Probing DNA Dynamics: Stacking-Induced Fluorescence Increase (SIFI) versus FRET

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Stacking-induced fluorescence increase (SIFI) was introduced recently as a method to probe DNA structure and dynamics using only a single fluorescent label. Here we show that the same DNA hairpin dynamics can be recovered, at the single-molecule level, using either SIFI (with Cy3 as the label) or FRET (with Cy3 as donor and Cy5 as acceptor). We also measured FRET using a donor that cannot undergo SIFI, Cy3B, in the presence and absence of a molecular crowding agent (PEG). Although crowding increases hairpin hybridisation to the same extent with either Cy3 or Cy3B as the donor, the absolute rates are affected by the choice of donor dye. This work shows that SIFI can be used to measure single-molecule dynamics, which could offer advantages over FRET in some cases. It also illustrates how local dye interactions can influence biomolecular dynamics, which should be considered when designing experiments.

Förster resonance energy transfer (FRET) is a powerful tool for measuring the structure and dynamics of DNA,[1] revealing molecular details far below the diffraction limit. Furthermore, the extension to single-molecule FRET (smFRET) has produced a step-change in the information attainable.[1b,2] FRET requires the presence of two labels, a fluorescent donor and an acceptor, which is normally also fluorescent; it also requires inter-dye distances in the 2–10 nm range. These requirements can limit the applicability of the method. In contrast, we recently demonstrated that both local and long-range information on DNA structure and dynamics could be obtained using only a single fluorescent dye.[3] We showed that the fluorescent dye Cy3 is able to stack site-selectively in DNA with a resultant enhancement in fluorescence, which we termed stacking-induced fluorescence increase (SIFI).[3]

Earlier work by several other groups had shown that interactions of Cy3 with proteins and nucleic acids can influence its quantum yield by hindering or promoting the photo-induced isomerisation around the central C=C double-bond.[4] The interaction with proteins is known as protein-induced fluorescence enhancement (PIFE)[4a,b] while with nucleic acids it is known as nucleic acid induced fluorescence enhancement (NAIFE)[4d] or nucleic acids induced fluorescence quenching (NAIFQ).[4f] SIFI also works via modulation of photoisomerisation, and can be considered as a subset of NAIFE, exploiting the specific interaction between Cy3 and a nick or gap in the double-stranded DNA (dsDNA) structure. We previously used SIFI to detect hybridisation of a DNA hairpin at the single-molecule level, and to sense a long-range perturbation in the form of an abasic site up to 20 base pairs (bp) from the site of dye stacking.[3] Here we directly compare SIFI and FRET (using Cy3 as the donor and Cy5 as the acceptor) at the single-molecule level (termed smSIFI and smFRET, respectively) as tools for probing the dynamics of DNA. We study the opening and closing of a DNA hairpin, and show that the same kinetic parameters are recovered by both methods. We then examine the effect of the Cy3 dye on the dynamics by comparing FRET in hairpins labelled with a donor that does not undergo photoisomerisation, Cy3B.

As a model system, we chose a DNA hairpin design that we had previously employed (Table S1).[3,5] The hairpin consists of a 32 nucleotide (nt) dA loop and a 6 bp stem that allows the hairpin to frequently open and close under ambient conditions; an 18 bp dsDNA handle positions the hairpin away from the surface when immobilised via a biotin moiety at the 5’ end. For FRET experiments, the hairpin was labelled with Cy3 and Cy5, and the position of the dyes was designed to give a high FRET efficiency when the hairpin was in a closed conformation, whilst also avoiding dye-dye interactions. When the hairpin is in a closed conformation, there is a 2 nt gap between the dsDNA stem and dsDNA handle, which allows the Cy3 dye to stack and SIFI to occur.[3] Once the hairpin opens and the restriction on rotation around its double bond is lifted, photoisomerisation is more efficient and the quantum yield is correspondingly lower. Immobilisation of the hairpin on a passivated microscope slide allowed observation of the fluorescence intensity of the dyes at single-molecule resolution using total internal reflection fluorescence (TIRF) microscopy (see Supporting Information).

