Two-Photon Microscopy of Cells and Tissue

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Abstract—Two-photon excitation fluorescence imaging provides thin optical sections from deep within thick, scattering specimens by way of restricting fluorophore excitation (and thus emission) to the focal plane of the microscope. Spatial confinement of two-photon excitation gives rise to several advantages over single-photon confocal microscopy. First, penetration depth of the excitation beam is increased. Second, because out-of-focus fluorescence is never generated, no pinhole is necessary in the detection path of the microscope, resulting in increased fluorescence collection efficiency. Third, two-photon excitation markedly reduces overall photobleaching and photodamage, resulting in extended viability of biological specimens during long-term imaging. Finally, localized excitation can be used for photolysis of caged compounds in femtoliter volumes and for diffusion measurements by two-photon fluorescence photobleaching recovery. This review aims to provide an overview of the use of two-photon excitation microscopy. Selected applications of this technique will illustrate its excellent suitability to assess cellular and subcellular events in intact, strongly scattering tissue. In particular, its capability to resolve differences in calcium dynamics between individual cardiomyocytes deep within intact, buffer-perfused hearts is demonstrated. Potential applications of two-photon laser scanning microscopy as applied to integrative cardiac physiology are pointed out. (Circ Res. 2004;95:1154-1166.)

Key Words: two-photon excitation • laser scanning microscopy • calcium imaging

Two-photon excitation (TPE) microscopy has evolved as an alternative to conventional single-photon confocal microscopy and has been shown to provide several advantages. These include three-dimensionally resolved fluorescence imaging of living cells deep within thick, strongly scattering samples, and reduced phototoxicity, enabling long-term imaging of photosensitive biological specimens. The inherent three-dimensional resolution of TPE microscopy has been exploited in a number of studies wherein spatial discrimination of fluorescence signals at the micrometer and submicrometer scale within thick biological specimens proved critical. For example, TPE of the calcium-sensitive fluorophore rhod-2 has been used to resolve differences in the kinetics of intracellular calcium ([Ca^{2+}]_{i}) transients in donor myocytes and juxtaposed host cardiomyocytes deep in Langendorff-perfused mouse hearts following intracardiac transplantation of fetal cardiomyocytes and skeletal myoblasts. For neuroscientists, TPE microscopy has become an invaluable tool for studying calcium dynamics in thick brain slices and live animals and for long-term imaging of neuronal development. The spatial confinement of TPE has also been used for three-dimensional photolysis of caged compounds in femtoliter volumes or diffusion measurements by two-photon fluorescence photobleaching recovery.
Principles of TPE

In conventional confocal laser scanning microscopy, absorption of a single photon delivers sufficient energy for the fluorophore to reach the excited state from which it returns to the ground state by emitting a photon of fluorescence. As illustrated in Figure 1a, this technique causes excitation of fluorophores above and below the plane of focus, resulting in blurring of the image. Confocal microscopes increase the spatial resolution of the image by using an adjustable pinhole in front of the detector to reject out-of-focus fluorescence.

The excited state can also be reached by the near simultaneous absorption of two longer wavelength photons, resulting in the squared dependence on laser light intensity rather than the linear dependence of conventional, single-photon fluorescence imaging. Neglecting excitation saturation, the average rate of TPE per molecule is given by the following equation:

\[ \text{rate} = \frac{1}{2} \delta \langle F^2 \rangle \]

where \( \langle F^2 \rangle \) is the time average of the second power of local laser intensity in units of photons cm\(^{-2}\) s\(^{-1}\) and \( \delta \) is the two-photon absorption cross section in units of cm\(^4\) s photon\(^{-1}\) (10\(^{-50}\) cm\(^2\) s photon\(^{-1}\) equals 1 GM, or Göppert-Mayer).

The TPE cross section is a quantitative measure of the probability of a molecule to absorb two photons simultaneously. It is the product of the molecular absorption cross section and the fluorescence emission quantum efficiency. The intensity-squared dependence of TPE gives this technique its intrinsic three-dimensional resolution (see Figure 1b). Because the energy of a single long-wavelength photon is insufficient to excite commonly used fluorescent dyes, linear absorption by fluorophores above the focal plane does not occur. Excitation (and thus emission) is confined to a small ellipsoidal volume around the focal point, where photon flux is sufficiently high to give rise to two-photon absorption events. Because out-of-focus fluorescence is never generated, TPE microscopy provides optical sectioning without the need to introduce a pinhole in the detection path of the microscope, as in confocal microscopy. Thus, all of the signal generated by the sample can be collected by a large-area detector and contribute to the final image (nondescanned acquisition mode; see the online data supplement, available at http://circres.ahajournals.org).

The size of the TPE volume (and thus the optical resolution of the TPE system) critically depends on the numerical aperture of the objective lens and the illumination wavelength. To illustrate the effect of numerical aperture size on the spatial scale of the \( \langle F^2 \rangle \) distribution, isointensity contour plots in the \( x \)-\( y \) and \( x \)-\( z \) planes were simulated using an ellipsoidal Gaussian approximation to the diffraction-limited focus (Figure 2a). A 4-fold reduction in the numerical aperture of the objective lens will increase the spread of the excitation volume (measured at the 0.5 isointensity contours) 22-fold axially and 4-fold laterally, amounting to an increase in TPE volume by more than two orders of magnitude (Figure 2b), whereas increasing excitation wavelength from 700 to 1000 nm will increase TPE volume by only 3-fold. Thus, using TPE microscopy with a uniformly illuminated, high numerical aperture objective, fluorescence excitation is confined to less than femtoliter volumes around the focal point of the objective, with \( <1 \mu m \) resolution in the \( z \) direction. For comparison, the size of a mitochondrion in an eukaryotic cell is \( \approx 1.5 \) to 2 \( \mu m \) in length, and 0.5 to 1 \( \mu m \) in diameter. Thus, TPE microscopy provides fluorescence imaging with subcellular resolution. Although calculated dimensions of TPE volumes may deviate from their actual values within the specimen, knowing them is helpful to estimate the thickness of an optical section or to predict the number of caged calcium ions that one expects to release during two-photon photolysis (see below). Besides using a high numerical aperture objective and shorter excitation wavelengths, the optical resolution of the TPE system can be increased by using descanned detection in conjunction with a confocal pinhole in the emission path (see also the online data supplement). However, a confocal pinhole will reject scattered emissions, even though they originated in the focal plane, thereby reducing fluorescence collection efficiency, and thus signal-to-background ratio.

