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RAPID COMMUNICATION

Exploring the Nature of Photo-Damage in Two-photon Excitation by Fluorescence Intensity Modulation

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Abstract We investigate the relative photo-damage effects during one- and two-photon excitations and demonstrate that there exist fundamental differences in the damage induced by a high repetition rate laser as compared to that of a CW laser. This difference is evident from the degree of enhanced fluorescence intensity achieved by blanking the excitation with an optical chopper. Such an enhancement in fluorescence intensity provides better signal-to-noise ratio that could have immediate applications in multiphoton imaging of live specimens.

Keywords Two-photon cross-section · Photo-damage · Fluorescence enhancement · Multiphoton imaging

Introduction

Although fluorescence microscopy is an indispensable tool for imaging bio-samples for decades, it often suffers from photo-damage induced by the excitation. By photo-damage we mean any detrimental effect that leads to reduction in fluorescence intensity. In the context of the advantages of two-photon fluorescence microscopy over one-photon confocal microscopy, photo-damage has been argued to play a pivotal role; the confined fluorescence generation from very small focal volume (~10^{-15} l while using a tight-focussing objective with high numerical aperture) within the specimen during two-photon absorption (2PA) in contrast with the illumination over the whole optical depth of the specimen during one-photon absorption (1PA) makes the latter more suitable for live cell imaging [1, 2]. However, it has been shown that total exposure required for generating same fluorescence intensity is an order of magnitude more in two-photon absorption (2PA) than in one-photon absorption (1PA) [3]. Photo-damage induced by 2PA has been studied in detail for many fluorophores [4–7]. In both types of excitations photo-damage is brought about by various mechanisms, *e.g.* fluorophore saturation (and subsequent bleaching), photochemical reactions, excited state absorption *etc*. Also, environmental effects are known to play significant role in molecular fluorescence. We have shown in this paper that light-induced damage may also arise from the temperature rise of the 'transparent' (*i.e.* having linear absorption coefficient of 10^{-4} or less) solvent which in turn affects the fluorescence from the solute (chromophore) through complex solvent–solute interactions, even when the solute concentration is appreciably high (10^{-2} *M*). This kind of 'photo-thermal damage' happens to be more prominent for 2PA; indeed we have found that it is almost insensitive for 1PA. Thus, although there are contrasting view-points in the literature regarding whether to treat the photo-damage induced by a high repetition rate (HRR) laser in the same way like that induced by a CW source [8] or not [9], our results show that they are quite different. In addition to this, the effective removal of photo-thermal damage during 2PA and thereby enhancement in fluorescence intensity at low average laser power, simply by blanking the excitation with an optical chopper, provides higher signal-to-noise ratio and offers better condition for the viability of a living specimen under observation in two-photon fluorescence microscopy.

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Experiments

The schematic of the experimental set-up is shown in Fig. 1. In our experiment, the laser system was a mode-locked Ti:saph laser (Mira900-F pumped by Verdi5, Coherent) for two-photon excitation while we used a CW Nd:vanadate laser (Verdi5, Coherent) in case of one-photon excitation. We used ~150 fs pulsed excitation centered at 800 nm and having 76 MHz repetition rate for the former case, while 532 nm CW excitation for the latter. For blanking the excitation, a rotating-disk optical chopper (MC1000A, Thorlabs) with 50% duty cycle (*i.e.* having 1:1 mark/space ratio) was introduced in the excitation path. The average power of excitation was controlled by using an ND filter wheel (Newfocus) and was measured by a power-meter (FieldMate, Coherent). ~10^{-2} and ~10^{-4} M methanolic and *N*,*N*′-dimethylformamide (DMF) solution of rhodamine-6G (R6G) and ~10^{-2} M rhodamine-B (RB) solution in methanol were used as the samples. The laser beam was focused into the sample by an objective (20× 0.5NA, Olympus) and the fluorescence was collected in perpendicular direction by another objective (10× 0.25NA, Newport). The latter 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 using LabVIEW programming.

