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Cardiovascular Imaging Using Two-Photon Microscopy

John A. Scherschel^{1,2} and Michael Rubart^{1,*}

¹*Department of Pediatrics, Division of Cardiology, Wells Center for Pediatric Research, 1044 West Walnut Street, Indianapolis, IN 46202, USA*

²*Department of Medicine, Division of Cardiology, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202, USA*

Abstract

Two-photon excitation microscopy has become the standard technique for high resolution deep tissue and intravital imaging. It provides intrinsic three-dimensional resolution in combination with increased penetration depth compared to single-photon confocal microscopy. This article will describe the basic physical principles of two-photon excitation and will review its multiple applications to cardiovascular imaging, including second harmonic generation and fluorescence laser scanning microscopy. In particular, the capability and limitations of multiphoton microscopy to assess functional heterogeneity on a cellular scale deep within intact, Langendorff-perfused hearts are demonstrated. It will also discuss the use of two-photon excitation-induced release of caged compounds for the study of intracellular calcium signaling and intercellular dye transfer.

Keywords

two-photon excitation; laser scanning microscopy; second harmonic generation; calcium imaging

Introduction

Two-photon excitation (TPE) microscopy has become the standard technique for high-resolution deep tissue and intravital imaging. The intrinsic three-dimensional (3D) resolution of nonlinear optical microscopy combined with the greater imaging penetration depth compared to single-photon confocal microscopy has been widely used to study structure and function of a number of tissue types with cellular/subcellular resolution. For example, neuroscientists have exploited TPE microscopy to accurately map neuronal activity in three dimensions with high spatial and temporal resolution (Helmchen & Denk, 2005). By contrast, nonlinear optical microscopy has only recently begun to be used for imaging of intact cardiovascular tissue (Rubart et al. 2003a, 2003b, 2004b; Zipfel et al. 2003a), despite a great need for assays capable of characterizing functional properties of individual cardiac/vascular cells within their natural environment. For example, cardiomyocyte action potential properties are very likely to be different in intact cardiac muscle compared to single myocytes obtained by enzymatic digestion. The peak of the action potential is typically more positive in the isolated myocytes, because there is less passive outward current to oppose the depolarizing effect of locally activating inward sodium current. Nonmyocytes (e.g., fibroblasts, endothelial cells) may modulate the electrical properties of cardiomyocytes *in situ*. Finally, the precise mechanisms underlying 3D propagation of regenerative Ca^{2+} waves both at the cellular and multicellular level within the intact heart are not well understood, despite their alleged causal

*Corresponding author. E-mail: mrubartv@iupui.edu

involvement in fatal cardiac arrhythmias. Most recent work by Cerrone et al. (2007) suggests that events involving the entire heart (e.g., arrhythmias) can originate from events occurring at the single cell level (e.g., Purkinje myocytes). Thus, cardiac physiology/pathophysiology mandates development of imaging assays capable of resolving dynamic events with cellular/subcellular resolution deep in cardiac tissue. This article will highlight the potential of TPE microscopy to address some of these issues. It describes the basic physical principles of TPE and reviews the advantages and current limitations of its use in laser scanning microscopy of cardiovascular cells and tissue. Where possible, illustrative examples will demonstrate how the use of this technique can enhance our understanding of cardiovascular structure and function.

Principles of TPE

Whereas in conventional confocal laser scanning fluorescence microscopy absorption of a single photon provides sufficient energy for the fluorophore to reach the excited state from which it returns to the ground state by emitting a photon of fluorescence, fluorophore excitation can also be achieved by the near simultaneous absorption of two longer wavelength photons, as in two-photon fluorescence microscopy. For a two-photon process, the excitation rate is proportional to the product of the average squared light intensity (in units of photons $\text{cm}^{-2} \text{s}^{-1}$) and the TPE cross section of a molecule (in units of $\text{cm}^4 \text{s photon}^{-1}$; $10^{-50} \text{cm}^4 \text{s photon}^{-1}$ equals 1 Göppert-Mayer [GM] unit). The TPE cross section is a quantitative measure of the probability of a molecule to absorb two photons simultaneously. Values of TPE cross sections at the peak absorption wavelength vary from 10^{-2} GM for NAD(P)H (Xu et al, 1996b) to ~50,000 GM for cadmium selenide-zinc sulfide quantum dots (Larson et al, 2003). The values for most of the commonly used fluorophores are in the range of 1 to 300 GM (Xu & Webb, 1996a; Larson et al, 2003). Green fluorescent protein and its blue and red-shifted variants have relatively large TPE action cross sections in the range of ~100 to ~200 GM, making them well suited for intravital two-photon microscopy (Potter et al, 1996; Rubart et al., 2003a, 2004a).

The dependence of the TPE process on the second power of local light intensity gives this technique its intrinsic 3D resolution (see right panel in Fig. 1). Because the energy of a single long-wavelength photon is insufficient to excite commonly used fluorescent dyes, linear absorption by fluorophores above and below the plane of focus does not occur. Excitation (and thus emission) is confined to a small ellipsoid volume around the focal point, where light intensity is sufficiently high to give rise to TPE events. Importantly, because out-of-focus fluorescence is not generated, TPE microscopy provides optical sectioning without the need to introduce a pinhole in the emission path of the microscope, as in confocal microscopy. Thus, all of the signal generated within the sample can be collected by a large-area detector and contribute to the final image (nondescanned acquisition mode in contrast to descanned mode in single-photon laser scanning confocal microscopy).

If image acquisition is performed in nondescanned mode, the 3D resolution of a two-photon imaging system largely depends on the dimensions of the TPE volume within the sample. In turn, the size and shape of the TPE volume are determined by the numerical aperture of the objective lens and the illumination wavelength. For a uniformly illuminated, high numerical aperture, water immersion objective and an excitation wavelength of 850 nm, previous calculations based on an ellipsoidal Gaussian approximation (Zipfel et al, 2003b) to the diffraction-limited focus yielded TPE volumes of less than 1 femtoliter, with $<1\text{-}\mu\text{m}$ resolution in the z -direction. Thus, TPE microscopy theoretically provides fluorescence imaging with subcellular resolution. Importantly, however, optical aberrations (e.g., spherical aberration resulting from refractive index mismatch between the immersion medium and the specimen)

may cause an increase in the effective TPE volume within the specimen, causing a decrease in spatial resolution (see below).

