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Photon Upmanship: Why Multiphoton Imaging Is More than a Gimmick

Techreview

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Introduction

The unique niche that light microscopy occupies in biology is based on the ability to perform observations on living tissue at relatively high spatial resolution. This resolution is limited by the wavelength of light and does not rival that of electron microscopy, which is, however, a fundamentally nonvital form of observation. Other vital microscopies, such as MRI, can neither resolve subcellular structures nor provide the exquisite molecular selectivity that allows the detection of even single molecules in a background of billions of others.

Three-dimensional light microscopy inside living tissues has been hampered by the degradation of resolution and contrast caused by light scattering, which is due to refractive index inhomogeneities present to a varying degree in every tissue. Deeper into the tissue, image degradation becomes progressively more severe and high resolution imaging eventually becomes impossible. A major step toward overcoming this problem was the invention of confocal microscopy (Minski, 1961), which uses the resolving power of the objective lens twice: first, the illumination light is focused to a diffraction-limited spot; second, the signal photons are focused onto a detector pinhole that rejects scattered and out-of-focus light. Practical confocal microscopes (Amos et al., 1987) had to wait for the development of reliable laser sources and, equally important, for the advent of digital data aquisition, storage, and processing (for a comprehensive selection of reprints, see Masters, 1996).

The main drawback of confocal microscopy is its wasteful use of excitation, since absorption occurs throughout the specimen, but information is obtained only from a thin slice around the focal plane (henceforth called the focal slice). This is a major problem particularly in vital fluorescence microscopy where the ability to observe is usually limited either by the photodestruction of the fluorophore (photobleaching) or by photodynamic damage to the specimen mediated by the fluorophore or by endogenous chromophores (photodamage). In confocal microscopy, only ballistic fluorescence photons (i.e., photons that have not been scattered on their path out of the tissue) contribute to the signal, while scattered photons, often the majority, are rejected by the detector aperture. The increase in excitation necessary to compensate for this signal loss further exacerbates photobleaching and photodamage.

Virtually all of these problems can be solved by the use of multiphoton optical absorption to mediate excitation in laser scanning microscopy (Denk et al., 1990). (Multiphoton absorption is often called nonlinear because of the absorption rate's dependence on a higher than first power of the light intensity.) The possibility of absorbing more than one photon during a single quantum event had been predicted more than 60 years ago (Goeppert-Mayer, 1931), but experimental confirmation was delayed until after the invention of the laser (Kaiser and Garrett, 1961) because of the extremely high light intensity necessary to generate appreciable 2-photon excitation. The following comparison illustrates this point. In bright sunlight, a molecule of rhodamine B, an excellent 1- or 2-photon absorber, absorbs a photon through a 1-photon process about once a second, a photon pair by 2-photon absorption every 10 million years; no 3-photon absorption is expected throughout the entire age of the universe.

During the decades following its first demonstration, 2-photon absorption was mainly used for the spectroscopic study of excited molecular states (Friedrich, 1982). This makes sense in spite of the large experimental effort required for 2-photon spectroscopy (Birge, 1986) because different quantum–mechanical selection rules make 2-photon spectra genuinely complementary to and not just wavelength-scaled versions of their 1-photon counterparts. This is highly relevant for 2-photon microscopy where 2-photon spectra are needed for choosing appropriate excitation wavelengths. Fortunately, reliable data are now available for a large number of biologically relevant fluorophores (Xu and Webb, 1996; Xu et al., 1996). Odd (e.g., third) order excitation spectra are expected to be more similar to the linear spectrum (Xu et al., 1996).

Most 2-photon spectroscopy experiments were carried out using nanosecond laser pulses with high pulse energies but low repetition rates. Only the development of mode-locked lasers (for a selection of reprints, see Gosnell and Taylor, 1991) with pulse durations below 1 ps and repetition rates of about 100 MHz made 2-photon laser scanning microscopy (2PLSM) feasible in practice (Denk et al., 1990). Short pulses boost multiphoton absorption because average absorption is no longer simply proportional to average power, as in the 1-photon case, but benefits greatly from packing the available excitation photons into short temporal intervals. In this respect, multiphoton absorption behaves just like a chemical reaction with a large Hill coefficient, equal to the number of photons absorbed simultaneously. Mode-locking a Ti:sapphire laser, for example, boosts the 2-photon excitation rate 100,000-fold, compared to continuous-wave (cw) operation at the same average power.

