

The Long Shot: Multiphoton Microscopy Offers Deeper, Sharper, Safer Imaging Than Ever Before

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Introduction

When living cells are far below the surface, they pose particularly complex problems for researchers trying to view dynamic life activities in the laboratory. Many fluorescence imaging systems rely on short-wavelength ultraviolet (UV) or blue light, which is then absorbed by the specimen and emitted as visible light. But living tissue scatters so much short-wavelength light that some of the emitted fluorescence from the region of interest does not reach the detector. The deeper the area of interest, the more severe this problem becomes. Indeed, for every specimen, there is a point at which so much scatter occurs that traditional fluorescence imaging techniques are no longer effective. Raising the intensity of the excitation light in order to get more light out of the system can itself be lethal for living systems, as it causes increased photobleaching and phototoxicity. Adding to the problem is the fact that in deep imaging, regions of the specimen above and below the focal plane that are not of interest are exposed to light, causing unwanted fluorescence. Finally, excessive scattering of the excitation light when imaging deep below the specimen's surface results in an image with poor signal-to-noise ratio. These images tend to look soft and dull instead of crisp and full of contrast. Assuming that the scientist's research protocol will not allow the use of thinner-cut sections, deep imaging is still possible via multiphoton microscopy.

Confocal microscopes

Confocal microscopes are used for three-dimensional fluorescence imaging of living specimens because they are designed to optically remove out-of-focus light coming from above and below the focal plane, reducing background haze and also reducing the thickness of optical sections. Workhorses of today's life science research laboratories, confocal systems are powerful because they allow the viewing of dynamic life processes as they occur, even in dimly fluorescing specimens, in ways that could never be achieved until the 1990s. But confocal systems have limitations as well. Because the excitation light generates fluorescence throughout the entire depth of the sample, confocal microscopes can damage or bleach the entire volume of the specimen not only at the plane of focus, but above and below it as well. When sequentially imaging down through a three-dimensional sample, there can often be bleaching and damage to the lower regions before the researcher works his way down to them.

Another potential issue with confocal microscopy happens when collecting the emitted signal of the fluorophore. The emission must travel from the sample, through the microscope

optical system, and then be precisely directed through a small confocal aperture. Any deflection or scattering of the emitted fluorescence in the sample results in its rejection by the confocal aperture. The farther that emitted photons have to travel (from deeper within the specimen), the higher the odds that they will be scattered. In addition, a certain percentage of out-of-focus light that should be rejected will get scattered into the confocal aperture. This also contributes to degradation of the signal-to-noise ratio and leads to less than optimal images. Thus, while confocal microscopy holds an extremely important place in three-dimensional imaging, there may be trade-offs when attempting to view living specimens, especially when imaging at greater depths and/or for long time periods. This is just as important as the excitation side of the equation in evaluating the quality of the overall imaging system.

Multiphoton microscopes

Multiphoton microscope systems, which have some similarities with traditional confocal systems, have alleviated many of the problems arising during both dye excitation and signal collection [1-3]. This is because these systems rely on much longer wavelengths of light to excite fluorescence specimens than their confocal counterparts, and long wavelengths are subject

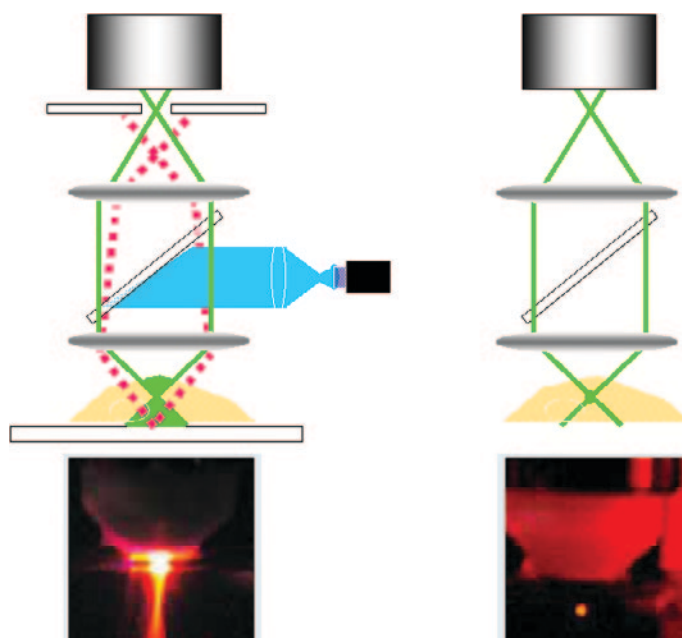


Figure 1: Comparison of image collection in single photon vs. multiphoton systems. Fluorescent dye is excited by single photon excitation (left image) and multiphoton excitation (localized spot indicated by white arrow on the right), illustrating that two-photon excitation is confined to the plane of focus.