Results

The fluorescence spectra of 10^{-2} M R6G in the range from 550 to750 nm for different values of average laser power are shown in Fig. 2. The total fluorescence intensity was obtained by integrating the area under each spectrum using a fixed base-line. The logarithmic plot of total fluorescence intensity against average laser power and the linear fit to it are shown in Fig. 3.

We investigated the modulation of fluorescence intensity with changing the frequency of chopping the excitation beam. We performed both single- and two-photon excitation and measured the spectra and intensity of fluorescence that followed excitation. Due to 1:1 duty cycle of the chopper, the average excitation power was halved during the blanking. The fluorescence spectra of R6G under at different chopping frequencies are shown in Fig. 4.

The plot of integrated fluorescence intensity obtained from these spectra versus the chopper frequency in the range 0.5–5 kHz is shown in Fig. 5, along with the integrated fluorescence intensities with un-blanked excitation having same (300 mW) and double (600 mW) average laser power. This was repeated for much concentration (10^{-4} M) of the solute (Fig. 6) and under four different values of average laser excitation power (Fig. 7). Three characteristic features were noted from these plots: firstly,

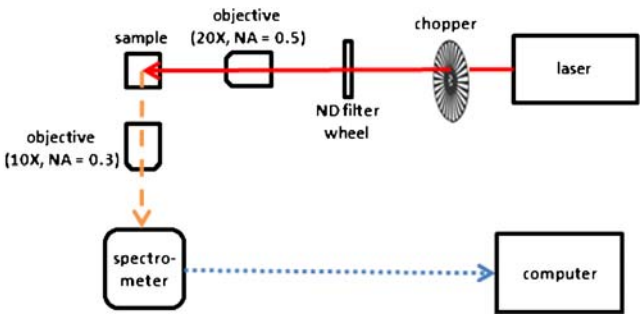

Fig. 1 Schematic of the experimental set-up: the laser is either a mode-locked Ti:saph laser or a CW Nd:vanadate laser. The excitation beam path is shown in solid line, the fluorescence collection path in dashed line, and the electrical cable connections in dotted line

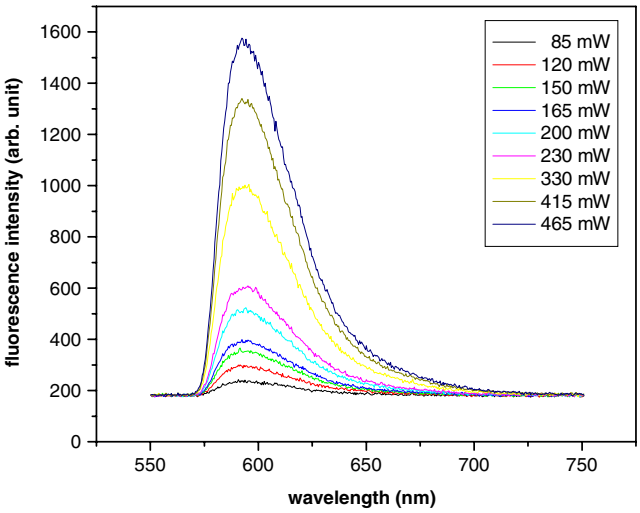

Fig. 2 Modulation of fluorescence spectra of R6G with average laser power during two-photon excitation

