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Fluorescence advantages with microscopic spatiotemporal control

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Abstract

We present a clever design concept of using femtosecond laser pulses in microscopy by selective excitation or de-excitation of one fluorophore over the other overlapping one. Using either a simple pair of femtosecond pulses with variable delay or using a train of laser pulses at 20-50 Giga-Hertz excitation, we show controlled fluorescence excitation or suppression of one of the fluorophores with respect to the other through wave-packet interference, an effect that prevails even after the fluorophore coherence timescale. Such an approach can be used both under the single-photon excitation as well as in the multi-photon excitation conditions resulting in effective higher spatial resolution. Such high spatial resolution advantage with broadband-pulsed excitation is of immense benefit to multi-photon microscopy and can also be an effective detection scheme for trapped nanoparticles with near-infrared light. Such sub-diffraction limit trapping of nanoparticles is challenging and a two-photon fluorescence diagnostics allows a direct observation of a single nanoparticle in a femtosecond high-repetition rate laser trap, which promises new directions to spectroscopy at the single molecule level in solution. The gigantic peak power of femtosecond laser pulses at high repetition rate, even at low average powers, provide huge instantaneous gradient force that most effectively result in a stable optical trap for spatial control at sub-diffraction limit. Such studies have also enabled us to explore simultaneous control of internal and external degrees of freedom that require coupling of various control parameters to result in spatiotemporal control, which promises to be a versatile tool for the microscopic world.

Keywords

Fluorescence imaging; microscopy; femtosecond pulses; spatiotemporal control; two-photon excitation; nanoparticle; multi-photon process

1. INTRODUCTION

Despite poorer resolution as compared to electron microscopes, fluorescence microscopy continues to be the best choice for experimenting with live specimen as the energy deposited

in electron microscopy adversely affects the viability of live specimens. This practical compromise, therefore, begets resolution enhancement as one of the most important developmental aspects of fluorescence microscopy.¹ Implementing multi-photon excitation in laser scanning microscopy for better depth resolution, for example, is an important achievement. However, in such two-photon fluorescence (TPF) microscopy, the broad spectral window of an ultrafast laser pulse and the overlapping multi-photon absorption spectra of common fluorophores lead to simultaneous excitation of many different fluorophores. So, selective enhancement or suppression of fluorescence is important in keeping the resolution of the TPF microscopy. Quantum control approaches using ultrafast laser pulse-shaping² holds the potential to discriminate nearly identical fluorophores³⁻⁵ with applications in microscopy.⁴⁻⁹ Precise control over inter-pulse delay and phase in pulse-pair^{10,11} (or pulse-train¹²) excitation can manipulate excited state population and the corresponding spontaneous emission (and/or fluorescence) through coherent quantum interference. This was earlier demonstrated through solution phase fluorophore discrimination.¹³ On the other hand, pulse-pair fluorophore excitation can also occur by manipulation of the excited state photo-physics, i.e. incoherent population dynamics, where the only “control factor” is the time delay between the pulse pairs. Earlier our group reported selective TPF suppression using a pulse-pair excitation scheme which was explained based on selective stimulated emission by a time-delayed second pulse following the excitation pulse.¹⁴ Control is achieved by simultaneous two-photon absorption (TPA) by two different fluorophores followed by selective one-photon stimulated emission for one particular fluorophore.¹⁵ 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.¹⁶ Here the first pulse leads to TPA while one-photon stimulated emission takes over TPA for successive pulses. We have also explored the effect of different polarization states of the pulses used under such incoherent schemes, wherein their effect, as expected, is only minimal unlike in the coherent case.¹

