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LETTERS

Probing Photoinduced Intersystem Crossing by Two-Color, Double Resonance Single Molecule Spectroscopy

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A photoinduced effect on the rate of reverse intersystem crossing (isc) in single sulforhodamine 101 (SR101) molecules has been observed using a novel two-color, multipulse, double resonance technique. The observed photoinduced intersystem crossing can be modeled by a four-electronic-state scheme ($T_1 \rightarrow T_N \rightarrow S_1 \rightarrow S_0$) in which one of the excitation colors is in resonance with the $S_0 \rightarrow S_1$ transition and the other is resonant with the $T_1 \rightarrow T_N$ transition. Synchronous averaging over many cycles of the two-color pulsed excitation experiment provides transient data that can be analyzed in a straightforward manner that is analogous to a conventional chemical kinetics experiment. Separate single color pulsed experiments were used to evaluate whether single color excitation (543 nm) alone could be responsible for photoinduced reverse isc (as previously reported for other molecules). However, results from the single color, pulsed excitation experiments show no evidence of this effect for two dyes, DiI and SR101.

Introduction

One of the best known features in the single molecule spectroscopy (sms) of organic aromatic dye molecules is fluorescence "triplet blinking".[1−7] This extreme example of fluorescence intensity fluctuations (during cw irradiation) is due to intersystem crossing (isc) resulting in an intermittent occupation of the relatively long-lived triplet "dark state". Triplet blinking is commonly described by a three-state electronic model including the ground-state singlet (S_0), first excited singlet (S_1) and first excited triplet (T_1), all shown in Figure 1B. Although isc yields are low for most organic fluorescent dyes used in sms, the rapid excitation rates required of sms produce triplets at rates of 10^2-10^4 s^{-1}.[5,7,8] By removing oxygen, triplet lifetimes can be increased dramatically,[7−10] even exceeding 100 ms, adding to the ease and reliability of investigating isc and triplet states in single molecules.

The kinetic parameters necessary to completely describe intensity fluctuations due to triplet blinking within this model are: τ_{on}, the average lifetime of the kinetic on state, during which the molecule is cycling between S_0 and S_1; τ_{T1}, the average lifetime of the triplet dark state; and I_{on}, the single molecule fluorescence intensity during the on cycle. The relationships of these quantities to the usual photophysical parameters for a dye molecule are given in Figure 1B. The remaining parameters are E_{det}, the fluorescence detection efficiency (10%), and ϕ_{isc} and ϕ_{fl}, the quantum yields of intersystem crossing and fluorescence, respectively. Most papers on triplet blinking identify τ_{off} with the spontaneous lifetime of the lowest energy triplet state of the molecule under investigation, which in turn is the inverse of the $T_1 \rightarrow S_0$ isc rate constant, $k_{isc'}$. *A few papers, however, have reported single molecule evidence that at high irradiation intensity an additional photoinduced channel (e.g., $T_1 \rightarrow T_N \rightarrow S_1 \rightarrow S_0$) for isc becomes significant.*[11,12] Experimental evidence for the photoinduced isc (pii) effect has also been observed in ensemble experiments in liquid solution.[13−18]

We report here a novel two-color, multipulse, double resonance technique that is designed to investigate the effect

