Control of Single Molecule Fluorescence Dynamics by Stimulated Emission Depletion.

R. J. Marsh, M. A. Osborne+ and A. J. Bain*, Department of Physics and Astronomy, University College London, Gower Street, London WC1E 6BT, UK
+School of Chemistry, Physics and Environmental Science, University of Sussex, Falmer, Brighton BN1 9QJ, UK

ABSTRACT

The feasibility of manipulating the single molecule absorption-emission cycle using picosecond stimulated emission depletion (STED) is investigated using a stochastic computer simulation. In the simulation the molecule is subjected to repeated excitation and depletion events using time delayed pairs of excitation (PUMP) and depletion (DUMP) pulses derived from a high repetition rate pulsed laser system. The model is used to demonstrate that a significant and even substantial reduction in the occurrence of 'dark states' in the fluorescence emission can be achieved using stimulated emission depletion. Variation in the PUMP-DUMP window allows precise control of the fluorescence yield with substantial increases in the fluorescence intensity observed at early PUMP-DUMP delays.

INTRODUCTION

In this work we show how the dynamics of the single molecule fluorescence cycle can in principle be controlled by the use of picosecond stimulated emission depletion (STED). Single molecule fluorescence has several advantages over conventional ensemble averaged techniques including the ability to measure the underlying dynamic and static disorder in a (possibly inhomogeneous) molecular population. However the apparent clarity that single molecule fluorescence affords is accompanied by complications unique to this novel technique. These arise from the random nature of the spontaneous relaxation processes possible in an excited molecule, in particular the non-radiative pathways that give rise to so-called "dark states" where the molecule is unable to either absorb or emit electromagnetic radiation[1]. Triplet state formation is believed to be one of the main routes leading to dark states. Triplet state blinking is a considerable problem in technological applications of single molecule spectroscopy such as single molecule fluorescence DNA sequencing[2] leading to extended data collection times and false negative errors in the identification of nucleotide incorporation events[3]. In this context the ability to manipulate the single molecule absorption-emission cycle is of crucial importance.

STED is a technique that has a wide variety of applications, from work to reduce the spatial resolution beyond the diffraction limit in fluorescence microscopy[4,5], to the study of ground and excited state relaxation dynamics[6]. Recently we have achieved STED from two photon excited states of a variety of fluorescent probes in bulk solution[7,8]. We have presented models and reported measurements of ground states vibrational relaxation times and stimulated emission cross-sections. We have also recently demonstrated how STED may be used to circumvent normal dipole selection rules allowing the observation of higher order degrees of molecular alignment[9].

An example of time-resolved stimulated emission depletion is shown in Figure 1. An excited state population is prepared by the PUMP laser pulse with stimulated emission depletion to the molecular ground state being induced by a time delayed DUMP pulse. Optimisation of the DUMP process (pulse width, energy and wavelength) allows depletion of up to 90% of the excited state population to be achieved.

* Corresponding author, email: a.bain@ucl.ac.uk

Proceedings of SPIE Vol. 5222 Nanocrystals, and Organic and Hybrid Nanomaterials, edited by David L. Andrews, Zeno Gaburro, Alexander N. Cartwright, Charles Y. C. Lee, (SPIE, Bellingham, WA, 2003) · 0277-786X/03/$15.00

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

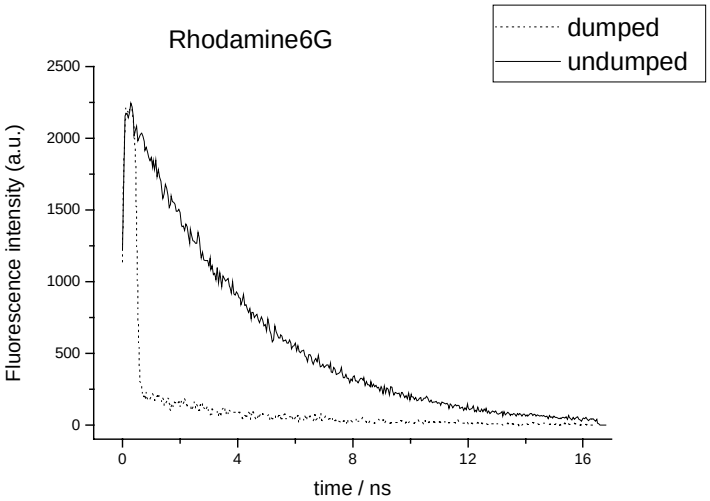

Figure 1: Stimulated Emission Depletion in Rhodamine 6G, optimisation of the STED process allows significant excited state depletion to be achieved. A 90% DUMP probability is achieved in Rhodamine 6G at 645nm with a DUMP pulse of 1.36ps, energy E=28nJ and a spot size of c.a. 1.8 $\times 10^{-6}$cm^2.