TPE events are exceedingly rare at light intensities typically used for epifluorescence microscopy. To generate enough fluorescence practical for TPE laser scanning microscopy, sample illumination is typically provided by pulsed lasers such as the mode-locked Ti:Sapphire laser, which generates pulses of \( \approx 100 \) fs duration at a repetition rate of \( \approx 80 \) MHz. When pulsed illumination is used, the instanta-
Figure 2. Simulated effects of excitation wavelength and numerical aperture on the dimensions of the TPE volume. a, Normalized distributions of laser intensity-squared in the x-y and x-z plane for three different numerical apertures of water-immersion objective lenses at an excitation wavelength of 850 nm. Intensities-squared at lateral (x,y,0) and axial (x,0,z) positions were calculated using an ellipsoidal Gaussian approximation to the diffraction limited focus7,11 and expressed as the fraction of the intensity-squared at the focal point [I(0,0,0)2=1]. Color-coded contour plots depict isointensity lines in the x-z and x-y plane at the levels of 0.1, 0.3, 0.5, 0.7, and 0.9 of [I(0,0,0)2]. Note different scales for each panel. b, Dependence of TPE volume on numerical aperture of the objective lens and illumination wavelength. Values were obtained by approximating the intensity-squared distribution as a three-dimensional Gaussian volume.12 For all calculations, it is assumed that the objective lens is uniformly illuminated (overfilled) and that no saturation of the fluorescence excitation process occurs. NA indicates numerical aperture.

neous light intensity is extremely high, whereas the average energy received by the sample remains relatively low. Estimating the instantaneous focal intensities achieved during pulsed illumination is useful to minimize the occurrence of irreversible photodamage14,15 as well as to prevent fluorophore saturation, which will increase the size of the TPE volume and thereby reduce the optical resolution of TPE imaging.12 For a two-photon process, the excitation rate is proportional to the average squared intensity ⟨I⟩2 (see Equation 1) rather than the average intensity squared ⟨I⟩2. Because the average intensity ⟨I⟩ equals the product of the pulse frequency, f, and the integrated intensity during a pulse of duration τ (full-width-at-half maximum), the dependence of ⟨I⟩2 on average intensity is given (for τ≪1/f)12 by the following equation:

\[
⟨I⟩^2 = g(⟨I⟩)/τ
\]

where g is a dimensionless factor (0.5916, 0.57615). Compared with continuous illumination, pulsing will increase ⟨I⟩, and, consequently, TPE probability by a factor of (g/τ), but, at the same time, reduce ⟨I⟩2 by the same factor. Thus, the yield of nonlinear excitation processes can be increased by shortening of the pulse width τ and/or by reducing the pulse repetition frequency f. For example, for 100-fs pulses and f=80 MHz, the TPE probability at the focal point is increased by approximately five orders of magnitude, whereas reduction of the pulsing frequency to 200 kHz without changing the pulse width will theoretically increase the probability of two-photon absorption events by a factor of 3×107. However, the peak laser intensity at the lower repetition frequency will be unusually high, resulting in degradation of the optical resolution caused by excitation saturation12 and in an increase in the relative contribution of higher-order (more than second-order) excitation processes to the fluorescence output, which in turn increases the risk of photobleaching17 and photodamage.14,15 The lowest usable repetition frequency is dictated by the pixel rate of the scanning module because at least one pulse must be delivered per image pixel. Fluorescence emission would be further increased if the pulse repetition rate were maximally increased to the inverse fluorescence lifetime of typically 1 to 2×10−9 s−1.19 Shortening of the pulse duration below the standard value of ≈100 fs is possible,19 but is limited by the appearance of group velocity dispersion.12

Comparison of TPE Microscopy and Single-Photon Confocal Imaging

TPE in conjunction with laser scanning microscopy offers several advantages over conventional single-photon confocal laser scanning microscopy. One major advantage is its capability to provide optical sectioning with subcellular resolution from deeper within scattering biological specimens than single-photon confocal microscopy, making this technique particularly attractive for intravital fluorescence imaging. Systematic observations on a variety of biological samples have provided experimental evidence that TPE improves imaging penetration depth by at least 2-fold relative to confocal imaging.20–22 Several reasons account for the increase in penetration depth. First, the dependence of fluorophore excitation on the second power of laser light intensity confines photon absorption to a narrow region at the plane of focus, where photon flux is highest (see Equation 1 and Figure 1b). Thus, unlike single-photon confocal microscopy, TPE microscopy lacks linear absorption of the excitation beam by fluorophores above the plane of focus, which can significantly reduce excitation light before it reaches fluorophores within deeper tissue regions.20 Second, the longer wavelengths used for TPE are scattered by the tissue much less than the shorter wavelengths used for confocal microscopy,23 resulting in deeper penetration of the focused laser beam. Third, scattered light emitted from an excited fluorophore within the focal volume does not contribute to the final image in confocal microscopy because it is indistinguishable from fluorescent light generated in out-of-focus areas and is rejected by the pinhole in the emission path. By contrast, because TPE never generates out-of-focus fluorescence (see Figure 1b), scattered photons from fluorophore emission can be used to generate the TPE image, resulting in increased fluorescence collection efficiency and thus greater signal intensity at any given tissue depth.23 With common mode-locked Ti:Sapphire lasers, imaging depths in a variety
of tissues are typically \(<500 \mu m\) depending on the labeling procedure. Oheim et al have extended the penetration depth of two-photon microscopy by \(\approx100 \mu m\) by means of a low-magnification, high numerical aperture objective lens.\(^{23}\)