The steep dependence of the absorption rate on the photon concentration, i.e., light intensity, gives multiphoton excitation laser scanning microscopy (MPLSM) its optical sectioning properties because fluorescence is generated virtually only in the vicinity of the geometrical focus (henceforth called the focal volume) where the light intensity is high (Figure 1). While scanning the laser focus in both lateral (x and y) directions, fluorescence excitation is limited to the focal slice, and a crisp image of a single section through the specimen is generated. No detector pinhole is necessary since no fluorescence

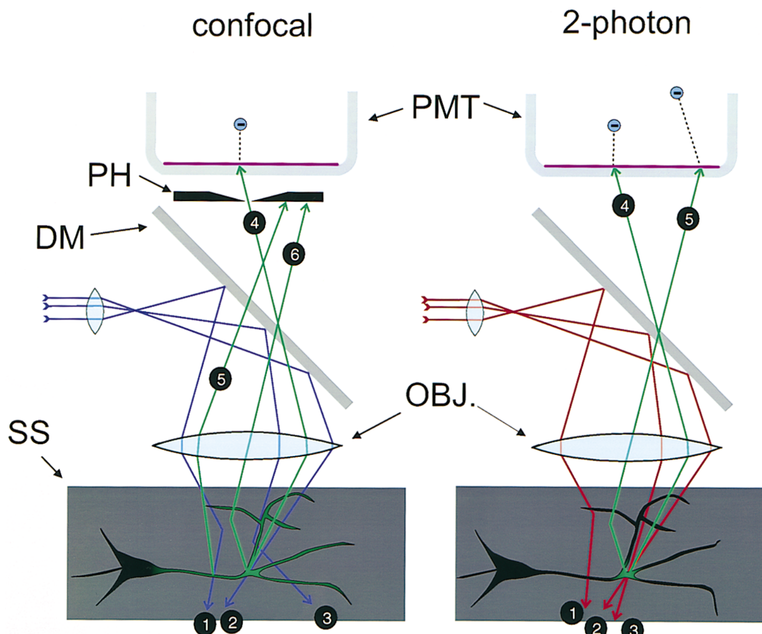

Figure 1. Imaging in Scattering Media

Without multiphoton excitation, one has to choose between resolution and efficient light collection when imaging in scattering samples. Nonlinear excitation imaging lifts that constraint as is illustrated here in a comparison to confocal 1-photon imaging (the scan optics are omitted for clarity, but see Figure 2). Typical fates of excitation (blue and red lines) and fluorescence (green lines) photons. In the confocal case (left), the excitation photons have a higher chance of being scattered (1 and 3) because of their shorter wavelength. Of the fluorescence photons generated in the sample, only ballistic (i.e., unscattered) photons (4) reach the photomultiplier detector (PMT) through the pinhole, which is necessary to reject photons originating from off-focus locations (5) but also rejects photons generated at the focus but whose direction and hence seeming place of origin have been changed by a scattering event (6). Excitation, photobleaching, and photodamage occur throughout a large part of the cell (green region). In the multiphoton case (right), a larger fraction of the excitation light reaches the focus (2 and 3), and the photons that are scattered (1) are too dilute to cause 2-photon absorption, which remains confined to the focal volume where the intensity is highest. Ballistic (4) and scattered photons (5) can be detected, as no pinhole is needed to reject fluorescence from off-focus locations.

is generated outside the focal volume, and all fluorescence photons, whether leaving the sample on scattered or ballistic trajectories, constitute useful signal.

This unique combination of optical sectioning and efficient use of molecular excitations permits one to peek deeply into brain slices and lets one record, with virtually undiminished spatial resolution, functional signals, for example, from single dendritic spines (Yuste and Denk, 1995; Denk et al., 1995c). Tissue penetration is further helped by the longer excitation wavelengths (roughly twice what they would be for single-photon excitation of the same fluorophores). Scattered excitation light, on the other hand, is just harmlessly bouncing around, too dilute to 2-photon excite, too long in wavelength to be absorbed otherwise (Denk et al., 1994; Denk, 1996).

