Vital imaging: **Two photons are better than one**

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The recently developed technique of two-photon fluorescence microscopy causes much less photodamage than conventional confocal microscopy, expanding the possibilities for imaging living specimens.

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Many potentially interesting biological imaging experiments are foiled by the damaging effects of the illumination used, namely photobleaching of the label and phototoxicity [1]. The researcher is forced to make compromises to minimize the total light dose, such as reducing the number of sections in a three-dimensional section series, the average number of scans per image or the number of time-points when observing an on-going process. A new player in the confocal microscopy arena, two-photon laser-scanning microscopy (TPLSM), alleviates many of these concerns, and will allow more researchers to consider imaging living, rather than fixed, specimens.

What is TPLSM?

This technique of TPLSM, first introduced by Watt Webb and co-workers in 1990 [2], is similar to confocal laser-scanning microscopy (CLSM). A laser beam is raster-scanned across a focal plane within the labeled specimen, and fluorescence is detected by a photomultiplier tube to produce a digital image on a computer. The most important difference is that, in TPLSM, the label is excited only at the focus of the beam. There is no out-of-focus fluorescence (hence no need for a confocal aperture), so dye bleaching and phototoxicity are limited to the plane of focus. With CLSM, the whole thickness of the specimen is harmed by every scan (Fig. 1).

This clever trick relies upon the phenomenon of two-photon excited fluorescence. Two infrared photons must collide with a fluorophore (fluorescent molecule) simultaneously. Their combined energy excites the fluorophore to a state virtually identical to that caused by a single visible photon of about half the wavelength. The fluorophore relaxes back to the ground state, emitting a visible photon. Because a fluorophore must absorb two photons per excitation event, fluorescence depends on the square of the infrared light intensity. This non-linear effect, combined with the sharp focusing of a microscope objective lens, allows one to excite only a diffraction-limited spot within the specimen.

Unfortunately, a very large instantaneous flux of infrared photons is necessary to make two-photon excitation happen. This requires the use of exotic infrared lasers that compress all of their output into very short (~10^{-13} sec), high-energy (~2 kW) pulses. These 'mode-locked' lasers space the pulses relatively far apart (~10^{-8} sec), so that a peak power in the kilowatt range is reduced to a mean

Figure 1

With CLSM (left), fluorescence, and hence, photodamage, occurs throughout the hourglass-shaped path of the excitation beam. Two-photon fluorescence (right) is limited to a spot at the focus of the scanning pulsed-infrared laser beam, resulting in a much less harmful light dose during repeated scanning. The infrared illumination used for TPLSM also penetrates deeper into the specimen than visible excitation. (For excellent, in-depth tutorials on TPLSM, see [9,13].)

