Stimulated emission depletion following two photon excitation.

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ABSTRACT

The technique of stimulated emission depletion of fluorescence (STED) from a two photon excited molecular population is demonstrated in the S_1 excited state of fluorescein in ethylene glycol and methanol. Two photon excitation (pump) is achieved using the partial output of a regeneratively amplified Ti:Sapphire laser in conjunction with an optical parametric amplifier whose tuneable output provides a synchronous depletion (dump) pulse. Time resolved fluorescence intensity and anisotropy measurements of the fluorescein emission are made using picosecond time-correlated single photon counting. Pump–dump time delayed fluorescence intensity measurements are used to characterise the response of the system and to provide additional data on saturation dynamics of the dump transition. Two photon STED is modelled using both approximate analytical techniques in the weak dump limit and by numerical solutions to the appropriate rate equations. The latter are used to fit experimental data from which it is possible to determine the cross-section for the stimulated transition and lifetime of the upper vibrational levels of the ground state.

Keywords: two photon, stimulated emission, fluorescence lifetime, anisotropy, vibrational relaxation, fluorescein.

1. INTRODUCTION

Stimulated emission depletion (STED) from excited states prepared by single photon excitation has proved to be both a valuable tool in high resolution molecular spectroscopy[1], in time resolved spectroscopy as a means of orientational photoselection[2] and in the study of ultrafast vibrational relaxation within electronic ground states[3]. There has been considerable interest in the use of single photon STED in fluorescence microscopy[4] where sub wavelength image resolution has been recently demonstrated[5]. The advantages of employing two-photon excited fluorescence in both time resolved photochemistry and optical microscopy are similarly well established[6-7]. The potential advantage of combining these techniques in both spectroscopy, imaging and dynamics makes the development of two photon STED an attractive goal.

1.1 Stimulated emission from two photon excited states

A schematic representation of the two photon STED process in a condensed phase molecular probe such as fluorescein is shown in figure 1. Initial excitation from low lying vibrational levels in the S_0 ground state is via the simultaneous absorption of two (non resonant) near infra red photons (c.a. 800nm) with rapid relaxation to vibrationally excited levels in the S_1 excited electronic state. Sub picosecond collisional relaxation with solvent molecules leads to the rapid population of lower vibrational levels in S_1. In the absence of external perturbations the population in S_1 decays by spontaneous emission (c.a. 10^{-9}s) to upper vibrational levels of S_0 consistent with the Franck Condon principle. Solvent deactivation of the vibrationally hot ground state population is correspondingly rapid.

In STED following the establishment of the fluorescent population in S_1, a visible laser pulse resonant with the $S_1 \rightarrow S_0$ emission is applied to induce transitions to the upper vibrational levels of S_0. The net result of this process is a sharp reduction in the excited state population and a loss in fluorescence intensity (the stimulated emission being collinear with the dump pulse). The efficiency of the STED process depends on a number of factors, firstly the dump wavelength whilst resonant with the emission must be sufficiently red shifted from the single photon absorption maximum so as to avoid the excitation of fluorescence from unexcited molecules within the interaction volume of the sample. A second consideration is the polarisation dependence of the STED process, two photon excitation in an isotropic medium creates a significantly more sharply peaked angular distribution of molecular transition dipole moment directions in the laboratory frame than in single photon excitation ($\cos^4\theta$ vs. $\cos^2\theta$).

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The interaction between the pumped population and the dump field is obviously maximised for parallel pump and dump field polarisations. In addition the pump-dump time separation needs to be sufficiently short to minimise the effects of orientational averaging within the excited state distribution. More critical however are the constraints imposed by the intrinsic molecular photophysics, as will be seen the degree of excited state depletion is strongly dependent on the dump photon flux (i.e. pulse energy). However the time over which the dump field is applied is of critical importance, molecules placed into the upper vibrational levels of S_0 can be re-pumped into S_1 if there is insufficient time for their subsequent relaxation. Optimisation of the dump pulse width to avoid saturation is crucial for efficient STED. As will be seen fluorescence intensity and polarisation measurements can yield information on both the stimulated emission cross section and the relaxation time of the ground state levels.

