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Monitoring the excited state of a fluorophore in a microscope by stimulated emission

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Abstract. A two-pulse experiment is described, using stimulated emission to reduce the fluorescence of 1-ethyl-4- (4-(p-dimethylaminophenyl)-1,3-butadienyl)-pyridinium perchlorate (Pyridine 2) in a microscope of high numerical aperture. The experiment employed a 130 fs pulse at 375 nm for excitation and a 20 ± 5 ps pulse at 750 nm for stimulated emission. The pulses were provided by a mode-locked Ti:sapphire laser. The 375 nm excitation pulse was obtained by frequency-doubling and the 20 ± 5 ps infrared pulse by optical grating dispersion. The population of the excited state was monitored by varying the temporal delay between the excitation and stimulating pulse. This method enabled the measurement of the lifetime of the dye. For Pyridine 2 in glycerol, we determined a lifetime of 0.86 ± 0.2 ns. The decrease of fluorescence was due to stimulated emission, as was established by measurements of the temporal behaviour of the fluorescence signal upon amplitude modulation of the stimulating beam. We present a theoretical analysis of the temporal behaviour of depletion by stimulated emission in a two-pulse experiment.

Keywords: fluorescence microscopy, confocal, stimulated emission, lifetime, STED.

1. Introduction

The depletion of the excited state of a dye by stimulated emission offers a new perspective in fluorescence microscopy. As in the case of multi-photon excitation, stimulated emission is likely to become a new tool in this rapidly developing field [1, 2], with the first and perhaps most fascinating application being the improvement of resolution beyond the diffraction limit [3]. Given that the product of two offset point-spread-functions (PSF) is narrower than a single PSF [4], offset beams for excitation and stimulated emission can be used to decrease the effective focus field of a scanning fluorescence microscope. In our proposal for a STED-fluorescence microscope [3] we showed the possibility of depleting the excited state of a dye in a two-beam arrangement with consecutive pulses for excitation and depletion.

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Stimulated emission is the basis of laser action and one of the most widely applied physical phenomena. First reports of stimulated emission in organic fluorophores go back to Sorokin and Lankard [5] and to Schäfer and co-workers [6], who pioneered the development of the dye laser. The operational requirements in a laser are somewhat different than those for depletion of fluorescence. In a laser, the role of stimulated emission is to strengthen the beam by collecting stimulated photons, whereas in microscopy one is primarily interested in the depletion of the excited state by stimulated emission, irrespective of the population of the excited state.

Experiments showing an influence of stimulated emission on fluorescence signals were first reported by Galanin *et al* [7], who studied fluorescence by two-photon excitation with giant pulses of a ruby laser. At high pulse energies they observed that the fluorescence yield exhibited a more linear instead of the expected quadratic dependence; i.e. the fluorescence excitation was less efficient than

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expected. Galanin and co-workers attributed the decrease in the efficiency of fluorescence excitation to stimulated emission. In spectroscopy this phenomenon has also been referred to as *light quenching*. The experiments of Galanin *et al* were single-pulse experiments. Such experiments, however, do not allow for a *depletion* of the excited state, as we have described in [3], since the fluorescence signal increases with greater pulse intensity. In studies independent of ours, stimulated emission has been shown to be of importance in fluorescence spectroscopy. Gryczynski *et al* [8] demonstrated that the increase in fluorescence was less than linear upon excitation with a subpicosecond pulse centered in the anti-Stokes region of the emission spectrum of a dye. In their development of light quenching with picosecond lasers, Lakowicz and Gryczinski [8–11] showed the potential of stimulated emission for opening up new fields in spectroscopy, offering control of the polarization anisotropy, fluorescence emission intensity, and lifetime of fluorophores. These phenomena are also of great interest in fluorescence microscopy and will surely stimulate further research on such applications of the STED concept.

After outlining the basics of depletion by stimulated emission, we outline an experiment implementing this process in a microscope with two pulses. In particular, we describe the dependence of the efficiency of depletion on the temporal separation of the pulses and show how the stimulating pulse can be used to monitor the population of the excited state.

