Fundamentals and Applications in Multiphoton Excitation Microscopy

Two-photon excitation microscopy (also referred to as **non-linear**, **multiphoton**, or **two-photon laser scanning microscopy**) is an alternative to confocal and deconvolution microscopy that provides distinct advantages for three-dimensional imaging. In particular, two-photon excitation excels at imaging of living cells, especially within intact tissues such as brain slices, embryos, whole organs, and even entire animals. The effective sensitivity of fluorescence microscopy, especially with thick specimens, is generally limited by out-of-focus flare. This limitation is greatly reduced in a **confocal** microscope, through the use of a confocal pinhole to reject out-of-focus background fluorescence and produce thin (less than 1 micrometer), unblurred optical sections. Alternatively, **deconvolution microscopy** employs a conventional microscope to digitally reconstruct an image using the measured point spread function of the optical system.

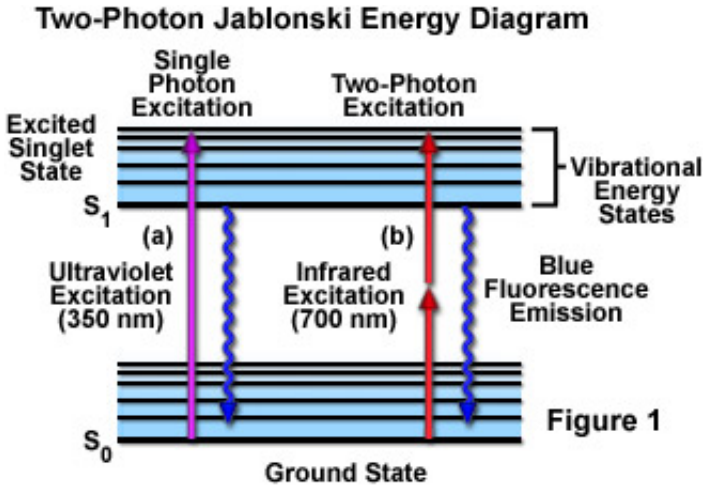


Figure 1

This article presents a description of the basic physical principles of multiphoton excitation and discusses the advantages and limitations of its use in laser-scanning microscopy. Practical considerations are highlighted in order to illustrate the utility of this technique and demonstrate some of its physical limitations. Finally, selected applications of two-photon excitation microscopy are discussed in order to illustrate how this technique has allowed experiments that could not have been performed otherwise.

Before performing any optical sectioning experiment, careful consideration should be given to choosing the technique that is best suited to provide answers to the problem being investigated. For fluorescence microscopy on relatively thick specimens, two-photon excitation often provides the most appropriate solution, although complementary three-dimensional fluorescence microscopy methods have particular benefits that give each of them advantages in certain experiments.

Confocal microscopy utilizes a pinhole to exclude out-of-focus background fluorescence from detection. Thus, this technique allows three-dimensional sectioning into thicker tissues. However, the excitation light generates fluorescence, and thus produces photobleaching and phototoxicity throughout the specimen, even though signal is only collected from within the plane of focus. This large excitation volume can cause significant photobleaching and phototoxicity problems, especially in live specimens. Furthermore, the penetration depth in confocal microscopy is limited by absorption of excitation energy throughout the beam path, and by specimen scattering of both the excitation and emission photons.

Deconvolution techniques often provide the best solution for specimens with relatively low out-of-focus background or for specimens with low overall signal levels. Because deconvolution methods employ conventional widefield microscopes for image acquisition, the excitation intensity is generally kept low. Consequently, deconvolution is usually effective for imaging monolayers of living cells. It is important to realize, however, that many so-called deconvolution methods are simply non-linear data filters that do not generate quantitative data. Only true **constrained iterative deconvolution** techniques produce quantitative data that can be used for further analysis. However, deconvolution performed on widefield fluorescence microscopes provides limited penetration into thick specimens, due to increased out-of-focus background and light scattering. Additionally, due to the heavy computational requirements, the deconvolved images cannot provide immediate feedback during an experiment.

Two-photon excitation, as discussed further in this article, provides three-dimensional optical sectioning without absorption (which would lead to photobleaching and phototoxicity) above and below the plane of focus. Consequently, the technique offers increased depth penetration as compared to confocal microscopy, and can be less phototoxic to live specimens. Thus, two-photon excitation microscopy is used, in preference to other techniques, for experiments that require deep penetration into living tissue or intact animal specimens. However, because the photophysics governing two-photon excitation is different from that of conventional fluorescence excitation, deleterious effects are occasionally observed with two-photon excitation of certain fluorophores, which in turn limits the applicability of this method for optical sectioning in thin specimens.

Principles of Two-Photon Excitation

Two-photon excitation is a relatively old theoretical concept in quantum optics. It was first proposed by Maria Göppert-Mayer in her doctoral dissertation and observed experimentally some thirty years later, shortly after the invention of the laser. Consequently, a substantial well-understood theoretical and experimental background exists. The phenomenon of two-photon excitation arises from the simultaneous absorption of two photons in a single quantitized event. Since the energy of a photon is inversely proportional to its wavelength, the two absorbed photons must have a wavelength about twice that required for one-photon excitation. For example, a fluorophore that normally absorbs ultraviolet light (approximately 350 nanometers wavelength) can also be excited by two photons of near-infrared light (approximately 700 nanometers wavelength) if both reach the fluorophore at the same time (see Figure 1). In this case, "the same time" means within an interval of about 10 × E(-18) seconds.

Because two-photon excitation depends on simultaneous absorption, the resulting fluorescence emission varies with the square of the excitation intensity. This quadratic relationship between excitation and emission gives rise to many of the significant advantages associated with two-photon excitation microscopy (discussed in more detail below). In order to produce a significant number of two-photon absorption events (in which both photons interact with the fluorophore at the same time), the photon density must be approximately one million times that required to generate the same number of one-photon absorptions. The consequence is that extremely high laser power is required to generate significant two-photon-excited fluorescence. This power level is easily achieved by focusing mode-locked (pulsed) lasers, in which the power during the peak of the pulse is high enough to generate significant two-photon excitation, while the average laser power remains fairly low. In this situation, the resulting two-photon-excited state from which emission occurs is the same singlet state that is populated when carrying out a conventional fluorescence experiment. Thus, fluorescent emission following two-photon excitation is exactly the same as emission generated in normal one-photon excitation. Figure 1 presents a Jablonski diagram illustrating absorption of a single (ultraviolet) photon (Figure 1(a)) and the simultaneous absorption of two near-infrared photons (Figure 1(b)), producing the identical excited state.

