Selective two-photon microscopy with shaped femtosecond pulses

Igor Pastirk, Johanna M. Dela Cruz, Katherine A. Walowicz, Vadim V. Lozovoy, and Marcos Dantus

Department of Chemistry, Michigan State University, East Lansing MI 48824
dantus@msu.edu

Abstract: Selective two-photon excitation of fluorescent probe molecules using phase-only modulated ultrashort 15-fs laser pulses is demonstrated. The spectral phase required to achieve the maximum contrast in the excitation of different probe molecules or identical probe molecules in different micro-chemical environments is designed according to the principles of multiphoton intrapulse interference (MII). The MII method modulates the probabilities with which specific spectral components in the excitation pulse contribute to the two-photon absorption process due to the dependence of the absorption on the power spectrum of $E^2(t)$ [1-3]. Images obtained from a number of samples using the multiphoton microscope are presented.

OCIS codes: (180.0180) Microscopy (180.2520) Fluorescence Microscopy (320.0320) Ultrafast Optics (320.5540) Pulse Shaping

References and Links

1. K.A. Walowicz, I. Pastirk, V.V. Lozovoy, M. Dantus, "Multiphoton intrapulse interference. 1. Control of multiphoton processes in condensed phases," J. Phys. Chem. A **106**, 9369-9373 (2002)
2. V.V. Lozovoy, I. Pastirk, K.A. Walowicz, M. Dantus, "Multiphoton intrapulse interference. 2. Control of two- and three-photon laser induced fluorescence with shaped pulses," J. Chem. Phys. **118**, 3187-3196 (2003)
3. J.M. Dela Cruz, I. Pastirk, V.V. Lozovoy, K.A. Walowicz, M. Dantus, "Multiphoton intrapulse interference 3: Probing microscopic chemical environments," J. Phys. Chem. A (in press) (2003)
4. W. Denk, J.H. Strickler, W.W. Webb, "2-Photon laser scanning fluorescence microscopy," Science **248**, 73-76 (1990)
5. K. Konig, "Multiphoton microscopy in life sciences," J. Microsc. -Oxf. **200**, 83-104 (2000)
6. P.T.C. So, C.Y. Dong, B.R. Masters, K.M. Berland, "Two-photon excitation fluorescence microscopy," Annu. Rev. Biomed. Eng. **2**, 399-429 (2000)
7. K.W. Dunn, R.M. Sandoval, K.J. Kelly, P.C. Dagher, G.A. Tanner, S.J. Atkinson, R.L. Bacallao, B.A. Molitoris, "Functional studies of the kidney of living animals using multicolor two-photon microscopy," Am. J. Physiol. -Cell Physiol. **283**, C905-C916 (2002)
8. J.W. Wang, A.M. Wong, J. Flores, L.B. Vosshall, R. Axel, "Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain," Cell **112**, 271-282 (2003)
9. T.G. Oertner, "Functional imaging of single synapses in brain slices," Exp. Physiol. **87**, 733-736 (2002)
10. K.A. Kasischke, H. Vishwasrao, A.A. Heikal, W.W. Webb, "Two-photon redox-fluorimetry: A new functional imaging technique for visualizing energy metabolism in brain tissue," Biophys. J. **82**, 2420 (2002)
11. C.R. Rose, Y. Kovalchuk, J. Eilers, A. Konnerth, "Two-photon Na^+ imaging in spines and fine dendrites of central neurons," Pflugers Arch. **439**, 201-207 (1999)
12. A.M. Weiner, "Femtosecond pulse shaping using spatial light modulators," Rev. Sci. Instrum. **71**, 1929-1960 (2000)
13. C540 is 3-(2'-Benzothiazolyl)-7-diethylaminocoumarin. R6G is a mixture of rhodamine 590 (2-[6-(ethylamino)-3-(ethylimino)- 2,7-dimethyl-3H-xanthen-9-yl]-ethylester, monohydrochloride), DOCI (3,3'-Diethyloxacarbocyanine Iodide) and DCM 4-Dicyanmethylene-2-methyl-6-(p-dimethylaminostyryl)-4H -pyran).
14. T.H. Tran-Thi, T. Gustavsson, C. Prayer, S. Pommeret, J.T. Hynes, "Primary ultrafast events preceding the photoinduced proton transfer from pyranine to water," Chem. Phys. Lett. **329**, 421-430 (2000)