2. Theory

Figure 1 displays the energy levels of a typical fluorophore. S_0 and S_1 are the ground and first excited singlet states, respectively. S_0^{vib}, and S_1^{vib} are higher vibronic levels of these states. The excitation of the dye takes place from the relaxed state S_0 to the state S_1^{vib}, and fluorescence by the radiative transition $S_1 \rightarrow S_0^{vib}$. The transition $S_1 \rightarrow S_0^{vib}$ can also be induced by stimulated emission, which is of particular interest here. The transitions $S_1^{vib} \rightarrow S_1$ and $S_0^{vib} \rightarrow S_0$ are vibrational relaxations. In the discussion of stimulated emission we can ignore the triplet state. Detailed reviews of dye properties are given by Lakowicz [12] and Schäfer [13].

Figure 1 also displays the transition rates. The rates of the spontaneous processes are given by the inverse of the lifetimes τ of the source states. The fluorescence rate is $k_{fl} = 1/\tau_{fl}$, the rate for vibrational decay is $k_{vib} = 1/\tau_{vib}$, and the non-radiative quenching rate of S_0 is $k_Q = 1/\tau_q$. The rates for excitation and stimulated emission are given by the product of the photon fluxes of the beams and molecular cross sections, i. e. $h_{exc}\sigma_{exc}$ and $h_{sted}\sigma_{sted}$ for the excitation and stimulated emission, respectively. Typical values of σ_{exc} and σ_{sted} are 10^{-16}–10^{-20} cm^2, respectively. The fluorescence lifetimes τ_{fl} and τ_q are on the order of 1 and 10 ns, respectively. The lifetimes of the vibrationally

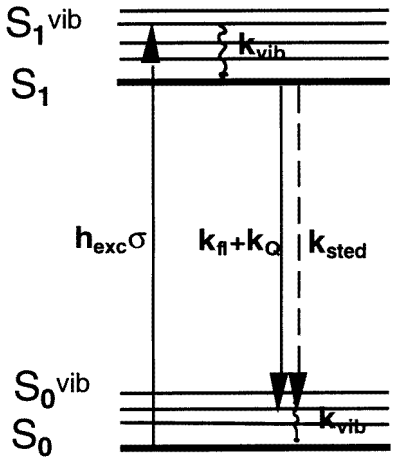

Figure 1. Energy states (Jablonski diagram) of an organic fluorophore.

excited states are very short, $\tau_{vib} \leq 1$ ps [12, 13]. Thus, the vibrational relaxation $S_0^{vib} \rightarrow S_0$ and $S_1^{vib} \rightarrow S_1$ are three orders of magnitude faster than fluorescence. The rapid vibrational decay causes the molecules in S_1^{vib} to relax to S_1 before emitting a photon. The state S_1 is the actual source of fluorescence photons, and the effective number of emitted fluorescence photons is directly proportional to the S_1 population. To a good approximation and at room temperature, S_1 represents a bottleneck every molecule has to pass before emitting a fluorescence photon.

When considering the lifetime and the transitions of figure 1, it is evident that for sufficiently high intensities of a stimulating beam a significant depopulation can occur. Fluorescence has a broad spectrum extending over several tens of nanometers in wavelength but the stimulated photons have the same wavelength, polarization and direction of propagation as their stimulating counterparts. Therefore, stimulated photons are not distinguishable from the photons of the stimulating beam, but the effect of stimulated emission can be observed as a loss of fluorescence intensity in the remaining part of the fluorescence spectrum.

As a first step, we investigate theoretically the behaviour of the fluorescence molecules excited by a focused beam, $h_{exc}(t)$, and depleted by a second focused beam $h_{sted}(t)$ [3]. The variable $h(t)$ denotes the photon fluxes, i.e. intensity point-spread-functions divided by the photon energy $\hbar\omega$, with $\omega = 2\pi c\lambda$ and $\hbar$ being Planck's constant. The excitation wavelength λ_{exc} is preferably at the absorption maximum, whereas the wavelength of the stimulating beam, λ_{sted}, is in the emission spectrum of the dye. The population probabilities $n_i^{(vib)}(t)$ are found by considering the photon fluxes as functions of time [3]. The following set of differential equations describes the interplay between absorption,

