Selective excitation between two-photon and three-photon fluorescence with engineered cost functions

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Abstract: Coherent control based on a feedback-controlled self-learning loop was applied to enhance the ratio of two-photon (2P) fluorescence from a fluorescent label enhanced green fluorescence protein and three-photon (3P) fluorescence from the essential amino acid L-Tryptophan. The two biosamples were mixed in a buffer solution contained in a quartz cuvette and exposed to near-infrared laser pulses from a femtosecond (fs) oscillator. However, the enhancement of the 2P/3P fluorescence ratio was always accompanied by a significant loss of the valuable 2P fluorescence. To achieve a trade-off between the 2P/3P fluorescence ratio and the 2P fluorescence intensity, we then engineered the cost function in the self-learning algorithm. The optimal pulse shape obtained by use of the engineered cost function could be useful for 2P fluorescence imaging of living cells with reduced phototoxicity, because DNA and protein can be directly damaged by 3P absorption of fs laser according to an excitation band ~270nm.

OCIS codes: (140.7090) Ultrafast lasers, (320.5540) Pulse shaping, (320.7160) Ultrafast technology, (190.7110) Ultrafast nonlinear optics

References and links

1. W. Denk, J. H. Strickler, and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," Science **248**, 73-76 (1990).
2. S. Maiti, J. B. Sher, R. M. Williams, R. W. Zipfel, and W. W. Webb, "Measuring serotonin distribution in live cells with three-photon excitation," Science **275**, 530-532 (1997).
3. G. H. Patterson and D. W. Piston, "Photobleaching in Two-Photon Excitation Microscopy," Biophys. J. **78**, 2159-2162 (2000).
4. H. Rabitz, R. de Vivie-Riedle, M. Motzkus, and K. Kompa, "Whither the future of controlling quantum phenomena?" Science **288**, 824-828 (2000).
5. A. M. Weiner, "Femtosecond pulse shaping using spatial light modulators," Rev. Sci. Instrum. **71**, 1929-1960 (2000).
6. H. Kawano, Y. Nabekawa, A. Suda, Y. Oishi, H. Mizuno, A. Miyawaki, and K. Midorikawa, "Attenuation of photobleaching in two-photon excitation fluorescence from GFP with shaped excitation pulses," Biophys. Biochem. Res. Commun. **311**, 592-596 (2003).
7. K. Konig, P. T. C. So, W. W. Mantulin, and E. Gratton, "Cellular response to near-infrared femtosecond laser pulses in two-photon microscopes," Opt. Lett. **22**, 135-136 (1997).
8. V. Shafirovich, A. Dourandin, N. P. Luneva, C. Singh, F. Kirigin, and N. E. Geacintov, "Multiphoton near-infrared femtosecond laser pulse-induced DNA damage with and without the photosensitizer Proflavine," Photochem. Photobiol. **69**, 265-274 (1999).
9. K. Konig, T. W. Becker, P. Fischer, I. Riemann, and K.-J. Halbhuber, "Pulse-length dependence of cellular response to intense near-infrared laser pulses in multiphoton microscopes," Opt. Lett. **24**, 113-115 (1999).

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10. R. Y. Tsien, "The green fluorescent protein," Annu. Rev. Biochem. **67**, 509-544 (1998).
11. W. H. Press, B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling, *Numerical Recipes, 2nd Ed.* (Cambridge Univ. Press, Cambridge, 1989), Chap. 10.
12. R. J. Levis, G. M. Menkir, and H. Rabitz, "Selective bond dissociation and rearrangement with optimally tailored, strong-field laser pulses," Science **292**, 709-713 (2001).
13. T. Brixner, N. H. Damrauer, P. Niklaus and G. Gerber, "Photoselective adaptive femtosecond quantum control in the liquid phase," Nature **414**, 57-60 (2001).
14. Y. Barad, H. Eisenbery, M. Horowitz, and Y. Silberberg, "Nonlinear scanning laser microscopy by third harmonic generation," Appl. Phys. Lett., **70**, 922-924 (1997)
15. N. Dudovich, B. Dayan, S. M. G. Faeder, and Y. Silberberg, "Transform-limited pulses are not optimal for resonant multiphoton transitions," Phys. Rev. Lett. **86**, 47-50 (2001).

