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Abstract. Controlling two-photon molecular fluorescence leading to selective fluorophore excitation has been a long sought after goal in fluorescence microscopy. In this letter, we thoroughly explore selective fluorescence suppression through simultaneous two-photon absorption by two different fluorophores followed by selective one-photon stimulated emission for one particular fluorophore. We achieve this by precisely controlling the time delay between two identical ultrafast near infrared laser pulses. © *2011 Society of Photo-Optical Instrumentation Engineers (SPIE)*. [DOI: 10.1117/1.3645082]

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1 Introduction

Selective enhancement or suppression of fluorescence is important in two-photon fluorescence (TPF) microscopy where the broad spectral window of an ultrafast laser pulse and the overlapping multiphoton absorption spectra of common fluorophores lead to simultaneous excitation of many different fluorophores. Quantum control methods based on ultrafast laser pulse-shaping[1] have been shown to discriminate between nearly identical fluorophores[2–4] with applications in microscopy.[3–8] Precise control over interpulse delay and phase in pulse-pair[9,10] (or pulse-train[11]) excitation can manipulate excited state population (and hence the spontaneous emission, i.e., fluorescence) through (coherent) quantum interference. This has been recently demonstrated for solution phase fluorophore discrimination by us.[12]

Pulse-pair excitation can also lead to selective fluorophore excitation by manipulating the excited state photophysics, i.e. (incoherent) population dynamics, where the only "control knob" is the time delay between the pairs. Earlier our group reported selective TPF suppression using a pulse-pair excitation scheme[13] which was explained based on selective stimulated emission by a time-delayed second pulse following the excitation pulse;[14] the control is achieved by simultaneous two-photon absorption (TPA) by two different fluorophores followed by selective one-photon stimulated emission for one particular fluorophore. Here we further explore the mechanistic detail of such one-color control scheme in depth. Recently we have also demonstrated selective fluorescence suppression by pulse-train excitation where, instead of two time-delayed identical pulses, many pulses with gradually decreasing pulse intensities (in geometric progression) having a controllable delay between successive pulses, were employed;[15] here the first pulse leads to TPA while one-photon stimulated emission takes over TPA for successive pulses.

2 Methodologies

Mode locked ~180 fs pulses centered on ~730 nm at 76 MHz repetition rate from a Ti:sapphire oscillator (Mira900-F pumped by Verdi5, Coherent, Santa Clara, California) were split and recombined in a Mach–Zehnder interferometer coupled to the confocal-ready multiphoton microscope system (FV300 scan-head coupled with IX71 inverted microscope, Olympus, Japan); we used an oil-immersion objective (UPlanApoN 40×1.4 NA, Olympus) for imaging. Precise delay steps in one of the two arms of the interferometer were introduced by a motorized stage (UE1724SR driven by ESP300, Newport, Irvine, California) interfaced with a personal computer through a GPIB card (National Instruments, Austin, Texas). The delay between a pulse-pair was varied and for each delay a two-dimensional image was collected by scanning the diffraction-limited focal spot across the focal plane with a pair of galvo-scanning mirrors. Specimen slides of bovine pulmonary artery endothelial cells having nuclei stained with DAPI and F-actin stained with Mito Tracker Red CMX Ros (F14781, Molecular Probes, Invitrogen, Carlsbad, California) were used. The scanning and image analysis were performed using FLUOVIEW software (Olympus, Japan).

3 Results and Discussion

In stimulated emission [Fig. 1(a)], a first pulse (wavelength-tuned to one-photon absorption maximum) launches population from the ground vibrational state of the ground electronic state to excited vibrational manifolds of an excited electronic state; this is followed by a fast (typically ≤1 ps) relaxation to the ground vibrational state of the same electronic state; a time-delayed second pulse (wavelength-tuned to the red-edge of steady-state fluorescence) sends this relaxed population back to the ground electronic state. Thus fluorescence is decreased if one monitors the backscattered fluorescence (or "epi-fluorescence") but enhanced while monitoring the forward-scattered fluorescence (or "trans-fluorescence"); the former method is employed in techniques that use the suppression of fluorescence, e.g., STED microscopy[16] or ultrafast dynamical microscopy,[17] while the latter has been recently implemented to increase the fluorescence gain from very weakly fluorescing molecules, thereby making them suitable candidates for imaging.[18]

From the preceding discussion it is evident that pulses of two different colors are required for any fluorescence microscopy

