

Thermal-lens-induced anomalous solvent's effect on fluorescence produced by two-photon continuous-wave laser excitation

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Measurements of two-photon-excited fluorescence (TPF) of fluorescein and Rhodamine 6G in various solvents were performed with a continuous-wave (cw) laser for excitation and an acousto-optic tunable filter for spectral dispersion. Interestingly, the cw laser excitation produced an unwanted thermal-lens effect when the measurements were performed in solvents that absorb the excitation laser light (e.g., alcohols and water, because these solvents absorb the 780-nm excitation light through the overtone and combination transitions of the O—H group). The defocusing effect of the thermal lens leads to a decrease in the TPF signal. Because the strength of the thermal lens depends on the thermo-optical properties (dn/dT and thermal conductivity) of the solvent, its interference makes the effect of solvents on the TPF much different from those on one-photon-excited fluorescence. However, the thermal-lens interference will not limit the application of this cw laser excited TPF technique because, even when measurements were performed in solvents that absorb cw excitation laser light, the thermal-lens interference was observed only in solvents such as nonpolar organic solvents that have relatively better thermo-optical properties. Interference was not observed in water, which is the most widely used solvent for the TPF technique (because water has poor thermo-optical properties). © 2000 Optical Society of America

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1. Introduction

It was theoretically predicted in 1931 that an atom or a molecule could absorb two photons simultaneously in the same quantum event.¹ The first experimental verification that this is so came 30 years later.² Since then, the technique has been the subject of theoretical and experimental studies. Its popularity increased significantly when it was demonstrated in 1990 that the two-photon-excited fluorescence (TPF) technique can be used in biological imaging.³ The popularity stems from the advantages of the technique, including its ability to excite UV-absorbing dyes with the low-energy red or near-infrared photons, which reduces the possibility of photodecomposition of the sample. The quadratic relationship between the signal and the excitation intensity provides the technique with a three-dimensional capa-

bility. This capability, in turn, makes it possible to use the technique for three-dimensional scanning microscopy without a pinhole.^{4–12}

The probability for instantaneous absorption of two photons is rather low. As a consequence, pulsed lasers are often used for excitation.^{4–8} However, even when they are excited by such a high-power, high-repetition-rate (picosecond or femtosecond) laser, the two-photon-excited fluorescence signals are still so low that they are difficult to detect. Furthermore, such intense excitation laser pulses often induce other processes, including stimulated scattering, that make the detection of the small fluorescence signals difficult because the excitation pulses contribute to the unwanted background signals. As a consequence, elaborate and sophisticated detection methods including photon counting, time discrimination, and second-harmonic detection must be used to extract the small signals in the presence of a large background.^{4–8}

Continuous-wave (cw) lasers are relatively cheaper and easier to operate and maintain than are pulsed lasers. Additionally, they provide radiation with narrower bandwidth and a wider wavelength range than do pulsed lasers. TPF with cw laser excitation would increase the accessibility and application of the technique. TPF with cw laser excitation has, in fact,

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been reported.^{9,10} However, because of its relatively low peak power compared with that of pulsed lasers, a TPF signal generated by cw laser excitation will be much smaller than the corresponding pulsed laser excitation. Because it is relatively difficult to measure such small signals, TPF with cw laser excitation has not been studied in detail.

Our aim in this study was to investigate systematically TPF with cw laser excitation. An acousto-optic tunable filter (AOTF) was used as the dispersive device for this study. The AOTF was selected because this electronically driven, spectrally tunable filter is known to have substantially higher throughput than conventional monochromators; i.e., more than 90% diffraction efficiency can be achieved with an AOTF.^{11–13} Because the diffracted light is spatially separated from the transmitted light, the AOTF can provide better rejection of background signals than can a conventional monochromator.^{11–13} Additionally, because the light diffracted from the AOTF can be readily amplitude modulated (by modulation of the corresponding rf signal applied to the AOTF), a phase detection technique can be used to enhance the signal further and reduce the noise.^{11–13} As a consequence of these advantages, TPF with cw laser excitation has been obtained. Interestingly, we observed that cw laser excitation produces not only a TPF signal but also a photoinduced thermal lens signal and that interference by the latter leads to a decrease in the intensity of the former.