SINGLE MOLECULE FLUORESCENCE DYNAMICS

Experimental observation of single molecule fluorescence can be readily observed using continuous wave (CW) illumination microscopy. The experimental set-up is based on an inverted optical microscope (Nikon TE2000) to which a CW excitation laser source (Ar^+, Spectra-Physics) and a single-photon avalanched diode (SPAD, SPCM-AQR-14, EG&G) are coupled. The 200mW, 514.5nm Ar^+ output is spatially filtered to obtain a uniform Gaussian cross-sectional intensity profile and attenuated to 1mW using a neutral density filter. The beam entering the microscope is directed through a high numerical aperture, infinity corrected, oil immersion objective lens (Plan Apo 1.4 NA 100X, Nikon, UK) by a 45° dichroic mirror (540DRLP02 or 505DRLP02, Omega Optics Inc.). The laser is focused to a diffraction-limited spot, whose depth of focus is determined primarily by spherical aberrations introduced by high angle focusing and in practice cannot be made less than 1µm[10]. The $1/e^2$ intensity along the optical axis of the laser focus was determined to be l=1.76±0.06 µm by sampling fluorescence from a monolayer of tetramethylrhodamine (TMR). The focal area and depth define a probe volume of approximately $V=0.25 \times 10^{-15}$L which contains an average of 0.15 molecules at 10^{-9}M solute concentrations. Fluorescence is back collected by the illuminating objective (~30% collection efficiency), passed through a dichroic mirror (T~80% transmission) and directed to an observation port by a 45° prism. A 50µm precision pinhole (Elliot Scientific, UK) is placed in the image plane of the microscope to reject fluorescence emerging from above or below the plane of focus of the laser. The transmitted fluorescence is spectrally filtered to remove residual laser light and scattered photons from Rayleigh and Raman processes by a bandpass filter (580DF30 T~90%, Omega Optics Inc., USA). The remaining photons are detected by the high QE~50%, low dark count (100 s^{-1}) SPAD. The overall detection efficiency of the system, including diffraction losses at the pinhole is about 1%. TTL pulses derived from the photon counting module were analysed using a multichannel scalar (Ortec MCS-PCI, Ametek) configured within a personal computer. 10^{-9}M analyte samples of 5-(and-6)-TMR biocytin (Molecular Probes, USA) were prepared by successive dilution of stock solutions with 0.1M PBS buffer. The 10µM stock solution was prepared in DMSO (Sigma). The detection volume was monitored at 1 kHz (1 ms integration per channel) over 10,000 channels and single molecule photon bursts were recorded with a signal to noise as high as 40:1. This is consistent with the estimated emission rate $I=4.5\text{x}10^6$ s^{-1} for TMR at an off-maximum excitation wavelength of 514.5nm with 1mW of laser power. Higher acquisition rates of 1 MHz (1 µs per channel) were used to monitor the trail of photons arriving at the detector as a single molecule transits the laser focus. At this duty rate the observed fluorescence consists of single-photon events and dark gaps representing the combined effect of the non-fluorescent triplet state and the photon detection efficiency. In our case the dark gaps are predominantly due to the latter 1% detection efficiency[11], where a photon is only expected to be detected every 22µs on average whilst dark states due to the triplet lifetime (2 µs) would to be an order of magnitude shorter.

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

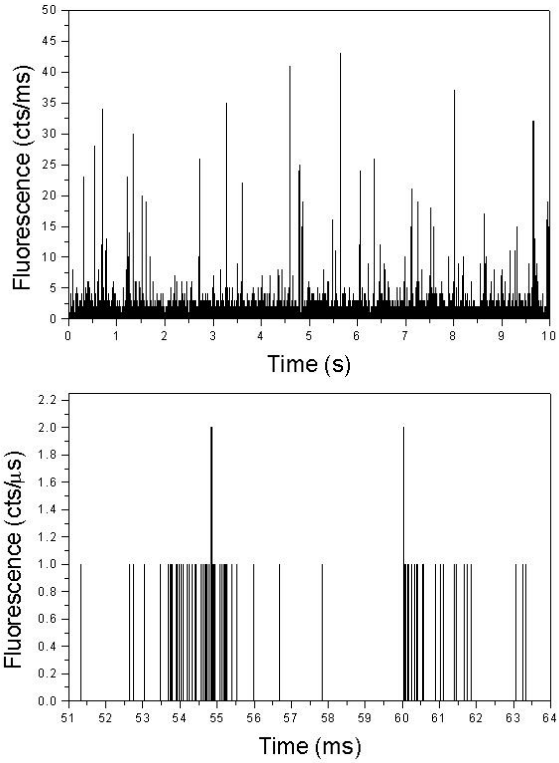

Figure 2: (A) Fluorescence bursts from a 10^{-9} M biocytin-TMR solution diffusing through a 0.25 fL observation volume recorded at an acquisition rate of 1 kHz. Discreet single molecule photon bursts are observed on average for 13% of the time. The detection zone is empty 86% of the time and is only occupied by 2 molecules or more about 1% of the time in accordance with Poisson statistics and an average of 0.15 molecules per volume element.
(B): Data as in (A) recorded at an acquisition rate of 1 MHz. The 13 ms window shows photon trails consisting of single-photon events and dark gaps that represent the combined effect of the microscope detection efficiency and the non-fluorescent triplet state.