Photochemistry, destructive during bleaching or photodynamic damage, can be put to good use in uncaging (reviewed by Corrie and Trentham, 1993). Again, multiphoton excitation confines the uncaging volume and allows, for example, the mapping of receptors on cell surfaces (Denk, 1994). By disturbing the equilibrium of fluorophore distribution using 2-photon uncaging or photobleaching, and then watching it relax, transport properties can be measured on a microscopic scale (Svoboda et al., 1996b).

Instrumentation

Beam scanning and data aquisition in a multiphoton microscope are essentially identical to those found in most confocal microscopes (Figure 2; for a more detailed discussion of many technical issues, see Denk et al., 1995b). In fact, commercial laser-scanning (but not Nipkow-disk) confocal instruments can easily be converted for 2-photon operation (Denk et al., 1990). The different excitation wavelengths require replacement of the dichroic beam splitter (separating excitation and fluorescence) and sometimes of other dielectric mirrors in the excitation path. We normally use protected silver coatings on the scanning mirrors because of their superior reflectivity in the infrared wavelength range. A variety of modalities for fluorescence detection are possible in MPLSM (Denk et al., 1995b) and can be implemented as modifications of a standard instrument (Figure 2). De novo instrument design offers, however, an opportunity for simplification since emission descanning is rarely necessary or even desirable (Denk et al., 1995b).

Fluorescence Detection

In experiments in this laboratory, mostly performed on neural tissue, the "whole-field" approach has been used where the photodetector always "sees" all the light (in the wavelength region admitted by the detection filter) that enters the objective lens. Aside from simplifying the optical path considerably, this allows the detection of fluorescent photons that were generated at the focus but subsequentially scattered and now appear to come from a different location (see Figure 1). In a confocal microscope, these scattered photons would be rejected by the detector aperture (Denk et al., 1994) because the field seen by a typical confocal aperture, even if "opened up", subtends only several microns, while the scattered light halo has a diameter on the order of the focal depth inside the tissue. One drawback of whole-area detection is its sensitivity to ambient light (Denk et al., 1995b), which requires a light-tight microscope enclosure or operation with the room lights off. Under some circumstances, it may be important to exploit the additional resolution that can be obtained by using descanned detection in conjunction with a confocal pinhole (Stelzer et al., 1994). This resolution improvement, however, comes at the expense of decreased collection efficiency and increased sensitivity to chromatic aberration (Denk, 1996).

