EXCITING INSTRUMENTS

Defining therapeutic mechanisms of action using single-molecule FRET and FCS on the EI-FLEX system

Application Note

Produced in collaboration with the Robb Lab - University of Warwick

Defining therapeutic mechanisms of action using single-molecule FRET and FCS on the EI-FLEX system

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In this application note, we demonstrate how Groves et al. performed both single-molecule Förster Resonance Energy Transfer (smFRET) and fluorescence correlation spectroscopy (FCS) on the EI-FLEX, investigating the mechanism of action of two viral SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) inhibitors: a nucleotide competitive inhibitor (remdesivir) and a non-nucleotide inhibitor (sumarin)[1]. They designed a doubly labelled RNA hairpin that would act as a reporter for RNA extension by RdRp when analysed using smFRET.

Overview of this application note:

- smFRET can distinguish non-extended from extended RNA species and the effect of RNA-dependent RNA polymerase inhibitors on this process
- FCS provides further context in the form of identifying RdRp stalling on RNA
- These techniques provide complementary data alongside gel-based assays, distinguishing the mechanisms of action for a nucleotide competitive inhibitor and a non-nucleotide inhibitor

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.

Autocorrelation: How similar a fluorescent signal is to itself after a given time delay (lag time); molecules that are diffusing slowly will produce similar signals over longer periods of time, shifting the autocorrelation curve to the left relative to smaller species that are diffusing through the confocal volume quicker.

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?

- Enabled generation of smFRET and FCS data on one platform, providing single-molecule and ensemble complementary insights to resolve the mechanism of action of remdesivir
- smFRET can precisely distinguish between non-extended and extended RNA species in the authors' hairpin model, while also resolving partially and fully extended molecules
- smFRET was highly suited to understanding the mechanism of action of sumarin, which showed clear shifts from low to high-FRET states with increasing inhibitor concentration

The EI-FLEX single-molecule spectrometer

smFRET can detect the extension of a doubly labelled RNA hairpin structure

To investigate the mechanism of action of the RdRp inhibitors, Groves et al. first made a reporter RNA that contained a hairpin structure labelled with Cy3 and Cy5 fluorophores. smFRET was used to assess extension by RdRp in the presence of different nucleotides. When the RNA had not been extended, the fluorophores were in close proximity, producing a high-FRET state (Figure 1A). As a control, the authors annealed a double-stranded RNA to mimic the fully RdRp extended RNA, which produced a low FRET efficiency (Figure 1b). Upon addition of RdRp and ATP, both a high and low-FRET state were observed, suggesting that RdRp partially extends some RNAs (lower FRET efficiency population) while stalling on others (high FRET efficiency) (Figure 1c). Stalling is resolved by the addition of UTP, which permits full extension and results in the expected low FRET efficiency (Figure 1d). Finally, mutation of the RdRp does not permit RNA extension even in the presence of ATP/UTP, as indicated by the high FRET efficiency in the presence of the RdRp mutant (Figure 1e).

