Ultrafast pulse-pair control in multiphoton fluorescence laser-scanning microscopy

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Abstract. In multiphoton fluorescence laser-scanning microscopy, ultrafast laser pulses [i.e., light pulses having pulse width ⩽1 ps (1 ps=10^{-12} s)] are commonly employed to circumvent the low-multiphoton absorption cross-sections of common fluorophores. Because of the broad overlapping two-photon absorption spectra of fluorophores and the large spectral bandwidth of a short pulse, simultaneous excitation of many fluorophores is common, which justifies a persistent demand for selective excitation of individual fluorophores. We describe the use of pulse-pair excitation with possibilities of controlling molecular fluorescence in laser-scanning microscopy and compare it with coherent control using pulse sequence [De and Goswami, "Coherent control in multiphoton fluorescence imaging," *Proc. SPIE* **7183**, 71832B (2009)]. © *2009 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.3268440]

1 Introduction

Coherent control or quantum control refers to controlling light-matter interactions using phase-coherence of the laser.[1–3] Early attempts in frequency domain control by precise excitation of a single vibrational mode using near-monochromatic continuous wave lasers were largely doomed by the fact that rapid energy dissipation among other vibrational modes (known as "intramolecular vibrational-energy redistribution") results in loss of coherence. However, owing to the fleeting existence, ultrashort laser pulses can launch coherent vibrational wave packets because the vibrational time period is much longer than the temporal width of the pulse. Ultrafast laser pulses are generated by constructive interference among different longitudinal optical modes sustained by the laser cavity, which is known as "mode-locking".[4–6] This results in the generation of a train of laser pulses with a repetition rate equal to the inverse of the round-trip time of a pulse within the laser cavity. Femtosecond (1 fs=10^{-15} s) laser pulses have been shown to probe the vibrational "dephasing" (i.e., intramolecular loss of coherence) in real time by precise tuning of time delay between a pair of pulses; this is known as pump-probe spectroscopy and has been pioneered by the research groups of Shank, Mathies, Hochrasser, Fleming, and Zewail, to name a few.[7] Control over the time delay between pump and probe pulses imparts control over the time evolution of the wave packet, and this is known as the pump-pump (or pump-dump) scheme of quantum control, originally proposed by Tannor, Kosloff, and Rice.[8] A somewhat different but equivalent control scheme, proposed by Brumer and Sha-piro, exploits the idea of controlling the phase of different optical frequency components (or laser modes) within a pulse and thereby achieving the control by quantum interference between multiple light-matter interaction paths.[9] This is achieved by "laser pulse shaping," which makes use of a grating-spherical lens (or a grating-concave mirror to eliminate chromatic aberration) combination for maximum spatial separation of the spectral components in a plane (known as the "Fourier plane") and subsequent recombination by using another grating-spherical lens combination. A spatial light modulator (SLM) kept at the Fourier plane can modulate the phases (and/or amplitudes) of various frequency components and thereby giving a pulse shaped in time owing to the inverse Fourier relationship between the time and frequency components of a mode-locked pulse.[10] By using a programmable SLM, one can generate pulses of various shapes and attain the desired pulse shape calculated by iteratively solving the time-dependent Schrodinger equation as described by the "optimal control theory"[11] or iteratively search for the optimal pulse shape by using learning algorithms, where a series of experiments are carried out in a feedback loop until the optimal condition is reached.[12] For a detailed discussion on quantum control schemes by laser pulse-shaping, see Ref. 13.

Because mode-locked ultrafast lasers are used in multiphoton fluorescence microscopy,[14] to make use of the gigantic instantaneous peak power for circumventing low-multiphoton absorption cross sections of common fluorophores, all the above-mentioned methods of coherent control have exciting applications in imaging with laser pulses in numerous ways. In this paper, we present our recent work, showing possible control by ultrafast pulse-pair excitation with applications in multiphoton imaging. Starting with a discussion on the valid-

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ity of using highly repetitive pulse train excitation for studying ultrafst dynamics in real time, we demonstrate selective chromophore excitation exploiting dynamics beyond coherence time-scale with comparison to control scheme that exploits coherent dynamics.

