Time-Varying Triplet State Lifetimes of Single Molecules

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It is found that triplet state lifetimes and intersystem crossing yields of individual molecules embedded in a polymer host at room temperature are not constant in time. The range over which the triplet lifetime of a single molecule varies during long observation times shows a strong similarity with the distribution of lifetime values obtained during short observation times of many individual molecules dispersed in space. The similarity is an elegant manifestation of the ergodic principle of statistical physics.

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Single molecule microscopy and spectroscopy give new and unique insight in the complex behavior of individual emitters on the nanometer scale [1–16]. Measurements on individual molecules have two distinct advantages. First, phenomena that are usually hidden due to ensemble averaging can be directly observed, such as spectral and rotational jumps [1–8], photon (anti-)bunching [7,9–13], and discrete photobleaching. Second, the monitoring of single molecule fluorescence forms a powerful way to probe the dynamics of the local environment on a nanometer scale. Therefore, single molecule detection allows the inhomogeneity of the ensemble to be directly related to the real time dynamics of the heterogeneity of the environment. In contrast, alternative techniques to study the composition of an inhomogeneously broadened ensemble like spectral hole-burning or pump-probe spectroscopies still average over a subset of the ensemble.

For the purpose of this Letter, fluorescent molecules are considered as a three-level system. Besides the repetitive transitions between the singlet ground state (S_0) and the lowest singlet excited state (S_1) giving rise to fluorescence, the molecule has a small chance to undergo intersystem crossing (ISC) from S_1 to the lowest excited triplet state (T_1). As long as the T_1 state remains occupied, the S_0 – S_1 transition does not occur and the fluorescence is interrupted temporarily. After decaying to S_0 the molecule starts fluorescing again. As a result, the fluorescence photons are emitted in bunches separated by dark periods when the molecule is in T_1. This so-called photon bunching can be investigated in two ways. First, autocorrelation of the time intervals between detected photon counts yields the typical duration of the dark periods and thus the T_1 lifetime [7]. The disadvantage of this indirect method is that it yields only a mean value for the T_1 lifetime. Second, integration of the detected fluorescence photons over time intervals shorter than the duration of the dark periods can identify the time length of each excursion to T_1 in a direct way [11–13]. We have used the second method to obtain time-resolved T_1 state dynamics.

The first measurements on the T_1 state of individual molecules were performed at cryogenic temperatures [1,7–9,11]. It was found that T_1 lifetimes and intersystem crossing rates could vary among different molecules, which was attributed to local static disorder in the crystal host. At room temperature and under ambient conditions, due to increased dynamics, molecules exhibit increased dissociation probability, reduced absorption cross section, and shortening of the T_1 lifetime when compared to cryogenic conditions. Not surprisingly, due to the severe experimental demands on the determination of single molecule T_1 lifetimes, only a few experiments at room temperature have been reported [12,13].

For the first time, the conditions to observe T_1 state excursions of single molecules at room temperature have been fulfilled with near-field scanning optical microscopy (NSOM). We describe measurements on the T_1 state of single molecules which show that T_1 lifetimes vary in time. We find a suitable length of time of about 10 s over which the distribution of T_1 lifetimes of one molecule becomes similar to the distribution of constant T_1 lifetimes obtained from many individual molecules during short observation times.

In our experiments the individual molecules are excited by a local probe with a subwavelength aperture light source in a NSOM configuration [4,5,14,15], enabling subnanometer accuracy for locating molecules. Moreover, the three-dimensional orientation of individual molecules can be determined [14,15]. Through the use of high brightness probes [15] (typical excitation intensity 10 kW/cm^2) we obtained a single molecule fluorescence signal up to 10^6 photon counts/s (close to saturation of the S_0 – S_1 transition), pushing the time resolution of the apparatus down to 30 μs. Furthermore, with a lateral position feedback system, nanometer accuracy for positioning the probe above a molecule of choice was achieved. We have used the same 135-nm-diameter probe emitting circularly polarized light for all measurements described in this Letter. The measurements were performed on DicC_{18} molecules embedded in either of two different polymer hosts (10 nm...
thin films of PMMA and polystyrene, respectively) and for two different oxygen concentrations. DiI has been studied before at the single molecule level, yielding information about the fluorescence spectrum [5,6,16], fluorescence lifetime [16], orientation [4,14,16], quantum jumps to $T_1$ [13], and intensity fluctuations [6].

Figure 1 shows two typical fluorescence gray-scale images of DiIC$_{18}$ molecules in PMMA obtained by raster-scanning the probe at a height of 10 nm above the sample surface. Each image shows the collected fluorescence of one single DiIC$_{18}$ molecule with an integration time of 200 $\mu$s per pixel. The presence of several isolated dark pixels within the bright spots indicates that the fluorescence is not continuous in these images. The dark periods (different length for both molecules) are attributed to a temporal residence of the molecule in the $T_1$ state.