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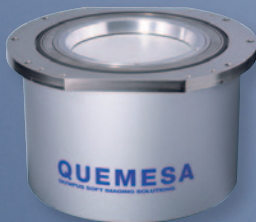
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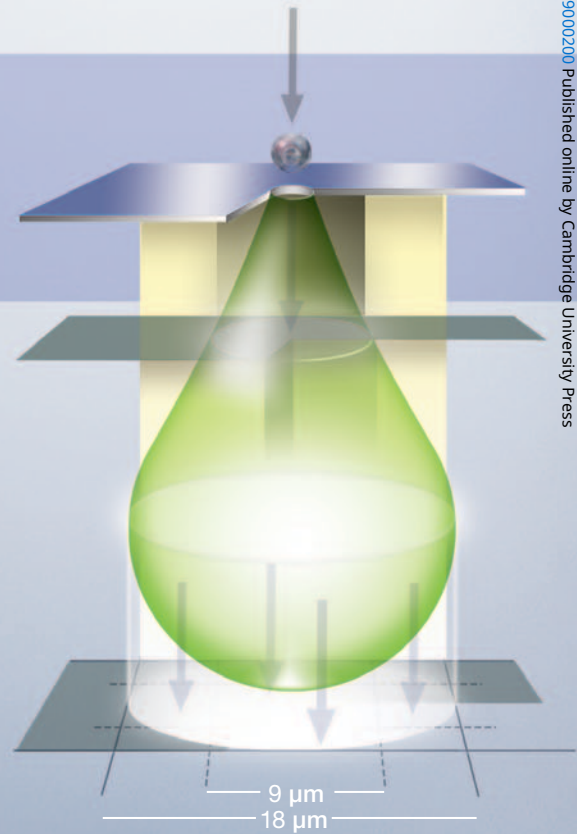
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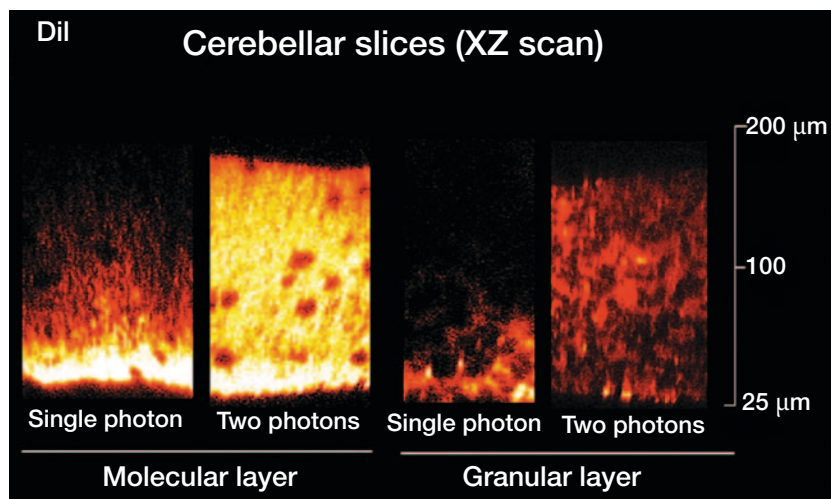


Figure 2 : Vertical cross-sections of the mouse cerebellum molecular layer (left two images) and granular layer (right two images) marked with Dil and obtained using single-photon (488 nm confocal) and two-photon (720 nm) excitation. Multiphoton excitation's superiority to confocal observation is apparent in deep observation of brain slices. Image courtesy of Yasuyuki Hayakawa, Haruo Kasai, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo.

