Single-molecule analysis of protein-mediated ssDNA conformational control

Application note

This application note was produced in collaboration with the Antony lab at St Louis University School of Medicine

In this application note, we demonstrate how single-molecule Förster Resonance Energy Transfer (smFRET) can be performed using the EI-FLEX to measure the end-to-end distances of single-stranded DNA (ssDNA) molecules. Here, Chadda et al. explore ssDNA molecules of varying lengths, investigating the impact of binding to replication protein A (RPA)[1]. Existing models using crystal structures or bulk methods have failed to reach consensus on whether binding causes DNA wrapping or stretching, demonstrating that in-solution and single-molecule methods are crucial for elucidating dynamic conformations and nanoscale distances.

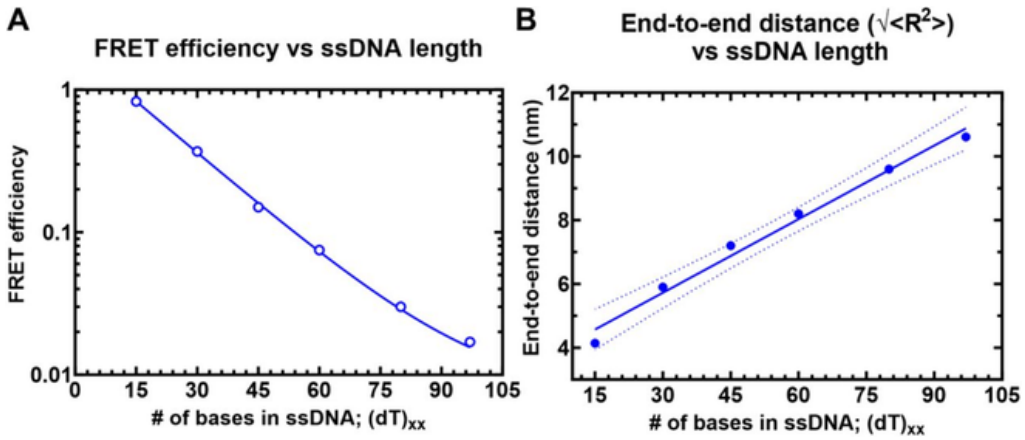


Figure 1 – FRET efficiency and end-to-end distance show linear relationship to ssDNA length
A) FRET efficiency plotted against number of bases in ssDNA molecules
B) End-to-end distance (nm) plotted against number of bases in ssDNA molecules

Overview of this application note:

- End-to-end distances of a variety of ssDNA molecules were calculated from FRET efficiency values, showing linear relationships between number of bases and distance
- Data captured on the EI-FLEX was in good agreement with distances calculated using the Picoquant MT-200 instrument
- A reduction of end-to-end distance of 2.6 Å was observed upon addition of RPA, which was maintained irrespective of ssDNA length, indicating that DNA wraps around RPA, rather than being stretched

Glossary of terms used in this application note

FRET efficiency (E): A measure of how effectively energy is transferred from a donor dye to a nearby acceptor dye. It is determined from the ratio of acceptor emission to total emission detected when only the donor laser is active. High FRET efficiency indicates that the labelled sites are closer to each other, low FRET efficiency indicates they are further apart.

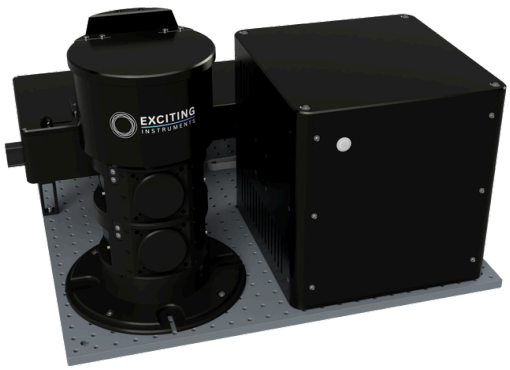
Alternating Laser EXcitation (ALEX): A measurement scheme in which a donor and an acceptor laser are switched on and off in rapid sequence, hitting each molecule multiple times with either laser. This allows emission arising from donor and acceptor excitation to be distinguished. This enables the calculation of stoichiometry, which is used to identify and separate doubly labelled molecules from donor-only and acceptor-only species.