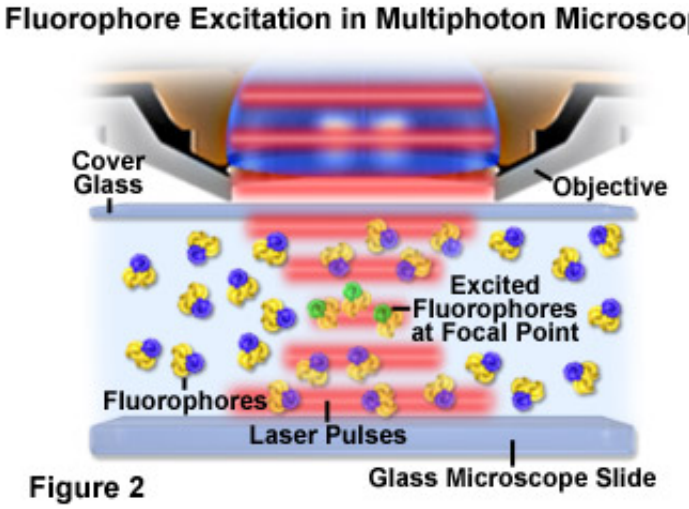


Figure 2

Another closely related nonlinear optical process, **three-photon excitation**, may also prove to be beneficial for biological experiments. Three-photon excitation occurs in much the same way as the two-photon process except that three photons must interact with the fluorophore simultaneously to produce emission. Due to the quantum-mechanical properties of fluorescence absorption, the photon density required for three-photon excitation is only about tenfold greater than the density needed for two-photon absorption (rather than another million-fold greater). Three-photon excitation can therefore be considered an attractive option for some experiments. As an example, an infrared laser (of about 1050-nanometer wavelength) can produce three-photon excitation of an ultraviolet-absorbing fluorophore (at 350 nanometers) and simultaneously cause two-photon excitation of a green-absorbing fluorophore (at 525 nanometers). Additionally, three-photon excitation can be employed to extend the region of useful imaging into the deep ultraviolet (for example, use of 720-nanometer light to excite a fluorophore that normally absorbs at 240 nanometers in the ultraviolet). This is a valuable enhancement to the capability of the conventional microscope since ultraviolet wavelengths below approximately 300 nanometers are very problematic for regular microscope optics. Higher-order non-linear effects, such as four-photon

absorption, have been experimentally demonstrated, although it is unlikely that these phenomena will find any immediate application in biological research.

Two-Photon Excitation in Laser-Scanning Microscopy

The considerable advantages of using two-photon excitation in laser-scanning microscopy arise from the basic physical principle that the absorption depends on the square of the excitation intensity. In practice, two-photon excitation is generated by focusing a single pulsed laser through the microscope optics. As the laser beam is focused, the photons become more crowded (their spatial density increases), and the probability of two of them interacting simultaneously with a single fluorophore increases. The laser focal point is the only location along the optical path where the photons are crowded enough to generate significant occurrence of two-photon excitation. Figure 2 illustrates diagrammatically the generation of two-photon excitation in a fluorophore-containing specimen at the microscope focal point. Above the focal point, the photon density is not sufficiently high for two photons to pass within the absorption cross section of a single fluorophore at the same instant. However, at the focal point, the photons are so closely spaced that it is possible to find two of them within the absorption cross-section of a single fluorophore simultaneously.

In practice, two-photon excitation microscopy is made possible not only by concentrating the photons spatially (by focusing of the microscope optics), but also by concentrating them in time (by utilizing the pulses from a mode-locked laser). The combined effect allows generating the necessary photon intensities for two-photon excitation, but the pulse duty cycle (the duration of the pulse divided by the time between pulses) of 10 × E(-5) limits the average input power to less than 10 milliwatts, which is just slightly greater than that used in confocal microscopy. Although the laser pulse durations are considered ultra short, typically ranging between approximately 100 femtoseconds and 1 picosecond (10 × E(-13) to 10 × E(-12) seconds), in comparison to the fluorophore absorption event of about 10 × E(-18) second, they are relatively long in duration.

The narrow localization of two-photon excitation to the illumination focal point is the basis for the technique's most significant advantages over confocal microscopy. In a confocal microscope, although fluorescence is excited throughout the specimen illuminated volume, only signal originating in the focal plane passes through the confocal pinhole, allowing background-free data to be collected. By contrast, two-photon excitation only generates fluorescence at the focal plane, and since no background fluorescence is produced, a pinhole is not required. This dramatic difference between the excitation regions of confocal and two-photon excitation microscopy can be demonstrated by imaging the photobleaching patterns of each method. Figure 3 illustrates the photobleaching pattern that arises in the x-z direction from repeated scanning of a single x-y plane (the image plane) in a fluorescein-stained formvar film. The laser of the confocal system excited fluorophores above and below the focal plane (shown by the white box in Figure 3(a)), contributing to the bleaching observed in these extensive areas. In contrast, two-photon excitation occurs only at the focal plane, and bleaching is therefore confined to this area (Figure 3(b)).

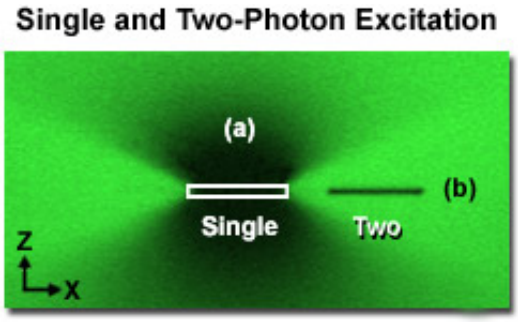


Figure 3

Many advantageous effects result from the localization of excitation in the two-photon microscopy technique. Perhaps the most important is that the three-dimensional resolution of a two-photon excitation microscope is identical to that achieved in an ideal confocal microscope. Additionally, because there is no absorption in out-of-focus specimen areas, more of the excitation light penetrates through the specimen to the plane of focus. The result is greatly increased specimen penetration, which is generally two or three times greater than is possible with confocal microscopy. Another benefit of utilization of two-photon excitation (illustrated in Figure 3) is the minimization of photobleaching and photodamage - two of the most severe limitations in fluorescence microscopy of living cells and tissues. Although cell damage caused by interactions with light is poorly understood, it is clear that decreasing photodamage will lead to extended viability of the biological specimens being investigated. The evidence from practical experience is that the red excitation light alone does not affect cell viability, and it is probable that most of the observed photodamage is associated with two-photon absorption, and therefore is confined to the focal plane.

