6

Two-Photon Excitation Microscopy for Three-Dimensional Imaging of Living Intact Tissues

David W. Piston

Washington University in St. Louis, Department of Cell Biology and Physiology, School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110-1093, USA

6.1 Introduction

Two-photon excitation microscopy is an alternative to confocal microscopy that provides attractive features especially for optical sectioning deep into intact tissues or whole animals. Two-photon excitation microscopy is the most commonly used type of a wider class of imaging approaches called *multiphoton* or *nonlinear* microscopies. These nonlinear microscopies include two-photon excitation (2PM) and three-photon excitation (3PM) microscopies, second-harmonic generation (SHG) and third-harmonic generation (THG) microscopy, as well as coherent anti-Stokes Raman spectroscopy (CARS) and stimulated Raman spectroscopy (SRS) imaging. All these approaches offer similar advantages in terms of inherent optical sectioning, but two-photon excitation yields the most efficient signal generation and is therefore the most common. So this chapter will focus on the 2PM technique. As detailed throughout this chapter, the advantages of 2PM over linear optical sectioning techniques are greater imaging depths and reduced overall phototoxicity. As is true for all fluorescence microscopy approaches, 2PM enables dynamic cellular measurements, so the extra depth penetration opens up a broad range of experiments that rely on noninvasive intravital imaging of cellular and subcellular processes. While 2PM provides the capability to perform quantitative imaging in tissues at depths well beyond those that can be reached with confocal microscopy, inherent difficulties with the nonlinear optics underlying 2PM limit its main usefulness to deep-tissue imaging and a few other specialized applications.

For investigations of samples thicker than a couple of micrometers – that is, anything thicker than a monolayer cell culture – out-of-focus background limits the usefulness of fluorescence microscopy, especially for quantitative measurements. For many years, researchers overcame this limitation by embedding and sectioning cells, typically in conjunction with immunofluorescent labeling. The advent of commercially available optical sectioning microscopes, including

Fluorescence Microscopy: From Principles to Biological Applications, Second Edition.
Edited by Ulrich Kubitscheck.

confocal, deconvolution, and more recently light sheet (or selective plane illumination) microscopy, allowed diffraction-limited imaging studies to move from single cells to the tissue level, and even to whole animals. These optical sectioning methods allow investigators to acquire three-dimensional (3D) image data from intact, live samples without the need for fixation and mechanical slicing.

The most widespread optical sectioning microscopy used is confocal microscopy. As detailed in Chapter 5, the confocal microscopy concept relies on the fluorescence signal generated everywhere throughout the sample (whether the fluorescence arises from above, below, or within the plane of focus), and then uses a conjugate focus (i.e., confocal) pinhole to act as a spatial filter and reject the out-of-focus background fluorescence. As such, clean optical sections of the region near the focus are produced with minimal background contamination. However, imaging live samples with confocal microscopy can create deleterious effects in the sample, in particular due to photobleaching of the probes and the phototoxicity of the excitation light. The fluorescence signal is detected from only a single optical plane, but background fluorescence is excited throughout the sample. Most of the background fluorescence is rejected by the pinhole. This out-of-focus excitation can be particularly damaging in causing rapid photodestruction of the fluorophore in question as well as exciting autofluorescent compounds that can contribute to the killing of the live sample. As described below, the physics underlying 2PM allows much deeper imaging with 2PM since it is much less susceptible to degradation by out-of-focus absorption and scattering. A good rule of thumb is that 2PM can provide useful image data about 6 times deeper into tissue than can confocal microscopy.

While 2PM was initially demonstrated over 25 years ago [1], for the first ~15 years, its use was limited to specialist laboratories due to the expertise needed to control the sophisticated ultrafast laser technology required for 2PM. Nowadays, reliable "turn-key" laser systems have been developed, allowing a hands-off approach to 2PM and rendering it as easy to use as commercial confocal microscopes. This has allowed 2PM to transition from a novelty of the laser laboratory into a useful tool in a true biological research setting, where investigators can concentrate on studying the biological question at hand without worrying about aligning, operating, and maintaining the microscope system. Beyond the instrumentation advances, though, greater understanding of 2PM and its advantages has allowed this technique to be effectively utilized in biological research.

This chapter will cover the basics of 2PM and how the underlying photophysics leads to both its advantages and limitations. To take full advantage of its strengths, the instrumentation design needs to be optimized for 2PM, so these points will also be described in detail. The other nonlinear microscopies will be briefly described, with emphasis on the particular advantages of each. Finally, some recent examples of 2PM will be presented to highlight the wide range of physiological studies enabled in fields such as neuroscience, developmental biology, immunology, cancer, and endocrinology.

6.2 What is Two-Photon Excitation?

Given the plethora of optical sectioning methods, such as confocal, deconvolution, and light sheet microscopies, a good question to ask is why is yet another approach needed. To answer this question, we can consider the limitations of the other approaches, which are all based on linear absorption. That is, fluorescence can occur when a single excitation photon (typically of ultraviolet (UV), blue, or green wavelengths) is absorbed by a fluorescent molecule (fluorophore), which leads to a transition of the molecule into an excited state. The molecule then relaxes back to the ground state by emitting a single photon with less energy (or longer wavelength than the excitation wavelength), see Figure 6.1. In this case, which holds true under low to moderate excitation intensities, one photon is absorbed by the fluorophore, which can in turn emit a single fluorescent photon. If the excitation power is doubled, then twice the fluorescence is generated. This linearity is useful for quantitation but also leads to significant limitations, especially in confocal microscopy. This limitation is exacerbated in thick samples, as schematized in Figure 6.2. Because of focusing, the laser intensity is highest at the focus (focal plane) so a fluorophore there (f_1) is strongly excited. Fluorophores

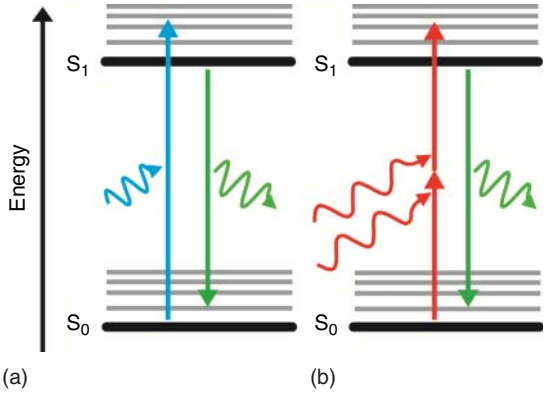

Figure 6.1 Jablonski diagram illustrating (a) one-photon versus (b) two-photon excitation of a molecule, and the subsequent fluorescence emission (shown in green). Two-photon-excited fluorescence results from the simultaneous absorption of two photons (red), each of half the energy of that from single-photon absorption (blue).

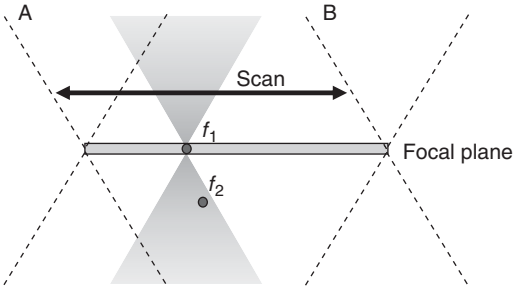

Figure 6.2 Photobleaching in confocal microscopy occurs above and below the plane of focus as shown in this axial photobleaching diagram. In confocal microscopy, as the beam is scanned from A to B to form an image, fluorophore f_2 is photobleached equally as f_1. Data is only acquired from f_1, which is in the focal plane, while data from f_2 is excluded from detection by the confocal pinhole.

above or below the plane of focus are illuminated less strongly at any given time, but as the laser beam is scanned across the field of view from A to B to form an image, out-of-focuses fluorophores are illuminated for a concomitantly longer time. During collection of an image, the entire sample is irradiated equally by the excitation light. That is, the regions above and below the focal plane see just as much excitation as does the focal plane. As a result, the entire thickness of the sample is subjected to photobleaching and associated photodamage similar to what is seen at the focus, but given the confocal pinhole, data is collected only from the focal plane. The fluorescence that arises from out-of-focus fluorophores is rejected by the pinhole, but photobleaching and photodamage still occur despite the lack of data collected from those regions. Deconvolution microscopy uses the out-of-focus fluorescence to increase the signal-to-noise ratio, and though this mathematical analysis approach works best for samples with low background, it has not been shown to function well for imaging of intact tissue. The more recently developed optical sectioning microscopies based on light sheet or selective plane illumination microscopy (SPIM) are methods where only the observed section of the sample is illuminated. Thus, SPIM approaches not limited by out-of-focus photobleaching and photodamage, and they may evolve as a replacement for confocal microscopy. However, both the deconvolution and SPIM imaging techniques are limited in their imaging depth by out-of-focus absorption of the excitation light and scattering of both the excitation and emission light. For SPIM, the excitation light is absorbed as the light sheet passes through the sample, so the penetration depth is limited by the concentration of fluorophores, which is usually quite high for intact tissue imaging.

6.2.1 Nonlinear Optics and 2PM

In contrast, two-photon excitation microscopy relies on nonlinear interactions between light and matter. Under very high excitation intensities, it is possible that a fluorophore can simultaneously absorb two excitation photons, each at half the excitation energy required for a single-photon absorption event (typically red to infrared wavelengths, see Figure 6.1). Because of the inverse relationship between photon energy and wavelength, a photon with half of the energy will have twice the wavelength. For example, a photon in the blue range (~450 nm) will have twice the energy as one in the near-infrared range (~900 nm). Thus, fluorescent probes that are normally excited in the UV (~350 nm) can be excited in 2PM with red light (~700 nm), while probes that are normally excited by blue, green, or orange light (450–550 nm) can be excited in 2PM with near-infrared light (900–1100 nm). Probes that normally absorb red light (>600 nm) require deeper infrared excitation (>1200 nm) where the photophysical properties of water can become a limitation. As detailed below, this nonlinear absorption means that in a microscope, two-photon excitation occurs only in the region of the focus. Since the excitation photons do not have sufficient energy by themselves to excite fluorescence, they pass through the sample with minimal interactions. This has two direct major implications. First, fluorophores above and below the microscope's focal plane are not excited by the illumination laser, and thus they are not photobleached, nor do they cause photodamage. It is important to realize

that fluorophores in the plane of focus are still subject to photobleaching and the associated photodamage – in fact, these deleterious events are often accelerated under the high laser intensities needed for two-photon excitation. Second, the excitation light is not attenuated by out-of-focus absorption as it travels through the sample. This allows stronger excitation deep into thick samples and more uniform illumination as a function of the focus depth. As detailed below, however, an additional practical consequence of this localized excitation causes 2PM images to be degraded less by light scattering in the sample, and as a result 2PM is much better than other optical sectioning approaches for deep-tissue imaging.

6.2.2 History and Theory of 2PM

The possibility of two-photon absorption was initially predicted by Maria Goppert-Mayer in her Ph.D. thesis in 1931 [2], but the high photon fluxes needed to achieve such nonlinear effects were not available at that time (Dr Goppert-Mayer went on to win the 1963 Nobel Prize in physics for her development of the nuclear shell model). Even the brightest arc lamps focused through the best objective lenses available at that time could only have created a single two-photon event every few minutes, well below the detection limits. Soon after the invention of the laser, however, Kaiser and Garrett first observed two-photon excitation in 1961, but the number of two-photon excitation events generated by those early lasers was still too low to permit imaging. It was not until the invention of ultrashort mode-locked lasers (see Box 6.1) that 2PM became practical, as was first demonstrated by Denk *et al.* [1] in 1990. Over the last 25 years, continuous improvements in instrumentation have made 2PM a practical alternative for optical sectioning microscopy, especially for deep-tissue imaging.

We now know that Goppert-Mayer correctly predicted the simultaneous absorption of multiple photons, and set the level of photon density (light intensity) that would be needed for this effect to occur. According to her original theory, which has been verified by many measurements over the last 50+ years, the intensity needed for efficient two-photon excitation is *about one million fold greater* than what is needed for the same number of one-photon excitations. Reaching such high intensities would not have been possible for biological experiments but for the advent of ultrashort pulsed lasers that provided high peak power (well suited for two-photon excitation) but have sufficiently low average power (which permits imaging samples without undue heating or other effects that come from linear absorption). In fact, development of ultrafast pulsed lasers is what initially enabled 2PM. These details are presented in Box 6.1.

Box 6.1 Practical Excitation Parameters for 2PM

To define the requirements of appropriate laser excitation sources for two-photon excitation microscopy, it is useful to understand the underlying photophysical parameters. In any fluorescence experiment, the number of excitation events depends on the strength of absorption and the intensity of the incoming light. As shown in the first row of Table 6.1, for the linear absorption used in confocal

(Continued)

Box 6.1 (Continued)

microscopy this number (N_{ex}) depends on the absorption cross section (σ) times the exciting laser intensity (I). The cross section is directly related to the extinction coefficient, which is typically measured in an absorption spectrometer, and for typical fluorophores it has a value of $\sim 10^{-16}$ cm^2 (row 2). For a typical image size of 512×512 pixels and fluorophore concentration in the submillimolar range, the excitation intensity needs to be in the range of 1 MW cm^{-2} (row 3). While this may seem like a large intensity, the focusing down to a submicrometer spot allows this level to be easily reached with a continuous wave (CW) laser with powers <1 mW, which is less than the intensity of a standard laser pointer. In fact, only 1 mW of laser power (P_{sat}) is sufficient to saturate the fluorescence (i.e., to excite all of the fluorophores) in a typical confocal microscope. For two-photon excitation (right-hand column in Table 6.1), the number of excitations (row 1) depends on the square of the excitation intensity and the two-photon cross section (δ). Since both photons must interact with the fluorophore at "the same time," δ has both a spatial component (as does σ) and a temporal overlap component (row 2). If we assume that the spatial components are similar to the 10^{-16} cm^2 measured in linear absorption, and factor out that amount for each photon, we are left with 10^{-18} s/photon, which gives a good estimate for what is meant by "the same time." That is, both photons must arrive within 10^{-18} s. Given the speed of light, the photons must be closer than 3 Å, which is consistent with the $\sim$10 Å size of typical fluorophores. In this case, the excitation intensity needed for imaging is roughly a million-fold higher than what is needed in linear absorption (row 3). Using a CW laser, this would require kilowatts of power, which is comparable to what is used in laser welding. To reduce the total power needed, ultrashort-pulse, mode-locked lasers are used (row 4). These lasers output pulses with a duration of $\sim 10^{-13}$ s at a repetition rate of 100 MHz, which means that the lasers are actually off 100 000-fold longer than they are on. While these pulses seem short to us, the 10^{-13} s is also 100 000 times longer than the simultaneous absorption time (10^{-18} s), so two-photon excitation has plenty of time to occur during each pulse. The use of lasers with a duty cycle of 10^{-5} means that, instead of kilowatt powers, fluorophore saturation can be reached with about 10 mW, or only 10-fold more laser power than is needed in linear absorption microscopy (row 5).

