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Coherent control improves biomedical imaging with ultrashort shaped pulses

Johanna M. Dela Cruz [a], Vadim V. Lozovoy [a], Marcos Dantus [a,b,*]

[a] *Department of Chemistry, Michigan State University, East Lansing, MI 48824, USA*
[b] *Department of Physics and Astronomy, Michigan State University, East Lansing, MI 48824, USA*

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Abstract

Although ultrashort pulses are advantageous for multiphoton excitation microscopy, they can be difficult to manipulate and may cause increased sample damage when applied to biological tissue. Here we present a method based on coherent control that corrects phase distortions introduced by high numerical aperture (NA) microscope objectives, thereby achieving the full potential of ultrashort pulses. A number of useful phase functions are recommended to gain selectivity that is similar to that which can be achieved by tuning a longer laser pulse; however this one involves no moving parts and maintains perfect optimization. This capability is used to demonstrate functional imaging by selective two-photon excitation of a pH-sensitive chromophore. Finally, we show that phase functions can also be introduced to minimize multiphoton excitation damage, while maintaining a high efficiency of two-photon excitation.

Keywords: Coherent control; Phase modulation; Shaped pulses; Femtosecond; Two-photon imaging; Three-photon

1. Introduction

Since the 1990s, imaging modalities, such as two-photon microscopy, based on nonlinear optical excitation have shown great promise [1–3]. Since nonlinear excitation scales linearly (for two-photon) or quadratically (for three-photon) with the inverse of the pulse duration, the availability of pico and femtosecond lasers has accelerated the development of multiphoton microscopy. Femtosecond lasers, especially, have been promising excitation sources for nonlinear optical imaging because of their high peak power and greater penetration ability when compared to lasers required for linear excitation. However, the expectation that a factor of ten reduction in pulse duration would lead to one order of magnitude greater signal for two-photon excitation in biological tissue was not realized, and shorter pulses were actually observed to cause greater laser induced damage [4]. This has been explained by the fact that DNA has a large absorption cross section at 260 nm, a wavelength region which can be significantly affected by three-photon excitation from a Ti:Sapphire femtosecond laser, the most common excitation source. The most frequent pulse duration used for two-photon microscopy today, therefore, is not at the lower limit of the source capability. Instead, a compromise between excitation efficiency and damage prevention is realized at ~80–100 fs.

Not all pulses with spectral bandwidths of about 20 nm are the same, unless they are transform limited (TL). A TL pulse is one whose pulse duration satisfies the energy-time uncertainty relationship $\Delta\nu\Delta\tau = 0.44$, a relation derived for pulses with a Gaussian spectrum with bandwidth $\Delta\nu$, and pulse duration $\Delta\tau$. Unfortunately, no commercial laser source produces TL pulses. Deviations from TL are usually given as a time-bandwidth product (TBP), where TL pulses have a TBP = 1. This value increases as the deviations increase. Typically femtosecond lasers have a TBP ~ 1.5. Deviations from TL cause a decrease in nonlinear optical excitation efficiency. A laser pulse with TPB = 1.5 yields 33% less two-photon and 56% less three-photon signal than a TL pulse. What is not widely appreciated is that these deviations may be strongly wavelength dependent. Therefore, similar laser sources could see different relative intensities when exciting a number of chromophores. This is especially important when the laser wavelength is tuned, and this severely impacts the quantitative prospects of multiphoton microscopy.

* Corresponding author. Tel.: +1 517 355 9715x314; fax: +1 517 353 1793.
E-mail address: dantus@msu.edu (M. Dantus).

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The nonlinear properties of a pulse are very sensitive to spectral phase distortions that are either inherent to the laser system or are introduced by lenses, optical fibers, mirrors, or microscope objectives. Eliminating these distortions is especially critical in cases where reproducibility is an important criterion for femtosecond laser applicability. These distortions need to be corrected to ensure the fast progress of femtosecond laser excitation in biomedical imaging applications. The method presented here, multiphoton intrapulse interference phase scan (MIIPS) [5–7], not only characterizes pulses, but corrects spectral phase distortions and delivers accurate and reproducible phase information to a sample. MIIPS is a single beam technique that takes advantage of the effects of phase modulation on nonlinear optical processes to analytically acquire the spectral phase of a pulse. Spectral phase compensation through MIIPS has allowed us to perform reproducible functional imaging through scattering biological tissue. In addition, it has increased its usefulness to a wide range of areas [8] including chemical and molecular identification [9,10], multiphoton microscopy [11] and chemical microenvironment probing [6].