#2502 - $15.00 US Received May 21, 2003; Revised July 06, 2003

15. T.H. Tran-Thi, C. Prayer, P. Millie, P. Uznanski, J.T. Hynes, "Substituent and solvent effects on the nature of the transitions of pyrenol and pyranine. Identification of an intermediate in the excited-state proton-transfer reaction," J. Phys. Chem. A **106**, 2244-2255 (2002)
16. B. Broers, L.D. Noordam, H.B.V. Vandenheuvell, "Diffraction and focusing of spectral energy in multiphoton processes," Phys. Rev. A **46**, 2749-2756 (1992)
17. D. Meshulach, Y. Silberberg, "Coherent quantum control of two-photon transitions by a femtosecond laser pulse," Nature **396**, 239-242 (1998)
18. R. Wolleschensky, T. Feurer, R. Sauerbrey, I. Simon, "Characterization and optimization of a laser-scanning microscope in the femtosecond regime," Appl. Phys. B- Lasers Opt. **67**, 87-94 (1998)
19. V.V. Lozovoy, I. Pastirk, K.A. Walowicz, M. Dantus, "Multiphoton intrapulse interference. 4. Characterization of the phase of ultrashort laser pulses.," Opt. Lett. submitted
20. M.W. Berns, Z. Wang, A. Dunn, V. Wallace, V. Venugopalan, "Gene inactivation by multiphoton-targeted photochemistry," Proc. Natl. Acad. Sci. **97**, 9504-9507 (2000)
21. V. Shafirovich, A. Dourandin, N.P. Luneva, C. Singh, F. Kirigin, N.E. Geacintov, "Multiphoton near-infrared femtosecond laser pulse-induced DNA damage with and without the photosensitizer proflavine," Photochem. Photobiol. **69**, 265-274 (1999)
22. D.R. Larson, W.R. Zipfel, R.M. Williams, S.W. Clark, M.P. Bruchez, F.W. Wise, W.W. Webb, "Water-soluble quantum dots for multiphoton fluorescence imaging in vivo," Science **300**, 1434-1436 (2003)
23. D.A. VandenBout, W.T. Yip, D.H. Hu, D.K. Fu, T.M. Swager, P.F. Barbara, "Discrete intensity jumps and intramolecular electronic energy transfer in the spectroscopy of single conjugated polymer molecules," Science **277**, 1074-1077 (1997)
24. C. Xu, W. Denk, "Two photon optical beam induced current imaging throught backside of integrated circuits," Appl. Phys. Lett. **71**, 2578-2580 (1997)
25. D.L. Osborn, S.R. Leone, "Spectral and intensity dependence of spatially resolved two- photon conductivity defects on a GaAsP photodiode," J. Appl. Phys. **89**, 626-633 (2001)
26. T. Brixner, N.H. Damrauer, P. Niklaus, G. Gerber, "Photoselective adaptive femtosecond quantum control in the liquid phase," Nature **414**, 57-60 (2001)
27. S. Maiti, J.B. Shear, R.M. Williams, W.R. Zipfel, W.W. Webb, "Measuring serotonin distribution in live cells with three-photon excitation," Science **275**, 530-532 (1997)
28. D. Yelin, D. Oron, E. Korkotian, M. Segal, Y. Silbergerg, "Third-harmonic microscopy with a titanium-sapphire laser," Appl. Phys. B- Lasers Opt. **74**, S97-S101 (2002)
29. T. Watanabe, M. Akiyama, K. Totani, S.M. Kuebler, F. Stellacci, W. Wenseleers, K. Braun, S.R. Marder, J.W. Perry, "Photoresponsive hydrogel microstructure fabricated by two-photon initiated polymerization," Adv. Funct. Mat. **12**, 611-614 (2002)
30. M. Dyba, S.W. Hell, "Focal spots of size lambda/23 open up far-field fluorescence microscopy at 33 nm axial resolution," Phys. Rev. Lett. **88**, 163901 (2002)

---

1. Introduction

Two-photon microscopy has provided researchers with unique possibilities for fluorescence imaging and photochemistry. It offers attractive advantages, including higher resolution, background-free signal, lower background scattering, better penetration in thick samples, and reduced photon-induced damage [4-6], which arise from the basic physical principle that the absorption depends on the square of the excitation intensity. Two-photon microscopy has been shown to be amenable to multiple probe staining, whereby two-photon transitions excite different probe molecules that emit at different wavelengths [6,7], and for functional imaging of living cells [8-11]. Here, we demonstrate how phase-modulated femtosecond pulses can selectively excite one type of probe molecule only, leaving the others in their ground state. Selectivity in multiphoton excitation is achieved by multiphoton intrapulse interference (MII) as outlined below [1-3]. Selective excitation can be used to enhance contrast and to achieve functional imaging of samples stained with fluorescent probes sensitive to their microscopic chemical environment.