Theer et al were able to increase the reach of multiphoton microscopy to \(\approx1\) mm in brain tissue by using regenerative amplification of 200-kHz pulses to achieve high peak powers while maintaining reasonable average powers.\(^{19}\) However, optical resolution degraded substantially at greater depths, possibly resulting from excitation saturation.\(^{12}\) Finally, Webb and colleagues recently showed that multiphoton microscopy through gradient index lenses enables subcellular resolution imaging several millimeters deep in the brain of anesthetized, intact animals.\(^{24}\) Importantly, the spatial resolution of TPE microscopy is maintained with increasing tissue depth,\(^{20,25}\) provided that excitation saturation is negligible.\(^{12}\) Although increasing incident average laser power is one common means to achieve greater penetration depth of the excitation beam,\(^{19,23,26}\) it also increases the probability of both excitation saturation and higher-order (more than second-order) excitation processes, resulting in decreased optical resolution and more extensive photodamage, respectively.\(^{14,15,17}\)

Future strategies to improve tissue penetration depth include development and use of dyes having a larger two-photon absorption cross section, use of longer wavelengths of the excitation light, and measures to increase collection efficiency of the microscope (such as better transmittance of filters, beam splitters, etc). Because the fraction of scattered photons in the emitted fluorescence signal increases with increasing imaging depth, optics with a larger effective angular acceptance should markedly augment the contribution of scattered emission photons to the fluorescence image, thereby further improving depth resolution.\(^{23}\)

In comparison with confocal imaging, TPE fluorescence microscopy reduces overall photobleaching and photodamage by limiting it to the narrow region around the focal plane.\(^{1}\) This reduction becomes important when collecting three-dimensional data sets (z-series) in thick specimens. By contrast, in confocal microscopy, all focal planes are exposed to excitation light every time an optical plane is collected. Therefore, confocal laser scanning fluorescence microscopy can cause more extensive photobleaching and photodamage than TPE fluorescence microscopy if z-stacks are taken repeatedly over time (time-lapse imaging). For endogenous (eg, nicotine adenine dinucleotide [NADH]) and exogenous fluorophores (eg, ion-sensitive indicators) that require UV excitation, conventional confocal imaging has only limited potential because of extensive UV-induced photodamage and photobleaching.\(^{27}\) Through the use of TPE microscopy, it has been possible to perform spatially resolved quantitative measurements of NADH levels and ion concentrations in single cells and intact tissue.\(^{28,29}\)

Finally, the spatial confinement of TPE provides unprecedented opportunities for three-dimensionally localized photochemistry at a subfemtoliter scale, such as photoactivated release of caged calcium ions\(^{7-9}\) or neurotransmitters,\(^{30,31}\) as well as for measurements of three-dimensional mobility of fluorescent molecules using fluorescence photobleaching recovery.\(^{10,11}\)

Optical resolution is linearly dependent on wavelength of the excitation light. It is therefore surprising that the difference in effective resolving power between near-infrared light TPE microscopy and visible light confocal microscopy has been shown to be much less than one would predict from the differences in excitation wavelength.\(^{13}\) This finding results from the nonlinear nature of the two-photon absorption process, which in turn limits the spread of the excitation volume, thereby preventing a significant decrease in resolution resulting from the use of longer-wavelength excitation light.

### TPE Cross Sections and Two-Photon Fluorescence Excitation Spectra

The linear dependence of TPE probability on the TPE cross section \(\delta\) (see Equation 1) underscores the importance of knowing the TPE cross sections and spectra for biologically useful fluorophores. Knowing the absolute values of TPE cross sections and their wavelength dependence is useful to design a particular experiment. For example, use of a calcium-sensitive fluorophore with a relatively high TPE cross section will facilitate the study of intracellular calcium dynamics deep in biological specimens compared with a fluorescent calcium indicator with lower two-photon absorption; and in studies using two-photon fluorescence resonance energy transfer (FRET), the wavelength exciting the donor molecule may not excite the acceptor molecule.\(^{32}\) Methods to determine the TPE action cross section are technically demanding and are described in detail elsewhere.\(^{16,33,34}\) TPE spectra for a number of fluorophores in the spectral range of \(\approx700\) to 1050 nm have been published.\(^{34,35}\) TPE cross section values at the peak absorption wavelength vary from \(10^{-2}\) GM for NAD(P)H\(^{35}\) to \(\approx50,000\) GM for cadmium selenide-zinc sulfide quantum dots.\(^{33}\) The values for most of the commonly used fluorophores, including those of conventional calcium indicators, are in the range of 1 to 300 GM.\(^{16,35}\) TPE action cross sections and their wavelength dependence may differ in vitro and in vivo, because of protein binding\(^{28}\) or pH changes.\(^{36}\) Green fluorescent protein (GFP) and its blue- and red-shifted variants have relatively large TPE cross section values in the range of \(\approx100\) to \(\approx200\) GM, making these proteins well suited for two-photon microscopy in living specimens.\(^{2,3,37}\) Although large TPE cross section values are desirable for TPE fluorescence microscopy, the probability of excitation saturation increases with increasing action cross section, resulting in an increase in TPE volume and, consequently, a decrease in optical resolution with increasing illumination intensity.\(^{12,33}\)

No differences between one- and two-photon--excited fluorescence emission spectra have been found so far,\(^{16}\) and there is usually substantial overlap of the two-photon-action cross section and the single-photon excitation spectrum when plotted at twice the wavelength. However, large blue shifts have been observed for rhodamine and several ion-sensitive fluorophores. As one consequence, UV-light excitable indicators such as fura-2 (and other members of the fura family)\(^{34}\) and indo-1\(^{38}\) for Ca\(^{2+}\) and SBF for Na\(^{+}\) have become accessible for two-photon fluorescence microscopy. As another consequence, a number of fluorophores with disparate...
one-photon excitation spectra can be excited simultaneously by TPE at a single wavelength, thereby avoiding chromatic aberration (Figure 3). Combined with the large separation between the excitation and emission light, this feature facilitates multicolor fluorescence imaging, provided that the emission spectra of the dyes used are well separated. Figure 3 shows simultaneous TPE of the calcium indicator rhod-2 and enhanced GFP (EGFP). When simultaneously exciting multiple fluorophores, the unwanted existence of FRET between them should be examined carefully.

