A Sensitive Technique for Two-Photon Absorption Measurements: Towards Higher Resolution Microscopy

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Abstract. High repetition rate (HRR) lasers are essential in multiphoton microscopy for satisfactory signal to noise at low average powers. However, HRR lasers generate thermal distortions in samples even with the slightest single photon absorption. Using an optical chopper with HRR lasers (“blanking”) we demonstrate a femtosecond z-scan setup that effectively eliminates thermal as well as small linear absorption effects and precisely measures two-photon absorption (TPA) cross-sections of chromophores. Accurate measurement of TPA cross-sections in biologically relevant chromophores is especially important since the TPA spectrum is considerably different in regions with even minute linear absorption. Such blanking measurements with chopper also show enhanced fluorescence efficiency of the chromophores.

1. Introduction

Two-Photon Absorption (TPA) is a quantum mechanical process, wherein an atom or a molecule absorbs two-photons simultaneously[1]. This is of great significance in biological research, especially in the field of two-photon bio-imaging[2,3]. In standard two-photon imaging studies, a detailed TPA spectrum of chromophores is necessary for choosing the suitable excitation and detection wavelength region where the TPA cross-section is fairly high so that two-photon transitions can occur efficiently. We have successfully measured large[4-7] TPA values for various chromophores as well as very small[8] TPA values for neat solvents by an open aperture Z-scan technique[9] with femtosecond lasers.

In multiphoton microscopy it is essential to use high repetition rate (HRR) lasers for satisfactory signal to noise for images at low average powers. HRR lasers generate thermal distortions, especially in samples with the slightest single photon absorption. It is, therefore, important to efficiently overcome such thermal effects. In this paper, we extend our sensitive TPA cross-section measurement techniques to chromophores with small linear absorption using an optical chopper for HRR femtosecond oscillators that effectively eliminate the thermal and linear absorption effects.

The experimental results of our improved experimental scheme of using “blanking” with the help of a chopper for a HRR laser at 76MHz presented in this paper demonstrate the removal of thermal effect as they compare favourably to those at much lower repetition rate of 1kHz laser. We also show that our experimental technique can accurately recover the two-photon spectra of a chromophore Cis-Bis-Ruthenium (II) bipyridyl chloride in dichloromethane (DCM) which has a small amount of 1-

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photon absorption at certain parts of the irradiating wavelengths. Finally, we show that the two-photon fluorescence efficiency of the dye Rhodamine-6G in methanol (MeOH) also increases as we use our “blanking” technique with HRR laser. Thus, our results presented here could have a profound impact on the two-photon imaging applications since the sensitivity of the images are expected to be enhanced.

2. Materials and method
Spectroscopy grade Cis-Bis-Ruthenium (II) bipyridyl chloride hydrate \([\text{Ru(bpy)}_2\text{Cl}_2\times\text{H}_2\text{O} \text{ (Rubpy)}\) and Rhodamine-6G (Rh6G) were purchased from Sigma-Aldrich and used without any further purification. TPA cross section values of these samples are measured by open aperture Z-scan (intensity scan) technique\([4-10]\). Our femtosecond experimental scheme (Fig. 1) involves mode-locked Coherent Mira Ti:Sapphire laser (Model 900) which is pumped by Coherent Verdi frequency doubled Nd:Vanadate laser.

![Figure 1.](https://example.com/figure1.png)

**Figure 1.** Experimental set up for the measurement of two-photon absorption cross-section. Use of chopper before the sample distinguishes it from a conventional z-scan open-aperture setup.

