Multi-Photon Microscopy: Seeing More by Imaging Less

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INTRODUCTION

Since the earliest microscopes of Leuwenhoek and Hooke in the 17th century, optical designers have strived to maximize a key performance characteristic of each new instrument model—that of contrast. Put simply, contrast is how well we can discern useful information about a microscopic specimen from unwanted background and noise.

Resolution (the ability to accurately measure features) and sensitivity (the ability to detect features) are intimately related to contrast. The Rayleigh criterion for resolution describes how far apart two tiny objects must be so that are clearly contrasted from the darker space between them. Sensitivity defines the faintest feature that can be contrasted from the background. Why is this seemingly philosophical discussion important? The answer is clear; we can improve both sensitivity and effective resolution by improving contrast between what we want to see and what we don't.

Scanning optical microscopes in general (23) deliver improved contrast in a number of different ways. A regular film or digital camera, mounted on a conventional microscope, takes a snapshot or a complete picture of the desired scene at the press of a button. The laser-scanning (optical) microscope (LSM) is quite different. The picture is built up in stages, rather as an artist might construct a work on canvas. The scene or field-of-view is surveyed, and the color and brightness of different regions are assessed and then represented by appropriate amounts of paint applied in small strokes, dots, etc. Surveying in the LSM is achieved by scanning a focused beam of laser light through the sample. One or more photodetectors are used to measure the brightness, color, or other properties at various points, and the resulting values are used to “paint” each corresponding point (or pixel) on a display screen.

The final part of this analogy is the most important. The painter does not make an exact copy of the scene on the canvas but makes an interpretation according to her artistic tendencies. While the LSM is somewhat more impartial, it too has a tendency to inexactitude. This is called the microscope's “response”, and since we are building-up our picture point-by-point, we can easily engineer our microscope's “point-response” to get the kind of pictures we want. More precisely, we can increase the contrast of selected features or parts of the specimen.

SHEDDING LIGHT ON THE SAMPLE

With a non-scanning microscope (as with a regular camera), the whole scene is illuminated and stray light (flare) can easily be scattered from one part of the scene to contaminate another (like pointing a camera towards the sun or using a simple flashgun to light a shiny object). In the LSM, the very act of scanning a finely focused laser beam means that we only light up each point as we measure it. By imaging single points at a time, we reduce unwanted flare and obtain more contrast.

If we consider light as a random stream of particles, then the arrival of each photon is a little like getting a six when throwing one or more dice. Sixes can be obtained more quickly by repeating the throws more frequently, just as more photons can be obtained by turning up the laser power.

FLUORESCENT PAINT FOR CELLS

Fluorescent probes and tagged antibodies have greatly increased our ability to contrast specific structures, even single molecules, in biological specimens (7,11,19,21,25). Like the artist, we can selectively highlight features of interest, and the use of single or multiple narrow-band laser lines, combined with modern interference filters, allows great flexibility in visualizing several different probes in the same sample. However, there is a problem with delicate or living specimens. The interaction of light with any fluorescent molecule can give rise to highly reactive photoproducts that rapidly destroy other molecules, including those of the biological material itself.

We can also consider exciting a fluorescence probe to be like getting a six when throwing dice. Increasing the throwing rate (equivalent to turning up the laser power) will increase the chance of exciting each molecule.

STAYING FOCUSED

Two things happen when a camera lens is “opened” to a wider stop or aperture; the picture becomes brighter, and out-of-focus features become progressively more blurred. Objective lenses in modern microscopes are particularly prone to this out-of-focus blur, especially in fluorescence work, as they are designed with the maximum possible apertures. This is necessary to collect as many light rays as possible from the specimen (to reduce illumination and photodamage), especially those at high angles of incidence that come from fine details.

The confocal LSM (15,16,27) uses a simple trick to engineer the microscope response so that the out-of-focus rays and background flare are removed. A small aperture in front of the photodetector is accurately co-focused with the laser illumination and effectively blocks the unwanted light, giving high-contrast optical sections (3). Unfortunately, some biological specimens tend to scatter both in-focus (useful) and unwanted light (Figure 1). This problem can only be partly overcome by increasing the size of the confocal aperture, at the expense of some loss in contrast and more blurred optical sections, or by image processing.

All microscope lenses focus some different colors (wavelengths) of light to different positions, so it is difficult to arrange the focused excitation light and (longer wavelength) emissions

to overlap correctly (8,26). This is a major problem for UV-excited dyes that emit fluorescence at visible wavelengths. It is also unfortunate that only the out-of-focus fluorescence emissions are blocked by the pinhole (aperture) and not the effect of the laser illumination away from the optical section.