Stoichiometry: A measure of the balance between donor and acceptor emission from a single molecule. It is determined from the ratio of emission detected under donor excitation to the total detected under both donor and acceptor excitation. Stoichiometry values near 0.5 indicate doubly labelled molecules, while values closer to 0 or 1 indicate acceptor-only or donor-only species, respectively.

Förster radius (R_0): The distance between a pair of fluorophores that gives a 50% FRET efficiency; usually given in Ångströms (Å). It is used to determine distances between fluorophores using measured FRET efficiencies. The Förster radii for fluorophore pairs can be determined experimentally or found online.

The EI-FLEX System

The EI-FLEX brings a biophysics professor into any lab with one simple, confocal benchtop solution that rapidly reveals physiologically-relevant behaviour without immobilising targets or requiring large sample volumes, all at single-molecule precision. With easy-to-use acquisition and analysis protocols and fully automated, high-throughput options available, high-quality data and publication-ready figures can be generated with ease.



The EI-FLEX single-molecule spectrometer

End-to-end length of ssDNA molecules can be calculated using smFRET data

While smFRET has been used to calculate distances between two fluorophores on double stranded DNA molecules, its use for ssDNA is emerging, with other techniques such as single-molecule total internal reflection microscopy previously reporting the analysis of DNA overhangs immobilised on glass slides. Here, Chadda et al. labelled ssDNA molecules of varying lengths with Cy3 and Cy5 fluorophores on opposite ends and performed smFRET to determine whether FRET efficiency is a suitable readout for measuring end-to-end distances.

FRET efficiency is related to the distance between fluorophores in the following manner:

$$R = R_0 \times \sqrt[6]{\frac{1-E}{E}}$$

whereby
R = distance between the fluorophores
R_0 = Förster radius (distance at which the FRET efficiency is 50%)
E = FRET efficiency

Figure 1 shows both FRET efficiency and end-to-end distance plotted against the number of bases in ssDNA molecules, with both graphs showing good linear fits of the data. FRET efficiency decreases as the number of bases and therefore distance between the ends increases.

smFRET data generated on the EI-FLEX platform is in good agreement with data from the Picoquant MT-200 instrument

Data collected on the EI-FLEX system were compared with data collected on the Picoquant MT-200, a well-known confocal FRET instrument. Under the same conditions, the FRET efficiency histograms and linear relationship between end-to-end distance and number of bases in ssDNA molecules were in good agreement between the instruments (Figure 2). Of note, the FRET histograms produced by the EI-FLEX had tighter distributions formed of greater photon counts, indicating improved data quality compared to the MT-200 instrument. Better linearity between parameters was also observed for EI-FLEX data.