In this article we present a method based on coherent control that has been used to correct phase distortions in the pulse, including the significant phase distortions introduced by high NA microscope objectives, rendering TL pulses at the sample. We describe a new approach to attain excitation wavelength tunability based on phase shaping that achieves optimum efficiency, quantitative wavelength tunability, and reduces photodamage. This method has no moving parts and can even be used for imaging through thick scattering biological tissues. Finally, we demonstrate experimentally the enhancements possible through phase correction and phase control.

We focus on the sensitivity of nonlinear imaging methods to the spectral phase of the pulses and we show how controlling this phase can lead to significant improvements and new possibilities in biomedical imaging.

2. Experimental

All experiments were carried out with a 250 mW, 97 MHz Ti:Sapphire oscillator operational for sub-10 fs pulse delivery (110 nm full width at half maximum, FWHM). The pulses were shaped by introducing phase functions into the spatial light modulator (SLM) of a pulse shaper [12].

2.1. Spectral phase distortion and MIIPS

Spectral phase characterization, correction of unwanted phase distortions and the introduction of accurate phase functions were carried out with a MIIPS [5–7] setup (Biophotonic Solutions Inc.). To test the viability of pulse compensation on imaging, a mouse kidney section (FluoCells® prepared slide #3 mouse kidney section with Alexa Fluor® 488 WGA, Alexa Fluor® 568 phalloidin, DAPI) was imaged with uncompensated and with compensated TL pulses.

2.2. Tunability and selective excitation

For these experiments a sinusoidal phase function with a period corresponding to the inverse pulse duration of the laser was used to shape the pulses. Alternatively, a binary phase function, where the phase values were limited to 0 and π retardation, was used. In both cases, the point of inversion (or reflection) symmetry is where maximum two-photon excitation is expected. The laser was then focused on a thin (0.01 mm) beta-barium borate second harmonic crystal and the resulting frequency doubled spectrum was analyzed in a compact fiber-optic coupled spectrometer.

2.3. Functional imaging

Selective excitation was carried out on 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), a pH-sensitive probe which has applications in the biological field, including monitoring intra-organelle pH in endosomal and lysosomal pathways [13]. An acidic solution of HPTS was placed in three capillary tubes, which were then immersed in a 2-mm cuvette filled with an alkaline solution of the same dye. Selective imaging of the capillary tubes required laser pulses designed to selectively excite HPTS either in acidic or alkaline solution. To achieve these shaped pulses, the unwanted phase distortions were first corrected, using MIIPS, as described above. Binary phase functions [14] were then applied with the SLM. The design of the phase functions was based on the two-photon cross section of HPTS in acidic and alkaline environments, the spectrum of the fundamental laser pulse, and the known dependence of two-photon excitation on spectral phase [15,16] and in particular to binary phase functions [14]. The laser, attenuated to 1 nJ/pulse and centered near 820 nm, was focused on the capillaries by a 20×/0.45 NA objective (Nikon Plan Fluor, extended long working distance). The two-dimensional (6 mm × 8 mm) images were obtained by scanning the sample at the focal plane of the laser; the resulting fluorescence was recorded by a spectrometer with detection wavelength set at 515 nm (24 nm spectral resolution) and averaged point by point with a lock-in amplifier.

To demonstrate the viability of selective excitation for biomedical imaging, a 500 μm slice of scattering biological (chicken breast) tissue was placed in front of the capillary tubes in the imaging setup described above. The same binary functions that were used for the selective imaging experiments generated localized two-photon excitation of HPTS at specific frequencies. Fluorescence from the excited HPTS solutions was collected from the back of the sample.