Our goal was to determine whether the same DNA dynamics could be measured using smFRET and smSIFI. The previous report of SIFI used DNA labelled with a single dye.[3] Here we label hairpins with Cy3 and Cy5, which allows both FRET and SIFI to be measured for the same molecules. When both dyes are active, we detect FRET. We note at this stage that the FRET process is itself affected by SIFI, since the donor dye’s quantum yield and lifetime increase upon stacking. This

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convolution of SIFI and FRET is analogous to the combination of FRET with PIFE. In our case, we postulated that SIFI might only affect the absolute intensities of donor and acceptor emission but not the FRET dynamics. The rationale for this is that the local stacking/unstacking dynamics are much faster than the resolution of the TIRF experiment so the FRET dynamics reflects hairpin opening and closing, not dye stacking/unstacking. As mentioned, we are able to study SIFI and FRET in the same molecule because after the acceptor dye bleaches, we detect only SIFI. In fact, we first became aware of the SIFI phenomenon when we recorded longer FRET movies that increased the chance of observing acceptor bleaching during the acquisition time. This enabled us to apply the smFRET and smSIFI analyses to the time trajectories of the same molecules, before and after the acceptor dye had photobleached, respectively (Figure 1).

With both donor (Cy3) and acceptor (Cy5) fluorophores active, the opening and closing of the hairpin produced anti-correlated changes in donor and acceptor emission, typical of a single FRET pair. Photobleaching of the Cy5 prohibits it from accepting energy from the Cy3. If Cy3 is still photoactive after Cy5 photobleaches, its fluorescence is restored to a donor-only intensity. As the emission of these Cy3 fluorophores is still sensitive to the hairpin conformation, it is possible to follow the dynamics of these hairpins using smSIFI (Figure 1).

To follow the opening and closing of individual DNA hairpins, we analysed the trajectories by adapting an existing hidden Markov modelling (HMM) program. Previous smSIFI studies used a simple thresholding method to determine the opening and closing rates of hairpins, whereas smFRET trajectories are typically subjected to a more rigorous analysis, such as HMM analysis. However, smFRET trajectories consist of both donor and acceptor intensities in contrast to smSIFI, which uses a single fluorophore. We therefore adapted our smSIFI analysis method to make it compatible with open source programs commonly used for smFRET experiments by adding a dummy acceptor trace (Figure S1), similar to the method of Pokhrel et al. using PIFE. Here the dummy acceptor trace is perfectly anti-correlated to the smSIFI trajectories, allowing the generation of a pseudo-smFRET trace, maintaining the same signal to noise ratio in the experimental data, the dummy acceptor trace and the resulting pseudo-smFRET traces.

Figure 1 also shows how the single-molecule trajectories can be separated into two sections that represent the fluorescent intensities of Cy3 and Cy5 before and after Cy5 photobleaches. These traces can then fitted to an idealised path, illustrated in Figure 2A, where the raw data is shown in blue and the idealised trace is in red. After photobleaching of the Cy5 dye we found that the relative populations in the open and closed states were the same (Figure 2B). Specifically, we show that the distribution between the open and closed states is not significantly affected upon photobleaching using a paired sample T-test. The single-molecule traces were then analysed both by simple thresholding analysis and by HMM. The thresholding analysis recorded significantly faster opening and closing rates for smSIFI than smFRET, an artefact due to the relatively lower threshold that distinguishes between the open and closed values (Figure 2B–E). FRET trajectories are typically less noisy due the requirement of anti-correlation between the two dyes averaging out the smFRET signals. This effectively increases the threshold relative to the noise of the trajectories, so the thresholding analysis of smFRET traces did not identify open/closed transitions as frequently as smSIFI traces. This is shown in Figures 2C–D, which illustrate that firstly the gap between the centre of open and closed signals is larger for FRET trajectories (Figure 2C) and that the full width half-maximum values are also smaller for the FRET traces (Figure 2D), resulting in a smaller signal-to-noise ratio and therefore fewer false transitions being detected by the thresholding analysis and therefore slower kinetics (Figure 2B).

