocal Microscopy

The development of confocal microscopy and its ability to study thick samples with minimal interference has proceeded in parallel with an increasing interest by life scientists in the study of living material. The extent to which the developments in instrumentation have stimulated this trend or vice versa is imponderable. What is very clear is that following the rapid progress in isolating

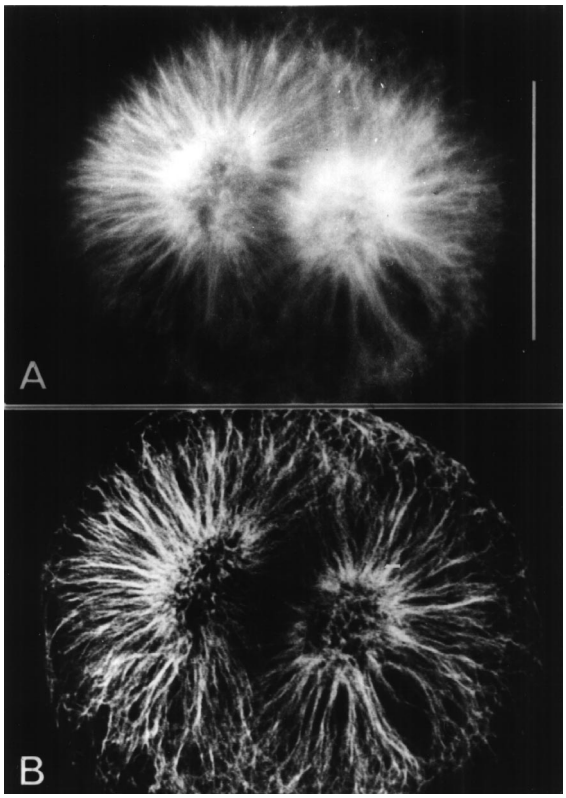

Fig. 2. Comparison of confocal and widefield fluorescence images of a fertilised egg of a sea urchin (*Psammechinus*) in which the microtubules have been immuno-labelled to show tubulin. The confocal image (B) shows much more detail than the widefield image (A). The scale bar is 50 μm. [From White *et al.* (1987).]

specific genes there is increasing interest in studying the function of genes in living cells or tissue or (if possible) whole organisms. This is the emerging field of functional genomics, sometimes also called integrative biology, a term which emphasises a desire to study biological function at as high a level of integration as possible. The study of live samples makes even more stringent demands on optical microscopy. For one thing it is required that the optical probe should not interact harmfully with the sample. For another, one wants to look ever deeper into biological tissue in its normal state. This is often a complex matrix of material which is not easy to image.

Light is not completely harmless to biological samples. Whenever fluorescence labelling is used individual fluorochrome molecules may be disabled through the process of fluorescence bleaching. This is most likely due to the fluorochrome molecule interacting with oxygen while in a highly reactive excited state and being chemically transformed into a dysfunctional compound. Bleaching is a problem for two reasons. Firstly, it limits the time available to image a sample, which can be important if one wants to study a sample over a long period of time. Secondly, fluorescent molecules are highly reactive while in an excited

state, more so if they become trapped in a long lived metastable state. Thus, even a probe as seemingly benign as light may be toxic to cells. It should be added that, even in the absence of fluorescence labelling, blue or (especially) UV light can interact directly with DNA and other cellular components to harm or even kill the cell.

One cannot eliminate the interaction of the optical probe with the sample. One must certainly ensure that the fluorescence collection efficiency is as high as possible to maximise the image brightness and minimise the illumination intensity that is used. Yet, however good the design, a confocal (or conventional) fluorescence imaging system still has a problem with bleaching and toxicity effects which, as we shall see, are worse than they need be.

Consider what is happening in a confocal microscope. The confocal optics certainly ensure that only in-focus fluorescence is collected. However, fluorescence excitation nevertheless occurs throughout the thickness of the sample. This is not what one wants since it means that the probe is interacting with regions of the sample from which no information is being collected. Ideally one wants a system in which the probe beam excites fluorescence *only* at the focus region. According to classical linear optics this is not possible. However, in 1989 a group at Cornell University, USA showed that by using a nonlinear mode of fluorescence excitation localised excitation was indeed possible. This is the basis of multi-photon fluorescence imaging.

4. What is Multi-photon Fluorescence Microscopy?

Multi-photon fluorescence imaging can truly be described as a paradigm shift in optical microscopy. It is a nonlinear technique in which fluorescence excitation occurs only at the focus region of the illuminating probe. No longer does the concept of 'out-of-focus' fluorescence excitation apply. Multi-photon imaging is therefore a technique which, conceptually at least, offers the ideal probe; one which interacts with the sample only in the region from which significant (i.e. in-focus) information is being collected.

Multi-photon fluorescence exploits the fact that under certain circumstances two or more photons of light may combine their energy and interact co-operatively (and simultaneously) with a fluorochrome molecule to excite fluorescence emission. The excitation process is shown in Fig. 3. If we take the example of a fluorochrome which would normally be excited by a single photon of UV light (e.g. ∼350 nm), then excitation is possible by two photons of red light of wavelength ∼700 nm. Illumination at near IR wavelengths of 900–1050 nm can correspondingly be used to excite visible wavelength probes that would normally be excited by light in the wavelength range 450–525 nm. The description multi-photon is used because it is also possible to achieve three-photon excitation. As an example three-photon excitation at 700 nm is equivalent to one-photon illumination in the deep UV at 233 nm.

The theoretical possibility of two-quantum transitions was first considered by Maria Goppert-Mayer in her doctoral thesis from the University of Goettingen submitted in 1930. She showed that even though the intermediate state is a virtual state, two-quantum processes are nevertheless an allowed solution of Dirac's equation. Maria Goppert-Mayer went on to become a distinguished theoretical

physicist studying the nuclear shell model. She and J. H. D. Jensen were jointly awarded the 1963 Nobel Prize in Physics.