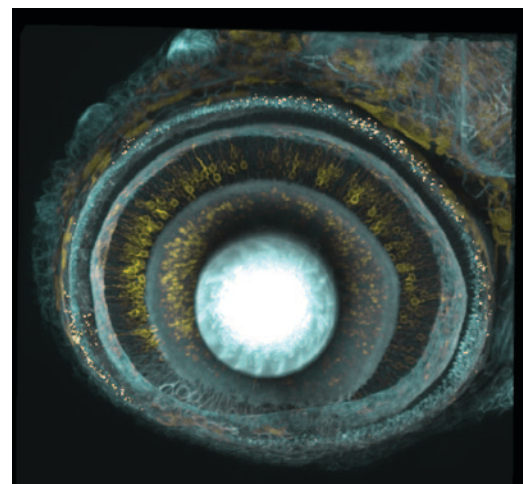


Figure 3: Multiphoton image of a living zebrafish: CFP and YFP expression during embryonic eye development (25x, 1.05 NA objective). Image courtesy of Phillip Williams and Rachel Wong, University of Washington.

to far less scatter as they move through tissue. In contrast to confocal microscopy, where fluorescence is generated throughout the entire 3-D volume, multiphoton fluorescence excitation only occurs at the plane of focus (Figure 1), resulting in lower levels of phototoxicity and photobleaching and allowing tissue to be imaged for much longer periods of time. Multiphoton microscopy also allows investigations of living tissue that is so thick it cannot be penetrated with conventional imaging techniques. Multiphoton methodologies are especially useful for neuroscientists, cell biologists, and other researchers who wish to study dynamic processes over time in living cells and tissues while retaining viable specimens for as long as possible, sometimes over hours, days, or even weeks.

The principle behind multiphoton microscopy is fairly straightforward. Two or more excitation photons can be simultaneously absorbed by a single fluorophore, combining their energies to bring the fluorophore to an excited state. For instance, green fluorescence protein (GFP), typically excited in fluorescence at 488 nm, can be excited using multiphoton imaging at wavelengths ranging from 850-960 nm. Whereas traditional fluorescence results in fluorophore emissions at longer wavelengths than the excitation light, the nearly simultaneous absorption of two longer-wavelength photons allows the fluorescence emission to occur at a shorter wavelength, typically in the visible light range.

Because multiphoton excitation requires the nearly simultaneous absorption of two or more photons, anything that increases photon density will increase fluorescence. This is why a burst of short pulses of highly focused light is directed at the focal point. A laser is used of such high peak energy that, during its ultra-short, femtosecond-long pulses, two photons from the same pulse excite a fluorophore essentially simultaneously, as if they were a single, short-wavelength, high-energy photon. The key

is that the statistical chance of two or more photons interacting with the same fluorophore molecule occurs only at the plane of focus where there is a high density of photons. Thus, fluorescence excitation is effectively limited to the plane of focus, eliminating photobleaching and phototoxicity in the areas above and below the focal plane. This is among the most important benefits of multiphoton microscopy.

By successfully localizing the excitation fluorescence, and by using near-infrared wavelengths, researchers can achieve deep-tissue imaging with significantly reduced photodamage and significantly enhanced, higher-contrast imaging results (Figure 2). Even such difficult specimens as ultra-thick brain slices, eye tissue, and developing embryos can be imaged (Figure 3).

As with confocal microscopes, three-dimensional multiphoton imaging is possible by building stacks of individual sections from each optical slice collected at sequential z-axis locations. However, even though multiphoton microscope systems have much in common with confocal instruments, they are not identical. One key difference is that multiphoton systems do not use confocal apertures in front of the detectors. Instead, optical sectioning is accomplished as a result of the excitation process.

Benefits in emission detection

Another key distinction of multiphoton systems is the detection system. With any live imaging system, the signal produced, which may originate deep within the specimen, must be detected with greater sensitivity. Since multiphoton excitation only occurs at the point of focus, and there is no out-of-focus light produced, every photon is a usable photon, and all the signal can be collected close to the specimen without passing through any kind of aperture, enhancing the efficiency of signal collection from deep within the tissue. The closer the detectors are to the specimen, the greater the efficiency of signal collection. In the

Olympus (Tokyo, Japan) FluoView® FV1000-MPE laser scanning multiphoton system, for instance, these non-descanned detectors are positioned immediately behind the objective lens, resulting in optimum collection of emitted signal. There are also noncommercial systems with multiple detectors near every objective that have excellent efficiency, but these are extremely complex and not particularly user-friendly. Often, these are designed by and for the person using them and can be difficult for anyone else to use.

A third area of differentiation involves how deep within tissue the systems can accurately image. A good multiphoton system can image efficiently from 2 to 6 times deeper within a given specimen than a comparable confocal system. The thicker and deeper the living specimen's area of interest, the more dramatic this contrast becomes.

Pulse width and resolution issues

Despite its potential, multiphoton microscopy has several limitations. First, the equipment required is highly specialized, and the lasers for the system can be quite expensive. Most systems use the Ti:sapphire laser, which is tunable over a wide wavelength range. In addition, if the specimen is highly pigmented, infrared light can boil the pigment, whereas shorter confocal wavelengths don't necessarily warm tissue in this way.

