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Advantages of ultrashort phase-shaped pulses for selective two-photon activation and biomedical imaging

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Abstract Two-photon excitation using ultrashort laser pulses can selectively activate nanoparticles or excite fluorophores within thick biological samples. We show how the use of methods such as multiphoton intrapulse interference phase scan (MIIPS) to compensate phase distortions caused by microscope objectives with a high numerical aperture increases signal intensity and reproducibility in two-photon imaging. Using phase shaping of our compensated pulses, we demonstrate selective excitation of fluorophores within a mouse kidney sample, increasing the contrast between different subcellular structures compared to unshaped pulses.

Key words: Femtosecond laser; Pulse shaping; Two-photon imaging; Selective excitation

Introduction

Two-photon microscopy, introduced by Denk and Webb in 1990 [1], can be used to achieve selective two-photon excitation of nanoparticles or chromophores for high-resolution imaging or for activating photodynamic therapy agents. Because two-photon excitation is highly localized, it can be performed through thick biological samples with minimal damage. The intensity of two-photon excitation depends nonlinearly on the peak intensity of excitation light, so two-photon excitation is usually performed with ultrashort (usually a few hundred femtoseconds) laser pulses, which have very high peak intensities. Although two-photon excitation continues to increase as pulse length decreases, pulse durations below 100 fs are rarely used, because shorter laser pulses are increasingly subject to phase distortions introduced as they travel through optics to the sample. These phase distortions lengthen the pulses, decreasing their peak intensity, and therefore also decreasing two-photon excitation in the sample. Although peak intensity can be recovered by increasing the intensity of the beam increasing the laser intensity, sample damage is also increased, usually to unacceptable levels. Moreover, the phase distortions vary significantly from laser to laser as well as with the day-to-day changes of a femtosecond laser's optical alignment, posing a significant challenge to reproducibility.

Several methods have been introduced to characterize the pulses emerging from an objective with a high numerical aperture [2-4]. However, to address the problems of peak intensity and reproducibility, it is important to correct high-order phase distortions (dispersion compensation) at the sample. As described in detail elsewhere [5], the multiphoton intrapulse interference phase scan (MIIPS) method for measuring and correcting phase distortions (dispersion compensation) is to our knowledge the only method currently available that accurately characterizes laser pulses at the sample and shows the best performance in eliminating high-order phase distortions. MIIPS is considerably simpler to incorporate than other characterization methods and requires no overlapping of beams; it requires only a thin frequency-doubling crystal (or another source for second-harmonic generation, such as starch [6]), a spectrometer, and a pulse shaper. Pulse shapers programmed to provide automated MIIPS pulse correction are now commercially available.

In addition to compensating pulses to maximize peak intensity and reproducibility, the pulse shaper can be used to

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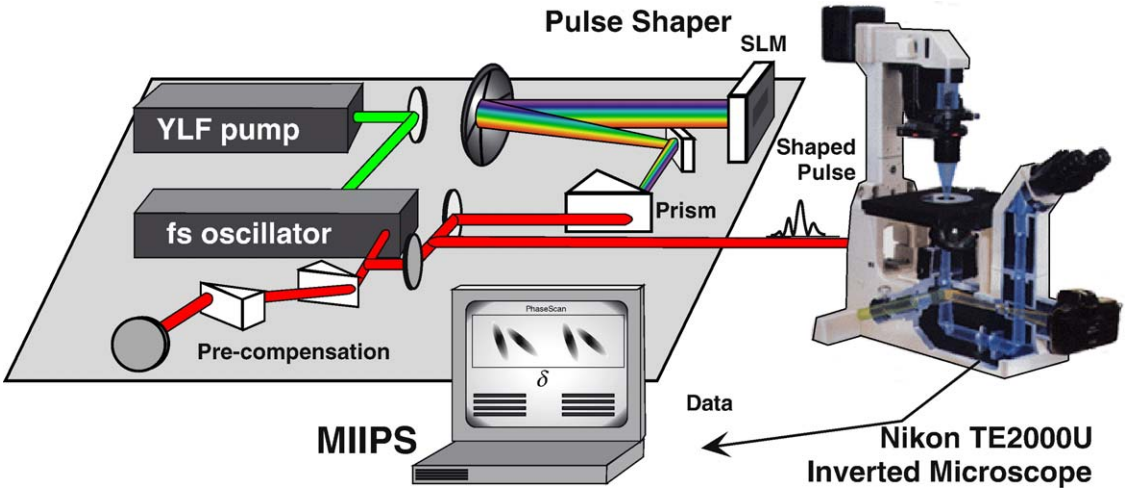