TPE events are exceedingly rare at light intensities typically used for epifluorescence microscopy. To increase the probability of TPE events to a level that is practical for TPE laser scanning microscopy, sample illumination is generally provided by pulsed lasers such as a mode-locked Ti:Sapphire laser, which generates pulses of ~ 100 -fs duration at a repetition rate of ~ 80 MHz. Although the instantaneous light intensity around the focal spot is extremely high, the average energy received by the sample remains relatively low. The probability of TPE events is inversely proportional to the product of pulse repetition rate and pulse duration. Thus, shortening of the pulse duration and/or reducing the pulse rate can increase the probability of TPE events. However, the peak laser intensity at the lower repetition rate will be unusually high, increasing the risk for photobleaching and photo-damage (Koester et al., 1999; Patterson & Piston, 2000; Hopt et al., 2001). The optical settings will thus be a compromise between maximizing TPE events and minimizing adverse photo effects. For more detailed descriptions of the technical aspects of multiphoton microscopy, specifically on imaging system parameters for deep high resolution imaging in living tissue, see previously published reviews (Zipfel et al., 2003; Helmchen & Denk, 2005).

TPE in conjunction with laser scanning microscopy has several advantages over singlephoton laser scanning confocal microscopy. One major advantage is its documented capability to provide optical sectioning from deeper within biological specimens than single-photon confocal microscopy, making two-photon microscopy the standard for deep tissue, high-resolution intravital imaging. Several factors account for the increase in imaging depth: First, TPE microscopy lacks linear absorption of the excitation beam by fluorophores above the planes of focus (see Fig. 1), which can markedly reduce excitation light before it reaches fluorophores within deeper tissue regions. Second, the longer wavelengths used for TPE are scattered by the tissue less than the short wavelengths used for single-photon microscopy. Third, because TPE never generates out-of-focus fluorescence (see Fig. 1), 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 (Oheim et al., 2001).

In addition to its greater penetration depth, TPE fluorescence microscopy has other advantages: First, it reduces photobleaching and photodamage by confining it to the narrow volume around the focal plane. This reduction becomes relevant when collecting z-stacks repeatedly over time (time-lapse imaging). By contrast, in one-photon confocal microscopy all focal planes are exposed to excitation light every time an individual x-y scan is obtained. Second, the spatial restriction of TPE provides the opportunity for three-dimensionally resolved photoactivated release of biologically active compounds, such as neurotransmitters, second messengers, and calcium ions. Third, a number of fluorophores with disparate single-photon excitation spectra can be excited simultaneously by TPE at a single wavelength (Rubart et al., 2003b), avoiding chromatic aberration effects. This feature enables multicolor fluorescence imaging, provided that the emission spectra of the dyes are well separated.

The ability of TPE fluorescence microscopy to image in real time dynamic events on a subcellular scale deep within living tissue is highlighted in the experiments shown in Fig. 2. In this study by Matsumoto-Ida et al. (2006), Langendorff-perfused rat hearts were loaded with tetramethylrhodamine ethyl ester (TMRE), a fluorescent mitochondrial membrane potential indicator, and subsequently subjected to an ischemia/reperfusion protocol. TPE microscopy was capable of resolving individual TMRE-stained mitochondria within cardiomyocytes *in situ* (Fig. 2C). Time series of two-photon scans revealed marked spatial (Fig. 2D) and temporal (Fig. 2E) heterogeneity in the loss of mitochondrial membrane potential during ischemia and

subsequent reperfusion, as indicated by the disappearance of red TMRE fluorescence in some cardiomyocytes (Fig. 2D). Thus, the study by Matsumoto-Ida and colleagues demonstrates the utility of two-photon fluorescence microscopy to monitor in real time dynamic changes in indicator fluorescence occurring on a cellular/subcellular scale within living cardiac tissue.

Spatial Resolution of Two-Photon Microscopy at Depth in Biological Tissue

Initial experimental observations (Centonze & White, 1998) suggested that the spatial resolution of tissue two-photon microscopy does not significantly decline with increasing imaging depth. Subsequent theoretical predictions (Gauderon et al., 1999), however, demonstrated that wave front aberrations caused by refractive index mismatches between the immersion medium and the tissue as well as within the tissue itself compromise 3D resolution in multiphoton laser scanning microscopy, even when water immersion objectives are used. Recent experimental observations confirm these calculations, as demonstrated in Fig. 3. In this study, Niesner et al. (2007) determined the dependence of the spatial resolution of a two-photon laser scanning microscope on the penetration depth in fixed brain tissue and freshly isolated lymph nodes. The spatial resolution of a laser scanning microscope is typically determined by the dimensions of the point spread function (PSF) of a punctiform object with dimensions below the optical resolution limit. Figure 3A illustrates the lateral (upper row) and axial (lower row) dimensions of the PSF of 100-nm fluorescent beads excited at 800 nm as a function of focal depth for brain tissue and lymph nodes. Both the lateral (y) and axial (z) dimension of the fluorescent profiles progressively widened with increasing imaging depth in either tissue type, indicating loss of spatial resolution. Interestingly, the spatial resolution was strongly influenced not only by the focal depth but also by the tissue type and constitution, as illustrated in Figure 3B. Whereas lymphatic tissue exhibited an approximately threefold reduction in both the lateral and axial resolution when the focal plane was moved 50 μm below the surface, the depth-dependent degradation of the spatial resolution in brain tissue was less pronounced over the same focal shift and was lowest in regions containing primarily axons. The study by Niesner et al. has two important implications for the application of intravital two-photon fluorescence microscopy. First, image deconvolution, a method to restore optical quality using mathematical postprocessing of image data, will have to take into account the depth- and tissue type-related changes in spatial resolution. Currently, 3D deconvolution of z -stacks of two-photon fluorescence images is typically performed using a general PSF either calculated based on microscope specifications or evaluated from the spatial fluorescence profile of subresolution beads in agarose. Based on the results by Niesner and coworkers, deconvolution using tissue type- and depth-dependent PSFs appears to be preferable to deconvolution based on general PSFs. Second, the findings by Niesner et al. (2007) suggest that the ability of two-photon microscopy to spatially discriminate dynamic changes in fluorescence progressively declines with increasing imaging depth. For example, based on data shown in Figure 3C, a decrease in the axial resolution from $\sim 1.5 \mu\text{m}$ at the tissue surface to $\sim 4 \mu\text{m}$ at a focal depth of 50 μm (Fig. 3C) would imply that fluorescence signals sampled from cell volume elements located within several microns from a neighboring cell represent variable contributions from both cells. This signal contamination could mask the existence of functional heterogeneities occurring at a cell-to-cell and/or sub-cellular level, unless measurements of the spatial resolution of the imaging system for the specific tissue type and imaging depth are provided. Approaches to enhance the spatial resolution at depth in tissue two-photon microscopy include the application of PSF engineering by means of deformable mirrors (Rueckel et al., 2006) and insertion of a confocal pinhole into the emission path of the microscope. The latter approach, however, typically causes a loss of the fluorescence signal at depth and is therefore impractical (Gauderon et al., 1999).