CONTROL OF SINGLE MOLECULE FLUORESCENCE VIA STED

Figure 3 shows the different excitation and relaxation pathways that are involved in the single molecule fluorescence cycle. The molecule can be promoted to an excited singlet state by either single or two-photon absorption using a high repetition rate (e.g. 76MHz) modelocked laser. This is followed by rapid, sub-picosecond, radiationless decay (internal conversion and/or collisional cooling) to the lower vibrational levels of the first excited singlet state (S_1). In the absence of external perturbations, relaxation back to the ground state occurs via spontaneous emission (fluorescence) or spin-conversion (intersystem crossing) and subsequent decay of the lowest triplet state (T_1). Conversion of electron spin in the absorption and emission of electromagnetic radiation is strongly forbidden and T_1 is consequently long lived ($\tau_{Tr} >> \tau_F$). As a result of long residence times in T_1 the molecule is effectively rendered 'dark' in that it is incapable of excitation within the singlet manifold (and thus fluorescence) by subsequent laser pulses. Transitions within the triplet manifold are however possible although for the purposes of this work we assume that all laser wavelengths are sufficiently detuned from $T_1 \rightarrow T_n$ resonances such that these excitations can be neglected. The number of 'missed' excitations that could lead to detection of a fluorescence photon therefore depends not only on the dark state recovery time, but also on the repetition rate of the pump laser and the excitation probability. A reduction in both the repetition rate and excitation probability would increase the likelihood of a molecule being available for excitation by subsequent laser pulses. However these conditions would lead to extremely low fluorescence detection rates and extended observation periods which given the finite lifetime of single molecules are both highly undesirable. Methods of reducing the triplet lifetime involving the addition of photochemical quenching agents such as oxygen containing species lead to the production of singlet oxygen and have the potential to induce chemical reactions which may irreversibly bleach the fluorophore or alter its environment.

To understand how STED can be employed to reduce the occurrence of transitions to the triplet state it is necessary to consider how the probability of making the $S_1 \rightarrow T_1$ transition varies with time after excitation. Consider a point in time shortly after excitation of the molecule that is significantly less than the fluorescence lifetime. If the molecule has not fluoresced in this time window there is a small chance this is because it is already in the triplet state. There is a greater probability however that the molecule remains unchanged in the S_1 state as inter-system crossing times are usually much longer than fluorescence lifetimes ($\tau_{ISC} \approx 10\text{-}100\tau_F$). In comparison, if at a point significantly longer than

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

the fluorescence lifetime after excitation no fluorescence has occurred the likelihood of this being due to inter-system crossing is much higher than in the previous case. Hence the majority of incidences where singlet to triplet conversion takes place are on average those where the molecule has spent a long time in S_1 state *without* fluorescing. The way in which STED may be used to hinder this process now becomes much clearer. If on a significant number of excitation-relaxation cycles the molecule was forced from the singlet excited state back to the ground state (figure 3) at a time short in comparison to τ_{ISC} the incidences of inter-system crossing would be greatly reduced compared to those of spontaneous emission. If a substantial number of potential excitation-emission events are 'missed' due to triplet trapping, the reduction in the number of detected fluorescence photons due to STED may be more than compensated for by the increased availability of the molecule for excitation from S_0. An additional consequence of a reduction of 'excursions' into T_1 is expected to be an increased photochemical lifetime for the molecule. Irreversible photobleaching (photochemical change) due to collisions with oxygen containing species is a significant limiting factor in determining the length of the fluorescence train from a single molecule[3]. The quantitative way in which STED modifies the single molecule fluorescence cycle depends on the molecular parameters along with the time delay and probability of simulating the $S_1 \rightarrow S_0$ transition dynamics induced by the DUMP pulse is complex[7,8]. A clear picture of the influence of STED on single molecule dynamics is approached using a stochastic computer simulation of the excitation-relaxation cycle with the induced and spontaneous transition probabilities as inputs. This approach and the results obtained are outlined below.

