Monitoring multiple distances within a single molecule using switchable FRET

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The analysis of structure and dynamics of biomolecules is important for understanding their function. Toward this aim, we introduce a method called 'switchable FRET', which combines single-molecule fluorescence resonance energy transfer (FRET) with reversible photoswitching of fluorophores. Typically, single-molecule FRET is measured within a single donor-acceptor pair and reports on only one distance. Although multipair FRET approaches that monitor multiple distances have been developed, they are technically challenging and difficult to extend, mainly because of their reliance on spectrally distinct acceptors. In contrast, switchable FRET sequentially probes FRET between a single donor and spectrally identical photoswitchable acceptors, dramatically reducing the experimental and analytical complexity and enabling direct monitoring of multiple distances. Our experiments on DNA molecules, a protein-DNA complex and dynamic Holliday junctions demonstrate the potential of switchable FRET for studying dynamic, multicomponent biomolecules.

Single-molecule methods have changed the landscape of biological physics and biochemistry by focusing on the fundamental unit of molecular analysis: an individual molecule[1,2]. A popular single-molecule method is fluorescence resonance energy transfer (FRET) spectroscopy[2–4], which relies on a distance-dependent dipole-dipole interaction between a donor and an acceptor fluorophore to report biomolecular structure, dynamics and interactions[5]. The versatility of FRET has recently been extended by alternating-laser excitation (ALEX) schemes that monitor the relative donor-acceptor stoichiometry[6].

Although single-molecule FRET usually involves a single donor-acceptor pair, one can also use multiple pairs to probe multiple intramolecular distances, enabling distance triangulation or multi-angulation[7] and multiperspective monitoring of conformational changes. Because of molecular heterogeneity and dynamics, obtaining multidistance information from a single molecule is preferable to combining information from populations of single molecules, each carrying a single FRET pair at different positions and measured in separate experiments. To this end, multicolor methods using three or more distinct fluorophores have been developed, including three-color FRET[8,9] and three-color ALEX[10] techniques. Such experiments are complicated: they require site-specific labeling with fluorophores exhibiting large spectral overlap for FRET yet having sufficient spectral separation to allow detection in three or more separate emission regions. Moreover, FRET between multiple fluorophores follows a cascade of energy-transfer processes, complicating data analysis and interpretation[9]. As a result, multicolor FRET methods cannot be easily extended, and as such, they have not realized their potential.

Here we describe switchable FRET, a method that can be used to probe two or more distances in a single molecule by using a single donor and two or more identical acceptors. Using only two types of fluorophores circumvents many problems of the multicolor approaches and allows the examination of multiple FRET pairs in a single molecule. Specifically, switchable FRET uses temporal confinement of fluorescence (that is, sorting fluorescence by time in addition to sorting by absorption or emission characteristics) to sequentially probe multiple FRET pairs. Temporal confinement is achieved by photoswitching[11,12] (that is, reversible modulation of fluorophore absorption), a process exploited in super-resolution imaging[13,14] and in ensemble FRET modalities[15,16]. As photoswitching can be induced in many fluorophores[17,18] our method is general and extendable. Moreover, probing of several switching states per molecule allows calculation of accurate FRET efficiencies, aiding efforts to deduce structural information from single molecules[7,19,20]. The number of distances measured by switchable FRET is fundamentally limited only by our FRET resolution.

Using switchable FRET, we measured two accurate FRET efficiencies and corresponding distances in a single DNA molecule. To demonstrate the general applicability of switchable FRET, we probed two distances within a protein-DNA complex. Finally, we used switchable FRET to directly monitor conformational changes in single dynamic Holliday junctions from two perspectives.

RESULTS

Concept

Switchable FRET is compatible with various types of fluorophores such as carbocyanine Cy5 and oxazine ATTO655 that undergo switching via different mechanisms[11,12,18] (**Fig. 1a**,**b**).

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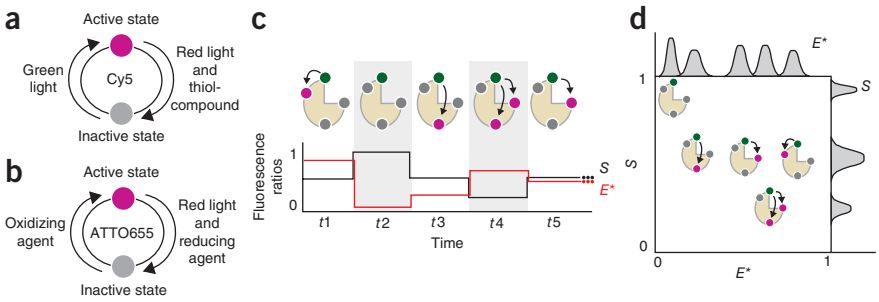

Figure 1 | Concept of switchable FRET. (**a**) Irradiation of Cy5 with red light reads out the fluorescence signal and switches the fluorophore from the active fluorescent state to the inactive dark state; this transition requires thiol-containing reducing agents and oxygen removal. Irradiation with green light supports the transition back to the active state. (**b**) ATTO655 reversibly switches in presence of a reducing agent and molecular oxygen upon irradiation at a single wavelength: the inactive dark state is reached by intersystem crossing and reduction from the active fluorescent state. Spontaneous oxidation restores the fluorescent state. (**c**) To probe multiple distances via FRET, a single molecule is labeled with one donor (green) and multiple identical photoswitchable acceptors (magenta). ALEX of individual immobilized molecules provides information on apparent FRET (*E**) and stoichiometry (*S*). Single-pair FRET states (*t*1, *t*3 and *t*5) are probed by switching the acceptors between an active state and an inactive state (gray) that does not interact with the donor. Transient donor-only states (*t*2) or states with more than one active acceptor (*t*4) can be identified by a change in stoichiometry. (**d**) The *E** and *S* data are summarized on a two-dimensional scatter plot with projected one-dimensional histograms at the sides. Different states appear as distinct clusters in the histogram and can be sorted using *E** and *S*.

