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Principles of Multiphoton Microscopy

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Key Words
Multiphoton microscopy · Fluorescence · In vivo imaging · Three-dimensional imaging · Intravital microscopy

Abstract
Multiphoton fluorescence microscopy is a powerful, important tool in biomedical research that offers low photon toxicity and higher spatial and temporal resolution than other in vivo imaging modalities. The capability to collect images hundreds of micrometers into biological tissues provides an invaluable tool for studying cellular and subcellular processes in the context of tissues and organs in living animals. Multiphoton microscopy is based upon two-photon excitation of fluorescence that occurs only in a sub-femtoliter volume at the focus; by scanning the focus through a sample, 2- and 3-dimensional images can be collected. The complex 3-dimensional organization of the kidney makes it especially appropriate for multiphoton microscopic analysis, which has been used to characterize numerous aspects of renal physiology and pathophysiology in living rats and mice. However, the ability to collect fluorescence images deep into biological tissues raises unique problems not encountered in other forms of optical microscopy, including issues of probe access, and tissue optics. Future improvements in multiphoton fluorescence microscopy will involve optimizing objectives for the unique characteristics of multiphoton fluorescence imaging, improving the speed at which images may be collected and extending the depth to which imaging may be conducted.

Introduction

Fluorescence microscopy has become a vital component of biomedical research, allowing investigators to localize specific molecules and characterize cell physiology at subcellular resolution. The extended reach and low photon toxicity of multiphoton microscopy now offers biomedical researchers the capability of characterizing cellular and subcellular processes deep into tissues in three dimensions and even in the context of tissues and organs in living animals. Here we present a basic introduction to the principles, biological applications, practical aspects and future of multiphoton fluorescence microscopy in biomedical research.

Principles and Development of Multiphoton Fluorescence Microscopy

Conventional fluorescence is stimulated by the absorption of a photon by a fluorophore, raising an electron to an excited energy state which, when it returns to ground state, results in the release of a photon. In contrast, multiphoton fluorescence microscopy is based upon the simultaneous absorption of two, low-energy photons by a molecule (fig. 1). While the energy of either of these low-energy photons is insufficient to excite an electron, their combined energy is enough to raise an electron to the excited state and thus stimulate fluorescence. This process was theoretically predicted in the PhD dissertation of Maria Goeppert-Mayer in 1931, part of the body of work that resulted in her receiving the first Nobel Prize in theoretical physics awarded to a woman [Goeppert-Mayer, 1931].

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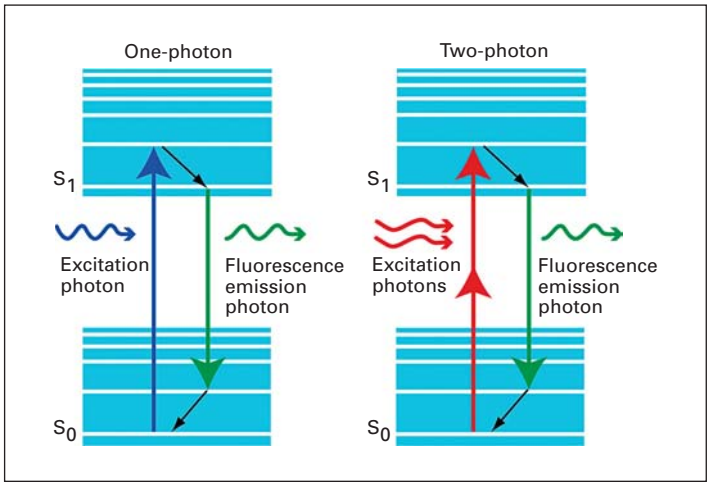

Fig. 1. Jablonski diagram demonstrating one- and two-photon fluorescence excitation. Two-photon excitation results from the simultaneous absorption of two low-energy photons by a fluorophore.