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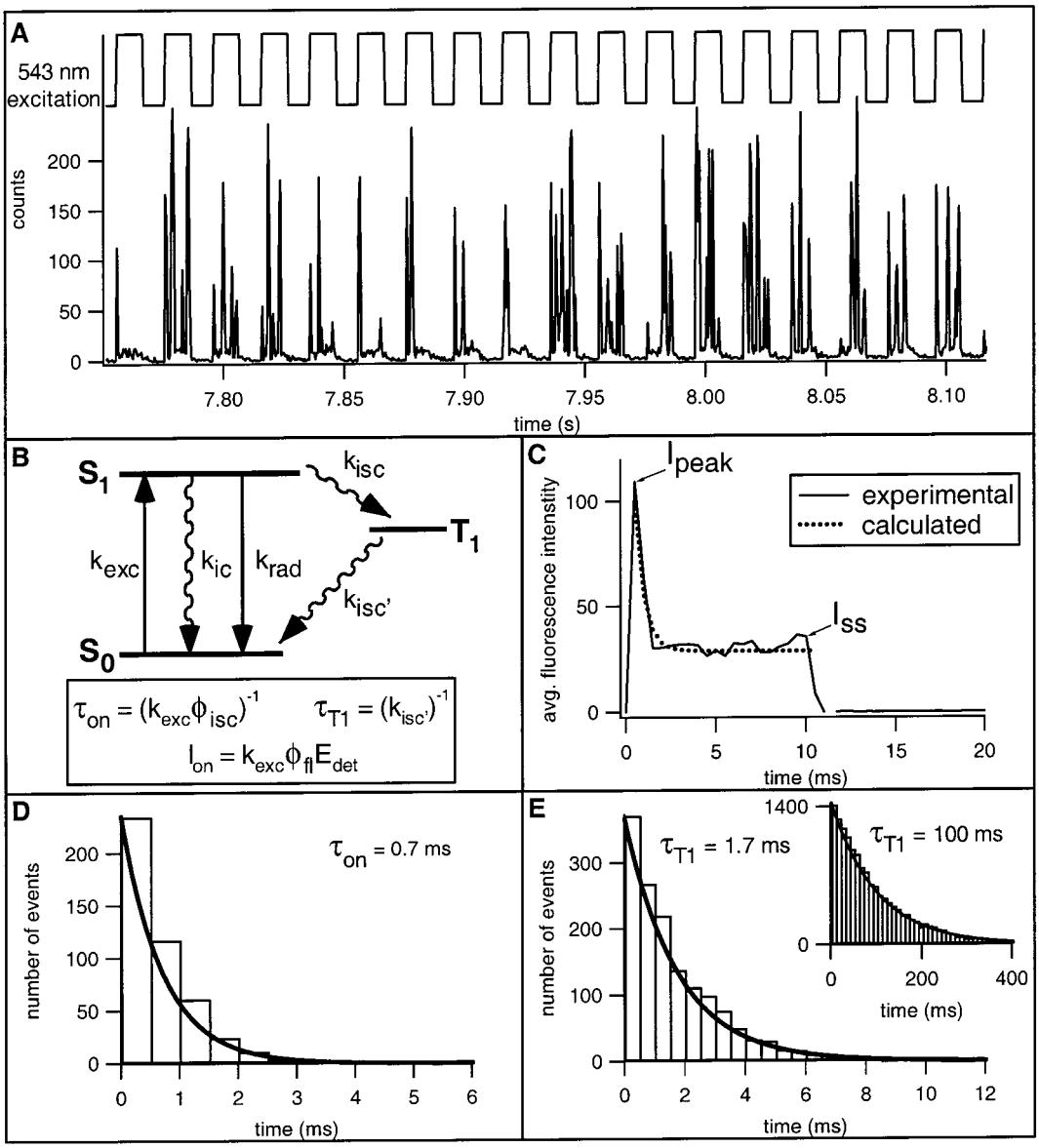

Figure 1. (A) Upper: sequence of 543 nm laser pulses modulated at 50 Hz (50% duty cycle). Lower: raw fluorescence data for a single molecule excited with 543 nm laser light pulses as shown above. (B) Jablonski diagram for a three-level system. (C) Average of 224 excitation cycles of the data shown in A. The dashed line represents the decay from the calculated peak value to the steady-state fluorescence intensity (see eqs 1−3): Intensity $= I_{ss} + (I_{peak} - I_{ss}) \exp[-(k_{isc'} + k_{exc}\phi_{isc})t]$. (D) On time histogram for the single molecule in A. (E) Off time histogram for the single molecule shown in (A). The inset shows an off time histogram for a different single DiI molecule with a longer, and more typical, triplet lifetime.