thermal quenching, vibrational relaxation, spontaneous, and stimulated emission [3]:

$$\frac{\mathrm{d}n_0}{\mathrm{d}t} = h_{\mathrm{exc}}\sigma_{\mathrm{exc}}\left(n_1^{\mathrm{vib}} - n_0\right) + k_{\mathrm{vib}}n_0^{\mathrm{vib}} \quad (1a)$$

$$\frac{\mathrm{d}n_0^{\mathrm{vib}}}{\mathrm{d}t} = h_{\mathrm{sted}}\sigma_{\mathrm{sted}}\left(n_1 - n_0^{\mathrm{vib}}\right) + \left(k_{\mathrm{fl}} + k_Q\right)n_1 - k_{\mathrm{vib}}n_0^{\mathrm{vib}} \quad (1b)$$

$$\frac{\mathrm{d}n_1}{\mathrm{d}t} = k_{\mathrm{vib}}n_1^{\mathrm{vib}} + h_{\mathrm{sted}}\sigma_{\mathrm{sted}}\left(n_0^{\mathrm{vib}} - n_1\right) - \left(k_{\mathrm{fl}} + k_Q\right)n_1 \quad (1c)$$

$$\frac{\mathrm{d}n_1^{\mathrm{vib}}}{\mathrm{d}t} = h_{\mathrm{exc}}\sigma_{\mathrm{exc}}\left(n_0 - n_1^{\mathrm{vib}}\right) - k_{\mathrm{vib}}n_1^{\mathrm{vib}} \quad (1d)$$

with $\sum_i n_i^{(\mathrm{vib})}(t) = 1$. Equations (1) also include re-excitation of the vibrationally excited ground state S_0^{vib} at λ_{sted}, and stimulated emission at the excitation wavelength λ_{exc}. (The functional notation (t) has been omitted for clarity.)

The lifetimes and transition rates of the fluorophore suggest that it is highly advantageous to employ pulsed lasers, as is evident upon consideration of equation (1c). Depletion by stimulated emission dominates for the condition

$$h_{\mathrm{sted}}\sigma_{\mathrm{sted}}n_1 \gg k_{\mathrm{vib}}n_1^{\mathrm{vib}}, \left(k_{\mathrm{fl}} + k_Q\right)n_1, h_{\mathrm{sted}}\sigma_{\mathrm{sted}}n_0^{\mathrm{vib}}. \quad (2)$$

Equation (2) reveals that depletion by stimulated emission faces three competing mechanisms, the first and second being the supply from the higher vibronic level S_1^{vib} and the spontaneous decay of S_1, respectively. Both can be excited by using *pulsed* excitation and *pulsed* STED beams. Employing an excitation pulse that is immediately followed by a stimulating pulse separates the steps of excitation and stimulated emission. Inasmuch as fluorescent level S_1 is not supplied by fresh molecules from S_1^{vib}, depletion is efficient. The excitation pulse width τ_{p} should be about $\tau_{\mathrm{vib}} \leq 1$ ps, thereby ensuring little fluorescence decay during excitation.

Another aspect of pulsed excitation is that the supply from the higher vibrational level S_1^{vib} to S_1 vanishes one picosecond after the excitation pulse has passed. To avoid spontaneous emission from S_1 the stimulating pulse has to closely follow the excitation pulse. This condition requires stimulating pulses in the picosecond range or shorter. Furthermore, to surpass the spontaneous decay a high intensity is required. Pulsed lasers are advantageous in this respect because they provide a high peak intensity at a relatively low average power. The latter is important for maintaining the total irradiation doses in the low range proposed for two-photon excitation microscopy [14].

