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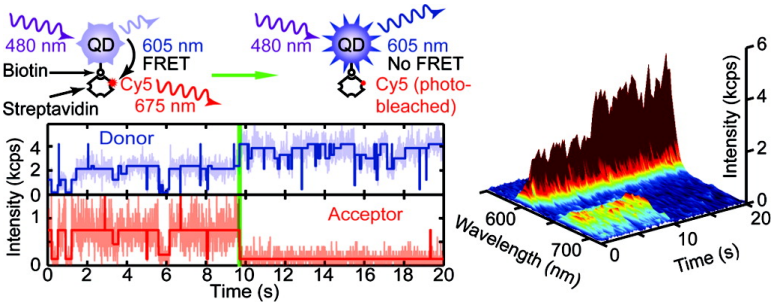

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Multiparameter Fluorescence Spectroscopy of Single Quantum Dot−Dye FRET Hybrids

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Förster resonance energy transfer (FRET) between donor and acceptor chromophores provides a sensitive mechanism for probing interactions between the chromophores and with their local environment. As donor chromophores, luminescent semiconductor quantum dots (QD) offer significant advantages over fluorescent dyes including superior photostability, broad absorption, and narrow, size-tunable emission. As a result, there is rapidly growing interest in QD−organic dye hybrids for FRET-based sensing.[1−4] At the single-particle level, these hybrids have potential for measuring the local environment of nanostructures and studying macromolecule conformational fluctuations. For most purposes multiple acceptors are bound to a single QD hub that serves as the sole energy donor. A major hurdle to using QD−dye hybrids in single-particle applications is the QD's complicated, intermittent emission, or blinking,[5−8] whose kinetic mechanism remains unknown.[9] The single-particle FRET signal in these QD-multiple-acceptor systems must be distinguished from acceptor dye photobleaching events, flickering dye emission caused by the QD donor blinking, and direct excitation of the acceptors. Here we demonstrate that the combined use of multiparameter fluorescence spectroscopy[10,11] and model-free statistical analysis[12] can address these issues. These methods should also be applicable to analyze the complex excited-state dynamics in other multichromophore systems, where various types of multiparameter single-molecule spectroscopy have previously been employed.[13−15] Our multiparameter single-molecule microscope measures the wavelength, emission delay relative to excitation (excited-state lifetime), and chronological time (intensity) for each detected photon. By measuring changes of multiple spectral parameters synchronized through correlated emission intensity jumps, high confidence event assignments can be obtained even in low signal-to-background environments. The model-free statistical analysis quantitatively determines the time of emission intensity changes, taking into account the magnitude of the change and the number of photons used in the evaluation.[12]

We have performed multiparameter FRET measurements for bulk samples of various QD605-Cy5 hybrids made with biotinylated QDs and Cy5 labeled streptavidin. Figure 1 panels a and b show results with, on average, about 5 and 1 dyes per QD. The FRET efficiency, indicated by the ratio of acceptor to donor emission in the spectra, reflects the number of acceptors. The time-resolved excited-state kinetics provides additional information about the energy transfer process (cf. Figure 1c,d). For example, with many acceptors present (cf. Figure 1c), energy transfer to the acceptors reduces the donor excited-state lifetime to ∼3.7 ns from its acceptor-free value of ∼10.5 ns. On the other hand, with only one acceptor (cf. Figure 1d), the decay of the acceptor emission is prolonged from ∼1.4 ns intrinsic lifetime to ∼7.4 ns because energy transfer can occur throughout the long donor lifetime. This result verifies that the