2. Experiment

A. Two-Photon-Excited Fluorescence Instrumentation

A cw Ti:sapphire laser (Coherent Model 890) was used for excitation. This laser was pumped by a 6.2-W multiline light from an argon-ion laser (Coherent Model Innova 70). The excitation beam was spectrally tuned from 680 to 850 nm (with a maximum output power of 1.2 W at 780 nm) by use of a short-wavelength optic set. The laser beam, which had a diameter of 0.6 mm and a full-angle divergence of 1.7 mrad, was focused by a lens with a 1-cm focal length onto a 1-cm path-length cell. The beam spot size at the focal point was approximately 20 μm , thereby providing a maximum photon flux of $\sim 1.5 \times 10^{24}$ photons/($\text{cm}^2 \text{ s}$). The fluorescence was collected at 90° with a 2.5-cm focal-length lens. To reduce any possible reabsorption of the emitted light, we focused the excitation beam close to the cell wall (on the detector side). A TeO₂ noncollinear acousto-optic tunable filter (Crystal Technology Model 97-01776), placed between two cross-axis Glan-Thompson prism polarizers, was used to disperse spectrally the TPF light emitted from the sample. A rf generator, similar to that used in our previous studies,^{14,15} provided the rf signal to the AOTF. The rf signal from this driver was amplitude modulated at 33.33 kHz by a home-built modulator and amplified by a rf power amplifier before being applied to the AOTF. The TPF light emitted from the sample was diffracted by the AOTF into monochromatic light,

and we could scan the wavelength of this diffracted light by changing the frequency of the applied rf signal. Furthermore, the diffracted (TPF) light was modulated at the same frequency as that of the applied rf signal (i.e., 33.33 kHz). This modulated light was detected by a red-sensitive photomultiplier tube (Hamamatsu Model 6060). The photomultiplier tube's signal was then amplified and demodulated by a lock-in amplifier (Stanford Research Systems Model SR810 DSP) whose output was connected to a personal computer by means of a 16-bit interface board. Programs written in C++ were used to scan the excitation wavelength, to control and to drive the AOTF as well as to acquire the data.

One-photon fluorescence spectra were measured with a Perkin-Elmer spectrofluorimeter (Model LS 5). Absorption spectra were taken on a UV-visible spectrophotometer (Shimadzu Model 1201).

B. Thermal Lens Instrumentation

The single-beam thermal lens instrument used in this study is similar to that used in our previous studies.^{16–18} Essentially, the same cw Ti:sapphire laser was used for excitation. The laser beam, which was set at a constant wavelength of 780 nm and a power of 60 mW, was amplitude modulated by an electronic shutter and focused with a 5-cm focal-length lens. The distance between the lens and the sample cell was adjusted to achieve the optimal thermal lens signal. The laser beam emerging from the cell was detected with a silicon P–I–N photodiode through a pinhole and placed at a distance of ~ 70 cm from the sample cell. The shutter was adjusted such that each signal was monitored for at least three times the thermal relaxation time t_c , which in this case was approximately 2–5 ms. The signal from the photodiode was amplified and recorded by a personal computer. The thermal lens signals (θ) and the thermal time constants (t_c) were calculated by procedures and programs similar to those used previously.¹⁸

C. Chemicals

Rhodamine 6G (R6G) and fluorescein (laser grade, 99%) were purchased from Acros Organic Chemicals (Fischer Scientific Corporation).¹⁹ We prepared stock solutions of the dyes (1.0×10^{-3} M) by dissolving R6G in absolute ethanol and fluorescein in distilled water. The pH of the aqueous fluorescein solution was maintained with a 1.0×10^{-2} M borate buffer (pH, ~ 9 – 10). Other chemicals and solvents were obtained from Aldrich Chemicals and used as received.