In switchable FRET, immobilized molecules labeled with a single donor and multiple identical acceptors are probed over time by reversibly switching acceptors between a dark state with an absorption spectrum that does not overlap with the donor emission spectrum (and does not participate in FRET) and a fluorescent state that participates normally in FRET (**Fig. 1c**). Stochastic switching separates the time segments of individual active acceptors, thus resolving multiple FRET pairs at different positions on a single molecule in a temporal fashion. The apparent FRET efficiency, *E**, reports the distance between the donor and the active acceptor. Reversible switching between active and inactive states interrogates each pair many times, a feature that permits monitoring structural changes in a molecule.

We used ALEX to probe FRET and the stoichiometry ratio (*S*) of active donor and acceptor fluorophores simultaneously[6,21]. Because the donor was not switchable in our experiments, *S* served as a 'counter' of the number of active acceptors at any time: donor-only states had high *S* values (**Fig. 1c**); states with a donor and a single active acceptor had intermediate *S* values, and states with one donor and two active acceptors display low *S* values. We summarized the FRET and stoichiometry information of an individual molecule on a two-dimensional *E**-*S* histogram (**Fig. 1d**).

Proof of principle

As a proof-of-principle experiment for switchable FRET, we prepared a 55 base pair (bp) DNA with a single Cy3B donor placed between two Cy5 acceptors (**Supplementary Table 1**). The distal and proximal donor-acceptor separations were 19 bp and 10 bp, respectively. One molecule could adopt four distinct photophysical states; namely (i) donor-only (denoted as 0-D0, in which 'D' stands for donor, '0' stands for an acceptor in its inactive state, and '-' denotes the 19-bp separation; 10-bp separation is implied between D and the second 0) when both acceptors were inactive, (ii) state A-D0 (low *E**, intermediate *S*) when only the distal acceptor (A) was active; (iii) state 0-DA (high *E**, intermediate *S*) when only the proximal acceptor was active; and (iv) state A-DA (high *E**, low *S*) when both acceptors were active.

We measured immobilized DNA molecules using total internal reflection fluorescence (TIRF) microscopy combined with ALEX[21]. A typical time trace of fluorescence intensity, *E** and *S* (an 'ALEX time trace'; **Fig. 2a**) from a single DNA molecule with one Cy3B and two Cy5 fluorophores showed that alternating red and green excitation caused stochastic photoswitching of the two acceptors, apparent from steps between three intensity levels in the acceptor emission upon acceptor excitation (F_{AA}). The intensity was maximal when both acceptors were active (~8,000 photon counts per 100 ms; state A-DA), intermediate during segments when only one acceptor is active (~4,000 photon counts per 100 ms; either state A-D0 or 0-DA) and low during donor-only segments (<100 photon counts per 100 ms; state 0-D0). Donor excitation yields donor emission (F_{DD}) and acceptor emission owing to FRET (F_{DA}). Because the donor transfers energy only to active acceptors, F_{DD} is maximal for 0-D0 but becomes substantially quenched when an acceptor switches on; each of the four states is adopted several times. The donor was not affected by the switching conditions and did not blink or bleach during the experiment (data not shown). We also observed that the photoactivation rate of Cy5 depended on the proximity to an activator fluorophore[11] and thus differed for the two acceptors in our system as indicated by their individual off-state dwell times in the ALEX time traces (data not shown).

The *E**-*S* histogram from the time trace in **Figure 2a** shows the expected four clusters representing the states 0-D0, A-D0, 0-DA and A-DA (**Fig. 2b**,**c**). To identify states and extract statistical information, we separated the clusters using a standard clustering algorithm (*k* means). The identified distributions were narrow ($\sigma(E^*)$ = ~0.03), compared to typical diffusion-based FRET histograms[19]. The two clusters of the single-pair FRET efficiencies (*E** was 0.256 for A-D0 and 0.793 for 0-DA) were clearly separated from states 0-D0 and A-DA, and identified as individual states.

Controls

To exclude the possibility that the single-pair FRET efficiency is altered by light absorption of the second acceptor in the inactive state, we studied the same DNA with a single acceptor either at 10-bp or 19-bp separation from the donor (**Supplementary Table 1**). The controls showed clusters identical to those from the two-acceptor DNA (**Fig. 2d**,**e**). To test the requirement for switching to probe multiple distances, we analyzed DNA with one donor and two acceptors under nonswitching conditions[22]; in this case, irreversible sequential acceptor bleaching reported on only one of the two pairs per molecule. If the proximal acceptor bleached first, the 0-DA state was missing from the *E**-*S* histogram (**Fig. 2f**);