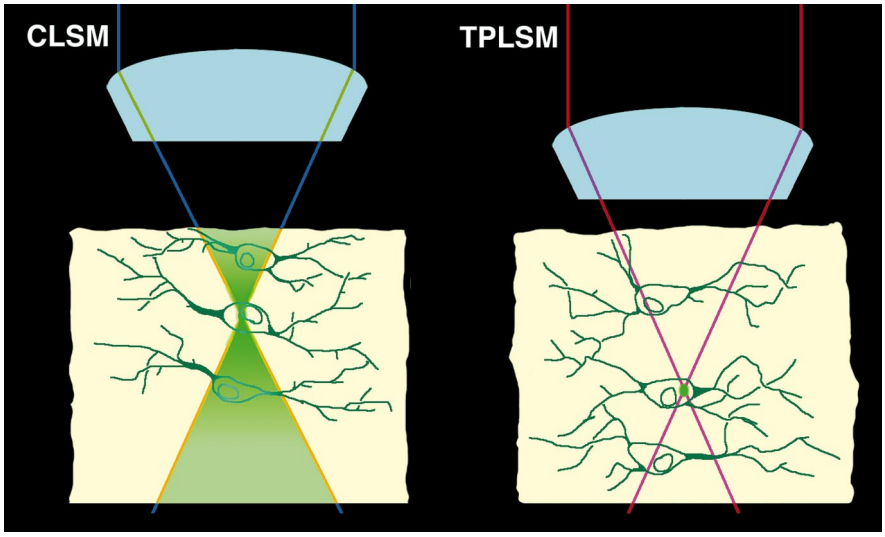
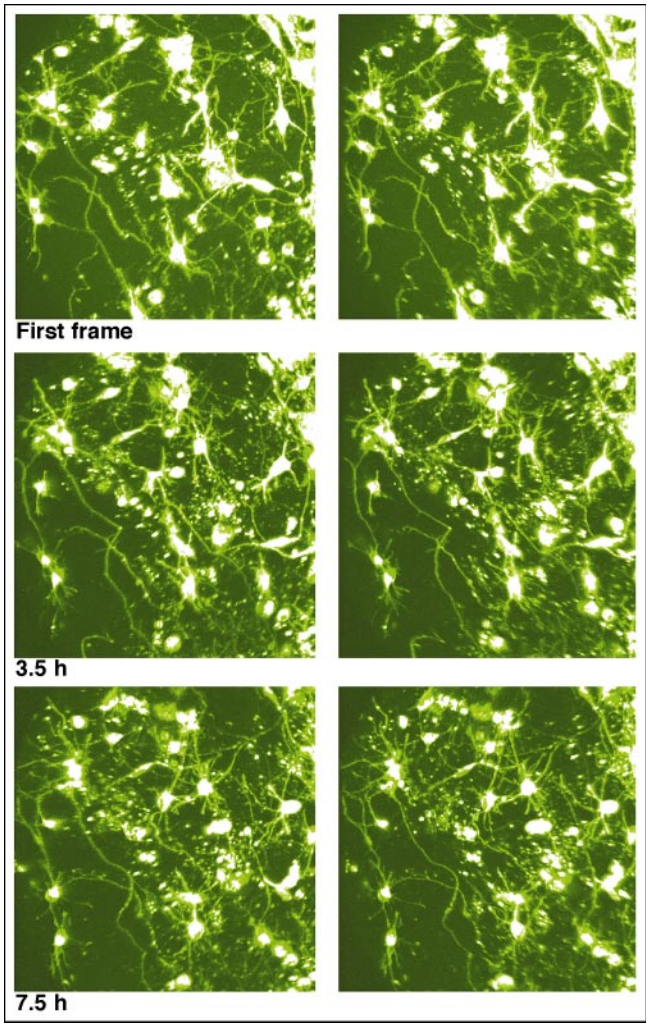


Figure 2



Four-dimensional imaging. Three representative stereo pairs from a time-lapse movie (available in the online version of this article via http://biomednet.com/cbiology/cub.htm) of an unlabeled, cultured hippocampal slice, seeded with DiO-labeled neurons. I imaged a 40 μm thick volume (20 sections) every 15 minutes for over 8 hours. The neurons were as active, and almost as fluorescent, in the final frames as in the initial frames. Section series were digitally projected using ImageSpace (Molecular Dynamics) software. The field is 250 μm across.

power of a few tens of milliwatts at the specimen. Most tissues are unaffected by this level of infrared illumination, but beware: some pigmented cells absorb infrared and are literally boiled by the infrared beam.

Four-dimensional imaging

The intrinsic sectioning capability of TPLSM gives its main advantage over CLSM: a substantial reduction of photodamage, especially when conducting a *z*-series of scans to create a three-dimensional rendering. With TPLSM, and recent advances in data storage and manipulation [3], it is now possible to image a substantial volume within a fluorescently-labeled living specimen in four dimensions: the three spatial dimensions, *x*, *y* and *z*, and time (Fig. 2) [4]. Four-dimensional TPLSM allows us to observe, for example, many dynamic aspects of neurite growth and retraction, as well as gross cell migration. These dynamics would be missed in studies using fixed specimens. Of course, the diminished photobleaching also makes TPLSM an appealing alternative for imaging fixed specimens, when other techniques would cause unacceptable dye fading.