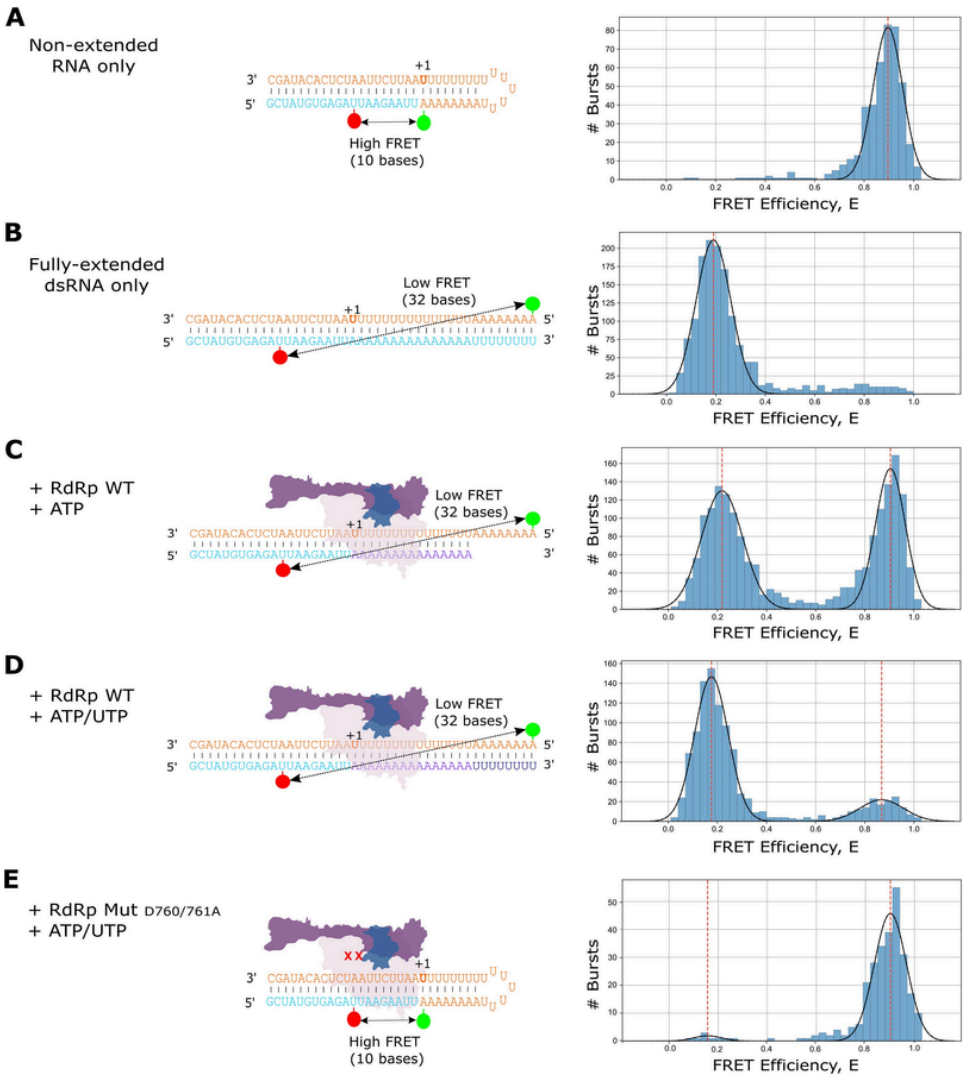

Figure 1 – smFRET data captures RdRp extension of a doubly labelled RNA hairpin structure

FRET efficiencies were measured for a range of conditions to demonstrate the validity of the model:
A) RNA only in its non-extended form shows a high-FRET state
B) Fully extended dsRNA shows a low-FRET state
C) Addition of the wild type RdRp and ATP shows non-extended (high-FRET) and partially extended (low-FRET) states
D) Addition of the wild type RdRp, ATP and UTP shows minimal non-extended (high-FRET), with the majority of RNA in the fully extended (low-FRET) states
E) Addition of mutated RdRP, ATP and UTP shows no RNA extension (high-FRET state only)

smFRET is well-suited for demonstrating the action of non-nucleotide inhibitor sumarin

With the model established, Groves et al. used smFRET to observe the effect of the non-nucleoside inhibitor sumarin on the extension of their labelled RNA hairpin model. The mechanism of action against SARS-CoV-2 is known to involve binding at two sites on the RdRp, preventing RNA template binding and disrupting RNA entry into the active site. The inhibition of RNA extension was clear: sumarin reduced the proportion of RNA molecules in the low-FRET state in a concentration-dependent manner, confirming the absence of RNA extension (Figure 2).