Two-photon excitation microscopy does not require a pinhole to obtain three-dimensional resolution, allowing flexibility in detection geometry. The geometries for both descanned and non-descanned detection of two-photon excitation are presented in Figure 4. In the descanned geometry, the emitted light (illustrated in blue) returns along the same path as the excitation

light, striking the scanning mirrors before passing through the confocal pinhole to the detector (Figure 4(a)). In confocal microscopy this geometry must be utilized to eliminate the detection of out-of-focus emission. Non-descanned beam paths provide more configuration alternatives: a **conjugate plane detector** arrangement locates a dichroic mirror immediately after the objective lens (Figure 4(b), and reflects the emitted light through a transfer lens to a detector placed in a plane conjugate to the objective rear aperture; the emitted light may be collected by an **external detector** directly from the specimen, without passing through the objective lens (Figure 4(c); or the emitted light is diverted by a dichroic reflector to a charged coupled device (**CCD**) camera at the intermediate image plane, in order to acquire the widefield image (Figure 4(d)). This latter geometry configuration is suited to fast data acquisition systems employing two-photon excitation.

Although it is possible to use descanned detection for two-photon excitation, in order to take full advantage of the depth penetration of this technique, use of a non-descanned alternative (external detector) is recommended. The non-descanned path enables the collection of more scattered photons, requires fewer optical elements, such as mirrors and lenses, and reduces the path length, along which dust particles in the air interfere with the fluorescence signal. Consequently, using the non-descanned detection approach with two-photon excitation dramatically increases collection efficiency, and is essential for maximal depth penetration into living tissue.

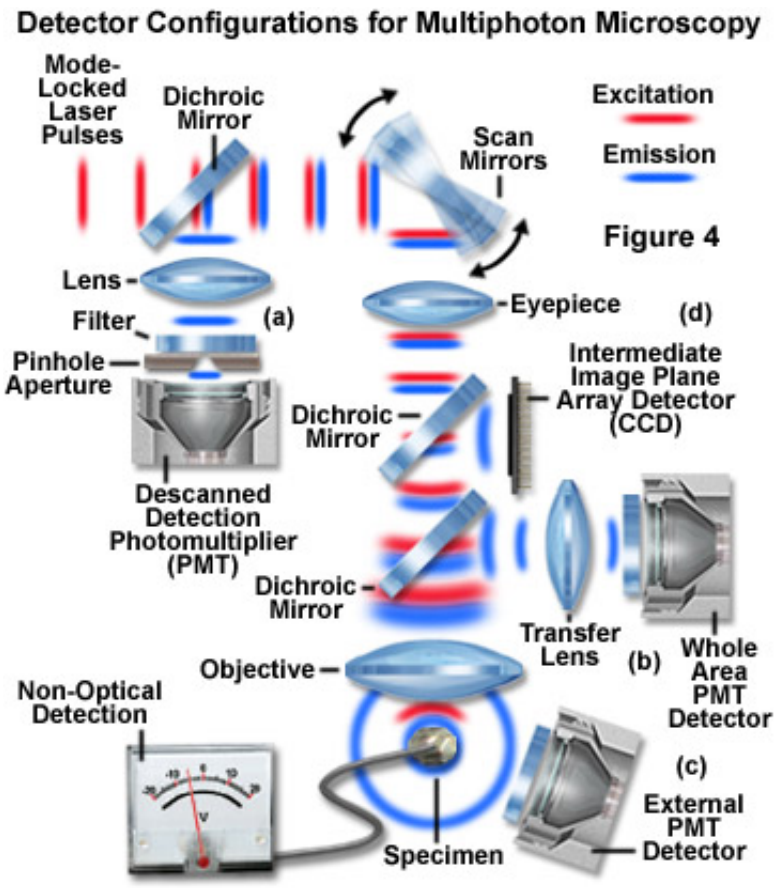
Mechanism of Deep Sectioning

As discussed above, the most powerful advantage of two-photon excitation microscopy is its ability to provide superior optical sectioning at greater depths in thick specimens than is possible by other methods. It is, therefore, important to understand the means by which this increased depth of penetration is achieved. Three physical mechanisms exist that function in combination to allow the increased effectiveness in thick specimens:

- Absence of out-of-focus absorption allows more of the excitation light photons to reach the desired specimen level.
- The red and infrared light employed in two-photon excitation undergoes less scattering than light that is bluer in color (shorter wavelengths).
- The effects of light scattering are less detrimental to two-photon microscopy than to confocal microscopy.

Although functioning in combination, it is useful to consider the three mechanisms separately. The two-photon microscope allows a greater proportion of the excitation illumination to reach the focal plane because the conditions for absorption are not met outside of the area of light concentration, and out-of-focus absorption is eliminated. In a confocal microscope, excitation photons are absorbed by any fluorophores that are encountered along the excitation light path. The consequence is that fewer photons reach the focal plane, thus decreasing the signal generated. This effect becomes more pronounced if the specimen contains fluorophores throughout, as illustrated in the images presented in Figure 5. The rhodamine-stained polymer film specimen presented in this figure is itself non-scattering, but contains a uniform distribution of a high fluorophore concentration.