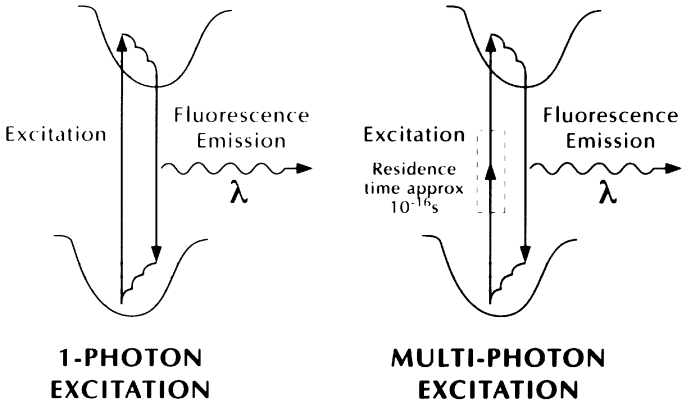

Fig. 3. Principles of one-photon and two-photon fluorescence excitation.

Because the intermediate state in multi-photon excitation is a virtual state it has a very short lifetime, so that excitation to the final state will only occur if the required two (or more) photons interact with the fluorochrome molecule within this short time of each other. Experimental demonstration and application of multi-photon transitions therefore had to wait until intense laser light sources became available. The first flourish of applications, from the early 1970s, was in spectroscopic studies of complex molecules where the two-photon process allowed excitation of energy states inaccessible (for parity reasons) by one-photon excitation. For reviews consult Friedrich and McClain (1980) and Friedrich (1982).

Multi-photon fluorescence is a nonlinear process. In two-photon fluorescence the signal increases as the square of the laser intensity and in three-photon fluorescence as the cube of the illumination intensity. It is this nonlinear dependence on intensity that causes excitation to be localised to the focus region of the illuminating probe. This is demonstrated in Fig. 4 which compares the cone of fluorescence excitation seen in conventional (i.e. one-photon) excitation by a focussed beam of green (540 nm) laser light with the 'pinpoint' of fluorescence produced by two-photon excitation with a focussed beam of infrared (1047 nm) light. This picture opens one's mind not only to the novelty of the multi-photon process, but also to the many new possibilities such a microscopic three-dimensional probe might have.

The first demonstration that multi-photon fluorescence could be used for microscopy was by Denk *et al.* at Cornell University using a modified laser scanning confocal microscope to which was coupled a colliding pulse mode locked dye laser at 630 nm. The first paper describing this work (Denk *et al.* 1990) highlighted the key features of the multi-photon process that it allows excitation of UV fluorochromes with visible wavelength light and that bleaching is localised to a narrow depth of focus 2 micron about the focal plane. Fig. 5 is a two-photon

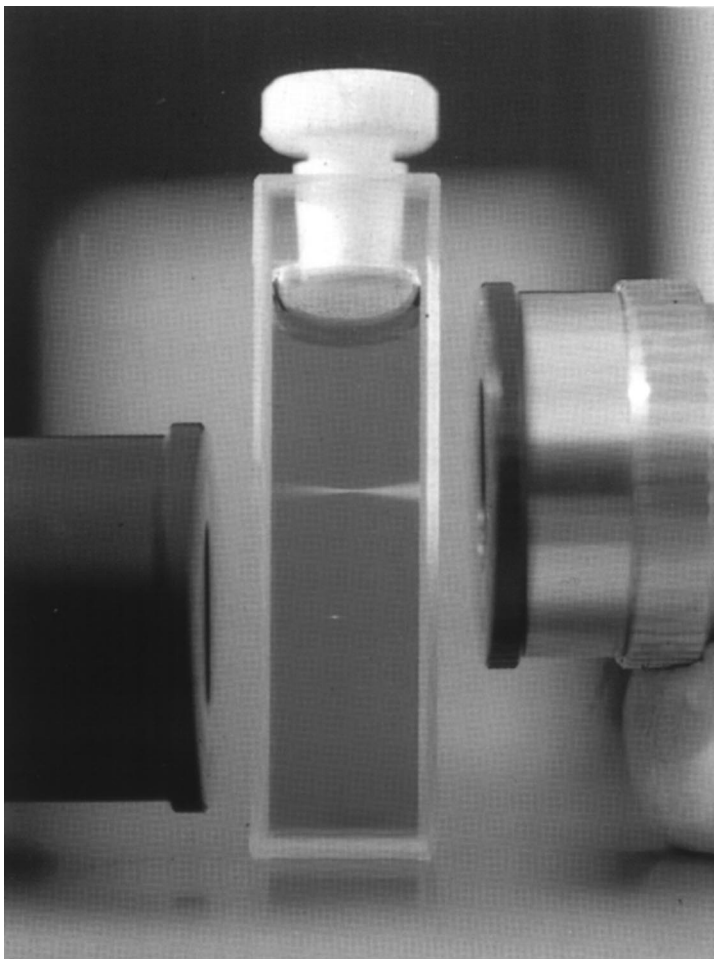

Fig. 4. Demonstration of the highly localised fluorescence excitation in two-photon illumination. The image shows a cuvette filled with fluorescent dye (safranin) which normally requires green light to excite its yellow fluorescence. To the upper right of the cuvette is a lens, which is being used to focus green light (543 nm) from a helium–neon laser into the dye. As expected, the emission is visible as a yellow beam coming to a focus in the centre of the cuvette and spreading out again, with some attenuation. To the lower left of the cuvette is another lens, focusing invisible infrared light (1047 nm) from a mode-locked NdYLF laser. This laser causes two-photon fluorescence producing a pinpoint of light at the focus of the beam in the middle of the cuvette. (With permission Brad Amos. Available from The Science Photo Library, London.)

image of live HeLa cells labelled with DAPI (a UV excited probe) showing the organisation of chromosomes during cell division. The paper speculated on the use of the technique for localised release by two-photon photolysis of biologically active chemicals, but did not comment on an additional feature of multi-photon imaging which is now well demonstrated by the Cornell group and others. This is the ability to image deeper into samples than is possible by confocal or widefield fluorescence microcopy. The reason for this will be explained shortly.