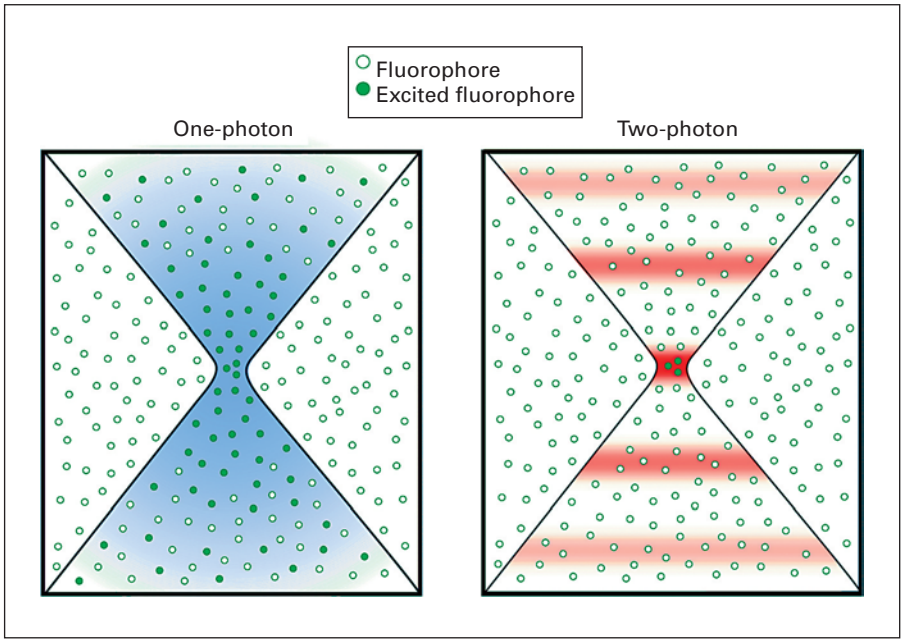

Fig. 2. Fluorescence excitation for one- and two-photon microscopy. In confocal microscopy, a continuous wave ultraviolet or visible light laser excites fluorophores throughout the volume. In two-photon microscopy, an infrared laser provides pulsed illumination such that the density of photons sufficient for simultaneous absorption of two photons by fluorophores only occurs at the focal point.

In order to stimulate an electronic transition, the two photons must arrive at the fluorophore within approximately an attosecond (10^{-18} s) of one another [McCain, 1970], which under most circumstances is a very unlikely event. Winfried Denk, one of the inventors of multiphoton microscopy, presented an illustrative numerical example that demonstrates this point [Denk and Svoboda, 1997]. A molecule of rhodamine, exposed to direct sunlight, will absorb a single photon around once per second but will experience a two-photon absorption around once every 10 million years. Thus, to generate detectable amounts of fluorescence, multiphoton fluorescence excitation requires an enormous photon flux. Consequently, it was not until sufficiently powerful lasers were developed 30 years later that Dr. Goeppert-Mayer's predictions of multiphoton fluorescence excitation were finally experimentally validated [Kaiser and Garret, 1961].

However, the high power that made these early lasers capable of stimulating multiphoton fluorescence excitation also made them incompatible with biological material. Consequently, the utility of multiphoton fluorescence to biological microscopy was not realized for yet another 30 years, with the development of ultra-short pulsed-laser systems [Denk et al., 1990]. These laser systems emit light in brief, intense pulses (typically between 100 and 2,000 fs in length), at a frequency of around

Table 1. Comparison of confocal and multiphoton microscopy

	Confocal microscopy	Multiphoton microscopy
Excitation source	Ultraviolet or visible laser Limited number of wavelengths Low cost	Pulsed infrared laser Continuously variable wavelength High cost
Effective imaging depth	Typically <20 μm	Up to 500–600 μm[a]
Spatial resolution (full width at half-maximum)	Theoretically up to 0.14 μm laterally 0.57 μm axially[b]	Theoretically up to 0.23 μm laterally 0.93 μm axially[b]
Sensitivity to scattering	High	Low
Photobleaching and phototoxicity	High – occurs throughout tissue volume	Low – restricted to focal plane
Image capture rate	Typically low, higher speeds possible via array scanning	Low, array scanning limited to thin samples

[a] See Kleinfeld et al. [1998] and Helmchen et al. [1999].
[b] Assuming 488 nm excitation for confocal and 900 nm excitation for multiphoton [Jonkman and Stelzer, 2002]. Note that the resolution of confocal and multiphoton systems are more similar in practice [e.g. Centonze and White, 1998].