Fig 1. Schematic diagram of experimental setup for two-photon imaging.

introduce special phase functions that produce selective two-photon excitation. Two-photon excitation depends on the two-photon cross-section of the molecule and the second-order component of the electromagnetic field $|E(\lambda)^{(2)}|^2$. Measuring this component of the field is simple; it only requires measuring the frequency-doubled spectrum of the laser. The second-order component of the field has been found to be very sensitive to the spectral phase of the laser pulses [7-10]. Certain phase functions can concentrate excitation at a particular wavelength while at the same time suppressing excitation at other wavelengths. Such phase functions can be used to selectively excite molecules whose two-photon cross-sections are large at these particular wavelengths. Presently, tunable femtosecond sources are used for selective excitation. However, because of the wavelength-dependent properties of the experimental optical components, problems with focusing, registration, and phase distortions negatively impact the quality of results. By using phase shaping to concentrate the energy of a pulse's second harmonic spectrum at different wavelengths, selective excitation can be achieved without changing the pulse's fundamental spectrum, avoiding deleterious wavelength-dependent effects. This method was first demonstrated by the Dantus group [11-16] and more recently has been used by Ogilvie et al [17].

Here we show experimentally how phase-corrected ultrashort pulses can be used for two-photon microscopy to increase signal intensity, contrast, and reproducibility. We compare the performance achieved by compensated and uncompensated pulses, and demonstrate further shaping of our compensated pulses to achieve selective excitation of different regions within a biological sample.

Materials and methods

The setup we used for two-photon imaging is shown in Figure 1. Our experimental setup resembles many typically used for two-photon imaging, except for the addition of a pulse shaper to compensate phase distortions and to introduce desired phase functions. For a review of pulse shaping methods, the reader is referred to the review by Weiner [18]. We performed our measurements with a titanium sapphire oscillator (K&M Laboratories) capable of producing 10fs pulses (100 nm full width half-maximum) centered near 800 nm, with a repetition rate of 97 MHz and average power of 250 mW. The beam was attenuated to an average power of 4 mW when focused on the sample, a fluorescently stained 15μm mouse kidney section (see Figure 3). The sample is stained with Alexa Fluor 488 (elements of the glomeruli and convoluted tubules), Alexa Fluor 568 (F-actin), and 4′,6-diamidino-2-phenylindole (nuclei). The laser beam was coupled through a 60× objective, through which fluorescence from the sample was also collected.

Because we are using such short pulses, it is very important to compensate for phase distortions at the sample. We first used a pair of prisms that compensated for 10^4 fs^2 of linear dispersion. This amount of dispersion is sufficient to lengthen 10fs pulses by a factor of a thousand. Note that 100fs pulses would only be lengthened by 3% by the same amount of dispersion. High-order phase correction at the sample was then achieved using MIIPS [5,11,13,19]. Without compensation, the signal obtained would have been four orders of magnitude smaller. Total integrated fluorescence, with no fluorescence wavelength filtering, was detected using an electron-multiplying charge-coupled device camera (iXon, Andor Technologies) as the sample was scanned across the focused laser excitation spot.

To show selective imaging, we used **binary-phase** pulse shaping (Figure 2, *A*) that enhanced two-photon excitation at shorter or at longer wavelengths, while suppressing two-photon excitation at the other end of the spectrum. The efficiency of these pulses can be confirmed by obtaining the frequency-doubled spectrum shown in Figure 2, *B* resulting from transmission through a thin 0.02-mm (±0.01 mm) potassium dihydrogen phosphate second-harmonic-generation crystal. The shaped laser pulses were used to image the