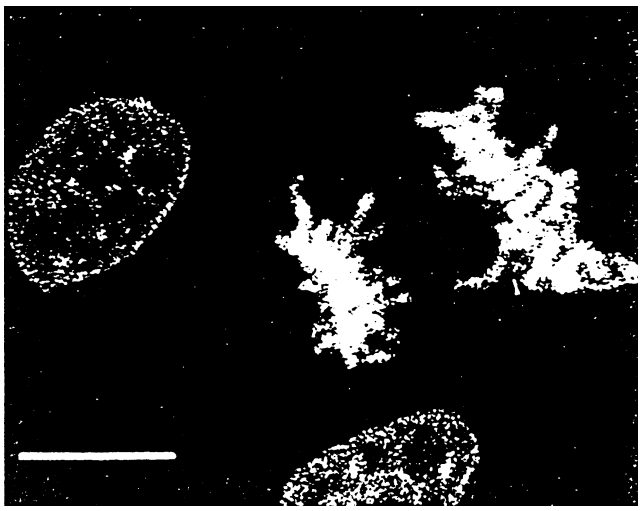

Fig. 5. Early two-photon image of chromosomes in live cells stained with the DNA stain DAPI. DAPI is excited in one-photon fluorescence by UV light. In the two-photon case it can be excited by red light (∼700–800 nm). (With permission Watt Webb.)

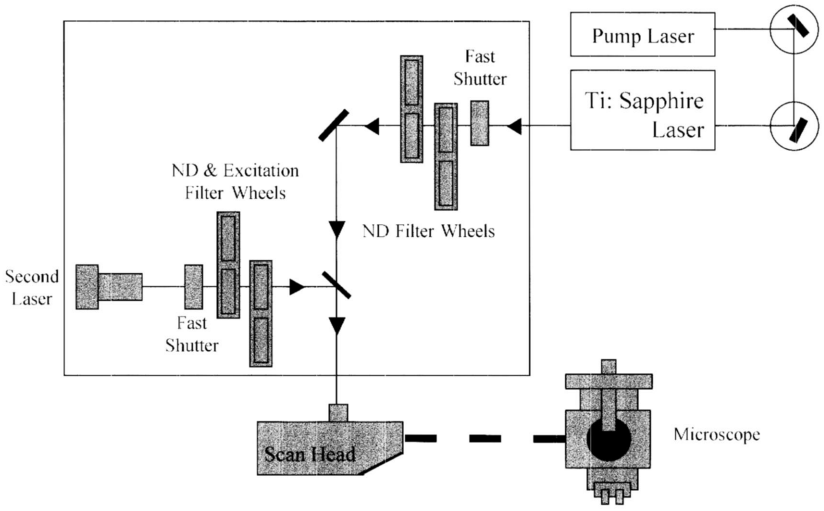

Fig. 6. Schematic diagram showing the layout of a laser scanning microscope system which can be used for both confocal and multi-photon imaging systems.

5. Design of a Multi-photon Fluorescence Imaging System

A multi-photon laser scanning system is generically very similar to a confocal laser scanning system in that the probe is a beam of laser light focussed to a diffraction limited spot and scanned in a raster pattern over the field of view. Fig. 6 shows a schematic layout of a laser scanning microscope system which can be used for either multi-photon or confocal imaging. There are two major

differences between what is required for confocal imaging and what is required for multi-photon imaging. These are in the choice of laser source and in the fluorescence collection system. The laser is a mode locked system giving out high intensity short pulses of light at high repetition rate. The fluorescence collection system does not include confocal optics but is designed to have the widest possible collection aperture. The reasons for these features are explained in the following sections.

6. Advantage of using a Short Pulse Laser

Although multi-photon fluorescence clearly achieves the benefit of localised fluorescence excitation there is concern that it requires so high a laser power that biological samples will be damaged by heating. As an example, if a CW laser is used to excite two-photon fluorescence a power of several hundred millwatts is required to produce a usable signal. Clearly to minimise the risk of heating one must attempt to maximise the fluorescence signal while minimising the laser power into the sample. The quadratic (or higher) dependence of fluorescence signal on laser intensity can be exploited to achieve this end by the use of a laser emitting its output in short pulses at high repetition rate.

Suppose that the laser output is a stream of pulses of width T_{p}, repetition rate F_{p} and average power P_{avg}. The fraction of time during which the laser is emitting (duty cycle) is $F_{\mathrm{p}}T_{\mathrm{p}}$. The peak power per pulse is therefore

$$P_{\mathrm{peak}} = P_{\mathrm{avg}}/F_{\mathrm{p}}T_{\mathrm{p}}\,.$$

The *average* fluorescence intensity I_{fluor} (per second) is then given by

$$I_{\mathrm{fluor}} = k\,P_{\mathrm{peak}}^2\,F_{\mathrm{p}}T_{\mathrm{p}} = k\,P_{\mathrm{avg}}^2/F_{\mathrm{p}}T_{\mathrm{p}}\,,$$

where k is a constant that depends on the cross section for multi-photon fluorescence excitation, on the numerical aperture (NA) of the focussing lens and on the wavelength of the laser light.