The infrared light used for TPLSM penetrates deeper into scattering specimens than commonly used visible excitation. In a variety of living and fixed specimens, including zebrafish embryos, *Drosophila* larvae and rat hippocampal slices, we are able to image two to three times deeper with TPLSM than with CLSM [5]. This allows three-dimensional reconstructions of larger structures (Fig. 3). In the case of brain slices, it allows one to avoid the cut surface, which may not be as healthy as deeper tissue.

Merits of TPLSM relative to CLSM

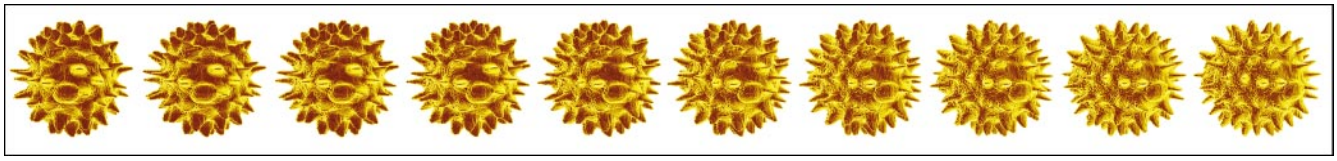
Advantages

- Dramatically reduced phototoxicity
- Dramatically reduced dye bleaching
- Greater depth of imaging
- Enhanced signal-to-noise ratio
- Ti : Sapphire laser is tunable across a broad range, for use with all common fluorophores
- Excitation of ultraviolet-excitable labels without an ultraviolet laser
- Photo-uncaging or photobleaching in a diffraction-limited volume
- Ultraviolet-transparent quartz optics not needed for ultraviolet excitation or uncaging
- Easy separation of excitation and emission light
- No chromatic aberration for multi-labeled specimens
- Simultaneous visible and ultraviolet excitation possible with three-photon system
- No pinhole aperture to align

Disadvantages

- Lasers needed are expensive, large, complicated and power-hungry
- No commercially available systems
- Two-photon effect only relevant for fluorescence imaging
- Not suitable for infrared-absorbing (pigmented) cells

Figure 3



Three-dimensional computer reconstruction of a pollen grain, imaged with TPLSM. The underside of this 25 μm diameter grain cannot be imaged with CLSM, because of light scattering and absorption. TPLSM provides a clear picture of the underside of the pollen grain, seen on the left side in the first frame. Adjacent pairs of images can be fused by crossing the eyes, to produce a rotating series of three-dimensional stereo images. I created these projections from 125 serial sections, using ImageSpace (Molecular Dynamics) software.

TPLSM equipment

Currently, there are no commercial turn-key TPLSM systems available. This fact, and the hefty power consumption (10–20 kW) and price of the mode-locked lasers used (~$135 000), have limited the widespread acceptance of TPLSM, despite its clear advantages over CLSM (see box). The most versatile pulsed infrared laser for TPLSM is the mode-locked, titanium-doped sapphire (Ti:Sapphire) laser (Coherent Mira900; Spectra Physics Tsunami), which requires an 8–10 W argon–ion pump laser. This system is tunable from 700–1000 nm, allowing two-photon excitation of fluorophores normally excited by ultraviolet, blue or green light (Fig. 4). This laser system uses three different mirror sets to span its output spectrum. Changing and aligning the mirror set takes several hours, but daily use requires only a few minutes of knob-twiddling to fine-tune the optics. Once the laser is correctly adjusted, its output remains stable for hours.

Rapid developments in solid-state (laser-diode-pumped) pulsed infrared lasers promise to provide much smaller, less power-hungry and eventually cheaper light sources for TPLSM. The success of such systems has been demonstrated, including three-photon imaging of ultraviolet fluorophores with excitation at 1047 nm [6]. Presently, such lasers are not tunable, and are less powerful than the Ti:Sapphire laser. A 5 W diode laser capable of pumping the Ti:Sapphire laser has been successfully used for TPLSM (W. Zipfel, personal communication). These compact devices (Spectra Physics Millenia; Coherent Verdi) require no cooling water, and run off a standard electrical outlet, making them an attractive alternative to the argon-ion pump laser.