Cardiovascular Applications of TPE Microscopy

Two-Photon Photolysis

Photoactivatable “caged” compounds are biologically inert precursors of active molecules that, when excited by light, release the active species. Photo release of the trapped species usually occurs within less than a millisecond. For example, release of calcium ions from the photolabile Ca^{2+} chelator DM nitrophen is complete after several microseconds and results in Ca^{2+} concentration jumps at the site of photoactivation. The property of photolyzable Ca^{2+} cages to rapidly and efficiently free trapped calcium ions upon illumination has been exploited by numerous investigators to study the regulation of calcium-sensitive intracellular signaling cascades, intercellular calcium signaling, or gating of ion channels (Hadley & Lederer, 1991; Parthasarathi et al., 2006). The more recent development of TPE-induced uncaging of calcium has provided the opportunity to photorelease caged compounds on a subcellular scale and maintain the high temporal resolution of conventional photoactivation. The dependence of TPE of a caged compound on the second power of laser intensity limits photorelease to small volumes around the focal point, giving this technique its intrinsic 3D resolution. Using TPE with high numerical aperture objectives and near-infrared light, it has been shown that rapid ($<50 \mu\text{s}$) release of calcium from its cages can be confined to less than femtoliter volumes, with an $\sim 1\text{-}\mu\text{m}$ resolution along the z axis (Brown et al., 1999). Consequently, the true three-dimensionally resolved excitation of cages using TPE has been exploited by numerous investigators to study the role of intracellular Ca^{2+} microdomains in the regulation of Ca^{2+} -sensitive cellular processes. For example, Ji and coworkers recently used TPE of caged Ca^{2+} (DMNP-EDTA) to determine the extent to which spatially restricted rises in $[\text{Ca}^{2+}]_i$ trigger Ca^{2+} release from SR calcium stores in isolated urinary bladder cells that were loaded with the fluorescent calcium indicator fluo-4 (Ji et al., 2006). Localized two-photon illumination reproducibly resulted in focal calcium transients (Fig. 4A), whose kinetics were very similar to those of the previously described spontaneous smooth muscle Ca^{2+} sparks, the highly localized transient increases in intracellular calcium resulting from the coordinated opening of a small number of colocalized SR ryanodine receptors (Nelson et al., 1995). To confirm that the triggered Ca^{2+} transients did not represent increases in $[\text{Ca}^{2+}]_i$ associated with the Ca^{2+} uncaging itself but rather occurred subsequent to TPE-induced localized rise in $[\text{Ca}^{2+}]_i$ (Ca^{2+} -induced Ca^{2+} release), photolysis experiments were repeated in the presence of specific inhibitors of SR Ca^{2+} release channels. Figure 4B demonstrates that inducibility of propagating calcium waves by two-photon flash photolysis was markedly altered by inhibition of the ryanodine-sensitive Ca^{2+} release channel (middle panel) and was completely ablated in the combined presence of ryanodine and the inositol 1,4,5-trisphosphate receptor (InsP_3R) antagonist xestospongin (Fig. 4B, lower panel). These results indicated that not only ryanodine-sensitive release channels in smooth muscle are activated by increases in the cytoplasmic calcium (similar to the Ca^{2+} -induced Ca^{2+} release process in cardiomyocytes), but that the gating of InsP_3 -sensitive Ca^{2+} release channels can also be augmented by local increases in calcium, independent of activation by the InsP_3R agonist phospholipase C. Although these studies were performed in urinary bladder smooth muscle cells, it is very likely that similar or identical processes exist in vascular smooth muscle cells. DelPrincipe et al. (1999) used TPE to induce artificial Ca^{2+} sparks in single cardiomyocytes. When TPE-triggered sparks were superimposed on electrically evoked global increases in cytosolic Ca^{2+} , they noticed an initial decrease in the Ca^{2+} spark amplitude, followed by a gradual recovery during the relaxation phase of the global transient. These results indicated that global Ca^{2+} signals can result in refractoriness of the local Ca^{2+} -induced Ca^{2+} release mechanism and were in agreement with earlier observations that cardiomyocyte calcium waves, which are thought to result from propagating activation of neighboring SR Ca^{2+} release channels, can be extinguished by colliding Ca^{2+} transients (Rubart et al., 2003b). Soeller and Cannell (1999) were able to trigger small-amplitude localized rises in $[\text{Ca}^{2+}]_i$ in isolated cardiomyocytes using TPE of DM-

nitrophen-caged Ca^{2+} . They subsequently used information gained from studying these artificial Ca^{2+} release events to validate numerical approaches to derive Ca^{2+} flux through SR release channels during a naturally occurring single Ca^{2+} spark, and thus the number of SR release channels within a cluster that contribute to a spark. Finally, Mulligan and MacVicar (2004) used TPE Ca^{2+} uncaging in astrocytes to examine the impact of local $[\text{Ca}^{2+}]_i$ elevations in regulating the diameter of nearby small arterioles.