The important conclusion of this analysis is that for a given average laser power and given repetition rate the fluorescence signal increases as the pulse width is reduced. This is the short pulse advantage. For three-photon excitation the advantage is even greater, such that the fluorescence signal increases as the inverse *square* of the pulse width.

The question might be asked whether the use of short high intensity pulses does not cause instantaneous heating in excess of what would be predicted for the same average power from a CW laser. Denk *et al.* (1995) have considered this question and concluded that: '$\cdots$ heating effects during high repetition-rate pulsed illumination are generally negligible and can be largely treated like CW illumination.' They gave the temperature rise T at time t after illumination with a focussed probe as

$$T = KP\ln[(2t/t_{\mathrm{c}}) + 1]\,,$$

where K is a prefactor depending on the absorption coefficient for light of the probe wavelength, P is the average laser power in mW and t_{c} is the thermal

time constant. For focussing with a high numerical aperture lens we have t_c ~ 70 ns in water.

In a scanning microscope system the time t is the pixel dwell time which is ~ 2 μs in a typical system. With this assumption the temperature rise in water for different wavelengths and laser power is shown in Table 1.

Table 1. Temperature rise in water for different wavelengths and laser power

λ (nm)	K	$P = 1$ mW	$P = 10$ mW	$P = 50$ mW	$P = 100$ mW
700	$2{\cdot}6\times10^{-4}$	$0{\cdot}9\times10^{-3}$	$0{\cdot}9\times10^{-2}$	$0{\cdot}045$	$0{\cdot}09$
1000	$4{\cdot}2\times10^{-3}$	$1{\cdot}4\times10^{-2}$	$0{\cdot}14$	$0{\cdot}7$	$1{\cdot}4$
1300	$1{\cdot}3\times10^{-2}$	$4{\cdot}5\times10^{-2}$	$0{\cdot}45$	$2{\cdot}25$	$4{\cdot}5$

The most widely used laser system for multi-photon fluorescence microscopy is the tuneable mode-locked TiSapphire laser. The TiS system can be operated over the wavelength range 680–1050 nm with sub 100 fs pulse width. Wavelength tuning is achieved by a prism dispersion unit combined with a movable slit to select the required pass wavelength. A typical pulse repetition rate from a commercially available TiS system (Spectra Physics Inc. USA, Coherent Inc. USA) is 80 MHz. This repetition rate, corresponding to $12{\cdot}5$ ns between pulses, allows the fluorochrome molecules excited during one pulse to emit fluorescence and return to the ground state before the next pulse.

An important development from the perspective of multi-photon microscopy is the introduction of all solid state TiS systems. In these systems the pump laser is no longer a large frame argon ion laser emitting blue light at 488 nm (and consuming prodigious amounts of water and electricity), but a high power laser diode pumped Nd:YVO_4 laser emitting 5 W or more of green light at 532 nm. The laser output from these systems is approximately ten times quieter than from an argon laser pumped system. Power consumption and cooling requirements are modest (single phase electricity, closed cycle chiller).

The TiS laser is unrivalled as a source of wavelength tuneable sub-picosecond pulses, which is why it is so widely accepted as a source for multi-photon fluorescence imaging. However, for certain well defined applications it may be appropriate to use a fixed wavelength laser. There are two main choices at the time of writing. One is a fixed wavelength version of the TiS laser (Coherent Inc. USA) emitting at 800 nm. The other is a diode pumped NdYLF laser emitting at 1047 nm. The NdYLF laser (Microlase Ltd., Scotland) emits $\sim$160 fs pulses at a repetition rate of 120 MHz. This system uses a fibre delivery/post fibre compressor system to produce short pulses at high average power output ($\sim$500 mW). There is intense interest in the possibility of using fibre optics for delivery of femtosecond pulses from other laser sources. The difficulty is to transmit pulses of good quality without sacrificing too much of the available power.

Laser specialists may be querulous that so few laser systems are presented as realistic options for multi-photon imaging. This is because most of the more experimental systems do not operate at sufficiently high average power to meet the practical requirement of imaging deep into absorbing and scattering biological tissue. In the future we will possibly see the development of a new generation of semiconductor based mode locked lasers. There is certainly a need for smaller, less expensive, more easy to use systems than those currently available.

7. Optimum Laser Pulse Width in Multi-photon Microscopy

We have shown that use of short pulse lasers gives a 'short pulse advantage' by enabling the average laser power to be kept low (typically 1–10 mW at the sample) while the fluorescence signal is increased. One may ask if there is any limit to this advantage? Should one use the shortest pulse possible? The answer in practice is that this is not the best strategy. The reasons are as follows. The first is that it is difficult (although not impossible) to deliver ultra-short pulses to the sample. The thickness of glass in the various optical elements broadens the pulses by the process of group velocity dispersion. Guild *et al.* (1997) have measured the group delay dispersion (GDD) of some typical microscope optical elements. High NA objective lenses were found to have GDD values in the range 1000 to 4500 fs^2. The effect of GDD in the range 1000 to 10,000 fs^2 on laser pulses of different pulse width in the range 50 to 200 fs is shown in Table 2.

The effect of GDD in the microscope optics can be corrected for by introducing an additional dispersion compensation unit into the optical system. With this unit the laser pulses are 'pre-chirped' before they enter the optical system in such a way that the group delay dispersion in the optical system re-compresses the pulses. This system is used by several of the pioneers in multi-photon microscopy (e.g. Soeller 1996). However, it introduces additional complexity into the optical system which in many cases is not justified.

