Identifying novel dynamic conformational states from crystal structures

Application note

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In this application note, we explore work by Howard et al., who used single-molecule Förster Resonance Energy Transfer (smFRET) and fluorescence correlation spectroscopy (FCS) on the EI-FLEX to reveal two previously unknown, salt-influenced conformational states on the bacterial helicase Rep[1]. X-ray crystallography had identified two conformational states, open and closed structures, respectively. Here, both techniques were performed on the EI-FLEX instrument, providing single-molecule insights that were complementary to several other biophysical techniques used in this work.

Overview of this application note:

- FCS identifies variations in hydrodynamic radius and diffusion time that are likely caused by salt-induced protein conformational changes
- smFRET uncovers two conformational states that were not identified in crystal structures, which are influenced by salt concentration and DNA binding
- Combining smFRET data with complementary techniques, such as ABEL trapping, resolved multiple interconversion conformational states that persisted on sub-millisecond to second timescales

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.

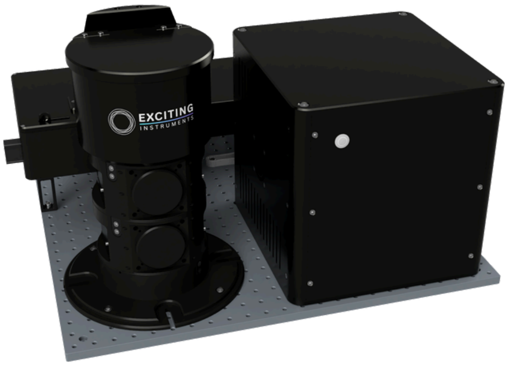
Shot Noise: Photon detection follows Poisson statistics, meaning photons arrive randomly even when the emission rate is constant. In single-molecule FRET, this sets the minimum width of FRET distributions expected for a static molecule due solely to limited photon counts.

Burst Variance Analysis (BVA): A hypothesis test for dynamic conformational changes occurring within bursts. This analysis compares the theoretically expected standard deviation in FRET efficiency with the experimentally observed one. This determines whether the FRET efficiency peak comes from static heterogeneity (multiple distinct, stable species) or dynamic fluctuations (species rapidly changing conformation).

Photon-by-photon Hidden Markov Modelling (H2MM): Analyses the sequence of individual photon arrivals to infer discrete FRET states and the transition rates between them, enabling the reconstruction of the underlying kinetic model with sub-millisecond temporal resolution.

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

smFRET identifies four conformational states that undergo dynamic interconversion

In FCS experiments, Howard et al. detected protein aggregation at low salt concentrations, potentially caused by salt-induced variability of Rep conformations. This prompted the use of smFRET to determine whether Rep was indeed sampling a range of conformations. TIRF-microscopy experiments identified surface-induced effects, such as dye photoblinking, and so the authors performed confocal smFRET on the EI-FLEX.

smFRET data were collected on low salt (10 mM) and high salt (500 mM) conditions, identifying two previously unknown conformational states in addition to the open and closed conformations known from crystal structures. Change in salinity was observed to alter the proportions of molecules in these four states (S1 (open), S2 (intermediate), S3 (intermediate), and S4 (closed); the high salt concentration induced a greater proportion of molecules in S1 and a lower proportion in state 3, compared to the low salt condition (Figure 1).