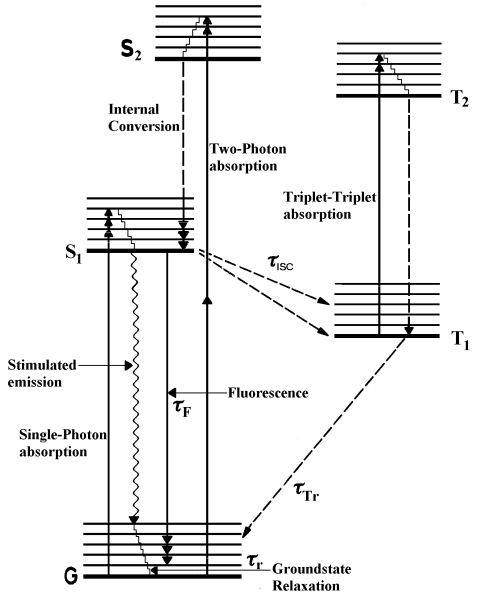

Figure 3: Jablonski diagram showing the possible relaxation pathways for an excited molecule. Once a molecule is excited (via single or two-photon absorption) it can either fluoresce or cross to the triplet state. Here, transitions to higher lying triplet states are strongly allowed given the appropriate laser pulses. Alternatively a DUMP laser pulse can be use to stimulate a transition from S_1 to the upper vibrational levels of the ground state, where rapid vibrational cooling occurs.

SINGLE MOLECULE STED SIMULATIONS

For a solvated molecular probe given pulsed excitation and time resolved detection, and particularly when considering the application of a second STED laser pulse, the fluorescence dynamics can be modelled by a sequence of Markov jump processes[12]. This implies that all the molecular transitions are effectively instantaneous when compared to the lifetimes of the states between which they occur; the molecule can also be taken to exist solely in one particular state with all coherence effects washed out by rapid (sub-picosecond) collisional dephasing. For simplicity, we assume the molecule remains located within the excitation volume and that fluorescence (if it takes place) is detected with 100% efficiency. In addition we do not consider the possibility of irreversible photo-bleaching or any other process leading to a permanent loss of fluorescence. It is also realistic to assume that the spontaneous transition rates (fluorescence, inter-system crossing and triplet relaxation) occur on very different time scales with $\tau_F \ll \tau_{ISC} \ll \tau_{Tr}$. At the start of each cycle (defined by the repetition rate of the laser) the molecule is subjected to the exciting (PUMP) laser pulse, the energy and temporal

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

width of which is simply that which will give a predetermined probability of inducing the $S_0 \rightarrow S_1$ transition. If at this time the molecule finds itself in the triplet state and thus unable to participate in the absorption-emission cycle, the probability P_{Tr} of relaxation back to the ground state by the time (t_{pp}) of the next PUMP pulse is given by

$$P_{Tr} = 1 - exp\left(\frac{-t_{pp}}{\tau_{Tr}}\right) \tag{1}$$

where t_{pp} is the time between PUMP laser pulses. If this probability exceeds a random number (between zero and one) the molecule transits to the ground state ready for excitation by the next PUMP pulse, otherwise it is left unchanged. In either case the simulation jumps to the next laser pulse and the cycle is repeated. Once in S_0 given a predetermined excitation probability (e.g.10%) the simulation (as above) determines whether $S_0 \rightarrow S_1$ transition takes place. If the molecule remains unexcited the simulation jumps to the next laser pulse and the process is repeated until the molecule is removed from S_0. Once this has happened it is necessary to decide whether either spontaneous emission or intersystem crossing has taken place in the time window between PUMP and DUMP pulses (τ_d). Assuming $\tau_F \ll \tau_{ISC}$ the probability of a fluorescence photon being emitted by this time P_F is given by

$$P_F = 1 - \exp\left(\frac{-t_d}{\tau_F}\right) \tag{2}$$

The decision process as to whether emission has taken place is based upon the following steps. Firstly a generated random number is required that is less than or equal to P_F. If this condition is met, a value for the emission time is randomly selected up to the value of the PUMP-DUMP delay, this is accepted with a probability $P_{em}(t_{em})$ related to the fluorescence lifetime

$$P_{em}(t_{em}) = \frac{t_d}{t_F} \exp\left(\frac{-t_{em}}{\tau_F}\right) \tag{3}$$

If this value is not accepted then a new emission time is chosen until this condition is met. Once an appropriate emission time is selected a determination must be made as to whether the molecule truly fluoresced or crossed to T_1 before this time. The probability, P_{ISC}, of this happening by the selected emission time (with $\tau_F \ll \tau_{ISC}$) is given by