1. Introduction

Multiphoton fluorescence microscopy offers distinct advantages in three-dimensional (3D) imaging of biosamples, such as improved longitudinal resolution, enhanced imaging depth, and reduced out-of-focus photobleaching and phototoxicity. Using a femtosecond (fs) laser as a light source to excite fluorescent labels, both two-photon (2P) and three-photon (3P) fluorescence microscopies have been demonstrated in the 3D imaging of biosamples [1-2]. However, since multiphoton fluorescence often demands the use of an ultra-high peak intensity to obtain sufficient excitation efficiency, it could induce stronger photobleaching and phototoxicity effects in the focal volume than one-phone excitation [3]. In fact, the photobleaching and phototoxicity have restricted the application of multiphoton fluorescence microscopy in many cases, such as living cell imaging.

Although great efforts have been devoted to investigations into photobleaching and phototoxicity, the mechanisms underlying these phenomena are still not very clear. Nevertheless, in a very general way, the excitation of fluorescence as well as the induction of photobleaching and phototoxicity can be regarded as the outcomes of some photophysical or photochemical processes. Therefore, a well-established technique, namely, the adaptive coherent control of physical and chemical processes with shaped fs laser pulses, could be applied to mitigate the photobleaching and phototoxicity effects [4-5]. In the coherent control process, a self-learning feedback loop iteratively produces new pulse shapes until the optimal outcome is achieved. Using this technique, optimal pulse shapes for reducing the photobleaching in the 2P excitation of the enhanced green fluorescence protein (EGFP) and its variants have recently been achieved, resulting in fourfold enhancement of the observation time of fluorescence [6]. Here, we attempt to apply the coherent control technique to reduce the phototoxicity in 2P fluorescence imaging of living biosamples by enhancing the ratio of 2P fluorescence from a fluorescent label EGFP and 3P intrinsic fluorescence from an amino acid – L-Tryptophan. We aim at reducing the 3P excitation because there ubiquitously exists a strong absorption band in the range of 260-280nm for DNA and proteins, which could induce 3P absorption of near-infrared (NIR) light with a center-wavelength near 780nm, and could in turn cause direct damage of living biosamples [7-9]. In addition, we show that the loss of useful 2P fluorescence, which often occurs as a price to be paid on an enhanced ratio of the 2P and 3P fluorescence, can be effectively diminished by use of engineered cost functions. Also we point out here that our technique could also be useful for reducing the THG generation in the practical fluorescence imaging of a living cell, and in turn the phototoxicity induced by the single-photon absorption of the third harmonic light. The enhancement of 2P fluorescence efficiency is important for high resolution, high speed imaging, in which strong fluorescence signal is necessary to ensure sufficient signal-to-noise ratio (SNR).

2. Experimental

The schematic of the experimental setup is shown in Fig. 1. In this work we used a mode-locked Ti:Sapphire oscillator (Femtosource, Femtolasers Produktions GmbH.) as the excitation source, which delivers pulses at a repetition rate of 75MHz, with a central wavelength of 785nm, a pulse duration of 12fs, and an average power of ~400mW. The laser beam was sliced using a chopper at a repetition rate of 100Hz for signal processing. The

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spectral phase of the laser pulse was modulated using a liquid crystal spatial light modulator (LC-SLM-128, Cambridge Research & Instrumentation, Inc.). We used a prism pair in front of the SLM setup to pre-chirp the fs laser pulses to compensate for dispersion of the objective lens. The shaped pulses, which had an average power of ~90mW and a spectrum width (FWHM) of ~90nm, were then directed into a long working distance (2.6mm) objective lens (40×, OLYMPUS) with numerical aperture (NA) of 0.6, and were focused into a quartz cuvette with a wall thickness of 1.25mm. The cuvette contained a mixed solution of EGFP (0.15mg/mL, Clontech) which is a variant of GFP from a jellyfish Aequorea Victoria [10], and L-Tryptophan (55mM, Kanto Chemical Co., Inc.) which is an intrinsic fluorescent aromatic amino acid that is abundant in many proteins and critical for the formation of many life-giving biomolecules. Both the EGFP and the Tryptophan were freely mobile in Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) NaOH buffer (20mM, pH 7.4). The 2P fluorescence (peak wavelength, 510nm) and the 3P fluorescence (peak wavelength 350nm) signals were detected by two photomultiplier tubes (PMT, H6780-06, Hamamatsu Photonics K. K.) which were installed separately on the two sides of the cuvette. The emissions from the EGFP and the Tryptophan were isolated using different color-glass filters that were inserted between the PMTs and the cuvette. The two analog signals from the PMTs were converted to digital signals using a digital oscilloscope (LeCroy 9350A) and then transferred to a personal computer in order to generate a cost function. The cost function provides a measure of the "quality" of the shaped pulse, and can thus be used as a feedback signal in the self-learning loop.

