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Monitoring biomolecular conformational dynamics with the EI-FLEX system: A quantitative smFRET approach

Application Note

Monitoring biomolecular conformational dynamics with the EI-FLEX system: A quantitative smFRET approach

In this application note, we demonstrate the use of the EI-FLEX system to recover rates of molecular conformational dynamics, using DNA hairpins as a well-characterised test system[1,2]. DNA hairpins reversibly transition between open and closed states on a millisecond timescale, with kinetics that are sensitive to NaCl concentration. This makes them an ideal model for validating kinetic measurements using single-molecule Förster Resonance Energy Transfer (smFRET). Our results highlight the utility of the EI-FLEX system in resolving fast dynamic events and underscore its potential as a powerful tool for probing biomolecular mechanisms in solution.

All data in this application note was produced in-house at Exciting Instruments on the EI-FLEX Pro.

Overview of this application note:

- DNA hairpins are used as a model system to demonstrate how smFRET can capture conformation dynamics for molecular structures
- Burst variance analysis separates static, heterogeneous populations from those that are dynamically undergoing conformational changes
- The influence of salt concentrations on the opening and closing rates of DNA hairpins can be determined by photon-by-photon hidden Markov modelling

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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 fluorescence to total detected fluorescence when only the donor dye is excited by a laser. 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.

How did the EI-FLEX benefit this work?

- Provided rapid distinction between open and closed hairpin structures, identifying the influence of salinity on these dynamic conformational changes
- ALEX and accurate FRET correction protocols enabled the calculation of correction factors to account for photophysical artefacts

The EI-FLEX Pro

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smFRET analysis of hairpins

To demonstrate how smFRET may be used to capture dynamic conformational changes in DNA hairpins, we performed smFRET on two samples: one for the labelled DNA hairpin as shown in Figure 1, and one containing a heterogenous population of double stranded DNA molecules that have fluorophore pairs at small, medium and large distances from each other, intended to give low-, mid- and high-FRET states.

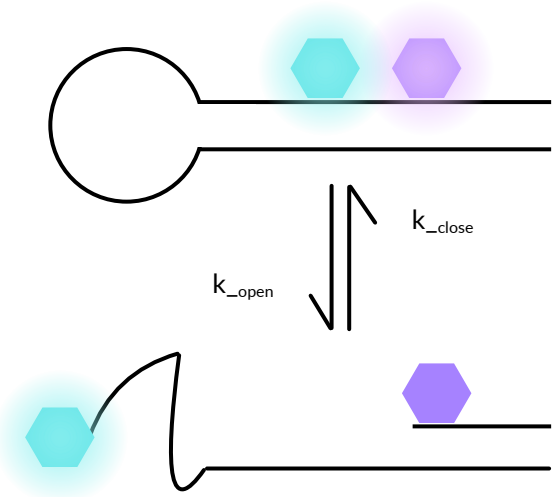

Figure 1 - Fluorophore proximity on open and closed DNA hairpins

The FRET efficiency histogram for the heterogenous DNA population revealed the presence of multiple states (low, mid, and high), caused by the different positions of the fluorophore pairs (Figure 2A, top). The DNA hairpin sample also shows the presence of multiple states (Figure 2B, top), however these results do not clarify whether these species are static or dynamically interconverting.

Therefore, we applied burst variance analysis (BVA) to assess changes in standard deviation beyond what is expected from shot noise, which would suggest the presence of dynamically interconverting molecules.

The black dotted lines in the bottom panel of Figure 2 represent the theoretical shot-noise limit of the standard deviation (σ_i) of FRET efficiency (E) in a static system. The curve indicates the expected relationship between FRET efficiency and the standard deviation when no dynamics are present, which would mean the fluctuations are only due to the shot noise.

The black triangles that fall on this line for the double-stranded DNA sample indicate that the experimentally observed standard deviation values behave close to the static or no dynamics model (Figure 2A, lower).