In addition to the temporal control effect of such highly repetitive pulses, they are also effective in terms of spatial control. Spatial control in terms of optical tweezers or single beam gradient optical traps came in 1986,¹⁷ which makes use of a tightly focused laser beam having a transverse Gaussian intensity profile to trap particles of various sizes ranging from a few micrometers to a few nanometers. The major advantage of laser tweezers over mechanical transducers is the accessible low force range (from 10^{-10} to 10^{-13} N), which makes them more suitable for biological applications.¹⁸ There can be certain further benefits when a pulsed laser is used in place of a continuous wave (CW) laser beam that is more typically used for optical trapping. In fact, we have shown that it is possible to exploit the huge pulse power of ultrafast pulsed lasers with a high repetition rate to efficiently hold tiny Rayleigh particles that are thought to be not easily trappable with CW lasers.¹⁹ Furthermore, pulsed laser tweezers offer clear observation of trapping events through nonlinear fluorescence signatures.²⁰

In terms of “spatiotemporal control” we essentially mean control over the spatial resolution in fluorescence microscopy by controlling temporal spacing between pulses or a control

over spatial manipulation through pulsed laser optical trapping. We discuss here the different facets of these two effective usage of fluorescence manipulation with laser pulses at microscopic level.

2. EXPERIMENT

The laser system used in all our experiments is a mode-locked Ti:sapphire oscillator (Mira900-F pumped by Verdi5, Coherent, Santa Clara, CA, USA) capable of producing femtosecond train of pulses (pulse-width ranging 120-180 fs depending on wavelength) at a high repetition rate (HRR) of 76 MHz centered in the tunable range of 720–980 nm.

2.1 Enhanced Resolution Fluorescence Microscopy

In a two-photon fluorescence laser-scanning microscopy (TPF-LSM), the image acquisition is done with point-by-point illumination with a HRR laser resulting in pixilated image construction. Microscopic images were collected in a confocal-ready multi-photon microscope system (FV300 scan-head coupled with IX71 inverted microscope, Olympus, Japan) using an oil-immersion objective (UPlanApoN 40×1.4 NA, Olympus). The high scanning speed obtained by scanning with a pair of mirrors as in our FV300 microscope system ensures laser dwell time on each pixel over very small time window (~1–10 ms). Thus, although the use of HRR lasers suffers from photo-thermal effect, this is not prominent under fast scanning conditions because each pixel is illuminated for a time period not sufficient for building up the photo-thermal effect. Effectively, in this LSM scheme, a pair of scanning mirrors quickly switches the focused laser beam among “spatially frozen” molecules located at different regions in the sample. Thus, HRR oscillators cause reduced photo-thermal effect in LSM.¹

For pulse-pair excitation, we used a collinear Mach-Zehnder-type interferometer where the laser beam was separated in two parts by using a beam-splitter and was recombined using another beam-splitter after passing the two split beams over almost equal distances. One of the beams was passed through a retro-reflecting mirror mounted on a mechanical stage (UE1724SR driven by ESP300, Newport) and the other through a fixed retro-reflecting mirror. The delay stage was interfaced with a computer controlled GPIB card (National Instruments) to provide precise delay steps. The collinearly propagating beams were sent into our Olympus microscope. Similarly, for the pulse train purposes, we used a Fabry-Perot etalon made from a pair of 50/50 beamsplitters that was 3 mm thick [Castech (Hamilton, New Zealand) BSP-254-030-780]. Due to multiple reflections between the coated surfaces, each pulse generates a pulse train; the intensity of the successive pulses decreases in geometric progression. We mounted one of the beamsplitters on a mechanical stage (UE1724SR driven by ESP300, Newport, Irvine, California). The average power of each beam entering the scan-head was ~10 mW.

The pulse pair as well as the pulse train used in our experiments were correlated individually with a reference pulse in a collinear interferometer setup to get the field autocorrelation traces. The separation between the pulse pair depended on the precise delay from the movable arm of the Mach-Zehnder interferometer. For the pulse train case, when the distance between the two coated surfaces was increased by a factor of two, the time delay