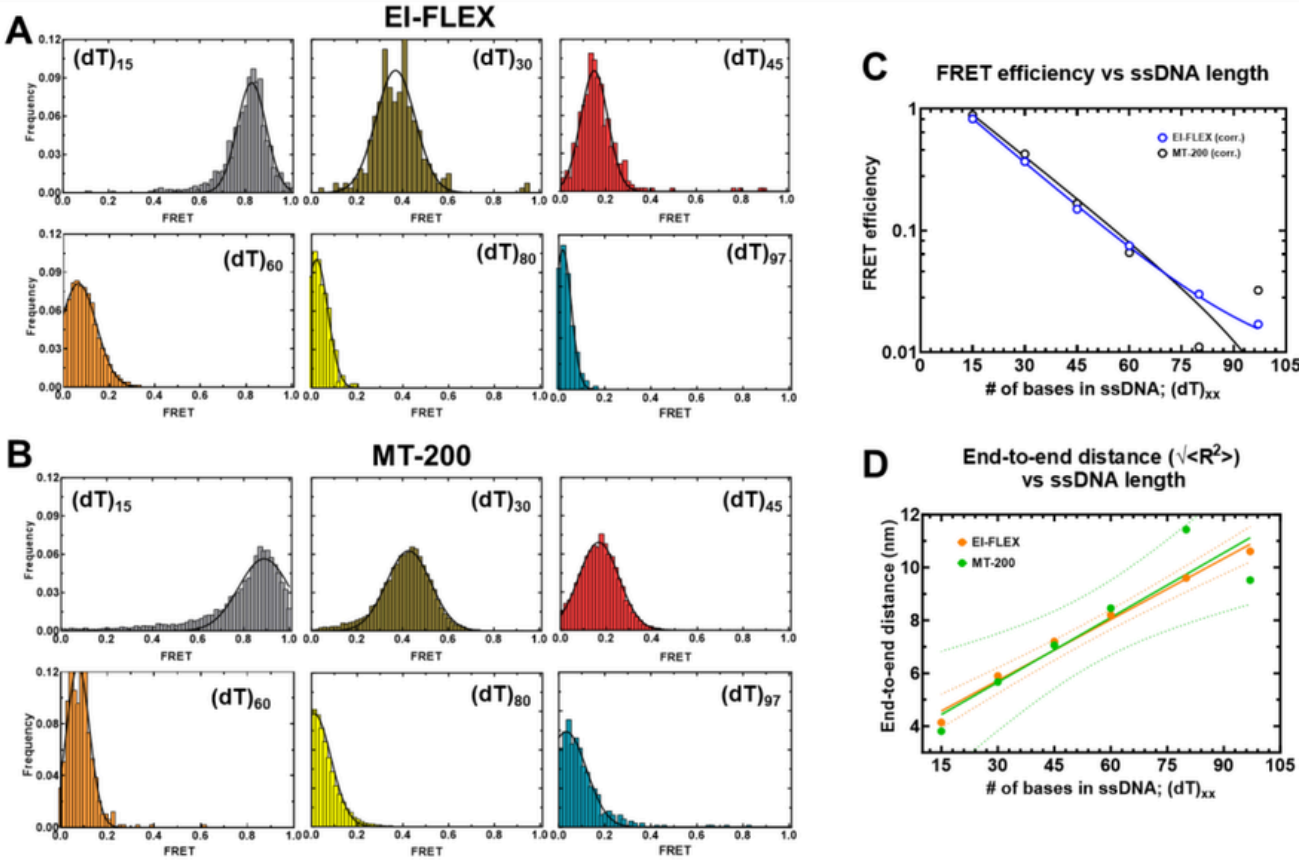


Figure 2 – Comparison of smFRET data generated on the EI-FLEX and Picoquant MT-200
smFRET data for a range of ssDNA lengths (15, 30, 45, 60, 80 and 97 bases)
A) FRET efficiency histograms generated from EI-FLEX data
B) FRET efficiency histograms generated from Picoquant MT-200 data
C) Linear fits for FRET efficiency plotted against base length for both instruments
D) Linear fits for end-to-end distances plotted against base length for both instruments

Binding of RPA increases ssDNA end-to-end distance in a concentration-dependent manner

Next, the authors incubated a doubly labelled ssDNA molecule of 25 nucleotides with increasing concentrations of RPA. FRET efficiency histograms were generated by plotting FRET efficiency against donor and acceptor label stoichiometry, using ALEX to identify and remove any donor-only or acceptor-only populations and perform appropriate FRET correction.

In the ssDNA-only condition, a mid-FRET population is present, representing unbound DNA. A dual population of mid and low-FRET efficiencies emerges at sub-nanomolar RPA concentrations, and the population shifts to an entirely low-FRET population at 1 nM RPA (figure 3), indicating that all the ssDNA molecules are bound by RPA. Mass photometry was used to ensure that only one RPA protein is bound to each ssDNA molecule.