Another limiting factor is pulse width. The energy of multiphoton excitation is improved by employing more intense, shorter pulses of light. Two or more photons must be absorbed simultaneously to excite a fluorophore, and fluorescence is generated only where the laser beam is tightly focused (the plane of focus). Pulse width is a determining factor in efficiency of excitation, and most Ti:sapphire lasers have short pulse widths

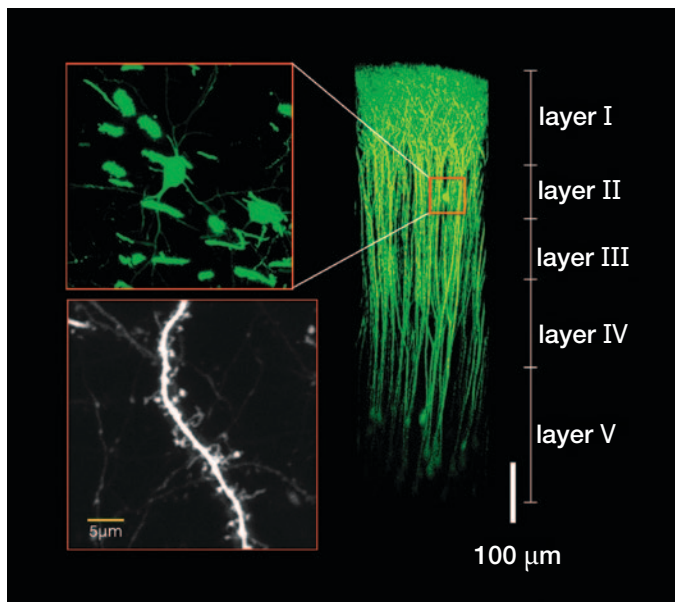


Figure 4: Three-dimensionally constructed images of neurons expressing EYFP in the cerebral neocortex of a mouse under anesthesia. Cross-sectional images down to 700 microns from the surface can be observed. Image captured using the Olympus FluoView FV1000-MPE multiphoton system with a 60x objective by Hiroaki Waki, Tomomi Nemoto, and Junichi Nabekura, National Institute for Physiological Sciences, National Institutes of Natural Sciences, Japan.

(typically 150 fs). However, the pulses in most commercial multiphoton systems are inadvertently broadened by components in the microscope itself, such as lenses and power control devices. This broadening degrades the efficiency of dye excitation. Recently, Olympus has developed a way of maintaining shorter, more tightly focused pulses with the FV1000-MPE system, optimizing the excitation efficiency of the system. By utilizing a process known as negative chirp, a specialized type of dispersion compensation, the laser's pulses are conditioned and kept short at the sample, further reducing the amount of average power necessary for imaging. This leads to less specimen damage and longer-term imaging of living tissue. The system incorporates the use of a femtosecond pulsed laser to achieve high photon density at the focal plane and is optimized for use with near-infrared wavelengths. Negative chirp ensures that the packet of light does not arrive already broadened. By being more efficient with the pulse, the system keeps power down, efficiently delivering a tight packet of light to the focal plane and resulting in the maximum return for the light placed into the system.

Perhaps the most often described limitation of multiphoton systems is their resolution, which generally has not been as high as with confocal systems. This assessment is based on a comparison with theoretical confocal systems that are only wavelength dependent. However, in the real world, the deeper the specimen, the less relevance this theoretical difference has. Indeed, being able to avoid confocal degradation of the image in deep scans means that investigators can image much more deeply into the tissue than is possible with even the best confocal systems. There are also the advantages of the enhanced signal-to-noise ratio and enhanced image contrast. In other words, multiphoton imaging is optimized for situations where scientists need to see deeply and clearly. The Olympus FV1000-MPE family of systems has resolved images up to 700 microns or deeper inside brain tissue—hundreds of microns deeper than possible using confocal microscopy (Figure 4).

Conclusion

Multiphoton methodologies allow scientists to see activity deeper and with less damage than ever before. Now that it has been commercialized by several companies, the technique is coming into much more widespread use, especially for repeated, long-range or time-lapse exposures. As scientists are seeing things they have never been able to view before, they are developing new and different research protocols. Microscope companies are working closely with the pioneers of this technique around the world to develop even more advanced research tools to meet tomorrow's needs. **MT**

References

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