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Tetherless, precise and extended observation of single-molecule FRET in an Anti-Brownian trap

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ABSTRACT

A comprehensive understanding of biomolecules calls for the ability to observe single-molecule dynamics at the nanometer scale without constraints. Single-molecule Förster resonance energy transfer (smFRET) is a powerful tool for probing nanoscale dynamics, but existing modalities have limitations. Solution based confocal measurements are restricted by the short (~1ms) diffusion limited observation time. Surface immobilized measurements can extend the observation window, but at the expense of the molecule's translational and rotational degrees of freedom. Moreover, there is always a concern that immobilization may perturb the biomolecule's function. We overcome these limitations by combining smFRET optics with the capability to isolate individual molecules in solution using an Anti-Brownian ELectrokinetic (ABEL) trap. Our new platform, ABEL-FRET, enables photon-by-photon recording of smFRET trajectories over tens of seconds in solution, without tethering the molecule to a surface. We first demonstrate ABEL-FRET using short (~10bp) DNA rulers and achieve near shot-noise limited precision of $\Delta E \sim 0.01$ for 5,000 photons, which enables resolution of single base pair differences in a mixture of FRET-labeled dsDNA molecules. We also demonstrate the capability to make simultaneous measurements of donor fluorescence lifetime and smFRET.

Keywords: Anti-Brownian Electrokinetic trap, single molecules, smFRET, multi-parameter detection

1. INTRODUCTION

Biomolecules are not static. A comprehensive understanding of how biological systems function and organize calls for the ability to observe their dynamics at the level of a single molecule. However, observing at this nanometer scale presents a technical challenge. Conventional light microscopy is restricted by the diffraction limit to several hundred nanometers and techniques which can resolve smaller structures (e.g. x-ray diffraction, electron microscopy) generally provide only static snapshots. There is still much room for developing techniques to access dynamics at the molecular scale. One powerful tool for observing nanoscale dynamics is single-molecule Förster Resonance Energy Transfer (smFRET), which uses an inter-dye distance dependent energy transfer efficiency between two fluorescent emitters to sense distances in the 2-8nm range[1–3]. smFRET has been widely used for many applications including (but not limited to): studying conformational and binding dynamics[4,5], following protein folding[6], and providing constraints for structural modelling[7].

When applying smFRET to biological systems of interest, it is highly desirable to observe the dynamics of a single molecule for as long as possible with minimal physical constraints. Unfortunately, these requirements are often not fulfilled simultaneously by the most commonly used smFRET modalities: burst based confocal[8–10] and surface immobilized[11] measurements (Figure 1). In burst based confocal measurements, labeled biomolecules rapidly diffuse through a ~fL detection volume (Figure 1 left, green ellipse). The dynamics of the molecules are unconstrained, but the observation window is limited by diffusion to ~1ms (for typical proteins), often too short for precise measurement of smFRET efficiency or observing dynamics. To extend the observation time, surface-immobilization based smFRET was developed and became the most popular modality. However, the act of immobilization complicates experimental design[12] and always introduces the concern that the tethered molecules, being restrained in translational and rotational degrees of freedom and close to a surface, might be perturbed. We aim to allow extended smFRET observation of freely diffusing/rotating molecules in solution by using an established platform: the Anti-Brownian ELectrokinetic

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(ABEL) trap[13,14]. The ABEL trap allows continuous monitoring of single molecules free in solution by actively suppressing Brownian motion using a feedback mechanism.

The idea of combining smFRET with the ABEL trap has been proposed since the inception of the trap [15]. Previous experimental implementations were focused on probing dynamics of the FoF1 system and not on improving the technique [16–18]. In this manuscript we describe our implementation of smFRET in the ABEL trap (ABEL-FRET). Using end-labeled short (11bp) double strand DNA (dsDNA), we demonstrate smFRET of freely diffusing molecules over several seconds in solution. We show that the precision of our measurements approaches the fundamental shot-noise limit, and that the ABEL-FRET platform is compatible with simultaneous measurement of excited-state lifetime.

