J Fluoresc (2009) 19:931–937
DOI 10.1007/s10895-009-0489-4

RAPID COMMUNICATION

A Systematic Study on Fluorescence Enhancement under Single-photon Pulsed Illumination

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Received: 21 September 2008 / Accepted: 3 April 2009 / Published online: 9 June 2009
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Abstract We present a detailed study on fluorescence enhancement by 'stroboscopic' illumination with light pulses having duration ranging from few milliseconds to sub-picoseconds. We show how a delicate balance between pulse width and pulse repetition rate can result in an unprecedented fluorescence enhancement that has immediate applications in fluorescence imaging.

Keywords Fluorescence enhancement · Reduced photo-bleaching · Photo-damage

Introduction

Nearly for a century fluorescence microscopy has been an indispensable tool for bio-imaging having applications ranging from observation of embryonic development to dynamic tracking of molecular motors within a live cell. This is based on the radiative relaxation (*i.e.* fluorescence) of an electronically excited state of a chromophore (more specifically 'fluorophore') inside a fluorescent molecule. Generally the light sources used in fluorescence microscopy are either xenon/mercury lamps for ordinary dark field fluorescence microscopes or lasers as in (confocal) fluorescence laser-scanning microscopes (LSMs). Recently light-emitting diodes (LEDs) have also been used as inexpensive light sources with broad range of wavelengths [1]. In all cases the illumination is continuous which imparts several deleterious effects on the fluorescence yield. Prolonged excitation may lead to irreversible photo-damage of the fluorophores. Also continuous exposure to light waves generates heat within the sample, which is dangerous for the viability of a living specimen. An obvious choice is to use pulsed illumination characterized by two parameters: the pulse width *i.e.* the duration of a light pulse, and the pulse repetition rate *i.e.* inverse of the time lag between two consecutive pulses. These two parameters dictate how the instantaneous pulse energy and power varies for a given time-averaged power. As opposed to continuous wave (CW) illumination, shorter pulse width imparts reduced illumination time and the finite time lag between two pulses make sure that all excited molecules non-radiatively relax to their ground states via non-rediative intra- (*e.g.* internal conversion, intersystem crossing etc.) and inter-molecular (*i.e.* conduction and convection) as well as rediative (*i.e.* flourescence and phosphorecence) de-excitation processes. Pulsed xenon lamps were shown to lessen photo-damage compared to continuous illumination due to reduced heating of the sample [2]. Millisecond pulsed illumination by LEDs have been reported to reduce photo-bleaching (as well as phototoxicity) [3, 4]. Photo-bleaching of Rhodamine 6G under CW and ultrashort pulsed excitation has been studied and explained by simple kinetic models by several groups [see ref [5] and the references therein]. Pulsed illumination is routinely used in multi-photon fluorescence microscopy [6, 7] but we restrict ourselves only within one-photon absorption studies. Here we describe a thorough investigation on fluorescence enhancement under one-photon pulsed excitation with pulse widths ranging from few milliseconds to hundreds of femtoseconds. We show that an optimum choice of pulse width and pulse repetition rates (as well as wavelength of excitation) results in dramatic enhancement in fluorescence intensity.

Experiments

The schematic of the experimental set-up is shown in Fig. 1. $10^{-4} M$ methanolic solution of the laser dye