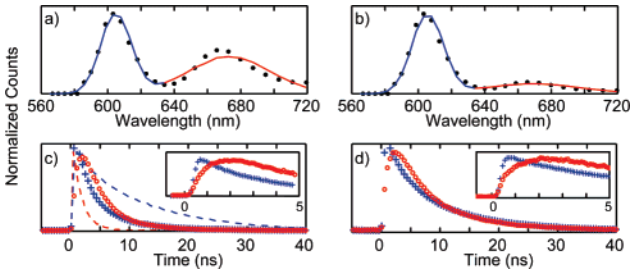

Figure 1. Emission spectra and decay profiles from bulk samples of QD605-Cy5 hybrids. Blue and red represent QD605 and Cy5, respectively. Data in panels a and c represent samples with a ∼1:5 QD605-to-Cy5 ratio. Data in panels b and d represent samples with a ∼1:1 QD605-to-Cy5 ratio. Spectral data are shown as black dots with maximum likelihood estimation (MLE) fits of two Gaussian profiles overlaid as solid lines. Emission decay profiles QD605 (blue "+") and Cy5 (red "o") binned at 480 ps are shown for clarity. Decay profiles of QD605 without acceptor (blue dashed) and that of Cy5 alone (red dashed) are shown for reference in panel c. The insets in panels c and d show close-ups of the histogram rising edge binned at 96 ps.

acceptor emission is due to FRET and not direct excitation. The apparent slow rise of the acceptor emission transient (cf. insets of Figure 1c,d) results from competition between the intrinsic de-excitation of the acceptor and energy transfer from the donor. A coupled four-state kinetic model involving the excited and ground states of donor and acceptor explains this process. This model uses a single exponential, $\exp(-t/\tau_D)$, to describe the donor emission decay, where τ_D is the donor decay with acceptors. The acceptor emission is described by $\exp(-t/\tau_D) - \exp(-t/\tau_A)$, where τ_A is the intrinsic de-excitation time constant of the acceptor without the donor. The bulk-level characterization, however, contains convoluted contributions from the static distribution of number of acceptor dyes around a QD donor, as well as those from the dynamic QD emission blinking and bleaching events. These issues are resolved in single-particle studies as discussed below.

Our single-particle measurements produce a time-stamped photon record that is subsequently divided into QD donor and dye acceptor channels on the basis of the time-integrated spectrum of that QD, determined from a time segment after dye photobleaching. Emission intensity time traces (trajectories) for a single QD-dye hybrid are displayed in Figure 2 panels a and b, which show complicated dynamics in both the donor and acceptor channels. To distinguish acceptor photobleaching events from fluctuations due to donor or acceptor blinking, donor and acceptor intensity change points are first identified. Causal relationships between donor and acceptor change-point pairs are then established on the basis of their overlap in time, which we quantify by the affinity functional.[16] Finally, the sign of the change points within each pair are examined. Opposite signs indicate photobleaching while identical ones suggest QD blinking. As an example, the change-point reconstructed donor and acceptor intensity trajectories are superimposed onto 10 ms

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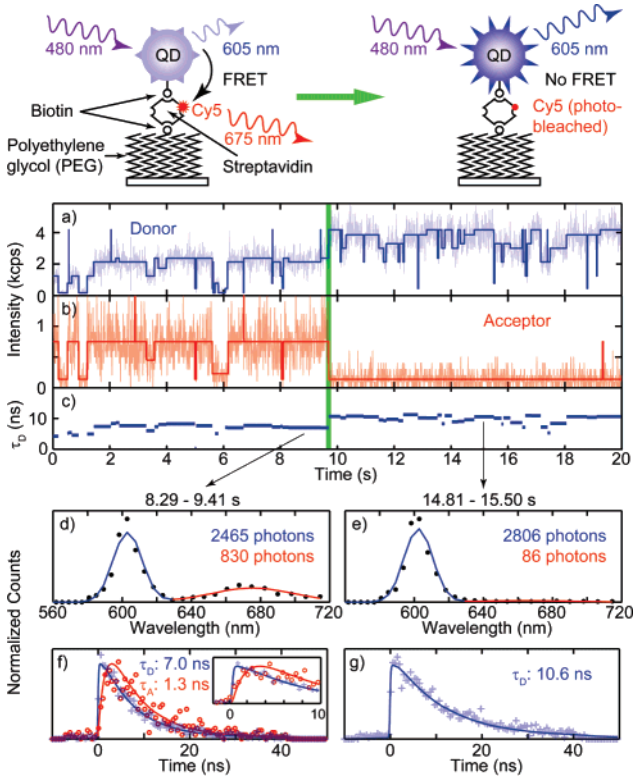