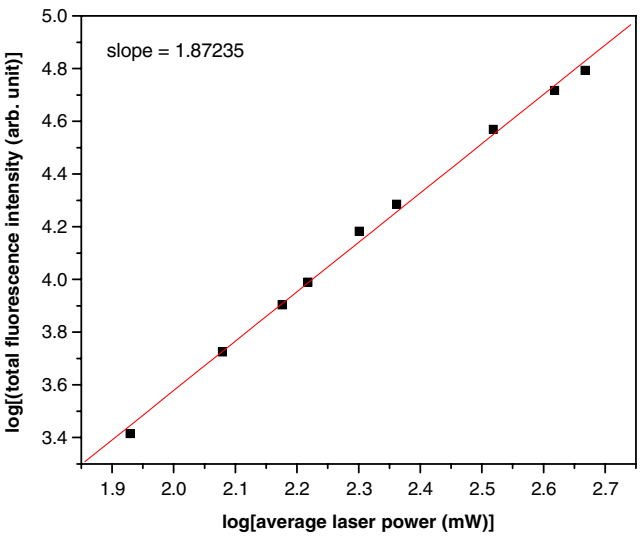

Fig. 3 Logarithmic plot of total fluorescence intensity against average laser power: the red line indicates the linear fit

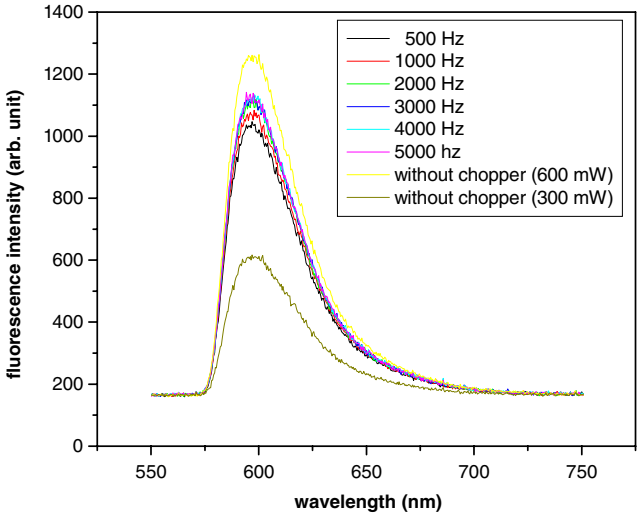

Fig. 4 Fluorescence spectra of R6G collected by a spectrometer: fluorescence intensity is increased when the IR excitation beam is chopped at the same average power as compared to the un-chopped situation, also note the variation of fluorescence intensity when the excitation was blanked at frequencies from 500 Hz to 5 kHz

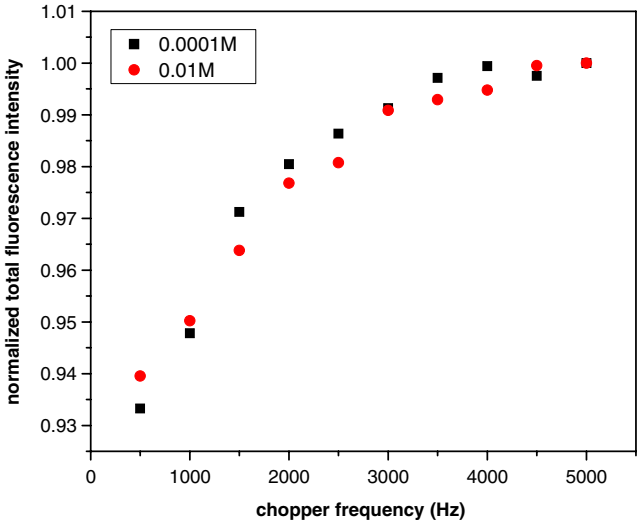

Fig. 6 Variation of total fluorescence intensity of R6G at different concentrations with chopper frequency

by blanking the excitation total fluorescence intensity is greatly enhanced (nearly two-fold, which is evident from the square-dependence of absorption on the input intensity) as compared to the un-blanked excitation with same average power (300 mW); and secondly, the total fluorescence intensity gradually builds up with the blanking frequency and then becomes almost constant when the blanking frequency crosses a certain value (~3.5 kHz). Also, the nature of this variation with chopper frequencies depends neither on the solute concentration nor on the average laser power.

The similar time scale was observed when we changed the solute (from R6G to RB) keeping the solvent (methanol) the same as shown in Fig. 8 but almost no modulation was seen while using solution of the same solute (R6G) in a solvent (DMF) of low thermal conductivity as evident from Fig. 9.