2. Experimental section

The experiments are carried out using a titanium-sapphire oscillator (KM Labs) laser system capable of generating pulses as short as 11 fs after a double pass prism compressor. The amplitude profile and spectral phase of the pulse is tailored using a computer-controlled pulse shaper of a design similar to those described by Weiner [12]. The shaper uses a pair of SF-10 prisms as the dispersion elements and 0.4 m cylindrical mirrors for collimation. A schematic of the experiment is shown in Fig. 1. Phase modulation is introduced by a computer-

#2502 - $15.00 US Received May 21, 2003; Revised July 06, 2003

controlled spatial light modulator (SLM-256, CRI Inc.), located at the Fourier plane of the shaper setup. For the experiments shown here, the pulses were centered near 800 nm. The spectral phase of each pulse was calibrated using the multiphoton intrapulse interference phase-scan method (MIIPS), first to obtain transform-limited (TL) pulses and then to introduce the desired phase [1,3]. The spectral phase function used for this experiment is given by

$$\phi(\Omega)=\alpha\mathrm{Cos}(\gamma\Omega-\delta), \quad (1)$$

where Ω is the frequency detuning measured in s^{-1} from the center wavelength of the laser pulse.

The shaped laser pulses, with an energy of 0.2-1 nJ per pulse and 87 MHz repetition rate, were focused mildly, to a spot size of ~ 200 microns in diameter, on the different samples. Two-photon induced fluorescence was collected by an infinity corrected apochromatic microscope objective (APO 100X, Mitutoyo) and imaged into a liquid nitrogen cooled CCD detector using an InfiniTube (Proximity Series, Infinity Photo-Optical). Experiments were carried out on three different types of samples. The first sample was prepared with acrylonitrile-vinylidene chloride polymer cured in acidic (pH 6) or basic (pH 10) buffered conditions and stained with the pH-sensitive fluorescent probe 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (10^{-3}M) (Sigma-Aldrich). The second sample consists of pieces of polymethylmethacrylate (PMMA) doped with C540 and R6G fluorescent probes (10^{-4}M) [13]. The third sample consists of a mixture of 10 μm blue and 15 μm green fluorescent FluoSpheres (Molecular Probes). The images are shown in black and white without optical filters in the collection optics or post image manipulation. The background, detector counts when the laser is off, was subtracted. The movies show images obtained as the spectral phase is scanned and are displayed with false color to highlight changes in intensity.

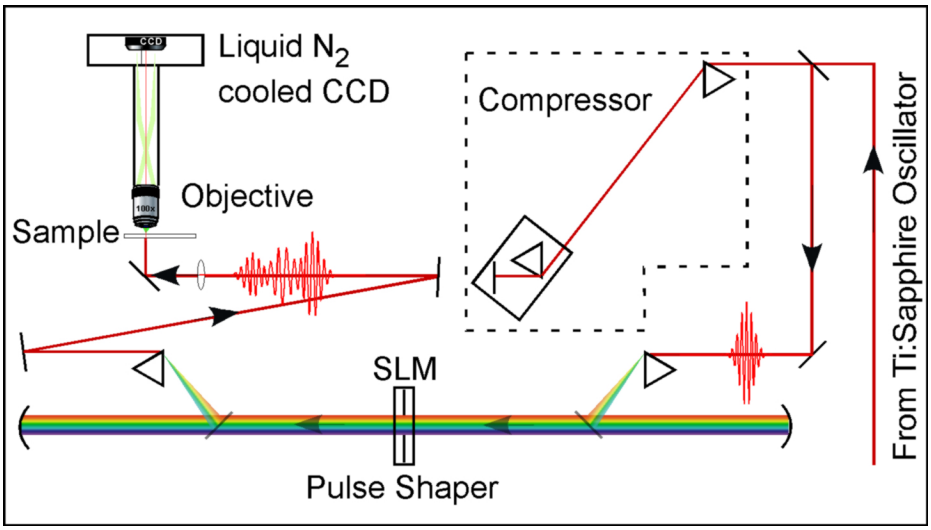

Fig. 1. Schematic experimental setup for selective two-photon microscopy. Femtosecond laser pulses are compressed and sent to the pulse shaper. The modulated beam is then focused on the microscope slide with the specimen. The two-photon induced fluorescence is collected by a microscope objective and imaged on a CCD.