The model 900 Mira is tunable from 730nm to 900nm and its repetition rate is 76MHz. We employ blanking by using a mechanical chopper (model SR540 from Stanford Research Instruments Inc.), whose frequency is finally optimized and set to 500Hz to allow equal ratio of illuminating to blanking of 1ms. The optimization of the frequency of the chopper is based on optimizing the elimination of local heating due to the small residual linear absorption of the chromophore (Rubpy) or for the thermal effect as evident between 1cm and 1mm sample-cell (Fig.2). Using a 20cm focal length lens, the pulses are focused into either a 1cm or 1mm long cell filled with sample, where it produces GW-level intensity at the focal point of the lens. This is achieved through appropriate choice of the beam-diameter (through an adjustable optical iris from Thorlabs Inc.) and average power of the laser with the help of a half-waveplate and polarizer combination attenuator. The waveplate and polarizer combination attenuator not only provides continuous intensity scaling capability but also provides well-defined linear polarization for the experiment. The 1mm long sample cell satisfies the condition that the cell-length is less than the Rayleigh range. So, the 1cm cell is only used as a means to ascertain the appropriate chopper frequency, all the reported TPA measurements in this paper are collected with the 1mm cell. We scan the sample through the focal point using a motorized translation stage (Newport Inc. model ESP 300), which can step with a minimum resolution of 0.1µm. This allows a smooth intensity scan for the samples in this wavelength. The transmitted beam through the sample is detected by focusing with a 7.5cm focal length lens into a UV-enhanced amplified silicon photo detector (Thorlabs DET210).
Figure 2. (a) UV-Vis spectrum of Cis-Bis Ru(II) bipyridyl chloride hydrate in DCM. (b) Chopper “blanking” at 500Hz wherein the mode-locked pulses are present for 1ms and absent for 1ms with an exact 50% duty cycle. (c) Effect of linear absorption on chopped signal as the focal point (z=0) approaches for a 1cm sample cell. (d) The same case as in (c) except that the sample cell is 1mm long. Thermal effects are minimized as compared to (c). Since we measure peak to peak values on the oscilloscope, our data distortion is further minimized. This process works best when the linear absorption is minimized with the 1mm cell and the instantaneous change is unaffected. (e) Mode-locked train of pulses that are 13.6ns apart is unaffected within the chopped area. (f) Open aperture z-scan for chopper versus without chopper and the corresponding fit to the Sheik-Bahai model.
The peak-to-peak value from the DET210 signal is measured with an oscilloscope (LeCroy Waverunner Model LR6100R), which is triggered by the chopper frequency. The delay stage and the oscilloscope are interfaced with the computer using a GPIB card (National Instruments Inc.) and the data is acquired using LabVIEW programming.

We obtain the nonlinear absorption coefficient $\beta$, by fitting our measured transmittance values to the equation:

$$T(z) = 1 - \frac{\beta I_0 L}{2 \left(1 + \frac{z^2}{z_0^2}\right)} ,$$

where $\beta$ = nonlinear absorption coefficient, $I_0$= on-axis electric field intensity at the focal point in absence of the sample, $L$= sample thickness, $z_0$ = Rayleigh range = $\frac{\pi w_0^2}{\lambda}$, $w_0$ is the minimum spot size at the focal point. The $\beta$ values are obtained by curve fitting the measured open-aperture traces with the above equation. Upon obtaining the value of $\beta$, the TPA cross section $\sigma_2$, of one solute molecule (in units of $10^{-40}$ cm$^4$ s photon molecule$^{-1}$) is generated from the expression: $\sigma_2 = \beta \nu \times 10^4/Nc$, where $\nu$ is the frequency of the incident laser beam, N is Avogadro constant, c is the concentration of the compounds in respective solvents. We take the known value of $\sigma_2$ for Rhodamine-6G in MeOH at 806nm as the reference for calibrating our measurement technique. At each wavelength, such $\sigma_2$ values are calculated based on the open-aperture intensity scan data and finally the TPA spectrum is generated as a function of different wavelengths. Such a knowledgebase makes it possible to choose the suitable excitation and detection wavelengths to a region wherein the TPA cross-section as well as the figure of merit $\frac{\beta \lambda}{n_2}$ of the particular chromophore is fairly high. We also performed the unchopped z-scan experiments at much lower repetition rate of 1kHz from an amplified Ti:Sapphire laser that has minimal thermal effects and compared those to our HRR laser results.

The two-photon fluorescence signal with Ti:Sapphire MIRA oscillator with and without optical chopper (Fig. 5) was generated with the help of 10cm focusing lens at the front surface of the 1cm fluorescence cuvette and was collected perpendicularly through the fiber-tip of the CCD based Ocean-Optics monochromater (HR-4000).

3. Results and Discussion

We have measured spectral dependence of TPA $\sigma_2$ of Rubpy in DCM with and without chopper (Fig. 3). We find that the TPA spectra look quite different in two cases. From the UV-Vis spectra of the compound in DCM (Fig. 2a) it is evident that there is a shoulder around 720-770 nm. The HRR laser induces sufficient linear absorption in this wavelength region and the light passing through the sample is absorbed to be converted into thermal load (Fig. 2c). A typical open-aperture z-scan data collected at 770nm without chopper, therefore, shows deviation from the expected Sheikh-Bahai[9] model (Fig. 3). Thus, in effect the TPA spectrum without the chopper is reminiscent of the single photon spectra in that wavelength range (Fig. 2a). In fact, it is not possible to extend further into the absorbing bluer wavelengths due to stronger absorption under this experimental setup. However, with the use of chopper with its frequency set to result in 1ms on and 1ms off time (“blanking”), this effect is completely eliminated which is reflected in the z-scan data conforming to the expected Sheikh-Bahai model (Fig. 2f) and the TPA spectra (Fig. 3) look much more valid. TPA experiments performed using ~1nJ/pulse energy from an amplified laser operating at 1kHz at 800nm also confirmed this elimination of the thermal effect. The mechanical chopper allows pulses to come at 13ns (76MHz) apart within the 1ms (500Hz) window allowing the averaging benefits of the high-repetition that would be needed for microscopy measurements. However, the chopper eliminates the thermal effects by blanking at a 1:1 duty-cycle of 1ms each at a frequency of 500Hz and provides the best of both worlds for microscopy.
We also address the issue of using the ultrashort (femtosecond) pulses with high repetition rate which is preferentially used with respect to continuous-wave (cw) lasers. This preference is to increase the probability of two-photon absorption since ultrashort (femtosecond) pulses with high repetition rate gives enough peak power for the transition as well as is a preferable situation for microscopic measurement due to the better signal to noise possible with the high levels of averaging possible at high repetition rates. We have also tried the same experiment in cw-condition, by unmodelocking the laser. As expected, we did not observe any two-photon absorption (Fig. 4) under the cw-condition even at higher average laser powers for the cw laser.