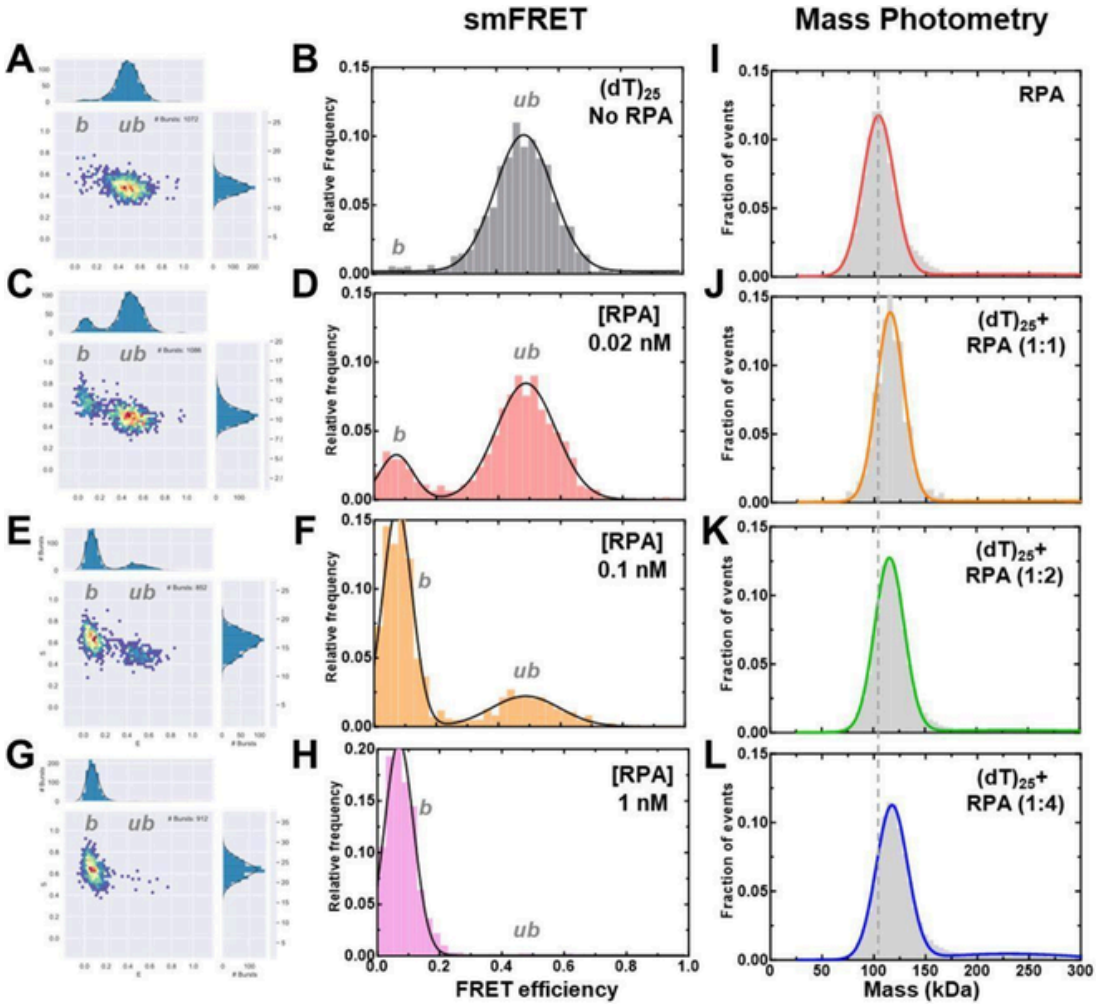


Figure 3 – RPA binding reduces FRET efficiency of labelled ssDNA in a concentration-dependent manner
FRET efficiency versus stoichiometry plots and FRET efficiency histograms for ssDNA with A and B) No RPA, C and D) 0.02 nM RPA, E and F) 0.1 nM RPA, G and H) 1 nM RPA
I-L), Mass photometry plots highlighting that one RPA molecule is bound to one ssDNA molecule

End-to-end distances ssDNA increase moderately when bound to RPA

To resolve the questions around the conformation of ssDNA when it binds to RPA, Chadda et al. used smFRET to calculate the end-to-end distances for RPA-bound ssDNA molecules across of range of sizes, comparing these to the distance of fully linearised and unbound ssDNA. Double Electron-Electron Resonance (DEER) spectroscopy was also used as a complementary technique to measure high resolution distances on ssDNA molecules using nitroxide labels instead of fluorophores.