Equation (2) also shows that it is not expedient to make the STED pulse as short as absolutely possible. That is, efficient depletion requires a small population n_0^{vib} of S_0^{vib}, the final state of the stimulated emission process. A STED pulse facing a high population n_0^{vib} would re-excite the dye to S_1 and depletion would thus be inefficient. For very short pulses one would expect that shortly after the STED pulse has passed, half of the molecules would be in S_0^{vib} and the other half in S_1 . If the rate $S_1 \rightarrow S_0^{\mathrm{vib}}$ is lower than the vibrational decay $S_0^{\mathrm{vib}} \rightarrow S_0$, this problem is avoided. Hence, at a given pulse energy the STED pulse should be long enough to allow the state S_0^{vib} to relax vibrationally. Since the lifetime of vibrational states is $\leq$1 ps, the duration of the STED pulses should be in the range of several picoseconds in order to allow S_0^{vib} sufficient time to relax [3].

It is of interest to first investigate the depletion process. The following parameters were chosen: $\tau_{\mathrm{fluor}} = 2$ ns, $\tau_{\mathrm{Q}} = 0.1$ ns, $\tau_{\mathrm{vib}} = 1$ ps, $\lambda_{\mathrm{exc}} = 500$ nm, $\lambda_{\mathrm{sted}} = 600$ nm, $\sigma_{\mathrm{sted}} = 10^{-16}$ cm^2, and a numerical aperture of 1.4 (oil). Let us assume that the fluorescence molecules are already excited, i.e. $n_1(t = 0) = 1$, and the excitation pulse is switched off. This can be accomplished, for instance, by subpicosecond pulsed excitation. Figure 2 shows the temporal dependence of the population probability of S_1 when subjected to a stimulating pulse of $\tau_{\mathrm{p}} = 100$ ps duration (FWHM), reaching its maximum at $t = 0.2$ ns after the subpicosecond excitation pulse has left the focal plane. We can safely neglect the triplet state of the dye since intersystem crossing is too slow to play a role on this time scale. $n_1(t)$ has been calculated for different peak photon fluxes $\tilde{h}_{\mathrm{sted}}$ of (a) 0, (b) 3.0×10^{25}, (c) 3.0×10^{26}, (d) 1.5×10^{27}, and (e) 3.0×10^{27} photons s^{-1} cm^{-2}), corresponding to peak powers of (b) 10, (c) 100, (d) 500, (e) 1000 MW cm^{-2}. Curve (a) describes the regular fluorescence decay of S_1 with the STED beam switched off, and curves (b–e) show how the STED beam depletes the population of S_1 . For low intensities (b, c) the depletion by stimulated emission is not complete. After the STED- pulse has passed, the decrease of $n_1(t)$ is governed by spontaneous emission and non-radiative quenching. For higher pulse intensities (d, e) depletion is strong, and for a peak intensity of 500 MW cm^{-2} the state S_1 is depleted after 200 ps. As long as the pulse length is much shorter than the lifetime of the state S_1 and assuming that stimulated emission has not reached saturation, depletion increases with increasing numbers of stimulating photons in the pulse. The cross section of stimulated emission is also of great importance since the required intensities are proportional to σ_{sted} . The depletion of the excited state S_1 can be interpreted as an enforced reduction of the lifetime of S_1 by stimulated emission [9, 11]. By altering the temporal delay between the excitation and depletion pulse, the lifetime of the dye is changed artificially. Hence, one of the benefits of stimulated emission is the selective alteration of the lifetime of the excited state. As two consecutive pulses are used, we can imagine that the delay Δt between the pulses is like a time window in which the dye is 'switched on'. Implemented in a scanning fluorescence microscope, a two-pulse beam arrangement offers enhanced temporal resolution for fluorescence investigations.

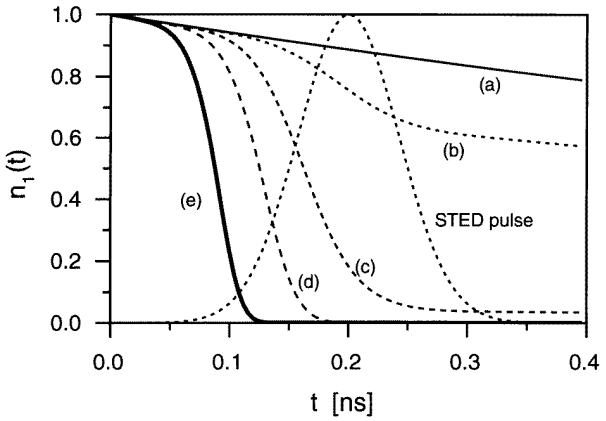

Figure 2. Population of the first excited state as a function of time when exposed to a STED pulse of 100 ps duration reaching a maximum 0.2 ns after the excitation pulse has left. For increasing peak intensities: (b) 10, (c) 100, (d) 500, (e) 1000 MW cm^{-2}, depletion by stimulated emission occurs increasingly faster, thus reducing the lifetime of the excited state. Curve (a) shows the regular spontaneous decay of the fluorophore.