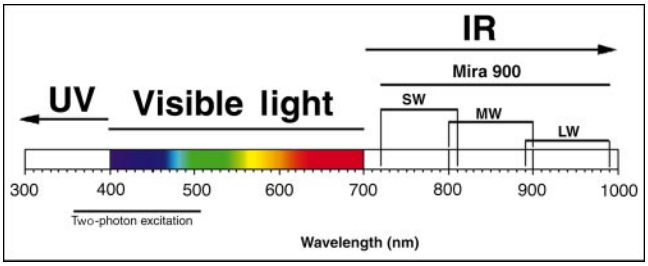
It is fairly straightforward to adapt an existing confocal microscope for TPLSM [5]. Scott Fraser and I converted a Molecular Dynamics Sarastro 2000 confocal microscope. We coupled it to a Ti:Sapphire laser, replaced three mirrors with broad-spectrum (infrared and visible) mirrors, and installed a beam-splitter to separate the infrared excitation from the visible fluorescence. We retained the ability to use the microscope in CLSM mode for comparison purposes. We can switch from CLSM to TPLSM mode in a few seconds by changing which light source is active, selecting the proper filters, and removing the confocal aperture.

Resolution

The resolution of a TPLSM system is entirely determined by the excitation spot. Unlike with conventional microscopy, there is no need to focus the emitted fluorescence, so TPLSM is inherently less affected by scattering and chromatic aberration. The infrared laser power must be adjusted to minimize the size of the excitation volume for optimal resolution. A large excitation volume will result in poor resolution and excessive photodamage.

The theoretical resolution of TPLSM is worse than that of CLSM by approximately a factor of two, because of the longer wavelength of excitation [7]. In practice, we have observed somewhat sharper images using TPLSM, in all cases where we compared the two imaging modes on the same specimen. This is because one seldom achieves the

Figure 4



Light spectrum, showing ultraviolet (UV), visible, and near-infrared (IR) regions. The ranges covered by the three mirror sets of the Mira900 Ti:Sapphire laser are indicated by SW, MW and LW. This broad tunability allows excitation of fluorophores normally excited by light from about 350–550 nm. (For all fluorophores tested, the two-photon absorption maximum is at a somewhat shorter wavelength than twice the one-photon maximum.) One beam-splitter can be used for all labels, as the emission and excitation wavelengths are hundreds of nanometres apart. Two-photon excitation in the SW range can be used to excite ultraviolet-excitable labels and caged compounds, eliminating the need for ultraviolet-transparent optics.

ideal resolution when imaging living specimens with CLSM. One must attenuate the laser power to reduce photodamage, and use a larger-than-optimal confocal aperture to obtain a reasonable signal-to-noise ratio [8]. As there is no out-of-focus fluorescence with TPLSM, all of the emitted light collected by the microscope is used to create an image. This gives an enhanced signal-to-noise ratio, providing slightly better resolution and depth discrimination in typical vital imaging situations, compared with CLSM.

Other TPLSM uses

The localized nature of two-photon absorption lends itself to a number of additional applications. Denk *et al*. [9] used two-photon uncaging of caged carbachol to map acetylcholine-receptor density, by measuring whole-cell current as a function of beam position. Summers *et al*. [10] used two-photon uncaging of caged fluorescein-dextran to map cell fates in the developing sea urchin embryo. Yuste and Denk [11] applied TPLSM to fast calcium imaging, so as to study the dynamic activity of dendrites. A high-repetition rate pulsed laser, like the Ti:Sapphire, allows one to carry out time-resolved fluorescence studies; this technique was combined with TPLSM by So *et al*. [12]. These and other recent applications of TPLSM make clear its benefits for probing the inner workings of living cells. I predict that, once the laser technology becomes less cumbersome and expensive, there will not be a confocal microscope that is not also a two-photon microscope. More information about TPLSM is available at my web site: http://www.caltech.edu/~pinelab/pinelab.html.

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