$$P_{ISC} = 1 - \exp\left(\frac{-t_{em}}{\tau_{ISC}}\right) \tag{4}$$

As before the decision as to whether the transition takes place is dependent on the probability being greater than a random number. Should the transition to the triplet state occur the simulation jumps to the next PUMP pulse, no fluorescence is recorded and the cycle is repeated but with the molecule initially in the triplet state. If fluorescence occurs the emission time is recorded and the molecule is returned to the ground state and the cycle repeated as above. If however the molecule remains in S_1 when the DUMP pulse arrives, a decision is made by random number generation (given a predetermined stimulated transition probability) whether or not the molecule undergoes the transition to S_0. If the molecule returns to S_0 the simulation returns to the start of the cycle. However as the stimulated photon is emitted co-linearly with the DUMP pulse and is not detected no emission time is recorded. If stimulated depletion does not take place the molecule must either spontaneously emit or undergo intersystem crossing to the triplet state. A similar approach (as above) is used to determine an emission lifetime and to decide whether ISC or spontaneous emission has occurred. Here the time interval is now taken as that between the PUMP-DUMP delay time to $5\tau_F$ (a number chosen for computational convenience; after $5\tau_F$ the probability of emission not occurring given no ISC is less than 1%). Again, if spontaneous emission is deemed to have taken place the emission time is recorded. The final stage of the simulation considers occasions where the molecule still remains in S_1 after $5\tau_F$. Here, the same procedure to determine the whether fluorescence or intersystem crossing takes place. However, to deal with computational limitations the time period now extends from $5\tau_F$ to infinity. If the molecule spontaneously emits within this interval the emission time is not recorded

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

but the photon is counted towards the total fluorescence emission. The cycle then repeats with the next PUMP pulse but with the molecular state determined by the outcome of the previous cycle. Throughout the simulation, the time spent in each of the electronic states is recorded and a histogram of excitation-emission coincidence times is built up. The total number of fluorescence photons emitted in each successive integration period (corresponding to a set number of laser shots) is also recorded to enable observation of real time emission events as in figure 2. A test of the simulation in the absence of DUMP transitions is shown in figure 4. A PUMP repetition rate of 76MHz and an excitation probability of 10% are used in this and all subsequent simulations. Taking a radiative lifetime of 2.1ns and an intersystem crossing time of 66ns corresponds to an excited state lifetime of 2.035ns, the simulation yields a distribution of emission coincidence times which are well fitted by an exponential decay of 2.023 ±0.005ns (figure 4A). A 10ms portion of the fluorescence trajectory is shown in figure 4B showing characteristic bursts of fluorescence photons separated by dark periods due to trapping in the triplet state.

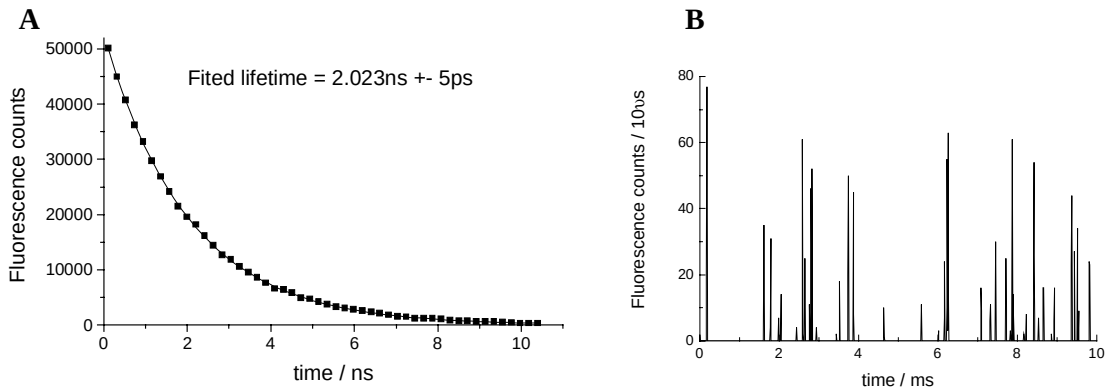

Figure 4 (A): Simulation of single molecule fluorescence dynamics, taking a radiative lifetime of τ_F=2.1 ns, an intersystem crossing time of τ_{ISC}=66ns yields a simulated lifetime of 2.023 ±0.005ns close to the expected value of 2.035ns. (B): Single molecule fluorescence trajectory assuming a triplet lifetime of 0.2 ms and an integration window of 10µs.

RESULTS

Simulations were performed using molecular constants of comparable magnitude to those of commonly used fluorophores such as tetramethylrhodamine (τ_F = 2.1ns, τ_{ISC} = 66ns). The first set of simulations investigates the degree to which STED can influence the single molecule fluorescence cycle given different degrees of triplet trapping. The DUMP probability and PUMP-DUMP delay were set at 90% and 2ns ($\cong\tau_F$) respectively. Simulations with triplet relaxation times of 2ms (negligible triplet quenching) and 2µs were performed. In addition, simulations with a DUMP probability set to zero are performed for comparison. The results of the simulations are plotted as histograms of excitation-emission coincidence times and displayed in figure 5. In both UNDUMPED simulations the distributions of photon arrival times correspond to single exponential relaxation dynamics as in figure 4A. In the DUMPED simulations there is a marked discontinuity in the arrival time distributions at 2ns (the PUMP-DUMP delay) with an enhancement in the relative yield of photons in this time window ($I_D(0)/I_U(0)>1$). The overall fluorescence yield for the DUMPED and UNDUMPED simulations (I_D/ I_U) is also calculated. It is clear from the results that STED is more effective in increasing the overall yield of fluorescence in molecules with a long triplet lifetime and where the effects of triplet blinking are more pronounced. With a 2µs lifetime I_D/ I_U is less than unity, depletion of fluorescence due to STED outweighs the increase in the excitation probability due to a more rapid return to the ground state. In both simulations however the enhancement in emission within the PUMP-DUMP time window is noticeable.