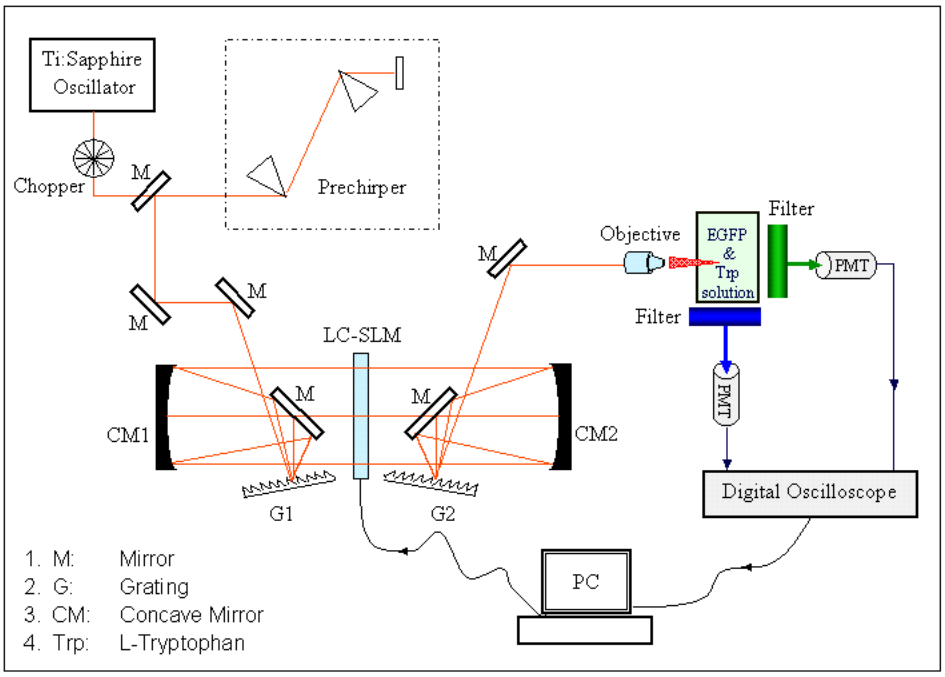

Fig. 1. Schematic of the experimental setup for selective excitation between 2P and 3P fluorescence with engineered cost functions.

3. Results

First, we simply defined the cost function as $C = F_{2P} / F_{3P}$, where F_{2P} and F_{3P} are the 2P and 3P fluorescence signals, respectively. The experiment began with the Fourier-transform-limited (FTL) pulse shape and with the initial value of the cost function adjusted to 1. The target of the self-learning loop was to increase the value of this cost function by iteratively

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applying new phase masks on the SLM. To find the best phase mask, we used a simulated annealing algorithm, which randomly produced a new phase mask for each iteration [11]. If the new phase mask caused an increase in the cost value, it is always accepted, and the self-learning process advances to the next iteration with the updated cost value. However, if the new phase mask caused a decrease in the cost value, it still had a probability of $\exp(-\Delta/T)$ of being accepted, where Δ is the change in the cost function and T is a parameter that needs to be determined for each experiment. Selecting a good value of T would help prevent the cost function from being trapped at some local minimum while still maintaining a fast search speed. After about 1000 iterations, the value of the cost function increased to 27, with F_{2P} reduced by 4.8 times and F_{3P} reduced by ~130 times. In this condition, the fluctuation of the 3P fluorescence signal was significant because of the background noise. We speculate that the value of the cost function could be further increased with an improved SNR of PMT. The temporal profile of the shaped pulse was reconstructed by a second-harmonic-generation frequency-resolved optical grating (SHG-FROG), as shown in Fig. 3(a). Roughly speaking, the pulse was split into a string of sub-pulses and the average pulse width was broadened significantly. A reduction of the peak intensity with such a pulse shape should be responsible for the enhancement of the fluorescence ratio, and additionally, the relatively greater decrease in the 3P fluorescence might be explained if the SLM throws the spectral components most responsible for 3P fluorescence into the parts of the pulse which are less intense.