In other studies, TP photolysis was used to study inter-cellular dye transfer through gap junctions in three dimensions. In one such study, dissected pancreatic acini were loaded with the green fluorescent dye calcein/AM (Fig. 5), to label individual acinar cells, and the cell membrane permeable caged coumarin dye NPE-HCC2.AM (Dakin & Li, 2006). The caged coumarin is not fluorescent and cannot cross gap junctional channels. Two-photon illumination of one acinar cell resulted in release of gap-junction permeable fluorescent coumarin from the caged state, and its diffusion into neighboring cells via gap junctions was followed in real time by acquiring two-photon z-stacks at defined time intervals post photoactivation. Because the wavelength used for imaging does not liberate the active species from the cage, two-photon uncaging and imaging can be combined. In another study, transfer of uncaged fluorescein across gap junctions of neighboring cells in the ocular lens was shown to be highly anisotropic and to occur predominantly in the radial direction in the lens periphery, whereas diffusivity appeared more isotropic in the central portion of the lens. This diffusion pattern correlated with the spatial distribution of gap junctional proteins along the radial axes of the cells, with clustering at the broad site of cells in the periphery and a more dispersed appearance in cells at the center of the lens (Jacobs et al., 2004).

The amount of caged compound that is released in a given photolysis experiment largely depends, among other factors, on the TPE action cross section. Values for TPE action cross section have been measured for a small number of cages previously and are usually $<2 \text{ GM}$ (Rubart, 2004b; Dakin & Li, 2006). Thus, relatively high average illumination energies are necessary to induce photoactivation, which in turn increases the likelihood of inducing photodamage (Ji et al., 2006). Careful titration of the incident laser power by adjusting, for example, the wavelength of the excitation light and/or illumination duration is necessary.

TPE uncaging has many potential applications to the cardiovascular system. The three-dimensionally resolved excitation of cages is ideally suited to study gap junctional communication in the normal and diseased heart. Redistribution of cardiac gap junctions in the diseased heart has been causally invoked in the genesis of arrhythmias (Akar et al., 2004; Danik et al., 2004; Ai & Pogwizd, 2005). Also, previous *in vitro* studies (Gaudesius et al., 2003; Miragoli et al., 2006; Chilton et al., 2007) showed that myofibroblasts electrically couple to cardiomyocytes via connexin43-gap junctional channels and modulate excitability, automaticity, and conduction of the coupled cardiomyocytes through electrotonic interactions. The question has remained as to whether electrical coupling between heterologous cells actually occurs *in vivo*, such as, for example, at the junction of scar tissue and surviving myocardium in the infarct border zone or at the transitional zone between the sinus node and atrial working myocardium. Combining TPE uncaging of gap junction permeable fluorophores and the optical sectioning properties of TPE microscopy could be utilized to prove or disprove, respectively, the existence of heterologous cell coupling as well as to directly determine the functional impact of gap junction redistribution in the diseased heart *in situ*. Further, the possibility to design caged compounds containing fluorophores with selective permeabilities for different types of gap junctional channels that exist in the cardiovascular system would provide the basis for future studies aimed at visualizing coupling pattern and coupling strength variations within the intact tissue (Eckert, 2006). Further, localized $[\text{Ca}^{2+}]_i$ elevations have been shown to regulate DNA transcription in cardiomyocytes (Wu et al., 2006). The capability of TPE uncaging to reproducibly generate highly localized Ca^{2+} elevations both in the nucleus

and cytoplasm could be used to unveil the mechanisms by which the cell decodes frequency, amplitude, localization, and duration of local Ca^{2+} elevations into transcription of genes. The availability of caged second messengers (e.g., cAMP, InsP_3) and neurotransmitters (e.g., glutamate) can be exploited to determine functional compartmentalization as well as distribution and sensitivity of agonist activated ion channels in the cardiovascular system, respectively. Progress in this area will largely depend on the availability of membrane permeable caged compounds with larger two-photon absorption cross sections and a greater variety of active species. Because the TPE volume may increase with increasing focal depth in living tissue, the volume of photorelease of caged compounds may similarly increase. Calculations of the two-photon photolysis volume for thick biological specimens should therefore be used with great caution.

TPE Fluorescence Imaging of Cardiovascular Tissue

TPE imaging of dynamic events (e.g., calcium transients) on a subcellular scale within the intact heart requires sufficient immobilization of the specimen to prevent regions of interest from moving out of the plane of focus. One type of immobilization approach entails pharmacological inhibition of the excitation-contraction mechanism. Cytochalasin D at a concentration of $50 \mu\text{mol/L}$ was previously shown to sufficiently eliminate motion artifacts in Langendorff-perfused rat and mouse hearts, respectively, during single- and dual photon-excitation imaging (Rubart et al., 2003a, 2003b, 2004b; Aistrup et al., 2006), but did not abolish action potential-induced $[\text{Ca}^{2+}]_i$ transients. Many spatial and temporal characteristics of spontaneous or electrically evoked rhod-2 transients are retained in individual ventricular cardiomyocytes within the Langendorff-perfused rodent heart in the presence of cytochalasin D when compared to transients obtained from single cardiomyocytes in the absence of the uncoupler but otherwise similar experimental conditions (Ito et al., 2000; Rubart et al., 2003b). These include the frequency-dependent shortening of the transients, as well as the spatial uniformity and the velocity of the rise of $[\text{Ca}^{2+}]_i$ in response to an action potential, reflecting synchronous activation of SR ryanodine receptors secondary to Ca^{2+} influx through activated L-type Ca^{2+} channels in the t-tubular membrane (Cannell et al., 1994). On the other hand, cytochalasin D at motion-eliminating concentrations has previously been shown to markedly prolong action potential duration in the buffer-perfused mouse heart (Baker et al., 2000). Thus, although this agent enables subcellular resolution $[\text{Ca}^{2+}]_i$ measurements within the *ex vivo* rodent heart, its additional electrophysiological effects have to be taken into account when interpreting experimental results. Similarly, 2,3-butanedione monoxime has been used by numerous investigators to uncouple contraction from excitation in isolated perfused hearts of a variety of species during optical mapping studies (Efimov et al., 2004). However, at concentrations that caused immobilization of the buffer-perfused mouse heart for TPE imaging, this substance also resulted in loss of electrical excitability (Rubart et al., 2003b). Naturally, TPE imaging of intracellular calcium dynamics precludes the use of uncouplers that specifically target intermediary steps of the cardiac-excitation contraction mechanism, including Ca^{2+} buffers and inhibitors of L-type calcium channels or SR Ca^{2+} release/uptake mechanisms (Salama & Morad, 1976). Blebbistatin, an inhibitor of myosin II isoforms, has recently been shown to effectively uncouple cardiac excitation from contraction in isolated perfused rabbit hearts without exerting additional effects on Ca^{2+} handling or action potential properties (Fedorov et al., 2007). However, blebbistatin can be inactivated by illumination with ultraviolet and visible blue light (Kolega, 2004; Sakamoto et al., 2005), and it fluoresces over the range of visible light. Thus, although blebbistatin shows great promise as a highly selective excitation-contraction uncoupler for microscopic optical imaging, its utility for TPE fluorescence imaging of the intact heart remains to be demonstrated. The typically high levels of local light intensity during TPE may readily inactivate the compound, and its fluorescence properties may interfere with the fluorescence signal of interest. Collectively, progress in the application of TPE fluorescence microscopy to imaging of dynamic events (e.g., Ca^{2+} handling,