80 MHz (fig. 2). In doing this, pulsed lasers provide the high peak photon flux necessary to stimulate detectable fluorescence, but because their duty cycle is so small (typically approximately 0.001%), the average power is low enough to avoid damage to tissues or fluorescent probes.

In a multiphoton microscope system the probability of multiphoton absorption is further increased by focusing the beam down to a diffraction-limited spot through a high numerical aperture microscope objective. Because the photon flux decreases with the fourth power of distance from the focus, multiphoton fluorescence excitation is effectively limited to a sub-femtoliter volume at the focus. Thus, one can collect optical sections from a sample volume by raster-scanning the focus across the sample, and by translating the focus through the sample, one can collect 3-dimensional image volumes.

The combined effects of pulsing and focusing the laser are such that under typical use, the average peak photon flux is more than a million times that at the surface of the sun. While this sounds ridiculous, the photon pulse is so brief, and its volume is so small, that estimates indicate that, even with 100 times this power, illuminating the focus for one full second would increase the temperature of water by only 0.2 K [Schonle and Hell, 1998]. Indeed, multiphoton microscopy has been found to be remarkably non-toxic. Squirrell et al. [1999] collected multiphoton fluorescence images of embryonic hamsters every 15 min over a period of 24 h without affecting their development.

Multiphoton microscopy thus provides a method of optical sectioning that compares favorably with confocal microscopy. Since the size of a diffraction limited spot is proportional to the wavelength of light, one might expect the resolution of multiphoton microscopy to be approximately 2-fold worse than that of confocal microscopy. In fact, due to the two-photon mechanism, the calculation is somewhat more complicated, but still predicts a resolution that is approximately 60% worse than that of confocal microscopy (table 1). However, resolution is influenced by a number of additional factors, including image noise and background, so that in actual practice, the resolutions of the two systems are similar [Centonze and White, 1998; Konig, 2000; Zipfel et al., 2003b].

Multiphoton microscopy has several advantages over confocal microscopy for collecting images deep within biological samples. First, since fluorescence excitation is limited to a single spot in the sample volume, no confocal aperture is needed to reject out-of-plane fluorescence. This is a significant advantage for imaging thick tissues where light scattering can redirect emissions away from the confocal aperture, reducing sensitivity and contrast. Since excitation is localized to a single point, fluorescence emissions can be collected using one or more detectors located close to the microscope objective to optimize collection of scattered light. In addition to more efficient collection of fluorescence, multiphoton microscopy also offers more efficient stimulation of fluorescence deep into biological tissues. As compared with the visible light used in confocal microscopy, the near-infrared light used to stimulate multiphoton fluorescence is both scattered and absorbed less by biological tissues, so that fluorescence can be excited deeper into biological samples [Konig,

2000]. Finally, since fluorescence is stimulated only at the focal point, photobleaching is likewise limited to this point. The lack of out-of-plane photobleaching makes multiphoton microscopy particularly amenable to collection of 3-dimensional image volumes, which when collected via confocal microscopy, suffer from the accumulation of photobleaching of the entire volume that occurs with the collection of each image plane.

Thus, for several reasons, multiphoton fluorescence microscopy is superior to confocal microscopy for imaging deep into biological tissues (a detailed demonstration can be found in Centonze and White [1998]). Examples of how biomedical researchers have utilized multiphoton microscopy for imaging deep into biological tissues are described in the next section.

