Resolving the dynamic structural mechanisms of protein-RNA complexes

Application note

Produced in collaboration with the Hill Lab - Department of Biology, University of York

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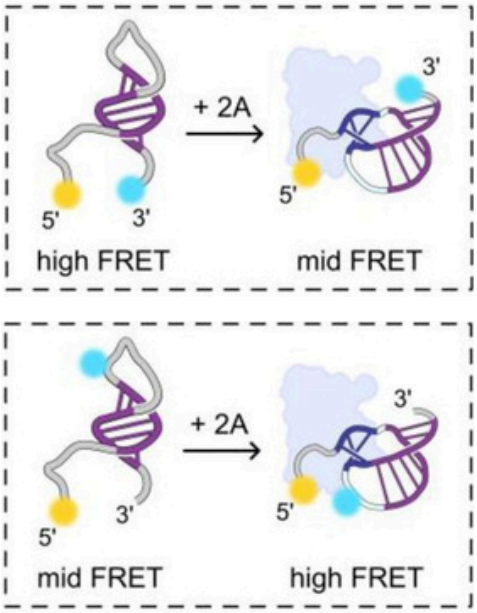
In this application note, we demonstrate how single-molecule Förster Resonance Energy Transfer (smFRET) was used to resolve a previously unknown RNA virus riboswitch mechanism. Here, Betts et al. performed smFRET on the EI-FLEX platform and used this data with insights from X-ray crystallography and small-angle X-ray scattering (SAXS), uncovering protein-dependent RNA pseudoknot formation that acts as a stimulatory element, regulating programmed −1 ribosomal frameshifting (PRF) in Theiler's murine encephalitis virus (TMEV)[1].



Overview of this application note:

- smFRET adds complementary information to structural techniques such as X-ray crystallography and SAXS to elucidate RNA conformational changes upon protein binding
- Burst variance analysis reveals that pseudoknot formation is a binary event dependent on protein 2A binding, rather than continuous conformational shifts
- smFRET provided insights alongside MST binding analysis that elucidated which RNA bases are crucial for protein 2A binding

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.

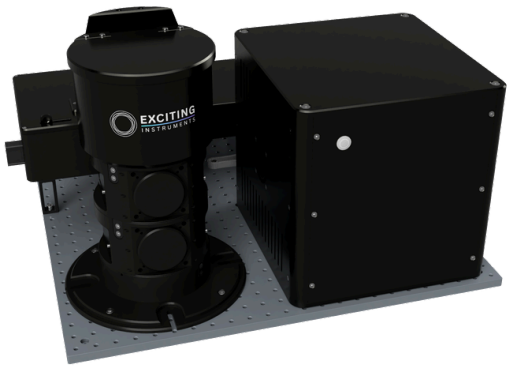
Shot Noise: Photon detection follows Poisson statistics, meaning photons arrive randomly even when the emission rate is constant. In single-molecule FRET, this sets the minimum width of FRET distributions expected for a static molecule due solely to limited photon counts.

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).

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.

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 adds complementary dynamics to PRF stimulatory element structures

It is known that the initiation of PRF in TMEV is dependent on the binding of viral protein 2A. To characterise this process, Betts et al. combined complementary biophysical approaches. X-ray crystallography data yielded a static 1.9 Å structure of the RNA-protein 2A pseudoknot complex, while SEC and SAXS provided in-solution, ensemble data suggesting the unbound RNA instead favours a seven-base-pair stem-loop. However, these techniques could not capture the dynamic structural transitions between these states.

Therefore, the authors used smFRET to determine these conformational changes upon protein binding. Dye pairs were placed in two configurations: one where the fluorophores were on the terminal ends of the RNA molecules, and the other where one had been moved to act as an internal reporter. This was designed to provide distinct FRET efficiencies between RNAs in either pseudoknot or stem-loop structures, as well as to minimise interference with RNA folding or fluorophore quenching from adjacent guanine bases.

smFRET data confirmed a shift in FRET efficiency upon functional 2A binding that was lost when 2A was inactivated (Figure 1).

The different dye placements also distinguished between the pseudoknot and stem-loop structures; the end reporter configuration captured the unbound stem-loop (high-FRET efficiency, dyes close together), which moved upon 2A binding (mid-FRET efficiency, dyes further apart). In contrast, the internal and terminal dye pair showed a mid-FRET efficiency when the RNA was unbound, which moved to a high-FRET efficiency upon 2A binding, indicative of pseudoknot formation.