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smFRET analysis of hairpins

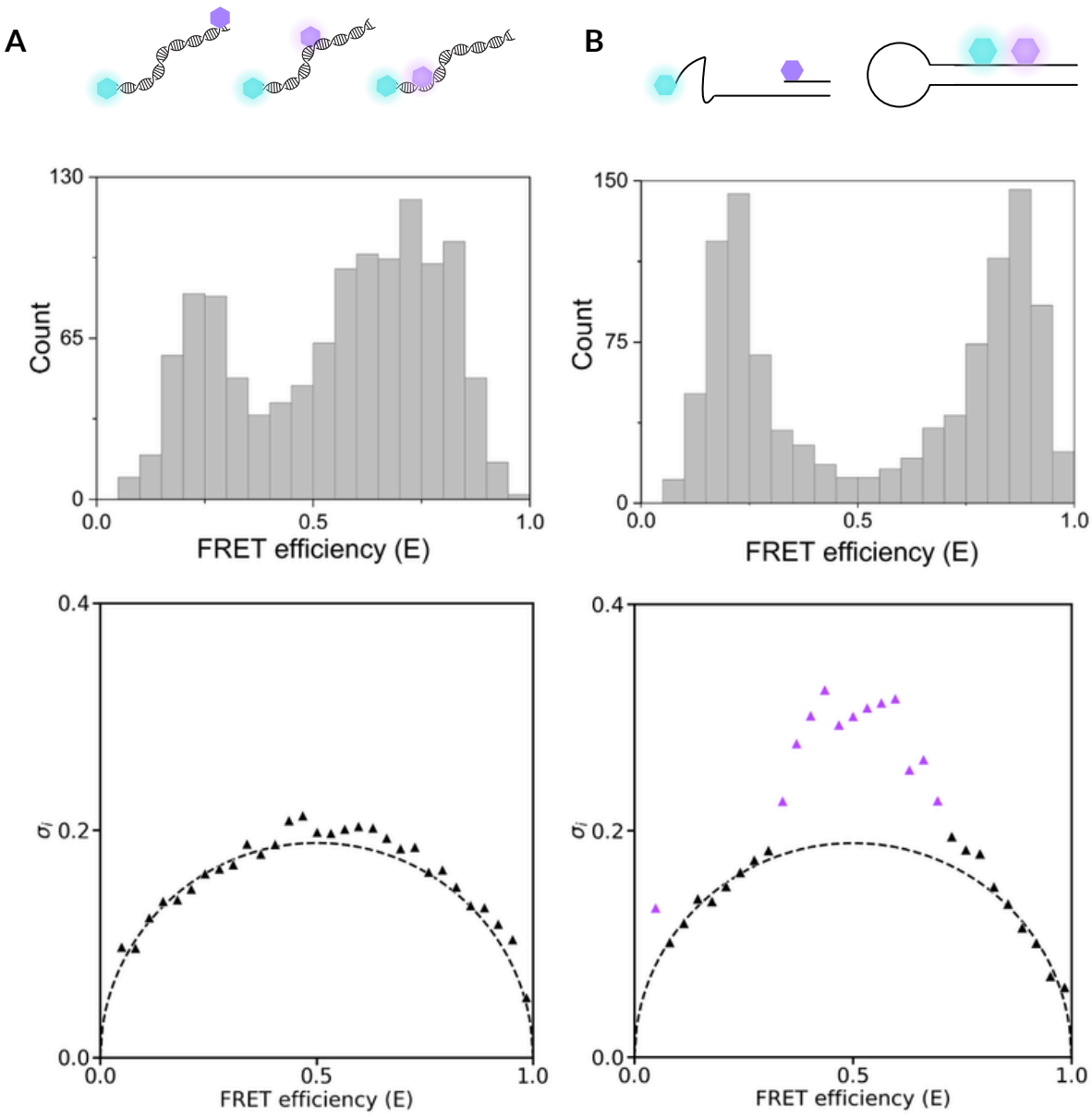

Figure 2 - FRET efficiency histogram and burst variance analysis of DNA duplex standards (A) and DNA hairpins (B) at 400 mM NaCl

FRET efficiency histograms (top). Burst variance analysis of DNA structures (bottom)

For the DNA hairpin, the constructs with low-FRET and high-FRET values adopt a static conformation and fall on the theoretical shot-noise limit. However, constructs that have a mid-FRET efficiency value (indicated by purple triangles) deviate from the theoretical shot-noise limit and therefore adopt dynamic conformational changes (Figure 2B, lower). This deviation suggests that these molecules undergo transitions between multiple conformational states within the timescale of a single photon burst.

