Review: Statistical Evaluation of Single Nano-Object Fluorescence (M. Orrit)

Coherent Control for Spectroscopy and Manipulation of Biological Dynamics (M. Motzkus)
Single-molecule dynamics: Semiconductor quantum dots can be used for studies of single-molecule dynamics. The authors used a quantum dot (QD) as donor and an organic acceptor for fluorescence resonance energy transfer (FRET) measurements and observed conformational dynamics from a DNA four-way junction tagged with this FRET pair (picture, top). The time trace of this composite shows anticorrelated fluctuations of signals in the donor (green) and acceptor (red) channels (bottom).
Single-Molecule Quantum-Dot Fluorescence Resonance Energy Transfer

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Colloidal semiconductor quantum dots are promising for single-molecule biological imaging due to their outstanding brightness and photostability. As a proof of concept for single-molecule fluorescence resonance energy transfer (FRET) applications, we measured FRET between a single quantum dot and a single organic fluorophore Cy5. DNA Holliday junction dynamics measured with the quantum dot/Cy5 pair are identical to those obtained with the conventional Cy3/Cy5 pair, that is, conformational changes of individual molecules can be observed by using the quantum dot as the donor.

Introduction

Various single-molecule fluorescence techniques have been used to reveal the inner workings of biomolecules.[1–5] Single-molecule fluorescence resonance energy transfer (FRET) is particularly popular because it is sensitive to distance changes in the center-of-mass frame of the host biomolecule but relatively uninfluenced by the overall tumbling. Typically, two organic fluorophores, the donor and the acceptor, are attached to a biomolecule to report on the changes in the interfluorophore distances induced by conformational changes of the biomolecule. While many pairs of organic fluorophores such as Cy3/Cy5[7] have been used successfully for various biological systems spanning DNA[8–10] RNA[11–17] and proteins, [18–20] one could always benefit from better probes. Brighter fluorophores can make it easier to carry out single-molecule experiments in live cells by reducing background fluorescence. [21–22] Long-lasting fluorophores may reveal both fast and slow processes in one molecule so that slow changes in kinetic properties of a biomolecule[22] may be studied in detail. Better probes may also make the single-molecule techniques more widely accessible by bypassing the need for expensive instrumentation or special skills.

Colloidal semiconductor quantum dots are attractive because they are much brighter and more photostable than organic fluorophores.[23, 24] Their wide, size-tunable color range, broad absorption spectrum, and narrow emission spectra are ideal for multicolor imaging. The same chemistry for different colors makes bioconjugation straightforward once established. There have already been efforts to utilize quantum-dot FRET for immunosensors[25–30] and scanning probe microscopy.[31] However, severe blinking, or emission intermittence, of single quantum dots has been an obstacle to their widespread application in single-molecule studies.[32–34] Recently, we discovered that the blinking of commercially available, water-soluble quantum dots can be suppressed very efficiently by adding small thiol-containing molecules in solution.[35] Here we report the observation of single-pair FRET between a quantum dot and an organic fluorophore. We further demonstrate that such a FRET pair can be used to measure the conformation changes of single biomolecules using the DNA four-way (Holliday) junction as a model system.

Results and Discussion

We used the streptavidin-coated quantum dot with the emission peak at 585 nm (called Q5S85 from now on, Quantum Dot Corp.) as donor and measured FRET to an organic fluorophore acceptor Cy5. Q5S85 has a number of potential advantages over Cy3 as the FRET donor. As Figure 1 a shows, Q5S85 has a narrow and symmetrical emission spectrum that greatly reduces its bleed-through into the acceptor’s spectral range. Furthermore, because Q5S85 is about 20 times brighter than Cy3 (on excitation at 532 nm), direct excitation of Cy5 can be reduced to an undetectable level. Overall, these two properties virtually guarantee that any signal appearing in the acceptor channel arises from energy transfer, provided that all quantum dots have the same spectrum. FRET is more efficient if the overlap between the donor emission and acceptor absorption spectra is larger. The smaller overlap between Q5S85 emission and Cy5 absorption, compared to that of Cy3 and Cy5, is compensated by the higher emission quantum yield of Q5S85 (45 % relative to rhodamine 101 in ethanol as reference vs. ≈ 25 % for DNA-conjugated Cy3). [36] To test how high FRET efficiency can be if the acceptor is brought as close as possible to the quantum dot surface, we placed Cy5 and biotin at the same end of duplex DNA. Two complementary DNA single strands were purchased from IDT DNA, one containing Cy5 at the 5’ end and the other with biotin at the 3’ end. Q5S85/DNA composites were immobilized on a quartz surface coated with biotinylated bovine serum albumin (BSA), as shown in Figure 1 b. Thus, the streptavidin-coated quantum dot is used both as the donor and as a bridge to the surface.