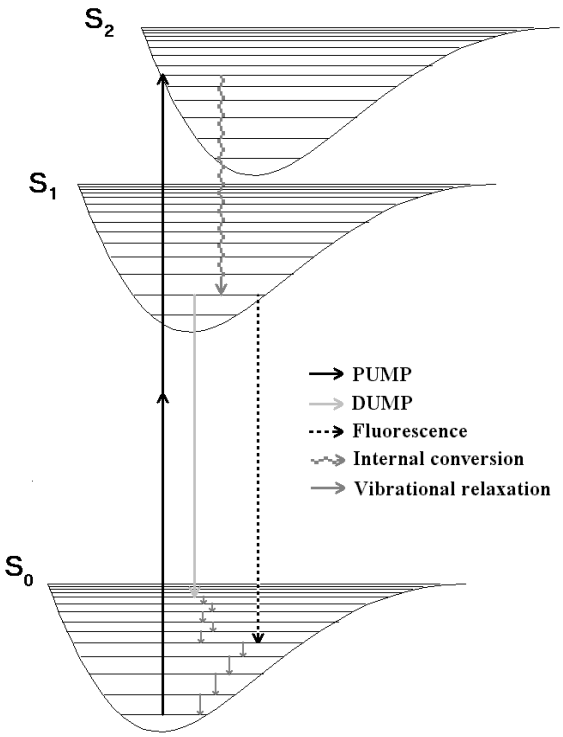

Figure 1. Excitation scheme for STED following two photon absorption. The dump laser is red shifted from the emission maximum to minimise contamination of the fluorescence signal by dump induced single photon fluorescence. Fluorescence depletion at shorter wavelengths is observed through a series of short pass interference filters to avoid scattered pump or dump photons reaching the detector.

2. EXPERIMENTAL PROCEDURE

The experimental apparatus for STED is illustrated in figure 2.The experimental sample, a 5×10^{-4} M solution of fluorescein sodium salt (Sigma Aldrich) in methanol or ethylene glycol was contained in a 1cm square quartz cuvette (Hellma) with four optical windows. Two photon excitation of the sample was achieved using the partial output of a regeneratively amplified Ti:Sapphire laser (Coherent Mira 900F, Coherent RegA 9000) which provided 800nm laser pulses with ca.60nJ energy and 140fs FWHM pulse width at a repetition rate of 250kHz. The majority of the 800nm output was used to pump an optical parametric amplifier (Coherent OPA 9400) which could provide a synchronous (c.a. 250fs) pulse train tuneable across the visible spectrum. By examining the emission spectrum together with the single and two photon absorption spectra of the experimental sample, a dump wavelength of 580nm was chosen. There is strong emission from fluorescein at this wavelength (c.a. 20% of that at λ_{MAX}) whilst having a low cross section for

single and two photon absorption. To prevent saturation at low dump energies (c.a. 1nJ) and dump induced two photon fluorescence, the OPA pulses were stretched using the material dispersion provided by passage through a distilled water cell. With this arrangement OPA pulses spanning the range from 250fs to 2.5ps could be produced. The pump and dump wavelengths were verified using a laser spectrum analyser (IST-Rees), and the pulse widths monitored using a scanning autocorrellator (APE). Pump and dump beam polarisations were controlled using broadband halfwave plates (Melles-Griot). The pump-dump separation could be set and scanned using a variable optical delay line (Time & Precision). Pump and dump powers were controlled by neutral density wheels and measured by a precision power meter (Anritsu). Prior to the experimental sample the pump and dump beams were spatially overlapped using a broadband dichroic beam combiner (CVI Optics).

