TWO-PHOTON EXCITATION IN FUNCTIONAL BIOLOGICAL IMAGING

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1 INTRODUCTION

Optical methods have always played an important role in the study of physiological phenomena. Light microscopes have been used for more than one hundred years¹ to gain insight into the structure of biological systems in general and the nervous system in particular. Anatomical studies had given us a first understanding of shapes and types of nerve cells.² Another example is the ubiquitous and rarely mentioned use of the light microscope to guide electrophysiological recording electrodes toward their targets. Only in the last two decades, however, has light microscopy become widely used to gather quantitative dynamic information about physiological processes. The list of examples includes: the study of diffusion in membranes with fluorescence methods;^{3–6} the detection of transmembrane potential changes using intrinsic signals⁷ or extrinsic fluorophores (for reviews see Refs. 8, 9, and 10); the measurements of nanometer displacements;^{11–14} and the quantification of changes in intracellular ion concentration using fluorescent indicator dyes (for a review see Ref. 15). More recently, light has also been used to trigger physiological processes by applying biological effector molecules with high spatial and temporal resolution via photochemical release, commonly called “uncaging.”^{16–19} In this article, I review the contribution that the combination of two-photon absorption with laser scanning²⁰ has made to functional biological imaging in recent years.

2 TWO-PHOTON EXCITATION: HISTORY AND BACKGROUND

Two-photon excitation is not a new idea. However, after being theoretically predicted²¹ in 1931 as a consequence of the then newly discovered and mathematically formalized quantum mechanics, it took 30 years before an experimental confirmation was obtained.²² The reason for this long delay is the miniscule size of typical two-photon absorption cross sections, which render two-photon excitation virtually nonexistent under ambient light condi-

tions. Even at the light intensity present inside the arc of a high-pressure mercury-vapor lamp (the brightest conventional light source), only about one two-photon absorption event occurs per fluorophore every one hundred seconds. This is the absolute upper limit for conventional light sources since, as a result of the brightness theorem, light cannot be concentrated beyond the source intensity (see Ref. 23, Sec. 4.8.3). The laser, on the other hand, when operating in a single lateral mode, allows the concentration of all its power into a single diffraction-limited spot. This has permitted the study of excited electronic states by two-photon absorption spectroscopy (for a review see Ref. 24).

An early proposal for two-photon excitation microscopy involved a resonant cavity,²⁵ which is, however, impractical for most applications and still suffers from the presence of a very high average power density at the sample. The development of the cw-mode-locked laser^{26–28} finally provided, at moderate average powers, molecular excitation rates that are large enough ($10^8/\text{s}$ for a single fluorophore, which is the saturation limit) to be useful for laser scanning microscopy.²⁰ While two-photon excited laser scanning images can be obtained with cw lasers,^{29,30} the speed of image acquisition at the same average power is about 100,000 times slower, which makes the study of dynamic physiological phenomena virtually impossible. Two-photon absorption by cw beams can, however, become important as a damage mechanism during optical trapping experiments.^{31–33}

3 USING FLUORESCENCE FOR PHYSIOLOGICAL MEASUREMENTS

3.1 ONE EXAMPLE: MEASURING Ca^{2+} CONCENTRATION

One of the most important applications of light to gather quantitative physiological data has been the use of fluorescent indicators to measure the dynamics of the free calcium concentration inside cells.^{15,34–37} The main reason for the importance of calcium ion influx is that it serves almost univer-

sally to couple membrane depolarization, which is detected by voltage-sensitive Ca^{++} -permeable ion channels (for an excellent introduction see Ref. 38), with biochemical processes, which are often dependent on calcium binding proteins.^{39,40} Crucial to functional fluorescence microscopy are highly sensitive instruments such as cooled CCD devices,^{35,36} or laser scanning confocal^{41,42} and two-photon microscopes.^{20,43–47}

3.2 IMAGING IN SCATTERING TISSUE

With widefield illumination and focal plane detection (intensified TV and CCD detectors), high resolution imaging inside intact tissue is severely hampered by light scattering, which can be particularly strong in nervous tissue, and by fluorescence background resulting from exogenous or endogenous fluorophores. In confocal microscopy, unwanted out-of-focus and scattered light is rejected by the detector pinhole. Two major obstacles remain: photodamage, also referred to as phototoxicity, and photobleaching (P&P). In fact, P&P can even be exacerbated, compared to, say, cooled CCD detection, as a result of the smaller detector quantum efficiency for photomultipliers and, more fundamentally, due to the loss of fluorescence photons that originate at the focus but are scattered in the tissue and then miss the detector pinhole (Fig. 1—see color plate on p. 301). In addition, while information is only collected for the focal slice, excitation and hence P&P occur throughout the sample.