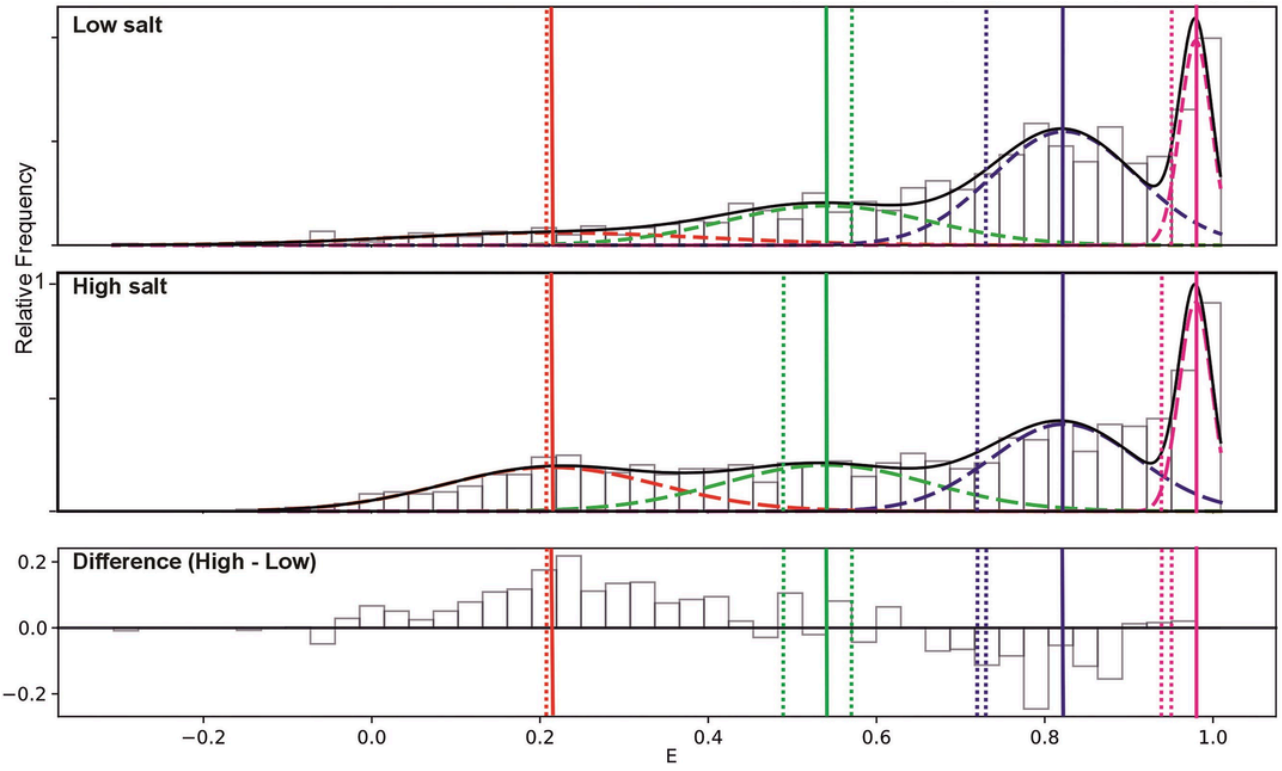


Figure 1 – FRET efficiency histograms comparing low and high salt conditions
Upper) 10 mM NaCl, Middle) 500 nM NaCl, and lower) The difference in FRET efficiencies between high and low salt conditions.
Low to high FRET efficiencies are separated into four distinct states: S1 (red), S2 (green), S3 (blue), S4 (pink).

smFRET identifies four conformational states that undergo dynamic interconversion

Following this, burst variance analysis (BVA) was performed to determine whether the multiple FRET efficiencies measured for Rep protein are attributable to a heterogeneous, static population or proteins that are dynamically interconverting between alternate conformations. Figure 2 shows a heatmap demonstrating BVA for the 10 mM NaCl condition, whereby the black dotted line represents the theoretically expected standard deviation of FRET efficiency if no dynamic changes were occurring; the standard deviation for the experimentally observed data was much greater than this, meaning that the conversions between the conformational states are dynamic and prompt further analysis.

Given that solution-based smFRET captures dynamic changes over a sub-millisecond time scale, complementary methods are required to resolve slower transitions. An anti-Brownian Electrokinetic (ABEL) trap confines a single molecule within a confocal volume, enabling data acquisition with a greater dynamic range; using this technique, the authors identified some FRET states that were stable for up to a second.