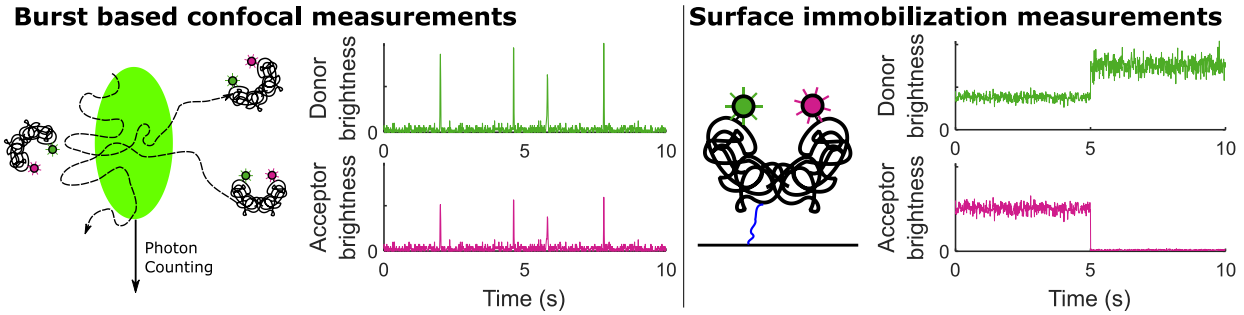

Figure 1: Existing modalities for smFRET measurements. Burst-based confocal measurements give access to the unconstrained conformations of molecules freely diffusing in solution, but the observation time is diffusion limited (~1ms for typical proteins). Surface immobilization allows an extended observation window, but incurs the penalty of having to engineer a tether for attachment to the surface, and calls for additional controls to check for perturbation to biological function.

2. CHARACTERIZATION OF ABEL-FRET BY MEASUREMENT OF DNA RULERS

2.1 ABEL trap setup with smFRET detection

To trap a single molecule in solution, the ABEL trap monitors the position of a fluorescent molecule in real-time, and applies feedback voltages to approximately cancel the Brownian motion displacement via electrokinetic forces[14]. In ABEL-FRET, the trapping hardware and algorithms are implemented as previously described[19]. Briefly, the position of a single labeled biomolecule is determined by fast scanning of a focused laser spot over a micron-sized field of view. On detection of a photon, the corresponding beam position gives a measurement of position, which is refined using a hardware implemented Kalman filter. Feedback voltages are calculated based on this refined position estimate to push the molecule back towards the center of the field of view. In this work, we used a 532nm laser line (Coherent Obis) to excite trapped Cy3-Cy5 labeled molecules. The laser beam is modulated by a pair of acousto-optic deflectors to produce a 32-point "knight's tour" pattern at the sample plane. The scanning pattern produces a homogeneous (time-averaged) excitation profile over a 3μm×3μm trapping region[19]. For the experiments presented here, we typically use an average intensity of 400 W/cm^2 (a peak intensity of ~7.5kWcm^{-2}) in the sample plane. The fluorescence signal is collected by a silicone immersion objective (100X, NA1.35, Olympus), focused through a 400μm pinhole (Thorlabs), and split with a 652nm long pass dichroic (Semrock) into donor and acceptor channels. The signal in each channel is then further filtered and focused onto an avalanche photo detector (Laser Components, COUNT T-series).

A custom-made microfluidic chip is used to hold sample solution and support strong electrokinetic responses. The ABEL trap suppresses Brownian motion in 2D (xy) only, with diffusion in z confined by the axial thickness of the microfluidic chip (~700nm). The chip is made of fused silica and fabricated in Princeton University's nanofabrication facility using a modified version of published recipes[20]. Before trapping experiments are conducted, the chip is first cleaned with Piranha solution (a 3:1 mixture of H_2SO_4 and hydrogen peroxide), activated by incubating for 15 minutes with 1M potassium hydroxide, and then passivated with polyethylene glycol (PEG) by 24h incubation with methoxy-PEG-silane (Gelest SIM6492.73-1GM).