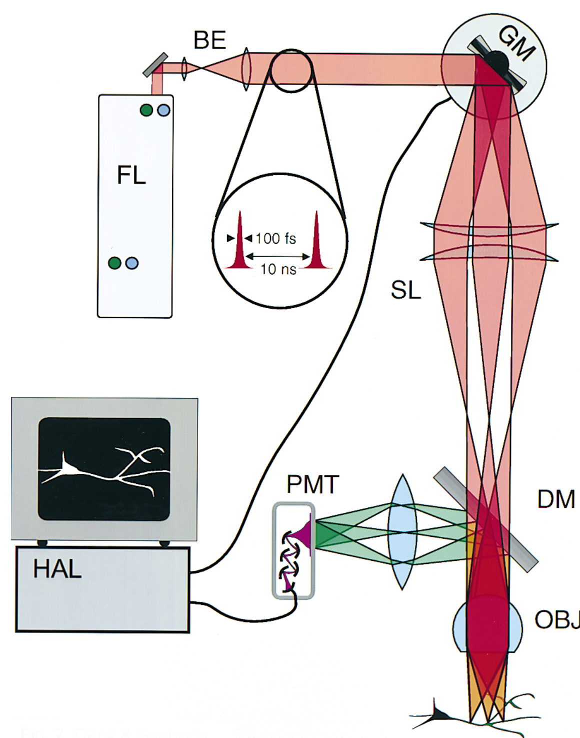

Figure 2. Generic Multiphoton Microscope

The excitation light, a train of femtosecond pulses from the mode-locked (FL) laser, passes the beam expander (BE), a pair of galvanometer scanning mirrors (GM, only one axis shown), the scan-lens (SL, essentially a low magnification eyepiece), intermediate optics inside the microscope (not shown), the objective lens (OBJ), and comes to a diffraction-limited focus inside the specimen. Some fraction of the 2-photon excited fluorescence passes back through the objective (detection of light entering the condensor or coming off to the side is possible as well) and is deflected by a dichroic mirror (DM) through a filter (blocking excitation light, not shown) into the photomultiplier detector (PMT). This is the whole-area detection mode (see text). Alternatively (not shown), but with loss of signal, the descanned detection path familiar from confocal microscopy can be used where the fluorescence light has to pass the scan lens and the scan mirrors to reach the dichroic beam splitter and, finally, detection pinhole and PMT. A computer (HAL) controls the scanners and synchronously records the signal from the PMT.

Detection filters and photodetectors are, of course, selected for high transmission and high quantum efficiency, respectively. In contrast to conventional 1-photon fluorescence microscopy, excitation and emission wavelengths are typically far apart in MPLSM. Therefore, colored glass filters (such as BG 39 or BG 40, Schott) sometimes offer better performance than interference filters, in terms of transmission of fluorescence and blockage of residual excitation light.

Recently, this laboratory has begun using a novel type of detector (La Rue et al., 1993), an "intensified photodiode" (Model IPD with GaAsP photocathode; Intevac), which offers much better quantum efficiency and lower noise than the traditionally used photo-multiplier tubes and a large enough sensitive area for whole-area detection (Denk et al., 1995b).

Light Sources

Efficient multiphoton excitation of fluorescence requires the short pulses and high repetition frequencies of mode-locked lasers. The light source of choice in this and other laboratories has been the Ti:sapphire laser (Curley et al., 1992). Reliable instruments are now available from several commercial manufacturers (Spectra Physics, Coherent, Clark MRX), most recently with diode-pumped frequency doubled NdYAG lasers as "all-solid-state" pump sources. Pulse widths (50–100 fs) and repetition rates (~100 MHz) are close to their optimal values as constrained by spectral width of dye absorption, acceptable pulse broadening due to group-velocity dispersion, and fluorescence decay times (Denk et al., 1995b; Denk, 1996). The spectral range covered by Ti:sapphire lasers (690–1050 nm) permits 2-photon excitation of many commonly used biological fluorophores (Xu and Webb, 1996; Xu et al., 1996). Fortunately, the frequently used xanthene dyes (fluorescein and rhodamine) and their derivatives, among them the Ca^{2+} indicators Calcium Green and Calcium Orange (Molecular Probes), are good 2-photon absorbers, albeit with blue-shifted spectra (Xu and Webb, 1996).

Wavelengths shorter than can be reached by Ti:sapphire lasers are needed for the excitation of certain uncaging groups (Denk, 1994). Dye lasers can provide ultrashort pulses at such wavelengths, but their operation requires considerable effort and expertise and should not be embarked upon lightly.

Mode-locked light sources pumped directly by laser diodes are beginning to be available for multiphoton excitation (Svoboda et al., 1996a; Wokosin et al., 1996) (Figures 3A and 3B). These lasers lack the tunability found in Ti:sapphire lasers, but their lower cost (initial and operational) should make them attractive for many applications. Due to the broad 2-photon absorption spectra of many dyes (Xu and Webb, 1996; Xu et al., 1996), the lack of tunability is less of a drawback than it may initially seem.

Imaging Modalities

As a general rule, the multiphoton laser scanning microscope can be used in a manner very similar to a laser scanning confocal fluorescence microscope. This includes the aquisition of image stacks for subsequent 3-D reconstruction (Figure 3A). When limited by bleaching or photodamage, the number of photons that can be aquired for each voxel is increased, however, by orders of magnitude compared to confocal microscopy. For quantitative measurements, this laboratory frequently uses the "line-scan" mode where one spatial dimension has been traded for time resolution, which is now in the millisecond regime, well matched to many neurophysiological phenomena (Yuste and Denk, 1995; Denk et al., 1995a, 1995c; Svoboda et al., 1996b).