2.4. Reduced multiphoton induced damage in two-photon microscopy

The efficacy of phase modulation for maximizing two-photon excitation while minimizing three-photon transitions was demonstrated by imaging a 30 μm × 30 μm section of a slide containing the chromophores HPTS and L-tryptophan mixed with an aqueous polyvinyl alcohol (4%) solution on glass cover slips. The laser was brought in the rear of a Nikon

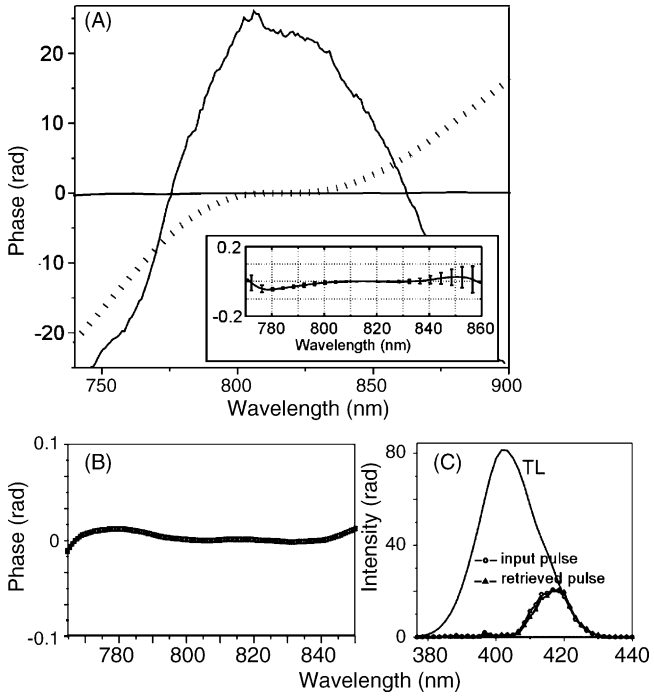

Fig. 1. Compensation of spectral phase distortions. (A) Spectral phase retrieved from a laser pulse transmitting a 60×/1.45 NA microscope objective before (large distortion, dotted lines) and after (minimal distortion, solid line) compensation. The inset in A shows the same compensated data, averaged over five independent measurements, over the FWHM of the laser pulse on a reduced scale. Note the proximity of the compensated pulses to zero (flat) phase structure corresponding to TL pulses. (B) Spectral phase retrieved after a laser pulse transmitted through 500 μm of scattering tissue. The initially TL pulse is recovered despite scattering in tissue. Note that the phase retrieved in both cases is almost zero, indicating successful generation of TL pulses as a result of spectral phase measurement and correction. (C) Binary shaped pulse designed for optimal excitation at 420 nm is retrieved after transmission through scattering tissue. The retrieved pulse intensity was multiplied by 20 to highlight its similarity with the input pulse.

TE2000-U inverted microscope, and focused on the sample with a 60×/1.45 NA objective. The emission was imaged onto a CCD camera (Andor, iXon DV887). To generate the final image, the sample was raster scanned in 0.3 μm steps with a digital piezo controller (Physik Instrumente, E-710.3CD). Sinusoidal phase functions were used for the selective imaging in this experiment.

3. Results and discussion

3.1. Spectral phase distortion and MIIPS

Ultrashort laser pulses, when they are focused by high NA microscope objectives, are subjected to substantial spectral phase distortions. Consequently, image resolution is degraded. Fig. 1A confirms our experimental findings that MIIPS can successfully measure and correct all orders of spectral phase distortions in a sub-10 fs pulse that goes through a 60×, 1.45 NA microscope objective. The data show an almost flat phase (0 ± 0.1 rad) retrieved from an initially highly distorted phase (~20 rad). The roughly zero phase that was recovered after measurement and compensation using MIIPS is an indication of the production of TL pulses which act as indispensable control pulses for assessing two-photon excitation selectivity. The corresponding reduced scale data over the FWHM of the fundamental laser spectrum is shown in the inset.