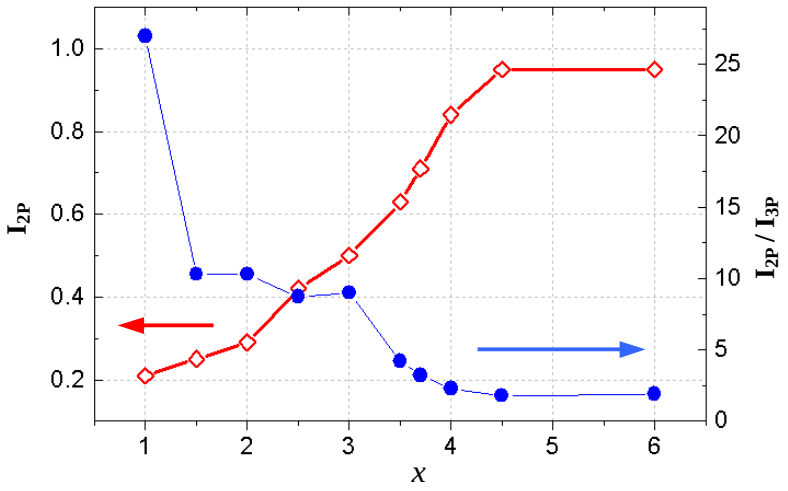

Fig. 2. Ratio between the 2P and 3P fluorescence signals (blue line) and the 2P fluorescence (red line) as functions of x. The signals of the 2P and 3P fluorescence are normalized by those excited by FTL pulses and denoted by I_{2p} and I_{3P}, respectively.

Although a significant enhancement of the 2P/3P fluorescence ratio was achieved by using the coherent control technique described above, the ~80% loss of F_{2p} that it caused could be a new problem for practical applications. In order to control the loss of 2P fluorescence, we designed a new group of cost functions with the form $C = F_{2P}^{x} / F_{3P}$, where x is a new control parameter. We then practiced the coherent control again for different values of x ranging from 1 to 6. It is interesting that the intensity of the 2P fluorescence indeed increases with the x value. Figure 2 shows the ratio between the 2P and 3P fluorescence signals, as well as the intensity of the 2P fluorescence as functions of x. It shows that the 2P fluorescence signal rises as the value of x increases, while, in contrast, the

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F_{2P}/F_{3P} ratio falls with increasing x. After x exceeds 4.5, the 2P fluorescence signal slightly decreases to ~95% of its initial level of FTL pulse excitation, whereas the 3P fluorescence can still be effectively reduced to ~53% of its initial value. Because different applications demand different 2P fluorescence intensities, one can freely select an optimal pulse shape from the two curves in Fig. 2 based on the individual experimental condition, namely, a pulse shape inducing minimal 3P absorption with an acceptable loss of 2P fluorescence.