3. Results

In Fig. 2 we show the experimentally measured and theoretically predicted dependence of the contrast ratio of intensity of two-photon laser induced fluorescence between samples with pH 10 and pH 6 for the solutions of pH sensitive dye in order to demonstrate the MII principle. The samples used were 10^{-5} M aqueous solutions of HPTS salt in different pH

#2502 - $15.00 US
Received May 21, 2003; Revised July 06, 2003

buffers. The laser spectrum remains unchanged throughout the experiments as spectral phase is modulated. The excitation spectra of HTPS exhibits large pH dependent differences while the emission spectrum centered at 515 nm is independent of pH [14,15].

These measurements were obtained using 23 fs transform-limited pulses that were shaped to have a Gaussian spectrum centered at 842.6 nm with a bandwidth of 48 nm and spectral phase defined by Eq. (1) with $\alpha = 1.5\pi$, $\gamma = 20$ fs. Spectral intensity $|E(\Omega)|^2$ and phase $\varphi(\Omega)$ of laser pulse are presented in Fig. 2(A). In Fig. 2(b) we show the calculated spectrum of the electric field squared

$$E^{(2)}(\Delta) = \int E(\Delta/2+\Omega)E(\Delta/2-\Omega)d\Omega \qquad (2)$$

where $\Omega=2\pi c(1/\lambda-1/\lambda_0)$ is the spectral detuning from the carrier frequency $2\pi c/\lambda_0$ of the laser pulse and $E(\Omega) = |E(\Omega)|e^{i\varphi(\Omega)}$. Simulations of the experimental signal were performed using the following equation with no adjustable parameters:

$$S^{(2)} \propto \int g^{(2)}(\Delta)\left|E^{(2)}(\Delta)\right|^2 d\Delta, \qquad (3)$$

where $g^{(2)}(\Delta)$ is two-photon absorption spectra of HTPS at different pH (single photon spectra are shown in Fig. 2(b)). The spectrum of the pulse $|E(\Omega)|^2$ and the absorption spectra $g(\Delta)$ of HPTS were measured experimentally. Intramolecular dynamics, such as molecular vibrations, are neglected because they have a minimum contribution to MII [1,2]. Because HPTS does not have inversion symmetry, its one-photon absorption spectrum $g(\Delta)$ is expected to be similar to its two-photon absorption spectrum $g^{(2)}(\Delta)$. In Fig. 2(c) the contrast ratio between the measured intensity of fluorescence at pH 10 and pH 6 is presented as a function of δ. The experimental data obtained by excitation of the dye solutions in methanol (10^{-3} M in water) generally agree with the calculated response [3].