Applications

Calcium Dynamics in Small Structures

Experiments in this laboratory have concentrated mostly on functional imaging in scattering tissue, such as living brain slices (Figure 2). There, the tissue penetration and reduced photodamage of 2PLSM is crucial for the observation of individual dendritic spines at a depth where the effects of the slicing trauma are no longer severe. In hippocampal CA1 pyramidal cells (Yuste and Denk, 1995), synaptic failures in individual spines and supralinearity of Ca^{2+} influx (a potential mechanism for Hebbian

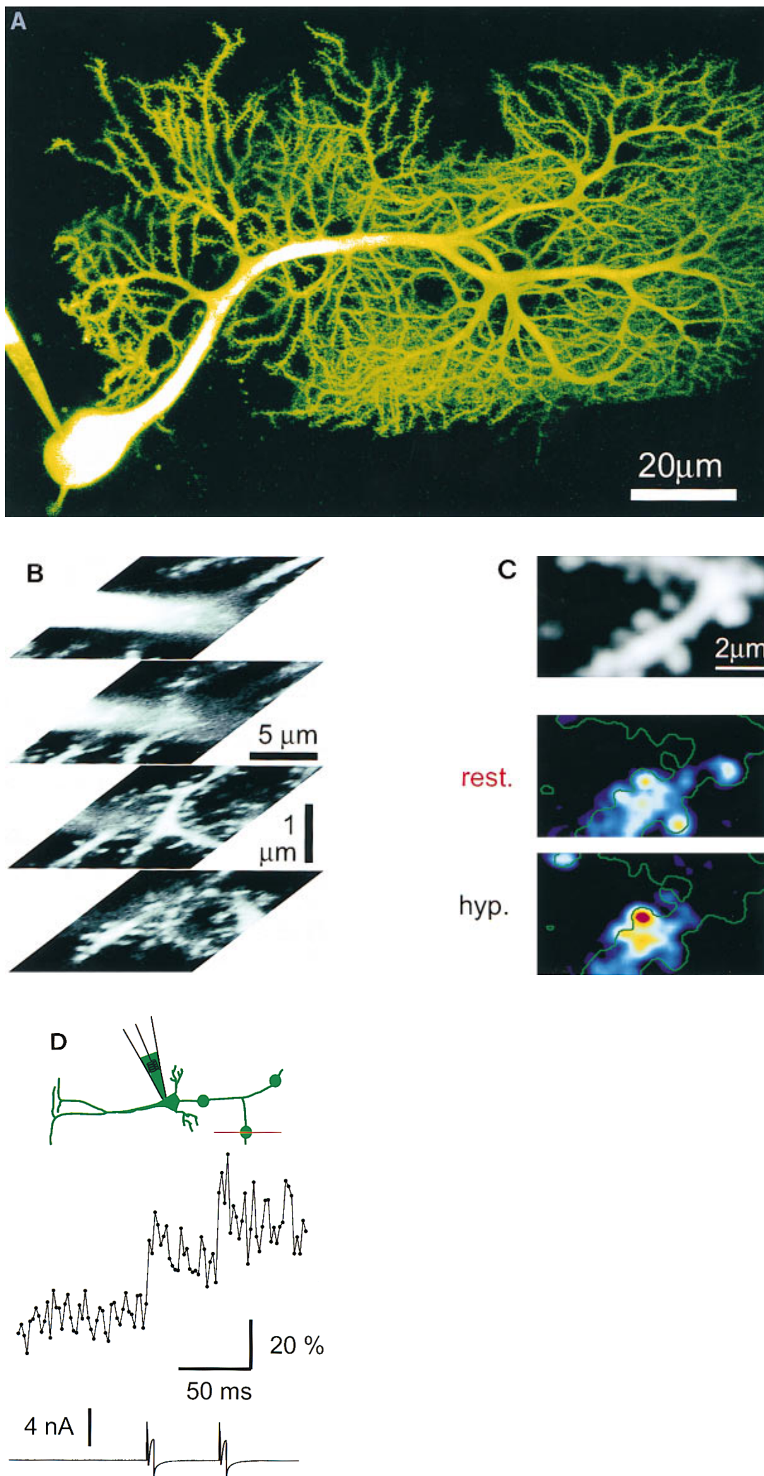

Figure 3. Three-Dimensional Functional Imaging in Brain Slices

2-photon excitation without and 1-photon excitation with confocal detection are (aside from the wavelength dependence of resolution) equivalent in their optical point-spread functions, including their optical sectioning properties (Sheppard and Gu, 1990). This figure shows images acquired using 2PLSM

(A) Maximum projection of a stack of optical sections through a living Purkinje cell filled with fluorescein dextran (400 μm).