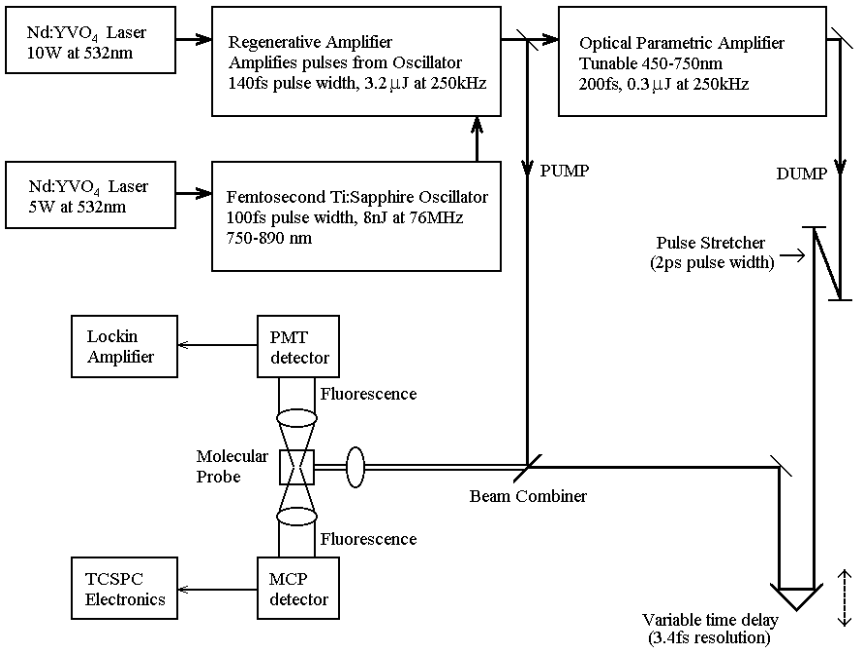

Figure 2. Diagram of the layout of the experimental apparatus showing different detection systems for the time averaged and time resolved STED measurements.

The pump and dump pulses were passed through an aperture of fixed diameter (φ=2.2mm) and focused into the sample using a 2.54cm focal length achromat (Melles Griot). Scattered laser light was blocked using an interference filter (Corion LS550) and glass cut-off filters (Corion BG36, Corion BG39). A 300 μm slit was used to isolate the waist region of the beams. Fluorescein emission was detected at 90° to the excitation and depletion beams, time resolved fluorescence lifetime and anisotropy measurements at fixed pump dump delays were performed using a picosecond time-correlated single photon counting system which has been described in detail elsewhere[8]. Total fluorescence intensity measurements as a function of the pump-dump separation were made using a conventional photomultiplier (Thorn EMI) in conjunction with a computer controlled lock in amplifier and optical chopper (Stanford Research Systems).

Picosecond TCSPC detection of STED in two photon excited fluorescein is shown in figure 2. Excited state population measurements were constructed from the vertical and horizontally polarised components of the fluorescence emission[9]. With a pump-dump delay of 636ps a 50% depletion in the excited state population of the excited state can be observed (figure 3a). With parallel pump and dump polarisations a significant change in excited state order following selective depletion of molecules oriented parallel to the dump polarisation is observed (figure 3b). The fluorescence polarisation is seen to suffer an abrupt change of 0.18. Measurements of total fluorescence depletion as a function of pump-dump delay can also be taken as described above, a typical result for fluorescein at below the onset of significant saturation in the dump transition is shown in figure 4. The total fluorescence intensity drops sharply over a 2 picosecond pump-dump delay. Differentiation of the transient is well fit by a Gaussian function of comparable width to the dump autocorrelation indicating that the relaxation processes within the S_1 excited state occur on a subpicosecond time scale.

As discussed above the efficiency of the STED process depends on a number of factors. Assuming no contamination of the fluorescence signal by dump induced single or two photon fluorescence and the absence of excited state absorption, all of which are in general minimised by the appropriate wavelength selection. These are, the degree of order remaining in the excited state when the dump pulse is applied, the dump pulse energy and crucially the degree of saturation of the dump transition resulting from the finite lifetime of the upper vibrational levels in the S_0 ground state. This will be discussed in detail later.