Utilization of Multiphoton Fluorescence Microscopy in Biomedical Research

The deployment of multiphoton microscopy in biomedical research has continued to be driven by technical developments in lasers. The original multiphoton microscope system utilized a fussy dye laser system that was too technically challenging for most biomedical researchers [Denk et al., 1990]. Thus, multiphoton microscopy was limited to very few laboratories until the development of solid-state titanium-sapphire lasers in 1992 [Curley et al., 1992]. Still somewhat challenging to the average biologist, these systems became progressively easier to operate in the past 10 years, culminating in the recent development of 'closed-box' computer-controlled systems that require almost no attention from the user.

With the development of commercial systems in 1996, multiphoton microscopy finally evolved from a method limited to laboratories specialized in advanced biophotonics to one accessible to a broad range of biomedical researchers. Since that time, biomedical researchers have applied multiphoton microscopy to a variety of studies, including some particularly exciting analyses of cellular and intracellular processes in living animals. Multiphoton microscopy has been used for in vivo analysis of dendritic spine development, cortical blood flow, senile plaque clearance and dendritic calcium dynamics in the brain, blood flow, angiogenesis and vascular permeability in tumors and T-cell trafficking in lymph nodes (summarized in Denk and Svoboda [1997] and Zipfel et al. [2003b]).

The complex 3-dimensional organization of organs such as the kidney makes it especially appropriate for multiphoton microscopic analysis. Phillips et al. [2001, 2004] have used multiphoton microscopy to characterize renal development in a mouse model of polycystic kidney disease. Because the kidney can be easily immobilized and apposed to a coverglass on the stage of a microscope, multiphoton microscopy can also be used to image renal physiology in living rats and mice [Dunn et al., 2002] and has been applied to studies of microvascular leakage in a rat model of renal ischemia [Sutton et al., 2003, 2005], folic acid uptake and transport [Sandoval et al., 2004] and organic anion transport in rat proximal tubule cells [Tanner et al., 2004]. Mik et al. [2004] have used multiphoton excitation to characterize local oxygen concentrations in kidney cortex of living rats. The laboratory of Peti-Peterdi has used multiphoton microscopy to characterize the function of isolated living glomeruli, arterioles, and cortical and medullary tissues [Peti-Peterdi et al., 2004; Peti-Peterdi, 2005].

Practical Multiphoton Microscopy

On first principles, one might expect that multiphoton fluorescence microscopy differs from conventional fluorescence microscopy only in the use of near-infrared illumination to excite fluorescence. Indeed, many current multiphoton microscope systems are simply confocal microscopes modified for infrared illumination. However, multiphoton microscopy is unique in several respects. In addition, the ability to collect fluorescence images deep into biological tissues raises unique problems not encountered in other forms of optical microscopy.

The first challenge concerns fluorescent probes for multiphoton microscopy. The process of two-photon excitation is generally introduced with the example that the fluorescence ordinarily excited by a single photon of a particular energy can also be excited by two photons, each with half that energy. Thus one would expect that efficient multiphoton excitation could be accomplished by simply doubling the wavelength used for one-photon excitation. However, the 'two-photon action cross section' (the property used to describe the probability of two-photon absorption) can differ dramatically from this simple relationship. So, for example, while the two-photon excitation profiles for probes such as lucifer yellow and Fura-2 closely approximate two times their single photon excitations, probes such as fluorescein and rhodamine show two-photon excitation optima that are not obvious from their corresponding one-photon excitation spectra. Thus before designing a multiphoton fluorescence microscopy

experiment, one should consult the analyses published by the Cornell research group [Xu et al., 1996; Zipfel et al., 2003a, 2003b] to identify the appropriate excitation wavelength.