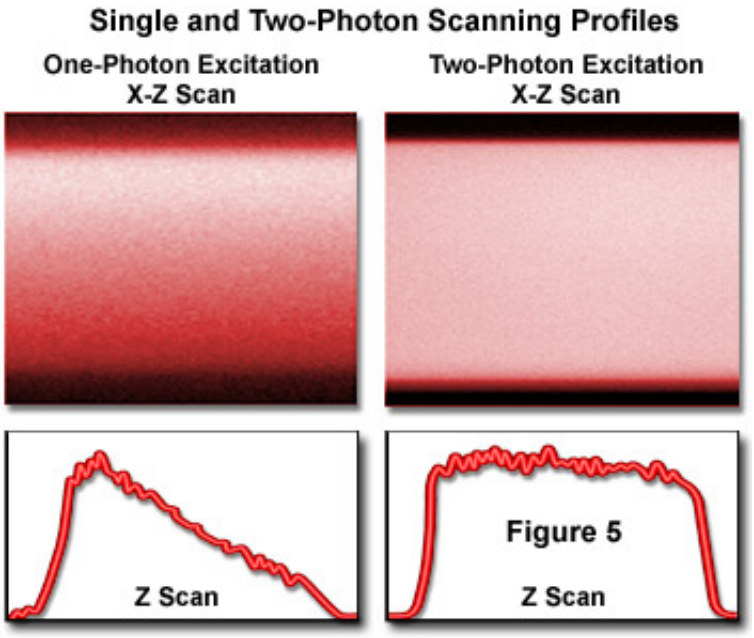
In Figure 5, the top of the x-z scan is nearest the objective lens, and the fluorescence intensity is plotted as a function of depth into the specimen (z distance) for each x-z scan. For the one-photon excitation mechanism (confocal microscopy, Figure 5(a)), the intensity exhibits a steady decrease with penetration depth as the excitation light is increasingly absorbed as it reaches the deeper focal planes. In distinct contrast, two-photon absorption occurs only in the focal plane, with no absorption of excitation light by fluorophores in the optical path between the objective lens and the focal plane. Thus, all of the excitation energy reaches the focal plane, which keeps the fluorescence signal constant throughout the depth of the specimen (the polymer in this example). Figure 5(b) illustrates the dramatic difference between confocal and two-photon excitation, in which the intensity is relatively constant with penetration depth.



The second mechanism contributing to the performance of two-photon excitation in thicker specimens is that the "redder" excitation light employed in two-photon excitation microscopy suffers less scattering by the specimen than does the "bluer" excitation light used in conventional excitation. Biological tissue can be considered as a non-uniform medium having a variable index of refraction. Light propagating through such a medium is multiply scattered in various directions. In fluorescence microscopy, excitation light incident on the specimen can be scattered to various degrees before it reaches the focal plane, and the resulting fluorescence can also undergo scattering as it returns through the specimen toward the detector. Both of these scattering effects combine to reduce the collected fluorescence signal.

Because of the irregular distribution of material having variable properties within biological specimens, it is not possible to calculate, or model precisely, the scattering behavior. However, the simplest approximation of Rayleigh scattering provides a minimal estimate of the fraction of light scattered in such a system. In this instance, the amount of scattered light is inversely proportional to the forth power of the light's wavelength. Applying this relationship in the estimation, 488-nanometer (one-photon) light would be expected to undergo approximately seven-fold more scattering than 800-nanometer (two-photon) light. Thus, this difference in scattering contributes additionally to the amount of two-photon excitation laser light that can reach the focal plane, and further increases the depth of penetration into the specimen. In practice, the observed scattering from interaction with tissue structures is always greater than is predicted by the Rayleigh approximation, but longer (redder) wavelengths are invariably scattered less than shorter (bluer) wavelengths. In the detection phase of fluorescence microscopy techniques, the emitted fluorescence is identical regardless of whether it was generated utilizing one- or two-photon excitation, and therefore, scattering of fluorescence emission affects both methods equally.

A third factor, listed above, is that any scattering of either the excitation or fluorescence light does not affect signal collection in two-photon techniques as significantly as it does in confocal microscopy. This difference can be explained by consideration of the physics of image formation in two-photon excitation microscopy. While the lack of out-of-focus absorption and the differences in scattering both contribute to increased excitation light reaching the focal plane deep within intact tissue, this third factor actually produces increased image contrast with two-photon excitation. The physical aspects contributing to this are illustrated in Figure 6.



In a confocal microscope (refer to Figure 6), the excitation light (blue) is focused into the specimen (a), and the fluorescence (green) from that focal spot is captured by the objective lens, passes cleanly through the pinhole, and reaches the detector (b). This fluorescence light is the desired signal, but some of it can be scattered as it passes back through the specimen (c). This scattered fluorescence does not pass through the pinhole, and is therefore lost and not detected. These losses greatly reduce the detected fluorescence signal. As the excitation light passes through the specimen, it may be absorbed (d) or scattered before it reaches the focus (e). If it is absorbed, it can generate fluorescence. Since this fluorescence does not arise from the focal spot, it does not pass through the pinhole, so it is not efficiently detected. However, a small portion of out-of-focus fluorescence can be scattered into the pinhole, and then be detected. This fluorescence will create a background fog that will be roughly constant across the image, as shown in the examples presented in Figures 7 and 8. This fog reduces the dynamic range of the image, thus reducing the image contrast. Likewise, the scattered excitation can generate fluorescence (e), and this fluorescence can also contribute to the background fog (f).

In the two-photon excitation method (also referring to Figure 6), the excitation photons (red) can be scattered (g) as in the confocal system. However, the probability of two photons being scattered simultaneously to the same specimen location is essentially zero, and consequently, the background fog that plagues confocal imaging in thick specimens is not generated in two-photon excitation. In addition, a greater proportion of the excitation light reaches the focal plane (h and i) due to the first two factors discussed above: the reduced out-of-focus absorption and the decreased scattering of the longer-wavelength two-photon excitation light. Importantly, the generated fluorescence (green), even if scattered, has an increased likelihood of being detected by the photomultiplier tube (j) because no pinhole is present to block it (k). This insensitivity to scattering effects and absence of out-of-focus absorption allow for the preservation of the full image contrast from considerable depth within specimens.