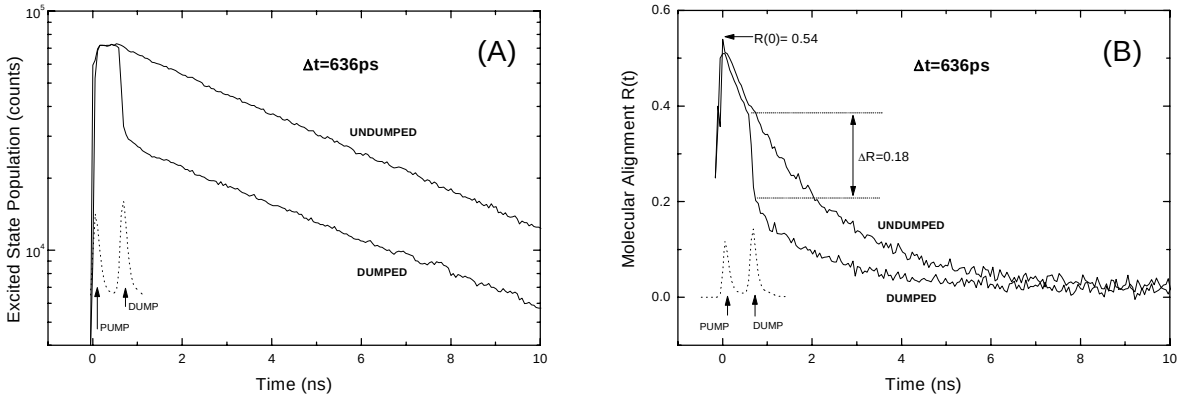

Figure 3. (a) Stimulated emission depletion (STED) in fluorescein molecules following two photon excitation with 212fs pump pulses (61nJ, 800nm). Half the excited state population is removed 636ps later by a 1.7ps dump pulse (16nJ, 580nm). (b): Modification of molecular alignment using STED. Selective removal of molecules oriented parallel to the dump polarisation results in an abrupt change to the degree of excited state alignment ($\Delta R = 0.18$).

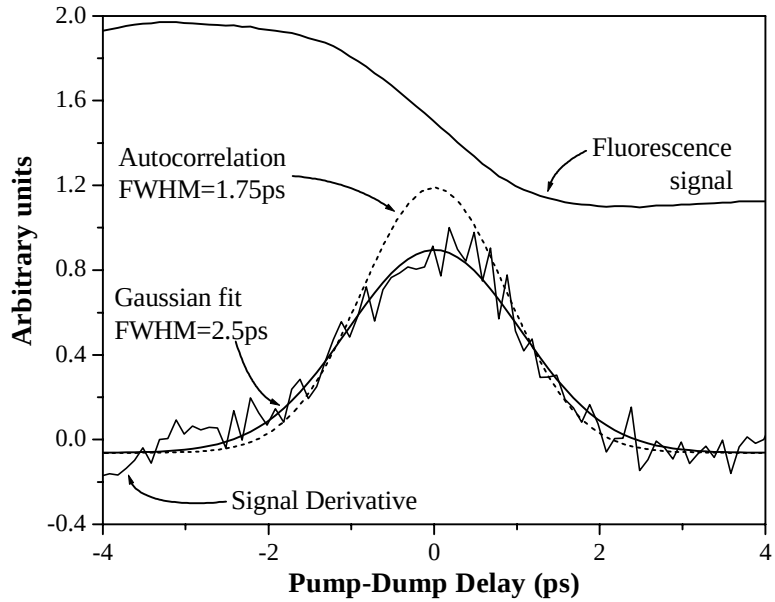

Figure 4: Variation in the total fluorescence emission from fluorescein as a function of pump-dump delay using a 1.75ps dump pulse at 580nm. The 'rise time' of the depletion is compatible with the with the dump pulse width, differentiation of the depletion signal yields an approximate Gaussian signal whose width is compatible with that of the dump pulse autocorrelation.

3. WEAK DEPLETION ANALYSIS

In the limit of weak excited state depletion and rapid relaxation of the ground state vibrational levels involved in the dump transition, the population distribution of molecules remaining in the excited state after the passage of the dump pulse $N_D(\theta,\phi)$ can be related to the distribution immediately preceding, $N_U(\theta,\phi)$, by [9,10]:

$$N_D(\theta,\phi) = N_U(\theta,\phi)(1-\alpha\cos^2\theta) = N_U(\theta,\phi)\left[1-\frac{\alpha}{3}\sqrt{4\pi}\left(Y_{00}+\frac{2}{\sqrt{5}}Y_{20}\right)\right] \quad (1)$$

Where α describes the strength of the interaction with the dump laser. Here the angular dependence of the interaction has been expanded in terms of the spherical harmonic functions Y_{KQ}. For a fluorescent probe molecule in an isotropic environment small step orientational relaxation is well described by the Debye equation[11], the time evolution of the excited state distribution after the ultrafast two photon excitation process is given by[9,10]:

$$N_U = N_U^0(\theta,\phi)\frac{1}{\sqrt{4\pi}}\left[Y_{00}+\frac{20}{7\sqrt{5}}Y_{20}\exp\left(-\frac{t}{t_{20}}\right)+\frac{8}{21}Y_{40}\exp\left(-\frac{2t}{t_{20}}\right)\right]\exp\left(-\frac{t}{t_f}\right) \quad (2)$$