3. Results and Discussion

Fluorescein and R6G were selected for this study because they belong to the xanthene type of dye, and these dyes are known to have high two-photon absorption cross sections.^{6,7} R6G was dissolved not in water but in an organic solvent such as ethanol, propanol, butanol, pentanol, and hexanol because this dye is known to undergo aggregation to form nonfluorescent dimers in water.²⁰ Fluorescein was dissolved in buffered aqueous solutions.

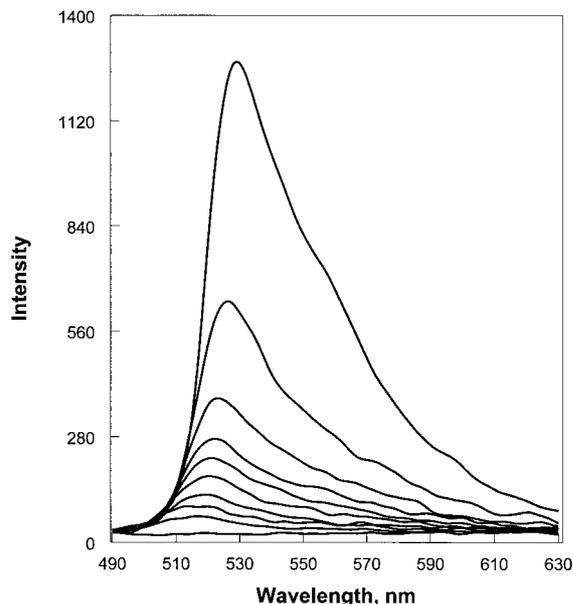


Fig. 1. TPF spectra of fluorescein at several concentrations (in 1.0×10^{-2} M borate buffer) excited with 1.1-W laser light at 770 nm.

TPF spectra of fluorescein in a borate buffer and of R6G in ethanol are shown in Figs. 1 and 2, respectively. We obtained these spectra by exciting the samples with 1.0 W of power from a cw excitation laser at 770 nm (for fluorescein) and 780 nm (for R6G). As expected, the spectra obtained are similar to those previously reported for these dyes obtained not with a cw laser but rather with a pulsed Ti:sapphire laser for excitation.⁶

Also shown in Figs. 1 and 2 are TPF spectra of fluorescein and R6G at several concentrations (from 1.0×10^5 to 1.0×10^{-3} M). As illustrated, fluo-

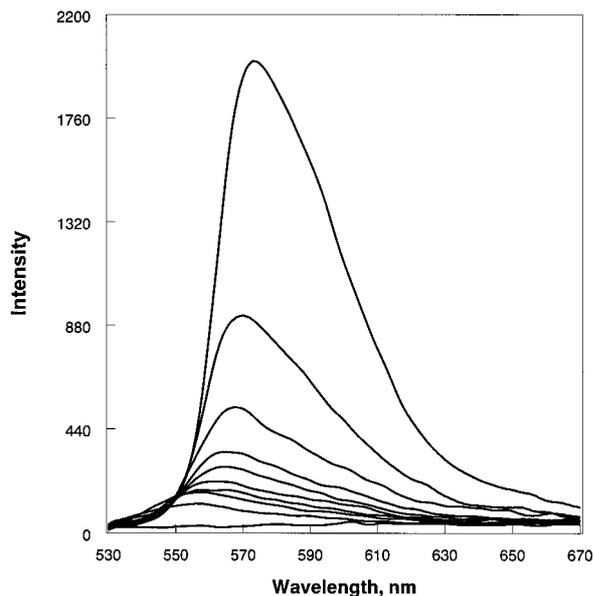


Fig. 2. TPF spectra of R6G in ethanol at several concentrations excited with 1.0-W laser light at 780 nm.