Table 6.1 Parameters for confocal and two-photon excitation microscopy.

	One-photon (linear) absorption	Two-photon (non-linear) absorption
1	$N_{ex} = \sigma I$	$N_{ex} = \delta I^2$
2	$\sigma = 10^{-16}$ cm^2	$\delta = 10^{-50}$ cm^4 s/photon
3	$I_{ex} = 1$ MW cm^{-2}	$I_{ex} = 10^6$ MW cm^{-2}
4	CW laser	$t = 10^{-13}$ s
		Freq. = 100 MHz
5	$P_{sat} = 1$ mW	$P_{sat} = 10$ mW

For laser scanning imaging, around one million pixels need to be acquired in about 1 s for each image, and to achieve a good signal-to-noise ratio in the image, over one thousand photons need to be detected in each pixel. To obtain powers at the sample sufficient for 2PM imaging, the excitation photons must be crowded not only in time with pulsed lasers but also in space using the focusing of a microscope objective lens. This critical point of crowding can be understood from the basic physical chemistry of a reaction that goes as $A + 2B \rightarrow C$ (where A is the fluorophore in the ground state, B is a photon, and C is the fluorophore in the excited state). This reaction will proceed with a probability that depends on the square of the concentration of B. As the excitation light is focused in the microscope, the photons are increasingly crowded so that their concentration increases, which in turn greatly increases the probability of two-photon excitation. Using a high numerical aperture (NA) lens, the laser beam is focused to a spot roughly 300 nm in diameter, which yields a photon density 10 million-fold greater than what is seen in the typical 1-mm laser beam diameter (or about 100 000-fold greater than the 100-μm diameter beam near the surface of the sample). As the two-photon excitation rates increase with the square of the photon concentration, this focusing enhances the probability several trillion-fold. Thus, through the combined crowding of photons in space by focusing and in time using pulsed lasers, it is possible to generate billions of the normally rare two-photon excitation events each second.

This requirement of photon crowding in space leads to a major advantage of 2PM – the localization of fluorescence excitation. This is schematized in Figure 6.3. As laser pulses pass through the sample, photons are crowded in time, but for most of their path they are not sufficiently crowded for photon pairs to interact simultaneously with single fluorophores. Only near the focus

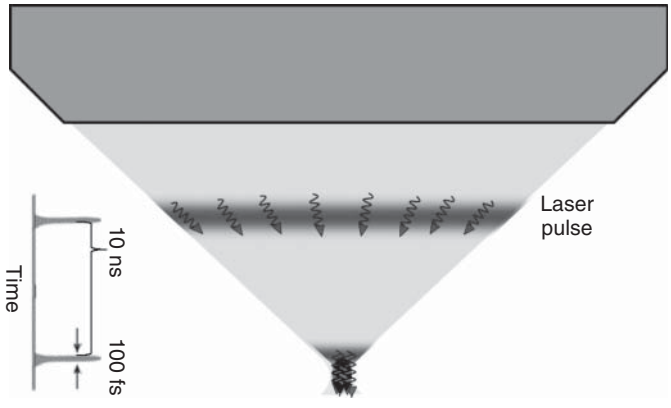

Figure 6.3 Excitation localization by photon crowding in time and space. Schematic representation of the fluorescence distribution in the focus resulting from single- and two-photon excitation. Under single-photon excitation, fluorescence is generated throughout the sample; in a confocal microscope only fluorescence generated at the focal plane is detected. Two-photon-excited fluorescence results in fluorescence being generated solely at the focal plane. The lack of out-of-focus excited fluorescence reduces overall phototoxicity in the sample and enhances contrast in deep-tissue imaging.

are photons sufficiently crowded to generate a significant number of two-photon absorption events. This region near the focus the two-photon excitation volume (often called the *focal volume*), and for a high NA objective lens it is ~0.1 fl. Two-photon excitation events fall off rapidly above or below the focal volume because of their dependence on the square of the excitation intensity, as shown in Figure 6.4. As can be seen from this figure, the only place where two-photon absorption occurs on a practical level is within the focal volume. This means that all the fluorescence comes from this small focal region and not from other depths through which the excitation light passes. This localization of excitation results in inherent optical sectioning, which is roughly equivalent to that achieved in confocal microscopy. However, in 2PM there is no need for a confocal pinhole to obtain optical sections, rather these are generated directly by the localization of the fluorescence. As an analogy, conventional fluorescence microscopy is equivalent to looking inside a house by shining a powerful spotlight from outside, while 2PM is shining a flashlight inside the house to examine its contents. The light needed is generated inside the sample of interest. This difference is illustrated in Figure 6.5, where two parallel laser beams are focused into a cuvette filled with a fluorescein solution. While the conventional one-photon absorption occurs throughout the sample, forming the hour-glass figure characteristic of a focused beam, the two-photon excitation is observed just at the focal point. In a confocal microscope, the pinhole is used to reject background fluorescence from outside this focal spot and permit detection of only the in-focus signal, but in 2PM there is no background created by the excitation, so there is no need for a pinhole to reject it. Since the pinhole is not needed to obtain optical sections, the detection geometry can be simplified, which also leads to further advantages in signal collection.

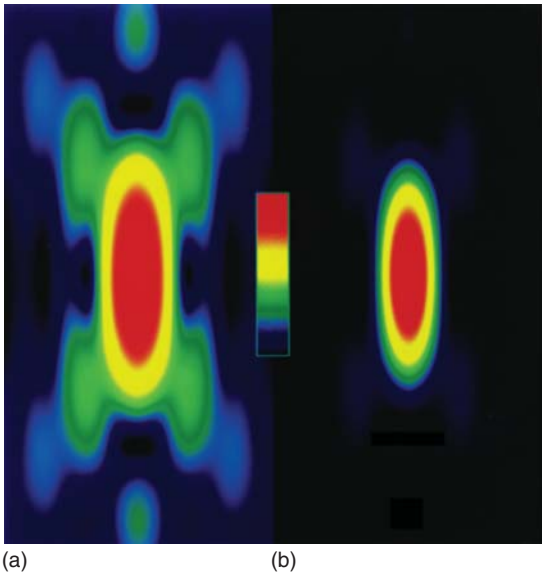

Figure 6.4 Axial image (*X*–*Z*) of the calculated point-spread functions for (a) one-photon and (b) two-photon excitation. For one-photon excitation, fluorescence is generated both above and below the focal spot, seen as the wings of the Airy disk profile. In two-photon excitation, the out-of-focus background created by these wings is not observed. (Sandison and Webb 1994 [3]. Reproduced with permission of The Optical Society.)

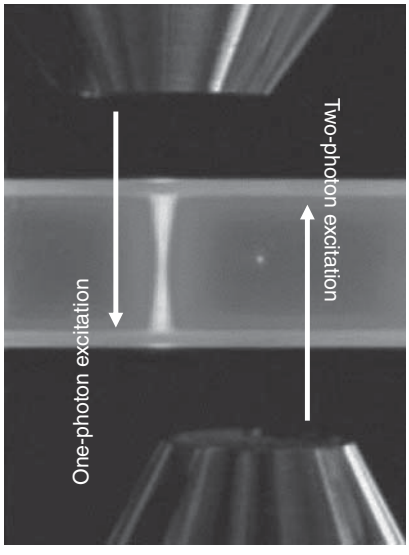

Figure 6.5 Experimental illustration of the difference between linear and two-photon excitation: 380 nm (confocal) and 760 nm (two-photon) excitation wavelengths were used to generate fluorescence in a 50 μM fluorescein solution. Two-photon excitation generates fluorescence in the focal volume exclusively, while conventional single-photon excitation generates fluorescence all along the light path. (Image used by permission, Kevin Belfield Research Group, University of Central Florida; Zhen-Li Huang and Ciceron Yanez.)

6.3 How Does Two-Photon Excitation Microscopy Work in Practice?

As described previously, the photophysical theory shows that 2PM excitation is limited to the focus. The strengths of 2PM for deep tissue imaging are all consequences of this effect [4]. The first consequence is that, as the laser focus is raster-scanned to form an image, two-photon excitation of the fluorophores as well as any photobleaching and photodamage associated with the probe excitation are also limited to the focal plane. This is demonstrated in Figure 6.6, which shows an axial (X–Z) scan through a polymer film that is uniformly stained with fluorescein. To create this image, a 20 μm × 20 μm square X–Y region was imaged repeatedly for an extended period, first using 488 nm and then, after moving the region 30 μm to the right, again using 780 nm 2PM. After these two regions were

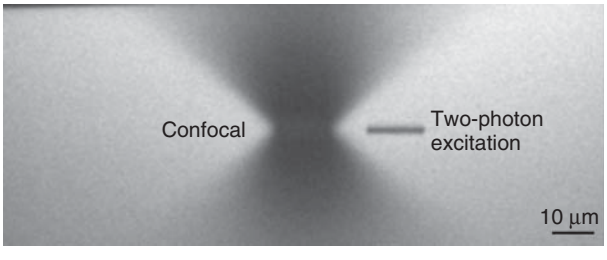

Figure 6.6 2PM limits photobleaching and associated photodamage. Axial photobleaching patterns produced by conventional confocal and two-photon excitation after imaging a single plane inside a polymer film containing sulforhodamine B. On the left, the effect of conventional confocal illumination (514 nm); on the right the effect of two-photon illumination (820 nm). Scale bar 10 μm.

photobleached effectively to zero, an axial X–Z scan was acquired to examine the 3D photobleaching profile. In the first case of linear excitation, as is used in confocal microscopy, photobleaching is seen uniformly throughout the sample: above, below, and at the focal plane. If the data from only the focal plane was acquired using a confocal pinhole to reject the out-of-focus background, all the other photobleaching (and associated photodamage) would be nil. That is, out-of-focus molecules would be photobleached and out-of-focus cells would be subjected to photodamage, but there would be no data collected in return. In the second case of 2PM, the photobleaching (and associated photodamage) is limited only to the focal plane. Thus, out-of-focus fluorophores are not damaged, and out-of-focus cells are not subjected to photodamage. It is important to note, though, that photobleaching and associated photodamage still occur at the focal plane, and as detailed in the following, the rates of bleaching and damage within the focus can actually be accelerated during 2PM.

6.3.1 The Role of Light Absorption in 2PM

The second consequence of 2PM excitation being limited to the focus is that the incoming excitation light is not absorbed as it passes through the sample, and therefore more excitation can reach deeper into the tissue. This effect is demonstrated in Figure 6.7, which shows axial (X–Z) scans through 250 μm of a colored, but otherwise clear and essentially nonscattering glass slide. This glass slide also has an index of refraction equal to that of the immersion oil used for imaging, so that there is no degradation of imaging quality from aberrations due to index of refraction mismatch. The first axial X–Z scan image was acquired using one-photon 488-nm excitation. As can be seen in the image, but especially in the accompanying intensity profile along the Z-axis, the intensity drops off as the imaging depth increases so that only 25% of the signal is observed at the 250 μm level. Since this glass is clear, there is minimal contribution from scattering. This means that the resulting signal loss at depth is entirely due to the absorption of the incoming light by out-of-focus fluorophores. In other words, 75% of the excitation photons are absorbed before reaching the focus 250 μm into the sample. This glass slide is labeled at a fairly high concentration of fluorophores, but losses due to out-of-focus absorption always place a critical limitation on confocal microscopy, and, in fact, also on the efficacy of light sheet microscopies, which also rely on penetration into the sample (see below). The second image shows an identical axial scan, except that this one was created using 780-nm two-photon excitation. In contrast to what is seen in the first case, the 2PM scan shows uniform brightness all the way down to 250 μm. The reason for this uniformity is that there is no absorption of the incoming light until the focus: the individual photons do not have sufficient energy to excite the fluorophores, and the photons are not crowded enough to generate two-photon excitation events until they reach the focus. Thus, one main reason for the increased imaging depth provided by 2PM is the lack of out-of-focus absorption of the incoming excitation light. The practical advantage of 2PM depends on the fact that the red and infrared excitation light is generally not absorbed by biological tissue. This is easily observed by covering a flashlight with a hand – it is the red light that comes

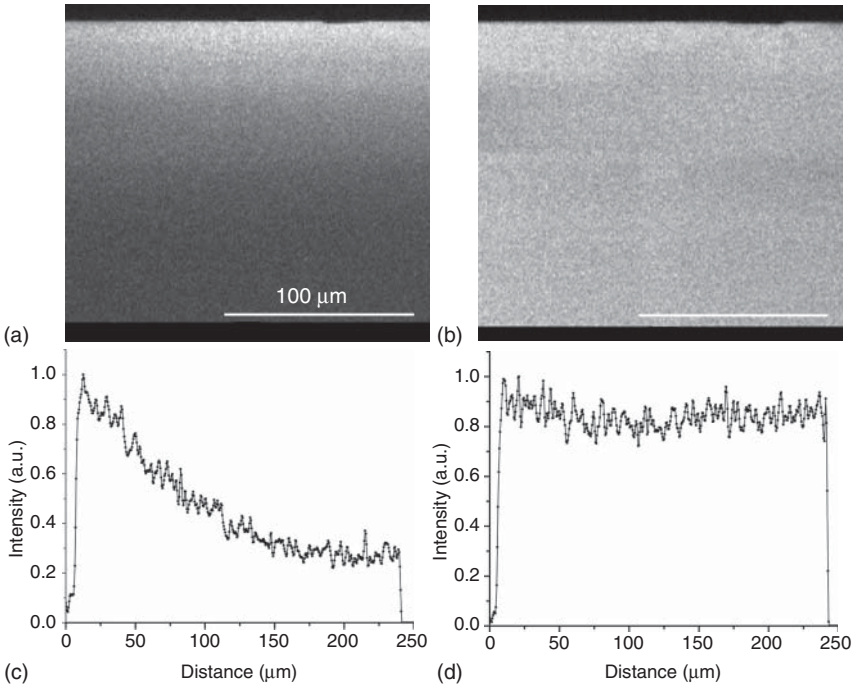

Figure 6.7 Out-of-focus absorption effects on fluorescence imaging with one- and two-photon excitation demonstrated using fluorescent glass (with negligible scattering). In one-photon excitation (a, c), excitation photons are absorbed throughout the sample, so fewer photons reach the focal plane. This can be seen as a decrease of the final fluorescence collected as a function of imaging depth. On the other hand, two-photon excitation light is not absorbed until the photons are sufficiently crowded at the focal spot (b, d). Thus, there is no loss of fluorescence signal as a function of imaging depth.

through – the other colors are absorbed by naturally occurring molecules, most strongly by flavins, lipo-pigments, and porphyrins (heme proteins) in the blue, green, and orange regions of the spectrum. While this assumption holds for most animal cells, it should be noted that many species, especially plants, can have very strong absorption in the red and near-infrared regions and that this absorption may limit or preclude the use of 2PM in such specimen. Finally, even though the excitation photons may not be absorbed as they pass through the sample, there is usually significant absorption of the resulting fluorescence as it comes out through the sample, and this contributes to limiting the maximum imaging depth of 2PM.