Upon excitation with a green laser (532 nm), 14 % of fluorescent spots in the donor channel were accompanied by spots in the acceptor channel. Since a small fraction of quantum dots in the long-wavelength tail of the size distribution may
have emission in the acceptor channel, we performed sequential two-color experiments (Figure 1 c) to ascertain the origin of the signal in the acceptor channel. The green laser excited the sample initially, and nonzero signal in the acceptor channel (red lines) indicated possible FRET. In the middle of the experiment, the green laser was blocked and a red laser (633 nm) was used to excite the sample. Increased acceptor signal and one-step blinking or bleaching indicated that the signal in the acceptor channel came from one Cy5. Finally, the green laser was unblocked and the red laser was blocked. In the time trace shown, Cy5 was inactive for FRET due to blinking and no signal appeared in the acceptor channel, and this confirms that the signal in the acceptor channel observed at the beginning of the trace was due to Cy5. Cy5 recovered from the blinking state and became active again for FRET at about 140 s with an accompanying drop in the donor-channel signal. In this set of experiments 11.4% (95 out of 836) of all observed quantum dots had a single Cy5, 2.4% had two Cy5s (20 out of 836), and 0.1% had three (1 out of 836). The number of Cy5 molecules was estimated on the basis of stepwise photobleaching or blinking on direct excitation with the red laser. Overall, these experiments show that signal in the acceptor channel arises from FRET between Q585 and Cy5.

Figure 1 d shows the FRET efficiency histogram built from 95 single quantum dot traces with one Cy5. FRET efficiency is expressed as $\phi_f/\left(\phi_d + \phi_f\right)$, where $\phi$ is a measure of how much the donor signal changes relative to the change in the acceptor signal when a FRET change occurs. The calibration factor $\phi$ can be obtained from anticorrelated changes of donor and acceptor signals that occur on acceptor bleaching or blinking. For example, $\phi$ is about 2.7 for the data shown in Figure 2b. The histogram was made from Cy5 active regions for FRET. The histogram peaks at 5.5%, a small but clear increase from 2.5% due to noise in the Cy5 channel measured when Cy5 is bleached. Interestingly, the FRET distribution is broadly extended toward the high-FRET region in the case of quantum-dot FRET. There are many possible biotin binding sites per quantum dot (15–20 streptavidin molecules per quantum dot and four biotin binding pockets per streptavidin), and higher FRET signals may arise when DNA happens to bind near the quantum-dot surface, not on the outer surface of the streptavidin layer. The quantum dot itself is not spherical but elongated (Quantum Dot Corp.) and this could possibly contribute to the broadening of the FRET efficiency histogram.

Next we tested whether single-molecule conformational changes can be observed using Q585/Cy5 FRET. The Holliday junction is an essential intermediate in homologous DNA recombination. It is composed of four DNA double helices in the form of a four-way junction which folds into stacked conformations in the presence of divalent ions. This junction is a good model system because its fluctuations between two stacked forms have been well characterized by using regular single-molecule FRET. [9, 38] Oligonucleotides of the following sequences (all written 5’ to 3’) and labeling were purchased from IDTDNA:

1) x strand: CCCAGTGGAGAGCTTGAGGG (unlabeled or 5’-Cy5)
2) b strand: CCCTAGCAAGCCGCTGCTACGG (unlabeled or 5’-Cy3)
3) r strand: CCCACCGCTCTTCTCAACTGG (5’-Cy5 or 5’-biotin)
4) h strand: CCGTAGACGACAGCGGATAGG (5’-biotin or unlabeled)

The first labeling configuration was used for the quantum dot experiments and the second for Cy3/Cy5 FRET experiments. Both configurations are expected to show the same transitions between high- and low-FRET states, with a bias toward the high-FRET state. [39, 40] The junction was assembled from four single-stranded DNA molecules (x, b, r, and h) by using the annealing procedures described in the Experimental Section. The final construct after surface immobilization is illustrated in Figure 2a.
Experiments were performed at 10°C in 10 mM Tris·HCl, pH 7.5, 50 mM MgCl₂, in which conformational transitions between the two stacked forms are slow enough to be clearly observed with the 100 ms time resolution used. Less than 1% of all quantum dot traces showed anticorrelated fluctuations of signals in the donor and acceptor channels (green and red, respectively). Both of these states had significant signals in the acceptor channel, clearly different from the donor-only segment (likely due to Cy5 blinking). A) An example of a time trace of Q585/Cy5/Holliday junction composite shows clearly anticorrelated fluctuations of signals in the donor and acceptor channels (green and red, respectively). Both of these states had significant signals in the acceptor channel, clearly different from the donor-only segment seen in parts of the traces (for instance from about 46 to about 57 s), which is likely due to Cy5 blinking.