of intense $T_1 \rightarrow T_N$ irradiation on the apparent overall reverse isc rate ($T_1 \rightarrow S_0$) of single molecules. One of the excitation wavelengths in the experiment is in resonance with the $S_0 \rightarrow S_1$ transition and is used as a saturation/monitoring pulse. The other excitation wavelength is resonant with the $T_1 \rightarrow T_N$ transition and induces isc by the sequence $T_1 \rightarrow T_N \rightarrow S_1 \rightarrow S_0$. The two-color experiment employs a repetitive pulse sequence involving a triplet saturation recovery period and a modified triplet saturation recovery period, during which the $T_1 \rightarrow T_N$ band is strongly irradiated. Synchronous averaging of the *single molecule fluorescence intensity* over many cycles of the two-color pulsed excitation experiment provides transient data that can be analyzed in a straightforward manner that is analogous to a conventional chemical kinetics experiment. The experiments also employ a sample preparation method that produces oxygen-depleted environments. This suppresses O_2-induced isc, allowing for a long average triplet lifetime, which can exceed 100 ms for ordinary aromatic fluorescent dyes.[9] Due to the extraordinarily long triplet lifetimes, the triplet state can be excited ($T_1 \rightarrow T_N$) many times, *thus increasing the likelihood of inducing low quantum yield effects including the pii effect.* Results are presented for two aromatic fluorescent dyes, DiI and sulforhodamine 101 (SR101). Separate single color pulsed experiments were used to investigate whether single color excitation (543 nm) alone could be responsible for photoinduced reverse isc (as previously reported for other molecules).[11]

Experimental Section

The sample preparation and single molecule microscopy setup have been described previously.[9] The apparatus was modified by the addition of an acousto-optic modulator (IntraAction Corp. AOM-40) to chop the excitation laser for the pulsed excitation experiments. For the two-color experiment beams from both a green HeNe (543 nm) and a diode laser (670 nm) were chopped by independent AOM's and coupled into the same optical fiber. Intensity−time trajectories were collected with 0.5 ms binning. A 670 nm notch and a 600 nm short-pass filter were placed in