6.3.2 The Role of Light Scattering in 2PM

The third consequence of 2PM excitation being limited to the focus is that 2PM is less sensitive to degradation by light scattering in the sample than confocal microscopy. Even though 2PM is less sensitive to this, light scattering defines the ultimate limit of the imaging depth. The relative roles of absorption and scattering can be demonstrated by a simple experiment using red and green laser pointers.

Covering the red laser pointer with a finger, the red light is observed coming through the finger (similar to what is seen using a flashlight as discussed above). The red light is not absorbed by the molecules and cells in the finger. However, the laser beam does not come through – the light is all heavily scattered as it passes through the finger and it comes out in all directions. In contrast to the red laser pointer, doing the same thing with the green laser pointer shows a much different result. None of the green light comes through the finger – it is all absorbed. A small amount of scattered green light may be observed close to where the laser beam impinges on the finger, but none traverses the finger and comes out on the other side. Still, even though the red light penetrates the tissue, it is highly scattered, which limits its usefulness for imaging.

The depth penetration of 2PM is limited by scattering in the sample of both the incoming excitation light and the outgoing fluorescence. Still, 2PM is less sensitive than confocal microscopy to degradation by light scattering, as demonstrated in Figure 6.8, which shows the ways light scattering affects both confocal microscopy and 2PM. Figure 6.8a depicts confocal microscopy with blue excitation and green fluorescence. The microscope focuses the blue laser to the focus (1), and the resulting fluorescence is collected and directed through the confocal pinhole to the detector (2). However, some of the fluorescence coming back through the sample can be scattered and miss the pinhole (3). This is fluorescence

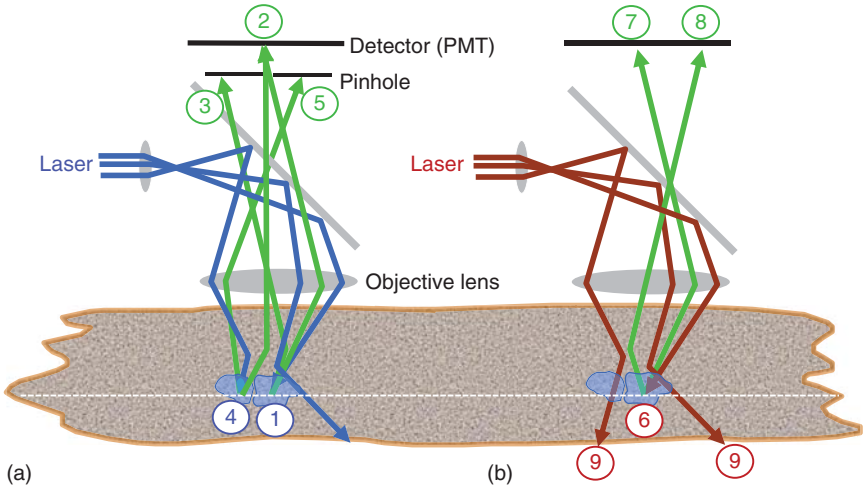

Figure 6.8 Scattering effects on fluorescence imaging with one- and two-photon excitation. In one-photon excitation (a), excitation photons may reach the focus (1) and generate fluorescence. Only the unscattered photons pass through the pinhole (2) and reach the detector (photomultiplier tube, PMT). However, some of the photons generated at the focus are scattered in the sample, and miss the pinhole (3). Excitation can also be scattered, which creates extra out-of-focus background (4). This background is largely rejected by the pinhole (5), but some of it can be scattered in the sample such that it passes cleanly through the pinhole and reaches the detector (2). In two-photon excitation (b), excitation light can also be scattered before it reaches the focus (6), but these scattered photons (9) are too few to generate two-photon excitation. For the fluorescence, both scattered (7) and unscattered (8) photons are detected since the pinhole is not needed to reject out-of-focus background.

that comes from the focus, and is thus signal that should be detected, but is lost due to scattering. In the same manner, the excitation light can also be scattered as it passes through the sample on the way to the focal spot (4). This light can interact with fluorophores outside the focal spot and generate extraneous background fluorescence. In a weakly scattering sample, most of this background will be excluded from collection by the pinhole, so only negligible amounts of it will be detected. In a more strongly scattering sample, or when imaging deeper into a sample, the increased amount of excitation scattering will result in significant amounts of background fluorescence being scattered into the pinhole (2). The detection of this extra background will be more or less constant over the image, causing a haze that reduces the image contrast (as shown in Figure 6.11). Thus, the scattering of both excitation and fluorescence reduces the detected signal, and scattering of background fluorescence reduces image contrast.

Figure 6.8b depicts 2PM with red (two-photon) excitation and the same green fluorescence. The laser is focused (6) to a point where the excitation photons are sufficiently crowded to generate two-photon excitation. As in a confocal microscope, the fluorescence is collected and directed to the detector. However, since we know that for 2PM the fluorescence is coming only from the focus, there is no need for a confocal pinhole to reject out-of-focus fluorescence. Therefore, fluorescence that passes cleanly back through the sample (7) as well as that which is scattered (8) can all be efficiently detected, which can greatly increase the signal. As described in the instrumentation section below, such a non-descanned detector can be used immediately after the objective lens, which further increases the efficiency of signal collection. As detailed in the next paragraph, the red and infrared excitation light used in 2PM is still scattered by the sample similar to what is seen in confocal microscopy. However, in 2PM these scattered excitation photons do not create additional background fluorescence since the probability of two photons scattering to *the same place at the same time* is essentially zero. Hence, both collection efficiency and imaging depth are increased over confocal microscopy by using 2PM.

Much has been written about how the longer wavelengths used in 2PM greatly reduce this scattering, but that is not really the reason behind the improved depth penetration. The reason for this confusion is that light scattering is best known in the atmosphere, which causes the sky to look blue. This effect is based on the Rayleigh scattering theory, which assumes that the scattering particles are much smaller than the wavelength of the light. This results in scattering strength that is inversely proportional to the fourth power of the light's wavelength. Thus, Rayleigh scattering is much stronger for violet and blue light than for the longer wavelengths toward the red end of the spectrum. However, in cells and tissue, the scattering particles (organelles, vesicles, chromosomes) are not much smaller than the wavelength of light; in fact, they can be larger than this wavelength. Scattering of light in tissue has been extensively studied in association with tissue imaging by diffuse optical tomography, which is used to measure tissue oxygenation and detect tumors. These studies show that tissue scattering can be much better predicted using the Mie scattering theory, which describes the scattering of light by spherical particles of any diameter. For particles on the order of the

wavelength of light or larger, the amount of Mie scattering is not strongly dependent on the wavelength but is proportional to the square of the particle diameter.

6.4 Instrumentation

A 2PM instrument is generally built similar to a confocal microscope, consisting of a laser, a scanning system, and a detector. In fact, most confocal microscope manufacturers offer an option to use their confocal microscope as a 2PM instrument. Two key components, though, differ significantly between confocal microscopy and 2PM: the laser and the location of the detector, as previously mentioned previously. A schematic of a typical two-photon microscope setup is shown in Figure 6.9, which is a basic laser-scanning microscope arrangement. The laser comes in and is scanned in a raster pattern to form an imaging field of view, which is focused onto the sample with a scan lens/objective lens system. The laser induces fluorescence, which is collected by the objective lens and directed back up the optical path.

6.4.1 Lasers for 2PM

The first major difference between confocal microscopy and 2PM is the laser. As described previously, for 2PM the laser must be "ultrafast," which is typically defined as subpicosecond pulses of laser light. Using these short laser pulses, it is

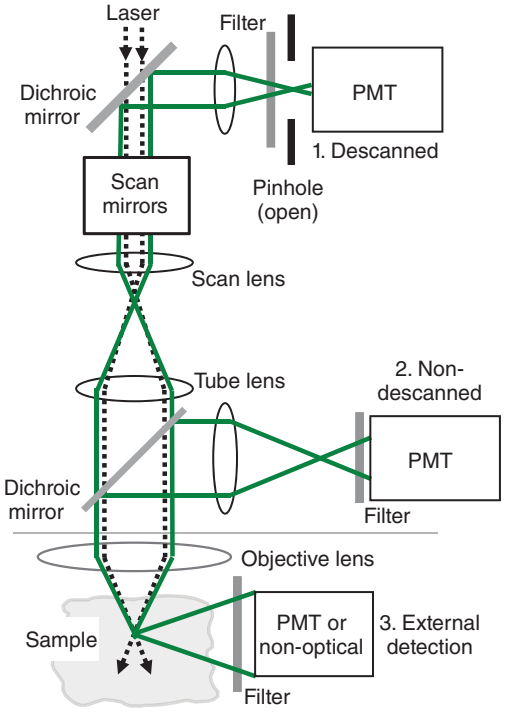

Figure 6.9 Schematic of a typical two-photon microscope setup with the different possible detection strategies. Most of the apparatus is similar to a confocal microscope with the exception of the laser source, the lack of detection pinhole, and the various possible detection geometries.

possible to deliver high laser intensities to the specimen, without simultaneously vaporizing it (see Box 6.1). 2PM is now a well-established technique largely due to the availability of easy-to-use, ultrafast, mode-locked laser systems that are appropriate for this approach. While 2PM first became possible with the invention of subpicosecond-pulse mode-locked lasers, these early versions were far from turn-key systems. The expertise required for daily laser operation was generally not available in cell biology or physiology laboratories. Over the last 15 years, however, laser manufacturers have developed Ti:sapphire and other laser systems in which the alignment and wavelength-tuning is automatic, and the laser system is essentially as much a turn-key system as a laser pointer. In addition to facilitating the use of 2PM in a nonspecialist laboratory, these lasers permit easy excitation wavelength scanning, which is needed for 2PM imaging of different fluorophores.

The laser source most commonly used is the Ti:sapphire laser oscillator, which typically provides tuning over the wavelength range 680–1050 nm (approximately equivalent to 340 and 525 nm single-photon absorption wavelength range). This wide tunable wavelength range brings with it a further advantage: a continuum of excitation wavelengths is possible to select in 2PM, whereas confocal microscope excitation is typically limited to several discrete wavelength lines. These mode-locked Ti:sapphire lasers generate ultrashort light pulses (~100 fs) with a repetition rate of ~100 MHz (which is equal to one pulse every 10 ns). Since the laser is essentially off 10^5-fold more time than it is on, the average power is kept low while providing high photon fluxes during pulsed operation. As described previously, this crowding of the laser photons in time is combined with focusing to a diffraction-limited focal volume in the microscope to achieve the high photon fluxes needed to generate sufficient nonlinear excitation events for 2PM imaging. A newer development is the broadly tunable optical parametric oscillator (OPO) laser, which is now commercially available in a hands-free automated package. These lasers provide pulse and power characteristics similar to those of the Ti:sapphire laser but are tunable from 680 up to 1300 nm. This broader tuning range opens up the possibility of using 2PM with fluorophores that are normally one-photon-excited in the red region (~650 nm). Fluorescence from these fluorophores is very weakly absorbed in tissue, which should allow imaging at greater depths than with blue, green, or orange fluorophores. At this point, though, the use of red fluorophores for 2PM has only begun to be explored. There are some practical limitations expected with using these probes, but none of these has been experimentally verified to date. First, we know that the absorption of water greatly increases throughout the infrared spectrum. Since this absorption is linear, the high powers needed to generate two-photon excitation can lead to heating (described in more detail below). Using 1300 nm light, a simple calculation suggests that heating could be as high as 5 °C during imaging. This would be sufficient to denature lipid membranes and greatly perturb cellular function. If this turns out to be the case, then care will be needed to keep laser powers low, which may force decreased spatial and temporal resolution. Other potential limitations may arise from the scarcity of red fluorescent proteins and the relatively poor quality of red fluorescence probes. There is considerable interest in developing such probes for

in vivo imaging, but still the quantum yield of even the best red and near-infrared fluorophores is ~10% (as compared to near 100% for green fluorophores). Finally, the use of deeper infrared wavelengths will require dedicated microscope optics. Fortunately, microscope manufacturers have developed objective lenses that are optimized for two-photon microscopy. These account for the need to efficiently transmit both infrared excitation and visible emission wavelengths and to image deep into a variety of tissues and other specimen without introducing significant image aberrations. While the Ti:sapphire laser wavelengths can be used to obtain reasonable results with regular microscope optics, the 2PM-optimized objective lenses will be crucial for extension of 2PM into the infrared region using the newest turn-key OPO lasers.