Nevertheless, at the very high peak power of the pulsed lasers, there could be a finite probability of the photo damage of the sample which we have successfully shown here to have minimized by using a mechanical chopper operating at 500 Hz.
Figure 5. Two-fold enhancement in the fluorescence intensity of Rhodamine-6G in MeOH with chopper compared to the one without chopper case (average power of 300mW with 120 fs pulses from Ti:sapphire Laser at 780 nm).

Enhancement in the two-photon fluorescence signal with the use of optical chopper (Fig. 5) also indicates that the enhanced TPA would further help in enhancing the two-photon image signal to noise as not only more photons will be effectively absorbed but also the fluorescence signal will increase.

4. Conclusion
We have reported a very sensitive technique for the measurement of two photon absorption cross section, which has a direct consequence in the two-photon bio-imaging. Though low repetition rate lasers can be utilized for TPA measurements, they are inappropriate for microscopy applications. In our present experimental setup, we have used the high repetition rate, high peak power from the femtosecond oscillators for the required intensity and signal averaging while at the same time eliminated the possibility of photo-bleaching of the samples by carefully using a mechanical chopper. The correct picture of TPA spectra is very important for a particular chromophore in use and our new setup obtains it precisely utilizing the best of both world—high repetition rate femtosecond laser source as well as minimization of thermal effects. Enhancement in the two-photon fluorescence yields with the use of optical chopper also indicates that these results have a very high potential of providing brighter and enhanced two-photon image. Further studies on these issues for many more systems are currently in progress in the authors’ laboratory.

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References
[1] Goppert-Mayer M. Ann. Phys 1931, 9,273.
[2] Xu, C; Williams, R M; Zipfel, W; Webb, W.W. Bioimaging 1996, 4,198.
[3] Albota, A.M; Xu, C; Webb, W.W. Appl. Opt. 1998, 37(31), 7352.
[4] Rath, H.; Sankar, J.; PrabhuRaja, V.; Chandrashekar, T. K.; Nag, A.; Goswami, D. J. Am. Chem. Soc. (Communication) 2005, 127, 11608.
[5] Das, S.; Nag, A.; Goswami, D.; Bharadwaj, P. K. J. Am. Chem. Soc. (Communication) 2006,
128, 402.

[6] Misra, R.; Kumar, R.; Chandrashekar, T. K.; Nag, A.; Goswami, D. Org. Lett. (Letter) 2006, 8, 629.

[7] Rath, H.; Prabhruraja, V.; Chandrashekar, T. K.; Nag, A.; Goswami, D.; Joshi, B. S. Org. Lett. (Letter) 2006, 8, 2325.

[8] Nag, A; Singh, S; Goswami, D. Chem. Phys. Lett. 2006, 430, 420–423.

[9] Sheik-Bahae, M.; Said, A.A.; Wei, T.; Hagan, D.J.; Van Stryland, E.W. IEEE J. Quantum Electron. 1990, 26, 760-769.

[10] Van Stryland, E.W.; Sheik-Bahae, M.; Characterization Techniques and Tabulations for Organic Nonlinear Materials, (Eds.: M. G. Kuzyk, C. W. Dirk), Marcel Dekker, Inc., 1998, pp. 655-692.

[11] Kim, D.; Osuka, A.; Shigeiwa, M. The Journal of Physical Chemistry A 2000, 109, 2996.

[12] Sengupta, P.; Balaji, J.; Banerjee, S.; Philip, R.; Kumar, G. R.;; Maiti, S. J. Chem. Phys. (Communication) 2000, 112, 9201.

[13] Tian, P; Warren, W.S. Optics Letters 2002, 27, 1634.

[14] Chen, Q; Sergeant, E. H; Leclerc, N; Attias, A J. App. Opt. 2003, 42(36), 7235.

[15] Hales, J.M; Zheng, S; Barlow, S; Marder, S.R; Perry, J.W. J. Am. Chem. Soc. (Communication) 2006, 128, 11362.