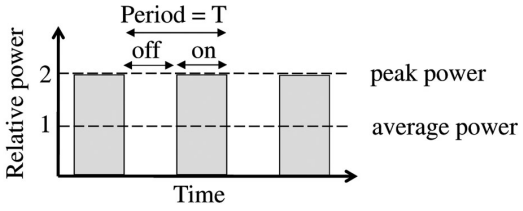
Signal enhancement in fluorescence microscopy by microsecond pulsed excitation

Although pulsed illumination is routinely used in multi-photon fluorescence microscopy[1,2], it is not quite common for its one-photon counterpart, i.e. (one-photon) confocal fluorescence microscopy[3]. However, prolonged illumination with continuous wave (CW) light source leads to excited (singlet) state absorption (as well as absorption from triplet state) leading to photo-damage. Here, by photo-damage we mean any detrimental (irreversible) effect that leads to reduced fluorescence yield. Stroboscopic or pulsed light illumination has been recently introduced to reduce photo-bleaching as well as photo-toxicity[4]. We studied in detail the relative fluorescence yield of the laser dye rhodamine-6G (R6G) under various pulsed conditions and noticed that significant fluorescence enhancement is achieved by using pulsed illumination instead of CW illumination[5]. This type of fluorescence enhancement is due to reduced photo-damage (as well as photo-thermal effects) and is extremely important in any fluorescence-detected method. In this correspondence we show how microsecond pulsed excitation, generated by simple blanking of a CW excitation at 1 MHz, leads to significant fluorescence enhancement for a fluorophore in solution and also demonstrate an application in confocal fluorescence laser scanning microscopy (LSM).

In our experiment, we used 488 nm CW beam from an argon-ion laser (IMA 10X, MellesGriot, Albuquerque, NM, USA). The laser beam was passed through an electro-optic (amplitude) modulator (EOM, Model No. 4101, New Focus, San Jose, CA, USA), driven by a fixed frequency driver (Model No. 3363, New Focus, San Jose, CA, USA) operating at 1 MHz. When a Glan-Taylor prism-polarizer (analyzer) was kept immediately after the amplitude modulator, the output signal intensity shows a sinusoidal modulation at 1 MHz frequency. The modulated laser beam was guided to the scan-head of the confocal microscope system (FV300 coupled with IX71, Olympus Inc, Tokyo, Japan) which focuses the beam onto the sample by a high numerical aperture (NA) oil-immersion objective (40X 1.4 NA, respectively). For fluorescence measurements, 10^{-4} M methanolic solution of R6G (Sigma-Aldrich, Switzerland) was used. The intensity count measurements as well as confocal image (of 512 × 512 pixels) acquisition of bovine pulmonary artery endothelial (BPAE) cells (F14781, Molecular Probes Inc., Eugene, OR, USA), revealing F-actin stained with Alexa 488 phalloidin (green fluorescent) were performed using the FLUOVIEW software.

Here any enhancement in fluorescence under pulsed illumination corresponds to the enhancement with respect to the fluorescence intensity under CW excitation at the same time-averaged power (the power levels indicated in the figures correspond to the values at the sample). The EOM was found to modulate only 60% of the beam entering the scan-head, leaving 40% unmodulated beam as constant background. We compared the fluorescence under intensity-modulated condition (after correcting for this background by subtracting the constant contribution) with the unmodulated condition at the



Chopper frequency = $^1/_T$	'on' time	'off' time
1 kHz	0.5 ms: prolonged excitation	0.5 ms: complete de-excitation
1 MHz	0.5 μs: reduced excitation	0.5 μs: near-complete de-excitation
1 GHz	0.5 ns: ideal excitation	0.5 ns: incomplete de-excitation

Figure 1. Schematic characterizing pulsed output obtained at various chopping frequencies with its effect on fluorescence.

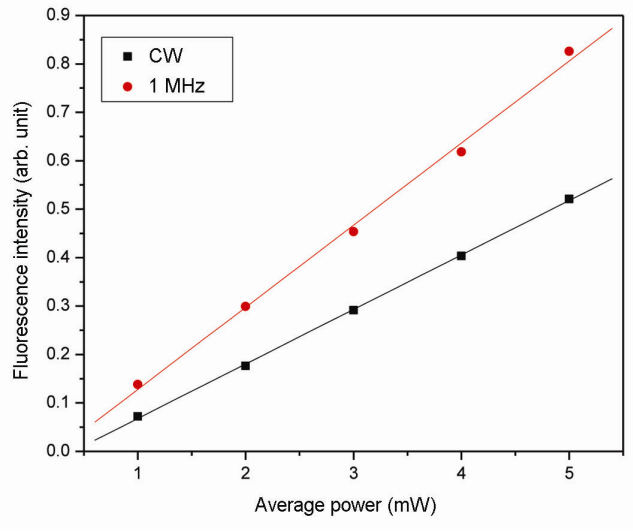


Figure 2. Variation of fluorescence intensity with average power under microsecond pulsed illumination.