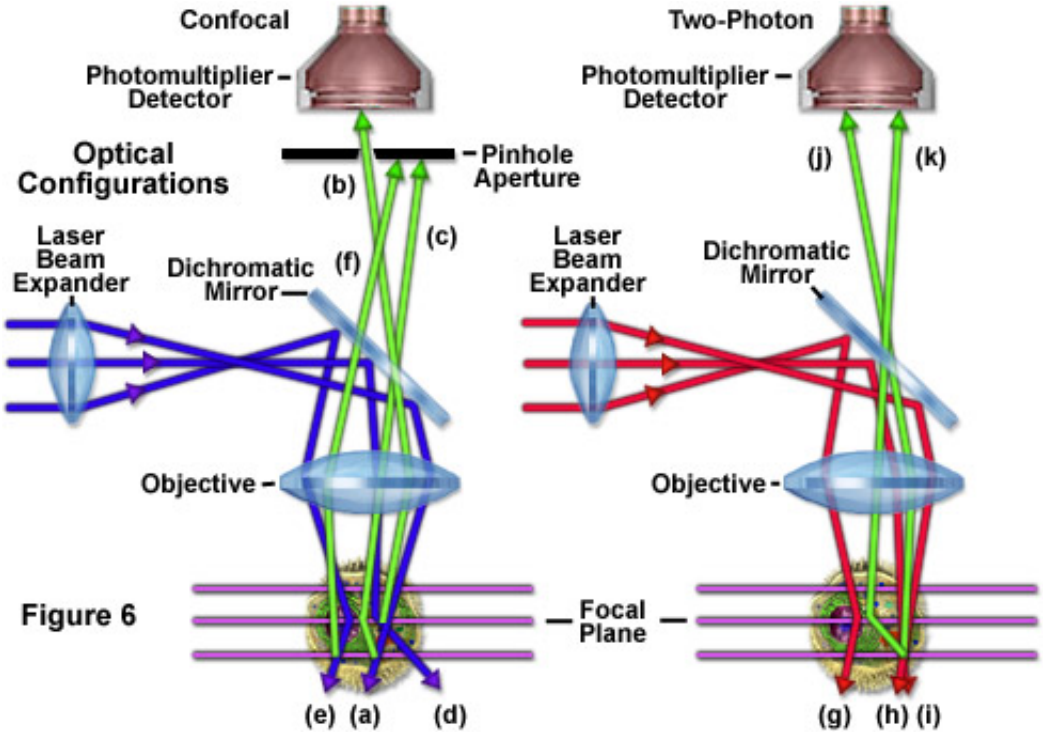


Figure 6

The images presented in Figure 7 (a shark choroid plexus stained with fluorescein) provide a comparison of confocal and two-photon microscopy imaging quality. These images were collected at 80-micrometers below the specimen surface, which is the maximal depth allowing sufficient image contrast from this specimen utilizing confocal microscopy. Although the signal level of the brightest features can easily be matched between the two methods, the overall image contrast in the confocal image (Figure 7(a)) is greatly reduced by the presence of background fog. By comparison, the two-photon excitation image (Figure 7(b)) exhibits excellent intensity contrast. Because scattering of the fluorescence is significant in thick biological specimens, the use of descanned detection, even with an "open" pinhole, is not sufficient to gain the advantages of two-photon excitation.

In order to achieve the full potential, a non-descanned detection scheme (see Figure 4), in which the fluorescence does not return through the scanning system as it must in a confocal microscope, is required to increase the fluorescence collection efficiency. Using the same shark specimen illustrated in Figure 7, a comparison of images employing descanned (pinhole "open") and non-descanned detection methods is presented in Figure 8. With each detection geometry, the same imaging optical components were used, including the dichromatic mirror, barrier filter, and photomultiplier detector. Initially the specimen was imaged using descanned detection at a depth (140 micrometers) near the maximum that provided some intensity contrast (Figure 8(a)). Keeping all settings fixed, and switching to non-descanned detection, the image presented in Figure 8(b) was collected. It is clear that this non-descanned image is fully saturated (maximum displayed brightness) in many areas, demonstrating the improved signal collection with this detection geometry. Because the excitation conditions were identical in both cases, this eight-fold increase in signal intensity can be attributed solely to the collection of scattered fluorescence photons, enabled by the non-descanned detection geometry. To obtain a full range non-saturated image (illustrated in Figure 8(c)), the photomultiplier tube voltage was reduced from 1000 to 750 volts, implying that this specimen can be scanned even deeper utilizing the non-descanned detection arrangement. In fact, the depth of penetration for acceptable imaging in this specimen was not limited by the tissue but by the working distance of the objective.

Image Resolution

The image resolution obtained with two-photon excitation is not better than that achieved in a well-aligned confocal microscope. The utilization of longer excitation wavelengths (such as red or infrared, instead of ultraviolet or blue), although an advantageous aspect of two-photon excitation, actually results in a larger resolution spot. If a biological structure cannot be resolved in the confocal microscope, it will similarly not be resolved in a two-photon excitation laser-scanning microscope. While this point is well understood by microscopists experienced in these techniques, prospective users in the biomedical research community often assume that the advantages of two-photon excitation include increased resolution.

Imaging Thick Specimens

As discussed previously, two-photon excitation is more effective for thick specimen imaging due to the combined effect of three factors: lack of out-of-focus absorption allows more of the excitation light to reach the intended specimen region; the red exciting light is scattered less; and the effects of fluorescence scattering are less detrimental to two-photon microscopy than to confocal microscopy. When long-working-distance optics and a non-descanned detection configuration are utilized, the

depth of penetration and image quality are often limited by the ability to effectively label the tissue. Introducing fluorescence labels into tissues becomes increasingly difficult at greater depths. Experiments taking advantage of the expression of green fluorescent protein (**GFP**) in transgenic animals is likely to enhance two-photon excitation imaging in vivo. Transgenic animals offer enormous promise in development of improved techniques to fluorescently label specific organs and proteins of living animals for detection using two-photon excitation. Certain tissue characteristics may impose an additional limitation on penetration depth in imaging thick specimens, and is especially a concern in either heavily pigmented tissue, such as liver, or highly scattering tissue, such as skin.

Imaging Thin Specimens

In general, the imaging of thin specimens does not necessarily benefit significantly from two-photon excitation techniques over conventional confocal microscopy. The reason for this is the slightly increased photobleaching that may occur in the focal plane (the total photobleaching in a thick specimen is greatly reduced compared to conventional techniques, as illustrated in Figure 3). There are applications, however, for which two-photon excitation is beneficial, even for thin preparations; one example is the imaging of ultraviolet-excited fluorophores such as NADH (further discussion follows). In such experiments, the damage caused by ultraviolet light is more significant than the two-photon induced photobleaching. In evaluating the potential benefits of two-photon excitation, it is always worthwhile to first carry out the experiments on a confocal microscope. Once it is known what limitations are imposed by the confocal for the desired imaging, it is then easily determined whether the use of two-photon excitation would be advantageous for completing the experiments.