As mentioned above, we have previously achieved excited state depletion levels approaching 90% from two-photon excited states (figure1). A second set of simulations was performed to investigate the influence of P_D on the fluorescence cycle. An intersystem crossing time of 1µs and a triplet lifetime of 200µs are assumed (all other experimental and molecular parameters were unchanged), together with values of P_D from 0 to 1. The resulting photon coincidence time plots are displayed in figure 6.

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

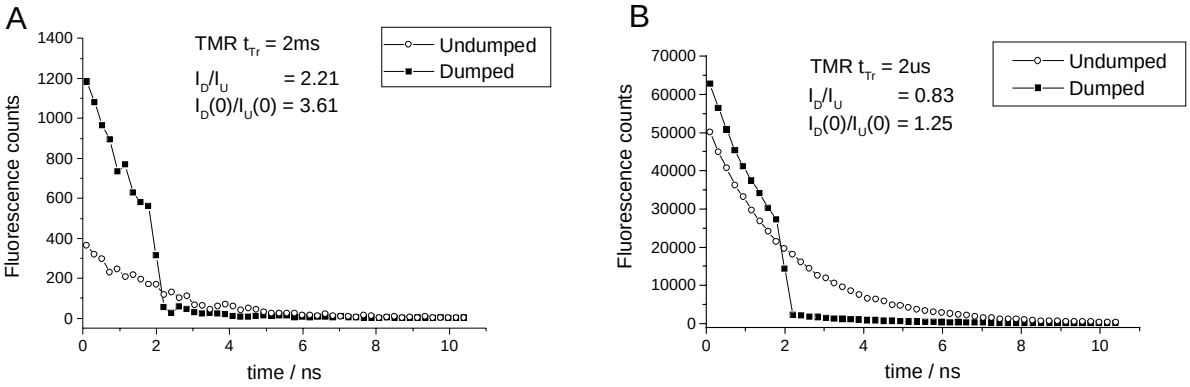

Figure 5: Plots of photon arrival times for DUMPED and UNDUMPED simulations of the fluorescence cycle for a single molecule with 2ms (A) and 2µs (B) triplet relaxation times. The enhancement of the total fluorescence yield I_D/I_U and the time zero intensities $I_D(0)/I_U(0)$ are clearly greater for the longer triplet relaxation time.

All the simulations show an increase in photon yield within the PUMP-DUMP window. However the variation in I_D/I_U with P_D is not linear, for a 50% DUMP probability there is a *decrease* in fluorescence (I_D/I_U=0.75). For higher DUMP probabilities an increase in fluorescence enhancement with P_D was observed. The results for the simulation with P_D=1.0 are included to show the upper limit of what can be achieved with the given molecular parameters; clearly this value cannot be realised experimentally.

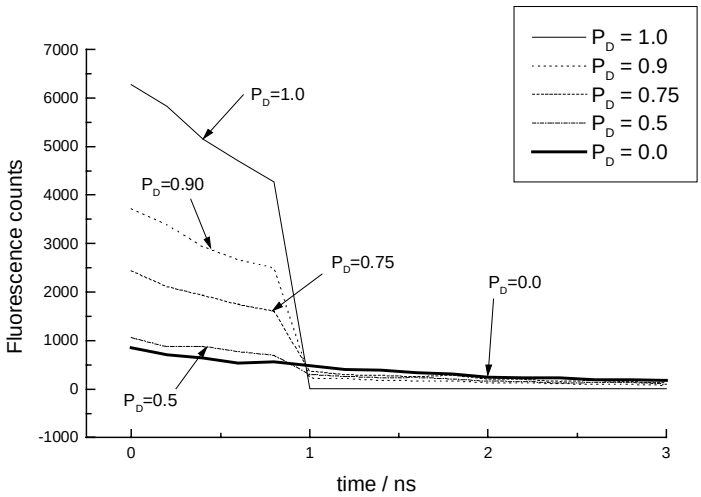

Figure 6: Simulated fluorescence photon distribution times for various DUMP probabilities compared with the UNDUMPED (P_d=0) case. Molecular parameters in this case are: τ_f = 2ns, τ_{ISC}= 1µs, τ_{Tr} = 200µs, PUMP-DUMP delay = 1ns. Significant enhancement is dependent on a high DUMP probability. Total enhancement I_D/I_U = 2.69, 1.80, 1.38, 0.75,1.0 for P_D = 1.0, 0.9, 0.75, 0.5, 0.0 respectively.