Here $N_U^0(\theta,\phi)$ is the initial excited state distribution, t_{20} is the isotropic rotational diffusion time (assuming single axis rotational diffusion) and t_f is the fluorescence lifetime. The presence of the Y_{40} term in (2) is as a direct consequence of the inherent quadrupolar symmetry of the two photon excitation process [12]. The proportion of molecules remaining in the excited state F_R is obtained by the angular average of the ratio [9,10]

$$F_R = \frac{\int N_D d\Omega}{\int N_U d\Omega} \quad (3)$$

Insertion of (1) and (2) into (3) yields

$$F_R = 1-\frac{\alpha}{3}\left(1+\frac{8}{7}\exp\left(-\frac{t}{t_{20}}\right)\right) \quad (4)$$

The Y_{40} contribution vanishes due to the orthogonality of spherical harmonics. The time varying contribution in (4) is proportional to the degree of excited state alignment (and in turn, the fluorescence anisotropy R(t)). For two photon fluorescence

$$R(t) = \frac{4}{7}\exp\left(-\frac{t}{t_{20}}\right) \quad (5)$$

Combining (4) and (5) and rearranging the depletion parameter α is given by.

$$\alpha = 3(1-F_R)/(1+2R_U) \quad (6)$$

where R_U is the fluorescence anisotropy (at time t) immediately before dumping. By comparing equation (6) with the solution to the full rate equations for stimulated emission depletion (see section 6) it can be seen that in the 'linear regime' where saturation can be neglected, α is simply the saturation parameter (S). S can be thus determined by experimental measurement of F_R and R_U as a function of dump energy. Outside the linear depletion regime limit α is expressed as a power a power series in both S and $\cos^2\theta$ [9,10].

4. RATE EQUATION ANALYSIS

For situations where the dump pulse is sufficiently intense that significant saturation of the dump transition occurs the above analysis does not hold. If significant depletion of fluorescence is required, say to cause large change in the excited state alignment or to suppress fluorescence from a specific spatial region such as in a microscope then an extremely long dump pulse is required to avoid saturation at the necessarily high pulse energy [7]. This may be difficult to realise experimentally. In these circumstances numerical solution or the rate equations is required to accurately model the processes involved. Provided the depleting laser pulse is sufficiently short that no significant reorientation of the molecules occurs during the STED process, the time evolution of the populations of the first excited (N_e) and upper vibrational ground (N_g) states can be described by;

$$\frac{\partial N_e(\theta,t)}{\partial t} = \frac{\sigma I_d(t)\cos^2\theta}{\hbar\omega}\left(N_g(\theta,t) - N_e(\theta,t)\right) - \frac{1}{t_f}N_e(\theta,t) \tag{7}$$

$$\frac{\partial N_g(\theta,t)}{\partial t} = \frac{\sigma I_d(t)\cos^2\theta}{\hbar\omega}\left(N_e(\theta,t) - N_g(\theta,t)\right) - \frac{1}{t_r}N_g(\theta,t) \tag{8}$$

where $I_d(t)$ is the time varying intensity of the dump pulse and t_f is the fluorescence lifetime and t_r the relaxation time of the vibrational levels in the ground state, for STED with parallel pump and dump polarisations cylindrical symmetry is preserved and there is no explicit ϕ dependence in the rate equations. Simplification of these expressions is achieved by assuming a 'square' shape to the dump laser pulse of duration t_p. Additionally the effect of fluorescence on depleting the excited state population can be ignored, as $t_f >> t_p, t_r$ in this limit (7) and (8) reduce to

$$\frac{\partial N_e(\theta,t)}{\partial t} = \frac{\sigma E_d\cos^2\theta}{\hbar\omega A\, t_p}\left(N_g(\theta,t) - N_e(\theta,t)\right) \tag{9}$$