Table 2 indicates that there are practical reasons to work with laser pulse widths above 100 fs since these are not broadened excessively by the microscope optics. There are also biological reasons not to become overzealous in the use of short pulses. This is to avoid unwanted three-photon effects. When three photons act cooperatively at say 750 nm their combined energy is equivalent to illumination in the deep UV at 250 nm. At this wavelength DNA absorbs strongly. Thus ultra-short pulses may cause direct damage to DNA and be harmful to cells. Wokosin *et al.* (1998) have considered these issues in more detail.

Table 2. Effect of group delay dispersion (GDD) in the range 1000 to 10,000 fs^2 on laser pulses of different pulse width in the range 50 to 200 fs

Pulse width from laser (fs)	Pulse width (fs) at sample broadened by GDD		
	2000 fs^2	6000 fs^2	10,000 fs^2
50	122	360	557
100	114	194	295
150	159	187	238
200	202	217	243

8. Advantage of using Non-confocal Collection in Multi-photon Imaging

Multi-photon microscopy is unique amongst fluorescence microscopy techniques in that the image resolution, both lateral and axial, is fully determined by the illumination probe. This is quite different from a widefield or confocal system. In widefield fluorescence microscopy the optical resolution is determined entirely by the signal collection optics. In confocal fluorescence microscopy the optical resolution is determined by a combination (the product) of the illumination and detection point spread functions (the 'psf' is the 3-D image of a point object).

The theoretical imaging performance of the two-photon system is given by the product of the illumination psf with itself. There is a similarity here with the confocal optical system since in each case it is the product of two psf (illumination and collection for confocal; illumination and illumination for two-photon) that sharpens up the axial resolution. However, as previously emphasised the multi-photon system differs from all other systems in that it is the fluorescence *excitation* that is confined to a small focal volume not the fluorescence *collection*.

The lateral and axial resolution in multi-photon imaging are determined by the wavelength of the illumination light and the numerical aperture (NA) of the microscope objective lens. In practice, if one compares confocal imaging with a probe wavelength of 488 nm (argon ion laser) and multi-photon imaging with a probe wavelength of 700 nm (TiSapphire laser), the lateral resolution is about the same in each case and the axial resolution is in proportion to the wavelength in each case.

Because the imaging performance of a multi-photon system is determined by the probe beam alone without reference to the collection optics, one can operate the multi-photon system with the confocal aperture wide open and still obtain high quality optical section images. The advantage of operating with the confocal aperture wide open (or as we shall see shortly, non-existent) becomes apparent as one images deeper into biological tissue. A demonstration of the improved performance at depth in a sample is shown in Fig. 7 which compares the image of the same focal plane obtained by two-photon and confocal imaging. What is happening is that as one probes deeper, scattering effects set in. These will affect both the probe light and the emitted fluorescence. However, since in multi-photon

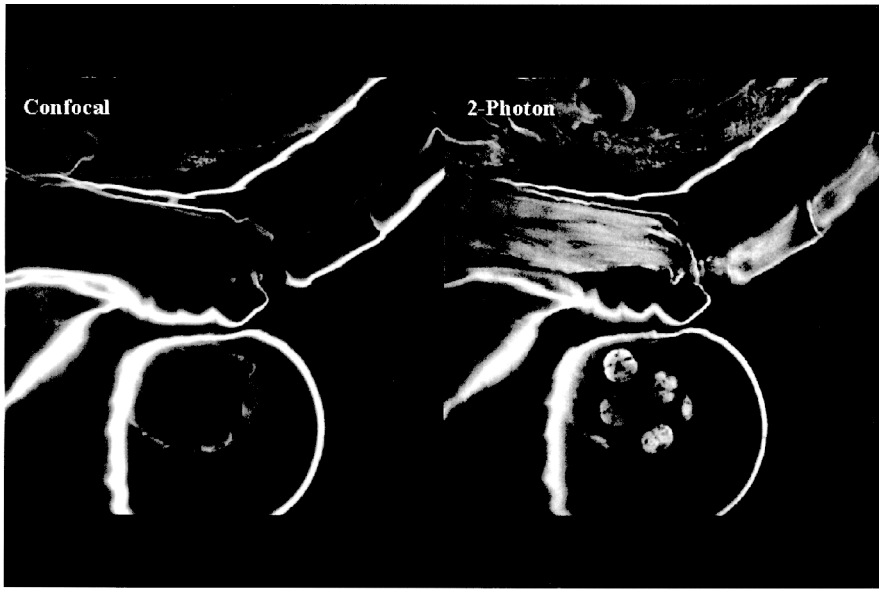

Fig. 7. Comparison of the confocal image (argon ion laser at 488 nm) and the two-photon image (NdYLF laser at 1047 nm) of the head region of an eosin stained Ceriodaphnia. The two-photon image shows significantly more detail at this depth into the sample.

imaging the probe light is of longer wavelength than the emitted fluorescence, Rayleigh scattering effects which are proportional to the inverse fourth power of the wavelength are significantly less for the probe than for the emitted light. Detailed studies show that scattering in biological tissue is more complex than this and that the dependence on wavelength is less strong. However, the general rule that scattering is less at long wavelengths does apply.

Scattering of the emitted fluorescence is a particularly serious problem in one-photon (conventional or confocal) imaging because the scattered fluorescence represents lost information; it cannot contribute to the in-focus image but appears only as out-of-focus flare. In multi-photon imaging, however, one knows that the fluorescence has originated from the focal region because this is the only place where multi-photon excitation occurs. Thus, even if the fluorescence emission undergoes scattering, provided it is collected, it can be imaged. This is now recognised as one of the most important capabilities of multi-photon imaging.