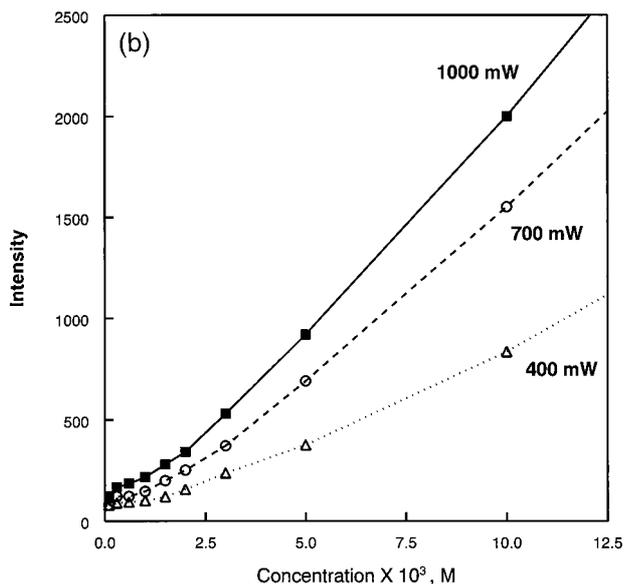
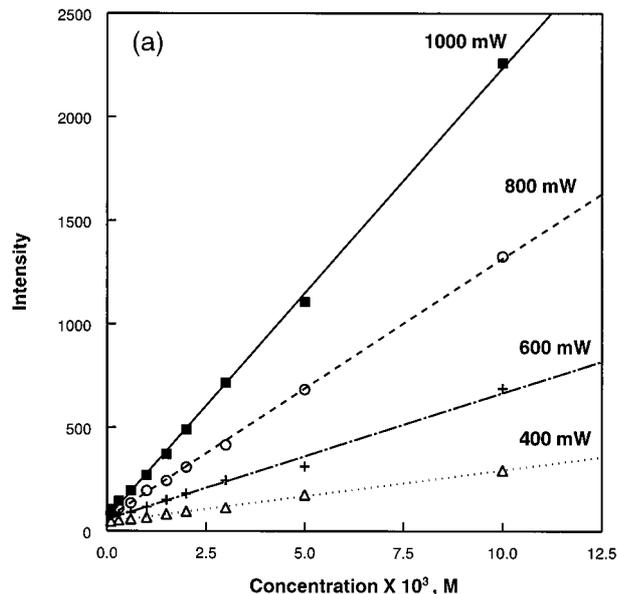


Fig. 3. (a) TPF intensity versus concentration for (a) fluorescein in a borate buffer at four constant excitation laser powers. (b) intensity versus concentration and (b) R6G in ethanol at three fixed excitation laser powers.

cein exhibited maximum intensity at 515 nm, whereas for R6G the maximum is at 555 nm. These values are in good agreement with reported values.^{21,22} Plots of the fluorescence intensity versus concentration for these dyes are shown in Figs. 3(a) and 3(b). As illustrated in Fig. 3(a), good linear relationships were obtained for fluorescein in water at several excitation powers (from 400 to 1000 mW). These results seem to indicate that, even at high concentrations (10^{-3} M), self-quenching, which would lead to negative deviation, was not present in this case. The close proximity between the cell wall (on the fluorescence detection side) and the excitation focal point may be partly responsible for the lack of self-quenching. It should also be noted that all

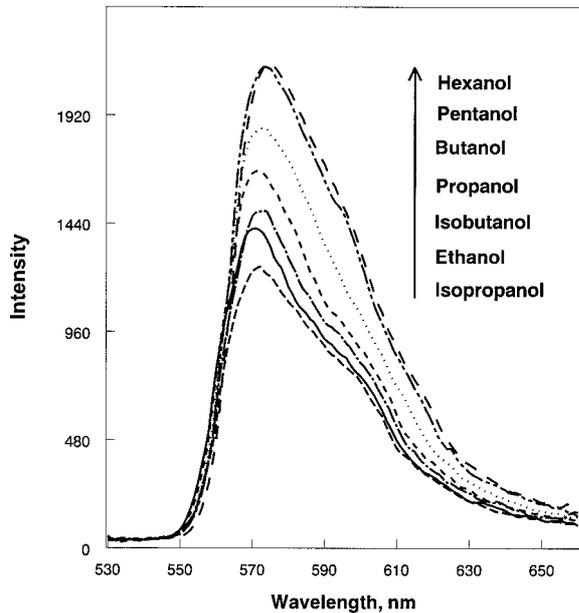


Fig. 4. TPA spectra of 1.0×10^{-3} M R6G in ethanol (— — —), propanol (— — —), isopropanol (— — —), butanol (....), isobutanol (— · —), pentanol (· — ·), and hexanol (— —) excited with 1.0-W laser light at 780 nm.

straight lines do not go through the origin; i.e., residual signals remain at low concentrations. As a consequence, it is relatively difficult to obtain a spectrum with a good signal-to-noise ratio at concentrations below 10^{-5} M.