In order to monitor the fluorescence of a single molecule continuously as a function of time, we positioned the NSOM probe directly above a molecule. All molecules in this study (a total number of 247) were illuminated and monitored until irreversible photobleaching occurred (typically after $10^6$ detected photon counts). Figure 2(a) shows a 15 ms interval of the fluorescence of a single DiIC$_{18}$ molecule, which emitted fluorescence for 2.8 s in total. The emission undergoes abrupt transitions from a high to a low intensity level and back due to quantum jumps of the molecule from $S_1$ to $T_1$ and from $T_1$ to $S_0$, respectively. From such a fluorescence time trace both a $T_1$ state lifetime ($\tau_T$) and intersystem crossing yield ($Y_{ISC}$) can be extracted. In good approximation, $Y_{ISC}$ is given by the ratio between the ISC rate from $S_1$ to $T_1$ and the fluorescence rate from $S_1$ to $S_0$. The duration of the dark periods was determined directly by selecting a signal level (defined as the 1% lower limit confidence level for the Poissonian photon noise distribution of a time-integrated fit of the intensity) to discriminate between emission (light periods) and dark periods. A histogram of the duration of all dark periods within a certain observation time interval yields an exponential decay with a typical decay time ($\tau_T$) for that interval. Figure 2(b) shows such a histogram that was obtained by evaluating the fluorescence during an observation time interval of 2.8 s. Similarly, a typical $Y_{ISC}$ for a certain observation time interval is obtained from the exponential decay of the number of photon counts in the light periods [Fig. 2(c)], as $Y_{ISC}^{-1}$ is the typical number of counts of the light periods divided by the detection efficiency.

We have determined $\tau_T$ and $Y_{ISC}$ of 80 different molecules embedded in PMMA and 72 molecules in polystyrene. For a fraction of these molecules, the histograms of the dark period duration and the number of counts of the light periods could be described by a single exponential relation. This indicates that both $\tau_T$ and $Y_{ISC}$ were constant during the entire observation time for each of these molecules at their specific location. In Figs. 3(a) and 3(b) the distributions of $\tau_T$ are given for these subsets in PMMA (51 molecules) and polystyrene (55 molecules), respectively. The uncertainty of the $\tau_T$ values (<10%) is smaller than the bin widths in these distributions. Note that both peak position and width of the distributions are different. In polystyrene, the distribution peaks at 40 $\mu$s, while in PMMA this value is 170 $\mu$s. The distributions of $Y_{ISC}$ for these sets of molecules in PMMA and polystyrene yield peak values of $3.3 \times 10^{-4}$ and $2.2 \times 10^{-4}$, respectively. The different peak values of both $\tau_T$ and $Y_{ISC}$ clearly demonstrate the

![FIG. 1](image1.png)

(a) and (b): Gray-scale NSOM fluorescence images (370 $\times$ 370 nm) of two different single DiIC$_{18}$ molecules embedded in a 10 nm PMMA layer (200 $\mu$s integration time per pixel). Dark pixels are caused by a temporal cease of fluorescence due to quantum jumps to the $T_1$ state. Molecule (b) has a longer $T_1$ lifetime than molecule (a).

![FIG. 2](image2.png)

(a) Single molecule fluorescence as a function of time (integration time per point = 57 $\mu$s). The fluorescence drops repeatedly to a low level due to transitions to the $T_1$ state. (b) Histogram of the length of the “dark” periods for the same molecule over an observation time of 2.8 s. The decay can be described with a single exponential giving a mean $T_1$ lifetime of $216 \pm 5$ $\mu$s. (c) Histogram of the number of photon counts of the “light” periods for the same molecule, resulting from an ISC yield of $(3.6 \pm 0.5) \times 10^{-4}$.
influence of the different environments on the molecular photophysical properties. The width of the distributions is caused by the spatial heterogeneity of each sample.

Surprisingly, we found that the histograms of $\tau_T$ and $Y_{\text{ISC}}$ for a considerable fraction of the molecules (35% in PMMA and 25% in polystyrene) could not be described by a simple single exponential decay. This could imply the presence of either multiple decay channels or values for $\tau_T$ and $Y_{\text{ISC}}$ that are not constant in time. To verify this, we have performed a time-resolved analysis of $\tau_T$ and $Y_{\text{ISC}}$. Figures 4(a) and 4(b) show, respectively, $\tau_T$ and $Y_{\text{ISC}}$ of a molecule embedded in PMMA as a function of the observation time. To evaluate a typical $\tau_T$ or $Y_{\text{ISC}}$, a minimum number of $T_1$ excursion events and thus a minimum observation period is required. A value for $\tau_T$ and $Y_{\text{ISC}}$ was evaluated with a single exponential in time intervals of 466 ms, containing approximately 600 $T_1$ state excursions each, resulting in an accuracy for $\tau_T$ of 5%–10%. The molecule indeed displays $\tau_T$ variations, ranging from 0.12 to 0.28 ms, and $Y_{\text{ISC}}$ variations between $2.3 \times 10^{-4}$ and $4.9 \times 10^{-4}$, until irreversible photobleaching occurs.