Although TPE at wavelengths twice the single-photon absorption wavelength in general results in fluorescence light sufficient for TPE laser scanning microscopy, evaluation of the TPE spectrum of a dye used in a particular experiment may be helpful to further enhance brightness for imaging. For example, Kuhn et al recently found that TPE of the novel voltage-sensitive dye ANNINE-6 at wavelengths more than twice the published single-photon excitation wavelengths increased voltage-sensitivity of fluorescence changes by a factor of \( \approx 2 \) without evidence for photobleaching.\(^3\) To exploit the full potential of TPE microscopy, it would be desirable to synthesize fluorescent molecules with large TPE cross sections, similar to those of C625 (744 GM)\(^4\) and water-soluble quantum dots (50 000 GM).\(^5\) Excitation and emission spectra of fluorescent molecules can be insufficiently distinct to be concurrently imaged by conventional means. Recent advances in imaging technology that exploit spectral and fluorescence lifetime fingerprinting to separate closely overlapping fluorescent indicators, such as linear unmixing,\(^6\)-\(^8\) allow signals from different fluorescent markers to be resolved. This approach offers the opportunity to simultaneously image multiple colored, spectrally overlapping markers within living cells and tissue.

**Applications of TPE Microscopy**

**Two-Photon Photolysis and Two-Photon Fluorescence Photobleaching Recovery**

Photolabile “caged” compounds are biologically inert precursors of active molecules that, when irradiated, free the active species at the site of action. The photochemical reaction can be very fast, with release of the active species often complete within less than a millisecond. For example, liberation of calcium ions from its photolyzable chelator DM-nitrophen generates \( \text{Ca}^{2+} \) concentration jumps at the site of photolysis which in turn can mediate a wide range of \( \text{Ca}^{2+} \)-dependent processes such as gating of ion channels or modulation of \( \text{Ca}^{2+} \)-sensitive enzymes.\(^8\)\(^4\)\(^4\)\(^5\) Highly localized, fast [\( \text{Ca}^{2+} \)] transients (eg, \( \text{Ca}^{2+} \) sparks)\(^8\) have been shown to play distinct roles in the regulation of variety of \( \text{Ca}^{2+} \)-dependent cellular processes. The availability of an uncaging technique with high spatial and temporal resolution would facilitate the probing of these \( \text{Ca}^{2+} \) microdomains. Similarly, localized photorelease of caged neurotransmitters would enable the study of single or small clusters of agonist-gated ion channels and their distribution and behavior in intact tissue. Although the lateral dimension of a tightly focused UV-laser beam in the plane of focus can be in the submicron range, the axial resolution is comparatively poor because of extension of the conically shaped excitation beam above and below the plane of focus, resulting in photoactivation throughout elongated cellular regions (see Figure 1a).\(^7\) One way to perform photolysis on a much smaller spatial, ie, subcellular, scale and at the same time maintain the high temporal resolution of conventional photolysis is TPE-uncaging. Neglecting excitation saturation, the dependence of TPE of a caged compound on the second power of laser intensity (see Equation 1) limits photoactivation to small volumes around the focal point, giving this technique its inherent three dimensional resolution. Using TPE with high numerical aperture objectives and near-infrared light from a Ti:Sapphire laser, it has been shown that rapid (within \( < 50 \mu \text{s} \)) release of calcium from its cages can be confined to less than femtoliter volumes, with \( \approx 1 \mu \text{m} \) resolution along the \( z \) axis.\(^7\) Quantitative approaches to predict the amount of caged compound released in a given photolysis experiment and to predict the temporal behavior of the unleashed compound have been published.\(^7\)\(^3\) Among other variables, these calculations require the knowledge of the TPE action uncaging cross section, the product of the TPE absorption cross section and the quantum yield. Action cross section values for only a

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**Figure 3.** Simultaneous TPE of fluorophores with disparate one-photon-absorption spectra. A Langendorff-perfused transgenic mouse heart expressing EGFP (peak one-photon excitation wavelength, \( \approx 490 \text{ nm} \)) in a mosaic pattern was loaded with the calcium-sensitive dye rhod-2 (peak one-photon excitation wavelength of the \( \text{Ca}^{2+} \) bound form, \( \approx 550 \text{ nm} \)). White line in full-frame image (a) denotes position of line-scan. Periodic increases in rhod-2 fluorescence in (b) correspond to action potential-evoked [\( \text{Ca}^{2+} \)] transients. Plots of the spatially averaged fluorescence intensities as function of time for an EGFP-expressing and nonexpressing cardiomyocyte (c) show cyclic changes in fluorescence only in the red channel, indicative of effective color separation in the detection path of the microscope.
small number of cages have been determined previously and are usually <2 GM.