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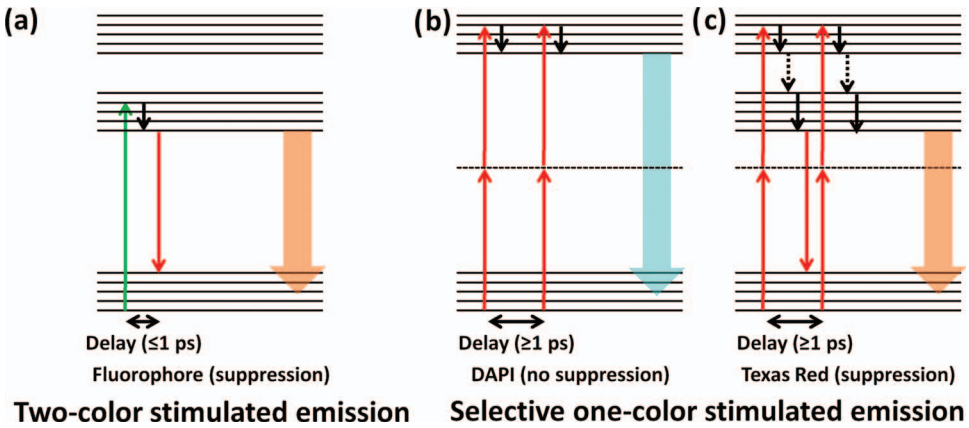

Fig. 1 Schematic of fluorescence suppression by stimulated emission in conventional two-color scheme (a) and one-color scheme discussed in this letter [(b) and (c)]. The excitation and stimulated emission are shown as upward and downward thin arrows, respectively, while fluorescence is shown as a broad downward arrow, which is at wavelengths corresponding to orange color for (a) and (c) and blue color for (b). In (a), thin upward arrow for electronic transition represent 532 nm, all other thin arrows [(a), (b), or (c)] representing electronic transitions are at 800 nm. Small downward black arrows within the vibrational manifold of the electronic states indicate either vibrational relaxation (solid) or internal conversion (broken). The colors of electronic excitation arrows are chosen to specify the different wavelengths.

method exploiting stimulated emission, as the excitation wavelength is much smaller than that of red-edge fluorescence. However, in multiphoton fluorescence microscopy the excitation wavelength is longer than the emission wavelength as energies of two (or more) photons are added up to cause an excitation; in TPF, two near-infrared photons are absorbed, energetically equivalent to absorption in the ultraviolet-visible region, followed by emission in the visible region. Thus, for a fluorophore whose emission tail happens to wavelength-match the excitation wavelength, we can selectively suppress the fluorescence in presence of other fluorophores using light pulses with just one color. However, one necessary condition for this to bring about is that the various fluorophores must be excited together at that wavelength; this is quite common in multiphoton fluorescence microscopy as the TPA spectra of commonly employed fluorophores are broad, hence overlapping, and the ultrafast laser pulse (required to circumvent the low multiphoton absorption cross-sections) itself has a broad spectral content. Note that two-color STED microscopy using TPA has already been employed.[19]

The photophysics for the fluorophore pairs (DAPI and Texas Red) under pulse-pair excitation is schematically shown in Figs. 1(b) and 1(c). The lower electronic excited state (or S_1 state) for each of these dyes may be assigned from one-photon absorption spectra and the ground vibrational state of this electronic state turns out to be the fluorescing state as evident from overlapping absorption fluorescence spectra.[20] For DAPI the S_1 state has a broad absorption profile (maximum ~360 nm) and TPA at ~730 nm launches the population to this state; the blue fluorescence (maximum ~460 nm) does not extend up to the excitation profile. Thus the two pulses execute the same TPA for DAPI and fluorescence yield must be independent on the delay between the pulse-pairs. In contrast, for Texas Red the S_1 state has a sharp absorption profile (maximum ~580 nm) and TPA at ~730 nm excites the population to a higher electronic excited state ($S_{n>1}$) which relaxes to the S_1 state; moreover, the red fluorescence (maximum ~620 nm) extends up to ~750 nm and overlaps with the excitation profile (maximum ~730 nm). Thus although the first pulse executes only TPA, the second pulse executes TPA as well as stimulated emission and thus fluorescence