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rhodamine 6G (R6G) was used for fluorescence generation. The laser light was focused onto a fluorescence quartz cell (1 c*m*×1 c*m*) containing the sample solution with a 0.5 numerical aperture (NA) microscope objective (20X, Olympus) and the fluorescence were collected at a perpendicular direction with a lens (of 5 c*m* focal length). This lens focused the fluorescence onto the tip of a multi-mode fiber-optic cable connected to a spectrometer (HR2000, Ocean Optics). The fluorescence spectral data were acquired by a computer using LabVIEW programming. As shown in Fig. 1(a), we chopped the continuous-wave (CW) green (532 n*m*) laser beam from a Nd:vanadate laser (Verdi5, Coherent). For chopping the excitation beam with 50% duty cycle at 1 k*Hz*, a rotating-disk optical chopper (30 slot, Thorlabs), having 1:1 mark/space ratio and triggered by a tunable frequency driver (MC1000A, Thorlabs), was introduced in the excitation path. For similar chopping at 1 M*Hz*, an electro-optic amplitude modulator (4101, New Focus), driven by a fixed frequency driver (3363, New Focus), was used keeping a Glan-Taylor prism polarizer next to it. For nano-second pulsed excitation, a Q-switched Nd:YLF laser (Corona, Coherent) producing 120 n*s* pulses at 532 n*m* was used (not shown in the figure); the pulse repetition rate was tuned to two different frequencies (1 and 10 k*Hz*). To get nano-second pulses at much higher repetition rate we modulated the CW output from the Nd:vanadate laser by an acousto-optic deflector (TECD-250-65-BR-1560, Brimrose) connected to a fixed frequency driver (FFJ-250-B3-F1.5, Brimrose). Figure 1(b) depicts the generation of 150 n*s* pulses at 532 n*m* having pulse repetition rate of 500 M*Hz* using the acousto-optic deflector; the modulated beam (*i.e.* the first-order diffracted beam) of the CW Nd:vanadate laser was collected by an iris. Fluorescence generation with sub-picosecond pulsed excitation is shown in Fig. 1(c); we used the second harmonic of the output from a mode-locked Ti:saph laser (Mira900-F pumped by the same CW Nd:vanadate laser) producing ~100 f*s* (measured by intensity auto-correlation technique) pulsed excitation centered on 780 n*m* at 76 M*Hz* repetition rate. We frequency-doubled the fundamental output by a second-harmonic generation (SHG) crystal of type-1 *beta*-barium borate (7 m*m*×7 m*m*×0.3 m*m*, Q136/02-01-ID, Newsphotons). After focusing the light onto the SHG crystal the resulted pulsed beam (centered on 390 n*m*) was collimated (with lenses of 5 c*m* focal length) and sent to the objective; before the objective we kept a 2 m*m* thick quartz cuvette containing saturated aqueous $CuSO_4$ solution that was used as an infra-red cut-off filter to ensure that no fundamental light is present at the sample. All chemicals were purchased from Sigma Aldrich.

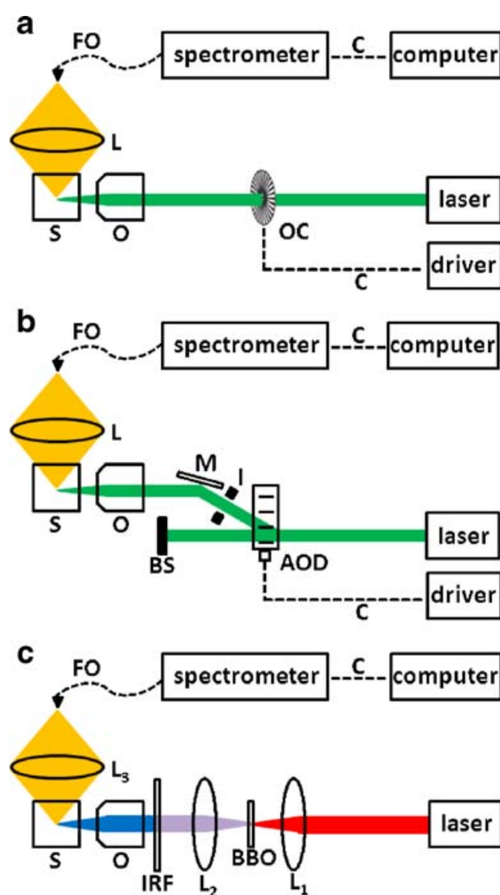

Fig. 1 Schematic of fluorescence generation and collection under pulsed illumination having pulse widths of (a) 1 milli- or micro-second at 1 k*Hz* or 1 M*Hz* respectively (both at 532 n*m*), (b) 150 nano-second at 500 k*Hz* (at 532 n*m*) and (c) 100 femto-second at 76 M*Hz* (centered on 390 n*m*). Abbreviations: AOD: acousto-optic deflector, BBO: *beta*-barium borate crystal, BS: beam stop, C: electrical cable, FO: fiber-optic cables, I: iris, IRF: infra-red cut-off filter, L: lens, M: mirror, O: objective, OC: optical chopper *i.e.* either a disk chopper (1 k*Hz*) or electro-optic modulator ((1 M*Hz*)), S: sample solution