3. Experiments

There is a major difference between the realization of high-intensity photophysical effects in a microscope and a spectrometer. In a microscope of high numerical aperture the light is focused onto a very small area about 300–500 nm in diameter so that intensities of the order of TW cm^{-2} are easily achieved with pulsed lasers. In standard spectrometry typical focal areas are about 20 μm so that the highest intensities are of the order of GW cm^{-2}. The availability of high intensities is advantageous when studying photophysical effects. However, the available fluorescence signal is generally much lower in a microscope due to the small volume being probed. In spectrometry one can avoid photochemical bleaching by stirring the solvent; this cannot be done in a microscope. Photophysical effects that are easily observable in a spectrometer might not be observable in a microscope because of the weakness of the signal and photochemical destruction. The exploration of photophysical effects, e.g. of stimulated emission, necessitates a careful study of the accompanying photochemical reactions.

A convenient approach for realizing depletion by stimulated emission in a microscope is to use the fundamental wavelength of a laser and its second harmonic [1, 2], which can be generated by focusing the laser light into a frequency-doubling crystal. The use of a single laser offers less flexibility than two lasers as far as wavelength selection is concerned, but the advantage of this approach is lower cost and the straightforward synchronization of the pulses. Currently, the most popular subpicosecond light sources in fluorescence light microscopy are mode-locked Ti:sapphire lasers. However, a serious disadvantage of such lasers for research on stimulated emission is their restriction to the infrared, for which only a few dyes are available.

An experiment based on the use of a mode-locked Ti:sapphire laser is sketched in figure 3. The Ti:sapphire laser provided 130 fs pulses at a repetition rate of 76 MHz and a central wavelength of 750 nm. The laser light was split into two beams, one of which was frequency doubled to 375 nm by a LBO crystal. The duration of the frequency-doubled pulses was largely unaffected by the LBO crystal. This was not the case with the infrared pulse, which underwent four encounters with a grating. As a result of dispersion the pulse was stretched to about 20 ps. This arrangement allowed the near UV femtosecond pulse to be used for excitation and the picosecond infrared pulse for stimulated emission. In addition, the adjustment of the grating allowed an arbitrary adjustment of the pulse length from a few picosecond to about 100 ps. This experiment differed from the one previously discussed [1] in that the excitation and stimulating light were focused by the same objective lens, which had a specified numerical aperture of 1.3 (oil) (Nikon). The stimulating beam passed two lenses, one of which, L_{scan}, was mounted on a precision scan stage (Light Line, Physik Instrumente, Waldbronn, Germany). The piezoelectric stage was capable of scanning the lens with a precision of about 10–20 nm. The optical arrangement was chosen so that the adjustment of the focused stimulating beam with respect to the focused excitation beam could be achieved with the lens L_{scan}. The magnification factor of about 50 enabled a calculation of the position of the stimulating focus with respect to the exciting focus with a precision of fractions of a nanometer. This arrangement permitted the study of depletion by stimulated emission in a microscope.

A suitable dye for excitation and stimulated emission is 1-ethyl-4-(4-(p- dimethylaminophenyl)-1,3-butadienyl)-pyridinium perchlorate (Pyridine 2, Radiant Dyes, Wermelskirchen, Germany) [1, 2, 15]. A small amount of Pyridine 2 was dissolved at high concentration in ethanol. A drop of this solution was diluted with glycerol and mounted between two cover slips so as to form a 10-15 μm thick layer. Pyridine 2 was excited at 375 nm and stimulated at 750 nm [1, 2, 15]. The fluorescence of Pyridine 2 was collected by the same objective lens and focused onto a pinhole. The collected light passed a stack of filters: a dichroic mirror with a transmission between 600 and 700 nm, a longwave pass dichroic filter with an edge at 500 nm, and a shortwave pass dichroic filter transmitting below 700 nm. The photomultiplier was blue sensitive and was operated in the photon-counting mode. The excitation beam filled the entrance aperture of the objective lens. The diameter of the detection pinhole was about the size of magnified back-projected Airy disk, thereby providing a pseudo-confocal operation of the setup. The total path length of the 375 nm pulse was matched to that of the 750 nm pulse. One mirror