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Effect of NaCl concentration on hairpin dynamics

Next, we explored the impact of NaCl concentration on the conformational dynamics of the model DNA hairpin. smFRET was performed on hairpin constructs at 100, 400 and 900 mM NaCl, producing a range of hairpin conformations (Figure 3). At 100 mM, the majority of the constructs are open, resulting in a major low-FRET population, with a small number of molecules with mid and high-FRET states.

As NaCl concentration increases, more hairpins adopt the closed conformation; at 400 mM, the proportion between open and closed molecules is equal, while at 900 mM NaCl, the majority of the hairpins are closed, resulting in a large high-FRET population. Burst variance analysis shows that molecules with low and high-FRET efficiencies adopt stable conformations, while those with mid-FRET states are rapidly interconverting, regardless of salt concentration.

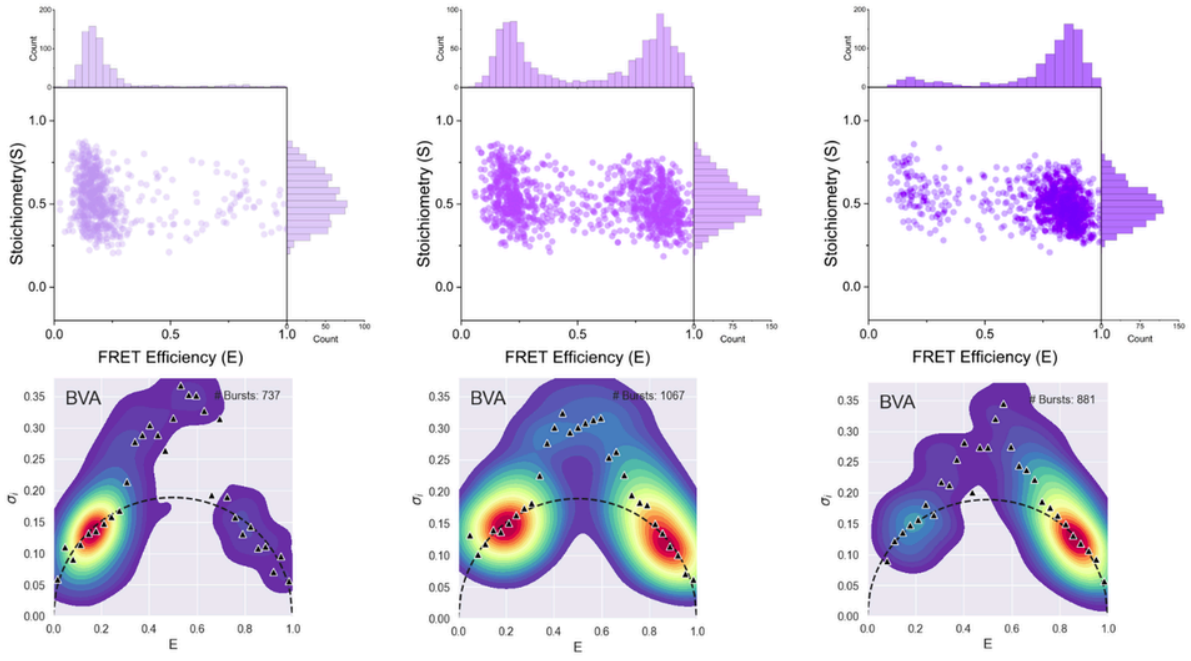

Figure 3 - FRET efficiency histograms and burst variance analysis of DNA hairpins at 100, 400 and 900 mM NaCl

FRET efficiency histograms (top). Burst variance analysis of DNA hairpins (bottom)

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Using photon-by-photon hidden Markov modelling to determine kinetic rate parameters

To quantify these conformational dynamics and extract kinetic information, we applied photon-by-photon hidden Markov modelling (H2MM), which allows us to resolve the underlying states and their interconversion rates with microsecond temporal resolution. We analysed the opening and closing rates across a series of NaCl concentrations (100 mM to 900 mM) using a H2MM method[3].