3.3 CONFOCAL VS TWO-PHOTON

The P&P problems encountered with confocal microscopy can be solved while retaining excellent optical sectioning capabilities by using two-photon excitation of fluorescence.^{20,43,44,46} Because optical sectioning occurs during excitation alone,⁴⁸ two-photon excited laser scanning microscopy (TPLSM) does not require confocal detection. A further consequence of the well-localized excitation is that P&P do not occur outside the focal slice,⁴⁸ as was convincingly demonstrated first by Piston and Webb,^{44–46} who compared directly the one-photon and the two-photon case, and, more recently, by Potter et al.⁴⁹

Another important advantage⁴³ of using two-photon excitation is that its longer excitation wavelengths are scattered less^{50,51} and hence penetrate deeper into the tissue (remember that we are using about twice the wavelength compared to one-photon excitation of the same fluorophore). This property of infrared light is also exploited in the visualization of living brain slices^{52,53} and to some extent in diffusive-wave imaging.⁵⁴ An experimental confirmation of the improved depth penetration was obtained recently by Potter et al.⁴⁹ and by Centonze et al.,⁵⁵ who compared directly on the same samples two-photon (non-confocal) and one-photon confocal microscopy.

Finally, as discussed later in detail, scattered fluorescence photons can contribute to the signal without any loss of spatial resolution. This is because in TPLSM, unlike in a confocal microscope, the detection process does *not* need to contribute to resolution or background rejection.⁴³ Scattered excitation light, on the other hand, is rendered harmless because it is diluted. As a practical matter, the comparative simplicity of the optical path when whole-area detection is used⁴⁶ helps to increase further the efficiency with which fluorescent photons are captured.

3.4 APPLICATIONS OF DEEP TISSUE IMAGING

Imaging deep inside tissue is particularly important when intact and complete cells are to be studied within their original environment, for example, to address questions of neural circuitry. When imaging in living brain tissue slices,⁵⁶ one traditionally had to make the choice between limited resolution⁵⁷ or the need to select cells near the slice surface where the integrity of the local circuitry and the health of individual cells can be questionable.^{58,59} Functional confocal imaging has generally been plagued with photodamage problems.⁶⁰ It has recently been demonstrated that TPLSM does overcome these problems and permits, for example, protracted imaging of synaptic spines,^{47,61} which are micron-sized protrusions from neuronal dendrites and serve as fundamental units of dendritic integration. Another example where depth penetration and damage minimization are crucial is imaging of developing embryos.^{62,63}

3.5 TWO-PHOTON EXCITATION IN SCATTERING TISSUE

High-resolution functional imaging in scattering tissue may well be where the strengths of two-photon excited fluorescence imaging are most effective.⁴³ As touched upon previously, imaging in scattering tissue can be accomplished by the use of either one-photon excitation combined with confocal detection or of two-photon excitation without the need for confocal detection. To compare both methods, several issues have to be considered⁴³ (Fig. 1). Scattering (1) considerably reduces the amount of excitation light that reaches the focus and (2) generates a diffuse background of excitation light. While one can compensate for the light loss at the focus by increasing the incident power, this has very different consequences for one- and two-photon excitation. In the one-photon case, increased P&P occur in the excitation path and throughout the diffuse background. Neither effect is significant for two-photon excitation, since the sharp (quadratic) drop of the absorption rate with the light intensity precludes significant excitation away from the focus. In addition, the diffuse background, which comprises light arriving along different path-

lengths, is temporally less coherent, thus reducing two-photon absorption further, at least for excitation with subpicosecond pulses. Another issue is the reduction of the effective numerical aperture, since light with a high transverse momentum, i.e., propagating at a large angle to the optical axis, traverses more tissue and thus suffers greater extinction. This effect is more severe for the shorter, more strongly scattered wavelengths that are used for one-photon excitation, resulting in a loss not only of excitation efficiency but also of resolution.

On the emission side, the fraction of fluorescence light that leaves the sample as "ballistic" photons (photons that are not scattered) is greatly reduced, as the depth of tissue that has to be traversed increases. In a confocal microscope, only ballistic photons can pass the detector pinhole and contribute to the signal, "diffusive" photons are lost. But every photon generated by a fluorophore, detected or not, uses up the valuable capital of a limited number of excitation events that organic fluorophores can undergo before suffering photodestruction.⁶⁴ In other words, to achieve a given signal-to-noise ratio under scattering conditions with a confocal microscope, a larger fraction of the available fluorophores will be destroyed and a larger amount collateral photodynamic damage to the biological tissue will be incurred.