In order to fully exploit the advantage of collecting scattered fluorescence in addition to the fluorescence which escapes 'ballistically' from the sample it is necessary to position the detector system as close to the sample as possible. The typical arrangement is to position the photomultiplier detectors between the laser scanning system and the microscope. This arrangement, illustrated in Fig. 8, is usually called the 'non-descanning' (or external) detector configuration

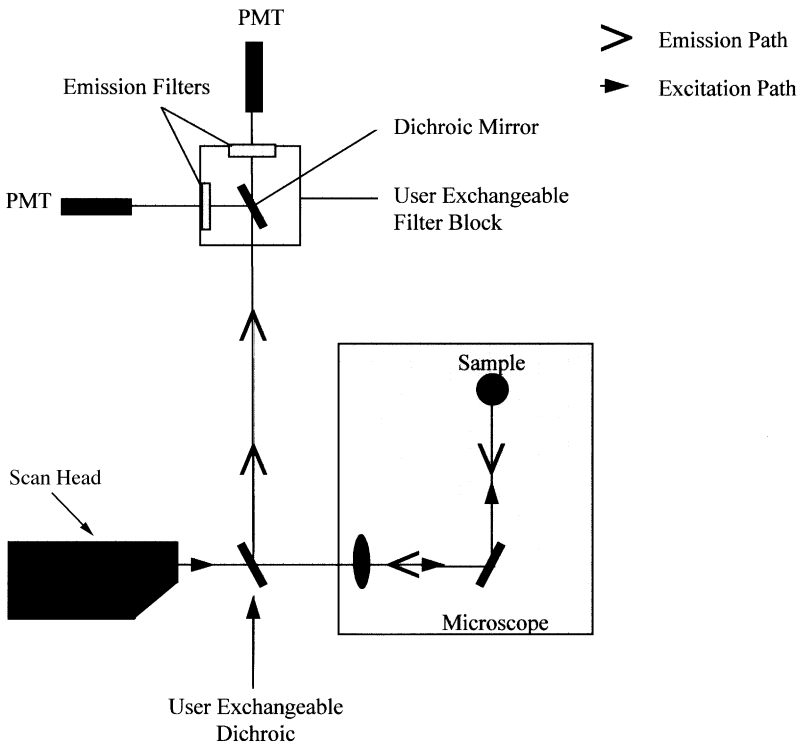

Fig. 8. Schematic diagram showing the non-descanned detection system in multi-photon fluorescence imaging. The detectors are mounted close to the microscope to collect as much of the emitted fluorescence as possible.

to distinguish it from the confocal system where the emitted fluorescence returns through the scanning system (i.e. is de-scanned) before being focussed onto the confocal aperture. The preferred optical arrangement is to image the back aperture of the objective lens onto the detector. This corresponds to collecting as much as possible of the fluorescence which is scattered and therefore presents itself to the imaging system as a diffuse source of light.

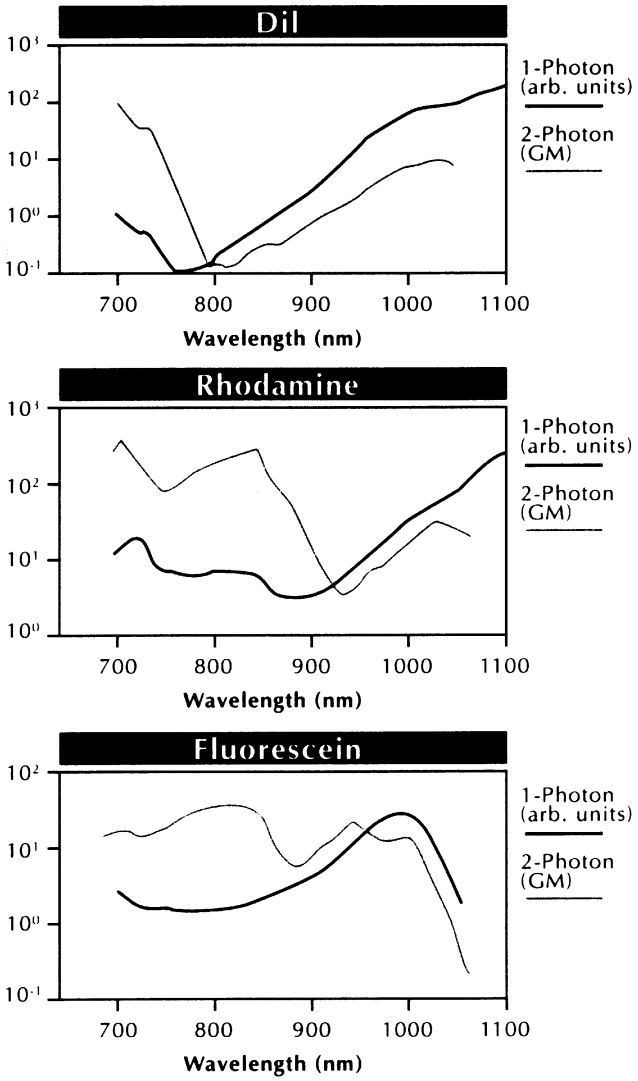

Fig. 9. Comparisons of one-photon excitation spectra and two-photon excitation spectra for three commonly used fluorophores. Two-photon cross sections are in Goppert-Mayer units. The one-photon cross sections are plotted x2 the actual excitation wavelength in arbitrary units. (With permission Watt Webb.)

Non-descanning detection gives its greatest sensitivity advantage when a significant proportion of the emitted fluorescence is scattered, as is the case when imaging deep ($\geq$100 microns) into samples such as brain tissue or skin. A second advantage of positioning the detectors very close to the microscope is that the transmission efficiency of the collection system is very high. This can be particularly important when collecting deep blue or UV fluorescence where the performance of lenses and mirrors is not optimal.