Uncertainties regarding the extent of spectral phase distortions that the laser pulse experiences as it propagates through several scattering lengths of tissue places limitations on the use of femtosecond lasers for medical diagnostics, therapeutics and imaging. The measurement and correction of such spectral phase deformations can, however, be accomplished with MIIPS. With this method, TL pulses at the sample are assured and any spectral phase function designed for selective excitation can be delivered even after transmission through 500-μm of chicken breast tissue. Based on our observations, with the exception of an exponential decline in overall signal intensity, the retrieved spectral phase showed very little dependence on the thickness of the scattering tissue. Phase distortion was minimal and the coherence of the femtosecond laser pulse was maintained in the ballistic photons. Fig. 1B shows the phase residue, averaged over five trials, of the pulse after going through tissue. The phase was compensated to within 0.02 rad over the entire FWHM of the pulse. In addition, Fig. 1C shows that a shaped pulse introduced to the sample is fully recovered (barring intensity relationship) after going through scattering tissue. Accurate phase compensation and delivery were confirmed from the experiments, and the completion of the necessary spectral phase measurement and correction makes it possible to perform selective functional imaging through biological tissue [17].

Fig. 2 validates the benefits of pulse characterization and compensation. A 30 μm × 30 μm image of a mouse kidney section stained with Alexa Fluor® 488 WGA, Alexa Fluor® 568 phalloidin, and DAPI is shown imaged with phase corrected TL pulses (left) and without phase correction (right). No fluorescence wavelength filters were used to separate contributions from each of the fluorescent labels. Comparing between the two images, it is clear that phase distortion leads to a significant loss of signal that cannot be compensated simply by increasing the laser power. This is because phase distortions cause changes in the wavelength region where multiphoton excitation takes place [16].

3.2. Tunability and selective excitation

Starting with pulses with a flat spectral phase allows us to explore the effects of precisely introduced phase functions on multiphoton processes. Our group has been developing strategies aimed at selective two-photon excitation based on multiphoton intrapulse interference (MII) [15,16]. Naively, one may think that a filter that blocks the undesired wavelengths of light and allows only the desired ones to transmit would provide selective two-photon excitation. Unfortunately, the loss of photons in this approach makes it impractical [14]. Our initial MII research led us to the use of sinusoidal phase functions to essentially tune the frequency where

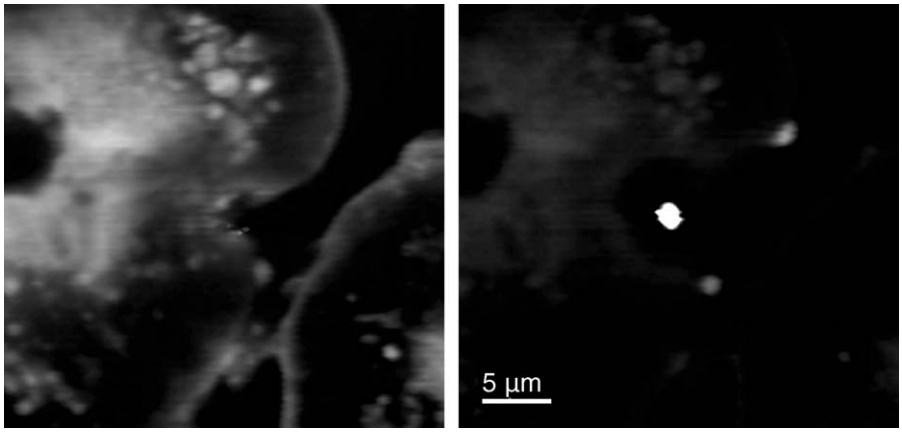

Fig. 2. Image quality improvement with spectral phase compensation. Left: fluorescence image of mouse kidney using femtosecond laser pulses with spectral phase deformations corrected. Right: fluorescence image of the same section of the sample when pulses are uncompensated. Both images were normalized to the same maximum intensity level. Image size is 30 μm × 30 μm.

constructive MII occurred [6,15,16]. Constructive interference enhanced two-photon excitation at a particular frequency with a reduced background at surrounding frequencies, this being accomplished without tuning the wavelength of the femtosecond laser source, and without affecting the overall number of photons per pulse. These observations led us to suggest and demonstrate selective two-photon microscopy based on pulse shaping a broad bandwidth femtosecond laser source [6,11].