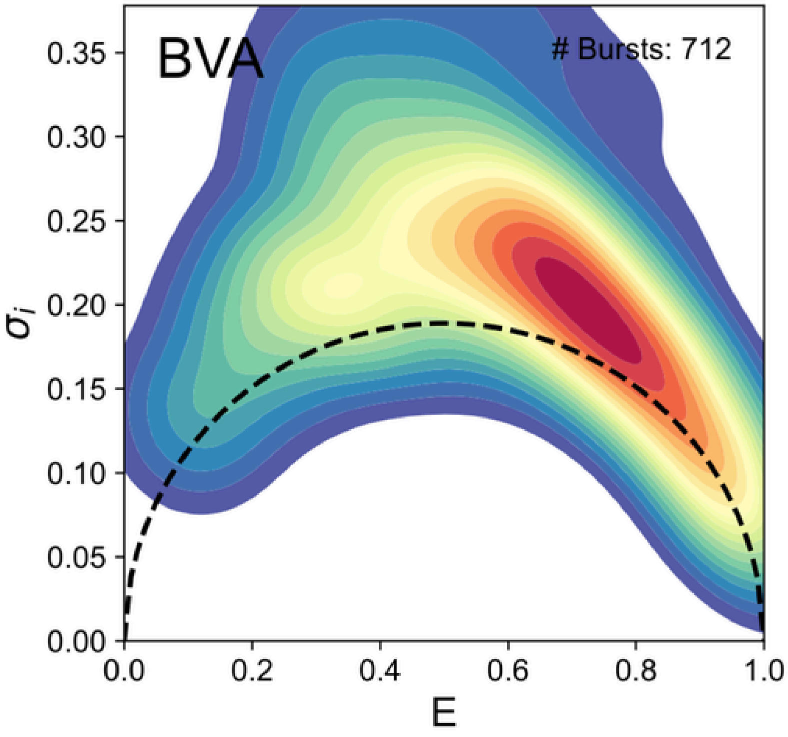


Figure 2 – BVA analysis of smFRET data at 10 mM NaCl without DNA
Dotted line indicates the theoretically expected standard deviation of FRET efficiency (taking shot noise into account), while the heatmap represents the standard deviation of the experimentally observed FRET efficiency.

smFRET identifies four conformational states that undergo dynamic interconversion

Hidden Markov modelling (H2MM) supported the conclusion that there are four interconverting conformational states, and that the proportions of these states (and which states interconvert) are influenced by salt concentration and the presence of DNA substrate (Figure 3). For example, interconversion between S1 and S3 is observed in the 10 mM NaCl condition, but not at 500 nM or 150 nM (with or without DNA). Additionally, the addition of DNA changes the rate of interconversion between S2↔S3 and S3↔S4, also permitting transitions of S1↔S4 and S2↔S4 that were not observed in any of the other conditions. Given that these are rare events compared to the rest of the interconversions, the most likely pathway that is taken between the open and closed state is S1↔S2↔S3↔S4 at physiologically relevant salinity.