Surprisingly, when the same experiment was repeated with 532 nm CW excitation, a completely different result was obtained. From Fig. 10, we see that even using a very high excitation power as 100 mW (*i.e.* 50 mW during blanking), upon blanking the excitation not only the integrated fluorescence intensities remain almost same as compared to the un-blanked excitation with same average power (which is evident from the linear dependence of

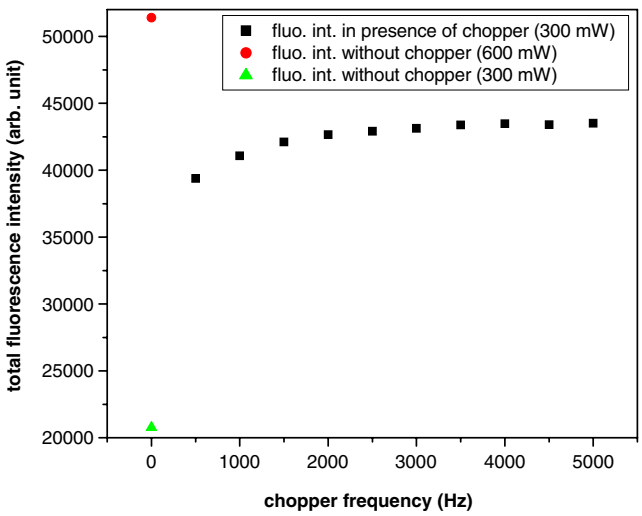

Fig. 5 Modulation of total fluorescence intensity of R6G with chopper frequency during two-photon excitation. Also note the fluorescence intensities in absence of the chopper

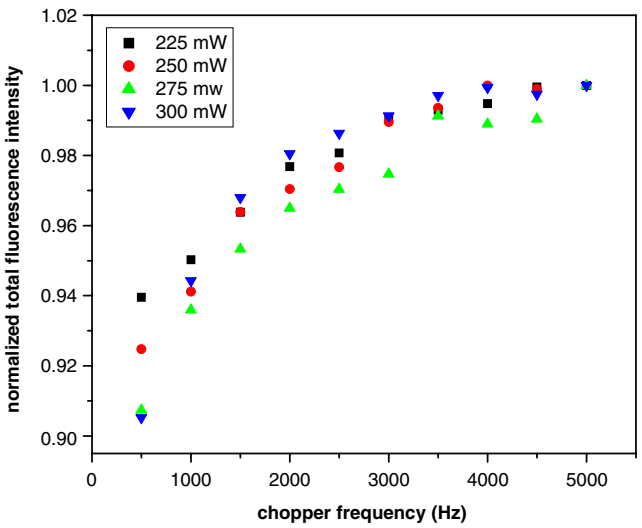

Fig. 7 Variation of total fluorescence intensity of R6G with chopper frequency during two-photon excitation under different values of average excitation power

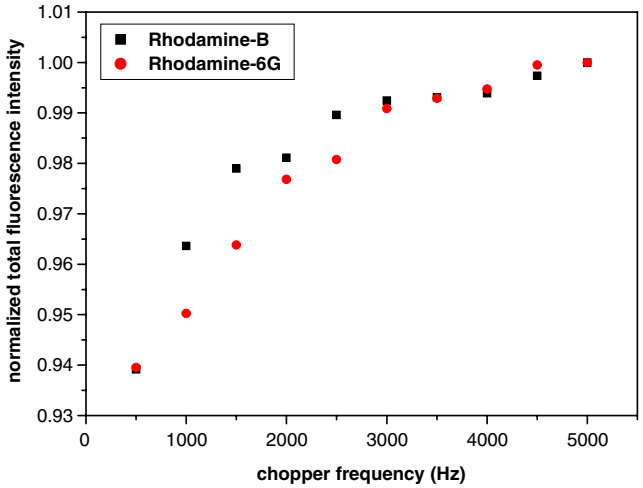

Fig. 8 Variation of total fluorescence intensity of R6G and RB in methanol with chopper frequency during two-photon excitation

absorption on the input intensity), the fluorescence intensity shows little modulation with the blanking frequency also.