2 Methodologies

In our experiment, the laser system was a mode-locked Ti:saph laser (Mira900-F pumped by Verdi5, Coherent) producing femtosecond laser pulse trains at a 76 MHz repetition rate having tunability in the range of 720–980 nm. We used ~120 fs pulsed excitation centered on 750 nm. The laser beam was separated in two parts by using a beamsplitter and recombined using another beamsplitter after passing the two split beams over almost equal distances. One of the beams was passed through a retroreflecting mirror mounted on a mechanical stage (UE1724SR driven by ESP300, Newport) and the other through a fixed retroreflecting mirror. The delay stage was interfaced with a personal computer using a GPIB card (National Instruments). The collinearly propagating beams were sent to a multiphoton-ready confocal microscope system (FV300 scan-head coupled with IX71 inverted microscope, Olympus). The average power of each beam entering the scan-head was ~10 mW. For imaging purposes, slides of bovine pulmonary artery endothelial (BPAE) cells were purchased from Molecular Probes Inc. (F36924 and F14781), and all images were taken using an oil-immersion objective (UPlanApoN 40X 1.4 NA, Olympus). The image acquisition and intensity counts were performed using FLUOVIEW software.

3 Results and Discussions

Several research groups have shown the applications of control schemes in various nonlinear imaging, e.g., multiphoton fluorescence microscopy[15–17] and coherent anti-Stokes Raman scattering microscopy.[18] All efforts are based on laser pulse shaping, where each pulse is shaped in phase (and/or intensity). At an equivalent control can be achieved by simple pulse-pair excitation with controlled time delay between phase-locked pulses (discussed later). Now, both low-energy (~1 nJ) pulsed excitation from a high (10–100 MHz) repetition rate (HRR) laser as well as high energy (1 μJ to 1 mJ) pulsed excitation using low (1–10 kHz) repetition rate (LRR) amplified sources have been implemented in microscopy. In laser-scanning microscopy (LSM), the image acquisition is achieved by point-by-point illumination resulting in pixilated-image construction and LRR systems are incompatible with the fast pixel integration times needed in the imaging process. Thus, fast scanning microscopes are equipped with HRR lasers, whereas slower stage-scanning systems are accompanied by LRR laser sources. In high-speed LSM, the high scanning speed (obtained by scanning with a pair of mirrors or "Nipkow disk") ensures laser dwell time on each pixel over very small time window ($\leq 10 \mu$s); therefore, each pixel is illuminated by $\leq 100–1,000$ low-energy laser pulses from an oscillator. Also, the radiative deexcitation of an excited electronic state (i.e., fluorescence) is nearly complete within the time delay between two successive pulses (~10–100 ns) of a typical HRR laser, making it suitable for microscopy applications.
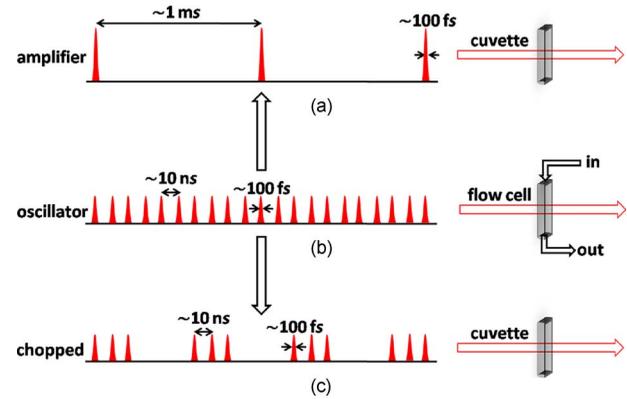
Fig. 1 Different experimental schemes to remove the pileup effect of pulse train: (a) low repetition rate amplified pulse train, (b) high repetition rate pulse train with flowing sample and (c) intensity modulated high repetition pulse train.