between two consecutive pulses is also doubled. The delay between the successive pulses in a pulse train was varied from 20 ps (corresponding to the minimum possible delay constrained by the physical separation between two mounts holding the beamsplitters) up to 50 ps in 1-ps step size, and we collected an image at every step. For imaging purposes in both the cases, slides of bovine pulmonary artery endothelial (BPAE) cells having nuclei stained with DAPI (blue fluorophore staining nuclei) and Texas Red-X phalloidin (red fluorophore staining mitochondria) or DAPI and F-actin stained with Mito Tracker Red CMX Ros (red fluorophore) were used that are available commercially (Molecular Probes, Invitrogen, Carlsbad, California). At ~730 nm both pairs of these dyes are simultaneously excited through two-photon excitation, which makes these fluorophore labeled samples a perfect choice for our selective excitation experiments. Among these fluorophores, the fluorescence from Texas Red extends up to ~750 nm, which suggests the possibility of its fluorescence suppression through stimulated emission in contrast to the fluorescence from Mito Tracker Red that almost dies out ~750 nm. The image acquisition and intensity counts were performed using FLUOVIEW software (Olympus, Japan).

2.2 Fluorescence Detection in Pulsed Optical Tweezers

We used ~120 fs mode-locked pulsed excitation centered at 780 nm from our Coherent Inc. Ti:sapphire laser that can also operate in a CW mode at the same center wavelength for constructing our optical tweezer. TPF was induced when the laser was operated in the pulsed mode. The expanded laser beam from a telescopic arrangement along with two steering mirrors was sent into a microscope objective (UPLSAPO 1.4 NA 100×O, Olympus Incorporated) of a homemade bench-top inverted microscope through a dichroic mirror placed just before the objective lens. At 780 nm, the dichroic mirror has ~95% reflectance while the objective transmits ~65%, which means ~60% of the incident light gets to the sample. This was confirmed by measuring the laser power with a powermeter (FieldMate, Coherent) as well as a silicon amplified photodiode (PDA100A-EC, Thorlabs) at the sample location. TPF as well as the backscattered light was collected from the dichroic mirror by a photomultiplier tube (PMT) using appropriate bandpass filters. The PMT signal was collected by an automotive oscilloscope (picoscope, Pico Technology) or, alternatively, a lock-in amplifier (SR830 DSP, Stanford Research Systems), triggered by a rotating-disk optical chopper (with a 30 slot wheel) run by a tunable frequency driver (MC1000A, Thorlabs) and operating at 800 Hz. This setup is capable of measuring the TPF as well as the backscattered light to observe trapping of micron size particles as well as tiny nanoparticles in real time. Simultaneously, video microscopy by bright-field illumination as well as dark-field fluorescence generation can also be acquired. We trapped polystyrene latex beads that were as small as 100 nm diameter (F8800, Molecular Probes) and were coated with fluorophore having single-photon absorption maxima at 540 nm and an emission maximum at 560 nm. A dilute and slightly alkaline (pH of ~8) solution of the sample was sonicated extensively and was used immediately for the trapping experiments to avoid aggregation. The 780nm pulsed excitation simultaneously traps as well as generates backscattered (under both pulsed and CW modes) as well as fluorescence signals only under the pulsed mode. For the case of 100 nm diameter, the wavelength of trapping light is nearly eight times larger than the particle diameter, which implies that the particle dimensions are in the Rayleigh regime.

3. RESULTS AND DISCUSSION

3.1 Fluorescence Microscopy

As discussed at the very outset, all our experiments are one-color experiments as they are more amenable with conventional microscopy. This is quite different from the typical pump-probe experiments^{21,22} where two different colors are used: one for pump and the second color for probe (Figure 1). The pump pulse excites a part of the ground state population to the excited state where it rapidly relaxes to the lowest vibrational level of the excited state from where the fluorescence emission occurs. However, if a time-delayed probe pulse that is wavelength-tuned to the red edge of fluorescence sends the population back to the ground electronic state by stimulated emission in competition with the fluorescence process, fluorescence suppression is observed. Fluorescence, being an incoherent emission, is omnidirectional. Stimulated emission, on the other hand, is a coherent process and, as such, photons generated by stimulated emission travel in the direction of the stimulating beam. So, if we collect only the back-scattered fluorescence (epi-fluorescence as in typical confocal microscopy) there is suppression in fluorescence signal as discussed.²¹ However, under the same experimental condition, if one collects the forward scattered photons, a gain in total measured signal was observed due to the additional stimulated emission which could even transform weakly fluorescent molecules into suitable candidates for microscopy applications.²² Our work, however, focuses on experiments that are of practical relevance to TPF-LSM and only restricts to one-color excitation scheme.²³