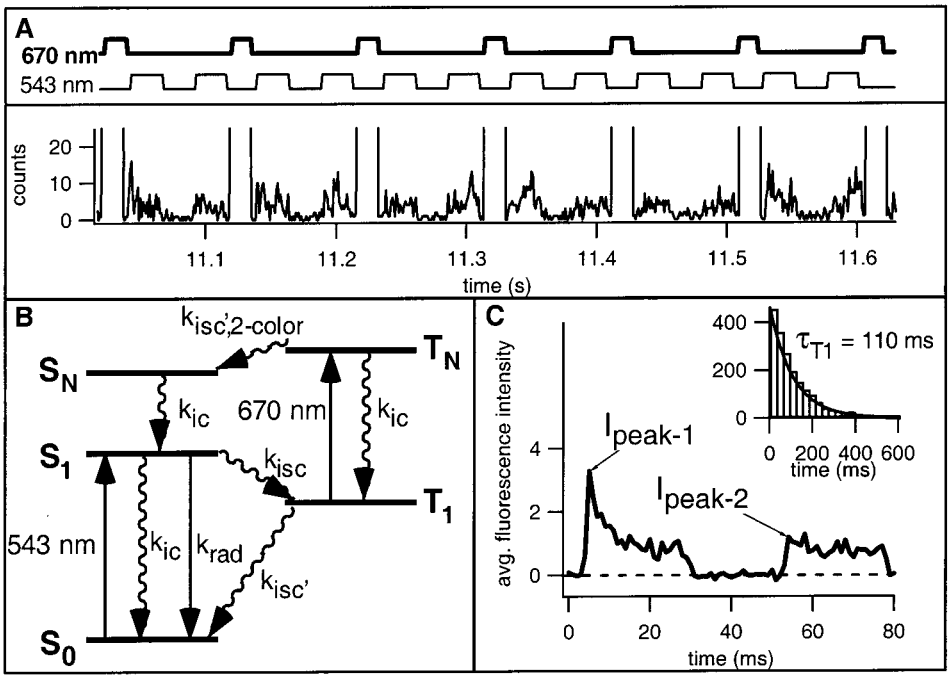

Figure 2. (A) Upper: schematic illustration of the two-color pulse sequence. In this example, the 670 nm laser light is modulated at 10 Hz (20% duty cycle) while the green laser light is modulated at 20 Hz (50% duty cycle). Lower: data from a single molecule transient collected using the two-color pulse sequence shown above. (B) Energy level diagram illustrating excitation, emission, and relaxation processes. (C) Background-subtracted averaged data of the 147 cycles prior to permanent photobleaching for the transient shown in (A). The inset is an off-time histogram for a typical SR101 molecule.

front of the detector to protect it from the red laser light. Overlap of the red and green spots was optimized and verified by sequentially imaging a sample of spatially isolated 100 nm spheres (Molecular Probes), which emit at 720 nm after excitation at either 543 or 670 nm. Different pulse sequence parameters (e.g., laser off-times) were used in order to optimize the pii effect. This was necessary due to sample variations in residual oxygen concentration, which in turn are responsible for sample-to-sample variations in the average triplet lifetime. Both dyes, 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) and SR101 (Exciton), were used as received.

Results and Discussion

The one- and two-color pulse sequences introduced in this paper are illustrated in Figures 1A and 2A, respectively. Both of the sequences are useful alternatives to the common cw irradiation sms approach. The pulse sequence approach allows for periods of different light intensity and color and is well suited for studying multiphoton effects such as photoinduced isc.

One-Color Pulsed Excitation. We begin with the discussion of the simpler, one-color pulsed excitation sequence, as summarized in Figure 1A. Within each of the green laser pulses, brief spikes of intense fluorescence are observed as the DiI molecule cycles between S_0 and S_1 before jumping to T_1. The average of 224 excitation cycles from the same molecule shown in Figure 1A is shown in Figure 1C with the background subtracted (the background signal was obtained after the molecule underwent permanent photobleaching). While a steady-state intensity is attained within the pulse duration (10 ms in Figure 1), the off-period of the laser permits a return to S_0, at a rate $k_{isc'}$. This provides a shift from the saturated steady-state triplet population, and thus this technique is a saturation recovery experiment.

The molecule described in Figure 1 has a short enough (1.7 ms) triplet lifetime that $T_1 \rightarrow S_0$ intersystem crossing is complete during the 10 ms laser off period. For most of the molecules, in contrast, the triplet lifetime exceeds 50 ms.[9] A representative off-time histogram for a typical molecule in the ensemble is shown in the inset of Figure 1E. For a typical molecule in the ensemble the triplet lifetimes significantly exceeds the laser off period, and very little fluorescence intensity recovery is observed immediately following the off period.

Two procedures were used to obtain estimates for the necessary parameters to model the data in Figure 1C. First, to obtain an estimate for τ_{T1}, conventional cw irradiation data were collected briefly for each single molecule. Panel E shows the off time histogram based on the cw data for this molecule, and τ_{T1} was calculated from this histogram fit. Second, an on time histogram (Figure 1D) was constructed (from the data in Figure 1A), and a τ_{on} value was obtained from a fit to this histogram. The raw data used in Figure 1 were chosen because the very good signal-to-noise ratio allowed for accurate determination of the kinetic parameters required to model the averaged data in Figure 1C, see below.