We observed that the rate of hairpin opening (low-FRET state) decreases with rising salinity; in contrast, the rate of hairpin closing (high-FRET state) increases with higher salt concentrations (Figure 4). The concentration at which the open and close rates are equal was found to be 400 mM, which correlates with the FRET histograms that indicated equal proportions of open and closed constructs at this concentration.

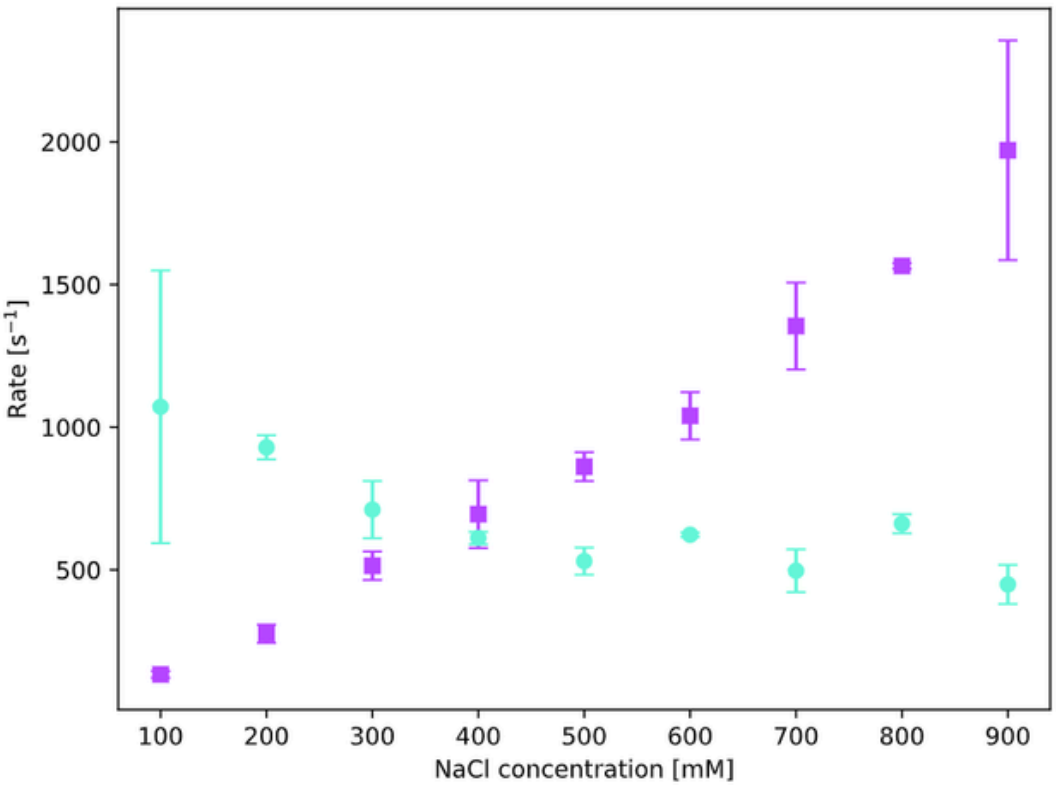

Figure 3 - Hidden Markov Modelling data showing k_{open} (circles) and k_{close} (squares) rates across a range of NaCl concentations (100 - 900 mM)
Error bars represent standard deviation around average values, N=3

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Summary

In summary, our study demonstrates the effectiveness of the EI-FLEX in capturing microsecond molecular conformational dynamics using DNA hairpins as a model system. By applying photon-by-photon hidden Markov modelling, we were able to resolve the salt-dependent switching between open and closed hairpin states, reflected in distinct FRET efficiencies. The observed trends in the rates of hairpin opening and closing with varying NaCl concentrations validate the EI-FLEX's capability to accurately recover kinetic parameters and conformational changes. These results underscore the system's utility for probing dynamic biomolecular processes with high temporal resolution.

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

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3. Pirchi, M. et al. Photon-by-Photon Hidden Markov Model Analysis for Microsecond Single-Molecule FRET Kinetics. J Phys Chem B 120, 13065–13075 (2016).

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