Figure 2 shows an example of an ABEL-FRET measurement. Here the sample is 11bp dsDNA, end-labeled with Cy3 as the donor and Cy5 as the acceptor. The trace begins with no molecule trapped and shows only background in each channel. At ~2 seconds (indicated by a red arrow) a DNA molecule diffuses into the trapping region and is captured by the rapid feedback forces (feedback panel). Photon counting on the donor and acceptor channels (green and red traces in the brightness panel) allows the FRET efficiency to be measured with high time resolution. At ~9 seconds, the molecule escapes from the trap. Between ~10.5s and 15.5s, another molecule is trapped and measured in the same way. In this second molecule, the acceptor bleaches before the molecule escapes. In the FRET efficiency panel (E), the apparent FRET efficiency is plotted every 5ms. The apparent FRET efficiency is calculated using

$$E = \frac{N_A}{N_A + N_D}, \tag{1}$$

where $N_D = N_{D,total} - B_D$, is the background corrected number of counts in the donor channel and similarly, $N_A = N_{A,total} - B_A - \alpha N_D$, where αN_D is an additional term to correct for donor leakage.

As can be seen from the trace, the mean FRET value remains constant during the trapping, but there is variability between consecutive time bins (5ms). Part of this variability comes from the unavoidable random noise associated with photon counting (e.g. shot-noise limited FRET variation). However, in addition to this photon counting noise there may be extra sources of experimental variation, which prevent the measurement from reaching the fundamental shot-noise limited precision. We next investigate the precision of ABEL-FRET and compare it to characterizations made using burst-based confocal and TIRF modalities[9,21], as well as the shot-noise limit.