The salient features of the averaged fluorescence transient are described here. Because the off-period of the laser ($t_{laseroff}$) allows time for singlet recovery, the averaged intensity trajectory shows a peak intensity (I_{peak}) as the excitation laser is turned on and a subsequent decay (τ_{equil}) to the steady-state intensity (I_{ss}). These parameters are defined as

$$I_{peak} = \left(1 - e^{-k_{isc'}t_{laseroff}}\left(1 - \frac{k_{isc'}}{k_{isc'} + k_{exc}\phi_{isc}}\right)\right)I_{on} \quad (1)$$

$$\tau_{equil} = \frac{1}{k_{isc'} + k_{exc}\phi_{isc}} \quad (2)$$

$$I_{ss} = \left(\frac{k_{isc'}}{k_{isc'} + k_{exc}\phi_{isc}}\right)I_{on} \quad (3)$$

Using the calculated τ_{on} (Figure 1D) and τ_{T1} (Figure 1E) values

as well as the mean on intensity ($\langle I_{on} \rangle$), the shape of the averaged transient was calculated and is shown in Panel C (dotted line). Two important points should be emphasized here. First, the good agreement between the calculated and experimental initial peak intensities (I_{peak}) indicates that the amount of average expected recovery to the singlet state occurs during the laser off-period. In this example the initial peak is relatively large due to the short triplet lifetime (1.7 ms) compared to the laser off-period (10 ms). Second, the good agreement for the steady-state intensity (I_{ss}), which is highly dependent upon values for τ_{on} and τ_{T1}, shows that the data is well described by a simple three-state model. Multiple experiments were conducted using a range of excitation powers from 1 to 73 μW. The results demonstrate that *k_{isc} is not dependent on excitation laser intensity*, which is consistent with Figure 1B but inconsistent with results presented in the literature for other dyes.[11,12]

Pulsed excitation experiments carried out for sulforhodamine 101 also show good agreement with the three-state model in most cases. However, it should be noted that difficulties in obtaining accurate model parameters and effects such as spectral diffusion may lead to the masking of a small deviation from the model when using the one-color pulsed excitation method. A more direct measurement can be made by placing a high-intensity perturbation pulse between the excitation pulses and measuring the effect with high signal-to-noise. This is the subject of the next section.

Two-Color, Double Resonance Excitation. Two-color, double resonance excitation can be used to access higher lying excited states as shown in Figure 2B. This study utilizes a two-color pulse sequence in which a high-intensity $T_1 \rightarrow T_N$ laser pulse (670 nm) occurs between each pair of $S_0 \rightarrow S_1$ pulses (543 nm), as illustrated in Figure 2A. A fluorescence intensity transient collected using this sequence on a single sulforhodamine 101 molecule is shown in Figure 2A. For the same single molecule, 147 excitation cycles prior to permanent photobleaching were averaged and are presented in Figure 2C. The excitation cycles following photobleaching were also averaged to yield a background intensity transient, which has been subtracted from the data in Figure 2C. The utility of synchronous data averaging can be seen by noting that, despite the low signal-to-noise in panel A, the pii effect is clearly apparent in the synchronously averaged data. It is important to note that for most of the SR101 molecules investigated the T_1 lifetime is >30 ms. An off-time histogram for a typical molecule in the ensemble is shown in the inset to Figure 2C. The long triplet lifetimes ensure that very little recovery occurs during the laser off period for most molecules in the absence of the $T_1 \rightarrow T_N$ pulse.

In the absence of any effect from the $T_1 \rightarrow T_N$ pulse, the initial fluorescence intensities from the two $S_0 \rightarrow S_1$ pulses in the averaged transient should be equal. The striking intensity difference between the two pulses shown in Figure 2C reports on the effect of the $T_1 \rightarrow T_N$ pulse on the triplet state of the molecule. *This effect appears as a shortening of the triplet state lifetime and can be interpreted as photoinduced intersystem crossing ($T_1 \rightarrow T_N \rightarrow S_N \rightarrow S_0$).* It should be emphasized that the yield (or magnitude) of the pii effect tends to be larger in molecules with long triplet lifetimes since a longer triplet lifetime allows for a greater number of $T_1 \rightarrow T_N$ excitations by the red pulse.