In a two-photon excitation microscope, both ballistic photons and diffusive photons (which can be the vast majority) contribute to the signal as long as nondescanned whole-field detection or descanned detection with a large enough detector⁴⁶ is used. "Large enough" here means to be roughly of the size of the scattering halo, which, in turn, has a diameter on the order of the imaging depth. As mentioned before, optical sectioning, equivalent to that of an ideal confocal microscope (with infinitesimal pinhole size⁵), results from the quadratic dependence^{48,66,67} (but note Ref. 68) of the excitation probability on the local light intensity.

Thus in scattering samples two-photon excitation reduces P&P significantly even in the focal plane, while fully retaining its out-of-focus advantage already familiar from optically clear samples.^{20,46} For example, the out-of-focus reduction of P&P amounts to a factor of roughly 1000 at a distance $\Delta z = 10 \mu\text{m}$ from the focal plane when imaging with a 1.3 NA objective lens. The reason⁴⁸ is that in a laser scanning microscope the time averaged light intensity is roughly independent of Δz . In the case of one-photon excitation, P&P are therefore not confined to the focal slice. In fact, they are almost independent of Δz (see e.g., Fig. 3 in Ref. 46). The average two-photon excitation rate and any excitation-linked damage, on the other hand, fall off quickly as the average of the squared instantaneous intensity falls off ($\propto \Delta z^{-2}$ for $\Delta z \gg \lambda/\text{NA}^2$).

3.6 TWO-PHOTON EXCITATION WITH CONFOCAL DETECTION

It is a quite natural idea to use a confocal detection pinhole in conjunction with two-photon excitation.⁴⁸ Such a combination has been implemented and characterized experimentally by Stelzer et al.⁶⁹ The benefits are significantly improved resolution and some additional background rejection. For physiology experiments, in particular when imaging in scattering tissue, these benefits have to be weighted carefully against the drawbacks: (1) using a pinhole that is small enough to provide significant resolution improvements⁶⁵ also entails, even for nonscattering samples, a significant loss of fluorescence light;⁴⁶ (2) the large wavelength difference between excitation and emission is likely to exacerbate problems resulting from lateral chromatic aberration, which, unless stage scanning is used, where optical axis always passes through the imaged pixel, leads to a misalignment of excitation and detection volumes; and (3) in scattering samples only ballistic (unscattered) fluorescence photons can contribute to the signal (see above).

3.7 WHERE IS TWO-PHOTON EXCITATION IMAGING APPROPRIATE AND NECESSARY?

The reduction and confinement of photodamage and photobleaching available with two-photon excitation is particularly relevant under the following circumstances: (1) whenever images at multiple focal planes have to be acquired such as for three-dimensional reconstruction of morphology; (2) when damage load on the whole tissue has to be reduced, for example when imaging in brain slices^{47,61} in developing tissue where damage to the genetic material has to be minimized to preserve the ability of the cells to divide properly,^{62,63,70} or when imaging intrinsic functional signals with low fluorescence yield, e.g., redox imaging using NAD(P)H,^{71,72} (3) when, even while imaging only a single focal plane, an out-of-focus pool of diffusible fluorophore exists that can replace those lost by bleaching, for example, when imaging cells filled with intracellular indicators.^{47,61,73,74}

4 PHOTOSTIMULATION

Another major use of light in physiology lies in stimulating a biological system rather than merely observing it. In the simplest case, photoreceptors are illuminated and a physiological response is observed. To stimulate with light when there is no intrinsic responsivity requires the introduction of a "sensitizer." One example are molecules that change their biological efficacy via a photochemical reaction either reversibly (by photo isomerization) or irreversibly (by photolytic cleavage).^{16,75-81} Such "caged" molecules have become readily available

during the last two decades and are now widely used to deliver effector molecules either when very high time resolution (below a millisecond) is required⁸² or when diffusional access is slow, for which the intracellular space is an extreme example.^{83,84}

Some spatial localization of release can be achieved optically,^{18,85,86} but release with high spatial resolution is difficult for several reasons: (1) most caged compounds require UV light for their activation, which makes high resolution release expensive, even in optically clear specimens, since special laser light sources and microscope optics are required;⁸⁷ (2) scattering, which is particularly pronounced at shorter wavelengths, makes it next to impossible to release with high resolution deep inside, for example, a brain slice; (3) release always occurs throughout the whole focus cone,⁸⁸ which makes true localization along all three spatial dimensions almost impossible. An additional aspect⁸⁹ of photostimulation is that, unlike in confocal fluorescence microscopy, the detection process, using, e.g., transmembrane current^{18,85,89} often cannot be exploited to provide additional resolution, optical sectioning, and background rejection.