Numerous previous studies have exploited the true three-dimensionally resolved excitation of cages using two-photon absorption. Denk was the first to show that the highly localized liberation of carbamylcholine from its bath-applied cage by two-photon absorption-mediated photoactivation can be used in conjunction with whole-cell current measurements to determine the distribution of functional nicotinic acetylcholine receptors in the cell membrane of cultured BH3 cells. Matsuzaki et al went on to use two-photon uncaging of glutamate in hippocampal slices as a means to achieve rapid, reproducible and fine three-dimensional spatial control of neurotransmitter concentration on a scale that faithfully mimics quantal release at the individual synapse. In another study, transfer of uncaged fluorescein between neighboring fiber cells in the periphery of the ocular lens was shown to be highly anisotropic and to occur predominately in radial direction, whereas transport appeared more isotropic in the central portion of the lens and occurred across cell columns (Figure 4c and 4d). The observed directionality of intercellular dye transfer was explained by a redistribution of connexin46 gap junctions along the radial axis, with clustering at the broad site of cells in the periphery and a more dispersed appearance in cells at the center (Figure 4a and 4b). Finally, TPE uncaging of Ca<sup>2+</sup> has become an important tool in probing the role of microdomain Ca<sup>2+</sup> in the regulation of Ca<sup>2+</sup>-sensitive cellular processes. Mulligan and MacVicar determined the impact of changes in Ca<sup>2+</sup> in astrocytes on the diameter of nearby small arterioles in brain slices by using two-photon Ca<sup>2+</sup> uncaging to increase [Ca<sup>2+</sup>]. They were able to demonstrate that vascular constrictions occurred when Ca<sup>2+</sup> waves evoked by uncaging propagated into the astrocyte endfeet, where they triggered the release of vasoactive substances. DelPrincipe et al showed that Ca<sup>2+</sup> signals in isolated cardiomyocytes resulting from TPE-mediated focal release of Ca<sup>2+</sup> shared many similarities with cardiac Ca<sup>2+</sup> sparks, the highly localized transient elevations of intracellular calcium resulting from the coordinated opening of a small number of colocalized SR ryanodine receptors (Figure 4e). When these local Ca<sup>2+</sup> signals were superimposed on global increases in Ca<sup>2+</sup>, their amplitude decreased initially, followed by a gradual recovery (Figure 4f and 4g). These results indicate that global Ca<sup>2+</sup> signals can result in refractoriness of the local Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release process. Soeller and Cannell were also able to produce small repeatable calcium release events using DM-nitrophen in isolated cardiomyocytes, which had spatial and temporal properties very similar to those of naturally occurring Ca<sup>2+</sup> sparks.

In a subsequent study from the same group, artificial sparks with known underlying SR Ca<sup>2+</sup> release fluxes were used to validate various numerical approaches to derive SR Ca<sup>2+</sup> release flux underlying Ca<sup>2+</sup> sparks and, thus, the number of SR ryanodine receptors contributing to a Ca<sup>2+</sup> spark.

Two-photon fluorescence photobleaching recovery is a technically related approach to study the three-dimensional mobility of fluorescent molecules with three-dimensional resolution at a micrometer scale. Short-duration TPE of fluorescent molecules with intense laser light photobleaches a fraction within the ellipsoidal TPE volume of known dimensions (see Figures 1 and 2). As unbleached fluorescent molecules diffuse in, the fluorescence is monitored with a lower laser power to measure fluorescence recovery. From the time course of fluorescence recovery, the diffusion coefficient of the fluorescent molecule can be measured. Svoboda et al used this technique to study the diffusional exchange between dendritic spines and shafts of CA1 neurons in rat hippocampal slices. Stroh et al determined the diffusion coefficient of rhodamine-conjugated nerve growth factor in solution using TPE fluorescence photobleaching. A detailed description of the mathematical equations used to...
Two-photon uncaging and two-photon fluorescence photo-bleaching recovery have many potential applications to the cardiovascular system. The temporal and three-dimensional spatial resolutions of two-photon uncaging are adequate for mediating a wide range of intra- and intercellular events. For example, a previous in vitro study showed that cardiac fibroblasts linking strands of cardiomyocytes are capable of relaying electrochemical activity between strands of cardiomyocytes through electrotropic interactions. Impulse propagation across fibroblast strands was slow and involved gap junctions that were composed of both connexin43 and connexin45. The important question has remained as to whether functional coupling between cardiomyocytes and cardiac fibroblasts actually occurs in vivo, such as, for example, at the interface of the myocardium and cardiac fibroblasts. Redistribution of connexin43 immune reactivity has been shown to occur in the diseased myocardium. Two-photon uncaging of gap junction permeable fluorescent compounds (eg, fluorescein) in combination with the optical sectioning properties of TPE microscopy could be exploited to directly probe the functional communication between heterogeneous cells in the heart and to image the functional consequences of gap-junction remodeling, respectively. Microscopic intracellular Ca\(^{2+}\) transients have been shown to be involved in a variety of cellular events, such as transcription.

The capability of TPE uncaging to reproducibly generate highly localized Ca\(^{2+}\) elevations both in the nucleus and cytoplasm can thus be used for quantitating the relationship between frequency, amplitude, localization, and duration of local Ca\(^{2+}\) elevations and transcription. The availability of caged second messengers (eg, IP\(_3\), cGMP, etc) and neurotransmitters (eg, glutamate) can be exploited toward a better understanding of functional compartmentalization as well as distribution and sensitivity of agonist-activated ion channels in the cardiovascular system, respectively. Toward this end, new cages with larger two-photon absorption action cross sections and with a greater variety of active species need to be developed to further enhance this unique and powerful tool. Finally, the technique of photobleaching recovery can be exploited to determine the mobility of biologically important ions (Na\(^+\), Ca\(^{2+}\)) and molecules in cardiovascular cells.