Fig. 3A and B illustrates the effect of sinusoidal phase modulation of ultrashort laser pulses. Here we highlight the constructive and destructive interference by looking into the spectrum of the frequency doubled light emerging from a very thin second harmonic generation crystal. TL pulses (dashed line) generate a broad bandwidth spectrum, however, phase modulated pulses generate a sharp feature. Notice that the wavelength where the sine function crosses zero corresponds to the location of the high intensity feature in the frequency doubled spectrum. The position of this feature is easily tuned by displacing the sine function across the spectrum. More recently, we have discovered that binary phase functions are much more efficient at controlling MII [14]. In Fig. 3C and D, we illustrate the effect of binary phase shaping for tuning ultrashort pulses. Comparison between Fig. 3B and D allows us to see that binary phases can be used to create much sharper spectral resolution, with a much lower level of unwanted background. In both cases (sinusoidal and binary

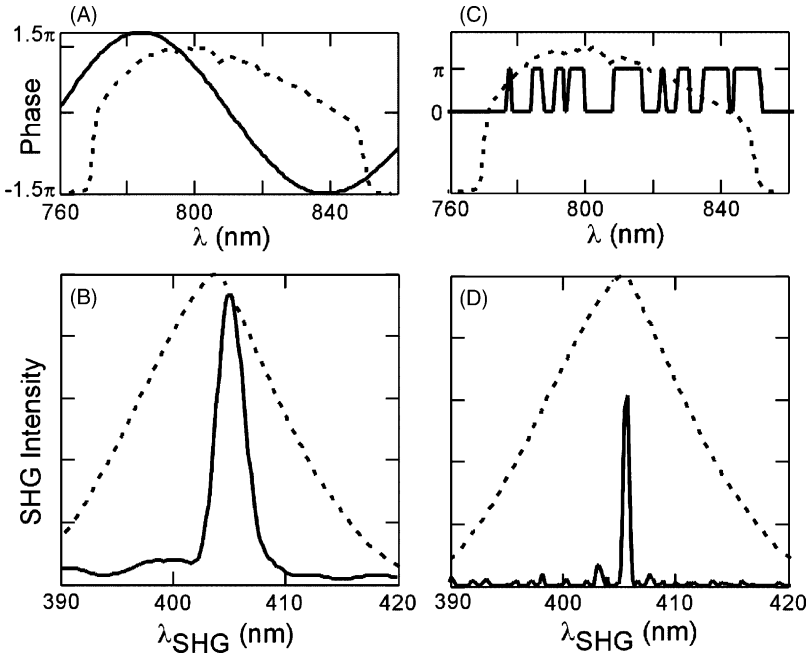

Fig. 3. Control of the wavelength where two-photon excitation takes place using phase shaped femtosecond pulses. (A) Diagram with a sinusoidal phase function superimposed on the broad-bandwidth femtosecond laser pulse spectrum. (B) Effect of the sinusoidal phase function on second harmonic generation. The frequency doubled spectrum of the TL pulses corresponds to the dashed line. (C) Diagram with a binary phase function superimposed on the broad-bandwidth femtosecond laser pulse spectrum. (D) Effect of the binary phase function on second harmonic generation. The frequency doubled spectrum of the TL pulses corresponds to the dashed line. Notice that binary phases lead to a sharper desired excitation feature with lower background signal.

phase modulation), it is possible to tune the frequency of two-photon excitation without tuning the laser, and without affecting optical alignment.

Recently Ogilvie et al. adopted the sinusoidal approach towards selective two-photon excitation microscopy to image live eGFP labeled Drosophila embryos. Pulse shaping, in this case, allowed selectively excitation of eGFP or selective excitation of endogenous fluorescence [18]. The ability to separate endogenous two-photon induced fluorescence, which is ubiquitous in biological samples, is an important achievement that greatly enhanced the contrast of the obtained images.

Although selective excitation can be carried out by wavelength tuning of narrower-bandwidth lasers, the more practical approach of phase modulation of a broad-pulse femtosecond laser has a number of advantages. In wavelength tuning, spectral phase measurements at every desired excitation wavelength are required and movement of optics causes problems when trying to overlap images obtained at different wavelengths (a pixel registration problem). By phase modulation, the whole process of spectral phase measurement and compensation is carried out in advance and excitation at different wavelengths within the bandwidth of the ultrashort pulse is achieved, requiring no physical movement of wavelength tuning optics. In fact, no change in the laser spectrum takes place, just its two photon excitation capability.