The observed pii effect can be quantified in the form of an enhancement factor, which describes the percentage intensity enhancement of the first peak relative to the second. This enhancement factor (E) is defined in eq 4, where I_{peak-1} and I_{peak-2} are the initial peak fluorescence intensities from the first and second $S_0 \rightarrow S_1$ pulses, respectively (Figure 2C). For the averaged transient shown in Figure 2C, the enhancement factor is 1.74. For this molecule, the triplet lifetime is especially long as indicated by the relatively small degree of recovery of the fluorescence signal during the laser off-period in the absence of the $T_1 \rightarrow T_N$ pulse. This is reflected in the second peak in Figure 2C. As mentioned above, molecules with long triplet lifetimes tend to have larger pii effects. Thus, the larger enhancement factor in Figure 2C is partly due to the longer triplet lifetime of this molecule. Of course, if the pii effect were not operating, the $T_1 \rightarrow T_N$ pulse would have no effect (i.e., $I_{peak-1} = I_{peak-2}$), and an enhancement factor of zero would be observed. In fact, a distribution of values centered on zero is expected due to random error, see below.

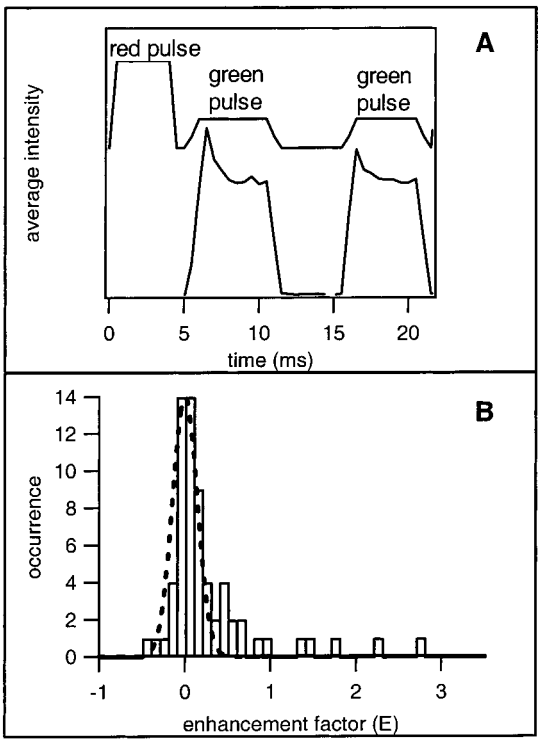

Figure 3. (A) Upper: schematic illustration of the two-color pulse sequence. Lower: sum of background-subtracted average intensity traces for 20 single molecules studied on the same day. (B) Histogram of the enhancement factors for 65 different single sulforhodamine 101 molecules. The dashed line represents an estimate of the random error.

$$E = \frac{I_{peak-1} - I_{peak-2}}{I_{peak-2}} \quad (4)$$

The pii effect is especially apparent by averaging single molecule data for several molecules. For example, in Figure 3A, an average of the intensity transient for 20 different molecules is portrayed. All molecules investigated during a day of experiments were included in this average. Therefore, Figure 3A is an ensemble (albeit small ensemble) average of the pii effect.