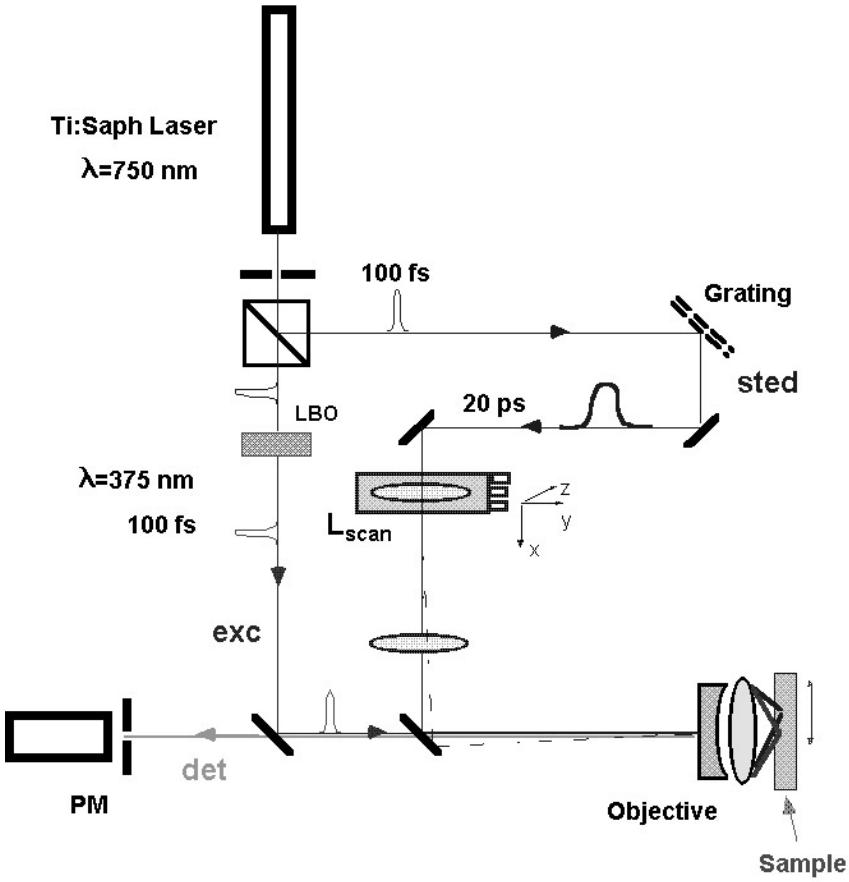

Figure 3. Experimental arrangement to study stimulated emission on a microscopic scale. The pulse from the Ti:sapphire laser is split by the cube, partly directed to a grating where it is dispersed to about 20 ps duration. The other part is frequency-doubled by a LBO crystal. The fluorescence light from the sample is collected by the objective lens and focused onto a pinhole in front of the photo multiplier. The lens L_{scan} scans the 750 nm light across the focus formed by the 375 nm excitation light.

was placed on a translation stage to allow a precise change in the path length.

The illumination and detection pinhole ideally define a confocal point-spread function of the lens so that, in principle, the arrangement of figure 3 featured a defined probe volume from which the fluorescence light was registered. When reconsidering the theoretical predictions of figure 2, we find that the effect of stimulated emission allows the population of the excited state to be monitored in a two-pulse experiment. The fluorescence yield P_{fluor} is proportional to the integral of the population of the excited state population

$$P_{\text{fluor}} = \text{constant}\,\frac{1}{\tau}\int_{t=0}^{t\geq 5\tau_{\text{fl}}} n_1(t)\,\mathrm{d}t. \qquad (3)$$

The STED pulse decreases the population $n_1(t)$ and therefore the total fluorescence signal. This is shown in figure 4 in the comparison between the fluorescence signal with and without the stimulating beam. The stimulating beam was chopped with a rotating-wheel chopper. The UV-excited fluorescence also dropped in the presence of the near- infrared stimulating photons.