3.3. Functional imaging

HPTS exhibits a pH-dependent absorption shift allowing selective two-photon fluorescence excitation. Fig. 4 demonstrates the viability of selective imaging by binary phase modulation. With TL pulses there is no difference between the two pH solutions. HPTS in acidic and alkaline media have approximately equal integrated excitation cross-sections, emitting at around 512 nm. Shaped pulses, however, preferentially excite either the protonated or deprotonated forms. This observation is depicted in Fig. 4, where the sample, which is exactly the same in both panels, displays variable fluorescence intensities based on pH environment. The walls of the capillary tubes (∼300 mm), which exclude any fluorescent solution, are clearly visible as black lines. This confirms the confocal effect attained by two-photon excitation [1,19]. The image displayed on the top shows fluorescence enhancement of the acidic HPTS solutions (inside the capillary tubes) and a suppression of excitation from the basic HPTS solution when one particular binary phase function BP06 (localized two-photon excitation at high frequencies) was scanned across the spectrum of the laser pulse. The opposite effect was observed when another binary phase function BP10 (localized two-photon excitation at low frequencies) was used on the sample. This is shown at the bottom of Fig. 4. It is worth noting that the fundamental spectrum of the laser pulse does not

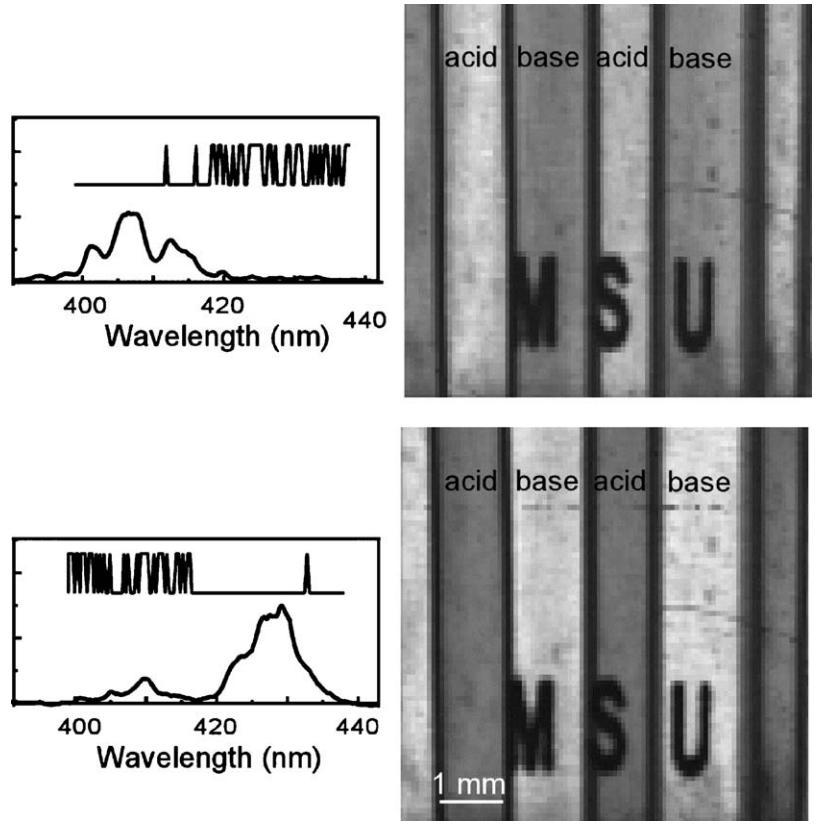

Fig. 4. Selective two-photon fluorescence of a pH-sensitive dye. Left: the effect of phase modulation with the phase functions BP06, top and BP10, bottom on the second harmonic generation spectrum. The maximum wavelength region is shifted to maximize fluorescence from either the acidic or alkaline dye solutions. The complicated structures of the binary phases are shown above the spectra. Top right: fluorescence from the acidic HPTS solution is enhanced while excitation from the alkaline dye is minimized. Bottom right: fluorescence from the alkaline HPTS solution is maximized while excitation from the acidic dye is diminished. The figure shows a 6 mm × 8 mm image of fluorescence from the experimental sample setup.

