Looking at the photodynamics of individual fluorescent molecules and proteins*

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Abstract: The photodynamics of individual molecules and fluorescent proteins has been investigated in real time. In the case of organic molecules, both the triplet state lifetime and intersystem crossing yield appear to vary in time and space. In the case of autofluorescent proteins, light-driven "on-off" fluorescence emission has been observed, with a behavior that is consistent with the existence of a long-lived dark state.

INTRODUCTION

In only a few years since its first observation [1], single-molecule fluorescence detection has evolved to a new frontier in science, with high impact and potential for a wide range of disciplines, such as material research, analytical chemistry, and the biological sciences [2]. Monitoring the fluorescent behavior of an individual molecule in time has essential advantages over ensemble-averaged experiments. Single-molecule time trajectories exhibit stochastic fluctuations that directly contain detailed statistical and dynamical information of systems under equilibrium conditions. Time-dependent processes in non-equilibrated systems can be followed without the need for synchronizing a population of molecules. Single-molecule detection reveals the full distribution of a given molecular property, instead of a statistical average. Thus, static and dynamic heterogeneity in a population of molecules in a complex condensed system can be identified and related to the molecular environment. As such, a single molecule can be viewed as a local reporter of its nano-environment.

In this paper we discuss recent results of the photodynamics of individual fluorescent molecules and autofluorescent proteins obtained using a single-molecule-sensitive, near-field scanning optical microscope (NSOM). In the case of organic molecules, we observed the influence of the nano-environment on the photophysical properties, with variations of the triplet parameters in time and as a function of the host matrix [3]. In the case of the green fluorescent protein (GFP), we have studied the Ser-65 $\rightarrow$ Thr (S65T) mutant. At the individual level, the photophysical properties of the GFP differ dramatically from those of ensemble measurements, with an intermittent, "on-off" emission. We have found that the on-off switching is light-induced and have discovered a nonemissive state—different from the triplet state—which is populated after photo-excitation [4].

EXPERIMENTAL METHODS

Carbocyanine ($DiIC_{18}$) molecules were immobilized in a thin polymethyl-methacrylate (PMMA) layer. $DiIC_{18}$ (Molecular Probes, D-282) was diluted in methanol to a final concentration of 5×10^{-8} M. The dye solution was added to a 0.5% weight PMMA in toluene. A 10–20-μl drop was spin-coated at 4000

rpm onto a 170-μm cover slip, resulting in a 5–10-nm layer with a surface coverage of typically ~25 DiIC$_{18}$ molecules per square micrometer.

Samples containing the S65T-GFP mutant were prepared by immobilizing the proteins in water-filled pores of polyacrylamide (PAA) gels. Proteins were diluted to a final concentration of 1×10^{-7} M using phosphate-buffered saline (PBS), pH 7.0. A 2-μl drop of the final solution containing the proteins was sandwiched between two cleaned silica glass cover slips, and the film was allowed to polymerize for a few minutes. The thickness of the resultant film was ~3 μm, with pore size ~3nm, sufficient to spatially confine the proteins while maintaining their naturally fluorescent native conformation.

Both types of samples were inspected using a homemade single-molecule-sensitive NSOM set-up. The technique yields an optical resolution of about 50 nm. This is achieved by scanning a small (sub-wavelength dimension) aperture probe in close proximity (within the near field, i.e., <5 nm) to the specimen under study. The probe is used as a near-field excitation source, while the fluorescence emitted by the sample is detected using conventional optics. In addition to its high resolution, other advantages of the technique are the simultaneous generation of a topographic image, its sensitivity for single-molecule fluorescence detection and the possibility to map the full 3D-dipole orientation of individual molecules. Details on the experimental set-up are described elsewhere [4]. DiIC$_{18}$ molecules were excited at 514 nm wavelength, and fluorescence >550 nm was detected. The S65T-GFP mutant was excited at 488 nm, and fluorescence >520 nm was detected.