(B) At higher resolution, a sequence of sections taken at 1.8 μm focus intervals (objective lens: 63×, 0.9 NA, water immersion, Zeiss) of a piece of dendrite belonging to the same cell.

(C) The anatomy of a spiny branchlet and its response to focal stimulation of parallel fibers while the cell is held at its resting potential (rest.) and at strongly hyperpolarized levels (hyperpol.; Denk et al., 1995c).

(D) The calcium dynamics in a presynaptic terminal during a pair of action potentials (bottom trace) in a neocortical pyramidal cell is shown (single trial). Calcium concentration was measured using fluorescence (100 μM Calcium Green 1) recorded in line-scan mode. The excitation source was a prototype diode-pumped Cr:LiSrAlFl laser (Svoboda et al., 1996a) in (A) and (B) and a Ti:sapphire laser in (D) and (D).

coincidence detection) were observed. In Purkinje cells (Denk et al., 1995c), distinct functional classes of spines were found, distinguished by their Ca^{2+} response at hyperpolarized levels. The different spine types occur in close spatial proximity and would remain unresolved by techniques with less spatial discrimination (Figure 3C). Similar challenges are posed by the measurement of presynaptic Ca^{2+} dynamics (Figure 3D).

Even in situations where scattering is not a problem, 2PLSM can be used to keep excitation away from sensitive regions of the cell. This property was exploited when measuring calcium influx into individual hair-cell stereocilia (Denk et al., 1995a), confirming that Ca^{2+} influx occurs at the tips of hair bundles and showing that mechanically gated channels can be located at both ends of tip links.

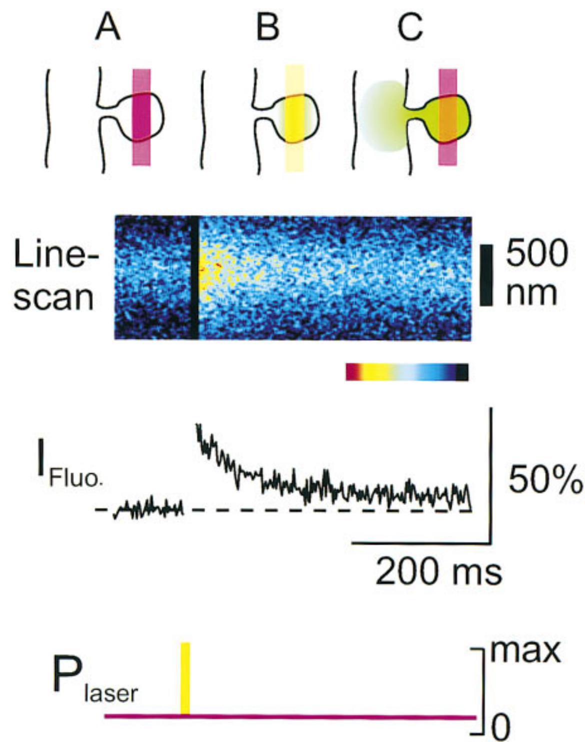

Figure 4. 2-Photon Uncaging

Photochemical activation of fluorophore, which can then be traced using 2-photon excited fluorescence, allows the measurement of diffusive transport with high spatial resolution. Shown here is an experiment (Svoboda et al., 1996b) that allows quantitation of how much the narrow neck of synaptic spines restricts diffusional exchange of small molecules (in this case, fluorescein conjugated to 3 kDa molecular weight dextran) between spine head and dendritic shaft. Long wavelength light, which does not consist of sufficiently energetic photons to excite the caging group even via 2-photon absorption, from a Ti:sapphire laser (λ = 830 nm, depicted in red) is used to image the cell and establish first (A) the baseline level of fluorescence in a spine. The same spine is then exposed briefly (B) to light from another Ti:sapphire laser, tuned to 700 nm (depicted in orange), which is sufficiently short in wavelength and hence high enough in quantal energy to 2-photon excite the caging group. This produces free fluorescein dextran that is initially confined to the spine head but then diffuses through the neck leading to a gradual decline of the fluorescence as measured using the monitor beam at 830 nm (C). The same information can be obtained by introducing free fluorophores into the cell and then selectively reducing the concentration of functional fluorophores inside a certain spine via 2-photon photobleaching (Svoboda et al., 1996b). The recovery of fluorescence is again a measure for the diffusive coupling between spine and shaft.