For all these and other reasons, adjustment of the confocal aperture can be an inefficient method of producing optical sections.

TWO PHOTONS ARE PROBABLY BETTER THAN ONE

If it were possible to excite the fluorescent probes only within the desired optical section, then we could avoid both the losses associated with having to use the confocal pinhole system and the “collateral damage” associated with photodamage outside of the section. To achieve this, we must get around a fundamental property of light; no matter how far a light beam travels, the total energy in any cross section of that beam is constant (it just gets spread out more when the beam diverges). So, if the amount of fluorescence excited in a molecule is proportional to the illumination, then we can never restrict excitation to a single optical plane. To do that, we must make the fluorescence intensity disproportional to the illumination. This can be achieved by arranging for each fluorescent molecule to absorb two or more photons of excitation light simultaneously.

When throwing a pair of dice, the probability of obtaining two sixes (1 in 36) is the probability of obtaining one six with one die squared. Predicting the properties of a system where one molecule could absorb two photons simultaneously is first attributed to Maria Goeppert-Mayer (who received the Nobel Prize in 1963 for atomic shell theory) in her PhD thesis at the University of Goettingham in the early 1930s. In general, this effect will increase by the square of the laser power, but for common fluorescent probes multi-photon absorbance is a very unlikely occurrence and therefore requires a high laser power. It was not until suitable lasers had been developed that the process was demonstrated in an LSM instrument (6).

Figure 1. Confocal microscopy of thick biological specimens—the problem. (a–f) Vertical (x,z) optical sections through an intact, living piece of leaf epidermis. The sections run through the stomatal complex at the positions indicated in (o). The lower part of each section in the left-hand panels is attenuated by scattering through the tissue. Digital correction has been applied to the sections in (g–l) to correct for this signal loss with depth. This helps to make the 3-D reconstruction in (n) a more faithful representation than with the uncorrected data (m) (26).

HOW CAN WE USE HIGH LASER POWER WITHOUT DAMAGING THE SAMPLE?

If we get a regular consignment of dice from the dice factory (our model for the output of a modest power laser) and throw them all once, but two at a time, then we get a constant double-six rate (two-photon excitation) of 1 in 36 for every throw. If we now throw them four dice at a time, then the chances of getting at least two sixes in each batch is about 1 in 15 for every two dice thrown. Batches of eight dice will give an even higher rate of about 1 in 10 for every two dice. For events with very low probability (such as multi-photon absorbance), the effect is more marked and continues to improve as the batch size increases. This means that delivering photons in “batches” should give a higher efficiency of two-photon excitation, compared to a constant stream, for the same average power (overall delivery rate from the “photon-factory” or laser).

LASERS FOR MULTI-PHOTON MICROSCOPY: MORE OFF THAN ON

The combined energy of the two absorbed photons must be approximately equal to that of the equivalent single photon by conventional absorption. This means that two-photon LSM lasers must operate at twice the wavelength of their single-photon counterparts. In general this is the near-infrared range of 690–1080 nm for probes normally excited in the far-UV and visible range (350–550 nm) (24,28). Three-photon excitation is possible for probes with single-photon absorbance peaks down to 250 nm (18,28).

So why does the laser cost over \$100 000? To answer this we need to look at how much power is required and how we can arrange to deliver the photons in batches. If we use a pulsed laser, instead of one with a continuous wave output (CW), then we find that a suitable figure-of-merit for our illumination system is:

$$F \propto P^n / (wr)^{n-1}$$

Where F = relative fluorescence excitation, P = time-averaged laser power, n = photons absorbed ($n = 1, 2$, or 3), w = pulse width (s), and r = repeat rate of pulses (Hz).

Table 1 shows some examples of pulsed lasers and their respective figures-of-merit for two- and three-photon excitation. Systems with the lowest duty cycle (ratio of “on time” or pulse-width to “off time”) for the same average power are best. This ratio can be as low as 10^{-5} , which is the same as a high-speed camera flashgun being fired just once a minute!