Eighty sulforhodamine 101 molecules were studied by the two-color, double resonance method. Molecules (19%) that did not survive long enough to yield meaningful averaged intensity transients were excluded from further analysis. Figure 3B is a histogram of enhancement factors for the remaining 81% of molecules. A Gaussian fit is shown to indicate the estimated random error (dashed line). Approximately 29% of molecules have enhancement factors lying outside the estimated random

error range on the positive side of the distribution. While most molecules show no pii effect, we have studied in detail those molecules outside of the random error estimate. Such analysis clearly reveals that there is an effect from the $T_1 \rightarrow T_N$ pulse. Furthermore, molecules showing the pii effect appear regularly throughout the data. The low occurrence of the pii effect is due to difficulties in focusing and overlapping two beams of widely different wavelengths in such a tightly focused spot. Since, in principle, molecules with long triplet lifetimes tend to have a large yield of the pii effect, an attempt was made to select only molecules with large τ_{T1} values, which were determined for each molecule by the initial cw illumination intensity fluctuation experiment. Nevertheless, some of the variations in the yield of the pii effect (as measured by the enhancement factor) may be due to variations in the triplet lifetimes of the molecules that were investigated. An additional potential source of molecule-to-molecule variations in the enhancement factor may be due to differences in the triplet−triplet absorption spectra for individual molecules. A similar number of DiI molecules was examined, and no significant effect from the red pulse was observed. This result suggests that the 670 nm light is not in resonance with the $T_1 \rightarrow T_N$ transition for DiI.

The effect of the $T_1 \rightarrow T_N$ pulse can be further quantified by determining the effective triplet lifetime $\tau_{T1,eff}$ from the enhancement factor (E) of the averaged transient:

$$\frac{1}{\tau_{T1,eff}} = \frac{-\ln\left(e^{-k_{isc'}t_{laseroff}}(1+E) - \frac{E}{1-A}\right)}{t_{laseroff}} = k_{isc'} + I_{red}\sigma_{T1\rightarrow TN}\phi_{isc',2-color} \quad (5)$$

where $t_{laseroff}$ is the off-period of the green laser, $k_{isc'}$ is the intersystem crossing rate with no $T_1 \rightarrow T_N$ pulse, $A = k_{isc'}/(k_{isc'} + k_{exc}\phi_{isc})$, I_{red} (~75 μW) is the intensity of the $T_1 \rightarrow T_N$ pulse, σ is the T_1-T_N cross section, and $\phi_{isc',2-color}$ is the quantum yield for the rate enhancement. On average, molecules showing the pii effect have a $\tau_{T1,eff}$ that is 42% of their respective $k_{isc'}^{-1}$ values. Assuming the $T_1 \rightarrow T_N \rightarrow S_N \rightarrow S_0$ pathway, values for the parameters $\phi_{isc',2-color}$ and $k_{isc',2-color}$ can be estimated using a σ_{T1-TN} based on literature values of rhodamine dyes[19] and a value of 200 fs for the T_N lifetime.[12] The molecule showing the largest observed pii effect ($E = 2.75$) gives a $\phi_{isc',2-color}$ of 10^{-5} and $k_{isc',2-color}$ of 10^8 s^{-1}.

This experimental approach allows a direct comparison of the magnitude of different isc rates: $k_{isc'} = 10$ s^{-1}; $k_{isc} = 10^6$ s^{-1}; and $k_{isc',2-color} = 10^8$ s^{-1}. The value for k_{isc} is typical for aromatic dyes,[20,21] and the $k_{isc'}$ value is comparable to those for dyes in oxygen-depleted polymers.[7,9] The large disparity between the k_{isc} and $k_{isc'}$ values has been attributed to differences in the magnitudes of the S_1-T_1 and T_1-S_0 energy gaps and the relative degree of spin−orbit coupling.[20] These reasons may also explain the difference between $k_{isc'}$ and $k_{isc',2-color}$.

In addition to photoinduced $T_N \rightarrow S_N$ isc, the observed enhancement could be attributed to other mechanisms including stimulated emission or photoionization from T_1. Stimulated emission can be eliminated based on literature values for the phosphorescence spectrum of SR101.[22] Photoionization from the red pulses and subsequent recombination could cause an enhancement similar to the one observed here. Photoionization occurring from sequential absorption of photons involving the triplet state is a well-known mechanism in organic dyes. For example, photoionization of fluorescein from the triplet state occurs at wavelengths shorter than 470 nm (2.6 eV).[23] Given the much longer wavelengths used here and the lack of evidence for increased destructive photochemistry compared to single color illumination, the likelihood that photoionization is occurring is very low. These arguments provide further evidence that the observed effect is due to photoinduced reverse intersystem crossing ($T_1 \longrightarrow S_0$).