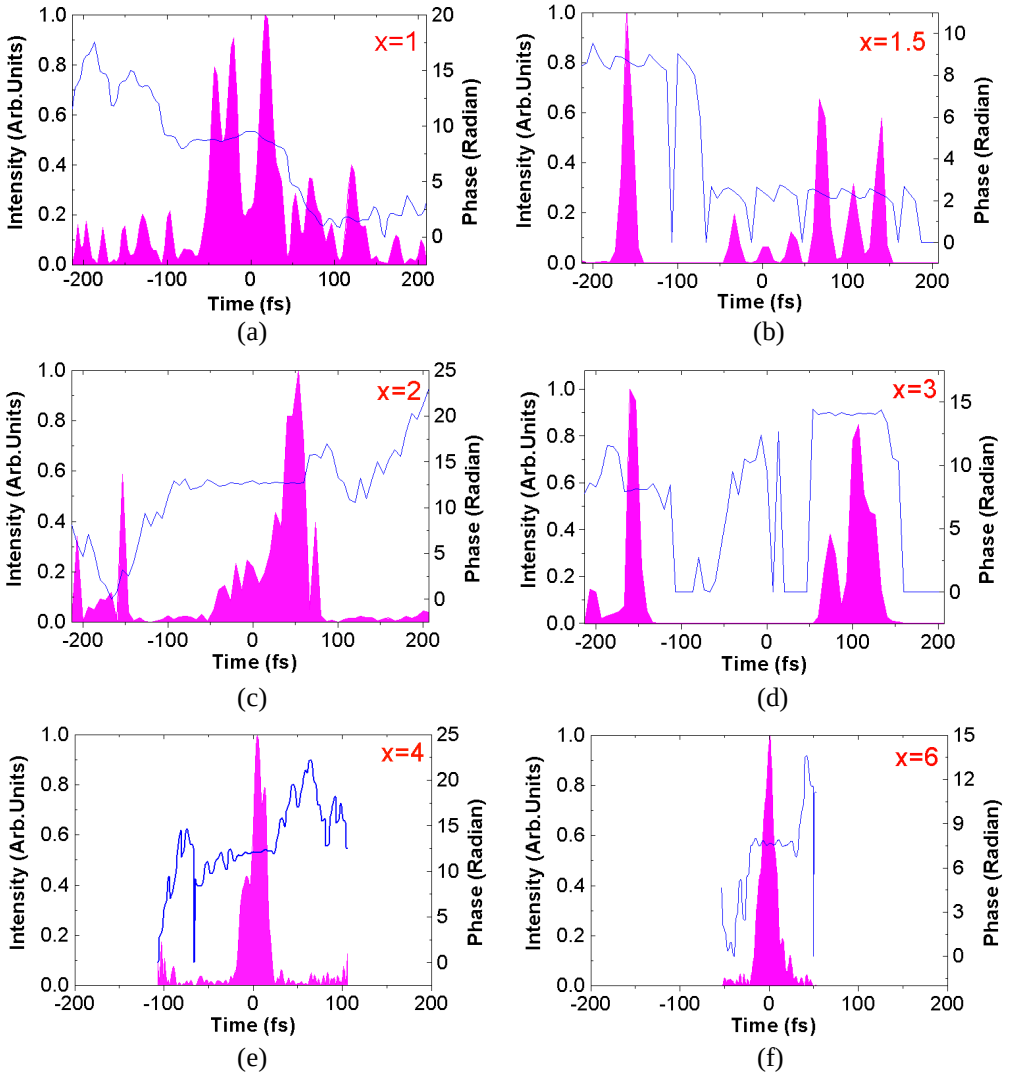

Fig. 3. SHG-FROG reconstructed temporal profiles (grid size is 64×64) of the shaped pulses obtained by using cost functions with (a) $x=1$, (b) $x=1.5$, (c) $x=2$, (d) $x=3$, (e) $x=4$, and (f) $x=6$. The red areas show the intensity profiles of the shaped pulses, and the blue lines show the temporal phases of the shaped pulses, respectively. The resulting FROG errors are 0.018, 0.015, 0.017, 0.017, 0.030 and 0.020 for Figs. (a) – (f), respectively.

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Figure 3 compares the temporal profiles of the shaped pulses reconstructed by SHG-FROG for different x values. For low x values, the shaped pulses were split into many sub-pulses so that the peak intensities were greatly reduced, leading to low excitation efficiencies for both 2P and 3P fluorescence. However, when we increased the value of x, the number of sub-pulses in the shaped pulse was gradually reduced, resulting in an increased peak intensity which in turn produced strong 2P fluorescence. Finally, when we chose x values larger than 4.5, the shaped pulses shown in Fig. 3(f) degenerated to a single pulse close to the FTL pulse. Therefore, the intensity of the 2P fluorescence could be maintained at almost the same value as when excited by the FTL pulse. However, some fine structures can be seen in the pulse shapes in Fig. 3(f) that could be responsible for the halved 3P fluorescence.