Absorption Spectrum

It is common for two-photon absorption spectra to bear little resemblance to the corresponding single-photon spectra. The experience obtained to date indicates that most fluorophores function fairly well when the two-photon excitation illumination has twice the wavelength of the fluorophore's one-photon absorption peak. For physico-chemical reasons outside the scope of this discussion, fluorophores with a non-symmetric chemical structure tend to adhere to this relationship more closely than symmetric ones. For example, fluorescent proteins (**CFP**, **GFP**, **YFP**, and others) are characterized by a non-symmetric fluorophore and absorb strongly at twice their one-photon excitation wavelength. For full advantage to be taken of the capabilities of two-photon excitation microscopy, however, the absorption spectra of the fluorophores must be measured. This is considerably more challenging technically than measuring conventional one-photon absorption spectra, and only a few sources for this information exist. As utilization of this microscopy technique increases, it is likely that two-photon absorption spectra will become more generally available.

Localized Photochemistry

An additional capability of two-photon excitation is the initiation of photochemical reactions in the focal region of the specimen. A variety of experimentally useful chemical processes involve ultraviolet light-induced reactions, for which two-photon excitation can be substituted. One category of reaction, **dye uncaging**, which is photochemically inducing a non-fluorescent molecule to become fluorescent, can be initiated in individual cells of a tissue using two-photon excitation. Similarly, biological stimulators or suppressants can be uncaged by the same excitation process. The variety of potential methods for two-photon-excited uncaging have yet to be fully developed, largely due to limitations resulting from the kinetics of the photoreactions, which can range from milliseconds to seconds. Thus, the target compound might diffuse over several micrometers within the specimen between its time of excitation and the time it becomes active. In spite of this difficulty, the technique is likely to be valuable in a number of interesting biological applications, some of which are discussed in the following sections.

Laser Sources

The instrumentation requirements for two-photon excitation microscopy are nearly identical to those for confocal microscopy, with the exception of the laser excitation source, which is considerably different. Two types of ultrafast mode-locked laser systems are in general use with current two-photon excitation microscopes: the **Ti:sapphire** laser and the **Nd:YLF** laser. Although these systems can be powered from regular electrical outlets and do not require water cooling, they are considerably more expensive than the small air-cooled lasers employed in confocal microscopy. The wavelength tunability of the Ti:sapphire laser (700 to 1100 nanometers) gives it greater versatility than the single-wavelength Nd:YLF laser (1047 nanometers), and current commercial models of Ti-sapphire lasers cover the wavelength range of 720 to 900 nanometers through easily implemented computer control. Further improvement in the ease of use and versatility of laser illumination systems is likely to continue in the foreseeable future.

Laser Power

The laser power required to excite a fluorophore-containing specimen has an optimal limiting value. With increasing laser power, the fluorescence intensity increases, up to the point of fluorophore **saturation**. The condition of saturation occurs at a laser power that is sufficient to cause a significant proportion of the fluorescent molecules to exist in their excited rather than ground state (this power level is about 1 milliwatt at the specimen for one-photon excitation, and 50 milliwatts at the specimen for two-photon excitation). At higher power levels, the additional photons are simply unable to excite more fluorescent

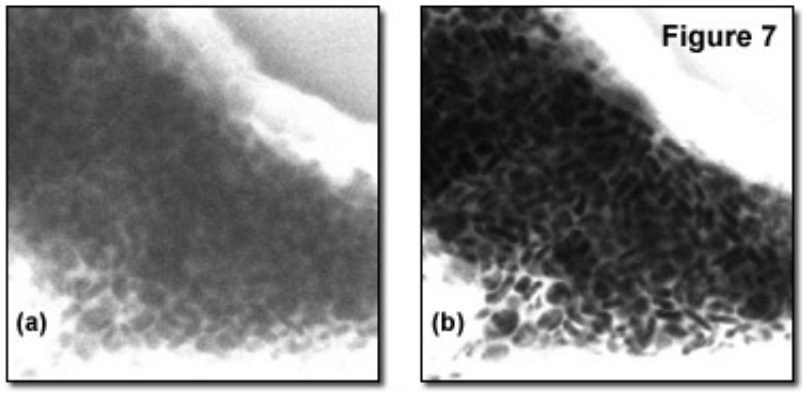
molecules. Any excitation energy increase beyond the saturation point contributes to increased photodamage and photobleaching. Each experimental setup must be assessed for damage imposed during beam scanning, and it is important to recognize that trivial cell viability tests (such as esterase activity or dye exclusion) do not always accurately reflect cellular photodamage. For many experiments, more rigorous functional tests may be more informative. As examples, in one recent published investigation, the viability of hamster embryos was confirmed by their continued development, and in another the viability of pancreatic islets was confirmed by their maintenance of normal glucose-stimulated NAD(P)H response.

Examples of Two-Photon Excitation Microscopy

A number of examples of recently published experimental results illustrate common situations in which two-photon excitation provides advantages over confocal imaging. Although the details of the experiments are not provided in this discussion, the highlights focus on the benefits of two-photon excitation resulting from its reduced phototoxicity, increased tissue imaging depth, and ability to initiate localized photochemistry.

Two-photon excitation microscopy is generally less phototoxic than confocal microscopy, as demonstrated by a recent study utilizing time-lapse imaging of hamster embryo development. In this example, the embryos' development was able to be monitored continuously for more than 10 hours using two-photon excitation of a vital mitochondrial dye. In comparison, when confocal methods were employed, normal embryo development ceased after only a few minutes of confocal laser exposure. The researchers concluded that the two-photon excitation laser's longer wavelength (1047 nanometers) permitted the greatly increased time of embryo viability. The same investigators also utilized two-photon excitation microscopy to assess the effects of inorganic phosphate on hamster embryo development. In this experiment, living hamster embryos were cultured in various amounts of inorganic phosphate, and their mitochondrial distribution imaged at 6 hours of culture using two-photon excitation microscopy. Following further embryo development, morphological assessment was performed after 27 and 51 hours of culture. Clear evidence was provided from these studies that the two-photon illumination was non-perturbing to the development of the hamster embryos, while they were damaged by parallel confocal imaging.