Results and discussions

An electronically excited molecule relaxes within 1–100 n*s* as the excited state lifetimes (*i.e.* the time constants associated with the single exponential decay) of most chromophores lie within this range; *e.g.* R6G has an excited state lifetime of 4 n*s* in water [8]. For this in every case we set the pulse repetition rate in such a way that the time lapse between two consecutive pulses is at least more than 10 n*s* to allow for nearly complete relaxation of the electronically excited fluorophore [9, 10]. Therefore, any enhancement arising out of such pulsed illumination is actually a consequence of pulse duration (*i.e.* how long the molecule is exposed to radiation) or/and pulse energy (*i.e.* how much energy is instantaneously dumped into the molecule). However, it is noteworthy that Donnert and co-workers [11] have demonstrated substantial fluorescence increase at pulse repetition rate ~0.5 M*Hz* and they have assigned it

due to 'dark-state' (*i.e.* triplet state) relaxation. Although we arrived at similar optimal condition for fluorescence enhancement (as discussed later), the present approach is quite different from that; while Donnert *et al* have looked at the total effect of exposure to same number of identical pulses (2.8×10^6 pulses) at various repetition rates (for that they illuminated the sample over different time durations), we looked at the total effect of fixed-time (134 m*s*, see below) exposure to pulses, having same time-averaged power, at various repetition rates (for that we had to change the pulse width and peak power *i.e.* pulses were not identical).

We compared the relative enhancement of fluorescence intensity (obtained by integrating the area under each fluorescence spectral curve using a fixed base-line) under pulsed illumination over CW illumination at the same average power; for this the integration time of the spectrometer (134 m*s*) was much slower than the slowest pulsing rate (1 k*Hz*) so that any enhancement in fluorescence intensity actually results from long time response. The average power levels were in between 10 and 50 m*W*. Although this is a much higher power compared to that used in fluorescence confocal microscopes (~1 m*W*), our deliberate use of a low NA (0.5) objective results in less fluence (*i.e.* energy per unit area) and irradiance (*i.e.* power per unit area) at the focal point; for a high NA (~1.4) tight-focusing objective used in confocal microscopy, the beam waist (*i.e.* the beam radius at the focus) becomes close to the diffraction limit while it is nearly an order of magnitude more for the present case (that results from a square dependence of spot size at focus on NA).

Effect of millisecond pulses As mentioned earlier, millisecond pulses were generated using a mechanical chopper operating at 1 k*Hz*. The relative peak power and average power due to 1:1 chopping are described in Fig. 2; it is evident that to keep the same average power the peak power during chopping must be fixed twice of the CW excitation power. Increasing the frequency results in decrease in both pulse width as well as the time lapse between pulses to the same extent (due to the 50% duty cycle). However, as shown in Fig. 3, no enhancement was noticed (burring a slight increase at much higher average power of ~50 m*W*) with millisecond pulses as reported earlier [12] which is in consistent with the absence of any dependence of photo-bleaching rate on chopping frequencies as noticed by others [13]. The reason for millisecond pulsed excitation behaving like CW excitation is that excited state lifetime is way too smaller, by five orders of magnitude, than the pulse duration; during such long time exposure to radiation, the molecule performs many absorption and emission cycles (*i.e.* Rabi oscillations in presence of spontaneous emission) and reach saturation

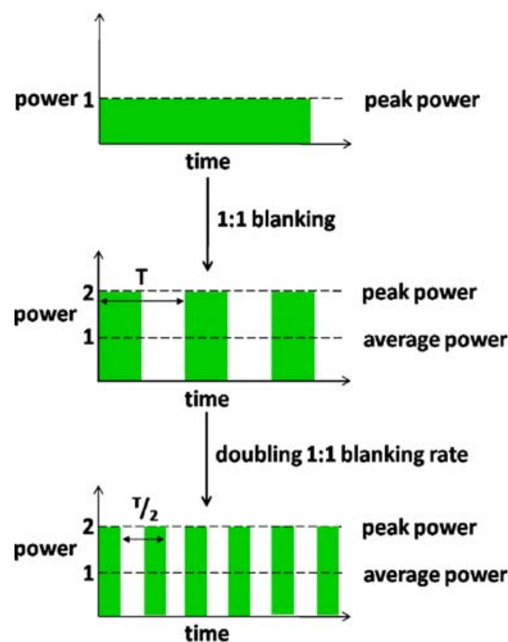

Fig. 2 Schematic (not to scale) of relative power levels (shown as dashed line) of a CW laser beam with a pulsed beam produced by 1:1 chopping at different frequencies (keeping the time-averaged power the same in all cases). The top panel shows the CW excitation, the middle one depicts the effect of chopping (at frequency $^1/_T$) and the bottom panel shows the resultant pulses due to increasing the chopping frequency twice that of the middle panel

equilibrium; simultaneous excited state absorption (or ESA, elaborately explained later) also takes place. However, we assume that the previous reports on removal of heating effects [2] and photo-bleaching [3, 4] by millisecond illumination is due to the presence of wide band of excitation by a mercury or xenon lamp and much higher power levels at the sample under tight focusing conditions. Contributions may come from solvent heating [12, 14] as delineated below.