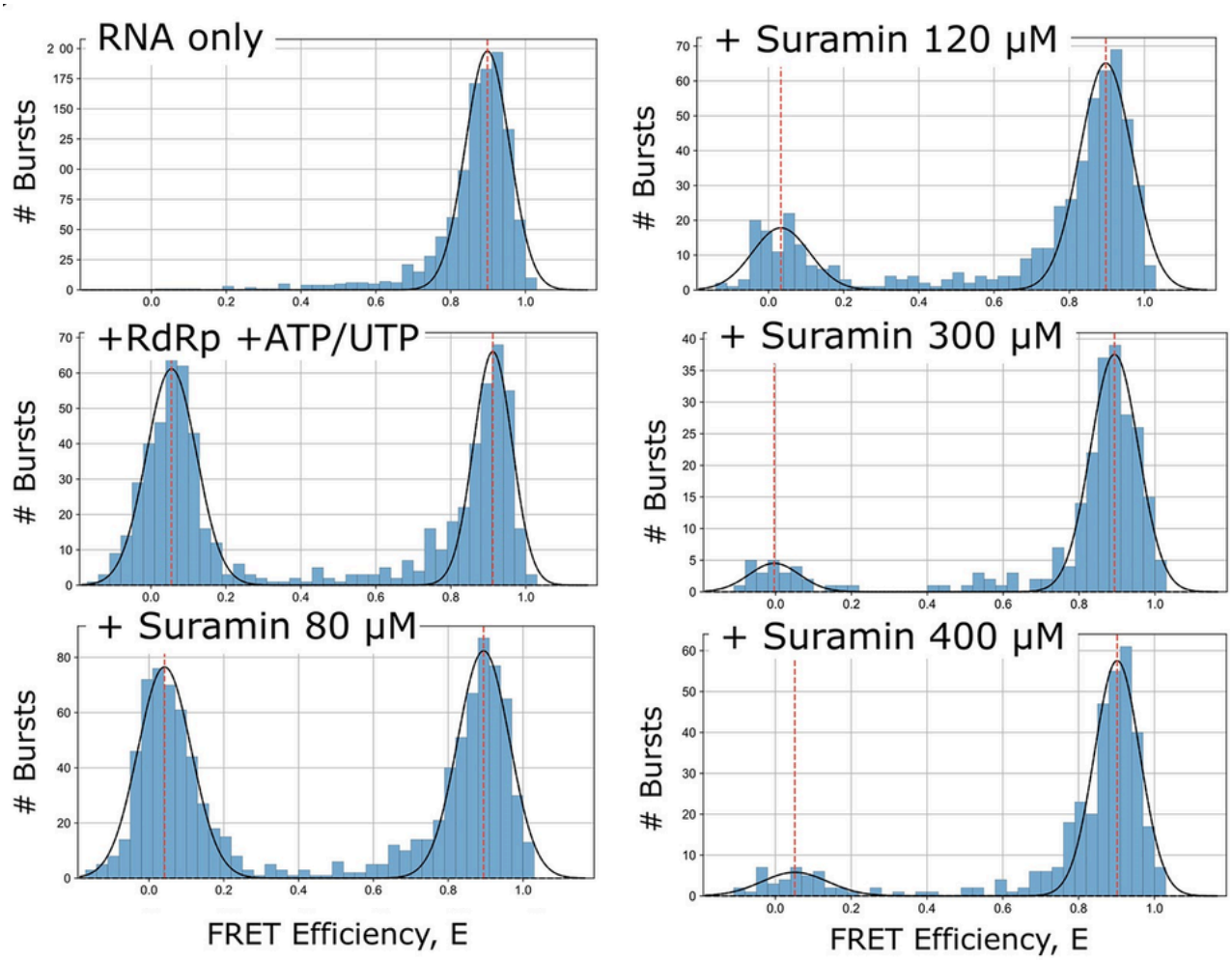

Figure 2 – smFRET data showing sumarin inhibition of RdRp extension of labelled RNA
FRET efficiency histograms across increasing sumarin concentrations, ranging from 0 to 400 µM.

smFRET and FCS data show that inhibition of RdRp is dependent on the concentration of remdesivir and NTPs