Histograms of the relative occurrence of the different values of $\tau_T$ and $Y_{\text{ISC}}$ for the long-lived molecule of Figs. 4(a) and 4(b) are shown in Figs. 4(c) and 4(d), respectively, over the entire observation period (13 s). Superimposed to the histograms are also the distributions of $\tau_T$ [as plotted in Fig. 3(a)] and $Y_{\text{ISC}}$, respectively, for spatially dispersed short-lived molecules. It is found that the relative occurrence in time of $\tau_T$ and $Y_{\text{ISC}}$ for the individual molecule matches a large part of the distributions of the constant $\tau_T$ and $Y_{\text{ISC}}$ for the set of spatially dispersed molecules. We find that this similarity is exhibited for all molecules with a time-varying $\tau_T$ and $Y_{\text{ISC}}$, both in PMMA and polystyrene. Furthermore, the longer the observation time, the larger the overlap becomes. We find that variations in $\tau_T$ and $Y_{\text{ISC}}$ for a single molecule occur on a time scale of 0.2 to 20 s. The combined histogram of the relative occurrence of $\tau_T$ and $Y_{\text{ISC}}$ for all molecules with a time-varying $\tau_T$ and $Y_{\text{ISC}}$ overlaps the entire distribution of $\tau_T$ [Fig. 3(a)] and $Y_{\text{ISC}}$, respectively, of spatially dispersed molecules.

The time-dependent behavior of $\tau_T$ and $Y_{\text{ISC}}$ can be understood if one considers that the polymer host constitutes a semirigid environment, which is dynamic in nature at room temperature [4,5,16,17]. As a result, photodynamical parameters such as $\tau_T$ and $Y_{\text{ISC}}$ can indeed vary with time. For instance, variations of the local oxygen concentration related to local conformational dynamics of the polymer host [17] could be involved in the observed variation in $\tau_T$. Oxygen is a well-known quencher of the $T_1$ state of many aromatic dyes [18]. The diffusion constant of oxygen in PMMA is 1 order of magnitude smaller than in polystyrene, while the solubility of oxygen is approximately the same [19]. For a single molecule in polystyrene at ambient conditions, the number of oxygen collisions in a 40 ns time interval is of the order of one to ten [19]. To verify if oxygen quenching plays an important role for the $\tau_T$ distribution in polystyrene, we have reduced the oxygen concentration by continuous flushing of the sample with argon. The
flashing indeed resulted in lengthening of $\tau_T$ to a mean value of 150 $\mu$s for a set of 95 different individual molecules. Thus, we propose that variations of the local oxygen concentration are involved in the observed variation in $\tau_T$, both in space and time.

However, we also find other mechanisms that change $\tau_T$ and $Y_{1\text{ISC}}$. We sometimes observe that an abrupt change in $\tau_T$ or $Y_{1\text{ISC}}$ of a molecule is directly correlated with a sudden change in emission intensity [12]. We can exclude orientational movements as a cause for these intensity jumps due to our orientation sensitive measurement. Therefore, we attribute the intensity jumps to spectral jumps [2,5,6,12] (probably related to changes of the local polarity of the environment) or abrupt changes of the fluorescence quantum yield [6] (probably related to twisting of the conjugated bridge of Dil molecules). Molecules exhibiting intensity or orientational jumps were excluded from the $T_1$ state analysis and will not be further discussed here.

Finally, a major conclusion can be drawn from the similarity of the two distributions of Fig. 4(c) for $\tau_T$ and Fig. 4(d) for $Y_{1\text{ISC}}$, one of them representing the behavior of one molecule in time, and the other representing the behavior of different molecules separated in space. The similarity indicates that all environmental sites become indistinguishable from each other in time. A molecule that would be observable for a sufficiently long period would indeed exhibit the same distribution of $\tau_T$ and $Y_{1\text{ISC}}$ occurrences as a set of molecules dispersed in space at a single moment in time. Therefore, we observe that our measurements satisfy the ergodic principle of statistical physics, stating that for a physical stationary system a time average is equivalent to an ensemble average. Moreover, we obtain a minimum time of approximately 10 s that is needed to observe this manifestation of ergodicity.

In summary, this Letter reports the first observation of time-varying $T_1$ lifetimes and ISC yields of single molecules at room temperature with NSOM. The observations illustrate the potential of single molecules to locally probe physical and chemical dynamics in time on a nanometer scale at ambient conditions. We have shown that $\tau_T$ and $Y_{1\text{ISC}}$ reveal the dynamical nature of the polymer host. The similarity between the distributions of $\tau_T$ and $Y_{1\text{ISC}}$ for different molecules dispersed in space, and for individual molecules in time is an elegant demonstration of the ergodic principle of statistical physics.

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