## PHOTODYNAMICS OF INDIVIDUAL DiIC$_{18}$ MOLECULES

Figure 1a shows a 40-ms interval followed by another 30-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 the lowest singlet excited state (S_1) to the lowest excited triplet state (T_1) and from T_1 to the singlet ground state (S_0), respectively. From such a fluorescence time trace, both a T_1 state lifetime (τ_T) and intersystem crossing yield (Y_{ISC}) can be extracted. In good approximation, Y_{ISC} is given by the ratio between the intersystem crossing (ISC) rate from S_1 to T_1 and the fluorescence rate from S_1 to S_0. A histogram of the duration of all dark periods within a certain observation time interval yields an exponential decay with a typical decay time τ_T (216 ± 5 μs for this particular molecule [3]). 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 ($3.6 ± 0.5 \times 10^{-4}$ for this molecule [3]).

We have determined τ_T and Y_{ISC} of 80 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. In Figs. 1b and 1c the distribution τ_T is given for a subset of molecules in PMMA and polystyrene, respectively. Note that both peak position and width of the distribution are different. In polystyrene, the distribution peaks at 40 μs, while in PMMA this value is 170 μs. Different values are also obtained for Y_{ISC} (data not shown). The different peak values of both τ_T and Y_{ISC} clearly demonstrate the influence of 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 τ_T and Y_{ISC} for a considerable fraction of the molecules could not be described by a simple exponential decay. This implies the presence of either multiple decay channels or values of τ_T and Y_{ISC} that are not constant in time. To verify this, we have performed a time-resolved analysis of τ_T and Y_{ISC}. Figure 2 shows a two-dimensional matrix containing the fluorescence emission in time of a single DiIC$_{18}$ molecule. Notice that the molecule indeed displays τ_T variations, ranging from 0.15 to 0.3 ms. The time-dependent behavior of τ_T and Y_{ISC} (data not shown) can be understood if one considers that the polymer host constitutes a semi-rigid environment, which is dynamic in nature at room temperature. As a result, photodynamic parameters such a τ_T and Y_{ISC} can indeed vary with time.
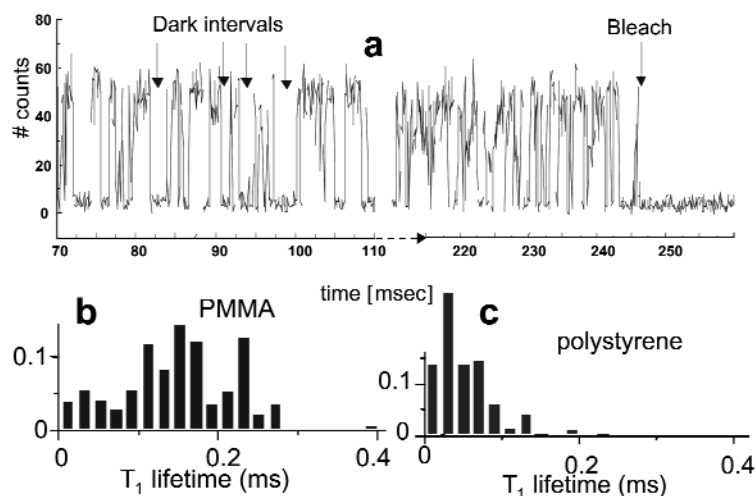
Fig. 1 (a) Single-molecule fluorescence as a function of time (integration time per point = 57 μs). The fluorescence drops repeatedly to a low level due to transitions to the T_1 state. (b) Distribution of τ_T of 51 spatially dispersed $DiIC_{18}$ molecules in PMMA and (c) 55 in polystyrene. The nano heterogeneity of the environment accounts for the range of different τ_T.
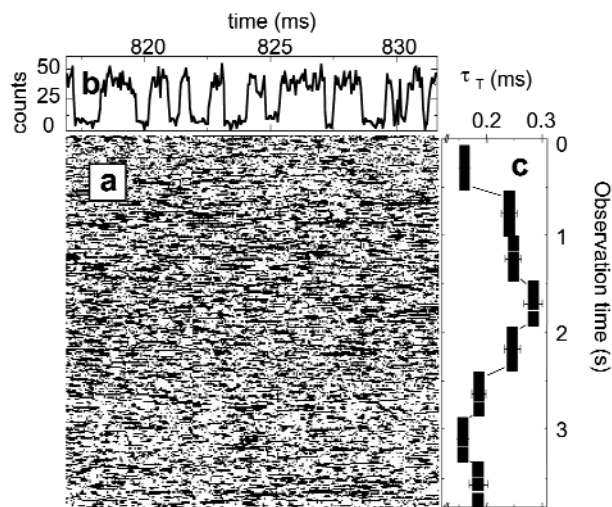

Fig. 2 (a) Two-dimensional matrix containing a fluorescence time trace of 3.75 s duration. Dwell time is 57 μs. Light pixels correspond to fluorescence emitted by the molecule. Dark pixels correspond to the molecule being shelved in T_1. (b) Time trace of 15 ms duration. (c) The total signal is divided in time windows ΔT, of 0.5 s. For each ΔT the triplet lifetime τ_T is statistically determined and plotted in time. Notice that the τ_T varies in time from 0.15 to 0.3 ms [3].