A major consideration regarding appropriate lasers for 2PM is just how short the pulses need to be. There is no hard and fast rule for this, but in general the shorter the pulse, the better. The original 2PM experiments were done using a colliding-pulse mode-locked (CPM) dye laser that provided pulses <100 fs ($<10^{-13}$ s), and similar pulses are available from the typically used Ti:sapphire and OPO lasers. Thus, the standard pulse width is ~100 fs, and this is known to work well in a wide range of applications. Pulses longer than 1 ps (10^{-12} s) have also been used effectively for 2PM. The trade-offs associated with different laser pulses are detailed in Box 6.2.

Box 6.2 Laser Pulse Widths for 2PM

The laser pulse width is a key parameter that defines the efficiency of two-photon excitation at a given laser power level. That is, for the identical laser power, more two-photon excitation will occur for a narrower pulse width. However, the dependence of two-photon excitation efficiency on pulse width is linear, so it is not as critical as might be initially thought.

Consider a 1 mW laser with 100 fs pulses coming at 100 MHz. In this case, the laser is "on" for 10^{-13} s and the pulses are spaced 10^{-8} s apart. This factor of 10^5 in duty cycle (the portion of time that the laser is "on") means that the peak laser power during each pulse will be 10 kW, or 10^5-fold greater than the average power of 1 mW. The rate of two-photon excitation will be proportional to the square of this peak power (100 kW^2), and the number of events per pulse will be this rate times the pulse duration (100 fs). For these parameters, the number of two-photon excitation events per pulse will be proportional to 100×100 fs $kW^2 = 10\,000$ fs kW^2. If we now consider a laser with identical parameters except for a longer pulse width of 1 ps (1000 fs), the laser is "on" for 10^{-12} s, which gives a 10^4 duty cycle and a peak laser power during each pulse of 1 kW. In this case, the rate of two-photon excitation will be proportional to 1 kW^2, but number of events per pulse will be proportional to 1×1000 fs $kW^2 = 1000$ fs kW^2. This is only a factor of 10 less than with the 100-fs pulses. While the efficiency drops with the square of the peak laser power, the longer pulse duration partially compensates for this lost efficiency. Using the 1-ps pulse, it is possible to obtain the same number of two-photon excitation events generated by the 100-fs pulses by simply increasing the average laser power by a factor 10. As the lasers used

in 2PM these days mostly provide average powers of >1 W, it is generally no problem to deliver the extra power needed for 2PM with a longer pulse width. However, some potential photodamage (such as laser heating, especially when using longer infrared wavelengths) depends linearly on the average laser power. This type of damage will be minimized by using shorter laser pulses. Another motivation for the use of shorter pulses is the loss of power due to scattering in deep-tissue imaging. In cases where the amount of input laser power is limited to a certain level, it is possible to image deeper by making the pulse widths narrower, thus making the two-photon excitation probability more efficient. For the deepest penetration depths (see Box 6.3), >1 W is needed at the surface of the tissue to generate sufficient two-photon absorption events at the focus. In this case, it is generally not possible to increase the laser power, but deeper penetration of imaging may be achieved by using shorter laser pulses.

It is best to use shorter pulses, but physical limits dictate the minimum practical pulse width. Light is governed by the Heisenberg uncertainty principle, which limits the precision of the product of energy and time. This means that photons confined within a short time window cannot have a precise energy (wavelength) but instead must have a spread of energies. The shorter the pulse, the more spread out this energy range. Light of different energies (colors) interacts with matter differently, so these ultrashort pulses with their color spread are affected not only by the focusing optics by chromatic aberrations but also by traveling through air where the speed of light varies as a function of wavelength. This variation of photon speeds leads to group velocity dispersion (GVD), which spreads the pulse width. While strategies exist to compensate for chromatic aberrations and GVD, in practice these factors are not a limitation on 2PM unless the laser pulses durations are <80 fs.

6.4.2 Detection Strategies for 2PM

The second major difference between 2PM and the confocal microscopy design is the location of the detectors. In a confocal microscope, the fluorescence passes back through the scanning mirrors, so that it is "descanned" and then focused onto the confocal pinhole to reject out-of-focus background fluorescence that would otherwise blur the image. While the pinhole leads to the desired optical sectioning in a confocal system, it does so at the cost of rejecting some in-focus fluorescence together with the out-of-focus background. This loss of "good" photons becomes more significant for deeper imaging into the specimen. In addition to losses at the pinhole, the fluorescence must also pass back through the scanning mirror optics before reaching the confocal pinhole. Passing back through this optics inevitably leads to further signal losses. In contrast, 2PM does not require the use of a pinhole since we know that the fluorescence originates solely from the focus. Thus, all the fluorescence emission can be collected without the use of a pinhole, which opens up a number of new possibilities for detector locations.

In 2PM, it is possible to simply open the pinhole (#1 in Figure 6.9), and thus capture some of the scattered fluorescence photons (Figure 6.8, photon (8)). While

this will increase the collection efficiency compared to confocal microscopy, it is still subject to the losses as the fluorescence passes back through the scanning system and is refocused onto the detector. Also, because of the optical system design, even a fully open pinhole still acts as a spatial filter and excludes many of the scattered fluorescence photons that should be collected in 2PM. Indeed, there is a simpler detector arrangement that be used, where all the photons collected by the objective lens are directed immediately to a photomultiplier detector. This strategy is called *non-descanned detection* (NDD, #2 in Figure 6.9) and is the most common detection arrangement for 2PM [4]. For NDD, the only selection applied is based on wavelength by using the appropriate optical filter to reject the excitation light and detect only the fluorescence of interest. Using an NDD, all the scattered fluorescence that is collected by the objective lens can be measured, which greatly increases the signal level. As for confocal microscopy, new photomultiplier detectors using GaAsP photocathodes can further improve the collection efficiency, and these should be used for 2PM if at all possible. Finally, it is possible to use an external detector that does not even rely on the objective lens (#3 in Figure 6.9). Such a detector could be a large-area photon detector, or a non-optical detector, such as the cell electrical response measured by electrophysiology (as given in the applications of 2PM example below).

6.4.3 The Advantages of 2PM for Deep-Tissue Imaging

Combining optimized NDD with the inherent depth penetration of 2PM permits maximal depth imaging. The actual maximum depth depends on the sample, but a good rule of thumb is that 2PM using an NDD can image 6–10-fold deeper than with confocal microscopy. As demonstrated in Figures 6.10 and 6.11, an increase in depth of 2–3-fold comes from the lack of out-of-focus absorption, and another factor of 3 in depth comes from improved collection of the scattered fluorescence with the NDD. Figure 6.10 shows a series of X–Z scans of a mouse kidney section that is heavily stained with eosin. The laser power was adjusted for each X–Z scan so that the signal level at the tissue surface was the same in each image. Using confocal microscopy, useful signal can be obtained only within the first ~10 μm from the surface. It is possible to collect fluorescence from deeper levels, but the contrast will be lost (as demonstrated in Figure 6.11). Switching from confocal to 2PM, but using descanned detection (detector strategy #1 in Figure 6.9) with the pinhole open, it is possible to collect useful data down to ~20–30 μm from the surface. This improvement is largely due to the lack of out-of-focus absorption in the case of 2PM, which allows more excitation light to reach the focal spot deep in the sample. Some scattered fluorescence is collected through the open pinhole, but many of these useful photons are lost as they stray from the optical path as it returns through the scan system and is focused on the pinhole. The NDD (detector strategy #2 in Figure 6.9) can be used immediately after the objective, which thus greatly increases the collection efficiency of the scattered fluorescence. Using the NDD, useful data can be acquired from >50 μm into the sample even without increasing the laser power. By increasing the laser power, it would be possible to obtain useful data down to 100 μm below the surface of this specimen. As was described for Figure 6.8, in the case of 2PM,

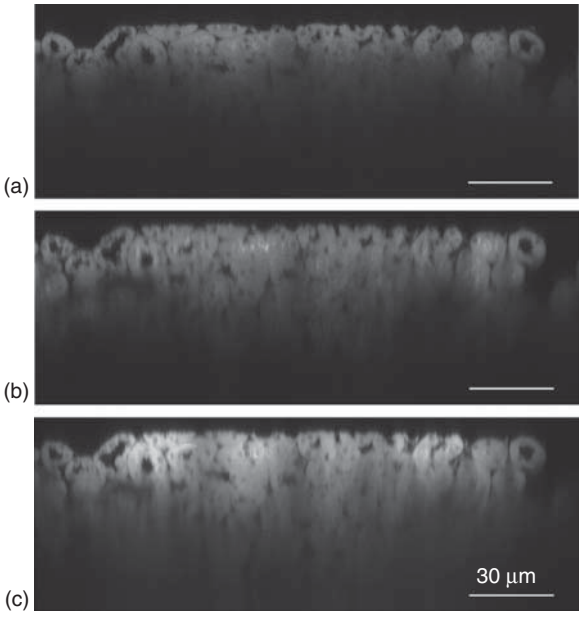

Figure 6.10 Comparison of imaging penetration depth between one- and two-photon excitation microscopy. *X–Z* profiles through an eosin-stained mouse kidney sample imaged through a depth of 70 µm with (a) confocal, (b) 2PM descanned, and (c) 2-PM with non-descanned detection microscopy. The imaging penetration depth with 2PM (descanned, b) is improved approximately twofold relative to confocal microscopy (a). Non-descanned detection (c) yields increased fluorescence collection, which further increases the effective depth of imaging. Scale bars = 30 µm.

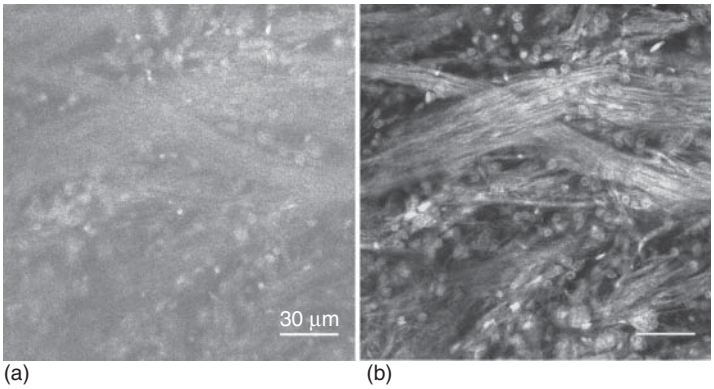

Figure 6.11 Comparison of imaging contrast between one- and two-photon excitation imaging at deep focal planes within a scattering sample. The two panels show the same *X–Y* plane imaged 50 µm into a rhodamine-stained slice of mouse brain with (a) confocal microscopy and (b) 2PM. In confocal microscopy, the laser power can be increased to obtain sufficient signal at depth, but this results in increased scattered background (see Figure 6.8), which appears as haze and reduces the image contrast. In 2PM, on the other hand, increasing the laser power creates more signal without decreasing contrast, so that fine structures can still be resolved and measured. Scale bars = 30 µm.

increasing the excitation power does not generate additional background fluorescence, which would create a haze and decrease the image contrast. This effect is demonstrated in Figure 6.11, which shows two comparable X–Y planes 50 µm into a rhodamine-stained slice of mouse brain. The first was taken using confocal microscopy, where the laser power was increased in an attempt to raise the fluorescence signal. Indeed, the fluorescence signal level is increased, but most of this fluorescence is seen as a background haze that smears out the contrast of the biological structure. On the other hand, with 2PM it is possible to increase the excitation laser power without creating additional background haze. In this case, the signal level is increased, and the fine biological structures are still visible. Importantly, areas in the image where no structure is present remain black, which indicates that the image contrast is a maximum.

In summary, there are several practical advantages to 2PM that derive from the photophysics of two-photon excitation in a microscope, where the excitation is limited to the focus. This leads to the inherent production of optical sections (often called *confocal for free* because no pinhole is required), and the lack of out-of-focus absorption of the excitation light allows more of it to reach the focus. Importantly, photobleaching and photodamage are also limited to the focal plane, and thus in a thick sample these deleterious effects can be greatly reduced. Because of the details of 2PM image formation, this approach is much less sensitive to degradation by light scattering in the tissue than with other optical sectioning approaches. Further, the red and infrared laser light used in 2PM is less absorbed by many biological samples, and so is less biologically damaging. Together, these factors give 2PM tremendous advantages for deep-tissue imaging with sub-cellular resolution.

6.5 Limitations of Two-Photon Excitation Microscopy

While all of the strengths described previously give 2PM significant advantages over other approaches for diffraction-limited imaging deep into intact tissue, 2PM is by no means a panacea. There are limitations and challenges to 2PM that must be appreciated to take full advantage of this approach. These include limits to spatial resolution, possible heating of the sample by the high laser powers used in 2PM, difficult to predict or measure excitation spectra, accelerated photobleaching (and associated photodamage) in the plane of focus, and the extra expense of appropriate laser systems, which limit the applicability of 2PM in spectral imaging. Despite these challenges, however, it is important to realize that many important studies have been enabled by 2PM, which would not have been possible using other imaging techniques.

6.5.1 Limits of Spatial Resolution in 2PM

Because of the longer wavelengths used in 2PM, in practice this approach yields somewhat poorer spatial resolution than confocal microscopy. In 2PM, the quadratic dependence on the excitation intensity reduces the size of the excitation volume by $\sqrt{2}$, but the use of light of double the wavelength broadens

the focal spot by a factor of 2. Use of a detection pinhole can improve the spatial resolution of 2PM, but this would negate most of the advantages of the approach (see Figure 6.8). Because of its use of shorter wavelengths, the resolution attained with a confocal microscope can be as small as half of that from a two-photon microscope. In biological fluorescence confocal microscopy, however, signal collection is typically optimized by opening the pinhole, which degrades spatial resolution. Thus, in practice two-photon microscopy provides spatial resolution that is only slightly worse than in confocal microscopy.