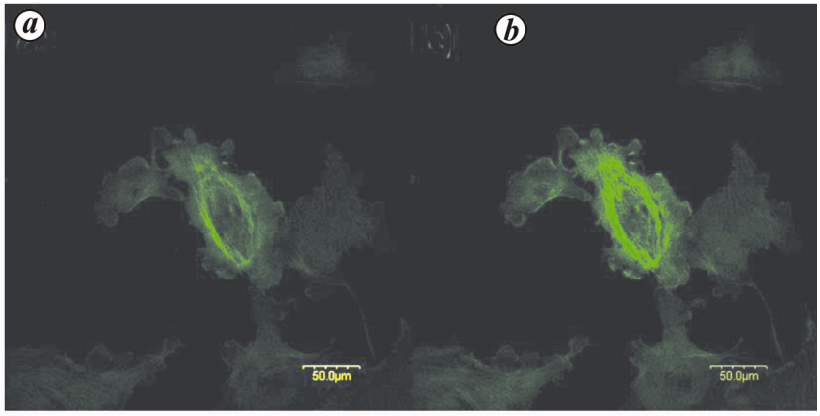


Figure 3. Images of BPAE cells taken with (***a***) intensity modulated (at 1 MHz) and (***b***) unmodulated pulse train at the same time-averaged power (1 mW).

same average power and each image was processed accordingly.

Pulsed excitation is always advantageous over CW excitation, mainly for two reasons. First, a short duration of the pulse ensures reduced re-excitation from excited states; in particular, a pulse width less than the excited-state lifetime is best suited. On the other hand, the finite time lag between consecutive pulses ensures that an electronically excited molecule relaxes via radiative as well as non-radiative intramolecular relaxation; the slowest de-excitation pathway is the triplet state (or 'dark state') relaxation. Thus, the 'ideal' illumination scheme turns out to be a pulsed excitation with the pulse width shorter than the excited-state lifetime, and the inter-pulse period longer than the triplet lifetime[6]. Experimentally this has been realized using picosecond pulsed excitation with inter-pulse time of a few microseconds[7] or by using 1–100 ns pulses with ≤1 MHz repetition rate[5]. However, such an 'ideal' illumination condition is somewhat impractical in LSM, as the typical laser dwell-time on each pixel is only between 1 and 10 µs. In other words, we need sufficient number of pulses illuminating each pixel for an optimal signal-to-noise ratio maintaining a reasonable scan speed.

One economical way of producing light pulses is by blanking (or 'chopping') a CW laser beam with a chopper; if the chopper has 50% duty cycle (i.e. has 1 : 1 mark/space ratio), the output consists of a pulse train having pulse width equal to the time lapse between pulses (which is inverse of the pulse repetition rate). Now, considering a dwell-time of 10 µs in our experiment, this would require a blanking at 1 MHz or higher repetition rate so that we have at least ten cycles per pixel (any slower modulation frequency would result in alternate bright and dark pixels[8]). However, increasing the blanking rate reduces the time lapse between pulses leaving the excited molecules less time for de-excitation. Thus a combination of 1 MHz blanking with 10 µs dwell-time turns out to be quite optimal. This is schematically shown in Figure 1.

Figure 2 shows comparative fluorescence enhancement for R6G solution under 1 MHz blanked excitation compared with CW excitation, while Figure 3 shows similar enhancement for Alexa 488 in confocal LSM.

Earlier, we reported fluorescence enhancement for R6G solution with 1 MHz blanked excitation and mainly attributed it to the reduced photo-thermal effects owing to heating of 'transparent' solvent[5]. In this experiment, laser-induced heating is supposed to have less dramatic effect under beam scanning (as the excitation beam is rapidly swept, leaving the beam only for 10 µs per pixel), particularly in a fixed specimen. Also, the low average power ensures absence of any appreciable effect arising from heating. However, we do not altogether rule out any role played by reduced photo-thermal effects in fluorescence enhancement. Note that solvent-mediated heating leading to decreased fluorescence is more prominent in the context of two-photon excitation, where short laser pulses with high peak power are employed to circumvent the very low nonlinear absorption cross-section of common fluorophores[9]. Apart from fluorescence enhancement, reducing photo-thermal effects are extremely important in the viability of a live specimen, for example, in live-cell imaging.

To conclude, we have demonstrated how simple optical blanking of a CW excitation at 1 MHz frequency leads to an enhanced fluorescence signal owing to enhanced triplet state relaxation (as well as to reduced photo-bleaching), which turns out to be quite useful in fluorescence microscopy. Further investigation of fluorescence enhancement pulsed illumination using different combinations of pulse widths and repetition-rates in other fluorescence-based analytical techniques will be interesting to explore.

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