action potential) within the intact heart will critically depend on the availability of an immobilization technique that will neither distort the morphology, time course, and propagation of the cardiac action potential and intracellular calcium dynamics, nor interfere with the fluorescence signal of interest.

Despite these limitations, TPE fluorescence imaging has previously been shown to be useful for tracking the functional fate of a variety of donor cells following their intracardiac transplantation. An example is shown in Figure 6. Atrial cardiomyocytes were isolated from embryonic day 15 transgenic hearts expressing enhanced green fluorescent protein (EGFP) under the control of the cardiomyocyte-specific α -myosin heavy chain promoter (Rubart et al., 2003a) and transplanted into the left ventricle of adult nontransgenic mice. Two weeks later, the heart was harvested, loaded with the calcium-sensitive fluorophore rhod-2, and subjected to two-photon laser scanning microscopy (TPLSM) imaging. While $[Ca^{2+}]_i$ transients in the atrial donor cardiomyocytes (which appear yellow due to the overlap of green EGFP and red rhod-2 fluorescence) and host ventricular cardiomyocytes were observed to occur synchronously, the $[Ca^{2+}]_i$ transients in the donor cells were of markedly shorter duration than those in the ventricular host cardiomyocytes (Fig. 6C,D). In fact, atrial donor cells engrafted in ventricular myocardium exhibit $[Ca^{2+}]_i$ transient kinetics that are indistinguishable from those of their *in situ* counterparts (Fig. 6D), suggesting that atrial myocytes retain their calcium signaling phenotype following transplantation into ventricular myocardium. Anticonnexin 43 staining revealed gap junction-typical pattern at the donor-host cell interface (Fig. 6E), indicating electrical coupling. Another study examined the ability of nascent skeletal myotubes to functionally integrate in the host ventricular myocardium following transplantation of EGFP-expressing skeletal myoblasts (Rubart et al., 2004a). In contrast to transplanted fetal cardiomyocytes, the majority of donor-derived myocytes did not develop $[Ca^{2+}]_i$ transients in response to propagating action potentials, suggesting that they were functionally isolated. A very small fraction of the EGFP-expressing myocytes at the graft-host border, most likely arising from skeletal myoblast-host cardiomyocyte fusion events, exhibited electrically evoked $[Ca^{2+}]_i$ transients in synchrony with their juxtaposed host cardiomyocytes. However, the kinetics of these transients could be distinctly different from those in their neighboring host cardiomyocytes. The ability of TPE-based laser scanning fluorescence microscopy to spatially and temporally resolve cell-to-cell heterogeneities in calcium signaling is further demonstrated in the line scan image shown in Figure 6F. The image was obtained from two neighboring cardiomyocytes within the infarct border zone of a Langendorff-perfused mouse heart during progressive increase in the electrical stimulation rate from 2 to 5 Hz. Both cardiomyocytes develop a frequency-dependent $[Ca^{2+}]_i$ amplitude alternans that is synchronous, but whose magnitude markedly varies between the two cells. Because Ca^{2+} alternans affects membrane conductance and therefore the cardiac action potential, it is possible that the cell-to-cell heterogeneity in calcium signaling translates into corresponding gradients in action potential duration and refractoriness. Issues pertaining to the electrophysiological effects of currently available pharmacological excitation-contraction uncouplers notwithstanding, simultaneous $[Ca^{2+}]_i$ and membrane potential measurements on a cellular/subcellular scale within the intact heart are highly desirable to better understand the cross-talk between these two parameters. However, the voltage sensitivities of currently available dyes may be insufficient to spatially resolve microscopic heterogeneities, and/or their TPE absorption cross sections are too small to enable micron-scale membrane potential measurements with acceptable signal-to-noise ratios. In this regard, it is noteworthy that TPE of the novel voltage-sensitive dye ANNINE-6 using wavelengths at the outer edge of the red spectrum yielded voltage sensitivities of 20–30% per 100 mV change in membrane potential in cultured HEK 293 cells (Kuhn et al., 2004) and neuronal tissue *in vitro* (Dombeck et al., 2005). Fisher and colleagues recently determined the TPE absorption cross sections of a number of commercially available and custom-made voltage-sensitive dyes and found that RH421, RH414, di-8-ANEPPS, and di-8-ANEPPDHQ had TPE cross sections of about 15 GM units with excitation wavelengths in the

790- to 960-nm range (Fisher et al., 2005). Although these results are encouraging, the ultimate utility of these dyes for TPE-based high-resolution optical recordings of membrane potential in the intact heart will require careful measurements of their potentiometric properties when excited by TP absorption.