The other experimental parameter that can be varied is the PUMP-DUMP delay, in relation to τ_F and τ_{ISC} this has a more pronounced effect on the level of enhancement than the DUMP probability. Simulations using the same molecular parameters as above and a DUMP probability of 90%were performed for PUMP-DUMP delays spanning the range from 200ps to 3ns. Figure 7 shows the variation in I_D/I_U together with the fraction of time spent by the molecule in S_0, S_1 and T_1 with PUMP-DUMP delay. The enhancement in the total fluorescence yield is seen to rise with decreasing PUMP-DUMP delay corresponding to a reduction in intersystem crossing due to STED, this is opposed by the reduction in fluorescence due to STED which becomes more pronounced at short PUMP-DUMP delays. The optimum PUMP-DUMP separation is found to be slightly below the excited state lifetime at c.a. 1.5 ns (I_D/I_U=2.6). At shorter time separations the reduction in fluorescence by STED is not compensated by the increased re-excitation frequency due to a reduction in

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

intersystem crossing and trapping in the triplet state. The time spent between the three electronic states is divided predominantly between S_0 and T_1 (ca 10% and 90% respectively at the optimum PUMP-DUMP separation). With increasing PUMP-DUMP delay the proportion of time spent in T_1 rises at the expense of that in S_0 whilst the time spent in S_1 is minimal remaining approximately constant (ca 0.03%).

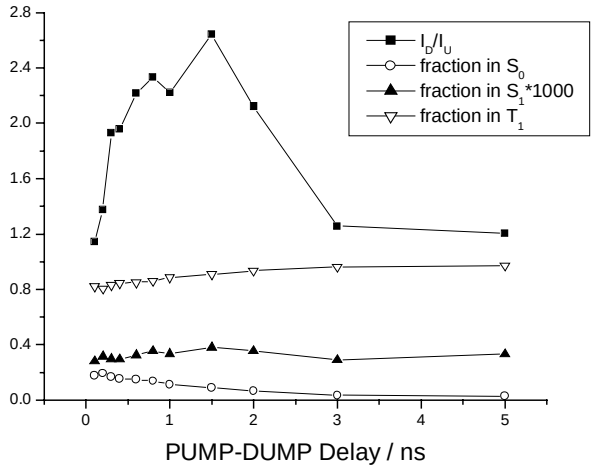

Figure 7: Upper plot: Variation in the fluorescence enhancement due to STED with PUMP-DUMP delay. The lower curves correspond to the proportion of time spent in T_1, S_1 and S_0. Molecular parameters in this case are: τ_f = 2.1ns, τ_{ISC}= 66ns, τ_{Tr} = 200μs, DUMP probability = 90%.

The nature of the changes STED brings to the single molecule excitation-emission cycle can be seen from the results of the next simulation (see Figure 8). In the case of no STED, the effects of triplet trapping can easily be seen. A high number of fluorescence photons are observed per 0.1ms integration period. However, intersystem crossings are frequent leading to dark periods while the molecule remains in T_1. In the DUMPED trajectory the fluorescence intensity is initially reduced as STED predominates over fluorescence. Nonetheless, as the molecule is removed from the excited state at a relatively early time (a PUMP-DUMP delay of 150ps), the chances of it undergoing transitions to T_1 are significantly reduced. Thus, on average, a much larger number of excitation-emission cycles will be observed before a triplet transition occurs. For this particular case the molecular parameters where chosen such that the total number of photons emitted in each simulation were approximately the same (I_D/I_U=0.9). However, the total time spent in the S_1 excited state was significantly less, being 116.5μs and 42.7μs for the UNDUMPED and DUMPED cases respectively.

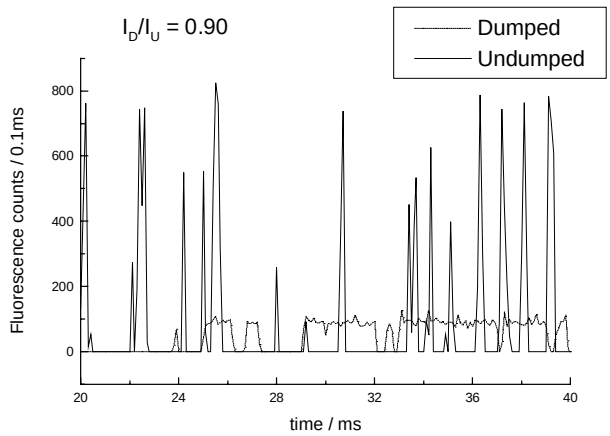

Figure 8: DUMPED and UNDUMPED trajectories with approximately equivalent photon yields (I_D/I_U=0.9). The free (UNDUMPED) trajectory consists of a series of infrequent high intensity fluorescence bursts separated by dark periods due to trapping in T_1 whilst the DUMPED trajectory consists of sustained low intensity periods of emission. The molecular and experimental parameters used were τ_F=2ns, τ_{ISC}=1μs, τ_{Tr}=1ms, a DUMP probability 95% and a PUMP-DUMP delay of 150ps.