Figure 2. Results for a QD605-$(Cy5)_1$ hybrid. Cy5 photobleaching event is identified with a green vertical line. Blue and red represent donor and acceptors, respectively. (a, b) The 10 ms binned and the change point reconstructed (1% false-positive) donor (a) and acceptor (b) intensity traces are shown in light and solid lines, respectively. (c) Donor lifetime from each donor change-point segment. (d, e) Representative emission spectra (black dots) and MLE fits (solid lines) of individual donor change-point segments. (f, g) Emission decay profiles (binned at 480 ps for clarity) from the corresponding segments in panels d and e; MLE fits are overlaid as solid lines. The inset in panel f shows a close-up of the histogram rising edge.

binned data (cf. Figure 2 panels a and b, respectively). Prior to 9.7 s, dips in the acceptor intensity synchronize with those in the donor trajectory, strongly suggesting they result from QD blinking. The intensity change-points at 9.7 s have opposite signs and are therefore assigned as a photobleaching event. After this event the acceptor intensity drops to baseline while the donor emission continues to fluctuate but with increased average intensity. Similarly, in a more complicated trajectory (see Supporting Information), two photobleaching events are successfully identified. In the above examples, although the acceptor and donor intensity changes are derived independently without assuming any kinetic model, they are closely correlated in time, demonstrating the robustness of the change-point method in extracting accurate state information from noisy intensity trajectories.

After identifying the nature of the change-points, we confirm the dynamics by extracting spectra (cf. Figure 2d,e) and decays (cf. Figure 2f,g) from the trajectory segments. Changes in the single particle emission decays as acceptor photobleaching occurs reflect results from bulk samples with different acceptor concentrations. Energy transfer in the single particle, as in the bulk, is verified by observing the slower rise time and the prolonged decay time of the acceptor emission matching the donor lifetime (cf. Figure 2f). The emission decay from a segment after photobleaching (cf. Figure 2g) shows the increased QD lifetime in the absence of energy transfer to the dye. The decays for all trajectory segments were fit using the MLE method to determine τ_D and τ_A in the kinetic model. The MLE method can determine lifetimes to 10% accuracy with less than 200 photons.[17−19] Here, single-exponential fits are used because the small number of photons contained in each segment cannot support more complicated models. The QD luminescence lifetime fluctuates throughout the trajectory (cf. Figure 2c), but the change-point analysis identifies when its lifetime increases owing to the bleaching of an acceptor dye. Similarly, an example with two dyes is shown in the Supporting Information (cf. Figure S2).

In summary, we demonstrated the unique capabilities of combining multiparameter spectroscopy and model-free statistical approach in the study of complex QD−dye hybrid FRET systems at the single-particle level. Multiparameter measurements that combine spectral and temporal information provide correlated signals to confidently identify the origin of measured fluorescence changes. Use of the model-free statistical approach to detect significant events in the multiparameter data successfully distinguished processes such as acceptor photobleaching from interfering donor and acceptor blinking. This study suggests a robust approach for using QD−dye hybrid FRET-based sensors in single particle applications.

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Supporting Information Available: Detailed description of sample preparation, instrumental configuration, determination of causal relationship between change points, discussion of change-point detection method, the 96 ps binned lifetime profiles of the QD605-$(Cy5)_1$ example, and the results for a QD605-$(Cy5)_2$ hybrid. This material is available free of charge via the Internet at http://pubs.acs.org.

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