The ability of TPE laser scanning microscopy imaging to temporally resolve intracellular calcium dynamics on a micron scale within the intact tissue should be of general utility to monitor the consequences of microheterogeneity at the whole organ level. Localized, spontaneous SR Ca release events (Ca^{2+} sparks) have been shown to precede or lead to Ca^{2+} waves (Wier et al., 1997), which in turn trigger arrhythmogenic afterdepolarizations via modulation of calcium-sensitive membrane conductances. TPE-based laser scanning fluorescence microscopy may allow spatially and temporally resolved visualization of Ca^{2+} dynamics that underlie the initiation, propagation, and termination of Ca^{2+} waves in intact cardiac tissue and, in combination with voltage-sensitive fluorescent dyes, should provide a better understanding of the role of intracellular calcium in cardiac arrhythmogenesis (Cerrone et al., 2007). However, implementation of pathophysiologically meaningful TPE-based $[\text{Ca}^{2+}]_i$ and membrane potential imaging studies will critically depend on the availability of EC uncoupling techniques lacking electrophysiological side effects, rapid-response voltage-sensitive dyes exhibiting improved signal-to-noise ratio, and fast 3D scanning techniques. The use of ratiometric calcium- and voltage-sensitive dyes (Bullen & Saggau, 1999; McMullen et al., 2006) for TPE imaging of cardiovascular tissue would enable quantitative determinations of dynamic $[\text{Ca}^{2+}]_i$ and membrane potential changes, respectively, facilitating direct comparisons across recording sites or across different time points.

Distinct differences in the action potential waveform, and magnitude and spatiotemporal properties of action potential-evoked $[\text{Ca}^{2+}]_i$ transients, have been reported between single ventricular cardiomyocytes and Purkinje cardiomyocytes (Cordeiro et al., 2001) and in the chronically ischemic myocardium (Heinzel et al., 2007). It will be important if and how this heterogeneity is modulated at the whole heart level, where cells are electrically connected and diffusion of calcium ions from cell to cell via gap junctions may attenuate intrinsic differences in cardiomyocyte membrane potential and Ca^{2+} cycling, respectively. Finally, TPE laser scanning microscopy is ideally suited to follow the functional fate of donor cells following direct intracardiac injection, or following homing to the site of injury, provided that the donor cells can be identified on the basis of fluorescent properties.

Other cardiovascular applications of TPE fluorescence imaging include measurements of capillary blood flow. For example, Kleinfeld et al. (1998) 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 and found a positive relationship between capillary blood flow and neuronal activity. Dunn et al. (2002) used intravital TPLSM of rats injected with fluid-phase fluorescent probes to assess renal capillary blood flow, glomerular filtration, tubular transport, and endocytosis.

Second Harmonic Generation Imaging

SHG imaging also provides inherent optical sectioning and has recently been used for intravital imaging (Zoumi et al., 2002; Zipfel et al., 2003a). In harmonic generation, multiple photons simultaneously interact with so-called noncentrosymmetrical structures (e.g., collagen fibrils; Campagnola et al., 2001) without absorption (Williams et al., 2005), producing radiation at exactly half of the excitation wave-length. The resulting second harmonic wave travels predominantly in the same direction as the incident light (Zipfel et al., 2003b) and therefore yields signals in reflectance mode only after backscattering. In comparison, emission of fluorescence photons following two-photon absorption is generally assumed to occur isotropically, as long as the TPE volume is small compared to imaging depth. Thus, the

backward-directed SHG signal strength is less than the forward-directed component, limiting its utility for thick tissue imaging and intravital microscopy. Despite these physical limitations, SHG microscopy in backscattering mode has been successfully used for structural and functional imaging of thick biological specimens (Zoumi et al., 2002; Zipfel et al., 2003a).

Zoumi et al. (2004) employed a combination of SHG and two-photon fluorescence microscopy to selectively visualize the changes of collagen, elastin, and smooth muscle cells in the walls of excised, fixed pig coronary arteries in response to progressive increases in transmural pressure as shown in Figure 7A. They demonstrated that forward-directed SHG signals derived from extracellular collagen fibrils can be spectrally separated from elastin and smooth muscle cell two-photon fluorescence. It is noteworthy that this study did not utilize exogenous contrast agents to produce the optical signals, but exclusively relied on the intrinsic scattering and fluorescence properties of the biological specimen for image formation.

Many cardiac disease processes are associated with abundant interstitial fibrosis, a process that involves excess deposition of collagen type I. These structural alterations have been hypothesized to profoundly alter cardiac functional properties, but studies directly demonstrating the functional consequences of interstitial collagen accumulation on a cellular scale within the intact heart have not been reported previously. SHG imaging of collagen fibrils in conjunction with TPE fluorescence microscopy of ion and/or voltage-sensitive indicators may constitute a feasible and unique approach to directly assess the functional impact of interstitial fibrosis, provided that backward-directed SHG signals are of sufficient magnitude for image formation. Figure 7B shows an example of a proof-of-concept experiment, wherein SHG imaging of collagen was combined with TPE fluorescence microscopy. The image was obtained from the infarct border zone within a buffer-perfused, rhod-2 loaded, transgenic mouse heart expressing EGFP exclusively in cardiomyocytes during continuous electrical stimulation at 3 Hz. The TPE wavelength was 810 nm. Cardiomyocytes can be readily identified by virtue of their green EGFP fluorescence. The backscattered SHG signal is encoded in magenta and reflects collagen distribution within the infarct scar. The second harmonic nature of the magenta signal was confirmed by demonstrating that (1) it can only be detected in a narrow range of ± 15 nm half the excitation wavelength, (2) the emission peak shifted with precisely one-half of the shift of the excitation wavelength (proving that the signal is not fluorescent in nature), and (3) that the signal intensity was proportional to the square of the power of the incident laser light. Action potential-evoked cardiomyocyte $[Ca^{2+}]_i$ transients are readily visible as periodic, synchronous increases in rhod-2 fluorescence in EGFP-expressing cells. Because SHG is nonlinear in nature, SHG imaging is, like two-photon fluorescence, intrinsically three-dimensional. Collectively, the images in Figure 7B demonstrate that SHG is uniquely suited to quantitate the spatial extension of interstitial collagen deposition in the living, intact heart without introducing another fluorescent marker. Further, it can be combined with TPE fluorescence microscopy of calcium indicators (or other ion-sensitive fluorophores, or other fluorescent markers or molecules) to correlate alterations in collagen fibril distribution with functional abnormalities.