Multiphoton Photochemistry

Combining localized 2-photon uncaging or photobleaching with 2-photon fluorescence microscopy (Figure 4) allowed us to measure diffusional transport in and out of individual spines (Svoboda et al., 1996b). We concluded that spines act as leaky chemical compartments and, using the measured cytoplasmic diffusion constant and specific resistivity, we also estimated the electrical resistance of the spine neck. The values that we found (mostly $<$100 MΩ) rule out that neck resistance acts as a major determinant of synaptic efficacy but leave open the possibility of selective activation of steeply voltage-dependent channels, as suggested by experiments on Purkinje cells (Denk et al., 1995c).

Multiphoton-induced photochemical release of agonist can also be used for subcellular spatial mapping of receptor distribution by "scanning photochemical microscopy" (Denk, 1994). A physiological signal, such as the transmembrane current, is recorded as a function of the position of the laser focus, which acts as a highly localized source of agonist by converting caged agonist inside the focal volume from an inert to a biologically active form. For example, the distribution of acetylcholine receptors on the surface of cultured BC3H1 cells has been mapped by liberating the nicotinic agonist carbamoylcholine photochemically from a caged precursor (Denk, 1994).

Developmental Biology

Living embryos are among those biological samples that are most sensitive to damage and require the imaging of whole specimens. 2PLSM has been used to study sea urchin embryogenesis (Summers et al., 1996). Various aspects of C. elegans development are beginning to be studied with MPLSM as well (Wokosin et al., 1996).

Biological Fluorophores

Green fluorescent protein (GFP), which can be expressed in transgenic animals under the control of various promotors (Chalfie et al., 1994), is becoming a widely used tool in monitoring gene activity and cellular trafficking. GFP also allows the convenient labeling of genetically distinct classes of cells for morphological imaging during development. Several groups have shown that GFP can be easily excited via 2-photon absorption at levels sufficient for imaging (Niswender et al., 1995; Potter et al., 1996). The 2-photon cross section of GFP has been measured to be around a quite usable value of 10×10^{-58} m^4s (Xu et al., 1996).

Intrinsic Fluorescence

Fluorescence of endogenous chromophores excited by multiphoton absorption, which is often a nuisance because it creates background noise, can sometimes be turned into a useful imaging tool. 2-photon excited NAD(P)H fluoresence has been used to assess the metabolic state of individual cells in the cornea (Piston et al., 1995) and in pancreatic B cells (Bennett et al., 1996). 3-photon excitation of serotonin fluorescence has been used to measure its concentration in secretory vesicles (Maiti et al., 1997).

In Vivo Functional Imaging

Recently, we have used 2PLSM to measure calcium dynamics elicited by sensory stimulation in neocortical pyramidal cells in vivo (Svoboda et al., 1997). In the intact brain (Figure 5), the need for tissue penetration is even greater than when imaging in slices, as the orientation of the cell axis with respect to the optical axis can no longer be chosen to suit the imaging method. For example, because even superficial cortical pyramidal cells extend to at least 400–500 μm below the cortical surface, tissue penetration down to such depths is crucial in order to map Ca^{2+} responses throughout the cell. Measurements of functional Ca^{2+} increases were reliably obtained with high spatial and temporal resolution using Calcium Green fluorescence excited via 2-photon absorption at 840 nm.