$$\frac{\partial N_{g(\theta,t)}}{\partial t} = \frac{\sigma E_d\cos^2\theta}{\hbar\omega A\, t_p}\left(N_e(\theta,t) - N_g(\theta,t)\right) - \frac{1}{t_r}N_g(\theta,t) \tag{10}$$

where E_d is the energy of the dump pulse and A is the interaction area. The adoption of the 'square pulse' approximation allows for an analytical solution of the rate equations. With the starting condition of a negligible population of the upper ground state vibrational levels ($N_g(\theta,0) = 0$), then the solution for the excited state population evaluated at $t = t_p$ is;

$$N_e(\theta,t_p) = N_e(\theta,0)\frac{\exp\left(-\left(t_p/t_r + 2S\cos^2\theta + d\right)/2\right)}{2d}\left[\frac{t_p}{t_r}\left(\exp(d)-1\right) + d\left(\exp(d)+1\right)\right] \tag{11}$$

where the saturation parameter S and the parameter d are given by;

$$S = \sigma E_d/\hbar\omega A \tag{12}$$

$$d = \sqrt{\left(t_p/t_r\right)^2 + 4S^2\cos^4\theta} \tag{13}$$

The degree of alignment of the molecules in the excited state is dependent on the pump-dump delay, the evolution of which is given is given in equation (2). This orientational probability distribution evaluated at t_d is then used to replace $N_e(\theta,0)$ in the solution of the rate equation (11) to give both the population and orientational distribution of excited molecules at the instant after STED. The relative intensities of the vertically (I_V) and horizontally (I_H) polarised components of the fluorescence can then be obtained by integrating over the orientational probability distribution weighted by the appropriate emission transition probability[13]

$$I_V = C\int_0^{\pi}\int_0^{2\pi} N_e(\theta, t_p, t_d)\cos^2\theta \sin\theta d\phi d\theta \quad (14)$$

$$I_H = C\int_0^{\pi}\int_0^{2\pi} N_e(\theta, t_p, t_d)\sin^2\theta \sin^2\phi \sin\theta d\phi d\theta \quad (15)$$

The proportionality constant in (14) and (15) contains transition probability factors and a component that decays with the fluorescence lifetime. From these expressions both the change in population and the change in anisotropy due to STED can be calculated for a variety of pump-dump delays, dump pulse widths and energies.

5. STIMULATED EMISSION DEPLETION IN FLUORESCEIN

In the weak depletion limit and assuming rapid ground state relaxation the saturation parameter S in (11) is given by[10]:

$$S \cong \alpha = 3(1 - F_R)/(1 + 2R_U) \quad (16)$$

Substitution of equation (12) yields

$$3(1 - F_R)/(1 + 2R_U) = \sigma E_d/\hbar\omega A \quad (17)$$

Figure 5 shows a plot of α against E_d for the fluorescein in methanol using a dump pulse width of 700fs and a pump-dump delay of 1ns. From the data it is clear that linear depletion holds for dump pulse energies up to a limit of 7-8 nJ after which the effects of saturation become obvious. From (17) a linear fit over this range will have a gradient equal to $\sigma/\hbar\omega A$. By fitting the fist four points of figure 5 in this way a value for σ of 2.78×10^{-16} cm^2 was obtained. As will be seen this is consistent with more detailed fits to both time resolved and total fluorescence measurements.

Time correlated single photon counting measurements of fluorescence intensity and anisotropy in the absence and presence of the dump pulse allow the detailed measurement of both the excited state population and anisotropy changes for a given set of dump characteristics (i.e. pump dump separation, dump energy and dump pulse duration). Plots of the population and anisotropy changes induced in fluorescein in methanol are displayed in figure 6 together with the corresponding simulations generated using (11). A dump pulse width of 700fs and a pump-dump delay of 1ns were employed as above. Rotational diffusion of fluorescein in methanol is rapid (c.a. 200ps). Given an evolution time of 1ns the rotational relaxation in the excited state is almost complete when the dump pulse was applied and the altered fluorescence anisotropy (R_D) at high dump energies was negative. Simulations based on a ground state relaxation time of 636fs and a stimulated emission cross section of $1.4\times10^{-16}cm^2$ are a good fit to the both the population and anisotropy data.