Beside imaging of collagen molecules, forward-scattered SHG signals of voltage-sensitive compounds have been used to monitor dynamic changes in membrane potential in cultured cells (Millard et al., 2004) and brain slices (Dombeck et al., 2005). For example, Millard and coworkers determined the sensitivity to transmembrane potential of forward-propagating SHG by di-4-ANEPPS in neuroblastoma cells in culture and found second harmonic sensitivities of up to 43% per 100 mV change in transmembrane potential, more than fourfold better than the nominal voltage sensitivity of this dye under one-photon illumination. Dombeck and coworkers used SHG microscopy to optically record changes in membrane potential in individual neurons of acute rat brain slices intracellularly loaded with the dye FM-64 and found linear responses of the SHG signal to changes in membrane potential of $\sim 7.5\%$ per 100 mV. They also directly

compared the strength of the forward- and backward-directed components of the SHG signal and found an approximately 2.6-fold reduction of the signal-to-noise ratio for membrane potential recordings with the backward-directed SHG component compared with the forward-propagating component of the SHG signal. Because the intact heart collection of the forward-directed SHG component is impractical, it appears unlikely that SHG microscopy will gain a more widespread application for high-resolution assessment of membrane potential changes within the intact heart. However, the apparently superior sensitivity of forward-scattered SHG signals to dynamic changes in trans-membrane potential compared to single-photon fluorescence signals may be of particular interest for micron-scale voltage mapping in single cardiomyocytes or thin cardiac tissue sections.

Conclusion

Technical developments are underway to improve two-photon dye absorption, enhance tissue penetration depth, increase 3D resolution, and optimize illumination of TPE microscopy. If used wisely, TPE microscopy with single cells and multicellular specimens will continue to be a valuable tool for generating a more integrated and comprehensive understanding of function and structure of cardiovascular tissue in health and disease.

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Figure 1.

Three-dimensional fluorescence distribution during single-photon (1P) excitation by focused laser light and during two-photon (2P) excitation using femtosecond pulses of infrared light. In single-photon microscopy, the fluorescence (marked in shades of green) is generated within an entire biconical volume with its center at the focal point of the objective, whereas TPE-induced fluorescence is confined to an ellipsoidal volume centered around the focal spot. Black lines indicate planes of focus. From Helmchen and Denk (2005) with permission from the Nature Publishing Group.

Figure 2.

TPE laser scanning microscopy imaging of changes in mitochondrial membrane potential (ψ_m) in individual cardiomyocytes within the buffer-perfused rat heart subjected to ischemia/reperfusion (I/R) injury. **A:** Schematics of the experimental setup. The heart was loaded with the fluorescent ψ_m indicator TMRE and perfused in the Langendorff-mode with a solution containing the excitation-contraction uncoupler butane dione monoxime. Series of X,Y scans were obtained at 0.5- μm Z-steps over a range of 50 μm . **B:** Representative X, Y scans obtained from a TMRE-loaded heart at the depths indicated (white numbers in images) before I/R injury. Scale bar, 20 μm . **C:** Typical mitochondrial staining pattern within an individual cardiomyocyte *in situ*. Scale bar, 10 μm . **D:** Real-time TPLSM imaging of changes in ψ_m within hearts subjected to prolonged ischemia (middle row) or combined I/R (bottom row), respectively. Upper row shows X, Y scans from a control heart. Arrows demark cardiomyocytes with uniform loss of TMRE fluorescence. Note the marked cell-to-cell heterogeneity in the loss of ψ_m . Scale bar, 50 μm . **E:** Time courses of ψ_m loss in individual myocytes in the imaged areas shown in **D**, measured at 5-min intervals. From Matsumodo-Ida et al. (2006) with permission from the American Heart Association.

Figure 3.

Decline of spatial resolution with increasing focal depth during TPE microscopy of brain tissue and lymph nodes. **A:** Lateral and axial profiles of PSFs in lymph nodes and para-formaldehyde-fixed brain slices. To determine the spatial resolution of a two-photon laser scanning imaging system, PSFs were measured by collecting the local 3D fluorescence signal of small green fluorescent polystyrene beads whose diameter (100 nm) was below the resolution of the imaging system. Measurements were performed at the surface, and 50 and 100 μm below the surface. Continuous lines are best fit to 1D Gaussian functions. Preparations were excited with 800-nm light, using a 20 $\times$ 0.95 NA water immersion objective. **B:** Dependence of the spatial resolution on the penetration depth in lymph nodes and brain slice regions containing either mainly axons or somata. Images show representative frame mode images of brain slices from transgenic mice expressing the enhanced yellow fluorescent protein (EYFP) in neurons exclusively and from lymph nodes from wild-type mice where red and green leukocytes had been transferred before imaging. Black areas in the left panel correspond to EYFP-negative somata. **C:** Plots of the axial and lateral resolutions, respectively, for three different tissue types in **B** as a function of focal depth. From Niesner et al. (2007) with permission from the Biophysical Society.

Figure 4.