**TPE Fluorescence Imaging Deep Within Scattering Tissue**

The combination of improved tissue penetration depth and true three-dimensionally resolved fluorophore excitation has made TPE microscopy a preferred tool to provide subcellular resolution images from deep within light scattering tissues in a contextual setting. In vivo two-photon laser scanning microscopy (TPLSM) has been used to probe coupling between neuronal activity and cerebral blood flow. Measuring blood flow in individual brain capillaries with millisecond temporal resolution, Chaingneau et al demonstrated that, in the olfactory bulb superficial layers, changes in capillary flow precisely outline regions of synaptic activation. Kleinfeld et al were able to resolve motion of red blood cells in individual capillaries that lie several hundreds of microns deep in the somatosensory cortex in rat (Figure 5a and 5b). They too found changes in the flux and velocity of red blood cells in individual capillaries in close response to stimulation of appropriate anatomical regions within the somatosensory cortex. Dunn et al have used intravitral multicolor TPLSM of animals injected with fluid-phase probes to characterize bulk fluid flow through the kidney of living animals (Figure 5c and movie in the online data supplement). The latter study demonstrates how bulk tracers may be used to assess capillary blood flow, glomerular filtration, fluid transport, tubular solute concentration, and endocytosis by proximal tubule cells. Further examples of intravitral TPE fluorescence imaging include visualization of lymphocyte trafficking in lymph nodes, reconstruction of tumor angiogenesis, and time-lapse studies of neuronal growth.

TPE fluorescence microscopy has been particularly successful in the imaging of intracellular Ca\(^{2+}\) dynamics in small cellular compartments of live brain tissue (reviewed by Helmchen and Waters). For example, using TPLSM in combination with the fluorescent calcium indicator calcium green-1, Helmchen et al were able to resolve fast-peaking Ca\(^{2+}\) transients in dendritic spines of pyramidal neurons down to a depth of 500 µm below the pial surface of the rat cortex in vivo (Figure 5d).

In contrast, TPLSM has only recently begun to be used for Ca\(^{2+}\) imaging in intact cardiac tissue. Subcellular resolution Ca\(^{2+}\) imaging in intact myocardium using single-photon confocal microscopy is typically restricted to regions less than ~40 µm below the surface. The capability of TPLSM to provide optical sectioning with subcellular resolution from deeper within scattering biological specimens than confocal microscopy has recently been exploited to more transients at depths of up to 200 µm below the epicardial surface. Direct measurements of the optical resolution, as well as calculations of the lateral (x,y) and axial (z) width of the TPE volume for a 1.2 numerical aperture objective lens (see Figure 2a), are compatible with the notion that under the imaging conditions used, both the lateral and axial dimension of the TPE volume are considerably less than the average depth, width, or length of adult murine ventricular cardiomyocytes (~13, ~32, and ~140 µm, respectively) (see Satoh et al). Thus, the system allows selective visualization of [Ca\(^{2+}\)], in a volume significantly less than that of a single cardiomyocyte within the intact heart without being affected by fluorescent signals from neighboring cells.

TPE imaging of single myocyte [Ca\(^{2+}\)], transients within the intact heart requires complete immobilization of the preparation to prevent regions of interest from moving out of the plane of focus. Cytochalasin D at a concentration of 50 μmol/L effectively eliminates movement artifacts in Langendorff-perfused mouse hearts during TPE imaging but does not abolish action potential-evoked [Ca\(^{2+}\)] transients. Importantly, many spatial and temporal properties of stimulated rhod-2 transients recorded from individual
ventricular myocytes within the buffer-perfused mouse hearts in the presence of cytochalasin D are qualitatively identical with those of electrically evoked fluo-3 transients previously reported for single, isolated mouse ventricular cardiomyocytes in the absence of cytochalasin D but in otherwise identical experimental conditions. These properties include shortening of the transient in response to increased stimulation rates (Figure 7),25,61 as well as rapid and spatially uniform rises of \([Ca^{2+}]_{i}\) (Figures 6 and 7), reflecting synchronous activation of SR ryanodine receptors secondary to \(Ca^{2+}\) influx through activated L-type \(Ca^{2+}\) channels in the t-tubular membrane.62 Thus, cytochalasin D-induced immobilization enables subcellular resolution \([Ca^{2+}]_{i}\) measurements in single cells within the intact, buffer-perfused heart using TPLSM.

TPE fluorescence microscopy has recently been exploited to probe the functional integration of donor cells following intracardiac transplantation (Figure 7).2,3 Using TPE laser scanning microscopy with a high numerical aperture objective (to minimize TPE volume), EGFP-expressing donor fetal ventricular cardiomyocytes (which appeared yellow because of the overlay of red rhod-2 and green EGFP fluorescence) were shown to exhibit action potential-induced \([Ca^{2+}]_{i}\) transients in synchrony with their neighboring EGFP-negative cardiomyocytes, indicating that they are functionally coupled.
and spatiotemporal properties of action potential-evoked myocytes. For example, distinct differences in the magnitude and/or functional heterogeneity between neighboring cardiomyocytes could be detected when the scan was approximately halfway across the field of vision. The stimulated [Ca\textsuperscript{2+}] transients appeared to rise quite uniformly as a result of intra- and intercellularly synchronized activation of SR calcium release. Red arrowheads, endothelial cells. Transverse line-scan mode images of action potential-evoked [Ca\textsuperscript{2+}] transients in three cardiomyocytes located at 10, 150, and 200 μm from the epicardial surface during stimulation at 1 Hz. Images were obtained by using a Zeiss LSM-510 Meta laser scanning microscope modified for two-photon illumination. The excitation beam (810 nm, ∼100 fs, 82 MHz) was scanned through a ×40, 1.2 numerical aperture water-immersion objective. Note the rapid and spatially uniform rise in [Ca\textsuperscript{2+}] in all three cells. c, Time courses of spatially averaged rhod-2 fluorescence from the cells in (b). d, Plots of normalized rhod-2 fluorescence as function of time derived from traces shown in (c).

(Figure 7a through 7c). Moreover, the kinetics of [Ca\textsuperscript{2+}] transients in transplanted ventricular cardiomyocytes were indistinguishable from those in host cardiomyocytes (Figure 7d). In contrast, the majority of donor-derived myocytes in hearts carrying EGFP-expressing skeletal myoblast grafts did not develop [Ca\textsuperscript{2+}] transients in response to propagating action potentials (Figure 7f), suggesting that they are functionally isolated. A small fraction of EGFP-expressing myocytes at the graft-host border, most likely arising from skeletal myoblast-cardiomyocyte fusion events, developed action potential-evoked [Ca\textsuperscript{2+}] transients in synchrony with their adjacent host cardiomyocytes (Figure 7f and 7g). The time courses of [Ca\textsuperscript{2+}], transients in these cells could be distinctly different from those in their neighboring host cardiomyocyte, as shown in Figure 7h. These observations demonstrate exemplarily that TPLSM is ideally suited to determine the functional state of individual donor cells following their intracardiac transplantation (provided they express a fluorescent marker) and is able to resolve spatial microheterogeneities of intracellular Ca\textsuperscript{2+} signaling within the intact, buffer-perfused mouse heart with high temporal resolution.