6.5.2 Potential Sample Heating by the High Laser Powers in 2PM

Red and infrared excitation light can cause localized heating effects in the sample when excessive powers are used. This heating is largely due to one-photon absorption of this excitation light by water. Since water is present in high concentrations in all biological systems, even a very small direct absorption can lead to significant numbers of excitation events. Water is not fluorescent, so most, if not all, of the absorbed energy is dissipated in the sample as heat. The absorption profile of water in the red and near-infrared region is shown in Figure 6.12 [5]. This is plotted on semilog scale, so the absorption at 980 nm is actually 100-fold greater than at 680 nm. Calculations of the localized heating during an imaging experiment suggest that the maximum heating even at 980 nm is <1 °C. For single-point experiments, though, where the laser beam is parked and not scanned, heating could be >1 °C s^{-1}. Using other wavelengths where the water absorption is lower (e.g., 800 or 1060 nm) can minimize any potential artifact from sample heating. Thus, for the laser intensities required for imaging using a Ti:sapphire laser, heating rarely causes any problem as long as one avoids parking the 980 nm beam. Use of the new OPO systems, which are tunable out to 1300 nm, though, could lead to the risk of more significant heating artifacts. Water absorption continues to rise throughout the infrared spectrum such that the absorption near 1300 nm can be almost 100-fold greater than at 980 nm. This means that a temperature rise of >10 °C might be possible for high laser intensities. As discussed previously, the spectral region from 1050 to 1300 nm has not been thoroughly explored so far, but it is clear that sample heating will be an important challenge in this spectral window.

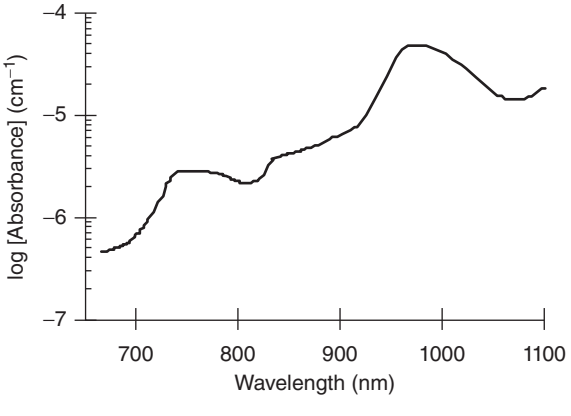

Figure 6.12 Semilog plot of the absorption coefficient for water in the near-infrared spectral region. Note that the water's absorption of light at 980 nm is over 100-fold greater than it is at 680 nm. (Data from Hale and Querry 1973 [5].)

6.5.3 Difficulties in Predicting and Measuring Two-Photon Excitation Spectra

Another challenge when using 2PM is that the two-photon excitation spectrum of a fluorophore can often be very different from the one-photon excitation spectrum due to the nature of the two-photon absorption process. This can be critical since choosing the wrong excitation wavelength can exacerbate other problems in 2PM, such as the sample heating and accelerated photobleaching. An example of the differences between the one-photon and two-photon absorption spectra is shown for the fluorophore diphenylhexatriene (DPH) in Figure 6.13. In this figure, the two-photon excitation spectrum has been plotted at half the wavelength for comparison (i.e., the two-photon absorption peak at 400 nm is actually at 800 nm). Knowing the one-photon spectrum, which is easy to measure, and doubling the absorption peak, an initial guess for the two-photon absorption peak would be 770 nm (2×385 nm). However, 770 nm is at a valley of the two-photon absorption. A much better choice would be 800 nm. This example, however, suggests a reasonable approach for optimizing the two-photon excitation wavelength. Beginning by doubling the one-photon peak, and then tuning the two-photon excitation wavelength above and below that initial wavelength, the maximum two-photon excitation can be found by maximizing the amount of fluorescence observed. In this case, the beginning wavelength would be 770 nm, but tuning every 10 nm in each direction would yield increased fluorescence signals at 750 and 800 nm.

One of the main reasons for the differences between the one-photon and two-photon absorption spectra is the differences in the strength of interactions with higher level excited states [7]. Figure 6.14 shows the relative absorptions in the fluorescent protein TagRFP by the lowest level singlet state (S_1) and higher order states (S_n) for both one-photon and two-photon excitation (in this case, the one-photon values are plotted at twice their wavelength for comparison – that is, the 550-nm one-photon absorption peak is plotted at 1100 nm). While the two-photon excitation efficiency of the S_1 state is greatly reduced in comparison with that of one-photon absorption, the two-photon excitation of the S_n states is greatly enhanced. This opens the possibility to use S_n excitation wavelengths (e.g., 750 nm) with 2PM, even though these transitions are too weak for efficient one-photon imaging. In this case, the transitions are well separated, which

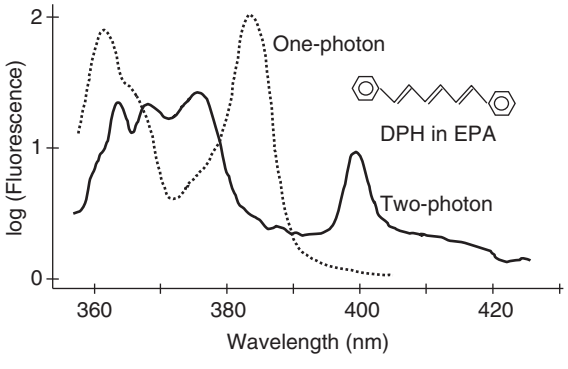

Figure 6.13 Two-photon excitation spectrum (solid line) of the membrane probe diphenylhexatriene (DPH) in an ether–isopentane–ethanol random glass matrix at 77 K. A portion of the one-photon absorption spectrum is shown by the dashed curve. (Adapted from Fang *et al.* 1978 [6]. Reproduced with permission of Elsevier.)

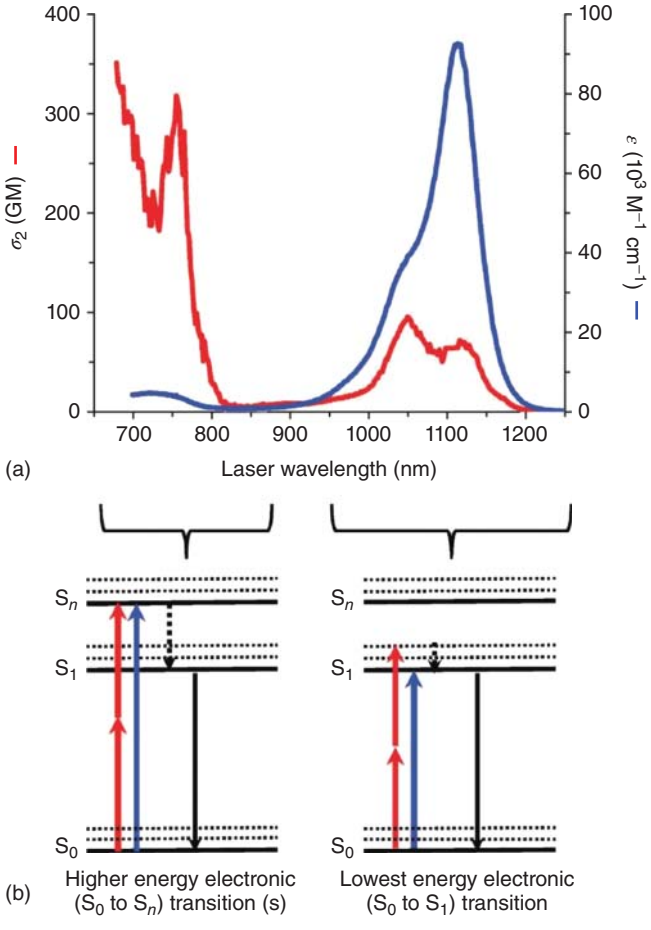

Figure 6.14 Structure of the two-photon absorption spectrum of a fluorescent protein. (a) One-photon absorption (blue) and two-photon absorption (red) spectra of TagRFP. (b) Jablonski diagram of 1PA and 2PA transitions. (Drobizhev *et al*. 2011 [7]. Reproduced with permission of Nature Publishing Group.)

makes sorting out these differences straightforward. However, even among the vibrational levels within the S_1 transition, there are differences between the one-photon and two-photon absorptions. This can be seen where the energy levels that form a shoulder in the one-photon spectrum at 525 nm are enhanced in the two-photon excitation spectrum. This leads to enhanced two-photon efficiency at 1050 over 1100 nm.

One common theme regarding the differences between one-photon and two-photon absorption spectra is the effect of the molecular symmetry of the fluorophore on the differences. The general rule of thumb is that symmetric fluorophores will exhibit a blue shift of the two-photon absorption peak. This means that the one-photon peak of 400 nm will appear in the two-photon spectrum at 700–750 nm rather than 800 nm (2×400 nm). Asymmetric fluorescent

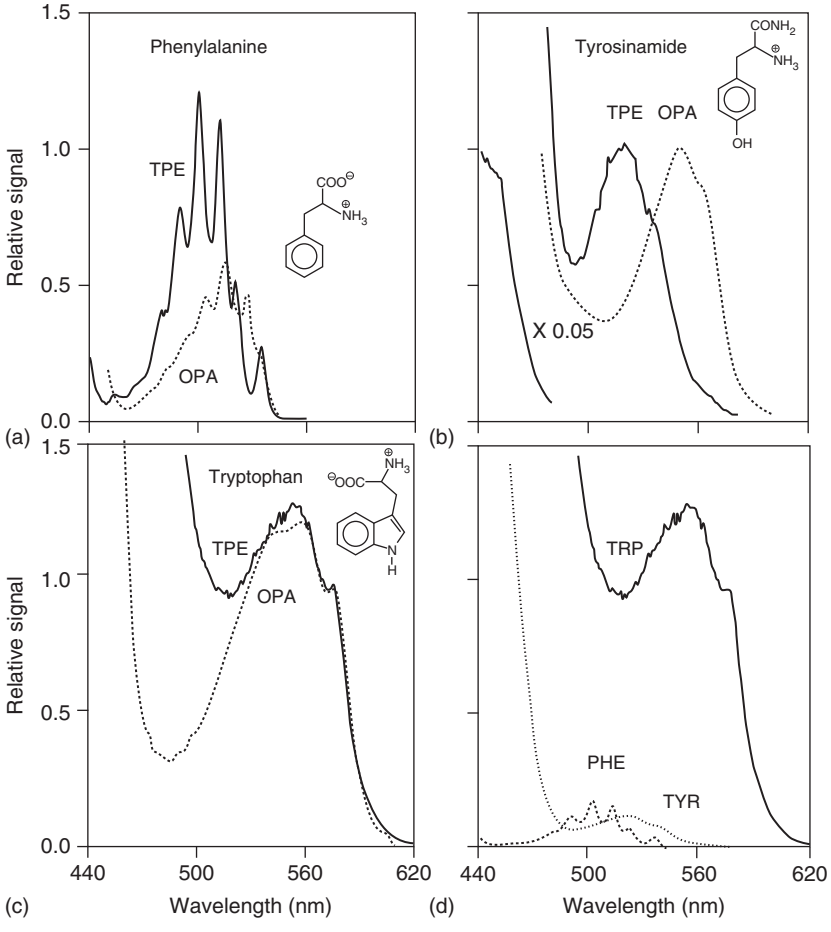

Figure 6.15 Two-photon excitation spectra (solid lines) with the corresponding one-photon UV absorption plotted at twice their actual wavelengths (dashed lines) for the aromatic amino acids (a) phenylalanine, (b) tyrosinamide, and (c) tryptophan in aqueous solutions at room temperature. (d) Comparison of relative two-photon signals of phenylalanine (Phe), tyrosinamide (Tyr), and tryptophan (Trp) observed at each compound's fluorescence maximum so that the relative signals are approximately in proportion to the relative product of the two-photon absorptivity and fluorescence quantum yield. (Reprinted with permission from Chemical Physics Letters, ©1993 [8].)

molecules are much more likely to show two-photon absorption peaks at exactly twice the one-photon peak wavelength. A good example of this is the different absorption spectra of the aromatic amino acids, especially tyrosine (symmetric) and tryptophan (asymmetric), as shown in Figure 6.15, where the one-photon wavelengths are plotted at twice their actual values [8]. The two-photon absorption spectrum of tryptophan almost perfectly overlays twice the one-photon absorption spectrum, while the two-photon absorption spectrum of tyrosine is blue-shifted by ~40 nm from the "expected" twice the one-photon absorption spectrum. This symmetry rule for two-photon excitation is very common among

fluorophores, and generally provides the best first guess at what an unknown two-photon absorption spectrum might look like.

Fluorophores are typically complex molecules, and it is not easy to predict their two-photon absorption spectra. Likewise, it is difficult to measure two-photon absorption, since this process utilizes a very small portion of the incident light. Thus, as described for the DPH example in Figure 6.13, excitation maxima are usually determined by tuning the laser and measuring the fluorescence of the fluorophore of interest normalized to a known two-photon excitation spectrum. Nowadays, the two-photon excitation spectra have been characterized for many commonly used fluorophores and fluorescent proteins, which can greatly facilitate their use with 2PM.