In our version of one color pulse-pair excitation, we found interesting control strategies that materialized even within a time window of 100 fs as shown in the region of 0.9 ps to 1.0 ps at 5 fs step-size as is seen from the suppression of Texas Red fluorescence in comparison to that of DAPI fluorescence as shown in Figure 3. In this case, the first pulse causes a TPA for both DAPI and Texas Red. The time-delayed second pulse causes similar TPA as in case of the first pulse but, in addition, could dump the population by (one-photon) stimulated emission if the red edge of fluorescence coincides with the excitation wavelength (as is the case for Texas Red) (Figure 2). Thus a distinction is seen between DAPI and Texas.Red. However, similar excitation scheme does not work for DAPI/Mito-tracker Red pair (Figure 4a). However, when pulse train excitation scheme is used, not only the distinction as seen in the pulse-pair scheme persists, even a distinction between DAPI/Mito-tracker Red pair becomes possible at longer delays (Figure 4b). The higher efficiency of pulse-train excitation over pulse-pair excitation may be understood from the fact that there exists a competition between TPA and stimulated emission process in such one-color excitation process. The intensity of successive pulses in a pulse-train decrease in geometric progression, both TPA and stimulated emission is decreased but the former decreases much more rapidly (due to square dependence on intensity) as compared to the latter (which depends linearly on intensity). Thus, the pulse-train excitation leads to better suppression than pulse-pair excitation and a noticeable fluorescence suppression of Mito-tracker Red relative to DAPI is seen (Figures 4), which was not present in the pulse-pair experiment in the 20 ps to 50 ps range. It is important to note in our one-color stimulated emission, TPA excites the red fluorophores to energetically higher electronic states as compared to the conventional two-color counterpart and as such, population relaxation to the state from

which the stimulated emission occur involves not only during the fast (1 ps) vibrational relaxation timescale but also rather slow (100 ps) internal conversion. This explains reason for the observed fluorescence suppression at a long pulse-pair delays of up to several tens of picoseconds in our one-color pulse pair or pulse experiments.²³

3.2 Pulsed Optical Tweezers

Although direct trapping of sub-micron objects, dimensionally, often similar to macromolecules, have been demonstrated using CW laser beam, it demands the use of quite high average powers. In our particular case where the particle dimension is at least an order of magnitude smaller than the wavelength of light, we are in the 'Rayleigh scattering limit' or the 'dipole limit' and under such condition, the trapping force is given by

$$F = \left(\frac{\alpha}{2} \right) E^2 \quad (1)$$

where α is the polarizability and E is the electric field of trapping light. Since the force depends on the polarizability, earlier efforts revealed that much higher power levels are required to stably trap latex nanoparticles.²⁴ In fact, the exertable force for latex nanoparticles is much smaller than for gold nanoparticles, making the former a poor choice for nano-scale applications. Additionally, as the size of the particle reduces, the Brownian motion increases making it harder to be held for any appreciable amount of time.²⁴ TPF detection turns out to be more advantageous than detection based on backscattering as TPF, being confined only within focus, is background-free and any persistent fluorescence signal corresponds only to the trapped particles that can be observed despite the presence of many other out-of-focus particles within the cone of illumination.²⁰ Using TPA detection scheme, we demonstrated direct trapping the 100nm latex (polystyrene) nanoparticles with femtosecond pulsed excitation at a low average power. Stable trap was observed when the average power was elevated to ~30mW as shown in Figure 5. It has also been clearly possible to show trapping of a single particle followed by another one as the relative strength of fluorescence has nearly a 1:2 ratio (after background subtraction), which confirms the number of trapped particles (the spikes with larger heights are due to other particles rapidly diffusing across the focal volume). Since the dimension of the focal volume dimension (diffraction limited focal volume created by ~0.8 mm light) is much larger (nearly three orders of magnitude larger) than the dimension of the particles (0.1 mm), the focus can accommodate many particles at a time. This is the cause of accumulation of particles in the trapping zone. Albeit a low signal-to-noise level, we managed to show similar trapping with CW excitation by observing backscattering signal at much higher average power (~200 mW). We could also extend the pulsed trap with TPA detection further for Q-dots as well, where we observed stable trapping as evident from Figure 5(b), however, no possible trapping signature was detectable for the Q-dots with CW lasers.²⁵