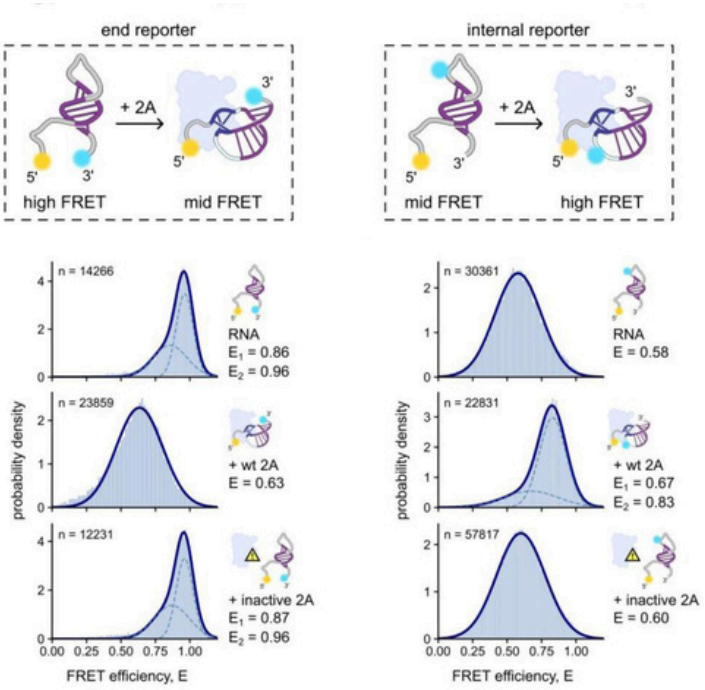


Figure 1 – FRET efficiency histograms for end reporter and internal reporter dye pairs

Schematics of dye placement and conformational changes (above). Corrected FRET histograms with Gaussian fits for RNA only, RNA + wildtype 2A, and RNA + inactive 2A (below)

Left) End reporter dye pair

Right) Internal reporter dye pair

Burst variance analysis confirms that conformational changes are not spontaneous

Next, burst variance analysis (BVA) was performed to determine whether these observed conformational changes arise specifically from protein 2A binding, or whether they exist at equilibrium through spontaneous adoption of several different conformations. BVA showed that the standard deviation of the bursts was consistent with that of the theoretically expected standard deviation, taking shot noise into account (Figure 2). This indicates that pseudoknot formation is not spontaneously occurring but rather is a direct consequence of protein 2A binding; the change is binary, not continuously shifting back and forth.

There was some notable deviation between dye pairings. The internal reporter dye pair appeared to be less dynamic than the end reporter dye pair, although the authors attributed this to the placement of the dye pairs rather than an intrinsic characteristic of the RNA.