9. Fluorescent Probes for Multi-photon Imaging

It has been found in practice that the fluorescent probes used in conventional and confocal (i.e. one-photon) imaging can mostly be used in multi-photon imaging. Xu *et al.* (1996) have measured the two-photon excitation cross sections for many of the most commonly used probes. An important, if unsurprising observation is that the shape of the excitation cross section curve is not simply the one-photon excitation curve displaced to longer wavelength.

The excitation spectrum for three commonly used probes is shown in Fig. 9. For comparison the one-photon curves have been displaced to twice the actual wavelength to which they apply. The curves show clearly that the one-photon and two-photon curves are different. They also show, at least for Rhodamine and FITC, that two-photon excitation extends over a wider wavelength range than one-photon excitation.

Table 3 lists many of the probes that have been used successfully for multi-photon microscopy. The list has been compiled from a review of the literature and from private communications. Absence of a probe from the list should not be read as implying anything about either its suitability or unsuitability. Multi-photon imaging is still at an early stage of use and many probes have not been investigated.

Table 3. Probes used successfully for multi-photon microscopy

Multi-purpose probes	DNA stains	Membrane stains	Ion probes	Autofluorescence
Bodipy	Hoechst 33258	Di-I	Calcium crimson	Flavins
Cascade blue	Hoechst 33342	Di-A	Calcium green	Serotonin
Coumarin 307	AMCA	Di-O	Calcium orange	Tryptophan
FITC	DAPI	FM4-64	Fluo-3	Collagen
GFP	Propidium	Mitotracker	FURA	Elastin
GFP S-65T	Iodide		Indo-1	NAD(P)H
Lucifer yellow			SNARF	Pharmaceuticals
Congo red				
Evans blue				
Rhodamine				
Rosamine				
Nile red				
Oregon green				
Safranin				
Texas red				
Eosin				

10. Biological Applications of Multi-photon Imaging

The importance of multi-photon fluorescence microscopy derives from its particular advantages over other microscopy techniques. These can be summarised as follows:

- Reduced photobleaching.
- Imaging deeper into biological tissue than possible by other fluorescence techniques.
- Excitation of UV probes or UV autofluorescence with visible wavelength light, so avoiding severe cytotoxic effects of direct UV illumination.
- Exact co-localisation of multiple label fluorescence imaging by using a single excitation wavelength.
- Ability to use the laser probe for localised interaction with the sample for applications such as photo-activated release of caged molecules or selective photobleaching.

These advantages are now being exploited, particularly for the study of live cells and tissue. The following paragraphs briefly review these biological applications.

In an early paper Summers *et al*. (1993) used two-photon microscopy to image living embryos of the sea urchin *Lytechinus variegatus* during early stages of cell division. The embryos were stained with Hoechst 33342 at metaphase of fourth cleavage. This work has recently been extended (Summers 1996) to the tracing of embryonic cell lineage by releasing ('uncaging') fluorescein in one of the cells at the two cell stage of development and then following further cell division by identifying the daughter cells which are fluorescent. Only multi-photon absorption allows photo-activation to be localised to a small *volume* in 3-D. Any other method would have resulted in a cone of activation extending throughout the thickness of the sample.

Potter *et al*. (1996) have used two-photon microscopy to study the process of neurite outgrowth and synaptic integration in embryonic rat hippocampal neurons growing on silicon 'neuroprobes'. For this work the neurons were labelled with DiO. In other work from this group, cultured neurons were imaged with a variety of labels including DiI. DiA, bodipy ceramide and green fluorescent protein.

Wokosin and White (1998) have used two-photon imaging at 1047 nm (NdYLF laser) to study development processes in sea urchin embryos and embryos of *C elegans* and more recently in hamster embryos. They report excellent viability of live cells in two-photon imaging and have studied cell division over periods of several hours. In developmental studies, it is often important to visualise DNA in the cell by counterstaining with DAPI or Hoechst. In a confocal microscope this requires a dual laser system with both visible wavelength and UV lasers. With such a system it is difficult to achieve exactly co-localised images. Multi-photon microscopy overcomes this difficulty by allowing, in many cases, visible wavelength and UV probes to be excited at the same two-photon wavelength. This guarantees that the separate images will be in exact registration.

Another possibility for multi-label imaging is to excite a UV probe by three-photon excitation. Wokosin *et al*. (1996) used the Nd:YLF laser to image the DNA counterstain DAPI by three-photon excitation. In this way light of wavelength 1047 nm achieved the equivalent energy of a 349 nm photon. By additionally labelling the sample with FITC and Rhodamine, which can be imaged

by two-photon excitation at 1047 nm, Wokosin *et al.* were able to produce a three colour image of a live biological sample.

Perhaps the central problem in studies of cell development is to directly follow gene expression during the development process. The use of green fluorescent protein (GFP) as a cell marker is a great technological advance for these studies (see the special issue of *Trends in Genetics* 1995). Potter *et al.* (1996) have investigated the relative performance of two-photon and confocal microscopy for intravital imaging of GFP. They studied axonal outgrowth and targeting in the visual system of the fruit fly *Drosophila melanogaster.* They concluded that single axons and terminals are better resolved in two-photon than in confocal imaging. One reason for this is that the low fluorescence signal from GFP (which is not amplified) requires the confocal aperture to be opened wider than its optimal setting. They also reported that two-photon fluorescence produces better images of deep structures. The wavelength used for these experiments was 900 nm at which they reported dramatically less photo-bleaching and no photo-toxicity when compared with confocal imaging.

Kohler *et al.* (1997) used green fluorescent protein as a marker to visualise the exchange of protein molecules between individual plastids in vascular plants. These plastids had previously been considered discrete isolated entities. By using the laser probe to selectively photobleach the GFP in an individual plastid they could observe the recovery of fluorescence as transport occurred through narrow tubules connecting with other plastids.