Provided the dump delay is sufficiently short so that there has been little population loss in the excited state from fluorescence, total intensity measurements (at the 'magic angle' polarisation) of the emission can be used to determine the fractional population loss in an equivalent manner to the TCSPC studies above. Given the statistical requirements of single photon counting[14] (maximum count rate 1% of laser repetition rate), a 250KHz repetition rate imposes a finite minimum collection time. Total intensity measurements when made in conjunction with modulation techniques allow for low noise data collection and background removal and a significantly reduced collection time[15]. Total fluorescence depletion measurements for fluorescein in ethylene glycol were made using a dump delay of 33ps, significantly shorter than the 2ns lifetime of fluorescein as determined by fits to the unperturbed intensity decay in figure 3(a). The fractional population loss arising from STED was calculated as a function of the dump energy for dump pulse widths of 710 fs 1.45ps and 2.5ps. The results are shown in figure 7 together with the results of numerical simulation of the fractional population removal using (11). For each pulse width data set the values of σ and t_r where varied for the best fit to the experimental points. Good agreement between experiment and theory is observed but there is some variation in the values of the 'adjustable' parameters (σ and t_r), these values are shown in table 1. Average values for σ and t_R of $3.6\pm1.0\times10^{-16}cm^2$ and 710 ± 90 fs respectively were obtained.

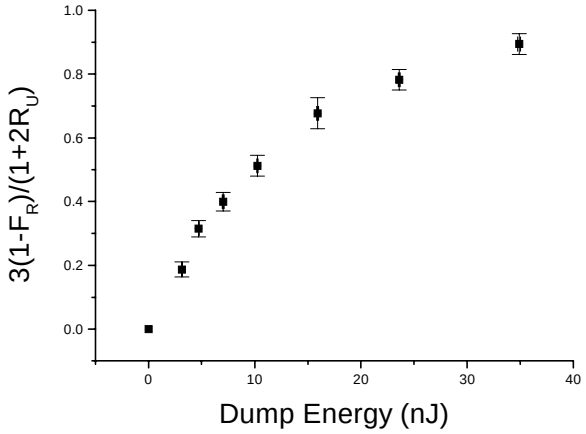

Figure 5. Variation in the 'dumping' parameter α with dump pulse energy for fluorescein in methanol. The data was obtained from TCSPC measurements as in figure 3. Depletion of the excited state is approximately linear in the dump pulse energy up to c.a. 7-8 nJ. A dump pulse width of 700fs and a pump–dump separation of 1ns were employed.

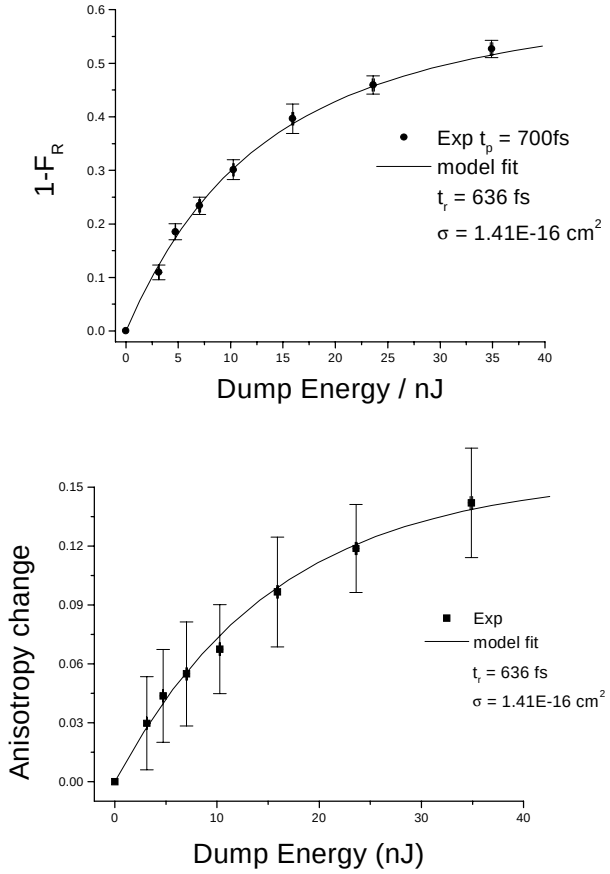

Figure 6. Comparison of experimental and simulated data of fractional population loss and anisotropy change for fluorescein in methanol. Values of the 'adjustable' parameters are indicated on the graphs. A dump pulse width of 700 fs and pump-dump delay of 1ns were employed.