Figure 4 suggests a useful definition of a depletion efficiency, ε, given by the relative difference in fluorescence yield with and without STED beam:

$$\varepsilon = \frac{P_{\text{fl}} - P_{\text{fl+depl}}}{P_{\text{fl}}}. \qquad (4)$$

The depletion efficiency ε can be interpreted as a probability that a fluorescence molecule is quenched by the stimulated emission. In Figure 4, ε was about 0.5. To a first approximation the depletion efficiency ε is proportional to the average population of the excited state and the average photon flux during the exposure to the STED pulse. Thus, ε is a function of the delay between the two pulses and constitutes a monitor of the excited state. That is, the STED pulse acts as a probe exploring the population of molecules that have not decayed. The population of the excited state is highest shortly after excitation. For longer delays many of the excited molecules have decayed before the stimulating pulse enters the focus. Thus, the fluorescence yield will increase. For delays that are of the order of the lifetime of the excited state, the STED pulse is not able to generate any

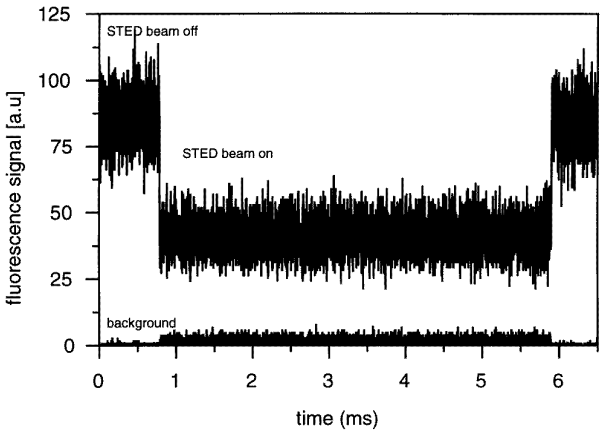

Figure 4. Fluorescence signal measured between 600 and 700 nm when exciting with 375 nm and switching on and off a stimulating beam at a wavelength of 750 nm. The 'background' signal is obtained when switching off the UV excitation and illuminating with the STED laser. It is probably due to backreflection of scattered laser light into the detector. The dwell time of each bin is 1 μs.

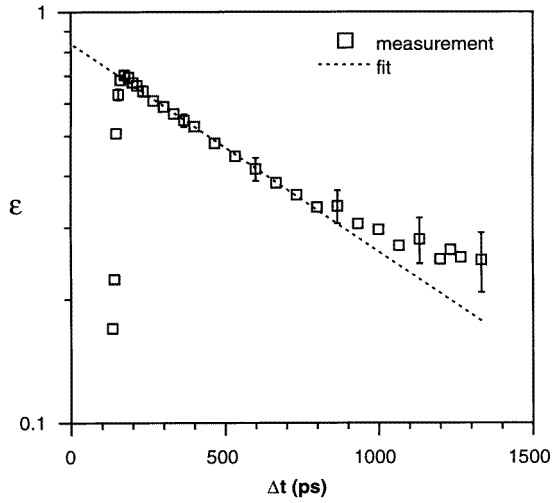

Figure 5. Depletion efficiency ε versus the delay of the stimulating pulse with respect to the excitation pulse.