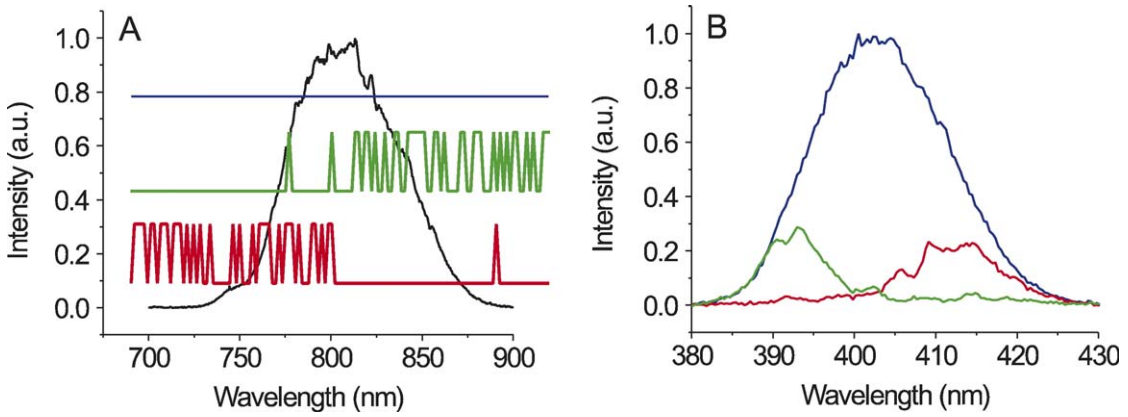

Fig 2. Pulse shaping for selective two-photon excitation. **A**, Spectrum of our femtosecond laser pulses and the three phases used for our experiments. The fundamental spectrum does not change by phase shaping, eliminating wavelength-dependent effects in the beam optics. The top line, when all phase values are equal, represents the phase function applied to achieve a transform limited (TL) pulse. The two other phase functions are applied to our laser pulse to produce selective excitation. The middle phase function is designed to maximize two-photon excitation at shorter wavelengths while suppressing two-photon excitation elsewhere. Similarly, the bottom phase function is designed to maximize two-photon excitation at longer wavelengths while suppressing two-photon excitation elsewhere. **B**, We tested the ability to induce selective two-photon excitation using a second harmonic generation crystal. The frequency-doubled output spectrum obtained for TL pulses changes as expected when introducing the short wavelength-optimizing phase function (middle phase), or the long wavelength-optimizing phase function (bottom phase).

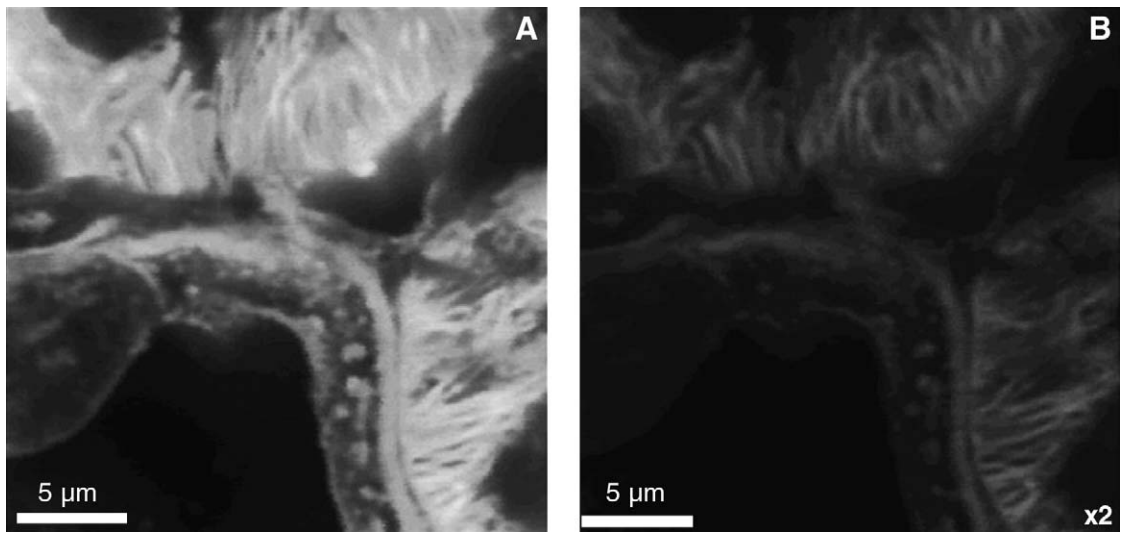

Fig 3. Photographs of the sample used. **A**, Fluorescently labeled mouse kidney sample (FluoCells prepared slide no. 3), imaged with transform-limited pulses. **B**, The same sample, imaged without compensation for high-order phase distortion. The intensity has been increased by a factor of 2 to show detail; actual signal intensity is decreased by a factor of about 7 as compared with the sample in **A**. Both panels show a portion of the convoluted tubules, with actin-containing microvilli structures on the top and right sides of the panels.

sample without further adjustment of the laser system. Each image was collected twice, in alternation, to check for reproducibility and photobleaching effects.