When both smFRET and smSIFI traces were examined using the adapted HMM analysis, fewer transitions were identified...
since HMM computes idealised trajectories based on probabilities rather than fluorescence intensity alone. It operates using a Viterbi algorithm to define the most likely path between open and closed hairpin conformations compared against the probability of observing emission intensities in each state. Figure 2E shows that the opening and closing rates determined by HMM from the smFRET and smSIFI trajectories are the same within experimental error, and both agree with the previously published values for the smFRET experiment.

Having established that SIFI and FRET can reveal the same dynamics, the next question is whether the stacking of the Cy3, which occurs in both the SIFI and the FRET experiments, has any effect on the hairpin dynamics. The issue of dye effects on DNA dynamics is an emerging topic and it is clear that the nature of the dye is important. To establish if donor dye stacking affects hairpin dynamics, we repeated the smFRET experiments after substituting Cy3 with Cy3B. Cy3B can also stack over terminal nucleotides similar to Cy3, but Cy3B does not have the required structure to permit enhancement by photoisomerisation, which precludes the occurrence of stacking-induced changes in intensity. We studied FRET in the same hairpin as above with either Cy3/Cy5 or Cy3B/Cy5 in buffer and in buffer containing 10% (w/w) of PEG as a molecular crowder. In both buffer and PEG, we find that Cy3B increases the rate of hairpin closing, in comparison to Cy3, but it has only a very small effect on the opening rate (Figure 3B). Cy3 and Cy3B have similar but not identical structures, which may affect the relative strengths of stacking to DNA structures. In particular, Cy3B has only a single sulfonate group, whereas Cy3 has two, which may explain the increased closing rate. Importantly, while the absolute rates of hairpin closing were different, the overall effect of the PEG crowder on the hairpin equilibrium was the same, irrespective of which donor dye was
used. The PEG crowder resulted in a two-fold decrease in the opening rates and a four-fold increase in the closing rates for the hairpin, which corresponds to a 7-8 fold increase in the equilibrium constant $K = K_{\text{close}}/K_{\text{open}}$ regardless of whether the donor was Cy3 or Cy3B (Figure 3C, and Tables S2 and S3), giving $\Delta \Delta G^\circ$ values of $-1.25$ kcal/mol and $-1.13$ kcal/mol for hairpins labelled with Cy3 and Cy3B, respectively. These opening and closing rates are in agreement with the changes observed previously for the Cy3-Cy5 hairpin, and show that the effects of PEG on hybridisation that were previously reported are independent of the donor dye.\[5\]

Reducing the number of dyes is critical in fluorescent experiments for two major reasons. Firstly, it simplifies the experiment and creates opportunities for other spectrally distinct fluorophores to be used in the same experiment. Secondly, fluorophores are generally artificial additions to natural systems; therefore experiments with fewer dyes are typically more faithful reconstructions of the native system under investigation. The dynamics of DNA structures are critical to many systems; DNA hybridisation occurs in essential biological processes such as replication, transcription and repair, while strand exchanges, breathing and fraying also play important roles in DNA nanodevices.\[2\] Here, we explicitly show that a single fluorophore is sufficient to investigate DNA dynamics, but also that the nature of the dye does influence the underlying biomolecular dynamics.

This work has established SIFI as an alternative approach to FRET for studying DNA dynamics, with the caveat that it requires the dye-stacking interaction. However, we also showed previously that the sensitivity of dye fluorescence to the stacking interaction could itself be useful to probe local DNA structure.\[3\] Therefore, we anticipate that SIFI will find use in many applications, and should be considered as complementary to FRET. In addition, if the same SIFI phenomenon can be extended to other, spectrally distinct dyes, then this could allow correlations between different parts of a molecule or between different molecules to be measured, without the distance limitations of FRET.

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Conflict of Interest

The authors declare no conflict of interest.

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