TPE-based laser scanning fluorescence microscopy allows spatially and temporally resolved visualization of Ca\textsuperscript{2+} dynamics from deeper within intact cardiac tissue than previously attainable with confocal imaging. The approach should be of general utility to monitor the consequences of genetic and/or functional heterogeneity between neighboring cardiomyocytes. For example, distinct differences in the magnitude and spatiotemporal properties of action potential-evoked [Ca\textsuperscript{2+}] transients have been found between single ventricular cardiomyocytes isolated from different transmural layers of the normal heart, between single ventricular cardiomyocytes and Purkinje cardiomyocytes, as well as between single ventricular cardiomyocytes adjacent to and remote from the infarct zone, respectively. It will be important to examine whether and how this in vitro heterogeneity is modulated at the whole heart level, where movement of calcium ions from cell to cell may attenuate intrinsic differences in cardiomyocyte Ca\textsuperscript{2+} signaling. Localized, subcellular sarcoplasmic reticulum Ca\textsuperscript{2+} release events (Ca\textsuperscript{2+} sparks) have been shown to precede or lead to Ca\textsuperscript{2+} waves, which in turn trigger arrhythmogenic afterdepolarizations via modulation of Ca\textsuperscript{2+}-sensitive transmembrane ion channels and transporters. Analysis of the mechanisms underlying initiation, propagation, and termination of Ca\textsuperscript{2+} waves in intact cardiac tissue should provide tremendous information concerning the role of intracellular calcium in cardiac arrhythmogenesis. TPLSM is also particularly well suited to follow the functional fate of donor cells following direct intracardiac delivery, or following homing to the site of injury, provided that the donor cells can be identified on the basis of fluorescent properties. The ability to assess functional aspects at the individual cell level (as opposed to global heart function) may also permit a better discrimination between a nonspecific effect on postinjury remodeling versus a direct contribution of transplanted donor cells to a functional syncytium.

TPE Imaging of Cellular Redox State

Two-photon microscopy provides metabolic imaging with subcellular resolution by using the inherent fluorescence of...
NADH as an indicator of both oxidative and glycolytic energy metabolism. Although its two-photon absorption cross section is approximately two to three orders of magnitude lower than that of conventional fluorophores (<0.1 GM at excitation wavelengths of ~700 nm), in vivo detection of NADH autofluorescence by two-photon microscopy is still feasible because of its high (millimolar) concentration and enhanced action cross section in the intracellular milieu compared with its aqueous solution. Kasischke et al exploited the inherent three-dimensional resolution and increased penetration depth of TPE fluorescence microscopy to monitor intrinsic fluorescence of NADH deep in hippocampal brain slices several hundreds of microns thick. They were able to demonstrate that the NADH response to focal neural stimulation is composed of an early dendritic dip (indicating increased NADH oxidation by the respiratory chain) and a late overshoot in juxtaposed astrocytes (indicating NADH production during nonoxidative glycolysis). This functional imaging study thus confirmed the ideal suitability of TPE microscopy to reveal spatiotemporal compartmentalization with high resolution in intact, highly scattering tissue.

Figure 7. TPE Ca\textsuperscript{2+} imaging in hearts carrying cellular grafts. a through e, Simultaneous imaging of rhod-2 and EGFP fluorescence in a nontransgenic mouse following transplantation of EGFP-expressing fetal cardiomyocytes. Red (rhod-2) and green (EGFP) fluorescent signals were superimposed. a, Full-frame mode image obtained during continuous stimulation at 2 Hz. Ripple-like wavefronts correspond to action potential-evoked [Ca\textsuperscript{2+}] transients. b, Line-scan mode image of the region in (a) demarcated by the white line. The line scan encompasses 7 cardiomyocytes. Cells 1, 4, and 7 are host (EGFP-negative) cardiomyocytes and cells 2, 3, 5, and 6 are EGFP-expressing donor cardiomyocytes. The preparation was paced at the rate indicated. Spont. indicates spontaneous [Ca\textsuperscript{2+}] transient. c, Spatially integrated traces of the changes in rhod-2 (red) and EGFP (green) fluorescence for cardiomyocytes No. 1 (host) and No. 2 (donor). Tracings were recorded during pacing at 2 and 4 Hz as indicated. d, Normalized and superimposed tracings of electrically evoked changes in rhod-2 fluorescence as a function of time from host (squares and triangles) and donor (circles) cardiomyocytes. e, Full-frame images taken from the same heart as in (a) at the depths indicated. Preparation was paced continuously at 2 Hz. f, Full-frame mode images of a rhod-2 loaded following transplantation of EGFP-expressing skeletal myoblasts. The heart was stimulated at a rate of 4 Hz. Asterisk denotes noncoupled donor cell. g, Line-scan images depicting [Ca\textsuperscript{2+}] transients at 2 and 4 Hz along the white line in (f). h, Superimposed tracings of normalized changes in rhod-2 fluorescence as a function of time from the EGFP-positive myotube (green traces) and neighboring host EGFP-negative cardiomyocytes, respectively.
The combined benefits of TPE fluorescence microscopy, including deeper penetration depth in thick, highly scattering biological specimens, and decreased overall fluorophore photobleaching and photodamage compared with confocal microscopy, are particularly useful for developmental studies that require the maintenance of viability to detect sequential events in the same specimen over extended periods of time. Thus, TPE laser scanning microscopy should enable numerous long-term imaging studies in living embryos to reconstruct dynamic processes such as gene and protein expression patterns in three dimensions\(^6\) and should enable imaging of three-dimensional cell migration. The advantages and disadvantages of TPLSM, however, for long-term imaging of developmental processes have to be compared with those of a novel optical sectioning technique, named selective plane illumination microscopy,\(^73\) which appears to generate even less photodamage/photobleaching than TPLSM at comparable spatial resolution.