4. Discussion

Since strong-field light pulses with peak intensities as high as 10^{11}W/cm^2 to 10^{12}W/cm^2 are used in the multiphoton excitation of fluorescence, coherent control of strong-field photochemistry can be performed in two fashions, namely, trivial control and nontrivial control [12]. In the case of trivial control, selective photoreactions can be achieved by simply varying the intensity or the pulse duration, whereas in nontrivial control, laser pulse shapes must be precisely tailored to cooperatively interact with the evolving molecule wave packet. Therefore in the nontrivial control, not only the intensity and the pulse duration, but also the phase modulation would affect the outcome of an experiment. In the selective excitation of two photochemical processes with the same nonlinearity, e.g., the 2P excitation of two materials which have similar one-photon absorption spectrum, trivial control cannot play a major role because both nonlinear processes will have nearly the same dependence on the pulse duration or intensity, but effective selective excitation is still attainable through nontrivial adaptive quantum control based on the self-learning loop, as has been demonstrated by some other research groups [13]. However, if we move to the selective excitation of two photochemical processes with different nonlinearities, such as the 2P and 3P excitations of fluorescence, then trivial control can contribute significantly to the cost function, since a high order nonlinear process is usually more strongly dependent on intensity and pulse duration than a low order one. Therefore, if we simply use a cost function with $x = 1$, trivial control could easily be used in the selective excitation of 2P and 3P fluorescence by reducing the peak intensity through the broadening of pulse duration. In principle, the highest 2P/3P fluorescence ratio would occur when both the 2P and 3P fluorescence decrease to their lowest values. However, this theoretical limitation is hard to achieve under experimental conditions because of the limited SNR of PMT. After the 3P fluorescence decreased to a level comparable to the noise, coherent control could not be executed properly due to fluctuation in the feedback signal, and so the cost function stopped increasing. From a scientific point of view, coherent control in such a trivial manner is not very interesting, because it doesn't take advantage of the differences in the energy level structures of different molecules. From an application point of view, the over-reduced excitation efficiency of the lower order nonlinear process (e.g., the 2P fluorescence) could be an important issue.

To make the coherent control work well in a nontrivial manner, the key point is to reduce the difference between the dependences on the intensity or on the pulse duration of the 2P and 3P processes. This can be achieved by promoting the nonlinearity of the 2P process in the engineered cost function with $x > 1$. For transform-limited pulses with a fixed energy, the integrated 2P signal is proportional to the peak intensity of the excitation light, I, and the integrated 3P signal is proportional to I^2. Then the x value can easily be determined as 2 because the cost function of F_{2P}^2 / F_{3P} would be independent of the intensity of the excitation light. However, if the excitation pulse shape deviates from the FTL shape, the nonlinear relationships of $F_{2P} \propto I$ and $F_{3P} \propto I^2$ cannot remain valid any more due to the destructive interference between different quantum paths. Therefore, predetermination of the

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best value of x for an arbitrary pulse shape is difficult. This problem can be solved by gradually increasing the x value until the optimal outcome is obtained. It is clearly shown that the large x values lead to the high peak intensities in the shaped pulses to maintain high excitation efficiencies for the 2P fluorescence, but we are still able to reduce the 3P fluorescence to a certain level. For example, in the case of $x = 3$, we achieved a ninefold-enhanced 2P/3P ratio with the 2P fluorescence only reduced by half. This result could be more suitable for practical bio-imaging application than that obtained by the use of $x = 1$, because in many cases, this 50% loss of GFP fluorescence is still acceptable. Moreover, in the case of $x = 6$, we found that the 2P fluorescence was only slightly decreased, whereas the 3P fluorescence was reduced nearly by half. Although clarification of the photochemistry of the result requires further investigation, it could be explained by reshaping in which spectral components most likely to undergo 3P absorption are thrown into the wings of what is, more or less, still a transform limited pulse.

It is noteworthy that in the practical fluorescence imaging of a living sample, strongly focused laser beams will produce significant quantities of third harmonic generation (THG) at interfaces, e.g., at the thin membranes of the cells [14]. In this case, linear absorption of 266nm light could easily be the dominate phototoxic mechanism. However, this THG can also be suppressed by the coherent control technique mentioned above. In fact, the fluorescence emission of Tryptophan, which is used for the feedback signal, could be produced from both the 3P absorption of IR light and the 1P absorption of the third harmonic light. When we suppress the fluorescence emission of Tryptophan at 350nm, we actually reduce both the linear and nonlinear absorption processes, and in turn the phototoxicity caused by them.

Finally, we expect that for some molecules with intermediate energy levels within the excitation laser spectrum, a properly selected value of x might give more interesting results due to resonant multiphoton transitions [15].

5. Conclusion

To conclude, we have shown that 2P and 3P fluorescence could be selectively excited by the use of shaped pulses. A trade-off between the fluorescence contrast and the 2P fluorescence excitation efficiency has been achieved by engineering cost functions with “artificially” increased nonlinearity of the 2P fluorescence. The shaped pulses could be useful for reducing the damage of biosamples in 2P fluorescence microscopy. Furthermore, our strategy for engineering the cost function would also be useful in the coherent control of photophysical and photochemical processes with other different nonlinearities, such as 2P and 4P, 3P and 4P, and so forth.

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