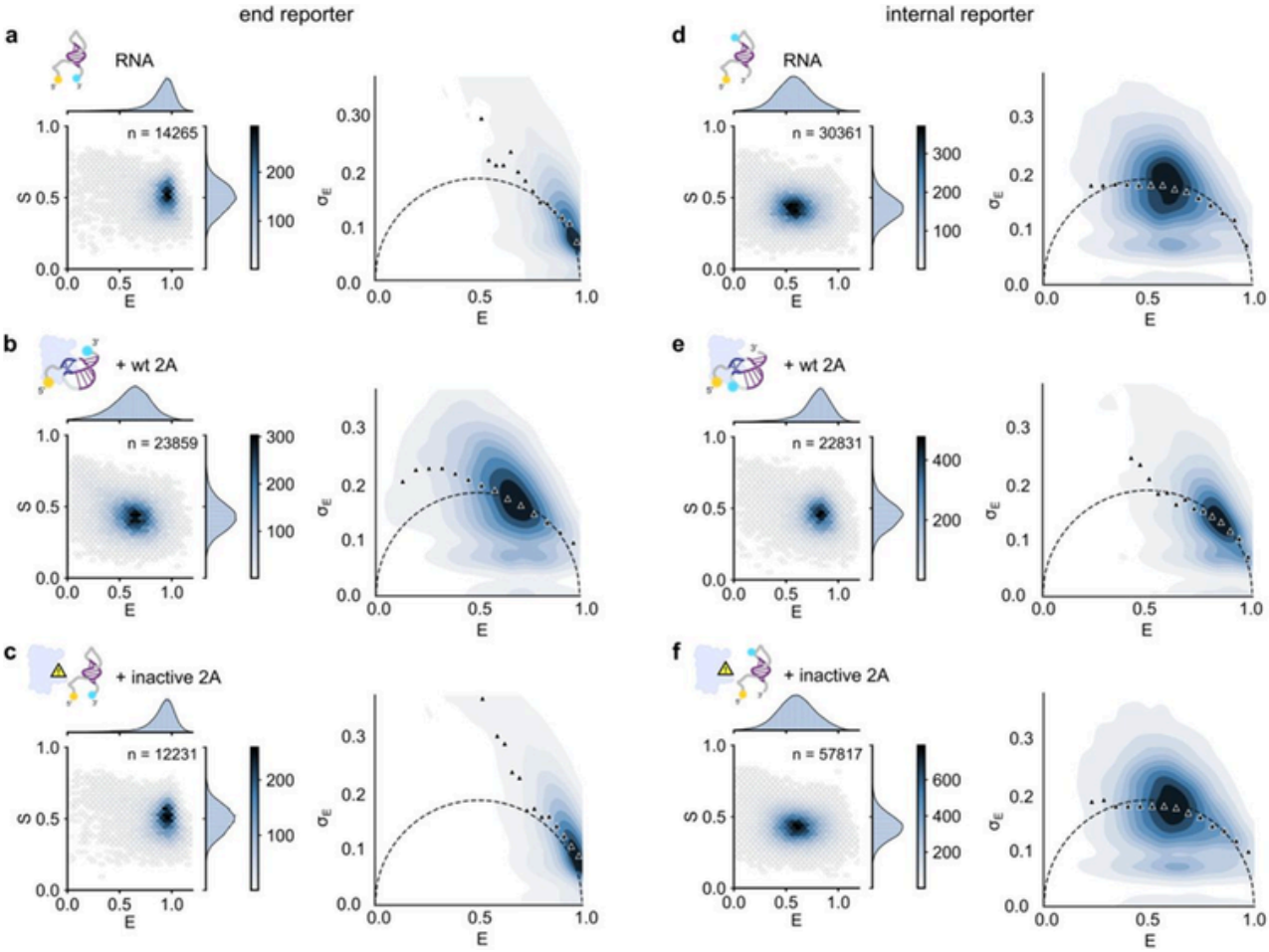


Figure 2 –BVA analysis indicates that pseudoknot formation is a static, binary process

FRET efficiency versus stoichiometry plots and histograms are given alongside BVA plots (dotted line represents the expected variance for a static species, the triangles show experimental results) for RNA only, RNA + wildtype 2A, and RNA + inactive 2A , A,B,C) End reporter dye pair, D,E,F) Internal reporter dye pair

Mutational analysis identifies key RNA base pairs for pseudoknot formation

To ascertain the key base pairs that enable protein 2A binding and pseudoknot formation, Betts et al. generated a series of RNA mutants in which nucleotides directly interfacing with key arginine residues on protein 2A were left unchanged, while the opposing bases on stem 1 were mutated. Electrophoretic mobility shift assays (EMSAs) and microscale thermophoresis (MST) were used to determine how these mutants affect protein 2A binding and indicated that mutation of any or all of the first three bases on stem 1 prohibited protein 2A binding. However, when smFRET was performed, a mid-FRET state was observed, suggesting the presence of a 'pseudoknot-like' population (Figure 3). It is likely that this complex is unstable and as such was missed by the ensemble binding methods, highlighting the importance of single-molecule resolution.