Possibilities

Several technological developments should be mentioned here that have not been applied to real biological problems but which could well become important in

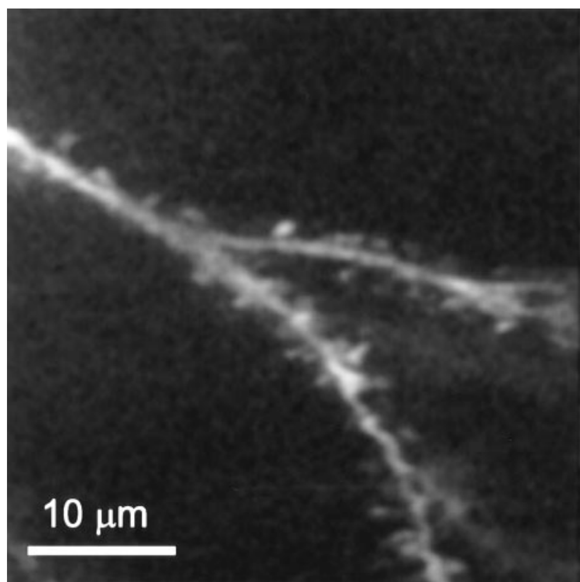

Figure 5. High Resolution Imaging In Vivo

A neocortical pyramidal neuron in layer 2–3 of the rat somatosensory cortex was filled iontophoretically with Calcium Green 1. Shown is a basal spiny dendrite approximately 200 μm below the pial surface (K. S., W. D., D. Kleinfeld, and D. Tank, unpublished data). The excitation source was a Ti:sapphire laser (840 nm wavelength).

the future. The fluorescence decay time, which is often measured in molecular spectroscopy because it is sensitive to the molecular environment, can provide image contrast in 2-photon imaging (Piston et al., 1992). Lifetime imaging can be implemented in a 2-photon microscope with little extra effort since the excitation light is already pulsed. One potentially important use of lifetime contrast in functional imaging of neurons is the measurement of absolute Ca^{2+} concentrations (Lakowicz et al., 1992) with indicator dyes that do not show a spectral shift and are therefore difficult to calibrate otherwise.

A potentially very important development is the synthesis of fluorescent compounds with significantly improved 2-photon absorption cross sections (Bhawalkar et al., 1996). It remains to be seen whether these fluorophores can be used in biological samples and whether indicator dyes can be constructed from these compounds.

Future Directions

The anticipated benefits (Denk et al., 1990; 1994) of multiphoton excitation in vital imaging, mainly reduced photodamage and improved depth penetration, have materialized, and no major unexpected obstacles have appeared. The crucial property of multiphoton absorption is the true localization of excitation, which occurs with the simultaneous absorption of at least two photons (Denk, 1996). Higher order nonlinear processes, such as 3-photon absorption, can provide some resolution improvement but, more importantly, access to different wavelength regions (Wokosin et al., 1996; Xu et al., 1996). This may be particularly useful for the excitation of endogenous molecules (Maiti et al., 1997), many of which 1-photon absorb only in the ultraviolet. On the other hand, spurious 3-photon absorption has to be considered as a possible damage mechanism during 2PLSM (Denk, 1996; Xu et al., 1996). For the excitation of fluorophores in the visible wavelength regime, the corresponding 3-photon absorption wavelengths lie far beyond 1 μm where the linear absorption by liquid water all but rules out the imaging of most biological specimens.

An essential condition for the successful use of multiphoton excitation is the lack of significant 1-photon absorption at the fundamental wavelength. Apart from the obvious case of melanocytes (Potter, 1996; our own observation), no problems have been reported that were traced to linear absorption of the excitation light.

Scattering processes not only reduce the available light intensity but can lead to a degradation of the laser focus as the depth into the sample increases. A better understanding of scattering in living specimens as a function of tissue type and wavelength is clearly necessary. Diffusing wave spectroscopy (Weitz et al., 1993) provides some of the relevant parameters, such as scattering and absorption lengths. However, multiple scattering processes are not very sensitive to the detailed angular dependence of the scattering probability, which is central to focus degradation. We find, for example, that a dense layer of cell bodies is almost impenetrable (possibly due to microlensing effects), but in neuropil-rich regions, imaging is possible down to several hundred microns.

The development of chromophores with especially large multiphoton cross sections would provide a welcome enrichment of the arsenal of quite usable fluorophores. More importantly, caged compounds with substantial 2-photon cross sections at Ti:sapphire wavelengths would open up fundamentally new ways of studying local biochemistry.

The availability of a commercial MPLSM instrument (Biorad) and the development of much more user-friendly laser sources providing ultrashort pulses of sufficient power will undoubtedly make multiphoton absorption techniques accessible to a much wider circle of users.

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