Effect of microsecond pulses Appreciable fluorescence enhancement was observed with micro-second illumination although the pulse width is nearly 100 times more than relaxation times. This enhancement also varies linearly with increase in input power; as shown in Fig. 4, at low average power (10 m*W*) this increase is ~16% while at higher average power levels (50 m*W*) it rises to ~22%. This may result from the fact that high power illumination imparts significant solvent heating that has a rather slow rise time [14] compared to excited state lifetime and hence can be effectively removed by M*Hz* chopping; in fact we have shown that such slow temperature build up is significant in two-photon excitation where higher pulse energy is needed due to low two-photon absorption probability as reported elsewhere [12].

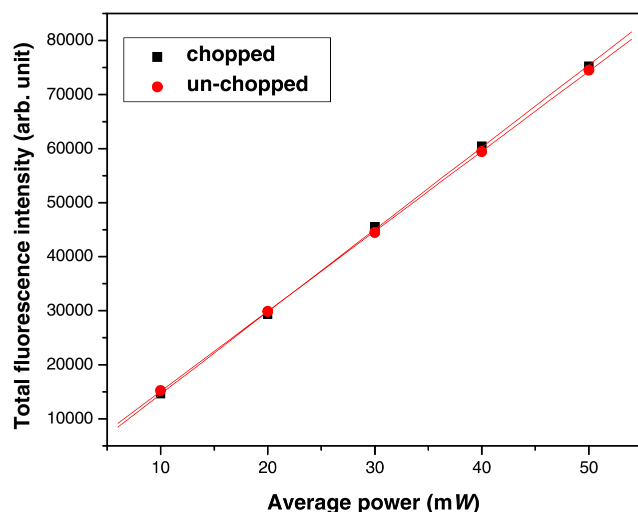

Fig. 3 Variation of fluorescence intensity with average power under milli-second pulsed illumination

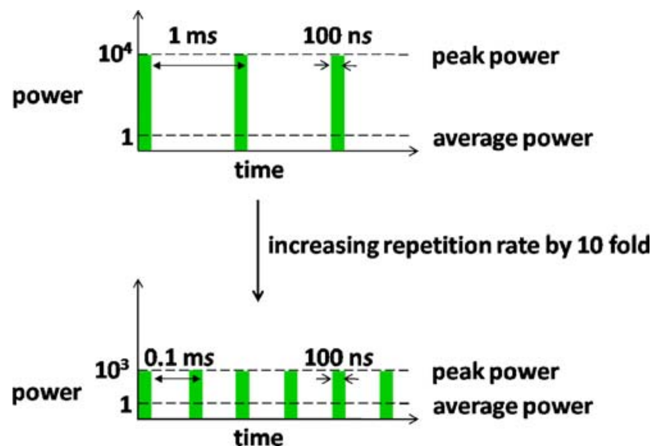

Fig. 5 Schematic (not to scale) of relative power levels (shown as dashed line) of nano-second pulses of same width (100 n*s*) but different repetition rate (both having the same time-averaged power). The lower panel shows the effect of increasing the pulse repetition rate ten times that of the upper panel

Effect of nanosecond pulses The average and peak power levels for a nano-second pulse (with fixed pulse width) at various repetition rates are depicted in Fig. 5. Just like the previous scenario with 1:1 chopping, if we use G*Hz* chopping to produce nano-second pulses, the time lapse between the pulses will be smaller than excited state relaxation time; however it will be interesting to look into the fluorescence intensity modulation studies at 10 and 100 M*Hz* chopping (not presented here).