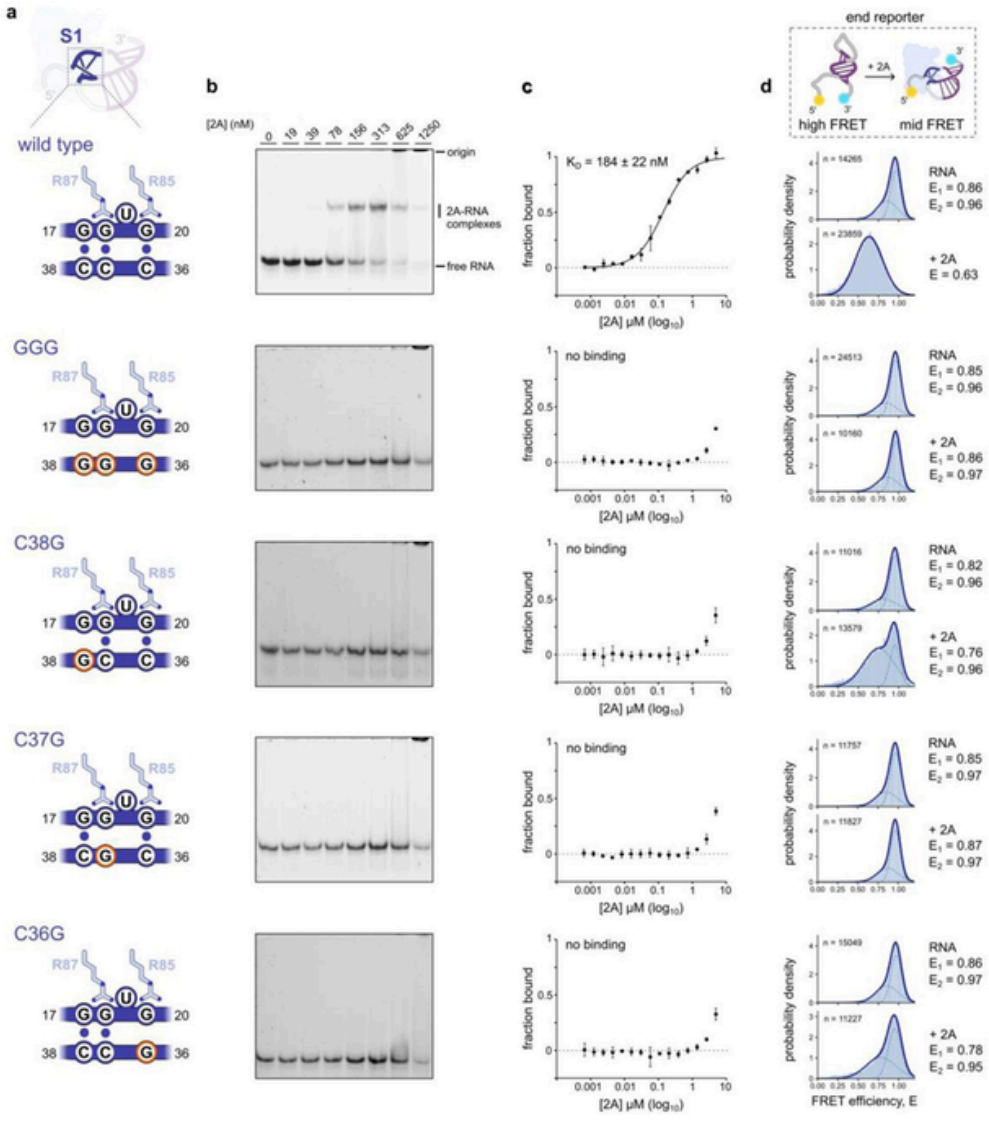


Figure 3 – Effect of stem 1 mutants on protein 2A binding (EMSA and MST) and FRET efficiencies
A) Wildtype or stem 1 mutant, B) EMSA analysis, C) MST binding curves for protein 2A (with KD values where possible), D) Corrected FRET efficiency histograms with RNA alone or with protein 2A

Summary

smFRET provided highly complementary analysis to static structural and ensemble techniques, resolving the previously undefined conformational shift of an RNA stem-loop into a pseudoknot upon viral protein 2A binding. Mutational analysis identified the first three nucleotides on stem 1 as crucial for protein 2A binding and pseudoknot formation, although ensemble binding methods (EMSA and MST) could not identify a 'pseudoknot-like' population that was only detectable in the smFRET data, indicating that an unstable complex was present. Betts. et. al. demonstrate how smFRET can be readily integrated into existing biophysical and structural workflows, highlighting the utility of smFRET for shedding light on dynamic structures and elucidating binding mechanisms.

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. Betts, J. K. et al. A new protein-dependent riboswitch activates ribosomal frameshifting. bioRxiv 2025.07.17.665365 (2025) doi:10.1101/2025.07.17.665365.

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

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