Even after one has selected a fluorescent probe, the next challenge may be to ensure that the probe has access to its targets in the sample. The ability to image deep into biological specimens raises unique issues of preservation and permeability of thick, fixed specimens. Careful experiments must be conducted to demonstrate that the distribution of the probe in the sample is not affected by the access of the probe to different parts of the sample. The problem of probe access is more complicated for studies of living tissues, especially within living animals. In this case, probe access depends upon physiological methods of probe delivery. So, for example, we have found that intravenous injection of a membrane-potential-sensitive probe, rhodamine R6, brightly labels the mitochondria of endothelia, but fails to label renal epithelial cells [Dunn et al., 2002].

One solution to the problem of labeling specific cells in living tissues is to express an exogenous fluorescent protein label. This can be accomplished by generating a transgenic animal expressing a fluorescent protein under the control of a tissue-specific promoter. For example, Sutton et al. [2003] examined injury to the renal microvasculature in a mouse expressing GFP under the control of an endothelial specific promoter. Alternatively, the gene may be introduced locally into a group of cells. For example, Tanner et al. [2005] have used micropuncture-mediated delivery of adenovirus to express various GFP protein chimeras in rat kidney cells.

Alternatively, one can circumvent the problem of delivering fluorescent reporters by using endogenous fluorophores. Many endogenous proteins, such as indoleamines, flavins, NAD(P)H and serotonin have fluorescence that can be excited by multiphoton excitation [Zipfel et al., 2003a]. The environmental sensitivity of NAD(P)H and flavin fluorescence has been used to characterize cellular redox in vivo in the rabbit cornea [Piston et al., 1995] and in human skin [Masters et al., 1997].

In order to realize the benefits of multiphoton microscopy for deep tissue imaging, special attention must be paid to the fluorescence detection system. While confocal microscope systems may be modified into multiphoton microscope systems, the collection system of a confocal microscope is poorly suited to collecting fluorescence from deep inside biological tissues. Since multiphoton fluorescence excitation is inherently localized to a single spot in the sample, all of the emitted fluorescence can be collected, unlike a confocal microscope where the out-of-plane fluorescence must be filtered out with the confocal pinhole. Consequently, multiphoton fluorescence emissions can and should be collected with detectors located as close to the microscope objective as possible. In addition to avoiding the inevitable transmission losses of the various filters and reflectors in the de-scanning mechanism of the confocal microscope, this design also more effectively collects scattered fluorescence emissions that are unlikely to successfully navigate the long optical path from the microscope objective to the photomultiplier detector in the confocal scanhead. A convincing demonstration of the benefit of using external, 'non-descanned' detectors is shown in Centonze and White [1998].

Multiphoton excitation can be accomplished with infrared lasers that provide pulsewidths in the range of 100–200 fs or in the range of picoseconds. However, picosecond lasers stimulate less multiphoton fluorescence than femtosecond lasers for the same average power. Since multiphoton fluorescence excitation is proportional to the square of the illumination power divided by duration of the pulse, a laser producing 1-ps pulses requires 3.16 times more power to produce the same amount of fluorescence as a laser producing 100-fs pulses. Because of patent issues, the choice of laser system is tied to the choice of commercial multiphoton microscope system, since 'sub-picosecond' multiphoton fluorescence microscopy is licensed to the Zeiss, whereas picosecond multiphoton fluorescence microscopy is licensed to Leica. The patent situation surrounding multiphoton microscopy has unfortunately limited its technical development since its first commercial introduction by BioRad in 1996.

Although their lower efficiency would seem to condemn picosecond systems, in many cases multiphoton microscopy is not limited by laser power. While the lasers of most systems are capable of delivering hundreds of milliwatts of power at the sample, powers above approximately 10 mW induce cell damage, as evaluated by a variety of criteria [Konig et al., 1997; Konig, 2000; Hopt and Neher, 2001] and powers in the range of 30–50 mW have been used to drill holes and sever intracellular structures [Konig, 2000]. As described previously, multiphoton microscopy conducted at lower levels of illumination can be remarkably non-toxic [Squirrell et al., 1999], but the damaging effects on cell viability appear to be nonlinear, so that damage is roughly proportional to fluorescence up to 10–15 mW, but then scaling at a higher rate above those levels [Koester et al., 1999; Hopt and Neher, 2001]. High power also appears to be disproportionately