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Figure 3a shows a typical time trajectory of a single S65T-GFP as acquired with our NSOM. In the case of the GFP, it is clear that the fluorescence emission does not occur in a constant fashion with the intermittent transitions to the T_1 state as observed for $DiIC_{18}$ molecules (Fig. 1a), but instead as bursts of fluorescence separated with very long dark intervals. To investigate the origin of dark intervals we have

performed *on* and *off* statistics over a total of 64 individual GFPs, as shown Figs. 3b and 3c [4]. Each histogram can be fitted with a single exponential decay with a characteristic decay time τ. For the particular excitation intensity used in these experiments (14 kW/cm^2) we obtain a relative "on" fraction $\tau_{on}/(\tau_{on} + \tau_{off})$ as small as 5%. In addition, the average number of counts obtained during each bright interval can be extracted from the data and (with a collection efficiency of 7%), we estimate ~6×10^4 photons per fluorescence burst.
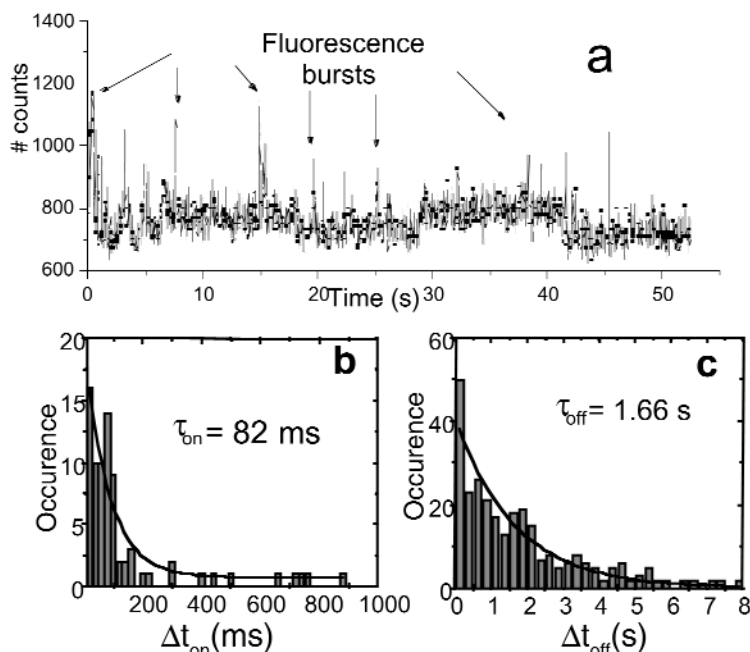
Fig. 3 (a) Real-time fluorescence trajectory of a S65T-GFP molecule (integration time per point = 12.6 ms). The time trace shows mainly background signal with occasional GFP fluorescence bursts. (b) Histogram of the length of the bright periods obtained for 64 molecules observed continuously during 60 s and excited at 14 kW/cm^2. The single exponential decay renders τ_{on} = 82 ± 18 ms at this excitation intensity. (c) Histogram of the length of the dark *off* periods obtained for the same set of molecules as in (b). The single exponential decay of the dark period distribution corresponds to τ_{off} = 1.6 ± 0.2 s.

We also performed statistical analysis over a large number of individual molecules at different excitation intensities. We found that the excitation intensity has a dramatic effect on the fluorescence *on-off* switching [4]. Whereas the time during which the fluorescence is *on* becomes shorter as the intensity is increased, the *off*-times are independent of excitation intensity. The observed behavior is consistent with the existence of a long-lived dark state D, in which the molecule is trapped and fluorescence is *off*. At present we are carrying out wavelength-dependent experiments with the aim of gaining more information on the origin of *on-off* switching of the GFP.

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