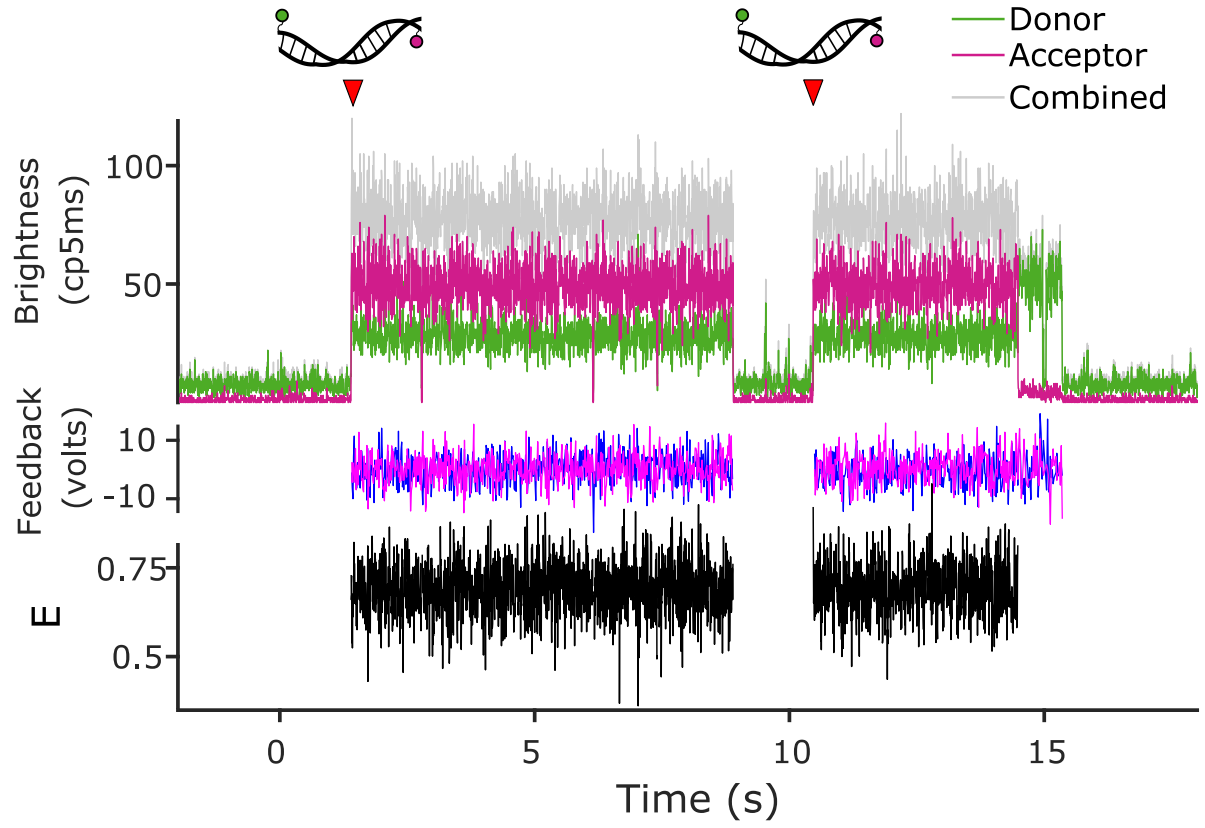

Figure 2: ABEL-FRET example traces. Two example trapping events of Cy3, Cy5 end-labeled 11bp dsDNA molecules with panels to show the donor and acceptor brightness (top), the feedback voltages applied to keep the molecule trapped (middle) and the FRET efficiency (bottom). The application of feedback in the ABEL trap has extended the observation time several orders of magnitude over burst-based confocal measurements.

2.2 Characterization of precision using DNA rulers

To characterize the precision of ABEL-FRET, we use the end-labeled 11bp dsDNA in Figure 2. Short dsDNA molecules provide a suitable sample for characterization of FRET precision due to their high rigidity and distance tunability. Here, we focus on how the uncertainty in measured FRET efficiency scales with the number of photons. After recording smFRET traces for several hundred 11bp-dsDNA molecules, we divide each trapping event into batches of N photons. We then calculate the apparent FRET efficiency of each batch and pool all efficiency values together to build up a FRET efficiency histogram such as those shown in Figure 3a. Precision is quantified by extracting the standard deviation of the distribution, fitted using a Gaussian function. As shown in Figure 3a, increasing the number of photons per batch (N) from 750 to 6000 significantly improves precision.