harmful to fluorescent probes; photobleaching rates increase disproportionately faster than fluorescence signals over this range of powers [Patterson and Piston, 2000]. It is noteworthy that below these threshold values, phototoxicity is apparently mediated by a two-photon excitation process [Konig, 2000], meaning that while picosecond systems excite less fluorescence for a given average power, they induce proportionally less photodamage as well. Thus, picosecond and femtosecond are effectively equivalent under conditions where fluorescence signals are limited by phototoxicity.

While these arguments suggest that laser power is available in abundance in multiphoton microscope systems, they ignore the fact that the photon flux delivered to the focal point deep in a biological sample may be significantly reduced by scattering. Thus, laser power may be limiting for the deep-tissue imaging applications most appropriate for multiphoton microscopy. In many cases, we have found that illumination levels must be increased in order to collect adequate images deep in tissues. Konig [2000] reports that laser power must be increased 10-fold to acquire images 100 μm into a tumor equivalent to those collected at the surface. Under these conditions, the additional efficiency of femtosecond laser systems may be required.

Other authors argue that the main factor limiting the collection of images deep into tissues is scattering or absorption of fluorescence emissions [Zipfel et al., 2003b]. To the degree that sufficient fluorescence is stimulated at depth, but the fluorescence emissions are scattered and thus undetected, increasing laser power will have a limited capacity for improving the signal. In fact, if adequate fluorescence is being generated but not detected, increasing laser power will probably rapidly incur fluorescence saturation. Increasing laser power under these conditions will not only fail to improve signal, but can also decrease the resolution of the image as the size of the excited volume is effectively increased [Zipfel et al., 2003b].

Future Developments in Multiphoton Microscopy

Although invented more than 15 years ago, multiphoton microscopy is still an immature technology, particularly as it exists in commercially available instruments. While some experimental systems have been specifically designed for the unique capabilities and requirements of multiphoton microscopy, commercial instruments still largely utilize designs only slightly modified from those used for conventional fluorescence and confocal microscopy.

Multiphoton microscopy places unique demands on microscope objectives. New designs optimized for transmission of near-infrared light improve the efficiency of multiphoton fluorescence excitation. However, opportunities for further improvements in multiphoton fluorescence performance may result from designs optimized for the fundamentally different role that the microscope objective in multiphoton microscopy plays. Objective lenses designed for conventional fluorescence and confocal microscopy are designed to focus visible wavelengths of light down to a diffraction limited spot in the sample plane. In contrast, objectives optimized for multiphoton fluorescence should be designed to focus a brief pulse of a range of infrared wavelengths of light to a point in the sample and to do so without broadening the pulse. Improvements are needed to decrease dispersion in objective lenses that broadens the laser pulse, decreasing the efficiency of multiphoton fluorescence excitation. Objectives designed for conventional fluorescence and confocal microscopy are designed to focus fluorescence emissions down to a diffraction limited spot in the image plane. In contrast, objectives used for multiphoton microscopy need not be designed to image fluorescence, but rather to efficiently collect fluorescence, both scattered and unscattered. Oheim et al. [2001] find that increasing the angle of acceptance of the collection optics improves the efficiency of fluorescence collection at depth up to 30-fold. Of particular note is the observation that a low-magnification, high numerical aperture objective provides a 10-fold improvement in the collection of emissions deep into brain tissues, as compared with 60–63× objectives with a similar numerical aperture, but smaller acceptance angles.