**Future Developments**

Two-photon microscopy excels at high-resolution imaging hundreds of microns deep in living tissue. The maximal imaging depth attainable with TPE is likely to increase even further in the future with the development of chromophores with higher TPE cross sections and implementation of techniques that will reduce fluorescence collection loss caused by absorption and scattering. The development of femtosecond lasers that are tunable to wavelengths in the 1000- to 1300-nm range will further improve laser-beam penetration depth and will enable TPE of longer wavelengths dyes and fluorescent proteins but, at the same time, lower the risk of photobleaching and photodamage. New signal processing algorithms will offer the opportunity to simultaneously image multiple colored, spectrally overlapping markers in living cells and tissue. Finally, the combination of TPE with time-resolved techniques including fluorescence lifetime imaging (which may be useful for analyzing FRET between engineered proteins), fluorescence anisotropy, and fluorescence correlation spectroscopy will provide new opportunities for studying cellular microenvironments and the behavior of fluorescent probes in the intracellular milieu.\(^14\),\(^15\) Thus, TPE microscopy will remain a valuable tool for imaging cellular and subcellular events within living tissue and, therefore, provide a technical basis for a much-needed integrative approach to biological problems.

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**References**

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**Components of a Two-Photon Laser Scanning Fluorescence Microscope**

In general, a two-photon microscope consists of a confocal laser scanning microscope converted for two-photon operation. Modifications include a pulsed laser as excitation light source, a large-area photomultiplier for direct (‘non-descanned’) fluorescence detection, a dichroic beam splitter for near-infrared reflection, and a spectral analyzer to monitor the laser spectrum. Images can be acquired in full-frame or line-scan mode. In order to perform TPE-mediated photolysis or two-photon fluorescence photobleaching recovery, it is important to be able to ‘park’ the laser beam at any specified \(x-y\) position. Detailed descriptions of the various components of a two-photon laser scanning microscope (TPLSM) can be found in references 1, 2, 3 and 4.

*Fluorescence Detection.* Since two-photon excitation (and thus fluorescence emission) is restricted to the focal plane, no confocal pinhole is required in the detection path of the microscope to reject out-of-focus fluorescence. Therefore, both scattered and non-scattered (‘ballistic’) emissions can be used to generate the two-photon image. The most efficient method of fluorescence collection is obtained by using ‘non-descanned’ or direct detectors. Large-area photomultipliers with high quantum efficiency offer a particular attractive approach to maximize collection efficiency in TPLSM, such as GaAsP photocathode photomultipliers (Hamamatsu Inc.) for fluorescence detection in the 400-650-nm range. It is possible to increase optical resolution by using descanned detection in conjunction with a confocal pinhole.5 However, a confocal aperture will reject scattered emissions, even though they originated in the focal plane, thereby reducing imaging depth.
Light Source. The most common and robust light source for two-photon illumination has been the mode-locked Ti: Sapphire laser with pulse width and repetition frequency of $\sim$100 fs and $\sim$80 MHz, respectively. Mode-locking is a technique used for generating ultra-short and highly intense laser light pulses. An ultra-fast laser simultaneously lases in many different modes, but the phases of these modes are uncorrelated, generating random fluctuations in the intensity over time. Mode-locking is a technique to create a known correlation between the phases and make it possible to predict when the intensity maxima will occur. Monitoring the laser spectrum is used to ensure proper mode-locking in a two-photon imaging system. The spectrum should exhibit a symmetrical Gaussian shape without evidence for spikes. Ti:Sapphire laser pulses have a large spectral bandwidth (spectral FWHM, $\sim$10 nm). The tuning range of the Ti:Sapphire laser ($\sim$700 to $\sim$1000 nm) permits two-photon excitation of many biologically relevant fluorophores.\textsuperscript{6,7}

**Movie 1:** Male Sprague-Dawley rats were infused with a 3,000 MW Cascade Blue dextran 24 hours prior to *in vivo* imaging via 2-photon microscopy. At the time of imaging, the rats were given Hoechst 33342 to label the nuclei, a 3,000 MW Texas Red dextran and a 500,000 MW Fluorescein dextran. The 500,000 MW Fluorescein dextran (GREEN) does not filter into the Bowman’s space and remains in the microvasculature, filling up the plasma volume. The dark oblong shadows are red blood cells that exclude the green dye. They appear as streaks because of the speed at which they circulate through the vasculature, in some cases exceeding 300 microns/second. The 3,000 MW dextrans rapidly filter into the Bowman’s space and travel through different tubule segments where they are eventually excreted. The Cascade Blue dextran (BLUE), given 24 hours prior, is localized towards the basal side of proximal tubule cells (PT) within mature lysosomes (thin arrow). The Texas Red dextran (RED), given minutes prior to
imaging, is localized to the early endosomes just below the apical region of the cells (thick arrow). Unlike proximal tubules, distal tubules (DT) do not accumulate these dextrans within the cells. The dextrans do, however, move through and become brighter as water is reabsorbed and the fluorescent dextran becomes more concentrated. Finally, the nuclei of all cells (CYAN-BLUE) are clearly visible. The nuclei of distal tubules (DT) generally fluoresce brighter than those of proximal tubules (PT). Other nuclei visible include endothelial and smooth muscle cell nuclei around the microvasculature and a circulating white cell in the upper right (asterisk, thin arrow). Imaging carried out by Dunn KW, Sandoval RM, and Molitoris BA, Indiana University.

References