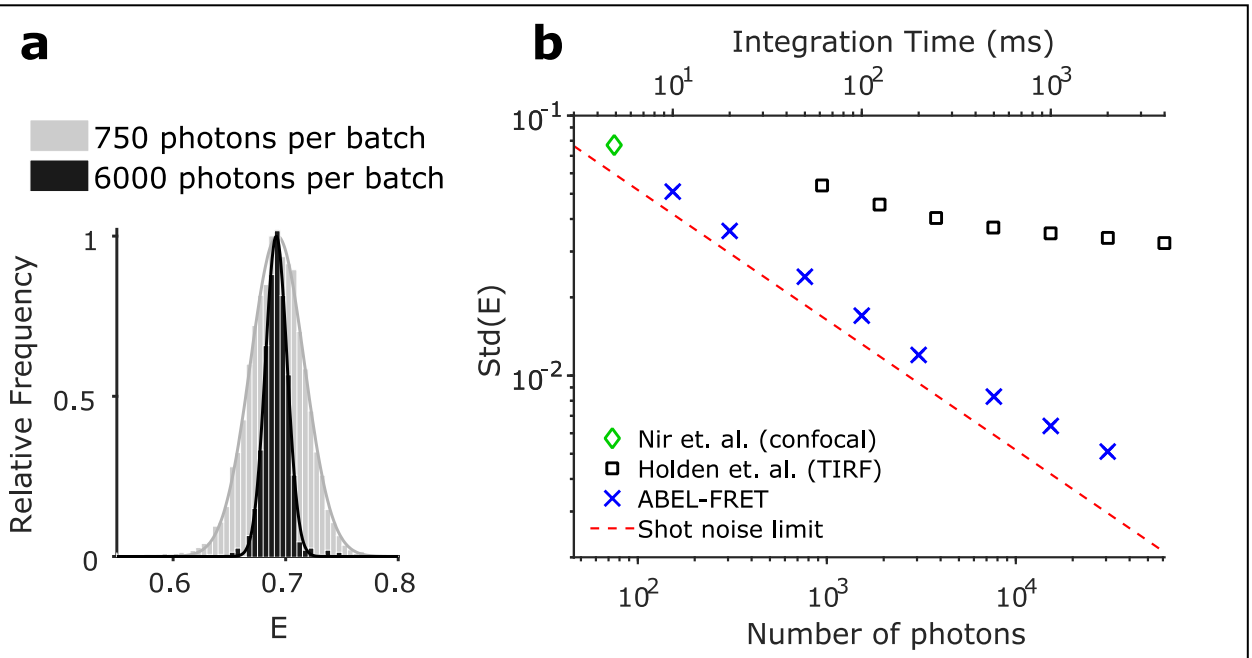

Figure 3: Precision of ABEL-FRET. (a) Histograms of FRET efficiency calculated with every 750 photons or 6000 photons. (b) FRET precision (quantified by the standard deviation of the efficiency histogram) versus the number of total photons per batch (N) for ABEL-FRET (blue X), a typical burst based confocal measurement (green diamond) and one previous characterization of a surface immobilized TIRF-based measurement (black square). The red dotted line shows the shot-noise limit (see main text for details). The upper x axis shows the integration time required to collect the number of photons on the lower x axis for a typical photon detection rate of ~$15kHz$.

To examine how FRET precision scales with the number of photons, we vary the number of photons per batch (N) and plot the resulting precision as a function of N (Blue "X" in Figure 3b) over two decades of photon numbers as shown in Figure 3b (Blue "X"). To estimate the shot-noise limit, we simulated ideal FRET experiments where the underlying efficiency is constant and photon counting in donor and acceptor channels follows Poisson statistics. We used realistic parameters extracted from experiments and the Gillespie algorithm for efficient simulation of stochastic events of photon detection [22]. The resulting shot-noise limit is plotted as the red dotted line. Evidently, the experimentally acquired precision in ABEL-FRET tracks the shot-noise limit with the same scaling (~$1/\sqrt{N}$) and an absolute value within 20%-75%. This comparison shows that ABEL-FRET enables precision smFRET close to the shot-noise limit.

Finally, we compare ABEL-FRET to other smFRET modalities. Precision in burst based confocal measurement has been extensively characterized and one example[9] is shown (green diamond). This measurement also approaches the shot-noise limit, but has poor precision due to the low number of photons (~50) limited by the short observation time. Furthermore, we also compare to one of the very few characterizations of a surface immobilized smFRET measurement [21]. Interestingly, their precision (black squares) shows significant deviation from the shot-noise limit and is at least 2 times worse than ABEL-FRET. We suspect that the immobilization process might introduce additional heterogeneity in their case.

With the ability to harvest large numbers of photons and a precision that is close to the shot-noise limit, ABEL-FRET is expected to provide resolving power for separating different populations with closely spaced FRET levels. To demonstrate this, we measured a mixture of three types of dsDNA molecules with inter-dye separations of 11, 12, and 13bp. In this experiment, we trapped molecules one-by-one and used the mean FRET efficiency of each molecule to construct an efficiency histogram (Figure 4). This histogram clearly shows three populations, which can be identified by comparison with samples of the individual strands. The ability to clearly resolve these three populations indicates that we have single base pair resolution, which is an exciting resolving power for biological application. To the best of our knowledge this is the first time that mixtures of dsDNA molecules separated by only 1bp have been resolved using smFRET.