Another factor that limits the depth at which imaging can be conducted by multiphoton fluorescence microscopy is the degradation of the shape of the illuminating spot, resulting from inhomogeneities in the sample. Recent results indicate that this problem can be ameliorated with adaptive optics. Widely used in astronomy, adaptive optics involves the specific shaping of lenses to compensate for specimen-induced aberrations. Optimized shaping of deformable lenses in the excitation light path of multiphoton fluorescence microscope systems has been found to improve axial resolution 2-fold at a depth of 50 μm [Marsh et al., 2003] and to extend the useful depth from 150 to 800 μm [Sherman et al., 2002] into an aberrating medium.

Technical developments may also increase the speed of image acquisition in multiphoton microscopy. Commercial systems collect reasonable sized images at a rate of 1–4 frames per second, a rate too slow to observe rapid dynamic processes in living tissues. For some applications, these slow capture rates can be compensated by collecting line scans, in which a single line in the sample is repeatedly scanned at millisecond frequencies, an approach that has been used to characterize calcium transients [Helmchen et al., 1999] and blood flow [Kleinfeld et al., 1998; Brown et al., 2001] in living rats. While improving speed, this technique provides limited spatial information.

Alternative designs of multiphoton microscopes, with faster scanning systems, are capable of significantly increasing the rate of image capture, supporting image capture at 15–100 frames per second [Fan et al., 1999; Kim et al., 1999; Nguyen et al., 2001]. However, the utility of these systems is limited to samples in which signal is not limiting, since as the speed of collection increases, the period of light collection proportionately decreases.

An alternative approach to high-speed scanning microscopy is to scan multiple foci simultaneously across the sample. In this way speed can be increased without necessarily sacrificing signal. One problem with this approach is that as the number and density of beams are increased, they begin to overlap with each other, resulting in excitation of fluorescence outside the focal plane, and corresponding decreases in axial resolution and image contrast. An elegant solution to this problem is to temporally offset the excitation of adjacent foci by 250–1,000 fs, thus preventing reinforcement of excitation between them [Andressen et al., 2001; Nielsen et al., 2001; Egner et al., 2002].

The major drawback of this approach is that it is poorly suited to thick, scattering samples. In order to realize the speed benefit, fluorescence is collected via an imaging detector, such as a CCD. Whereas unscattered fluorescence will be efficiently focused to the appropriate detector element, and thus sharply imaged, scattered fluorescence will be directed to incorrect detector elements, reducing both resolution and image contrast. In addition, dividing the illumination between multiple beams depends upon a surplus of power. As discussed above, this is likely to be reasonable for thin samples, but perhaps not for thick samples where illumination levels must be increased.

Thus, while there are solutions to the problem of slow image acquisition in multiphoton microscopy, they do not seem to be appropriate to the conditions most appropriate for multiphoton microscopy, deep tissue imaging. High-speed point scanning systems depend upon high levels of signal that may be difficult to achieve deep in tissues. It is unlikely that illumination levels can be increased to compensate for the low signal levels of these systems. A 30-fold increase in image capture rate will require more than a 5-fold increase in power that is likely to incur fluorescence saturation, and may not be available from the laser source. Multifocal multiphoton microscope systems may likewise be limited by power when imaging deep into tissues, and scattered emissions will compromise both sensitivity and image contrast.

Deployment of Multiphoton Microscopy

A survey of the published literature conducted in 2003 indicates that while the use of multiphoton microscopy has grown exponentially over the past 15 years, most publications come from a relatively few laboratories [Zipfel et al., 2003b]. To some extent this slow deployment of multiphoton microscopy may reflect the cost and/or technical complexity of multiphoton microscope systems. However, the biomedical application of multiphoton microscopy is unusual in that it involves many different kinds of expertise. Our experience indicates that fully realizing the unique potential of multiphoton microscopy requires an unusual degree of collaboration, not only between microscopists and cell biologists, but also with clinicians and individuals with skills in animal handling, tissue histology and digital image processing. Thus it may be difficult to achieve the critical mass of expertise necessary to fully exploit multiphoton microscopy. However, these difficulties are more than compensated by the enormous rewards of this technique, providing the ability to visualize and quantify cellular and intracellular processes in three dimensions and in living animals.

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