Results

A 25-μm $\times$ 25-μm image of our sample taken with compensated (transform limited, or, TL) pulses is shown in Figure 3, *A*. Bright regions correspond to sample areas that produced fluorescence under two-photon excitation. Figure 3, *B* shows an image of the same sample area taken after dispersion compensation but without high-order phase compensation. To make its features visible, its intensity has been increased by a factor of two; note that it is still considerably less intense than the image produced by compensated pulses. The difference in intensity caused by the high-order phase distortions was approximately a factor of seven on the day the images were taken but will vary from day to day and from laser to laser. The image obtained with uncompensated pulses shows some dark regions that should be bright. The reason for this difference is that the uncompensated high-order distortions can enhance two-photon excitation in certain wavelength regions [8]. Unfortunately, this enhancement is not reproducible,

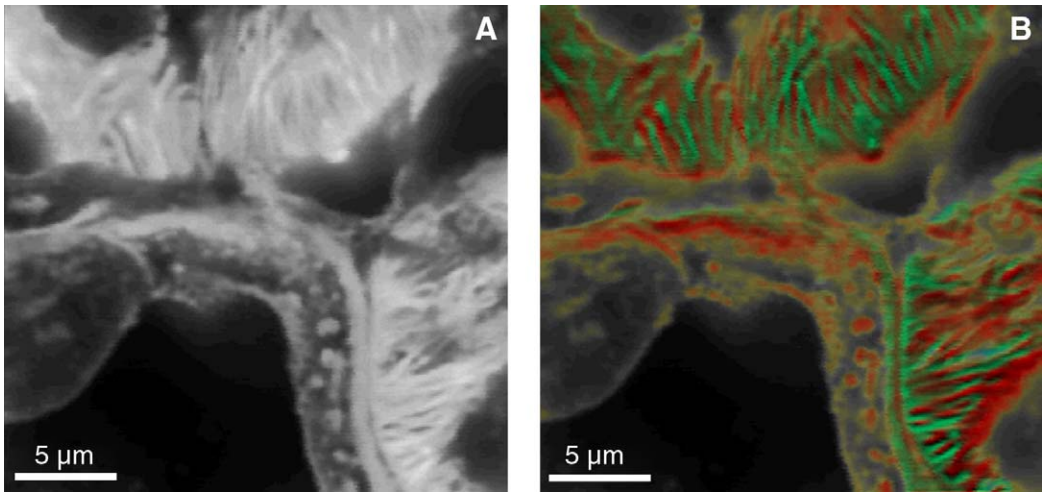

Fig 4. **A**, Fluorescently labeled mouse kidney sample (FluoCells prepared slide no. 3), imaged with transform-limited pulses (reprinted for comparison). **B**, Composite image of the same mouse kidney sample, imaged with the selective pulse shapes shown in Fig 3. Notice the greater detail and the ability to distinguish among different subcellular structures without resorting to fluorescence color filters.

because it depends on the laser alignment and the type of objective used. This is the reason that two-photon microscopy with uncompensated femtosecond pulses can have poor reproducibility.

In Figure 4, *B* we show reproducible selective excitation of different regions in our sample. This false-color image shows the excitation produced by pulses that enhance two-photon excitation at longer wavelengths (false-colored red), and by pulses that enhance two-photon excitation at shorter wavelengths (false-colored green). The image was obtained by combining three normalized images—one taken with TL pulses, one with red pulses, and one with green pulses. The excitation produced selectively by red pulses was determined by subtracting the image produced by green pulses from the image produced by red pulses, and finally subtracting the TL image as a constant reference point; a similar method was used to produce the selective excitation from green pulses. Figure 4, *A* shows the image obtained with TL pulses for comparison; note that the composite shaped image shows much more detail.

Discussion

Phase compensation methods such as MIIPS are useful tools for increasing the efficiency of two-photon excitation, and vital for ensuring reproducibility. We have shown a significant increase in fluorescence signal for two-photon imaging when TL pulses are used as compared with uncompensated pulses (four orders of magnitude) and linear dispersion compensated pulses (one order of magnitude). With a compensated pulse as a starting point, we used pulse shaping to tune the second harmonic wavelength, producing selective excitation that provides more contrast than TL pulses. Further pulse shaping can be used to target the two-photon excitation maxima of specific fluorescent molecules or nanoparticles within a sample, for imaging with other spectroscopic methods such as coherent anti-Stokes Raman scattering, or for selective activation of photodynamic therapy agents. In addition to selective two-photon excitation, pulse shaping can also be used to minimize three-photon damage [8,20]. The unscattered photons of a shaped pulse maintain their coherence as they propagate through scattering tissue, meaning that selective excitation can be performed through at least 1 mm of biological tissue [16]. The ability to selectively activate nanoparticles within a living organism opens exciting new possibilities for biomedical applications and research.

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