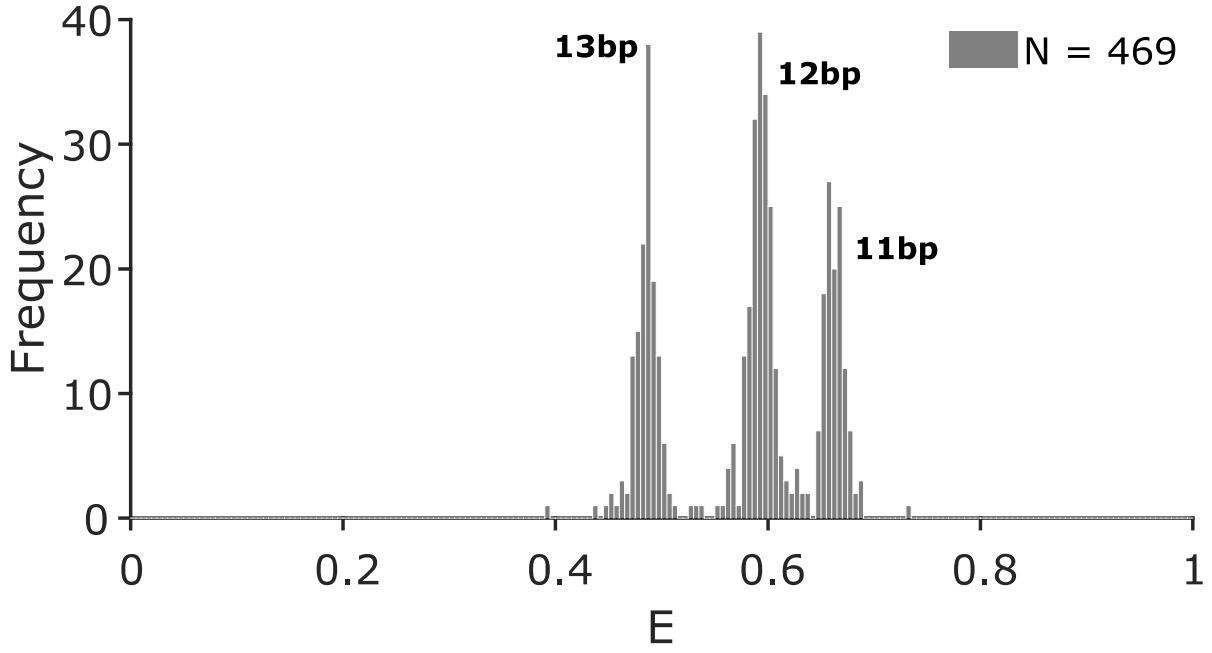

Figure 4: ABEL-FRET measurement of a mixture of dsDNA molecules. FRET efficiency histogram of a mixture sample composed of 11, 12 and 13 bp dsDNA molecules. Each type of molecule is end-labeled with Cy3 as the donor and Cy5 as the acceptor. Around 4% of the molecules show a brightness ~20% greater than the majority (96%) of trapped species and are excluded from the histogram. The donor-only population is also not shown.

3. SIMULTANEOUS MEASUREMENT OF FRET EFFICIENCY AND EXCITED-STATE LIFETIME

3.1 Excited-state lifetime measurements

Simultaneous measurement of FRET and other fluorescence parameters can potentially extract more information from biomolecules of interest. Our ABEL-FRET setup uses a point detector with sub-ns response time, making it compatible with simultaneous measurement of the excited-state lifetime using Time-Correlated Single Photon Counting (TCSPC). To measure the excited-state lifetime of trapped molecules we used 529nm pulsed excitation, generated by filtering the output of a supercontinuum fiber[23] (OFS Fitel) pumped by a Ti:Sapph oscillator (Mira 900, Coherent). TCSPC

was performed using a PicoHarp 300 module (Picoquant). To extract the lifetime, the decay histogram was fitted with the following function:

$$g(t;\tau,c) = (1-\gamma)[IRF(t-c)\otimes\exp(-t/\tau)] + \gamma g_{BG}(t)\ ,$$

where γ is the background fraction, $g_{BG}(t)$ is the experimentally measured decay histogram of background photons, $IRF(t)$ is the instrument response function (measured by back-scattering of the highly-attenuated excitation light from a glass coverslip), c is the time shift of the IRF and τ is the excited state lifetime. A maximum-likelihood approach was used for fitting[24,25].