As the pulse width gets closer to the excited state lifetime, significant fluorescence enhancement occurs. From Fig. 6 it was noticed that using the Q-switched laser, excitation with 120 n*s* pulses at 1 k*Hz* repetition rate results in 62% increase in fluorescence signal as compared with the CW illumination (obtained by integrating the area under the respective fluorescence spectral curve) while changing the repetition rate from 1 to 10 k*Hz* results in ~15% enhancement. This result shows that for the same pulse width increase in pulse repetition rate results in signal enhancement. To double check this inference, we examined the fluorescence intensity at almost similar pulse width (150 n*s*) but much higher repetition rate (500 k*Hz*) using the acousto-optic deflector; as shown in Fig. 7 an unprecedented near 10 fold fluorescence enhancement was noticed which, certainly, is not an effect arising out of pulse width alone. To explain the fact, we looked at the instantaneous pulse irradiance values. At 1 m*W* average power a ~100 n*s* pulse has, at 1 k*Hz* repetition rate, ~10 *W* pulse power while at 500 k*Hz* repetition rate it is ~20 m*W* pulse power. At lower repetition rate this high instantaneous

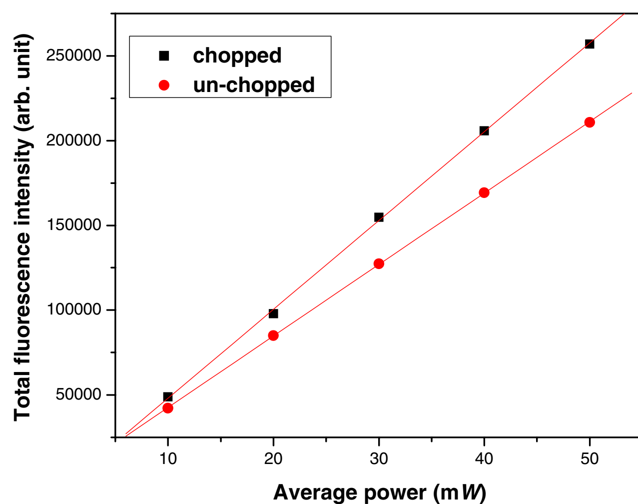

Fig. 4 Variation of fluorescence intensity with average power under micro-second pulsed illumination

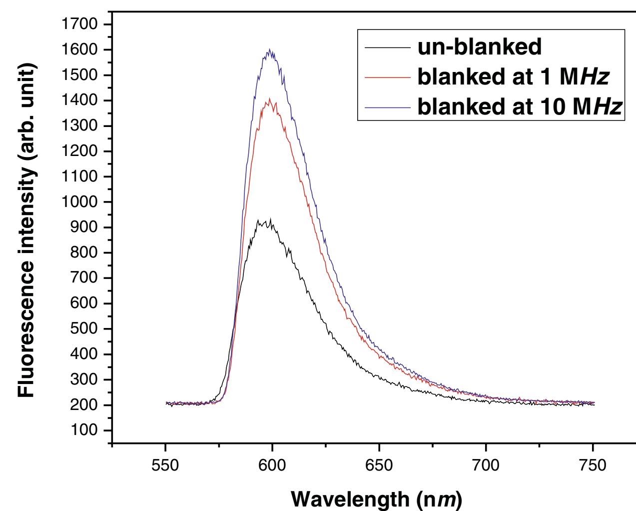

Fig. 6 Fluorescence enhancement with nanosecond pulsed illumination from a Q-switched laser

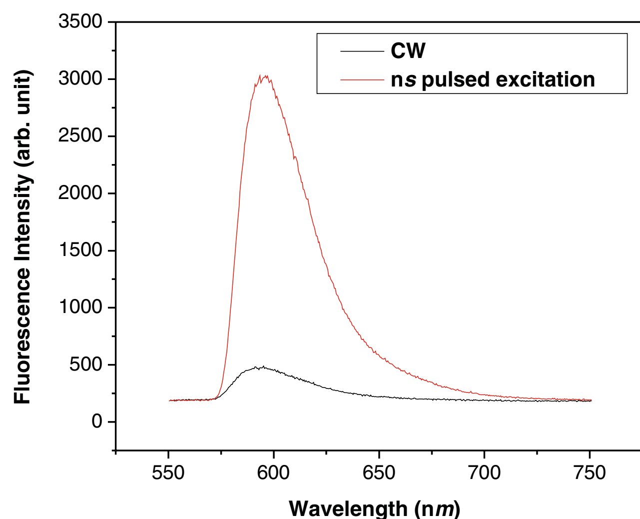

Fig. 7 Fluorescence enhancement with nanosecond pulsed illumination using an acousto-optic deflectorr