Differently from fluorescein, no linear relationships were obtained for R6G in ethanol [Fig. 3(b)]. This result is of particular interest and cannot be attributed to the aggregation of R6G in ethanol because R6G is known to have no aggregation in this medium [i.e., the absorption spectra of the dye concentrations shown in Fig. 2 have similar shapes (spectra not shown)]. Additionally, the residual signals at low concentration are relatively higher than those for fluorescein in water. It is therefore possible that the deviation from linearity is due not to R6G but rather to the differences between the properties of water (in which linearity was observed for fluorescein) and ethanol. To evaluate this possibility, we measured TPF spectra of R6G in various solvents including ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, and hexanol, (Fig. 4). It is of particular interest to observe that, even with the same dye concentration, the TPF intensity is highest in hexanol and lowest in isopropanol. The intensity seems to increase concomitantly with an increase in the length of the alkyl group. For comparison, one-photon fluorescence spectra of R6G in these solvents were also measured (not shown). It was found that, within experimental error, all solutions exhibit the same fluorescence intensity. This is as expected because the concentrations of R6G in these solutions were the same and any differences in the absorption at the excitation wavelength (as a result of the shifts of the absorption spectra in different solvents) were cor-

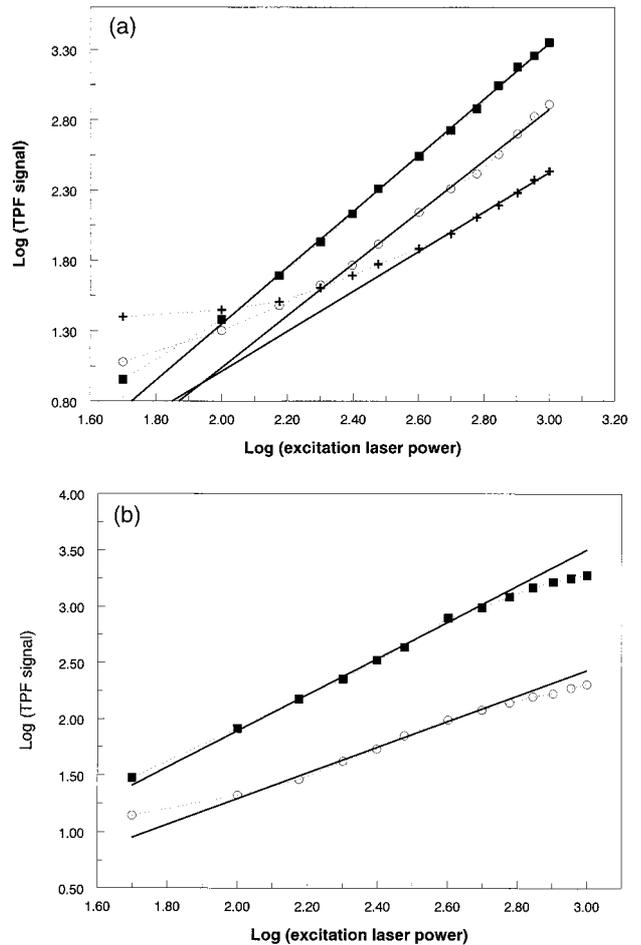


Fig. 5. (a) Log(TPF intensity) versus log(excitation laser power) for three solutions of fluorescein in water at three concentrations: 1.0×10^{-4} (+), 3.0×10^{-4} (O), and 1.0×10^{-3} M (■). (b) Log(TPF intensity) versus log(excitation laser power) for two solutions of R6G in ethanol at two concentrations: 1.0×10^{-4} (O) and 1.0×10^{-3} M (■). Solid lines, straight lines obtained by linear regression.

rected in these spectra. It should be noted that there are small differences in the viscosity of these solvents but that the differences seem to be too small to produce any observable effect in the fluorescence spectra. The fact that the one-photon fluorescence intensity is the same whereas TPF intensity changes among the various solvents seems to suggest that the differences may be due to the interaction of the solvents with a long and strong cw laser excitation laser beam.