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References and Notes

(1) Xie, X. S.; Trautman, J. K. *Annu. Rev. Phys. Chem.* **1998**, *49*, 441.
(2) Nie, S.; Zare, R. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 567.
(3) Ambrose, W. P.; Goodwin, P. M.; Jett, J. H.; Van Orden, A.; Werner, J. H.; Keller, R. A. *Chem. Rev.* **1999**, *99*, 2929.
(4) Basche, T.; Kummer, S.; Braeuchle, C. *Nature* **1995**, *373*, 132.
(5) Ha, T.; Enderle, T.; Chemla, D. S.; Selvin, P. R.; Weiss, S. *Chem. Phys. Lett.* **1997**, *271*, 1.
(6) Weston, K. D.; Carson, P. J.; Metiu, H.; Buratto, S. K. *J. Chem. Phys.* **1998**, *109*, 7474.
(7) Yip, W.-T.; Hu, D.; Yu, J.; Vanden Bout, D. A.; Barbara, P. F. *J. Phys. Chem. A* **1998**, *102*, 7564.
(8) Weston, K. D.; Carson, P. J.; DeAro, J. A.; Buratto, S. K. *Chem. Phys. Lett.* **1999**, *308*, 58.
(9) English, D. S.; Furube, A.; Barbara, P. F. *Chem. Phys. Lett.* **2000**, *324*, 15.
(10) Veerman, J. A.; Garcia-Parajo, M. F.; Kuipers, L.; van Hulst, N. F. *Phys. Rev. Lett.* **1999**, *83*, 2155.
(11) Fleury, L.; Segura, J.-M.; Zumofen, G.; Hecht, B.; Wild, U. P. *Phys. Rev. Lett.* **2000**, *84*, 1148.
(12) Eggeling, C.; Widengren, J.; Rigler, R.; Seidel, C. A. M. *Anal. Chem.* **1998**, *70*, 2651.
(13) Kamata, Y.; Akiyama, K.; Tero-Kubota, S. *J. Phys. Chem. A* **1999**, *103*, 1714.
(14) Lambert, C. R.; Kochevar, I. E.; Redmond, R. W. *J. Phys. Chem. B* **1999**, *103*, 3737.
(15) Larkin, J. M.; Donaldson, W. R.; Foster, T. H.; Knox, R. S. *Chem. Phys.* **1999**, *244*, 319.
(16) Murphy, S.; Schuster, G. B. *J. Phys. Chem.* **1995**, *99*, 8516.
(17) Redmond, R. W.; Kochevar, I. E.; Krieg, M.; Smith, G.; McGimpsey, W. G. *J. Phys. Chem. A* **1997**, *101*, 2773.
(18) Reindl, S.; Penzkofer, A. *Chem. Phys.* **1996**, *211*, 431.
(19) Carmichael, I.; Hug, G. L. *J. Phys. Chem. Ref. Data* **1986**, *15*, 1.
(20) Turro, N. J. *Modern Molecular Photochemistry*; Benjamin/Cummings: Menlo Park, 1978.
(21) Webb, J. P.; McColgin, W. C.; Peterson, O. G.; Stockman, D. L.; Eberly, J. H. *J. Chem. Phys.* **1970**, *53*, 4227.
(22) Chambers, R. W.; Kearns, D. R. *Photochem. Photobiol.* **1969**, *10*, 215.
(23) Lesclaux, R.; Joussot-Dubien, J. Electron Photoinjection From Aromatic Molecules in Condensed Media. In *Organic Molecular Photophysics*; Birks, J. B., Ed.; Wiley: New York, 1973; Vol. 1.