6.5.4 Accelerated Photobleaching (and Associated Photodamage) in the Focal Plane

One of the major advantages of 2PM is that photobleaching and any associated photodamage are limited to the focal plane. Over a thick specimen, this can greatly reduce the total photobleaching, especially in the acquisition of 3D data stacks. However, even if the total bleaching is reduced, there is a significant risk of actually increasing the local photobleaching within the focus. Because of the nonlinear absorption and high laser powers used in 2PM, there is an increased probability of photobleaching *at the focal plane.* The origin of this increased photobleaching remains unclear, but it is consistent with additional photons interacting with the excited state of the fluorophore and causing a transition to a state with a higher probability of photobleaching. Because of the high photon concentrations in the focus, it is easy to see how such transitions could be greater in 2PM. The practical manifestation of this accelerated photobleaching is demonstrated for fluorescein fluorescence and photobleaching in Figures 6.16 and 6.17. As described in Box 6.1, the amount of two-photon excited fluorescence is proportional to the square of the laser intensity ($F = I^2$). This intensity-squared dependence can be demonstrated clearly by a log–log plot ($\log(F) = 2\log(I)$), which should yield a straight line with a slope of 2 for two-photon excitation, as shown in Figure 6.16. It is possible to analyze the photobleaching rate in the same

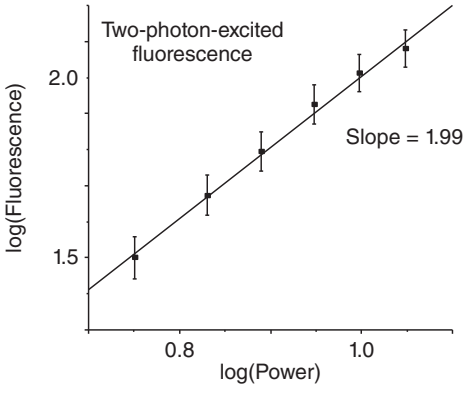

Figure 6.16 Log–log plot of the excitation dependence of fluorescein dextran fluorescence intensity for six levels of two-photon excitation with 710 nm laser light. The dependence exhibits a slope of ~2 indicating two-photon excitation. Data are displayed as mean ± SE ($n = 5$). (Data from Patterson *et al.* 2000 [9].)

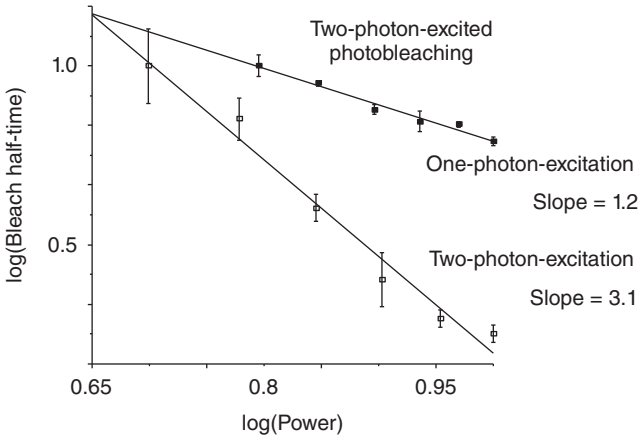

Figure 6.17 Log–log plot of the excitation dependence of fluorescein dextran average photobleaching rate as a function of laser power for one-photon excitation with 488 nm light (filled squares) and two-photon excitation with 710 nm light (open squares). The one-photon photobleaching slope is close to 1, indicating a near-linear photobleaching process, but the two-photon dependence is >3 (rather than the expected slope of 2). This higher slope generates accelerated photobleaching in 2PM. Data are displayed as mean ± SE ($n = 5$). (Data from Patterson and Piston 2000 [10].)

manner. If photobleaching is dependent only on the amount of fluorescence (as is thought to be the case for one-photon excitation), then the amount of photobleaching should be proportional to the square of the laser intensity ($P = I^2$). In turn, the log–log plot ($\log(P) = 2\log(I)$) should yield a straight line with a slope of 2 for two-photon excitation. However, measuring the amount of photobleaching for various laser intensities yields a different result, as shown in Figure 6.17. For one-photon excitation, the photobleaching dependence shown in this log–log plot is close to 1, as expected. However, the bleaching dependence on intensity for 2PM is not 2 but rather >3. This means that for fluorescein, the photobleaching is dependent on $I^{3.1}$. In other words, using twice the excitation power will yield fourfold more fluorescence but eightfold greater photobleaching. For this reason, minimization of excitation power is essential in 2PM to minimize not only any direct damage to the sample but also photobleaching (and any photodamage associated with the photobleaching).

It is also due to this accelerated photobleaching that 2PM will not always lead to significant improvements over confocal microscopy when imaging thin samples. Because of the accelerated photobleaching and photodamage, 2PM is poorly suited for most single-plane time-lapse imaging experiments. Of course, there are some cases (e.g., a single plane deep inside a tissue or use of UV-excitable fluorophores) where the experiments are just not feasible with other approaches, and this is where 2PM adds a novel approach.

6.5.5 Expensive Lasers Create a Practical Limitation for Some Experiments

A final practical limitation of 2PM is that the lasers needed for 2PM are expensive. Currently a state-of-the-art OPO system can cost as much as an entire entry-level

confocal microscope system. This can limit the access to the technology and also prevent some approaches that might be useful. For instance, because of the high cost of the lasers used, simultaneous multiple excitation wavelengths are rarely available. This makes multicolor imaging somewhat more difficult, although the tunability of most lasers used in 2PM allows one to find a single wavelength that comparably excites two fluorophores at the same time. So while two-color imaging is straightforward to implement in 2PM, hyperspectral imaging is more difficult to achieve, as compared to the multitracking (strobing on single excitations in sequence and detecting one fluorophore at a time) used in confocal and widefield fluorescence microscopy.

6.6 When is 2PM the Best Option?

The strengths and limitations of 2PM have been laid out, but it is useful to consider different scenarios regarding when it is better to use 2PM versus another approach, such as confocal microscopy. Since confocal microscopes are widely available, easy to use, and capable of delivering high-quality data, it makes good sense to use confocal microscopy first, and then try 2PM if the confocal approach is incapable of providing the needed data. However, there are three major situations where 2PM is almost always the better choice, and those are listed here along with a brief rationale explaining the advantages of 2PM for each situation.

6.6.1 Thick Specimen including *In Vivo* Imaging

The fact the 2PM is good for imaging deep into tissue has been stressed throughout this chapter, and the key advantages of 2PM for this work have been mentioned many times. Still, imaging structures that extend into the specimen or are buried in the specimen absolutely requires optical sectioning. Confocal microscopy can often accomplish the needed experiments by providing high-quality images at depths up to 100 µm or more into the sample. As detailed in Box 6.3, 2PM can extend this limit up to 1 mm. The reasons for this have been detailed previously. Similarly, 2PM is also the best option for *in vivo* imaging of structures within a living animal. First, it allows deeper imaging compared to confocal microscopy, which can be a major advantage, especially when excision of the specimen is not possible. 2PM also offers the advantage that it uses infrared light which is not seen by the animal. More importantly, though, reduced photobleaching and photodamage both contribute to reduced phototoxicity in the animal and thus make 2PM the preferred method for *in vivo* imaging. However, confocal microscopy is the better (and also cheaper) option when imaging thinner specimens, unless some other factors such as those listed below need to be considered. Further, for single cell layers, a conventional widefield fluorescence microscope is often an even better solution than confocal microscopy, since there might not be a need for optical sectioning in such a thin sample.

Box 6.3 Limits on the Depth of Imaging in 2PM

As discussed in the text, the potential limiting factors on the penetration depth in fluorescence imaging are the absorption of excitation light, absorption of fluorescence returning to the surface, and scattering of the excitation and fluorescence light. In the case of 2PM, the absorption of excitation light is quite small, so it does not play a limiting role. Similarly, scattering of the fluorescence is not a major source of signal loss, especially if NDD detection is used. Thus the two main sources of signal degradation are losses due to scattering of the excitation light and absorption of the fluorescence returning through the sample. The intensity of excitation will fall off as a result of light scattering in the sample as an exponential decay, e^{-ax}, where a is constant based on the amount of scattering and x is the distance the light travels through the sample. Similarly, the fluorescence intensity will also fall off exponentially, e^{-bx}, as it returns through the sample back to the microscope. Both these decreases can be described by a characteristic decay length related to the constants a and b. These constants vary between different tissues. To compensate for these losses and equalize the amount of signal collected at depth to that attained near the surface, the input laser power can be increased. However, there is a physical limit to how high this can be raised: at some point, the input laser power becomes high enough (even without the photon crowding at the focus) to generate two-photon excitation events at the surface of the sample. As described, the losses in the sample are proportional to e^{-x}, but the laser beam waist outside the focus is proportional to x^2. If we assume that the scattering coefficient a and absorption coefficient b are similar, then the loss of excitation due to scattering of the excitation light into the sample and the loss due to absorbance of fluorescence coming back out of the sample will be equal (this is a simplification, as the characteristic length of light scattering is likely much shorter than that for absorption in tissue). Imaging at a depth of 10 scattering lengths ($x = 10/a$) will attenuate the laser power by 450 000-fold, which will decrease the amount of two-photon excitation by a factor of $\sim 9 \times 10^9$. Under the assumption that $a = b$, the fluorescence will decrease by 450 000-fold as it returns through the sample, for a total signal decrease of $\sim 10^{15}$. To compensate for this loss, the excitation power would need to be raised by $\sqrt{10^{15}}$ or $\sim 3 \times 10^7$. At the same time, the area of the laser beam at the surface will have increased from the focus by only 10^2 or 100-fold. Thus, the laser intensity (power/area) at the surface would now be increased by 3×10^5 over what is needed to image through a nonscattering, nonabsorbing sample. This is comparable to the factor of 100 000-fold crowding by the objective lens focusing (see above). Since the tissue scattering length is typically $\sim 100\,\mu m$, this calculation is consistent with practical experience that the maximum depth of imaging is ~ 1 mm using green and orange fluorophores. Imaging red or near-infrared fluorophores using infrared 2PM may allow significantly greater imaging depths, but that has yet to be demonstrated.

6.6.2 Imaging Fluorophores with Excitation Peaks in the Ultraviolet (UV) Spectrum

Because of the large number of biomolecules that absorb UV light, it is often difficult to image UV fluorophores in live cells without compromising specimen integrity. UV photons have high energy, and they can easily produce cellular damage, especially in time-lapse imaging where the same region is imaged repeatedly over time. For optical sectioning, UV excitation becomes more problematic, as special objectives are necessary to properly transmit and focus UV light. 2PM using 680–800 nm light can excite fluorophores that are normally excited by UV light in the 340–400 nm range. The use of red and near-infrared light reduces scattering, and 2PM limits any photodamage to the focal plane, which greatly reduces it overall. Thus, 2PM can permit increased number of scans in a time-lapse experiment without incurring the amount of photodamage that UV light produces.

6.6.3 Localized Photochemistry

Two-photon excitation can stimulate any kind of optical transition, not just the excitation of fluorescence. In fact, 2PM is extremely useful for localizing photochemical reactions to selected regions of a specimen, since its nonlinear nature constrains excitation events to the focal volume (~1 μm^3). Using 2PM, it is possible to elicit a reaction inside a cell without any activation outside the cell or on the cell surface. The nature of the reaction depends on the photoactive chemicals used, but two-photon photoactivation is commonly used for uncaging photolabile compounds and photoswitching of fluorescent molecules (including fluorescent proteins). In all cases, the reactions could be triggered using one-photon excitation, but not with the same spatial confinement provided by 2PM. Similar to what is seen in one-photon photobleaching (Figure 6.6), photoactivation in confocal microscopy would occur throughout the sample – both above and below the target region. A further concern is that most photoactivation processes utilize UV light, so 2PM is even more preferred for these experiments as described previously. Several examples of 2PM photoactivation are given in the applications section.

6.7 Applications of Two-Photon Microscopy

Since the initial development of 2PM in 1990, the technique has expanded rapidly and impacted many fields of biological research. As described previously, the biggest application areas have been thick tissues, such as brain slices, and *in vivo* imaging, mainly in mice. A few of these applications are detailed in this section to highlight the role of 2PM in enabling them.

6.7.1 Imaging UV-Excited Fluorophores, such as NADH for Metabolic Activity

As discussed earlier, 2PM offers a convenient alternative to UV excitation, where single-photon excitation in the 340–400 nm range can be replaced by

two-photon excitation in the 680–800 nm range. This range is provided by commercial turn-key Ti:sapphire systems. There are many useful fluorescent probes that require UV excitation, but high phototoxicity associated with UV excitation can be problematic in live samples where relatively low fluorophore concentrations are necessary. One of the most successful applications of 2PM has been imaging metabolic activity via the endogenous fluorescent cofactor β-nicotinamide adenine dinucleotide (phosphate), abbreviated to NAD(P)H. NAD(P)H can be efficiently two-photon-excited at ~710 nm with much reduced phototoxicity compared with 355 nm UV excitation. In its oxidized form, NAD(P)H is fluorescent (it is one of the main components of UV-excited autofluorescence), but the reduced form, $NAD(P)^+$, is not fluorescent, so measuring NAD(P)H levels provides an assay of the *in situ* redox state. This assay has been used to elucidate the metabolic pathways in the islet of Langerhans, as highlighted in Figure 6.18. NAD(P)H is produced from NAD^+ in many metabolic processes such as glycolysis (in the cytoplasm) and citric acid cycle metabolism (in the mitochondria) [9]. Thus, as glucose is metabolized in the islet cells, the level of metabolic activity can be observed through NAD(P)H fluorescence. 2PM imaging of NADH levels has also been used to classify tissues as normal, precancerous, or invasive cancer, to determine how redox state regulates transcription, and to connect astrocyte metabolic activity and electrical activity *in vivo.*

Similar to imaging NAD(P)H, 2PM can be used to image other UV-excitable fluorophores with excellent 3D spatial resolution and overall reduced phototoxicity. Another useful UV-excitable fluorophore is Laurdan, which is a marker for membrane fluidity. 2PM of Laurdan has been used to investigate the importance of membrane order in cellular adhesion to the extracellular matrix, and how membrane order promotes acetylcholine receptor clustering in postsynaptic membranes.

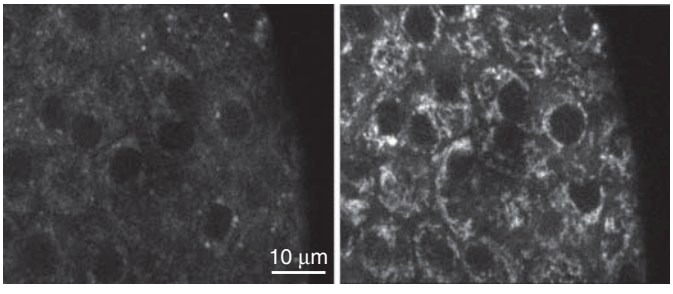

Figure 6.18 Imaging NAD(P)H with subcellular resolution in β-cells within a cultured pancreatic islet. Panel (a) shows the basal 1 level at 1 mM glucose and panel (b) shows the brighter autofluorescence reflecting the change in NAD(P)H-to-NAD+ ratio due to increased glucose metabolism. Both the cytoplasmic and mitochondrial NAD(P)H can be quantified representing cytoplasmic glycolysis metabolism and mitochondrial TCA metabolism, respectively. (Image adapted from data described in [9].)