To gain more insight into this interesting solvent effect we also investigated the dependency of the intensity of the TPF on the excitation power. Figures 5(a) and 5(b) show results obtained at several different concentrations and different excitation powers for fluorescein in water and for R6G in ethanol, respectively. As illustrated, a relatively good linear relationship (with a slope of 1.994 and correlation coefficient of 0.9993) was obtained for fluorescein at 1.0×10^{-3} M at excitation powers of 100–1000 mW [Fig. 5(A)]. As the dye concentration decreased, not only did the slope become smaller [slope of 1.83 ($R^2 =$

Table 1. Thermo-Optical Properties and Thermal Lens Signals of Several Solvents

Solvent	k (mW cm ⁻¹ K ⁻¹)	$-dn/dT \times 10^4$ (K ⁻¹)	A^a	$-A(dn/dT)/\lambda k$	θ
Ethanol	1.66	4.00	0.0238	7.35	0.31 ± 0.01
Propanol	1.57	3.72	0.0170	5.16	0.28 ± 0.01
Isopropanol	1.40	4.10	0.0191	7.17	0.310 ± 0.005
Butanol	1.51	3.90	0.0104	3.44	0.240 ± 0.008
Isobutanol	1.39	3.90	0.0172	6.18	0.28 ± 0.01
Pentanol	1.37	4.20	0.0127	4.99	0.21 ± 0.02
Hexanol	1.45	4.00	0.0088	3.11	0.197 ± 0.008
Water	5.95	0.81	0.0825	1.44	0.048 ± 0.005

^aMeasured in a 10-cm path-length cell at 780 nm.

0.9958) for 3.0×10^{-4} M and slope of 1.6 ($R^2 = 0.9957$) for 1.0×10^{-4} M] but the range of the powers in which linear relationships were obtained also became narrower (from 100 to 1000 mW for 1.0×10^{-3} M, from 250 to 1000 mW for 3.0×10^{-4} M, and from 400 to 1000 mW for 1.0×10^{-4} M).

For R6G in ethanol [Fig. 5(b)], the slope is generally smaller than 2, and a pronounced negative deviation is observed at a higher excitation power. Such a negative deviation was previously observed for TPF measured with a pulsed laser excitation. It has been suggested that a variety of processes including stimulated emission, excited-state absorption, and excited-state saturation could account for the negative deviation at higher excitation power. However, whereas these processes are important in pulsed laser excitation with high-power excitation [approximately 10^{31} – 10^{32} photons/(cm² s)], they are not so significant in the present study, which is based on cw laser excitation with a much lower photon flux [$\sim 10^{24}$ photons/(cm² s)].

Taken together, the results presented here suggest that the differences in the TPF intensity in different solvents and the deviation from linearity could be due to differences in interactions between the solvents and the long-duration and intense cw laser excitation laser beam. The photoinduced thermal lens effect may be responsible for this observation. In fact, it was reported recently that photothermal expansion is partly responsible for poor detection limits of TPF (excited by a picosecond laser) in capillaries.²³ All the solvents used in this study (water, ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, and hexanol) possess an O–H group, and this group is known to absorb the 780-nm excitation laser light (through the overtone and combination bands of the O–H group).^{15,24} It is therefore possible to produce a thermal lens effect in a sample when the sample is in a solvent with good thermo-optical properties and is excited by a high-power (and relatively long duration compared with that of a pulsed laser) cw laser. To evaluate this possibility we measured thermal-lens signals θ in water, ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, and hexanol; the results obtained are listed in Table 1. For comparison, $A(dn/dT)/\lambda k$ values for these solvents, calculated from dn/dT , thermal conductivity (k), and absorbance values, are also listed.^{25,26} As expected, the variation in measured

thermal-lens signal θ agrees well with that of the calculated $A(dn/dT)/\lambda k$. This is as expected because θ is related to A , dn/dT , λ , and k by

$$\theta = -2.303 \frac{AP_0(dn/dT)}{\lambda k},$$

where P_0 is the excitation laser power.^{16–18} Furthermore, the thermal-lens signal was found to be lowest in water and highest in isopropanol. Among the organic solvents, the θ value decreases as the alkyl group becomes larger.