Besides the TPF detection of trapping, the femtosecond laser advantage can be understood from its transitory existence, whereby the femtosecond pulse has enormous instantaneous peak power compared to its time averaged power, a typical ratio being 10⁵:1. This high photon flux increases the trap stiffness (i.e., force constant) to such an extent that a stable trap is observed at the same average power level of CW lasers that cannot trap nanoparticles.

When the particle dimension is at least an order of magnitude larger than the wavelength of light (i.e., “Mie scattering limit”), Brownian motion is sluggish and a shallow potential well created by a CW or time-averaged pulsed laser is good enough to trap the nanoparticles. In contrast, a rapidly moving Rayleigh particle cannot be trapped with a shallow force field, but it can be efficiently trapped when the well depth is steep, provided the particle does not diffuse away within the time period of the periodic force field. Thus the pulse repetition rate turns out to be a crucial factor for stably trapping Rayleigh particles. The 13.6 ns time lag between two consecutive pulses (coming from the 76 MHz repetition rate) is too short for the already trapped particle to leave the trapping region.^{19,25}

4. CONCLUSION

We have shown how clever use of relative delay between pulses in pulsed laser illumination can be utilized for resolution enhancement in fluorescence microscopy. Our experiments on pulse-pair and pulse-train control mediated by stimulated emission have interesting applications in microscopy. The control method is based on competition between fluorescence and stimulated emission and has been demonstrated for fluorophore discrimination. We have also demonstrated the role of gigantic instantaneous peak power of an ultrafast pulse excitation (at low average power) to efficiently trap objects of macromolecular dimension, which can be efficiently detected with the two-photon fluorescence technique.

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Figure 1.

Excitation scheme for commonly employed two-color pump-probe studies involving stimulated emission. The excitation and stimulated emission are shown as upward and downward thin arrows, respectively, while fluorescence is shown as broad downward arrow. Small black arrows pointing downwards indicate vibrational relaxation.

Figure 2.

Excitation scheme in our one-color pulse-pair and pulse-train methods described here (a)–(c). The excitation and stimulated emission are shown as upward and downward thin arrows, respectively, while fluorescence is shown as broad downward arrow; the colors are chosen to specify the different wavelengths. Little downward black arrows indicate either vibrational relaxation (solid) or internal conversion (broken).

Figure 3.

Change in the relative fluorescence intensity of (a) DAPI versus Texas Red and (b) DAPI versus Mito-tracker Red under one-color pulse-pair excitation. (c) Corresponding fluorescence suppression image of Texas Red relative to DAPI as a function of time delay between pulses under one-color pulse-pair excitation (similar suppression results for pulse train for this dye pair).

Figure 4.

Comparing the effect of pulse train with pulse pair: Change in the relative fluorescence intensity of DAPI versus Mito-tracker Red under one-color (a) pulse-pair excitation and (b) pulse train excitation. (c) Corresponding fluorescence suppression image of Mito-tracker Red relative to DAPI as a function of time delay between the trains of pulse under one-color excitation (no suppression for pulse pair excitation for this dye pair).

Figure 5.

Two-photon fluorescence for trapping of (a) 100nm particles and (b) 16 nm Q-dots. Inset in (a) shows the fluorescence from the 100 nm particle against a complete dark background. The red line in the both the panels is a guideline for the trapped fluorescence. The step in (a) represents the sequential trapping of two particles.