Compared to the Cy3/Cy5 pair (Figure 2 c), FRET efficiency of the Q585/Cy5 pair is lower, but the very similar timescale of two-state fluctuation suggests that the fluctuations represent conformational transitions of the Holliday junction. To test this interpretation further, we performed dwell time analysis. For the Q585/Cy5 pair, dwell time histograms were built from about 80 molecules selected from 20,000 quantum dot traces with a total of about 400 transitions. For the Cy3/Cy5 pair, about 150 molecules with a total of about 1500 transitions were used to build the dwell-time histograms. The average dwell times were obtained by fitting the histograms to single exponential decay. The first bin or first two bins were not included in the fitting to account for missing short events due to limited time resolution and signal-to-noise ratio. The Q585/Cy5 pair gave average dwell times of 1.2 and 0.6 s for the high- and low-FRET states respectively, while the Cy3/Cy5 pair gave 1.3 and 0.5 s (Figure 3). The results are essentially identical for both sets of probes, and we conclude that FRET fluctuations shown in Figure 2 b arise from true conformational dynamics of the Holliday junction. To our knowledge, this is the first time while imaging of single quantum dots has been used to study the internal motion of a biological molecule.

Since we are using an organic fluorophore as acceptor, the total observation time is still limited by photobleaching of Cy5, not of the quantum dot. Even in this incarnation, however, there are potential advantages over regular single-molecule FRET. Live-cell single-molecule studies using organic fluorophores suffer from high background fluorescence. This background issue should become insignificant if a quantum dot is used as the donor because 20 times lower excitation can be used. Lower excitation power also leads to negligible direct excitation of the acceptor. Combined with the narrow emission spectra of quantum dots it may also be possible to detect very low FRET.

An extremely long observation time may be achieved if FRET is measured between two quantum dots. However the size of quantum dots becomes more problematic for such a scheme because the minimal distance achievable is now given by the sum of the two radii. In addition, the acceptor quantum dot will be excited efficiently due to its continuum absorption spectrum, potentially masking acceptor emission arising from FRET.

While our results show that single-molecule conformational changes can be measured using quantum dot/organic fluorophore pairs, we conclude that FRET fluctuations shown in Figure 2 b arise from true conformational dynamics of the Holliday junction. To our knowledge, this is the first time while imaging of single quantum dots has been used to study the internal motion of a biological molecule.
replacement of the streptavidin layer with small bioconjugable molecules that can be used for data analysis highlight the majority of quantum dots without DNA, largely accounting for multiple DNA per quantum dot. This inevitably led to the vast majority of quantum dots without DNA, largely accounting for the low yield. Therefore, monofunctionality is also desired for future generations of quantum dots.

Conclusions

We observed single-molecule FRET between a quantum dot and an organic fluorophore and showed that single-molecule structural dynamics can be reliably probed. The low yields of molecules that can be used for data analysis highlight the need to develop smaller quantum dots with monofunctionality.

Experimental Section

Sample preparation: DNA molecules were prepared by mixing each strand (5 μM) in Tris·HCl (10 mM, pH 7.5) with Na+ (200 mM), heating to 90°C, and slowly cooling to room temperature over several hours. The DNA (2 μM) was then mixed with Q585 (2 μM) in Qdot buffer (Quantum dot Corp.) in a 1:1 ratio and incubated overnight. For single-molecule experiments, a narrow channel was made between a cleaned quartz microscope slide (Finkerbeiner) and a coverslip using double-sided adhesive tape. The surface was treated with biotinylated BSA (40 μL, 1 mg·mL⁻¹, Sigma) in Tris·HCl (10 mM, pH 7.4) with 50 mM NaCl (TN buffer). After 10 min incubation and washing out with TN buffer, DNA-Q585 complex diluted to 20 pM (40 μL) was injected into the channel.

Single-molecule measurements: A wide-field microscope with prism-based, total internal reflection excitation was used in combination with an intensified CCD camera. The microscope is based on an inverted microscope (Olympus IX70) with a 60× water-immersion objective with numerical aperture of 1.2 (Olympus, Melville, NY) and an intensified CCD camera (Intensified Pentamax, Roper Scientific, Trenton, NJ) that can record both the donor and acceptor intensities from several hundred single molecules simultaneously. A solid-state 532 nm laser (Crystalaser) and a HeNe laser (Coherent) were used to excite the molecules. Data were acquired using software written in Visual C++ (Microsoft). The experiments were performed in Tris buffer (10 mM, pH 7.5) with oxygen scavenger system (0.4% w/v glucose, 0.1 mg·mL⁻¹ glucose oxidase (Sigma), 0.02 mg·mL⁻¹ catalase (Roche), 1% v/v β-mercaptoethanol) and 50 mM MgCl₂.

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