Imaging Quality in Confocal and Multiphoton Microscopy



Two published studies have exploited the non-toxic nature of two-photon excitation to perform in vivo imaging of human skin. One investigation involved detailed spectroscopy of the autofluorescence signals collected from skin at various depths (0 to 50 and 100 to 150 micrometers) utilizing excitation wavelengths ranging from 730 to 960 nanometers. When used in conjunction with reflected light confocal microscopy, the combination of techniques nondestructively provides detailed reflected light and autofluorescence images of skin layers from the same region of skin.

Two-photon excitation provides the capability of avoiding the phototoxic effects of ultraviolet irradiation for fluorescent species that are activated in that spectral range. This feature is especially beneficial in imaging the naturally occurring reduced pyridine nucleotides [**NAD(P)H**] as an indicator of cellular respiration. Because NAD(P)H has a small absorption cross-section, a low quantum yield, and absorbs in the ultraviolet, it is difficult to excite and to measure and has the potential to cause considerable photodamage. NAD(P)H imaging has been utilized in studying the pathophysiology of cultured partially differentiated L6 myotube cells. The autofluorescence pattern manifested in cellular NAD(P)H images primarily reflects NADH in mitochondria as punctuate regions over a diffuse cytoplasmic signal. In differentiated cells, the fluorescence is evident as columns of mitochondria localized between muscle fiber striations, and an increase in fluorescence accompanied increased glucose concentration. This study demonstrated homogeneity in glucose metabolism, and that the kinetics of glucose utilization can be defined in real time for a single cell or can be averaged over several cells.

An additional area of investigation that has been reported in the literature is the quantitative two-photon imaging of NAD(P)H, centered on individual beta cells within the pancreatic islet, which is a quasi-spherical micro-organ consisting of about 1000 cells. Expanding on the overall nucleotide imaging, the spatial resolution of the two-photon technique allows the separation of the cytoplasmic and mitochondrial NAD(P)H signals. Figure 9 presents a typical image of beta cell NAD(P)H autofluorescence within an intact islet, displaying signals from both the cytoplasm and mitochondria, the latter being brighter and somewhat punctate. Single cell outlines are visible, as are the nuclei, both of which appear dark. By the separation of cytoplasmic and

mitochondrial signals in these regions of pancreatic islet beta cells, the detailed examination of the metabolism of glucose and pyruvate is made possible.

Current models for glucose-stimulated insulin secretion (**GSIS**) suggest that metabolites further along the signal transduction pathway should stimulate a similar cascade of signaling events and result in insulin secretion, although pyruvate potentiates GSIS but does not induce insulin secretion on its own. The study referred to utilized two-photon excitation imaging of NAD(P)H, and separation of cytoplasmic and mitochondrial signals, to demonstrate that beta-cells metabolize pyruvate, although transiently. Such a transient mitochondrial response suggests two separate models, which are currently being studied: either mitochondrial-pyruvate transport or the tricarboxylic cycle is inhibited during late pyruvate metabolism. Utilizing two-photon excitation techniques, living pancreatic islets were repetitively scanned to produce data at sampling intervals that are unobtainable by biochemical methods. Repetitive imaging of this type simply cannot be performed using confocal microscopy, because of the limitations imposed by photobleaching and ultraviolet light-induced photodamage.

Because two-photon excitation microscopy utilizes mode-locked (pulsed) lasers, it can be readily extended into combination with fluorescence lifetime imaging. Images based on nanosecond fluorescence decay times provide information that is independent of fluorophore concentration. One potential application is to utilize fluorescence lifetime imaging to obtain an unambiguous value of fluorescence resonance energy transfer (**FRET**) efficiency between two probes. In a recent investigation, two-photon-excited lifetime imaging microscopy of NAD(P)H was employed to quantitatively determine the NAD(P)H concentration in different subcellular compartments. Free NADH levels in the nucleus regulate the corepressor CtBP, which is a participant in cell cycle regulation and transformation transcriptional pathways. Through the combined use of two-photon excitation microscopy of NAD(P)H and lifetime imaging, it was demonstrated that the free NADH levels in the nucleus closely correspond to the half-maximal concentration for CtBP binding.