Discussions

As shown in Fig. 3 a slope of ~2 for the log–log plot indicates the quadratic dependence on the average laser power, as expected for a two-photon process. This shows the robustness of the present method of calculating the total fluorescence intensity as compare to the other methods (*e.g.* photomultiplier-based methods). Also the ~2 slope indicates that the power levels used in the experiments are below the damage threshold (for two-photon photo-bleaching this slope is >2 [5]). To further check this issue we calculate the probability of 2PA at wavelength λ is given by (under paraxial approximation) [1]

$$n_\alpha \propto \frac{\delta_2 p^2}{\tau f^2}\left(\pi \frac{\mathrm{NA}^2}{hc\lambda}\right)^2$$

where δ_2 is the 2PA cross-section, P is the average laser power, τ is the pulse width, f is the pulse repetition rate and NA is the numerical aperture of the focusing objective with h and c having their usual meaning. For P=500 mW using δ_2=15.3 GM for R6G at 800 nm [10] yields $n_a \approx 0.01$ *i.e.* the power levels used are far from photo-bleaching [11].

Although the repetition rate of the laser may be a serious problem in some cases, the ~10 ns time lapse between the pulses fits well with the excited state lifetime of most of the chromophores [11] (*e.g.* R6G has an excited state lifetime of 4 ns in water [12]). Since molecular de-excitation dynamics are much slower compared to the pulse duration (with FWHM ~100 fas), the fluorescence that follows has a 'δ-pulse response' with single exponential decay [13]; hence one pulse contributes little to the induced photo-thermal damage. The main source of heating is the accumulative effect arising out of small incremental rise in temperature due to each single pulse in the pulse-train [8] (also, in the context of thermal lensing due to 1PA, the induced change in refractive index has been shown to be dependent on the average laser power rather than the instantaneous pulse power [14]).

Chopping a train of pulses at few kHz frequency creates an envelope over a train of pulses (Fig. 11) resulting in a 'bunch of pulses' followed by a 'dark phase'; the duration of this 'bunch of pulses' being equal to that of the 'dark phase' due to the 50% duty cycle of the chopper. Therefore, blanking involves two widely different time-scales: one is associated with the time lag between the pulses (~10 ns)

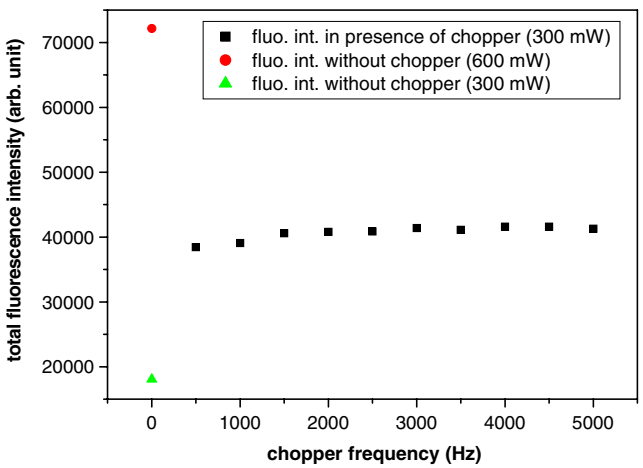

Fig. 9 Modulation of total fluorescence intensity of R6G in DMF with chopper frequency during two-photon excitation. Also note the fluorescence intensities in absence of the chopper