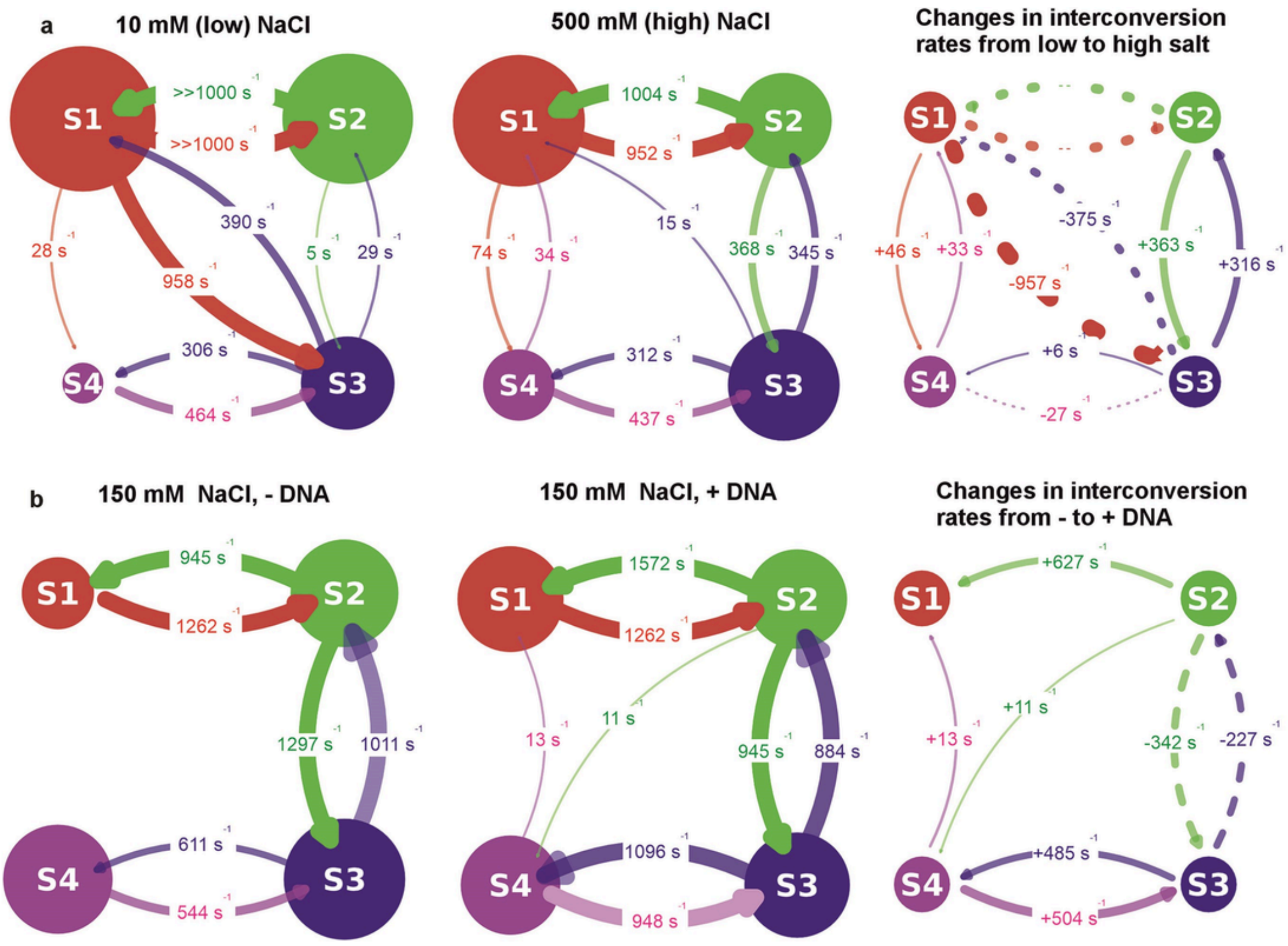


Figure 3 – Hidden Markov modelling data representing four conformational states and their interconversion rates

The states and their rate of interconversion is displayed for the low and high salt concentrations (a) and the data at 150 nM NaCl with and without DNA substrate (b)

Summary

FCS and smFRET were both performed on the EI-FLEX to identify salt-induced conformational changes for the bacterial helicase Rep. FCS showed that a rise in salt concentration increased diffusion time, indicating a more open state. The use of smFRET resolved surface-based artefacts that convoluted TIRF measurements. These data revealed two previously unknown conformational states, and the influence of salt concentration and DNA substrate on dynamic interconversion between them. By combining smFRET with complementary techniques such as ABEL trapping, the authors were able to measure slower transitions not typically accessible with solution-based FRET alone. The authors then used H2MM to identify the individual conformations and quantify the transition rates between them.

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. Howard, J. A. L. et al. The transitional kinetics between open and closed Rep structures can be tuned by salt via two intermediate states. Nucleic Acids Res. 54, gkaf1483 (2026).

All data used in this application note was generated by the authors cited in this publication.

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