4.1 TWO-PHOTON ABSORPTION MEDIATED PHOTOCHEMISTRY

For the same reasons that have been discussed above for fluorescence imaging, two-photon absorption provides true 3-D localization (i.e., optical sectioning) using the excitation process alone. Furthermore, at twice the one-photon wavelength, two-photon excitation avoids the need to use special objective lenses, even for photolysis energies in the UV. Two-photon excitation using red light has been demonstrated to uncage ATP²⁰ and caged fluorophores.^{90,91} The highly localized release of caged carbamoylcholine has been used to map neurotransmitter-receptor distributions on cultured cells.⁹² A remaining problem is that for currently available caged compounds, two-photon action cross sections for the release of agonist are typically more than 1000 times smaller (about 0.001–0.010 GM^{93,94}); than those for the excitation of fluorescence in common fluorophores such as fluorescein or rhodamine, which have cross sections between 5–100 GM.⁶⁷ It is worth mentioning here that two-photon cross sections cannot be calculated by simply rescaling the corresponding one-photon spectrum (discussed in more detail in Ref. 46 and in references cited therein). Small two-photon cross sections then leave only a narrow range where the excitation power is sufficient for release and still below the tissue damage threshold.^{92,94} Intrinsic absorption, in addition, is much more severe, in part due to two-photon absorption by NAD(P)H,⁷² for the wavelengths that are required for two-photon

uncaging⁹² (<700 nm) than for the wavelengths (830–860 nm) used, for example, for functional Ca⁺⁺ imaging in brain slices.^{47,61}

Finally, combining two-photon photobleaching or two-photon photorelease of fluorescence with two-photon fluorescence imaging allows the measurement of diffusional transport with high resolution inside scattering tissue. In this way the diffusional and electrical coupling between synaptic spines and their parent shafts has recently been measured.⁹¹

5 OPEN QUESTIONS

Many of the physical parameters relevant for two-photon excitation are not yet fully understood. For example, reliable and precise measurements of absolute two-photon cross sections for some common fluorophores are only now appearing in the literature.^{66,67} It is clear that two-photon excitation minimizes chromophore excitation, which, independent of the excitation pathway (one- or multiphoton absorption), always carries a finite probability of causing a damaging reaction. However, it is unclear whether and to what degree there are limits on the application of two-photon excitation that are imposed by damage mechanisms that depend on nonlinear processes driven by the high peak intensities present in tightly focused ultrashort pulses. The practical experience^{20,47,49,61,63,74,95,96} has been that quite extensive imaging at high resolution and high signal-to-noise ratios is possible without loss of physiological function. This has been demonstrated particularly clearly by the extended observation of synaptic transmission in single dendritic spines^{47,61} and by the protracted high resolution imaging of sea urchin embryos going through many cell division cycles.⁶³

5.1 POTENTIAL PROBLEMS

When imaging thin (1 to 2 μm) nonscattering samples, two-photon excitation is not expected to possess fundamental advantages over one-photon confocal or widefield microscopy, since no fluorescence photons are lost due to scattering and, for a given excitation rate per fluorophore, bleaching in the focal plane should be comparable for both methods. Surprises, in favor or disfavor of TPLSM, might result from differences in photophysics and photochemistry between one- and two-photon molecular excitation. For example, due to the difference in excitation wavelength, one- and two-photon absorption by the excited state⁹⁷ is bound to be different. Unintentional two-photon absorption by aromatic amino acids^{98,99} might even be responsible for some of the damage during high-intensity one-photon imaging but might be avoided using TPLSM. Similarly, during two-photon imaging, unintentional three-photon absorption^{55,100–102} may become a source of tissue damage. However, since the two-photon absorption rate R_n is proportional to τP_{peak} one can, by adjusting peak power (P_{peak}) and

pulselength (τ) appropriately reduce the rate of unwanted three or more photon processes without at the same time reducing the excitation efficiency for the desired two-photon processes. When the highest possible resolution is desired, the longer excitation wavelength of two-photon excitation is a disadvantage, but confocal detection in addition to two-photon excitation⁴⁵ can provide a remedy that is, not, however, without its own drawbacks (see Sec. 3.6). One practical problem with two-photon imaging is that optical sectioning is not controllable simply by opening a detector pinhole, making small isolated fluorescent objects more difficult to find.