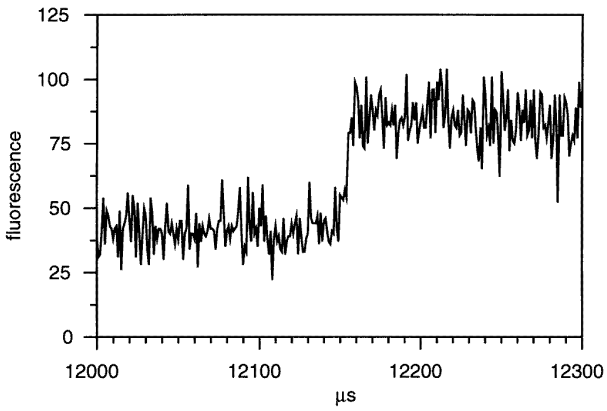

Figure 6. Change of the fluorescence signal when switching off the STED beam monitored at a timescale of tens of microseconds. The observed rise time of 20 μs is much shorter than possible changes due to recovery after bleaching.

reduction of the fluorescence signal by stimulated emission. A similar situation is encountered when the stimulating pulse overtakes the excitation pulse.

This expectation was confirmed in an experiment measuring the depletion efficiency ε as a function of the path lengths of the excitation and stimulating pulses (figure 5). For a negative delay Δt, a low value of ε was measured. With a delay of about the temporal duration of the STED pulse, ε reached an abrupt maximum. The steep slope was determined by the pulse length of the stimulating beam. With increasing delay Δt the depletion efficiency dropped continuously as a result of the spontaneous decay of the excited state, enabling a determination of the lifetime of Pyridine 2: $\tau = 0.86 \pm 0.2$ ns.

The dependence of the excited state on the depletion efficiency lifetime ε would seem to indicate stimulated emission, but this feature is not definitive in that it merely shows that the excited state participates in the decrease of the fluorescence signal. In principle, one could also imagine a photobleaching mechanism involving the excited state as an intermediate step. For instance, the STED pulse could excite fluorescence molecules to a higher state, leading to a photochemical decomposition of the molecule, and a corresponding drop in intensity would be due to photochemical decomposition rather than stimulated emission; the fluorescence recovery would reflect the back-diffusion of fresh molecules.

The model was investigated further by measuring the change of fluorescence signal when chopping the stimulating beam. Stimulated emission is expected to occur instantaneously, whereas back-diffusion should result in a noticeable rise of the signal on the timescale of back-diffusion in the solvent. Figure 6 shows the rise of the fluorescence signal upon blocking of the STED beam; the fluorescence rise time was on the order of 20 μs. Such an increase of fluorescence cannot be explained with a bleaching-recovery phenomenon, since the recovery time in viscous solvents such as glycerol should be at least two or three orders of magnitude larger. Thus, we would have expected a constantly rising signal rather than a step function. The rise and fall times were determined by the rotation speed of the chopper and the diameter of the beam. We found that the rise time of the fluorescence signal was of the same order as the 'fall time' of the STED beam. This fast recovery of the fluorescence signal was indicative of stimulated emission.

Although our experiments clearly proved that stimulated emission was the dominating process in the drop of

fluorescence intensity, we also observed a small but significant slow component in the recovery of the fluorescence signal. For average powers greater than 20 mW of the stimulating laser, a few percent of the fluorescence decrease was due to a slow effect with a recovery time of the order of 1–2 seconds. We also saw this slow component upon inverting the excitation and STED pulses, leading to an apparent ε of about 0.05–0.07. We attributed this phenomenon to bleaching of the sample by the infrared beam. It was not observable when the infrared beam was chopped and decreased with decreasing exposure time. It is apparent that bleaching is an effect that has to be considered and resolved when optimizing stimulated emission on the microscopic scale. This consideration certainly applies to the system Pyridine 2 in glycerol with a stimulating wavelength near 750 nm. However, it should be stated that all our measurements were carried out by focusing onto the same object point for several minutes; that is, the Pyridine 2-glycerol solution was not scanned. We have preliminary evidence that the influence of photobleaching decreases with reduced exposure time. For a depletion efficiency of $\varepsilon < 0.5$ bleaching can be neglected in this system.

In conclusion, we have described an experiment for realizing stimulated emission on a microscopic scale. In particular, we have shown how to monitor the population of the excited state of a fluorophore in a microscope by stimulated emission. Furthermore, we have shown that is possible to assess the lifetime of the dye by probing the excited state with stimulated emission. The measurements confirm the theoretical prediction that stimulated emission can significantly deplete the excited state of a population of fluorescence molecules in a microscope of high numerical aperture.

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