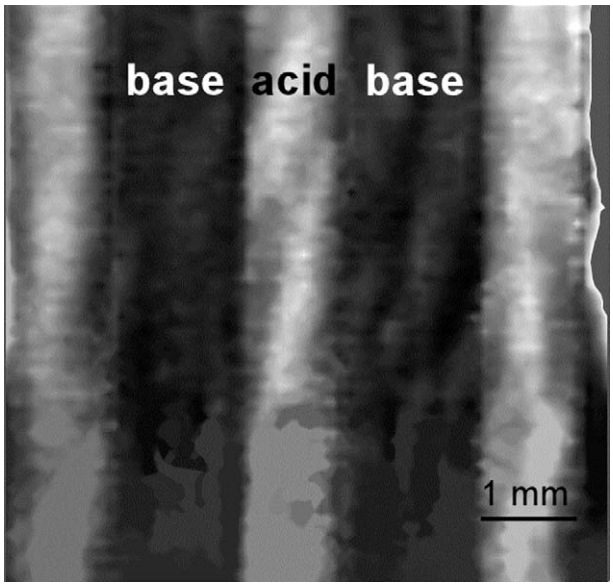

Fig. 5. Functional imaging through scattering biological tissue. Fluorescence from the acidic HPTS solution is clearly accented in this contrast image (6 mm × 8 mm). The surrounding alkaline portion of the sample shows minimal fluorescence.

change upon phase modulation. Modifying the spectral phase only affects the laser's potential to trigger two-photon excitation at specific wavelengths [15,16]. The complicated binary phase introduced in each of the shaped pulses used involves several frequencies where the phase of the light is retarded by $\pi\cdot$ These small variations lead to the observed changes in the SHG spectra, which in turn shift the excitation wavelengths to particular regions of the fluorophore's absorption spectrum. SHG has the same quadratic dependence on the intensity of the laser pulses as two-photon excitation and it is used here only for diagnostic purposes.

The full effect of coherent control by selective functional imaging using binary phase modulation can be appreciated in Fig. 5. The image, which was acquired as the laser transmitted through 0.5 mm of biological tissue prior to reaching the fluorescent solutions, is a "contrast image". Here, the image obtained using BP06 pulses was divided by that obtained using BP10 pulses. Despite a decrease in signal to background ratio caused by the scattering biological tissue, the contrast between the acidic and basic HPTS solutions is still apparent. It is obvious from this image that selective two-photon fluorescence after a scattering medium is possible because the phase characteristics of the shaped pulses were maintained. This is a confirmation of our earlier results (see Fig. 1) on phase retrieval.

3.4. Reduced multiphoton induced damage in two-photon microscopy

The laser power incident at a biological sample generally has to be limited to ∼10 mW to prevent the occurrence of highly nonlinear photodamage [20]. The favorable response of two-photon excitation in cell viability [1,21] when laser power is kept below this threshold has been observed before. Since two-photon photodamage is limited to a sub-femtoliter volume at the focal point, the reduction in photodamage volume results in a dramatic increase in viability of biological samples. However,