smFRET and DEER spectroscopy data showed good agreement regarding free and RPA-bound ssDNA and linearised ssDNA demonstrated an increase in end-to-end distance with additional nucleotides as expected. Interestingly, a stable increase of 2.6 Å was observed between free and RPA-bound ssDNA for each ssDNA substrate, irrespective of its length. This indicates that ssDNA wraps around RPA, rather than being stretched by the interaction, and given that this distance is preserved across ssDNA lengths, it suggests that RPA binding does not cause any extrusions or looping.

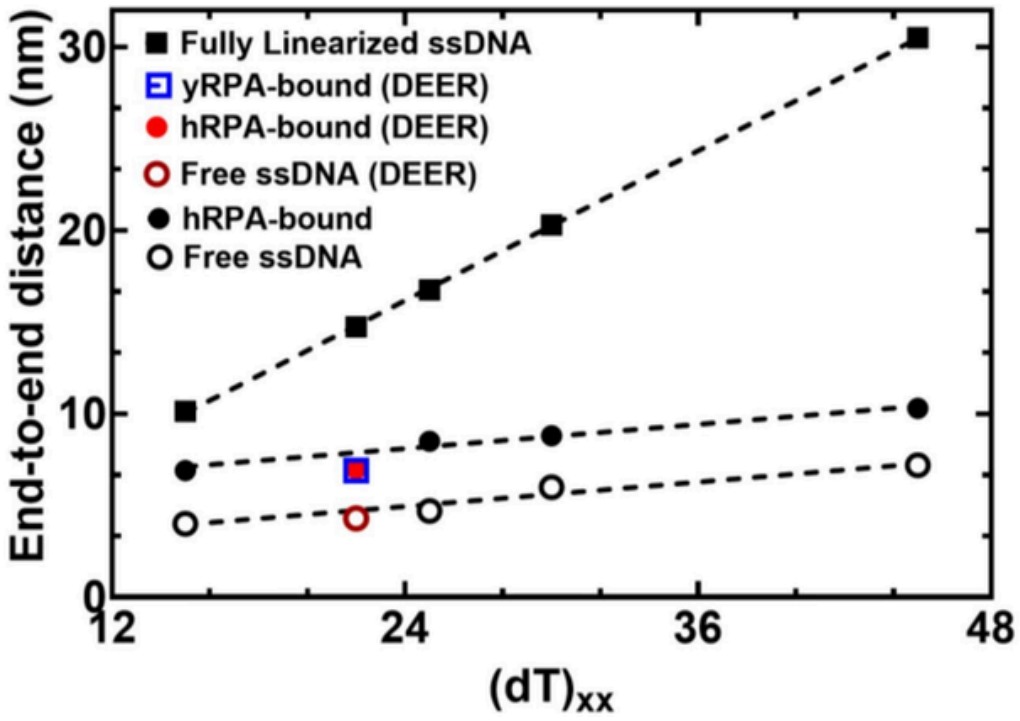


Figure 4 – Comparison of end-to-end distance versus ssDNA length
End-to-end distance was plotted versus ssDNA length for a range of conditions: linearised ssDNA, free ssDNA and RPA-bound ssDNA, both for data collected by smFRET and DEER spectroscopy

Summary

In summary, Chadda et al. performed smFRET on the EI-FLEX to measure end-to-end distances on ssDNA molecules, resolving conflicting models about how the conformation of the DNA changes upon binding to RPA. These distances were in good agreement with those generated via the Picoquant MT-200 instrument and by DEER spectroscopy, highlighting that the EI-FLEX benchtop instrument provides robust and high-quality in-solution single-molecule data.

For a deeper dive on the techniques used in this application note, we recommend exploring our Resource Library. Discover a range of applications for smFRET and the EI-FLEX system on our website.

References

1. Chadda, R. et al. Partial wrapping of single-stranded DNA by replication protein A and modulation through phosphorylation. Nucleic Acids Res. 52, 11626–11640 (2024).*

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