The final simulation demonstrates the limits of the modifications to the fluorescence cycle given optimum DUMP conditions (P_D=100%) and a short PUMP-DUMP delay (50ps). The first 20ms of the DUMPED and UNDUMPED fluorescence trajectories are shown in Figure 9, STED is seen to give rise to sustained fluorescence bursts which last

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx

longer than the dark periods that separate them. This gives rise to the dramatic increase in fluorescence yield (I_D/I_U=42.5) and an even larger increase in photons with early emission times ($I_D(0)/I_U(0)$=1700).

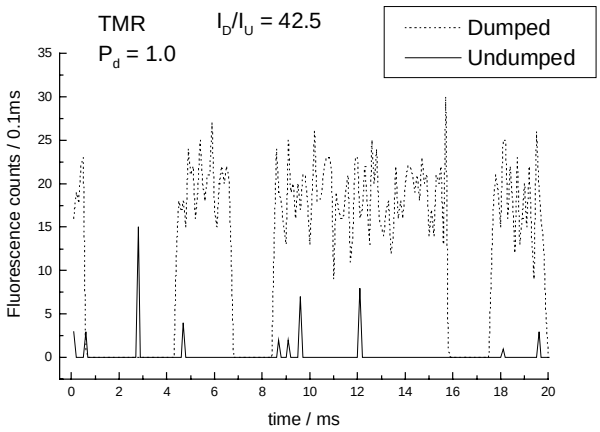

Figure 9: Demonstration of the very high increase in fluorescence possible using STED under optimum conditions. A DUMP probability of unity and a short PUMP-DUMP delay of 50ps are employed. The molecular parameters used are τ_F=2.1ns, τ_{ISC}=6.6μs, τ_{Tr}=2ms and an integration time of 100μs is employed. Although triplet blinking is still not quite eliminated the increase of fluorescence is substantial (I_D/I_U = 42.5 and $I_D(0)/I_U(0)$ =1700).

CONCLUSIONS

The stochastic simulations show that the single molecule fluorescence cycle can, in principle, be dramatically modified by STED. The effects are most noticeable in molecules where triplet blinking is marked. STED therefore has the potential to allow the use of fluorophores with intrinsic spectroscopic properties that would otherwise make them unsuitable for single molecule fluorescence applications. The differences between free and DUMPED fluorescence trajectories are marked by a transition to sustained periods of emission. The enhancement of fluorescence emission is shown to arise from increased re-excitation due to a reduction in intersystem crossing. The enhancement in fluorescence emission is most pronounced within the PUMP-DUMP time window with STED acting as an effective 'gate' to single molecule emission. These aspects of STED may be of significant use in time-resolved studies of single molecule dynamics where the resolution afforded by high quantum yield photo-detectors does not permit the observation of ultrafast emission events.

ACKNOWLEDGEMENTS

We would like to thank the Royal Society and the UCL Graduate School for financial support of this work

REFERENCES

[1] F. Vargas, O Hollricher O Marti G de Schaetzen and G Tarrach *J. Chem. Phys.* **117**, 866-871 2002
[2] S. Balasubramanian, D.Klennerman, C.L.Barnes and M.A. Osborne, PCT patent no. WO00/06770 (10th February 2000)
[3] M.A. Osborne, C.L. Barnes, S. Balasubramanian and D.Klennerman, J.Phys. Chem. B. **105**, 3120-3126 2001
[4] T A Klar, S Jakobs, M Byba, A Egner & S W Hell Proc Nat Acad Sci (USA) **97** 8206-8210 2000
[5] Ch Buehler, C Y Dong, P T C So, T French & E Gratton. Biophys. J **79** 536-549 2000.
[6] P. Changenet-Barret, C. T. Choma, E. F. Gooding, W. F. DeGrado & R. M. Hochstrasser, J. Phys. Chem. B, **104,** 9322-9329 2000 (and references therein).
[7] R J Marsh, D Armoogum and A J Bain, *Proc. SPIE*, **4812**, 45-54 2002
[8] R J Marsh, D Armoogum and A J Bain, *Chem. Phys. Lett*. **366,** 398-405 2002
[9] A J Bain, R J Marsh, D A Armoogum, L Porres, O Mongin and Mireille Blanchard-Desce BioChem. Soc. Trans. **31**, 1047-1051 2003
[10] R. Rigler, U. Mets, J. Windengren, P. Kask, *Eur. Biophys. J.* **22**, 169-175 1993
[11] S. Nie, D.T. Chiu, R.N. Zare, *Anal. Chem.* **67**, 2849-2857 1995
[12] R.L. Stratonovich. Conditional Markov Processes and their Application to the Theory of Optimal Control. Elsevier, New York 1968

Downloaded From: http://proceedings.spiedigitallibrary.org/ on 06/07/2016 Terms of Use: http://spiedigitallibrary.org/ss/TermsOfUse.aspx