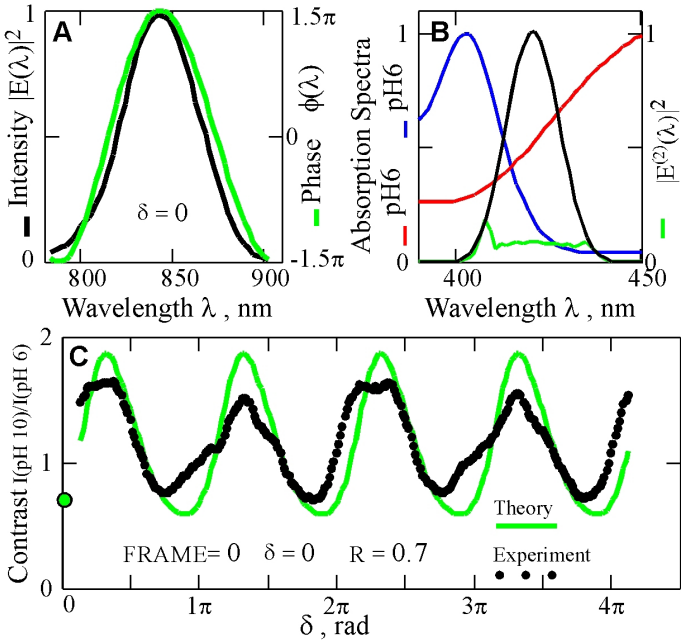

Fig. 2. (2.02 MB) Experimental results and theoretical predictions of selective two-photon excitation of HTPS solutions at different pH. For this image the pulses were shaped with $\alpha = 1.5\pi$, $\gamma = 20$ fs, and δ scanned from 0 to 4π. The movie shows the changes in the measured (black dots) and calculated (green line) contrast ratio as a function of the phase function parameter δ. (a) The laser spectrum (black line) and phase (green line) of the laser pulse. (b) Calculated power spectrum (green) of $E^2(t)$ phase modulated pulse at specific phase δ and for TL pulse (black line). Absorption spectra of HTPS at pH 10 (red line) and pH 6 (blue line) (c) Measured (black dots) and predicted (green line and big green dot) contrast ratio.

#2502 - $15.00 US
Received May 21, 2003; Revised July 06, 2003

MII modulates the amplitude of the power spectrum of $E^2(t)$ as the spectral phase of the laser pulse $\varphi(\lambda)$ is scanned (movie Fig. 2)[1-3,16,17]. Note that the maximum amplitude in the power spectrum in Fig. 2(b) shifts from longer to shorter wavelengths, giving the possibility for selectively exciting the fluorescent probe in different pH environments.[3]

We prepared a solid sample of HTPS with different pH regions and imaged it using a microscope. Fig. 3(a) shows an image obtained with TL pulses. The spectrum of the pulse and the spectral phase (flat line) are shown in the right panels. Figure 3(b) shows an image obtained with laser pulses optimally shaped for selective excitation of HPTS under acidic environment. The spectral phase used to acquire this image is shown in the middle panel. Figure 3(c) shows an image obtained with laser pulses optimally shaped for selective excitation of HPTS in a basic environment. The spectral phase used to acquire this image is shown in the middle panel. The contrast ratio between pH 10/pH 6 in these images varies from 1:1 in Fig. 3 (a) to 1:10 Fig.3 (b) to 64:1 in Fig. 3(c).