The mechanism of action of remdesivir is slightly different compared to sumarin. It is proposed to be through delayed chain termination, whereby it is incorporated in the place of adenosine and, following addition of the following three NTPs, induces RdRp stalling via steric hindrance. Here, the authors compared the effect of low and high ATP/UTP concentrations (5 and 500 µM) on the ability of RdRp to extend RNA with increasing concentrations of remdesivir.

smFRET data showed that full-length RNA (low-FRET population) is still produced even in the presence of remdesivir for both the 500 µM and 5 µM ATP/UTP conditions (Figure 3), although denaturing gel results suggest that some RdRp stalling is occurring in the latter. Remdesivir is likely being incorporated into the nascent RNA, as shown by smFRET results where ATP/UTP were absent; 0.5 mM remdesivir and above can produce high-FRET states even when present alone. The authors proposed that the antiviral activity of remdesivir may be delayed in this context, whereby inhibition occurs as a result of inefficient incorporation of UTP opposite remdesivir molecules in the template strand, acting as a potential secondary mechanism of action.

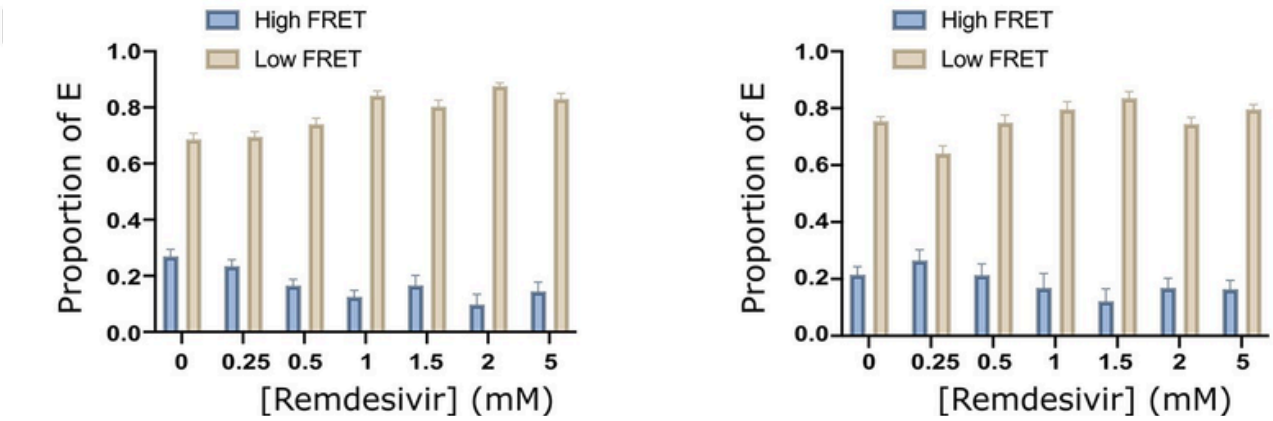

Figure 3 – Effect of increasing remdesivir concentration on the proportion of high and low-FRET efficiencies (E)
(Left) 500 µM ATP/UTP, (Right) 5 µM ATP/UTP
Error bars represent the standard error of the Gaussian fit of FRET efficiency histograms.

smFRET and FCS data show that inhibition of RdRp is dependent on the concentration of remdesivir and NTPs

To investigate the possibility of RdRp stalling further, Groves et al. performed FCS, which can measure the diffusion speed of molecules and resolve slower-moving complexes from unbound species. Here, Rhodamine 6G was used as a standard with a known diffusion coefficient. RNA alone diffused quickly, as demonstrated by the leftward shift on the autocorrelation plot (Figure 4).

For samples incubated with final concentrations of 500 µM ATP, UTP and remdesivir (in various combinations), diffusion times were slower than RNA in the presence of RdRp, indicating that they were in complex with the polymerase. However, no differences in diffusion times were observed between the combinations of NTPs and remdesivir, indicating that no polymerase stalling was occurring. However, for the samples with a final concentration of 5 µM, diffusion times were longer compared to RNA alone, particularly in the presence of remdesivir, confirming that RdRp stalling is indeed induced in limiting NTP conditions, while high NTP concentrations can overcome this and lead to fully extended RNA species.