6.7.2 Localized Photoactivation of "Caged" Compounds

2PM was originally conceived as a method for highly localized photoactivation of "caged" compounds such as caged calcium or the neurotransmitter caged glutamate. These caged compounds are initially inactive but can be activated by irradiation with light, typically in the UV region. The limitations of UV excitation were discussed earlier, but using UV light for uncaging cannot release the caged compound solely in a well-defined location because out-of-focus light will photoactivate the compound throughout the sample. Using 2PM, the uncaging is localized to the focus, and the use of red and infrared excitation light greatly reduces phototoxicity.

There are several general areas of photoactivation where 2PM has proven its worth. One of the simplest applications is the photoactivation of caged fluorophores. These molecules have a photolabile chemical moiety attached that quenches the fluorescence, but after stimulation of that moiety, the chemical dissociates and frees the fluorophore. Generally, these caged compounds are built around strong fluorescent molecules such as fluorescein and rhodamine, so the photoreleased signals are bright and robust. Fluorophore uncaging can be used for many purposes, such as cell coupling and flow measurements, intracellular diffusion measurements via an inverse FRAP (fluorescence recovery after photobleaching) approach where fluorescence is initially photoreleased in one spot and then its spread is measured, and cellular lineage tracing in cell growth and development. An example of this latter use is given in Figure 6.19, which shows a 3D reconstruction of an image stack acquired through a pre-gastrulation sea urchin (*Lytechinus variegatus*) embryo. This embryo arose from fertilization of an egg that had been injected with a caged fluorescein-10 kDa dextran, where one cell of the eight-cell embryo was scanned with the two-photon excitation beam. In the developing embryo, all descendants from the single photolabeled cell are fluorescent but the other cells are not. As seen in the two individual slices, cells in just one quadrant of the top half of the embryo are fluorescent, while no cells in the bottom half are fluorescent. The specificity of two-photon excitation along with its ability to penetrate deep within tissue allows this approach to be performed with any cell or group of cells anywhere within the specimen in an entirely noninvasive manner [11].

2PM has also found significant applications using photoswitchable fluorescent proteins, such as PA-green fluorescent protein (PA-GFP), which is efficiently converted to bright green fluorescence using two-photon excitation at 800 nm, and PAmCherry, which exhibits complicated photoactivation kinetics. For these genetically encoded labels, photoactivation is generally produced by photoisomerization between a cis and a trans state of the fluorophore within the fluorescent protein. It is often the case that the process of two-photon activation is complicated, as the laser light can cause transitions between multiple carboxylated and protonated states in addition to the photoisomerization. Further, the activation laser can trigger deprotonation within the decarboxylated states, while excitation of the photoactivated fluorescence simultaneously switches the fluorophores between their cis and trans forms and triggers protonation.

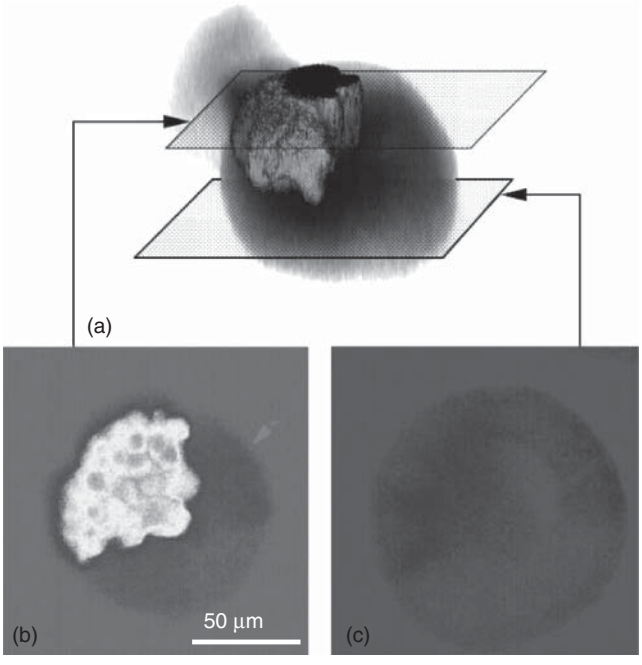

Figure 6.19 Two-photon uncaging for lineage tracing in embryo development. A caged fluorophore attached to dextran was released in a single cell of an eight-cell sea urchin embryo (*Lytechinus variegatus*). In the later pre-gastrulation embryo, shown as a full 3D reconstruction in (a), one-quarter of one hemisphere is brightly fluorescent (one-eighth of the total embryo). Two single optical sections of the 3D dataset are shown from the top hemisphere (b) which shows brightly labeled cells in one quandrant, and from the bottom hemisphere (c) which shows no fluorescence.

In contrast to photoactivation of fluorescence, 2PM photoactivation was first used to uncage carbamoylcholine for mapping of cell surface ligand-gated ion channel distribution in combination with electrophysiology, and the neuroscience field has continued to exploit this approach. One example combining two-photon photorelease of caged glutamate with 2PM imaging of neuronal structures is shown in Figure 6.20 [12]. This work used 2PM to activate excitatory potentials in well-defined locations along single dendritic spines in a brain slice culture. The precise photoactivation given by 2PM allowed the quantitative correlation of action potential strength and the spine length. This work expanded upon numerous previous studies using 2PM imaging and uncaging that defined how the network of individual dendritic spines and shafts processes excitatory inputs in terms of membrane potential summation and filtering. The reader is directed to reviews such as [13] covering the application of two-photon uncaging in neurosciences.

One area where the unique capabilities of 2PM are useful is optogenetics, where photoactivatable ion channels are used to manipulate cellular membrane potential. An elegant example of this approach is shown in Figure 6.21 [14]. Pyramidal cells in a brain slice from an optogenetic transgenic mouse can be patched and

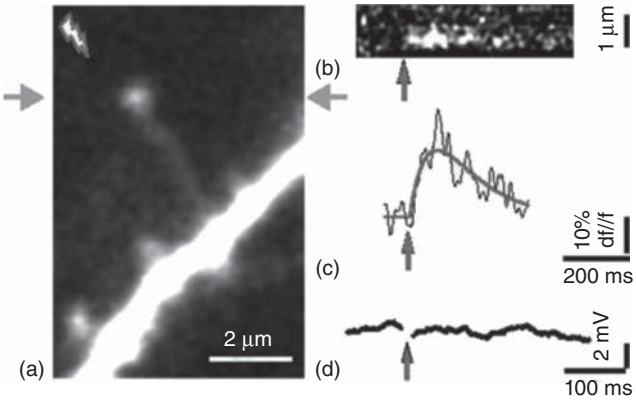

Figure 6.20 Inverse correlation between action potential amplitude and spine neck length in brain slice neurons stimulated with two-photon photoactivated caged glutamate. Synaptic activation by two-photon uncaging of glutamate near a single long-necked spine (a) generated a clear calcium response at its head (b,c), but no voltage deflection for uncaging at the soma (d). Arrows on either side of (a) mark the line scan intersecting the spine head for uncaging. Arrows on (b)–(d) denote the time of uncaging. (Reprinted with permission from Proceedings of the National Academy of Sciences, USA, ©2014 [12].)

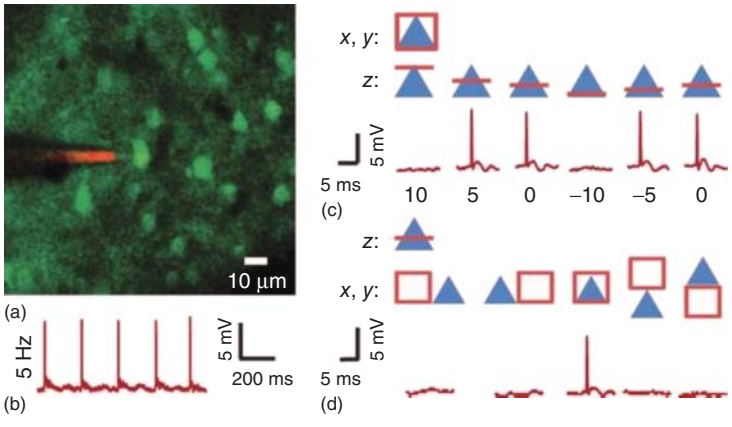

Figure 6.21 Optogenetics with 2PM. (a) *In vivo* two-photon image of layer 2/3 pyramidal cells (~150 µm from the surface of the brain) transduced with C1V1$_T$-p2A-EYFP (imaged during loose patch). (b) A trace of precise spike-train control with 5 Hz 1040 nm raster-scanning illumination; the amplitude and waveform of these evoked spikes recorded in cell-attached mode matched the spontaneous spikes in each cell. (c) The axial resolution of two-photon optogenetic control of spiking *in vivo*. Blue triangles indicate pyramidal neurons, and red boxes illustrate region-of-interest (ROI) positioning. Spiking of layer 2/3 cells as a function of raster scan position is shown (20×/0.5-NA objective; λ: 1040 nm; dwell time per pixel: 3.2 µs, scan resolution: 0.6 µm per pixel; line scan speed: 0.19 µm µs^{-1}; laser intensity: 20 mW). (d) Lateral resolution of two-photon optogenetic control of spiking *in vivo*. (Prakash *et al*. 2012 [14]. Reproduced with permission of Nature Publishing Group.)

imaged *in situ*. By two-photon stimulation of the optogenetic channels, action potentials can be triggered and observed by electrophysiology. As demonstrated in the figure, action potentials are observed only when the 2PM scan overlaps with the cell in all three dimensions, X, Y, and Z. No other optical approach offers this degree of precise specificity. Thus, two-photon optogenetics is expected to be a robust growth field over the coming years. More details are available in review articles, such as [15].

6.7.3 Imaging Electrical Activity in Deep Tissue

The principal advantage of 2PM is the increased penetration depth for thick-tissue imaging. One of the earliest applications of 2PM was in neuroscience, where this approach was used to map neuronal electrical activity in brain slices through imaging of cellular calcium dynamics. 2PM has become an indispensable tool in neuroscience for studying neuronal electrical activity and mapping neuronal architecture in brain slices as well as the brains of live animals. Imaging depths greater than 1 mm have been achieved in live mouse brains, which allows access to large swaths of the brain's information processing regions. An example of this superb depth penetration is shown in Figure 6.22, where a set of genetically labeled mouse neurons are imaged down to >800 µm into the brain [16]. To achieve useful images throughout this depth, the laser intensity was increased several times as the microscope was focused deeper into the brain. As described previously, however, this increase in intensity does

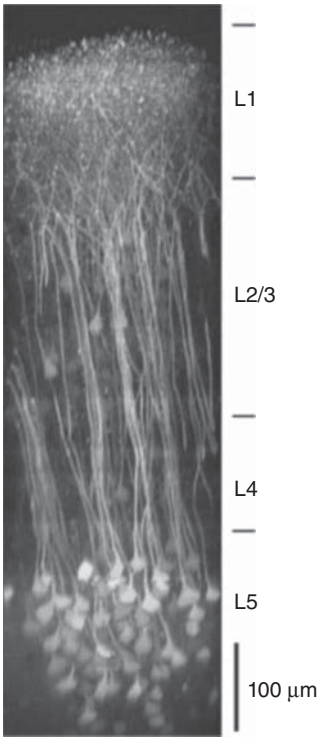

Figure 6.22 Deep 2PM in the brain of a live mouse expressing a genetically encoded indicator demonstrated with a maximum intensity *X–Z* projection of a fluorescence image stack. Nearly the entire depth of the neocortex can be imaged, but the laser excitation intensity needed to be increased several times (seen as lines of intensity change near the bottom of L2/3, and in L4 and L5). (Reprinted by permission from Macmillan Publishers Ltd, copyright (2005) [16].)

not compromise resolution and can be used to compensate for losses due to scattering of the excitation light and absorption of the resulting fluorescence.

2PM has also been applied to study electrical activity in the heart, whose tissue is denser than brain tissue, and whose movement also makes imaging more challenging. Studies of electrical activity through $[Ca^{2+}]$ imaging have been performed in excised hearts for the study of electrical synchronization. 2PM can enable subcellular studies of functions deep into excised hearts, for example, to measure mitochondrial responses to ischemia using a fluorescent indicator of membrane potential.

6.7.4 Light Sheet Microscopy Using Two-Photon Excitation

As described in the introduction, light sheet microscopy is a promising alternative to confocal microscopy, but it can be limited by penetration depth restrictions that arise from the use of single-photon excitation. This problem can be partially ameliorated by the use of two-photon excitation light sheets, where the combination of nonlinear excitation with the orthogonal illumination of light sheet microscopy can yield excellent performance for 3D imaging of thick biological samples. This is demonstrated in Figure 6.23, where equivalent images show that two-photon light sheets can image up to twice as deep as one-photon light sheet microscopy [17]. The geometry of light sheet microscopy does not permit NDD to be used, thus the depth increases only by a factor of ~2. Still, the hallmark advantages of 2PM are clear in the improved contrast and lack of background haze that degrades the one-photon light sheet image. Importantly, the light sheet approach was shown to collect images up to 10 times faster than point-scanning 2PM without compromising biological viability.