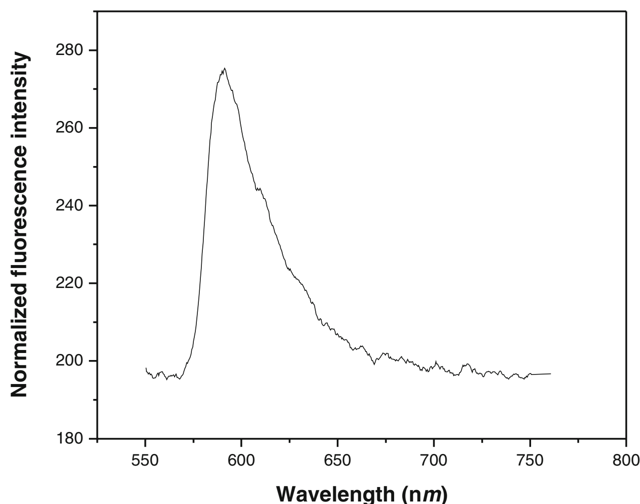

Fig. 9 Fluorescence spectra of R6G under femto-second pulsed illumination centered on 390 n*m*

pulse power produces too high instantaneous irradiance of ~10 M*W*/cm^2 (considering the spot size of ~10 μ*m* at the focus) which is far above the photo-bleaching threshold; on the other hand, at high repetition rate, this value goes down to ~20 k*W*/c*m*^{2}. The use of pulses with large irradiance also leads to pulse-saturation effects *i.e.* rapid and complete depletion of ground state population (which is well studied for multi-photon absorption processes [15]). An important decay channel, triplet state relaxation, may also have pivotal role leading to such gigantic fluorescence enhancement at 500 k*Hz* repetition rate owing to the rather slow (≥1 *s*) decay of the triplet states [11].

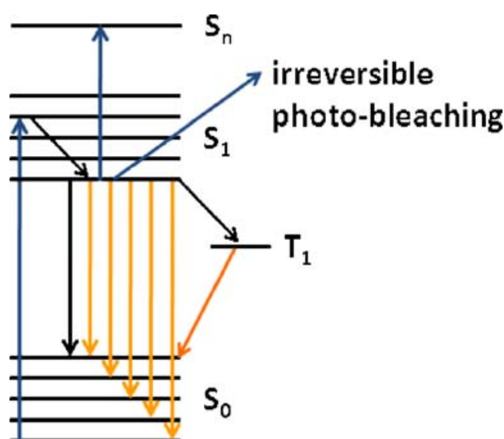

Fig. 8 Schematic of different photo-physical processes of a fluorophore. S_0 and S_1 are the ground and first excited singlet electronic states (shown with vibrational manifolds) respectively. S_n is any higher (n>1) singlet excited state and T_1 is a triplet state lower in energy than S_1. All radiative excitations are shown in blue arrows, non-radiative de-excitations by black arrows while fluorescence and phosphorescence are shown as brown and orange arrows respectively

Optimal choice for pulsed illumination To get more insight into the effects of pulsed illumination, consider Fig. 8. Fluorescence lifetime (τ_0) *i.e.* lifetime of an electronically excited state (S_1), is given by inverse of summation of radiative (fluorescence) and non-radiative (internal conversion and inter-system crossing) relaxation rate constants [16], *i.e.*

$$\tau_0 = \frac{1}{k_0} = \frac{1}{k_{rad} + k_{non-rad}} = \frac{1}{k_f + K_{IC} + k_{ISC}}$$

After excitation to higher vibrational levels of S_1, rapid (within nearly a pico-second) non-radiative decay to ground vibrational level of S_1 occurs (due to high density of states) following 'Kasha's rule'. However, if the illumination is still present, further absorption from S_1 to higher excited electronic states (S_n with n>1) may occur which is ESA. Irreversible population transfer from S_1, or photo-bleaching, may also happen in conjunction with ESA for prolonged exposure. These two pathways (along with triplet state dynamics, not shown in Fig. 8) compete with the above mentioned relaxation of S_1, reducing the