Three-dimensionally resolved flash photolysis. **A:** Two-photon flash photolysis (TPFP) of caged Ca (DMNP-EDTA) in a urinary bladder smooth muscle cell loaded with the fluorescent calcium indicator fluo-4. Delivery of two 88-ms trains of ultrashort light pulses (wavelength 730 nm, 100-fs pulse width, 4.7-mW average energy) to a highly localized volume within the cell does not result in an observed release of Ca^{2+} from intracellular stores. A third pulse train at the same location with no detectable delay results in a localized increase in cytosolic calcium (color encoded) that propagates partially throughout the cell and spontaneously terminates. Changes in fluo-4 fluorescence were monitored using single-photon laser scanning confocal microscopy. Levels of free calcium are encoded in color, with red being the highest and blue being the lowest. Numbers indicate time in seconds after obtaining the first frame image. Graph on the right shows the spatially integrated changes in fluo-4 fluorescence as a function of time. Vertical bars denote two-photon flashes. Note that the delivery of TPE alone does not result in detectable changes in cytosolic calcium levels, indicating that the observed increases result from secondary Ca^{2+} release from intracellular stores. **B:** Incubation of urinary bladder cells with ryanodine alone, which inhibits Ca^{2+} release through sarcoplasmic reticulum ryanodine-sensitive Ca^{2+} release channels, did not prevent TFPF-induced increase in intracellular calcium, whereas combined exposure to ryanodine and xestospongine C, an inhibitor of InsP3 receptors, completely eliminated Ca^{2+} release. Arrows indicate laser flashes. From Ji et al. (2006) with permission from The Rockefeller University Press.

Figure 5.

Two-photon flash photolysis to quantitate gap junction-mediated dye transfer in three dimensions. **A:** Dissected pancreatic acini from mouse were loaded with the green fluorescent dye calcein/AM and the cell-permeable caged coumarin dye NPE-HCCC2/AM. Images were constructed by volume-rendering two-photon z-stacks in the calcein (green) channel and the coumarin (magenta) channel. Images in **B** and **C** were taken 30 and 420 s after two-photon uncaging, respectively (730-nm excitation wavelength, <11 mW). The asterisk in **A** indicates the uncaged cell. Three cells in **B** outline the “donor” and two “recipient” cells (2 and 3). **E:** Time course of average coumarin dye fluorescence within the volume of the cells labeled 1 to 3 in **D**. From Dakin and Li (2006) with permission from the Nature Publishing Group.

Figure 6.

Monitoring heterogeneity in intracellular calcium signaling on a cellular scale within the intact mouse heart using TPE laser scanning microscopy. A-D: TPE laser scanning microscopy imaging of cellular $[Ca^{2+}]_i$ transients in Langendorff-perfused mouse heart carrying a graft of fetal atrial cardiomyocytes. Images were taken in frame-mode (A) and line-scan mode (B) during continuous electrical point stimulation at a site remote from the graft. Ripple-like periodic elevations in red rhod-2 fluorescence in the frame mode image and red bands in the line scan mode image correspond to action potential-induced increases in cytosolic free calcium. For line scan mode acquisition, the preparation was repeatedly scanned along the white line in A at a rate of 31 Hz, and the lines were stacked vertically to generate a line scan plot. Time is on the y-axis, distance along the scan line is on the x-axis. Line scan images depicts $[Ca^{2+}]_i$ transients at 2 and 4 Hz. To obtain the time course of rhod-2 and EGFP fluorescence of an EGFP-expressing and a nonexpressing cardiomyocyte, spatially averaged rhod-2 and EGFP intensities were plotted as functions of time (C). To compare $[Ca^{2+}]_i$ transient kinetics in donor and host cardiomyocytes, the spatially averaged rhod-2 intensities were normalized to their respective peaks (D). Anticonnexin43 immune staining (E, red signal) at the interface of an atrial donor (green signal) and ventricular host (blue signal) cardiomyocytes. Scale bars: 20 μ m in A; 20 μ m (horizontal) and 500 ms (vertical) in B. (In part from Rubart et al., 2003 with permission from the American Heart Association.) F: Development of frequency-dependent $[Ca^{2+}]_i$ transient alternans in infarct border zone cardiomyocytes. The heart of an adult transgenic mouse expressing EGFP exclusively in cardiomyocytes was subjected to coronary artery ligation. Two weeks later, the heart was harvested, retrogradely perfused in Langendorff-mode, and loaded with rhod-2. Line-scan mode images were obtained from two neighboring cardiomyocytes in the infarct border zone in the presence of the excitation-

contraction uncoupler cytochalasin D (50 $\mu\text{mol/L}$). Traces in the bottom part of F were obtained by first spatially averaging rhod-2 intensities in the line-scan images and then plotting them as function of time. Scale bars: 20 μm (horizontal) and 250 ms (vertical). Unpublished observations by the authors.

Figure 7.

Combined use of SHG and TPF. **A:** Visualization of changes in artery wall architecture during pressurization. Excised pig coronary arteries were fixed under the following loading conditions: zero-stress (a), zero (b), 30-mmHg (c), and 180 mmHg (d) transmurial pressure. The fixed arteries were then subjected to two-photon illumination using an excitation wavelength of 800 nm. Green color images correspond to SHG (400/10 nm) and red-color coded images correspond to TPF (520/40 nm). SHG signal is exclusively due to collagen, whereas the fluorescence signal results from elastic fibers and smooth muscle cells. Note that the preparation was not loaded with exogenous probes, i.e., the images rely on endogenous signals. The entire wall spanning from the intima (left) to the adventitia (right) was imaged. Application of progressively increasing transmurial pressure causes radial compression of the arterial wall and a marked decrease in collagen fiber thickness. Interestingly, the low transmurial pressure causes nonuniform thinning of collagen fibers in the inner layers, whereas the highest pressure results in homogenous decrease in fiber diameter across the vessel wall. From Zoumi et al. (2004) with permission from the Biophysical Society. **B:** SHG imaging of interstitial collagen in backscattering mode combined with TPE laser scanning microscopy imaging of rhod-2 and EGFP fluorescence in the infarct border zone of a transgenic mouse heart expressing EGFP exclusively in cardiomyocytes. Two weeks after coronary artery ligation, the heart was harvested, retrogradely perfused, and loaded with rhod-2. Imaging was performed during continuous point stimulation at 3 Hz. Excitation wavelength was 810 nm. SHG signal (magenta) was collected at 400–10 nm, and green EGFP and red rhod-2 fluorescence signals were collected between 500–550 nm and 560–650 nm, respectively. Blue arrows demark faint rhod-2 transients in the cells boxed in **B**. Scale bar, 20 μm. Unpublished observations by the authors.