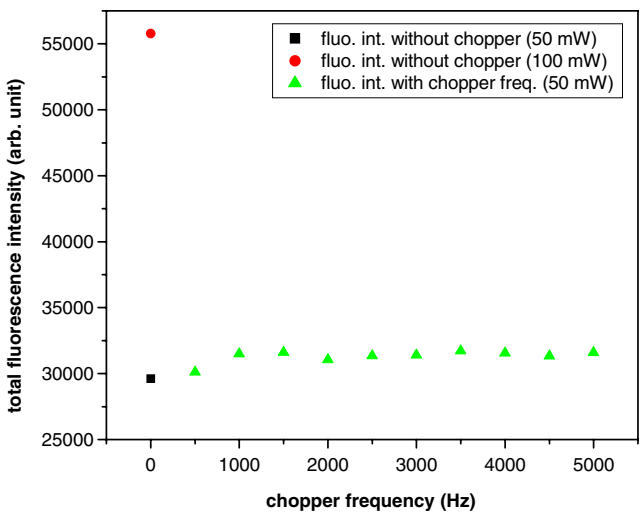

Fig. 10 Modulation of total fluorescence intensity of R6G with chopper frequency during one-photon excitation. Also note the fluorescence intensity in absence of the chopper

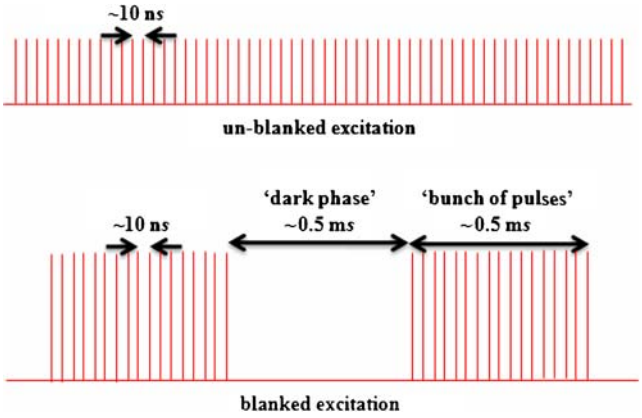

Fig. 11 Comparison of un-blanked and blanked excitation having the same time-averaged power. Each pulse (of nearly 100 fs duration) is shown as a spike

which is very small compared to the other time-scale (~1 ms) associated with kHz blanking.

Now during one 'dark phase' while blanking the excitation, the excited molecules have sufficient time for non-radiative relaxation via both intra- (*e.g.* internal conversion, intersystem crossing *etc.*) and inter-molecular (*i.e.* conduction and convection) processes; thus fluorescence intensity is enhanced compared to the un-blanked excitation as long as the average power remains the same (it must be noted here that for blanked excitation the peak-power is twice than that for un-blanked excitation due to 50% duty cycle of the chopper as shown in Fig. 11). With the increase in chopper frequency, both the duration of the 'bunch of pulses' and the 'dark phase' get reduced to the same extent, but the duration of the 'dark phase' still remains long enough for molecular relaxation even at high chopper frequencies (above 5 kHz). Therefore, it is intuitive to understand that the effect of blanking arises due to the reduction of duration of the excitation time and not the de-excitation time. Also, since the integration time of the spectrometer (134 ms) was much slower than the slowest blanking period (2 ms *i.e.* when the chopper operates at 500 Hz), any modulation in fluorescence (that resulted from this blanking) is actually time averaged signal.

We consider the heating effects arising only from the focal volume since two-photon fluorescence is generated only in that region and approximate this volume to be spherical. To explain the gradual increase and subsequent saturation of the fluorescence intensity with gradually increasing blanking frequency, we need a quantitative estimate of the thermal time constant (which is a measure of the time taken for the building up of steady-state conditions at the focal volume) given by [15]

$$\tau_c = \frac{\rho\omega_0^2}{4k_T}$$

where ρ is the heat capacity at constant volume, ω_0 is the beam-radius at the focal plane and k_T is the thermal conductivity. To calculate ω_0 we use the following relation [16]

$$\omega_0 = \omega \frac{f/z_0}{\sqrt{1 + \left(f/z_0\right)^2}}$$

where ω is the Gaussian beam waist falling on the lens with focal length f and z_0 is given by