Descanned and Non-Descanned Detection Techniques

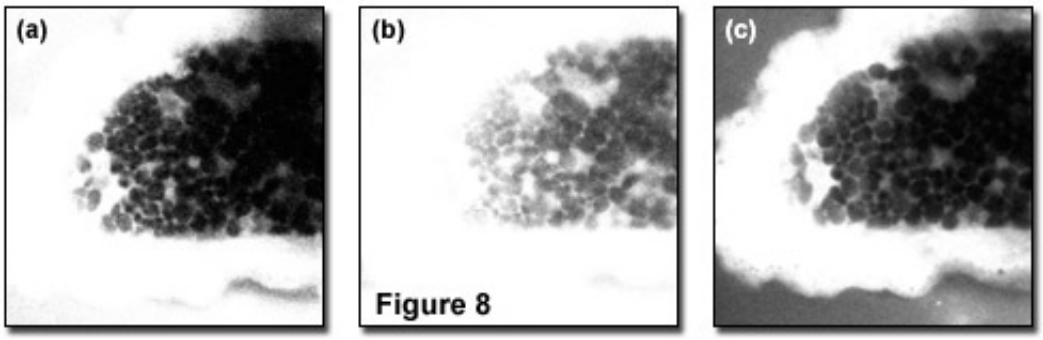


Figure 8

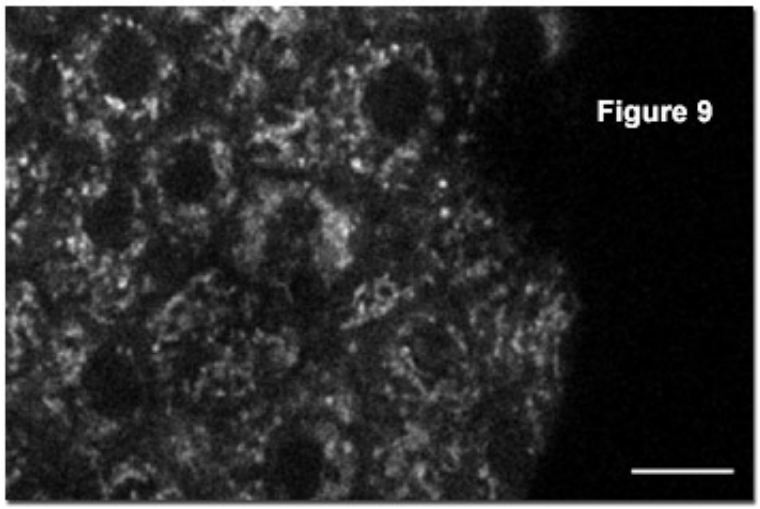
The technique of two-photon excitation can be combined with a wide range of other established biophysical techniques, including fluorescence correlation spectroscopy (**FCS**) and fluorescence recovery after photobleaching (**FRAP**). Each of these techniques generally utilizes stationary one-photon (continuous-wave) lasers. The FCS technique determines the occupation number and diffusion characteristics of fluorescent probes within the focal volume of the stationary illuminating beam, and has been successfully applied in molecular interaction and diffusion studies. By controlled photobleaching of fluorescence in the laser focal region, followed by observation of fluorescence recovery, FRAP has been employed to study macroscopic diffusion of fluorescent molecules. Both FCS and FRAP techniques have been used extensively to investigate the diffusion characteristics of fluorescent probes on cultured cell membranes. To date, the complexity of these techniques has limited their application to **in vitro** systems and to cell culture models. When quantitative data is required from either method, the well-defined excitation volume in two-photon excitation microscopy is an advantage. Additionally, FCS and FRAP can be expected to be highly valuable in studying bimolecular dynamics in thick living tissues, employing two-photon rather than one-photon excitation.

The deep specimen penetration obtainable with two-photon excitation allows **in vivo** imaging, although a number of challenges must be overcome when imaging living animals. In vivo fluorescence imaging may be accomplished by two-photon excitation through surgical openings in the skin of a living animal, or through coverslip "windows" placed onto the animal. An additional complication in working with live animals is the considerable difficulty in fluorescently labeling the specimen. One published study reports using a Ca2+ indicator for the specific labeling of neurons in living mice, allowing the monitoring of neural function employing two-photon excitation techniques. The expression of green fluorescent protein (GFP) in transgenic animals to fluorescently label specific organs and proteins is certain to lead to additional applications of two-photon excitation for in vivo imaging. Because of the likelihood that living specimens will move during the imaging process, the majority of in vivo studies are currently done with anesthetized animals, and imaging rates are increased in order to limit the effect of this movement. It is likely that future technological advances, such as the miniaturization of two-photon microscopes for attachment directly to a living specimen, will allow in vivo imaging of freely moving animals.

Several researchers have employed bulk loading of calcium indicator combined with two-photon excitation microscopy to map the microcircuitry of neurons in mouse brain slices. Their methodology was to trigger signal neurons, then map the calcium signal initiated in connecting so-called "follower" neurons. It was determined that the neocortex is composed of numerous

precisely organized microcircuits. The followers belonged to a few distinguishable anatomical classes and their positions were determined and could be predicted in different animals.

Pancreatic Islet Beta Cell NAD(P)H Autofluorescence



A potentially very powerful application of two-photon excitation microscopy is three-dimensionally resolved photorelease of caged compounds, referred to as **uncaging**. The quantitative two-photon excited uncaging of calcium, for example, is the focus of a number of techniques. As discussed previously, photochemical uncaging reactions are generally quite slow (ranging from milliseconds to seconds), which allows the target compound to diffuse over several micrometers within the specimen between the time it is excited and the time it becomes active. The diffusion does not present a problem in certain applications, such as "marking" cells by uncaging membrane impermanent fluorescent molecules. One group of investigators has successfully used two-photon excited photorelease, in combination with confocal microscopy, to track the development of sea urchin embryo cell lineages.

Faster-reacting uncaging molecules have been successfully utilized by other researchers in investigations of uncaging stimulants, in which they employed two-photon excitation to map neuron receptors. These studies expanded on the process of photochemical uncaging by taking advantage of the three-dimensional nature of two-photon excitation to control the location of uncaged stimulants in the imaging medium. When the stimulant was uncaged near the cell membrane, it stimulated receptors in close proximity, and the process was detected through a patch-clamp of the cell. In this type of imaging, measured excitatory response rather than emitted photons map the image. As a specific example, the uncaging of MNI-glutamate in conjunction with a whole-cell clamp for signal detection was successfully employed to map glutamate receptors. The specimen was acute slice preparations of cultured hippocampal neurons and hippocampal CA1 pyramidal neurons. The researchers conducting this study were able to obtain excellent lateral and axial full width at half maximum (**FWHM**) diameters of 0.6 and 1.4 millimeters, respectively, in the slice preparations, indicating rapid uncaging reaction times. They further determined that a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (**AMPA**)-type glutamate receptors are abundant in mushroom spines and that the distribution of these receptors is highly correlated with spine geometry.

Conclusions

Two-photon excitation microscopy offers great utility for dynamic imaging of living cells in thick specimens, such as intact tissue. The technique makes possible many experiments in which conventional imaging cannot be performed, or would not provide the information desired. Relying on mode-locked (pulsed) laser illumination to produce sufficient photon density at the focal point, two-photon excitation occurs only in the focal plane. The benefit of localized excitation is that emission is restricted to the narrow focal region, providing sectioning ability without the use of a pinhole. Furthermore, the limited excitation region reduces phototoxicity because photodamage is largely confined to the focal volume.

Although two-photon excitation microscopy does not produce images with higher resolution than confocal microscopy, it does allow for increased depth of penetration into thick specimens. The greater penetration depth is possible in part because of the open pinhole geometry of the two-photon microscope, the absence of out-of-focus absorption of the excitation light, and decreased scattering of the excitation light (because of its wavelength). In order to take full advantage of the depth of penetration, non-descanned detection geometries must be utilized, which effects a dramatic increase in collection efficiency of scattered fluorescence photons. The advantages of two-photon excitation are clearly established, and have allowed experiments to be performed that would not be possible using confocal microscopy. As this technique benefits from the predictable technological improvements and cost reductions, and becomes more popular, it is expected that an increasing number of exciting experimental results will be achieved.

Contributing Authors

David W. Piston - Department of Molecular Physiology and Biophysics, Vanderbilt University, 702 Light Hall, Nashville, Tennessee, 37232.

Thomas J. Fellers and **Michael W. Davidson** - National High Magnetic Field Laboratory, 1800 East Paul Dirac Dr., The Florida State University, Tallahassee, Florida, 32310.