THE LASER PULSE: KEEPING IT TOGETHER

It is tempting to think that further reducing the laser pulse width will give ever-increasing performance. There is, however, a serious limit. Narrower pulses of light contain a range of frequencies. When tuned to 800 nm, for example, the pulsed laser emits a range of wavelengths (perhaps 800 ± 7 nm). When these different wavelengths pass through optical components in the microscope, they can travel at different

Laser	Average Power (W)		Pulse Width (ps)	Repeat Rate (MHz)	Duty Cycle	Relative Power/ Duty Cycle	
	for 2-P	for 3-P				for 2-P	for 3-P
CW Argon Ion	10^{-1}	approximately 7 W	continuous	continuous	1	1	1
ps-pulsed TiSapphire	1.4×10^{-3}	2.3×10^{-2}	1	200	2×10^{-4}	70.7	17.1
100 fs-pulsed TiSapphire	3.2×10^{-4}	3.2×10^{-3}	0.1	100	10^{-5}	317	46.4

The continuous laser is not a realistic choice for most applications. The two pulsed lasers show that the shortest pulses allow for low average power but deliver high peak powers (shown as relative power/duty cycle) for efficient two-photon (2-P) and three-photon (3-P) excitation (the last two columns are independently normalized against the CW laser). All values are for approximately the same level of two- or three-photon fluorescence excitation.

speeds. This results in the pulse spreading out in time (i.e., getting longer) (4,10). The problem gets worse as the laser pulses get shorter, and there is little advantage below 100 fs. For critical work where the short-pulse advantage must be maximized, optical components with opposite dispersion can be inserted before the microscope to offset the pulse broadening. This “pre-chirping” adds additional complexity and requires frequent adjustments.

EVERY PHOTON COUNTS

The scanning excitation laser beam is now a single 3-D point in space (Figure 2), confined by the focusing lens in the horizontal plane and by multi-photon absorption in the axial direction. Unlike all single-photon excitation, including confocal arrangements, light cannot now be scattered from outside the focal volume into the detected signal to degrade contrast. Fluorescence light may be scattered away from within the focused spot, but as long as we can collect it, we know exactly at what pixel (or 3-D voxel) it should contribute to the final picture.

Detection in the multi-photon microscope can be designed to be very simple and highly efficient at collecting light rays from the specimen, even at high angles of scattering. This provides an ideal way to get fluorescence pictures from thick biological specimens without compromising the optical sectioning (5) (Figure 3).

ELIMINATING “COLLATERAL PHOTODAMAGE”

Photodamage will now only occur from molecules that are contributing to the collected signal, provided the specimen does not have significant single-photon absorbance at the near-infrared wavelengths used and the emitted fluorescence is efficiently collected. This can allow extended collection times for weak signals, or time-lapse observations, and reduced photo-toxicity with living specimens (22) (Figure 4). It also makes possible higher-illumination intensity to offset scattering of excitation light by thick specimens. All these benefits are particularly marked when two- or three-photon

energies equivalent to single photons of UV light are used for some DNA probes (9), ion imaging (12,20), and auto-fluorescing cellular components (13,14).

A LIFETIME FOR FREE

When a fluorescent molecule is excited by light, it jumps to a higher energy state. From here it loses this energy and is said to decay, back to the original or ground state. Some of the energy is lost thermally in one or more small jumps, and the bulk is emitted as a lower-energy photon of fluorescent light. The overall decay is a statistical process with a relaxation or fluorescence lifetime. Each type of fluorescent molecule has a characteristic lifetime (or a combination of component lifetimes). These are independent of the mean intensity of the emission but can be highly sensitive to the local environment

Figure 2. Multi-photon excitation is confined to a focal volume. A cuvette of fluorescent dye (from an image by Brad Amos, LMB, Cambridge, UK) shows the single-photon excitation beam (top arrow) lighting up the solution at the focus and also throughout the depth of the sample. Two-photon excitation (bottom arrow) only excites fluorescence at the point of focus.

(pH, molecular mobility, proximity to other molecules, etc.). For many fluorescent probes, the decay occurs over less than 10 ns, and so the pulse width and repeat rate of the best lasers for multi-photon excitation are ideal for recording of these processes (17). A fast photodetector is used to measure the decaying fluorescence after each laser pulse, and a decay curve is stored for each point of the scan (1). It is even possible to collect a separate decay curve at different wavelengths for each pixel (2). A picture composed of the fluorescence from the entire decay curve is simply the normal fluorescence signal we usually measure. However, the complete decay curve can be analyzed for one or more characteristic lifetimes. These lifetimes provide information on the environment or identity of the fluorescence molecules present (Figure 5) for each pixel with-

out compromising the regular "steady-state" fluorescence data.