It is particularly interesting to inspect Fig. 6, which plots the thermal-lens signal against the two-photon fluorescence intensity in several solvents. As illustrated, the thermal-lens signal seems to be inversely proportional to the two-photon fluorescence intensity, that is, in isopropanol the thermal-lens signal was highest but the TPF intensity was lowest, whereas hexanol provided the highest TPF but the lowest thermal-lens signal. This observation can be explained by the fact that, when a thermal lens is created in a sample, the excitation laser beam at the focal plane in the sample will be defocused; i.e., the beam spot size will be larger. This, in turn, will

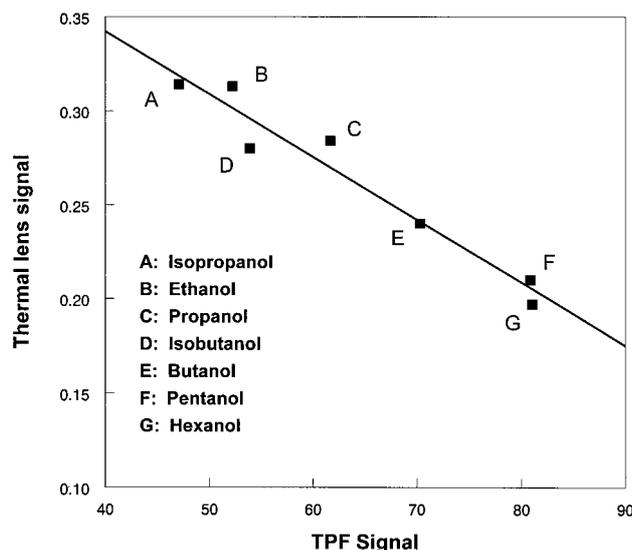


Fig. 6. Thermal-lens signal versus TPF intensity for R6G in seven solvents.

lower the intensity of the TPF because the two-photon fluorescence depends on the power of the excitation beam; i.e., the TPF intensity is inversely proportional to the beam spot size of the excitation beam in the sample. In a solvent such as ethanol or isopropanol with good thermo-optical properties, for the same excitation power the thermal-lens signal generated will be larger, and, as a consequence, the TPF intensity will be lower because the defocusing effect by the thermal lens is larger (i.e., the beam spot size will be larger). The TPF intensity is therefore inversely proportional to the thermal-lens signal. Such a thermal-lens effect is more pronounced when a cw laser is used for excitation. This is so because the time scales of these thermal effects agree within milliseconds, and therefore they can easily be generated and detected with cw laser excitation. Conversely, the thermal-lens signal will be small and difficult to detect when a picosecond or a femtosecond pulsed laser is used for excitation.

4. Conclusion

In summary, a cw excitation laser has been used for two-photon-excited fluorescence. However, there is a concern that cw laser excitation may produce a thermal-lens effect, which, in turn, may interfere with the TPF signals. The results of this study have shown that a thermal lens is produced only when measurements are performed in solvents that absorb the cw laser excitation light (through the overtone and combination transitions). Furthermore, even when such transitions are present, under the TPF measurement conditions a thermal lens is observed only in solvents (e.g., organic solvents) that have relatively better thermo-optical properties. For example, thermal-lens interference was not observed in water even though water absorbs at 780 nm (through the overtone and combination transitions of the O—H group). This is so because water has relatively poor thermo-optical properties. Inasmuch as water is the most widely used solvent for applications in which the TPE technique is used, it is therefore expected that thermal-lens interference will not limit applications of this cw laser excitation TPF technique.

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