Two-photon microscopy opens up important new possibilities in studying brain physiology. Denk, Svoboda and co-workers have carried out work of great technical virtuosity, studying such things as the coupling between dendritic spines and shafts and dendritic calcium dynamics. They have shown that two-photon microscopy can image deeper into brain tissue than is otherwise possible.

Most of the two-photon imaging of brain tissue so far has been done using a Ti:Sapphire laser. However, in a recent paper Svoboda *et al.* (1996) presented high quality images using a CrLiSAF laser emitting at 850 nm to excite calcium green.

Unpublished work at the MRC Laboratory of Molecular Biology, Cambridge UK (W. B. Amos, private communication) has shown the capability of a Nd:YLF two-photon system in studies of brain tissue. Applications include the 3-D arrangement of synapses in mormyriad fish cerebellum and the calcium ion control of excitable membranes in the cerebellum

Fluorescence microscopy is not used only as a passive tool to reveal structural organisation and developmental changes. It is also now a well developed tool for physiological studies with probes for *p*H, calcium ions and (less commonly) other ionic species. The most important application is a study of Ca^{++} activity which can occur on a timescale from ms to minutes.

The most used probe for calcium ion measurements in two-photon applications is Indo-1. Typically a wavelength in the range 690–720 nm is used. These wavelengths can only be reached at the moment with a Ti:Sapphire laser.

Sako *et al.* (1996) compared two-photon and confocal measurements of calcium ion concentration using Indo-1. They confirmed that live cells have increased viability in two-photon imaging. They also confirmed that two-photon fluorescence can be used to image deeper into live cells than confocal imaging.

Two-photon excitation can also be used to image visible wavelength calcium ion probes. Denk *et al*. (1994) imaged dendritic structure and spines in brain slices from turtle cortex labelled with Fluo-3. They reported that synaptic spines were easily resolved at depths greater than 100 microns in both rat and turtle preparations.

The Nd:YLF laser has also been used to image calcium levels during the period of second division in a *Caenorhabditis elegans* embryo. (J. White, private communication). The probe calcium green was used in a dextran conjugated form and was injected into the oocytes prior to fertilisation.

An interesting area of application that is opening up with multi-photon microscopy is autofluorescence imaging of biologically or pharmacologically significant components. Bennett *et al*. (1996) demonstrated the use of multi-photon imaging of NAD(P)H autofluorescence for subcellular imaging of glucose metabolism within intact pancreatic islets. They were able to demonstrate a difference in behaviour of isolated beta cells when compared with intact islets which latter could be imaged by two-photon fluorescence. This work demonstrates the value of a technique which allows the study of intact tissue, with all its optical difficulties, rather than individual cells.

Maiti *et al*. (1997) have used multi-photon excited auto-fluorescence to measure the distribution of serotonin, a neurotransmitter, in rat basophilic leukaemia (RBL) cells. The serotonin was excited by three-photon absorption with 700 nm light from a Ti:Sapphire laser. This achieved the equivalent energy of a 267 nm photon. This is a wavelength far below what is normally possible in optical microscopy. The serotonin fluorescence was detected in the UV centred on 350 nm using a non-descanned detector system.

Some recent, as yet unpublished work (Zipfel, Brown, private communication) has used autofluorescence of a pharmaceutical compound to study various aspects of drug delivery. Many pharmaceutical compounds are naturally UV autofluorescent. Two-photon microscopy with red light allows direct visualisation of the distribution of a drug in live tissue. The rate of metabolism can also be measured by using the shift in emission wavelength of the metabolised form.

11. Summary

Multi-photon fluorescence microscopy is set to become a major new technique in biological microscopy. The key reasons for this are that, as explained in this short review, it combines high sensitivity imaging deep into biological tissue with reduced photo-bleaching and photo-toxicity as compared with other fluorescence microscopy methods. The further ability to visualise UV excited fluorescence with visible wavelength illumination is also opening up completely new possibilities, such as the three-photon autofluorescence of selected proteins (e.g. serotonin) and visualisation of drug localisation in live cells by autofluorescence imaging. These new capabilities meet the new needs of biologists to understand what is happening in living cells and tissue and to study these structures with as little interference as possible.

The laser wavelengths required for multi-photon microscopy are now reasonably well understood. They cover the range 650–1050 nm. The shorter wavelength is required for work with photoactivated compounds, for exciting UV probes and the intrinsic autofluorescence of proteins and pharmaceutical compounds. The

longer wavelength limit is determined by the onset of significant absorption by biological material which will lead to direct heating and damage to live cells.

The only tuneable laser available commercially for multi-photon imaging is the Ti:Sapphire laser which can be tuned down to 690 nm. For many applications it is now becoming clear that a laser operating at a fixed wavelength would make for a less complicated instrument. At present the choice of fixed wavelength lasers is limited. We can expect further developments in this area in the future. From a practical point of view the requirement is for simplicity, small size and low cost.

Multi-photon microscopy today uses the same fluorescent probes as are used for conventional fluorescence microscopy. It is encouraging that this aspect of sample preparation has not had to be changed. However, there is a need for better understanding of the multi-photon excitation process to help the development of fluorescent molecules with greater excitation cross sections.

As a final remark I would say that multi-photon fluorescence microscopy, although now well characterised as a physical instrumentation method, is still at a very early state of development as a tool for serious biological research. Having lived through the early days of the 'confocal revolution' I am confident that multi-photon microscopy will also have a significant impact on biomedical research. Yet, while one can already see some of the areas where it is finding important applications, one must remain curious as to what unexpected applications are yet to be found. Almost every experiment that is attempted with multi-photon fluorescence today is being done for the first time. The opportunities for sweet success and bitter failure are both great!

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