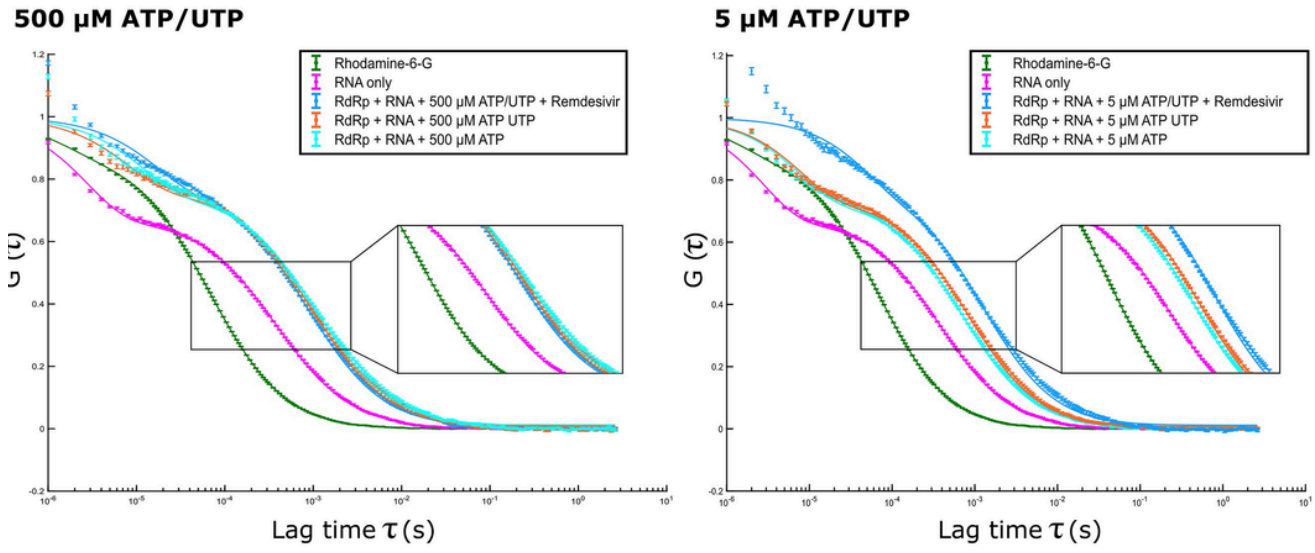

Figure 4 – FCS data demonstrates that RdRp stalls on RNA in low NTP conditions
FCS data showing autocorrelation plotted against lag time for final NTP concentrations of 500 µM (left) and 5 µM ATP/UTP (right) containing combinations of ATP, UTP and remdesivir.

Summary

smFRET and FCS were performed on the EI-FLEX to investigate the inhibitory mechanisms of two antiviral compounds that have different modes of action against the SARS-CoV-2 RdRp. Using smFRET with a doubly labelled RNA hairpin model, the authors demonstrated that high concentrations of NTPs can overcome the inhibitor effects of remdesivir, while high concentrations of remdesivir in the absence of NTPs can enable full extension. Interestingly, the second observation suggests a second mechanism of antiviral activity whereby sequences containing remdesivir molecules inhibit UTP incorporation in successive extensions.

Complementary gel and FCS data highlighted that limiting NTP conditions cause RdRp stalling on partially extended RNA molecules. Therefore, the precise method of action for remdesivir depends on both its own concentration and that of available NTPs. In contrast, the mechanism of action for the non-nucleoside inhibitor sumarin can be clearly observed using smFRET, whereby increasing concentrations prevent RNA extension through inhibition of RNA binding.

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 FCS on the EI-FLEX system on our website.

References

1. Groves, D. et al. Mechanistic insights into the activity of SARS-CoV-2 RNA polymerase inhibitors using single-molecule FRET. Nucleic Acids Res 53, gkaf351 (2025).

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

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