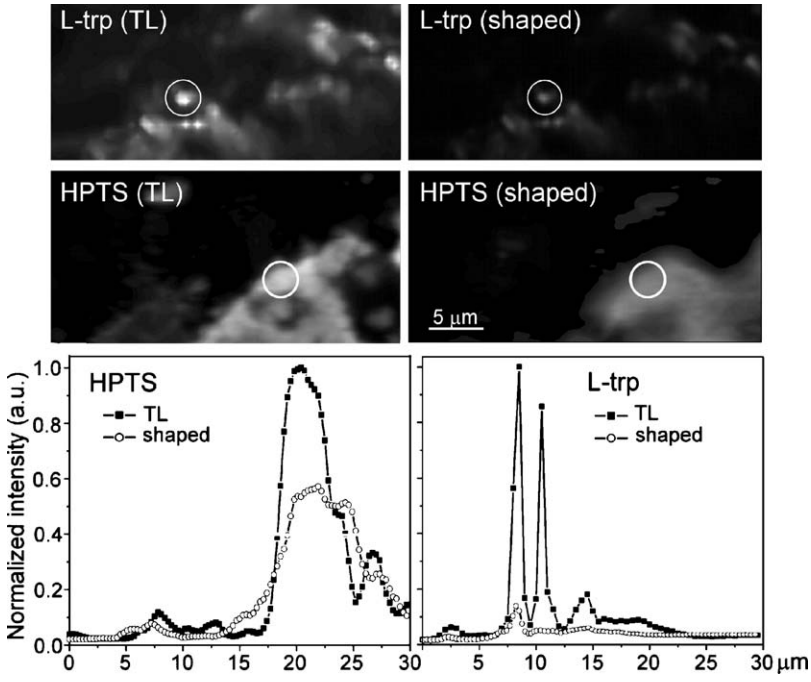

Fig. 6. Suppression of three-photon excitation by phase modulation. Top left: fluorescence image of L-tryptophan (L-trp) and HPTS deposited on a quartz cover slip and imaged with TL 800 nm pulses. Top right: fluorescence from the same samples when laser pulses are shaped with a sinusoidal phase function crossing zero at 0.25π. Bottom: Cross-section of the fluorescence intensities (encircled areas in images) of both dyes highlights the effect of phase modulation in minimizing three-photon excitation (right) while maintaining intensity of two-photon fluorescence (left). These intensities (normalized) are compared with those obtained using TL pulses. Each image size is 30 μm (width) × 15 μm (height).

the role of three-photon absorption in photodamage seems to be particularly relevant. This is plausible when the corresponding one-photon wavelength range for three-photon absorption with our laser system is considered. Three-photon absorption can result in the excitation of DNA and amino acids in proteins and this can lead to cell death.

With phase modulation, however, three-photon induced laser damage [20,22] can be suppressed by several orders of magnitude. This suppression results from lengthening of the pulse due to the phase structure and the destructive interference of three-photon excitation pathways [16]. In addition, the large two-photon excitation yield obtained by these pulses is preserved. Our early experimental findings showed the possibility of phase manipulation to inhibit excitation at the three-photon level while keeping two-photon activity at a relatively high level [16].

Fig. 6 shows how phase modulation can be used to achieve selective suppression of three-photon excitation while maintaining the efficiency for two-photon excitation. In this case three-photon excitation from L-tryptophan, an amino acid with absorbance maximum at 280 nm and fluorescence at 350 nm is suppressed by almost 90%. The two-photon dye (HPTS), on the other hand, maintains its intensity at the same phase where three-photon excitation is decreased. The effect of phase modulation on these two fluorescent molecules is clear when comparing them with the intensities obtained with TL pulses. Although phase modulation slightly decreases two-photon fluorescence intensity, its effect on three-photon excitation is significant.

The results presented here were generated using pulses that were modulated by sinusoidal phase functions. We have explored the possibility of yet enhancing the contrast achievable with two- and three-photon excitation by searching for optimal phase functions that would theoretically lengthen the excitation pulse and consequently focus two-photon excitation on desired frequency regions while defocusing three-photon excitation away from regions that can cause biological damage [8,16].

4. Conclusion and future directions

We have explored the detrimental effects of unwanted spectral phase modulation on multiphoton imaging, and we have also demonstrated the beneficial effects that can be achieved by judicious phase modulation. The first step in these experiments is the accurate characterization and correction of phase distortions in the laser pulse as it propagates through the high NA objective using MIIPS. The second step is to realize that TL pulses are not always ideal for multiphoton imaging because they may induce multiphoton damage. Here we suggest simple strategies to achieve selective two-photon excitation in environment-sensitive probes. We also show that selective suppression of three-photon processes can be achieved while maintaining the same efficiency of two-photon excitation. Finally, it is worth noting that it is possible to deliver the accurately shaped pluses that are required for selective two-photon activation through thick scattering biological tissue. Selective two-photon excitation and suppression of multiphoton induced damage are two capabilities that will enhance the potential applications of two-photon imaging for cancer detection and two-photon photodynamic therapy.

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