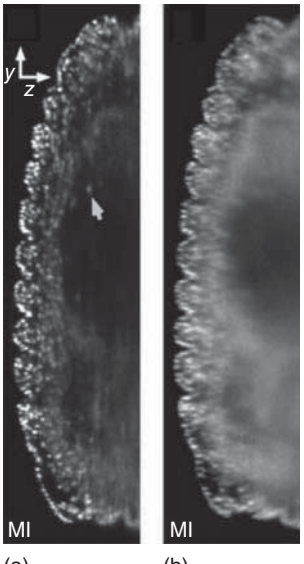

Figure 6.23 (a) Two- and (b) one-photon light sheet images of stage-13 embryos shown as *Y*–*Z* image slices of at $X = 90$ µm from embryo surface. The light sheet imaging was done with monodirectional illumination. Arrow in (a) indicates a deep cell at $X \approx 90$ µm and $Z \approx 70$ µm, which is clearly visible in the two-photon light sheet image, but not in the one-photon light sheet image. (Truong *et al*. 2011 [17]. Reproduced with permission of Nature Publishing Group.)

6.7.5 Other Applications of 2PM

There are numerous applications of 2PM, and the list grows longer each day. Here, a few of the other successful *in vivo* 2PM imaging experiments are briefly described. These include measurements of blood flow, kidney function, immune cell dynamics, and embryo development.

Functional intravital 2PM has been used extensively to study blood flow. These studies have been complementary to functional magnetic resonance imaging (fMRI), which can detect localized increases in blood oxygenation levels but does not have sufficient temporal and spatial resolution to resolve individual cellular events. 2PM allows resolution of individual capillaries in live mice to noninvasively study blood flow and quantitatively measure flow velocity with fluorophore-labeled red blood cells and blood plasma. As examples, this approach has been used to measure diverse physiological parameters such as blood flow variations in the olfactory bulb glomeruli as different smells activated specific neuronal units and in the islet of Langerhans as a function of glycemic state. 3D blood flow architecture has also been elucidated through 2PM-enabled targeted ablation of blood vessels, where single capillary ablation was possible with minimal collateral damage through the unique intrinsic confinement of two-photon excitation.

An obvious extension of 2PM blood flow measurements is for the investigation of renal tissue function. The kidney contains a complex network of vasculature, particularly in the glomerulus in which filtration occurs. 2PM imaging with fluorophores of different sizes and net charges can be used to quantify glomerular permeability, which is especially important in studying renal diseases.

The enhanced imaging depth provided by two-photon microscopy also allows for substantial improvements in 4D imaging (x, y, z, t) for observing *in vivo* dynamic processes. There has been a large body of work using two-photon microscopy for dynamically imaging immune cell motility, interactions, and clustering *in vivo*. These studies, many involving intravital imaging of lymph nodes and bone marrow, have all required high temporal and spatial resolution, 100 μm imaging depths, and with the minimal invasiveness to image for periods of hours, all afforded by two-photon microscopy.

Two-photon microscopy has many advantages when imaging live embryo development. One important demonstration of these improvements over confocal microscopy used time-lapse 3D imaging of hamster embryo development [18]. Since mammalian embryos are highly sensitive to culture conditions as well as exposure to light, the survival of these embryos to birth and normal development clearly showed the noninvasiveness of two-photon microscopy. Another strategy used with 2PM to study embryo development is the use of targeted ablations. For example, this approach has been used in Drosophila embryos to generate targeted deformation of specific tissue patterns. Quantifying these morphological changes yielded insight into the mechanisms of mechano-sensitive tissue development and gene expression.

Finally, intravital 2PM has been used to provide new insights into tumor pathology and physiology, since it can reveal gene expression and physiological function deep within regions of tumors that are not accessible with confocal microscopy.

This approach can be used to quantitatively resolve the vascular architecture, giving an insight into the mechanisms of angiogenesis in tumors, and to measure growth and localization of mutations in the tumor cells, such as those resistant to hypoxia-induced apoptosis. 2PM utilizing fluorescent semiconductor nanocrystals, called *quantum dots*, can also be used to image tumors. Quantum dots are very bright and resistant to photodamage, and, in combination with two-photon microscopy, have been shown to enhance imaging through highly scattering tissue such as skin and adipose tissue at 100 μm depth.

6.8 Other Nonlinear Microscopies

As mentioned at the outset, 2PM is a special case of a larger family of nonlinear microscopies. This family can be further divided into three sets of approaches: multiphoton absorption, harmonic generation, and Raman scattering. 2PM is the most prominent example of the multiphoton absorption approach, and the other utilized approach from this set is three-photon excitation. As the name implies, three photons can be absorbed simultaneously, and each of these provides one-third of the energy needed to reach the excited state. For example, infrared light at 1050 nm can be used to excite UV fluorescence that would normally be excited with 350 nm light. However, the higher order of nonlinearity means even weaker signals. Because this consequently requires higher photon fluxes, and does not add any additional advantages over 2PM, this technique has not been used as extensively as two-photon excitation.

Harmonic generation imaging is a fundamentally different nonlinear optical process. As for multiphoton absorption, the most common harmonic-generation-based approach is SHG imaging [19]. SHG imaging requires high photon densities just as 2PM, so the laser requirements for harmonic imaging are similar. The difference is in how the signal is created. In fluorescence, a photon is absorbed and then another is re-emitted. On the other hand, for optical harmonic generation, photons are not absorbed, but instead they interact with the sample via scattering. In SHG, incoming photons are simultaneously scattered and the two combine to generate a single photon that has double the energy of the incident photons (i.e., the scattered light has a wavelength that is half of the incident wavelength). SHG requires that the molecules in the specimen lack inversion symmetry and that they are spatially ordered, so this approach is useful for imaging ordered structures such as collagen fibers or microtubules, with the advantage that it does not require any labeling of the structures. In particular, the molecular structure of fibrillar collagen produces a strong SHG signal with minimal background from non-fibrillar components. Since collagen is an important structural protein in mammal, SHG imaging has become a useful tool for imaging its structural organization. THG, a three-photon analog of SHG, can also be observed, and used for imaging in some cases. Since the third harmonic signal does not require the lack of inversion symmetry in the specimen, this approach can be used to image more disordered molecules. For example, lipid bodies provide a major source of THG, allowing lipoid metabolism to be studied without the use of exogenous probes.

The third set of nonlinear microscopies fall into the category of light scattering approaches, based on Raman scattering. Two implementations that have proven feasible are CARS and SRS. The underlying photophysics, strengths, and limitations of these approaches are eloquently detailed in [20]. CARS is a third-order nonlinear optical process that is generally performed using three laser beams, which interact with the sample and generate a coherent optical signal at the anti-Stokes difference frequency. This anti-Stokes light is at a well-defined color that can be easily separated from the incident beams, and its intensity is resonantly enhanced at intrinsic vibrational modes of molecules in the sample. Leveraging the specific vibrational modes of lipid molecules, CARS can be used for noninvasive imaging of biological membranes, and implementations of CARS microscopy have been used to image myelin structures. The second Raman imaging approach uses SRS. In SRS, two laser beams are used to efficiently excite a specific molecular vibrational level, where for each quantum of the vibrational excitation being excited, one photon is annihilated from the pump beam and simultaneously a photon is created in the probe beam. The resulting intensity gain in the probe beam is called the *stimulated Raman signal*. Much like CARS, this signal can be used to detect intrinsic molecules within biological samples by targeting various vibrational bands. As an example, SRS imaging could reveal changes in the regulation of lipid storage in *Caenorhabditis elegans* resulting from genetic manipulation.

6.9 Future Outlook for 2PM

The outlook for 2PM is promising for further studies of *in vivo* physiology. Many important advances are currently under way that will enhance the utility of this approach. On the instrumentation front, the development of 2PM endoscopes has enabled 3D imaging with lateral resolutions of ~1 μm and axial resolutions of ~10 μm. Using these devices, 2PM imaging is no longer limited to 1 mm from the surface tissue, but may instead be used to image structures deep in living animals by introducing an endoscope via the esophagus, blood vessels, ear, or other entry points. Further developments in two-photon excitation light sheet or line-scanning microscopy will also expand the usefulness of 2PM by enhancing the speed of image acquisition. Finally, intravital 2PM can be improved through the use of adaptive wavefront corrections, which can compensate for image distortions caused by heterogeneous biological material. A second area of development is the optimization of fluorophores that are brighter and more red-shifted, including genetically encoded red and infrared fluorescent proteins. As described earlier, red-shifting the fluorescence reduces emission absorption and allows greater imaging depths.

6.10 Summary

Advances in our understanding of two-photon excitation microscopy (2PM), coupled with modern digital imaging instrumentation, have facilitated the use of this physical phenomenon whose existence was predicted 80 years ago.

Turnkey mode-locked laser systems have made 2PM straightforward for all researchers to use. Because of the nonlinear nature of two-photon excitation, it is confined to the focal plane, which results in inherent optical sectioning capability without the need for a confocal pinhole. Thus, photon collection can be more efficient, especially through the use of NDD. Further, confinement of the excitation reduces photobleaching and associated photodamage to the specimen. Because of the unique photophysics of two-photon excitation, 2PM imaging is not significantly degraded by light scattering in the sample, so it can be used to image thick specimens down to depths >1 mm, up to 10-fold deeper than with confocal microscopy.

Despite its strengths, it is important to keep in mind that 2PM is not a magical do-all replacement to the confocal microscope. Complications having to do with possible sample heating, unknown two-photon absorption spectra, and accelerated photobleaching and photodamage at the focal plane mean that, for many experiments, the confocal microscope is still a better instrument. 2PM provides much improved depth penetration for imaging, so it is usually the best option for *in vivo* fluorescence microscopy. Leveraging this advantage, many studies have used 2PM to determine 3D structure and morphology as well as to gain functional information on 3D motility, electrical activity, blood flow, and metabolic activity. 2PM also permits spatially localized perturbations to be introduced into systems through using targeted photo-uncaging or photoablations or optogenetics. Finally, endogenous UV-excited molecules, such as NAD(P)H or elastin that are difficult to image in live cells with conventional fluorescence microscopy, can be imaged by 2PM with minimal loss of cell viability.

As 2PM has become more accessible to biological research, it has been used for a wide range of challenging physiological studies. Successful 2PM applications span the gamut from neuroscience to immunology to cancer to developmental biology to endocrinology, all demonstrating the advantages that 2PM can bring to these and other fields of study.

Acknowledgment

I am grateful to Zeno Lavagnino for his collection of data for some of the demonstration images.

References

1 Denk, W., Strickler, J.H., and Webb, W.W. (1990) Two-photon laser scanning fluorescence microscopy. *Science*, **248** (4951), 73–76.
2 Goppert-Mayer, M. (1931) Elementary file with two quantum fissures. *Ann. Phys.*, **9** (3), 273–294.
3 Sandison, D.R. and Webb, W.W. (1994) Background rejection and signal-to-noise optimization in confocal and alternative fluorescence microscopes. *Appl. Opt.*, **33** (4), 603–615.
4 Denk, W. and Svoboda, K. (1997) Photon upmanship: why multiphoton imaging is more than a gimmick. *Neuron*, **18** (3), 351–357.

5 Hale, G.M. and Querry, M.R. (1973) Optical-constants of water in 200-nm to 200-μm wavelength region. *Appl. Opt.*, **12** (3), 555–563.

6 Fang, H.L.B., Thrash, R.J., and Leroi, G.E. (1978) Observation of low-energy Ag-1 state of diphenylhexatriene by 2-photon excitation spectroscopy. *Chem. Phys. Lett.*, **57** (1), 59–63.

7 Drobizhev, M., Makarov, N.S., Tillo, S.E., Hughes, T.E., and Rebane, A. (2011) Two-photon absorption properties of fluorescent proteins. *Nat. Methods*, **8** (5), 393–399.

8 Rehms, A.A. and Callis, P.R. (1993) Two-photon fluorescence excitation-spectra of aromatic-amino-acids. *Chem. Phys. Lett.*, **208** (3-4), 276–282.

9 Patterson, G.H., Knobel, S.M., Arkhammar, P., Thastrup, O., and Piston, D.W. (2000) Separation of the glucose-stimulated cytoplasmic and mitochondrial NAD(P)H responses in pancreatic islet beta cells. *Proc. Natl. Acad. Sci. U.S.A.*, **97** (10), 5203–5207.

10 Patterson, G.H. and Piston, D.W. (2000) Photobleaching in two-photon excitation microscopy. *Biophys. J.*, **78** (4), 2159–2162.

11 Piston, D.W., Summers, R.G., Knobel, S.M., and Morrill, J.B. (1998) Characterization of involution during sea urchin gastrulation using two-photon excited photorelease and confocal microscopy. *Microsc. Microanal.*, **4** (4), 404–414.

12 Araya, R., Vogels, T.P., and Yuste, R. (2014) Activity-dependent dendritic spine neck changes are correlated with synaptic strength. *Proc. Natl. Acad. Sci. U.S.A.*, **111** (28), E2895–E2904.

13 Svoboda, K. and Yasuda, R. (2006) Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron*, **50** (6), 823–839.

14 Prakash, R., Yizhar, O., Grewe, B., Ramakrishnan, C., Wang, N., Goshen, I. *et al.* (2012) Two-photon optogenetic toolbox for fast inhibition, excitation and bistable modulation. *Nat. Methods*, **9** (12), 1171–1179.

15 Oron, D., Papagiakoumou, E., Anselmi, F., and Emiliani, V. (2012) Two-photon optogenetics. *Prog. Brain Res.*, **196**, 119–143.

16 Helmchen, F. and Denk, W. (2005) Deep tissue two-photon microscopy. *Nat. Methods*, **2** (12), 932–940.

17 Truong, T.V., Supatto, W., Koos, D.S., Choi, J.M., and Fraser, S.E. (2011) Deep and fast live imaging with two-photon scanned light-sheet microscopy. *Nat. Methods*, **8** (9), 757–760.

18 Squirrell, J.M., Wokosin, D.L., White, J.G., and Bavister, B.D. (1999) Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability. *Nat. Biotechnol.*, **17** (8), 763–767.

19 Campagnola, P.J., Wei, M.D., Lewis, A., and Loew, L.M. (1999) High-resolution nonlinear optical imaging of live cells by second harmonic generation. *Biophys. J.*, **77** (6), 3341–3349.

20 Min, W., Freudiger, C.W., Lu, S., and Xie, X.S. (2011) Coherent nonlinear optical imaging: beyond fluorescence microscopy. *Annu. Rev. Phys. Chem.*, **62**, 507–530.