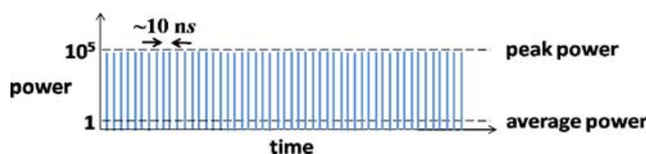

Fig. 10 Schematic (not to scale) of relative power levels (shown as dashed line) of a train of femto-second pulses at ~100 M*Hz* repetition rate centered on 390 n*m*. Each pulse (of ~100 femto-second duration) is shown as a spike

fluorescence yield; the kinetics of the S_1 state under such condition can be expressed as (neglecting the re-population of S_1 from S_n)

$$dS_{1/dt} = k_{01}S_0 - \left(k_f + k_{IC} + k_{ISC} + k_{1n} + k_{bleach}\right)S_1$$

Therefore, by reducing the light exposure time (*i.e.* pulse width) down to nano-seconds, we effectively shut down these two channels and get enhanced fluorescence. The use of even shorter (sub-n*s*) pulses results in pulse-saturation effects (which can be cleverly manipulated, as discussed in the following section). Hence, nano-second (1–100 n*s*, depending on fluorescence life-time) illumination at desired repetition rate (100 k*Hz* to 1 M*Hz*, ensuring complete triplet state relaxation) turns out to be the ideal excitation for maximum fluorescence output. It is worthy to mention here that this accounts for the reduced photo-bleaching (as well as photo-toxicity) in 'controlled light exposure microscopy (CLEM)' that exploits a programmable acousto-optic modulator [17]. In fact, the rapid bleaching of rhodamine 6G even at minimum pumping irradiance (~0.1 M*W*/cm^2 [18]) required for lasing action restricts the use of non-flowing dye media [19] and pulsed illumination with high repetition rate promises to be an alternate solution of using flowing dye solution. However, it should be kept in mind that photo-bleaching differs significantly with concentration; higher the concentration lower is the bleaching although there is no linear correspondence [13].

Effect of picosecond and femtosecond pulses Switching from nano-second to pico-second (and femto-second) pulses results in large instantaneous pulse power owing to light-matter interaction over an extremely short temporal window; for example, a 100 f*s* pulse with 100 M*Hz* repetition rate has a pulse power 10^5 times that of its time-averaged power. As mentioned earlier, this leads to significant pulse-saturation effects. This may also lead to non-linear effects via simultaneous two- (or more) photon absorption (TPA). However, if we use such intense pulses are used to probe at wavelength regions with very low one-photon absorption cross-sections, saturation must be lessened. With 100 f*s* pulsed excitation centered on 390 n*m* we could collect fluorescence spectra at 10 m*W* average power as shown in Fig. 9; although the total fluorescence intensity is nearly 14 times less than that with 532 n*m* CW excitation at the same average power (3,066 counts vs 42,162 counts), the molar excitation co-efficient at 532 n*m* is nearly 52 times larger than that at 390 n*m* (1.45425 vs 0.02809 in units of $M^{-1}cm^{-1}$ [20]). Despite negligible one-photon absorption cross-section, appreciable fluorescence generation is due to the extremely high photon flux offered by each pulse in a train of pulses temporally separated by ~13 n*s* (corresponding to 76 M*Hz* repetition rate) as shown in Fig. 10. Unlike low-repetition nano-second illumination such ultrafast illumination high-repetition rate is crucial for high-speed (~1 frame *i.e.* ~512×512 pixels per second) scanning fluorescence microscopy. Moreover, we have recently shown [21] that ultrashort pulsed one-photon illumination imparts intrinsic three-dimensional spatial resolution in fluorescence microscopy owing to the spatial extension of light wave-packets over few micrometers [22]. Thus clever the choice of excitation wavelength plays crucial role under ultra-short single-photon illumination.

Conclusions

To summarize, we have studied long time effects of fluorescence enhancement under various one-photon pulsed conditions in a systematic manner. We demonstrate that nano-second (1–100 n*s*) pulses with slow repetition rate (≤ 1 M*Hz*) are best suited for maximum fluorescence output while even shorter (sub-picosecond) pulsed illumination with high repetition (~10–100 M*Hz*) rate can be used at wavelengths of low absorption cross-section. The qualitative explanation presented here demands further detailed studies using real time probing of photo-physical phenomena under various pulsed illumination conditions by single-photon counting *etc* methods. Such stroboscopic illumination leads to reduced photo-bleaching (and also photo-toxicity), thereby having promising effects in live cell imaging.

Acknowledgements AKD thanks CSIR, India for graduate fellowship. The authors thank MCIT and DST, India and Wellcome Trust Foundation, UK for funding.

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