HORSES FOR COURSES

It was once widely believed that all fluorescence microscopes would one day be confocal instruments. Such predictions miss the obvious point that each innovation in microscopy design is usually in response to a range of specific problems and relevant to a limited range of applications. So too will multi-photon microscopy take its place alongside the growing range of laser scanning microscopy tools available to the biologist.

Many UV-excitable fluorescent probes have been used for ion imaging and following intracellular molecules in spectroscopic studies and flow cytometry for many years. Their use in laser scanning microscopy has been hampered by the need for high-quality UV optics and phototoxicity in living preparations. Multi-photon microscopy addresses both of these issues simultaneously by the use of near-infrared illumination (Figure 4). New ultra-fast laser designs may allow other well-characterized probes of choice such as Fura (for calcium imaging) to be used in laser scanning optical sectioning. Where optical sectioning is not needed or perhaps undesired (e.g., for whole-cell measurements), a conventional system with appropriate camera detection is clearly better.

Figure 3. Multi-photon microscopy of components in cartilage. Low-resolution optical sections of human intervertebral disc stained for connective tissues. Collagen fibres (A and B) pass through areas of extracellular matrix (ECM) (C). High-resolution optical sections of living rat intervertebral disc cartilage. (D) Autofluorescent ECM fibers 20 μm below the surface. (E and F) Living chondrocytes (cartilage cells), stained with CMFDA, between the autofluorescent fibres, 40 and 60 μm into the cartilage. PlanApo 60 $\times$ 1.2 NA water immersion objective. 770 nm TiS excitation, >400 nm emission, using direct scattered-light detectors.

Figure 4. Multi-photon microscopy of living plant roots. Optical section through epidermal cells (A) 5 min after labeling with mono-chlorobimane. The dye is non-fluorescent until it binds to glutathione in the cell cytoplasm. Cytoplasmic staining appears as a thin layer around the vacuole of each cell. (B) Longitudinal section through the midplane of a root tip, 30 min after labeling. The fluorescent conjugate has been transported into the vacuoles. Morphology of a young *Arabidopsis* root, imaged throughout the tissue by multi-photon excitation (C). 770 nm excitation. A.J. Meyer, M.D Fricker and N.S. White, BioRad Biological Microscopy Unit, Dept. Plant Sciences, Oxford.

Figure 5. Multi-photon lifetime imaging of fluorescent proteins. Multi-photon excited fluorescence lifetime images of cultured cells transfected with a GFP-tagged protein (a), yellow fluorescent protein (YFP)-tagged protein (b), and both GFP- and YFP-tagged proteins (c and d). The color bars show the calibration of fluorescence lifetime from approximately 2.1 (red) to 3.0 ns (dark blue). Although their fluorescence emissions overlap significantly in the green portion of the spectrum, the GFP tags show a significantly shorter lifetime (2.1 ± 0.3 ns) than the YFP tags (2.5 ± 0.3 ns) in the single labeled cells and can be distinguished in the co-transfected cells. Separation of these probes can be further improved by correcting for the instrument response of 0.2–0.3.

Thick biological specimens, perhaps several hundred micrometers in depth, usually scatter the illumination and fluorescence significantly. With fixed material, clearing agents and refractive index-matched mounting medium can assist the penetration of a confocal optical probe. Of course, this extensive preparation is not always possible, and so the less scattered infrared illumination and wide-angle direct detection gives multi-photon significant benefits for difficult tissues (Figure 3) and for thick living samples.

Under ideal conditions, even some non-scattering living preparations can benefit from multi-photon excitation. In general, these conditions usually require the longest wavelength illumination (>900 nm) (29) and fluorescent probes with good multi-photon cross sections. This improves the resolution of dynamic function, with less perturbation of cells, but the longer wavelength gives reduced spatial resolution compared to an optimized confocal LSM. The deciding factor is a balance of the resolution required, the number of sections to be collected, and the photodamage outside of the optical plane with single-photon excitation.

It should by now be apparent that multi-photon LSM finds a clear niche when several of the sample-related problems discussed here conspire together to cause difficulties when imaging biological specimens. However, it should not be forgotten that the unique nature of this illumination-based sectioning technique and the ultra-fast pulsed excitation offer new opportunities for image contrast (e.g., fluorescence lifetime, localized photomanipulation, and many other nonlinear optical techniques).

It is significant that the new nano- and molecular physics is already making use of nonlinear spectroscopy and imaging in forging the next generation of biological research tools.

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