3.2 Simultaneous FRET and lifetime measurements for a mixture of DNA rulers

Applying the capability to simultaneously measure the donor lifetime and FRET efficiency of the end-labeled 11, 12, 13bp dsDNA mixture allows us to build a 2D scatter plot of the donor lifetime and FRET efficiency of each molecule (Figure 5). The probability density is rendered by a 2D kernel density estimation algorithm [26]. In figure 5, there are four populations which can be identified, from highest to lowest FRET, as 11bp, 12bp, 13bp and donor only DNA molecules. As expected, the relationship between FRET efficiency (E) and donor lifetime (τ) follows the $E=1-\tau/\tau_0$ relation, where τ_o is the lifetime of the unquenched donor (here represented by the donor only DNA molecules). We have thus demonstrated exquisite resolution to separate FRET populations in efficiency-lifetime space.

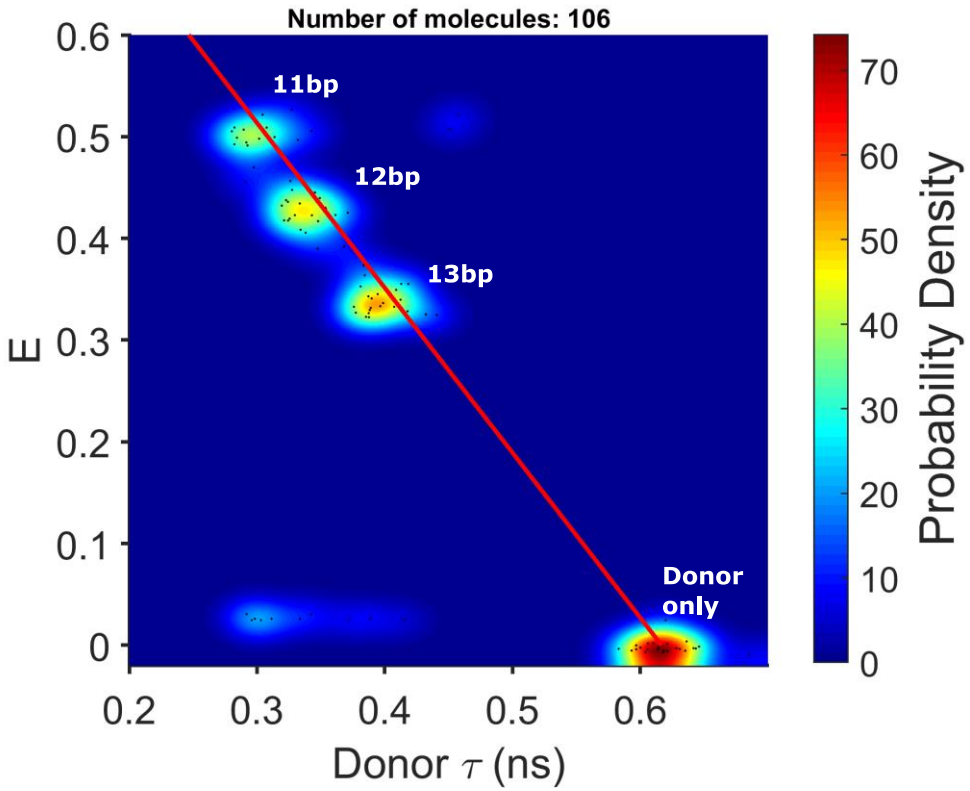

Figure 5: Simultaneous measurement of excited-state lifetime and efficiency with ABEL-FRET. Two dimensional scatter plot of FRET efficiency versus donor lifetime of a mixture sample composed of 11, 12 and 13 bp dsDNA molecules. Each type of molecule is end-labeled with Cy3 as the donor and Cy5 as the acceptor. The probability density is calculated using a 2D kernel density estimation algorithm. The red line shows the theoretical relationship $E = 1 - \tau/\tau_o$, where τ_o is the donor lifetime in the absence of FRET.

5. CONCLUSIONS

We have demonstrated a new modality, ABEL-FRET, which enables extended observation of smFRET in solution, without tethering to a surface. The measurement precision, characterized using FRET labeled short dsDNA molecules, approaches the shot-noise limit and is high enough to resolve single base pair differences. In addition we showed that ABEL-FRET measurements can be combined with simultaneous measurement of excited-state lifetime. We envision that ABEL-FRET will provide a platform for observing the nanoscale dynamics of unconstrained biomolecules and will become a powerful addition to the single-molecule toolkit.

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