Although the use of HRR lasers suffers from light-induced "heating" of the specimen under observation, this is not prominent under fast scanning conditions, as in LSM. We recently showed that, for multiphoton absorption, photothermal effects are manifested by a finite temperature rise resulting from the pileup effect of myriad laser pulses over a finite time window (much longer than temporal separation of pulses) and demonstrated that intensity-modulation of a train of pulses at ~1 kHz frequency results in complete removal of such deleterious effects, leading to significant fluorescence enhancement.[19,20] The results showed that laser-induced photothermal damage is largely governed by transparent solvent and the time scale for pileup effect depends on the heat transfer parameters of the solvent. These findings are crucial for live cell imaging, where photodamage can largely affect the viability of live specimens. This is precisely the reason why measurement of nonlinear absorption coefficients by the z-scan method using an intensity-modulated highly repetitive pulse train yields similar results using amplified low-repetition pulses.[21] Despite having no pileup effect as imparted by HRR lasers, amplified lasers suffer from poor signal-to-noise ratio and pulse-to-pulse carrier-envelope phase fluctuations. Also, the very high pulse energy ($\geq 1 \mu$J) results in pulse-saturation effects when tightly focused with a high numerical aperture objective. An alternative method is to use HRR laser excitation with the sample solution kept in a flowing condition. Another possibility is to use an intensity-modulated chopped excitation. Figure 1 shows a comparison among these different methods; the last two methods result directly from the fact that pileup effect is a long-time effect. An exactly similar condition occurs in laser scanning because each pixel is illuminated for a time period that is not sufficient for building up the pileup effect. In a flow cell, the molecules are rapidly swept across the focused laser beam fixed in space, whereas in laser scanning, a pair of scanning mirrors quickly switches the focused laser beam among spatially frozen molecules located at different regions in the sample. Thus, HRR lasers cause minimal photothermal damage in LSM; this allows us to perform ultrafast real-time experiments with HRR lasers under laser scanning.
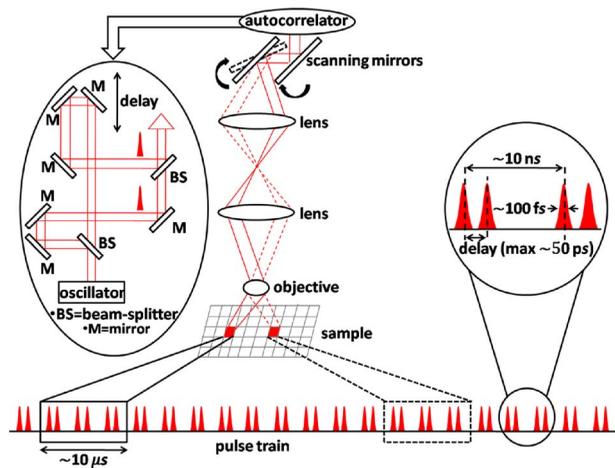
Fig. 2 Schematic of pulse-pair excitation in laser-scanning microscopy: the collinear interferrometric setup is shown in the left inset. The pair of scanning mirrors steer the laser beam to different points of the sample corresponding to different pixels in the image; two such beam paths are shown as solid and dotted lines. The exposure time on each pixel is nearly 10 μs, during which a train of pulse pairs (with one pulse precisely delayed with respect to the other, as zoomed within the right inset) illuminate each pixel of the sample.