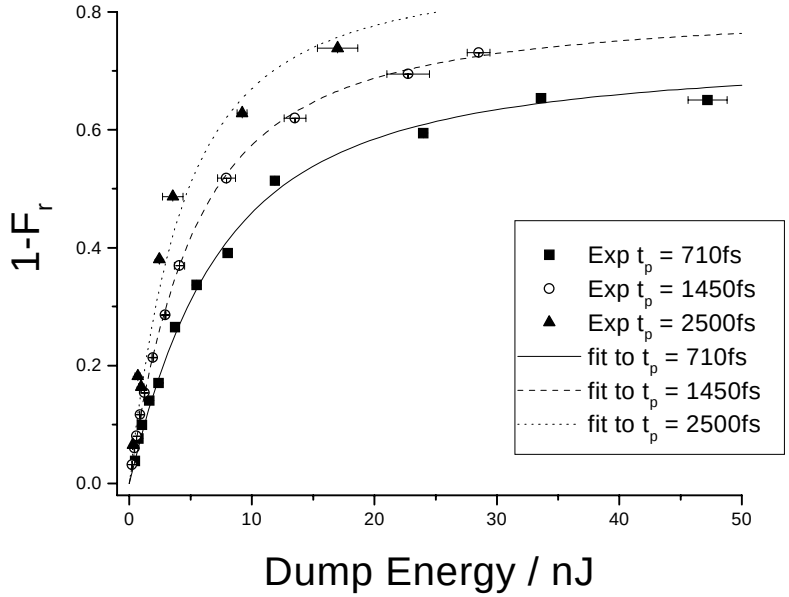

Figure 7. Experimental measurements of the fractional population removal for fluorescein in ethylene glycol as a function of dump energy for dump pulse widths of 700fs, 1.45ps and 2.5ps. The lines represent simulated data using equation (11). The transition cross-section σ and vibrational relaxation time t_r where adjusted for best fit in each case and are displayed in table 1.

$\sigma \times 10^{-16}(cm^2)$	$t_R \times 10^{-15}s$	$t_P \times 10^{-12}s$
3.26	690	1.45
3.55	725	1.45
2.32	591	0.71
5.10	833	2.50

Table 1. Summary of the values obtained for σ and t_R from fits of equation (11) to the fractional depletion–dump energy plots in figure 7. The average values for σ and t_R are $3.6\pm1.0\times10^{-16}cm^2$ and 710 ± 90fs respectively.

6. CONCLUSIONS

Two colour two photon stimulated emission depletion has been successfully demonstrated in fluorescein in both ethylene glycol and methanol solutions using both fully time resolved fluorescence intensity and polarisation measurements together with total fluorescence depletion measurements. Fluorescence loss measurements as a function of pump-dump delay indicate that relaxation within the S_1 excited state following two photon excitation occurs on a sub picosecond time scale. Theoretical analysis of the STED process has shown that in the linear regime fluorescence loss and anisotropy measurements can be used to determine the stimulated emission cross section σ. Application of a full rate equation analysis is needed to characterise stronger dump interactions with the excited state. From this approach it has been possible to analyse fluorescence intensity and anisotropy data and to determine both σ and the relaxation times for the upper ground state vibrational levels involved in the depopulation process. The cross sections obtained (1.4×10^{-16} cm^2 -2.78×10^{-16} cm^2 in methanol and $3.6\pm1.0\times10^{-16}cm^2$ in ethylene glycol) are commensurate with those expected for strongly allowed visible electric dipole transitions in an organic dye [16]. To within experimental error the values for the ground state vibrational relaxation t_R in both solvents are similar, 636fs for methanol and 710±90fs in ethylene glycol. The observed range of ground state relaxation times are somewhat longer than have been reported for other systems[3] however the dump wavelength (580nm) used in these studies was distant from the long wavelength tail of the emission spectrum. Selection of a longer wavelength would give rise to the population of the uppermost vibrational states of S_0

where collisional relaxation would be at a maximum. A study of the wavelength dependence of two photon STED in fluorescein and other systems is currently underway.

ACKNOWLEDGEMENTS

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