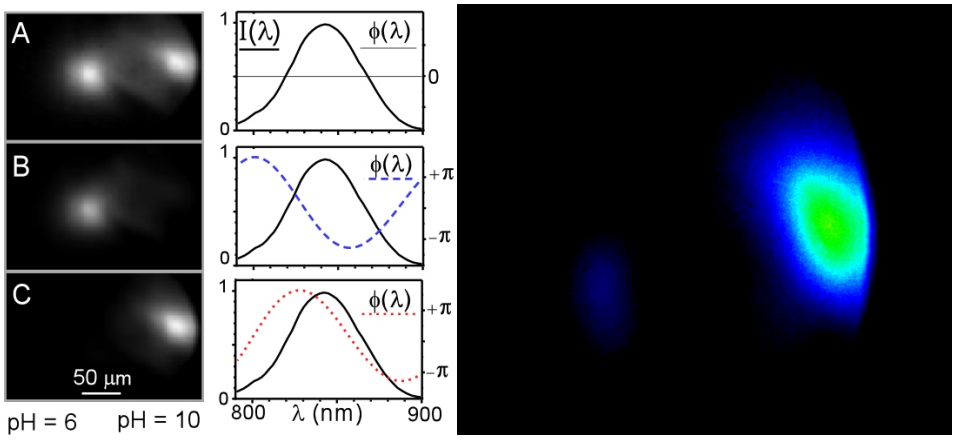

Fig. 3. (1.28 MB) Experimental demonstration of pH-sensitive selective two-photon microscopy. The sample being imaged has an acidic (left side of the frame at pH 6) and a basic (right side of the frame at pH 10) region, both labeled with HPTS. (a) Image of the sample obtained with transform-limited pulses. The diagrams on the right show the spectrum of the 21-fs laser pulses, centered at 842 nm, and the spectral phase of the pulse (blue dashed line or red dotted line, that maximize pH 6 or pH 10 fluorescence, respectively). (b) Image of the same sample and location obtained with pulses that have been optimized for selective excitation of HPTS in an acidic micro-environment. Notice that only the left region shows significant two-photon excitation. For this image $\alpha=1.5\pi$, $\gamma=20$ fs, and $\delta=0.75\pi$. (c) Image of the same sample and location obtained with pulses that have been optimized for selective excitation of HPTS in a basic micro-environment. Notice that only the right region shows significant two-photon excitation. For this image $\alpha=1.5\pi$, $\gamma=20$ fs, and $\delta=0.25\pi$. The movie shows an experiment where the phase function parameter δ is scanned from 0 to 4π. Selective two-photon excitation from the two pH regions in the sample is observed at specific values of δ.

The experimental results in Fig. 4 show selective excitation of different fluorescent probes. Figure 4(a) shows the data obtained using TL pulses. The two pieces of PMMA being imaged show similar amounts of two-photon laser induced fluorescence. When the spectral phase of the pulse is modified to optimize selective excitation of the C540-doped sample, we observe that only the top fragment fluoresces, as shown in Fig. 4(b). When the spectral phase of the pulse is optimized for selective excitation of the R6G-doped sample, Fig. 4(c), we observe strong fluorescence from the bottom fragment while the other side, containing the C540 probe, does not fluoresce. Note that the spectrum and intensity of the laser remain constant during the experiment for all three cases. The only change is the subtle modulation of the spectral phase of the pulse, which controls multiphoton intrapulse interference [1-3]. Contrast ratio (I_{R6G}/I_{C540}) (normalized intensities corrected to background) ranges from 1:9 for

#2502 - $15.00 US Received May 21, 2003; Revised July 06, 2003

pulses optimized for C540 to 8:1 for pulses optimized for R6G. Selective multiphoton excitation minimizes the cross talk between different fluorescent probes and does not require the use of multiple filters and detectors for imaging.

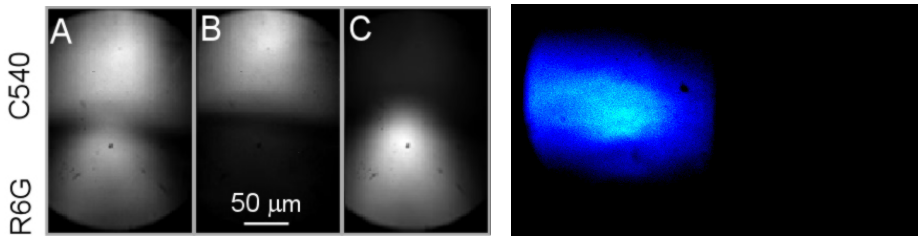

Fig. 4. (2.4 MB) Selective two-photon microscopy of pieces of PMMA doped with different fluorescent probes. (a) Image showing two-photon induced fluorescence from both pieces top-C540 doped PMMA and bottom-R6G doped PMMA, obtained with 17 fs transform-limited pulses centered at 790 nm. (b) Image obtained with pulses optimized for selective C540 excitation. For this image the pulses were shaped with α=1.5π, γ=20 fs, and δ=0.31π. (c) Image obtained with pulses optimized for selective R6G excitation. For this image the pulses were shaped with α=1.5π, γ=20 fs, and δ=0.74π. The movie shows an experiment where the phase function parameter δ is scanned from 0 to 2π. Selective two-photon excitation from the two PMMA pieces (left is C540 and right is R6G) is observed at specific values of δ.

The previous experiment showed selective two-photon excitation of different probe molecules. To further illustrate the potential of coherent control for functional imaging we show selective excitation of microspheres, typically used for targeted staining of biological samples. Fluorescence from two different microspheres is shown in Fig. 5. The top image shows the green-fluorescent microsphere which has an absorption maximum at 450 nm, while the bottom image represents the blue-fluorescent microsphere, which has an absorption maximum at 365 nm. The image in Fig. 5(a) was acquired with TL pulses. Under these conditions, large two-photon induced fluorescence signal is observed from both spheres. For the image in Fig. 5(b), we modulated the phase of the pulses such that the intensity of fluorescence from the blue microsphere is maximized and that of the green one minimized. For the image in Fig. 5(c), we modulated the phase of the pulses such that the fluorescence from the green microsphere is maximized and that of the blue one minimized. The observed contrast ratios (I_{BLUE}/I_{GREEN}) are 1:3 and 4:1 for the different phases.