With this logic of using HRR laser in LSM, we split each pulse of the pulse train into two pulses and delayed one pulse with respect to the other just as in pump-probe spectroscopy. As sketched in Fig. 2, the sample was illuminated by a "degenerate" (i.e., having same spectral content), isoenergetic and copropagating train of pulse pairs. The delay between the pairs was varied up to 50 ps (corresponding to the maximum possible temporal delay given by the motorized delay line), which is much shorter than the time lapse (13 ns) between the successive laser pulses (which is fixed by the laser cavity round-trip time). This means that instead of ~1000 pulses, each pixel in an image now results from excitation by ~1000 pairs of pulses (or "double pulses"). Because the duration of exposure was ~10 μs on each pixel, this took ~2.6 s to scan a whole area of 512×512 pixels. We collected series of images (each of 512×512 pixels) for different time delays between the pulse pairs. Figures 3(a) and 3(b) show the oscillatory time response of fluorescence (calculated from the intensity counts of the entire image) from two different samples used. Because at a short time delay, artifacts due to field-field correlation including spatial fringe ("coherent artifacts") appear due to interference between two Gaussian light wave-packets, the intensity values were normalized to that at 1 ps, where no spatial artifacts are seen; a temporal convolution of the two pulses at the sample produces third-order ($\chi^{(3)}$) interferometric autocorrelation trace. For one sample [F36924, cf. Fig. 3(a)], the time evolution of fluorescence shows little difference for the three different fluorophores (DAPI, Alexa Fluor 488 phalloidin and MitoTracker Red CMXRos; after correcting for "spectral bleeding" of DAPI into the green region); this is reflected in the corresponding images obtained at two different pulse-pair intervals as shown in Fig. 4. However, for the other sample [F14781, cf. Fig. 3(b)], a significant different temporal response of fluorescence was observed for one fluorophore (DAPI) as compared to the
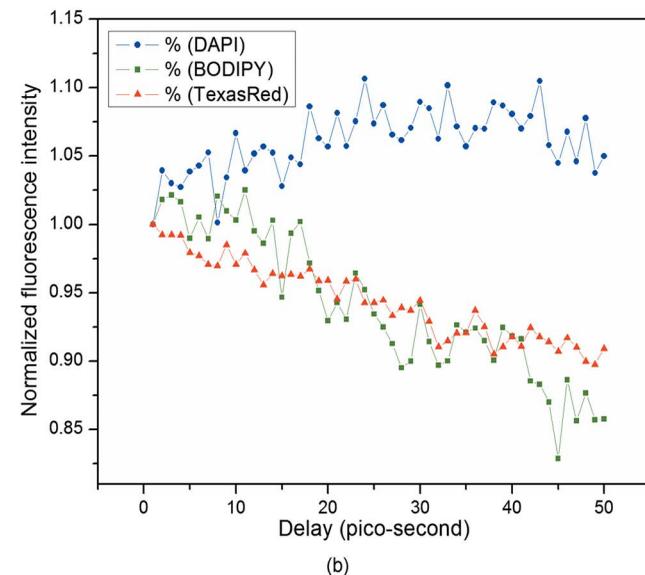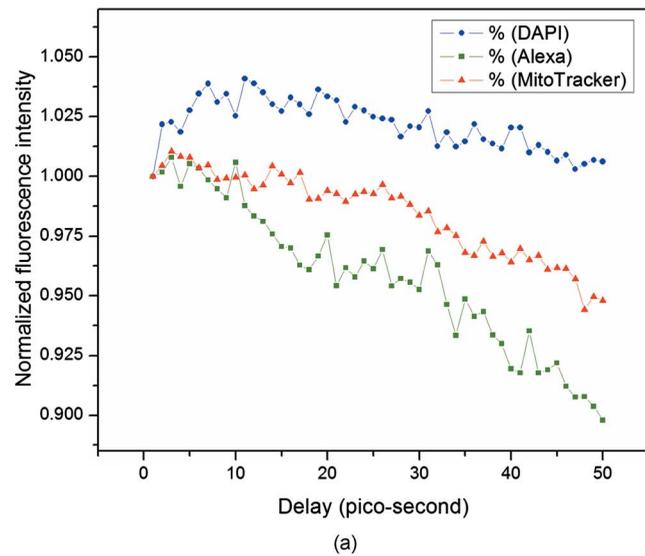
Fig. 3 Variation of fluorescence intensity with delay between the pulse pairs (normalized to that at 1-ps delay between the pulse pairs). The relative fluorescence intensity of (a) DAPI (blue circles), Alexa Fluor 488 phalloidin (green squares) and MitoTracker Red CMXRos (red triangles), and (b) DAPI (blue circles), BODIPY FL goat antimouse IgG antibody (green squares) and Texas Red-X phalloidin (red triangles) is modulated with the delay. (Color online only.)

other two (BODIPY FL goat antimouse IgG antibody and Texas Red-X phalloidin; this time also correcting for "spectral bleeding"); at 45 ps delay, the green and red fluorescence drops to 80% of that at 1 ps delay while the blue fluorescence is indeed slightly enhanced. The corresponding images, shown in Fig. 5, reveal the contrast arising due to this 20% fluorescence suppression, although this does not impart significant contrast, which demands further experiments with the pulse-pairs, as explained below.

Selective excitation of fluorophores has been demonstrated earlier with shaped ultrafast laser pulses[22] with applications to microscopy.[15–17] However, to the best of our knowledge, this is the first demonstration of pulse-pair control in microscopy
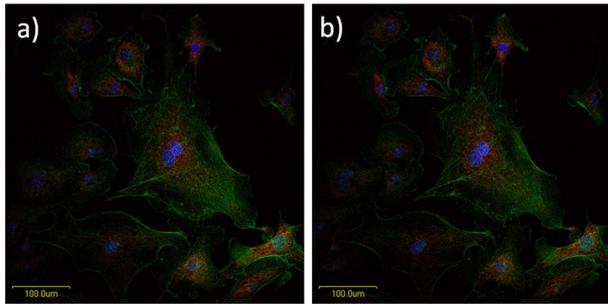
Fig. 4 Images of BPAE cells (F36924) showing nuclei stained with DAPI, F-actin stained with Alexa and mitochondria stained with MitoTracker at (a) 1- and (b) 45-ps pulse-pair delay [cf. Fig. 3(a)].