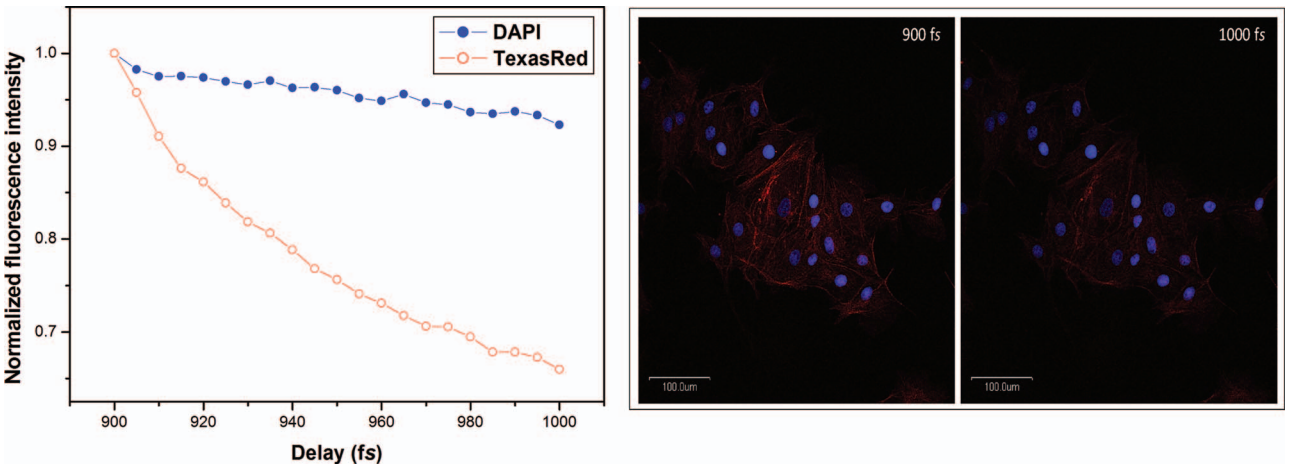

Fig. 2 Selective two-photon fluorescence suppression for Texas Red (open circle) compared with DAPI (solid circle) under pulse-pair excitation.

yield depends on the interpulse delay. Note that, unlike pulse-shaping methods, the phase coherence of laser pulse or relative phase between pulses is irrelevant here as this is a control over excited state population and not the coherence.

Now, if the red-edge of the fluorescence very poorly matches the excitation wavelength (as is the case for Mito Tracker Red), pulse-train excitation with decreasing intensity for successive pulses turns out to be a superior choice over pulse-pair excitation scheme.[15] To ensure better overlap between the fluorescence and the excitation beam, we tuned the laser to ~730 nm. The field autocorrelation trace measured at the sample position (pulse-width ~180 fs) showed that the two pulse interference zone extends up to ~900 fs. To avoid any "artifact" due to pulse-pulse temporal overlap, we set the minimum pulse-pair delay as 900 fs and zoomed into a 100 fs time window (i.e., 900 to 1 ps) with 5 fs time steps. At 1 ps delay Texas Red fluorescence drops to ~65% of its value at 900 fs delay as shown in Fig. 2 (left panel); the corresponding images are shown in Fig. 2 (right panel).

Note that for Texas Red, since the fluorescing state is populated not only by rapid ($\leq$1 ps) vibrational relaxation within an electronic state but by rather slow (typically $\geq$100 ps) internal conversion between two electronic states also, stimulated emission survives for a longer time scale [Fig. 1(c)] than usually encountered [Fig. 1(a)]. This is precisely the reason for our observation of fluorescence suppression over a long time scale (several tens of picoseconds, corresponding to experimentally maximum accessible time delay accessible with the delay stage[13–15]).

It is evident from the above discussion that in order to enhance the probability of stimulated emission, TPA by the first pulse or/and (one-photon) stimulated emission by the second pulse has to be enhanced. One possibility is to use a short ($\leq$100 fs) transform-limited pulse followed by a longer ($\geq$1 ps) transform-limited or chirped pulse; however, for one-color scheme this does not work due to optical interference as stated above. An alternative choice is to use a linearly polarized $\leq$100 fs pulse followed by a circularly polarized $\leq$100 fs pulse; this has been realized in further experiments (not presented in this letter). Note that a similar technique of using a stretched pulse with circular polarization for enhanced stimulated emission is routinely used in STED microscopy.

Selective one-color stimulated emission is only realized only through multiphoton excitation where simultaneous excitation of fluorophores is almost ubiquitous. Therefore, although this method is certainly limited to the choice of fluorophores (say, DAPI/Texas Red combination), which is also common with its one-photon counterpart (selective two-color stimulated emission[16,17]), the method can be potentially applied to large variety of experiments employing multiphoton fluorescence microscopy. In addition, if the red-edge of the fluorescence matches the excitation wavelength very poorly (as is the case for Mito Tracker Red), pulse-train excitation with decreasing intensity for successive pulses turns out to be a superior choice over pulse-pair excitation scheme.[15]

4 Conclusions

Thus we show how two different fluorophores can be excited simultaneously by two-photon absorption but selectively turned off by stimulated emission for a particular choice of fluorophore pairs. Further improvement of this technique is presently being pursued in the authors' lab.

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