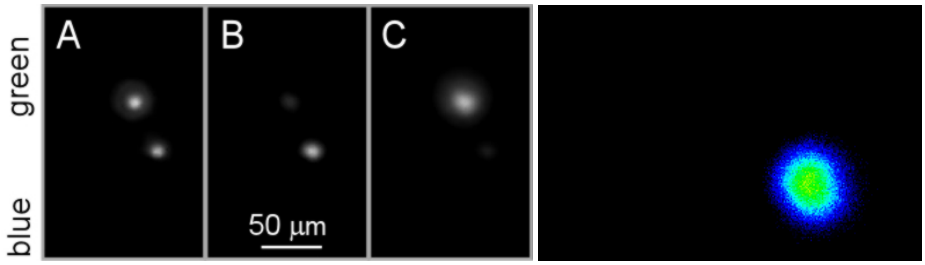

Fig. 5. (738 kB) Selective two-photon microscopy of 10 μm blue and 15 μm green fluorescent polystyrene microspheres. (a) Image showing two-photon induced fluorescence from both microspheres, obtained with 15 fs transform-limited pulses centered at 790 nm. (b) Image obtained with pulses optimized for selective excitation of the blue microsphere. For this image the pulses were shaped with α=2.5π, γ=10 fs, and δ=0.75π. (c) Image obtained with pulses optimized for selective excitation of the green microsphere. For this image the pulses were shaped with α=2.5π, γ=10 fs, and δ=1.25π. The movie shows an experiment where the phase function parameter δ is scanned from 0 to 2π. Selective two-photon excitation from the two microspheres is observed at specific values of δ.

#2502 - $15.00 US Received May 21, 2003; Revised July 06, 2003

4. Discussion

Selective excitation with significant contrast ratios has been achieved here by optimizing the overlap between the power spectrum of $E^2(t)$ and the two-photon absorption spectrum [1,2]. The addition of a computer-controlled pulse shaper to the multiphoton microscope provides a number of important advantages. First, the pulse shaper can be used to compensate unwanted phase distortions at the sample. Linear chirp, for example, has been shown to reduce signal intensity in two-photon microscopy [18]. With a pulse shaper, linear, quadratic, cubic and higher order chirp can be compensated to obtain the most efficient excitation [19]. Second, the pulse shaper can be used to control the output spectrum of the laser pulses by amplitude modulation. Here, for example, we used pulses centered at 790 or at 840 nm. Third, the pulse shaper can be used for selective probe excitation as shown here. Because the spectrum of the laser remains constant, phase modulation does not affect one photon processes such as absorption, reflection and scattering. Selective excitation minimizes possible cross talk between different fluorescent probes in the sample. Finally, the pulse shaper can be used to prevent three-photon and higher order nonlinear optical processes such as continuum generation. Higher order processes usually lead to sample degradation, and in the case of living samples to DNA damage [20,21]. Suppression of three-photon transitions of four orders of magnitude has been achieved using the MII method [2] and this suppression can be coupled with optimization of two-photon signal from living specimens.

We have demonstrated selective two-photon microscopy of fluorescent probe molecules using phase-modulated pulses as short as 15 fs. This method can be used to selectively excite either different probe molecules or identical probe molecules in different environments. In addition, this method can be used for selective excitation of luminescent probes such as quantum dots [22], metallic nanoparticles, and single molecules [23]. The same principle can be extended to achieve functional imaging of semiconductor microchips by two-photon laser induced conductivity [24,25]. Having a pulse shaper for multiphoton microscopy provides the flexibility of selective probe excitation or maximum signal enhancement by controlled modulation of the spectral phase of the femtosecond pulses. Even when for fluorescent labels with very similar absorption spectra, pulse shaping has been shown capable of selective excitation [26]. This level of selective excitation and enhancement can be adapted to different modes of two-photon and three-photon microscopy [27-30].

Acknowledgements

Funding for this research came from the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy. We gratefully acknowledge funds from the Office of Intellectual Property of Michigan State University for construction of the sub-20 fs pulse shaper. MD is a Camille Dreyfus Teacher-Scholar. We are very thankful to Professor Gary Blanchard for lending us his microscope for these measurements and for valuable scientific discussions.

#2502 - $15.00 US Received May 21, 2003; Revised July 06, 2003