Fig. 6 (a) Schematic of fluorescence generation (shown as green) followed by a pulse-pair excitation (red line): the two wave packets in the excited state interfere to modulate the fluorescence signal. (b) The relative pulse sequence and detection window is shown. (Color online only.)

with possibilities of controlling molecular fluorescence in microscopy using rather simple pulse-pair excitation. Now, to explain time-domain quantum control using phase-locked pulse-sequence, consider Fig. 6. Figure 6(a) shows that the first pulse triggers a two-photon absorption launching the population in higher vibrational levels of an electronically excited state (S_n). The time-delayed second pulse also executes the same excitation. The precise time delay of these two phase-locked pulses leads to modulation of the spontaneous emission (fluorescence) signal detected after a certain time; this is known as phase-locked spontaneous light emission (PLSLE[23,24]), as depicted in Fig. 6(b). Because these two wave packets interfere in the excited state, a constructive interference leads to enhanced fluorescence while a destructive interference leads to a decrease. The time delay between the pulse pairs controls the interference pattern. Thus, the fluorescence signal, detected after this wave-packet interference is over, is modulated with the delay between the pulse pairs. Now, for different fluorophores, the time delay for the wave-packet revival is different, which may manipulate the spontaneous emission channel for one particular fluorophore in the presence of the others. Because, at a specific pulse-pair delay, the fluorescence signal from different fluorophores are enhanced or suppressed to different extent, this can be successfully extended to selective visualization of a particular organelle inside a live cell. This is conceptually similar to laser pulse shaping in that a pair of pulses or many pulses also modulates the spectrum of a single pulse owing to Fourier transform; the spectral modulation leads to quantum interfer-

Fig. 5 Images of BPAE cells (F14781) showing nuclei stained with DAPI, α-tubulin stained with BODIPY and F-actin stained with Texas Red at (a) 1- and (b) 45-ps pulse pair delay [cf. Fig. 3(b)]. (Color online only.)
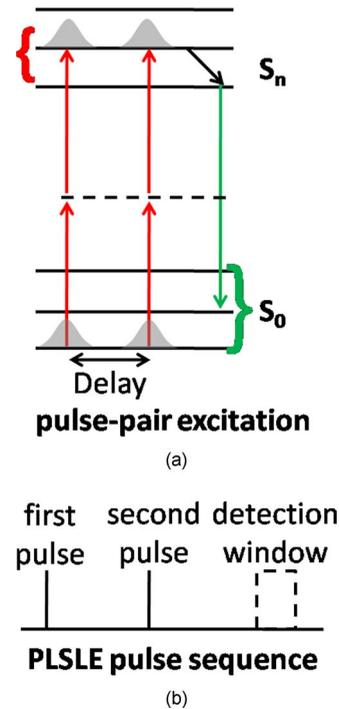
ence while in pulse shaping the interference is caused by the time delay of various spectral component within the pulse. In contrast, our experiment was based on pulse-pair excitation lacking coherent oscillations due to rapid loss (within ~1 ps) of vibrational coherence in condensed phase; for the second pulse there are several competitive pathways, e.g., two-photon absorption, excited state absorption, stimulated emission (discussed in the next section), etc., depending on the fluorophores. Although in the present case the relative suppression of fluorescence the fluorophores is not significant, for a particular choice of chromophores this may have interesting effects. Now, the crucial criterion to achieve this is precise time-delay between the pulse pairs, which is nicely maintained by the delay lines but, for robust and finer temporal delay as well as rapid data acquisition needs, one must use better methods (e.g. using acousto-optic modulators[25]) which are presently being studied in our group.

Dyba et al.[26] have earlier demonstrated ultrafast dynamics of relaxation in microscopy using pulse-pair excitation from an HRR laser; they have shown how stimulated emission can be used to deplete fluorescence of a particular chromophore, revealing the fast vibration, solvation, and orientation dynamics and furnishing useful visual information on local environments. The relative suppression of fluorescence for MitoTracker and Texas Red compared with other dyes may be due to the extension of red edge fluorescence within excitation wavelength. Note that this stimulated emission depletion (STED) microscopy has revolutionized optical microscopy by breaking the "diffraction limit," leading to possible subwavelength-scale spatial resolution.[27] The above-

mentioned work by Dyba et al.[26] has also revealed spatially localized distinct features of ultrafast relaxation of fluorescent dyes in different environments. Interestingly, a careful observation of the series of overlay images at different pulse-pair delay shows that these oscillations occur with different extents at different regions of space revealing the inhomogeneous nature of the surroundings that the fluorophores experience. This is also evident from different fluorescence response patterns for the same dye (DAPI) in two different environments.

4 Conclusions

Thus, we show how pulse-pair excitation, instead of usual pulse train excitation, can be directly implemented in multiphoton fluorescence laser-scanning microscopy to impart control via selective excitation. Further research in the field of coherent control in microscopy using phase-locked laser pulses to have selective fluorophore excitation is currently being pursued in the authors' laboratory.

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