5.2 PROMISING DIRECTIONS

Endogenous fluorophores⁹⁹ often pose a particular challenge to fluorescence imaging since they are not designed for minimal P&P as many synthetic fluorophores are. While still in its infancy, two-photon imaging of the intrinsic metabolic indicator NAD(P)H⁷² could become a useful tool for the study of dynamic physiological processes with high spatial resolution. Imaging or locally inactivating proteins with multiphoton excitation¹⁰³ is largely unexplored. Two-photon micro-photopharmacology has been demonstrated,⁹² but for a breakthrough into widespread applications, compounds with larger two-photon cross sections are needed. Fluorescent lifetime imaging is easy to implement,¹⁰⁴ since the excitation light is already pulsed. Fluorescence decay time contrast may become useful for the calibration of calcium indicators that change their excited state lifetime but not their excitation and emission spectra on binding of Ca^{++} .¹⁰⁵

Single fluorophore detection is clearly possible in purified biochemical samples,^{106–108} but in a cellular environment it is severely hampered by fluorescence and by Raman scattering from intrinsic chromophores. Two-photon excitation¹⁰⁹ may allow better discrimination against excitation light and reduce background for two reasons: (1) Raman scattering is negligible because detection occurs far in the antistokes regime and (2) two-photon absorption by weak intrinsic fluorophores, present at high concentration, might be strongly reduced compared to the one-photon case. The reason is that two-photon absorption cross sections depend on the fourth power of the typical transition matrix element between molecular electronic states, but the one-photon cross section depends only on the square (see Ref. 110, p. 64).

Similar considerations apply to fluorescence correlation spectroscopy,^{6,111,112} which may well be resuscitated by two-photon excitation.¹¹³

Using three-photon absorption allows the excitation of fluorophores, such as amino and nucleic acids and some Ca^{++} indicators, with excitation energies in the UV while still using the wavelengths

obtainable from Ti:sapphire lasers^{100–102} and Nd:YLF.^{113a}

In more complicated optical arrangements such as 4Pi^{114,115} and theta microscopy,⁶⁹ two-photon excitation³⁰ can either serve to avoid having to align a confocal detection path or it can be used to gain additional resolution and background rejection. By apodizing the illumination beam appropriately, the excitation volume can be reshaped: for example, using an annular aperture creates a volume elongated along the optical axis but with slightly narrower lateral extent.¹¹⁶ Since the full objective aperture can be used for detection in this case, the aperture entails no loss of fluorescent photons, quite different from the case of annular aperture confocal microscopy.¹¹⁷ By using nonlinear depletion or stimulated emission processes, far-field superresolution might be achievable.^{118,119}

6 OUTLOOK

The major obstacles hindering the spread of two-photon excited microscopy have been the expense and complexity of ultrashort-pulse lasers used for excitation. The commercial availability of the Ti:sapphire laser¹²⁰ has eliminated the requirement to keep a “laser jock” at hand, but not the significant capital expense and utility requirements.

The emerging alternative are all-solid-state, diode-pumped lasers,^{121–127} which can be used to acquire two-photon laser scanning images.^{55,128} Not only will diode-pumped systems ultimately be much cheaper, but they also consume about 1000 times less electrical power, are easy to cool, and require less than a tenth of the optical table space. Commercial diode-pumped systems are about to become available and for some (if not most) applications, such systems will undoubtedly dominate eventually.

It has to be kept in mind, however, that in research applications flexibility is essential and the combination of abundant power, optimal pulsewidth,⁴⁶ and broad tunability may make the Ti:sapphire laser the source of choice¹²⁹ for quite some time to come. The recent arrival of a diode-pumped frequency double Nd:YAG laser capable of adequately pumping the Ti:sapphire lasers has reduced the utility requirements but not yet the cost.

Where two-photon excitation will ultimately be applied, within and outside of physiology, will depend on its price and the degree of advantage it offers compared to other techniques. In the near future I see increasing use of two-photon excitation in those areas where it promises overwhelming advantages, i.e., where its depth penetration and low-photodamage properties are crucial.^{43,46,47,49,61,62,91}

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Fig. 1 Confocal one-photon excitation imaging compared with two-photon imaging in scattering tissue. Due to the longer wavelength, less excitation light is lost to scattering when using two-photon excitation. Ballistic and diffusing fluorescence photons can be used in the two-photon case, but only ballistic photons can be used in the confocal case.

COLOR PLATE

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