$$z_0 = \frac{\pi\omega^2}{\lambda}$$

Using $\omega \approx 1$ mm [17] and assuming the objective to act like a single convex lens of focal length 25 mm yields $\omega_0 \approx$ 10 μm. For methanol at 20 °C using $\rho = 2.5\times10^6$ $Jm^{-3}K^{-1}$ and $k_T = 0.21$ $Wm^{-1}K^{-1}$ [18] yields $\tau_c \approx 0.30$ ms. Therefore, blanking the excitation with an optical chopper above a frequency ~3.3 kHz should, in principle, remove this accumulative heating effect induced by the pulsed excitation. The thermal time constant (τ_c) is a property of the solvent only and does not depend on the average laser power. This is exactly what we have explored experimentally; we have demonstrated that this kind of fluorescence enhancement is independent of the solute concentration (Fig. 6) as well as of the average excitation power (Fig. 7).

We also double-checked our conclusion regarding the nature of steady-state temperature rise by changing the chromophore (rhodamine-B instead of rhodamine-6G) keeping the solvent the same (methanol). This also yields similar optimal chopping frequency (~3.5 kHz) required to reach an asymptotic increase in total fluorescence intensity (Fig. 8). Using R6G in a different solvent (DMF) renders almost no modulation with chopper frequencies as is evident from the very low thermal conductivity of DMF from methanol (Fig. 9). This unambiguously proves that the nature of two-photon photo-thermal damage can be easily understood by considering the heat-transfer properties of the solvent only.

Since fluorescence emission occurs from the same excited states of the fluorophore irrespective of the mode of excitation (1PA versus 2PA) [19], any difference in fluorescence intensity for single-and two-photon processes arises from the nature of excitation only. The nearly complete absence of any modulation with blanking frequency (Fig. 10) in fluorescence intensity during 1PA arises from the fundamental difference between 1PA and 2PA; for 2PA only one among ~10^6 photons are absorbed and the rest huge number of photons interacts with the solvent while a large fraction of the photons are absorbed during 1PA. Particularly in the present case, R6G has a very high 1PA at 532 nm (molar extinction coefficient of 114,441 $M^{-1}cm^{-1}$ [20]

compared to a relatively low 2PA at 800 nm (δ_2=15.3 GM [10]). This, in turn, means that during 1PA the photo-thermal damage arises due to strong light-chromophore interaction while during 2PA it is the solvent that undergoes photo-thermal damage and passes it to the chromophore.

To get more insight into the nature of blanking, consider Fig. 11; optically chopping the excitation (lower legend) reduces the average power to half, but the effect is completely different from that of an un-blanked excitation (upper legend) with the same average power. The enhancement of fluorescence with blanking promises to have potential application in two-photon fluorescence microscopy by better signal detection; particularly the low average power ensures less photo-thermal damage of any live specimen under observation also. For practical purposes, *e.g.* in laser-scanning two-photon fluorescence microscopy, the nearly diffraction-limited focal volume lowers the value of the thermal time constant (τ_c) down to ~100 ns [8]. Therefore, it can be logically anticipated that applying the above prescription of fluorescence enhancement will require high frequency (~10 MHz) optical chopping. However, it should be kept in mind that high-speed (~10 MHz) optical chopping (*i.e.* amplitude modulation) of a train of pulses introduces new optical side-band frequencies via self-phase modulation [10], but we do not take into account of such effects.

Conclusions

To summarize, we have demonstrated that the nature of photo-thermal damage induced by an HRR laser and a CW source are fundamentally different; in case of an HRR laser it is due to the accumulative heating of the solvent while for a CW laser heating effect is less pronounced due to very high relative 1PA by the solute compared with 2PA. However, to understand the mechanism of the temperature rise of the 'transparent' solvent in detail, further investigations need to be followed. During 2PA, the effective removal of photo-thermal damage and thereby enhancement in fluorescence intensity at low average laser power provides higher signal-to-noise ratio and offers better condition for the viability of a living specimen under observation with laser illumination. Further studies regarding the implementation of this idea in multiphoton laser scanning microscopy are presently being pursued in the authors' laboratory.

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