

Defining therapeutic mechanisms of action using smFRET and FCS

Application note

Produced in collaboration with the Robb Lab - University of Warwick

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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



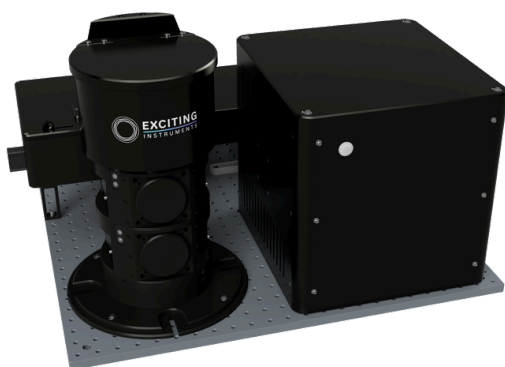
Glossary of terms used in this application note

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.



**The EI-FLEX
single-molecule
spectrometer**

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

With the model established, Groves et al. used smFRET to observe the effect of the non-nucleoside inhibitor suramin 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: suramin reduced the proportion of RNA molecules in the low-FRET state in a concentration-dependent manner, confirming the absence of RNA extension (Figure 2).

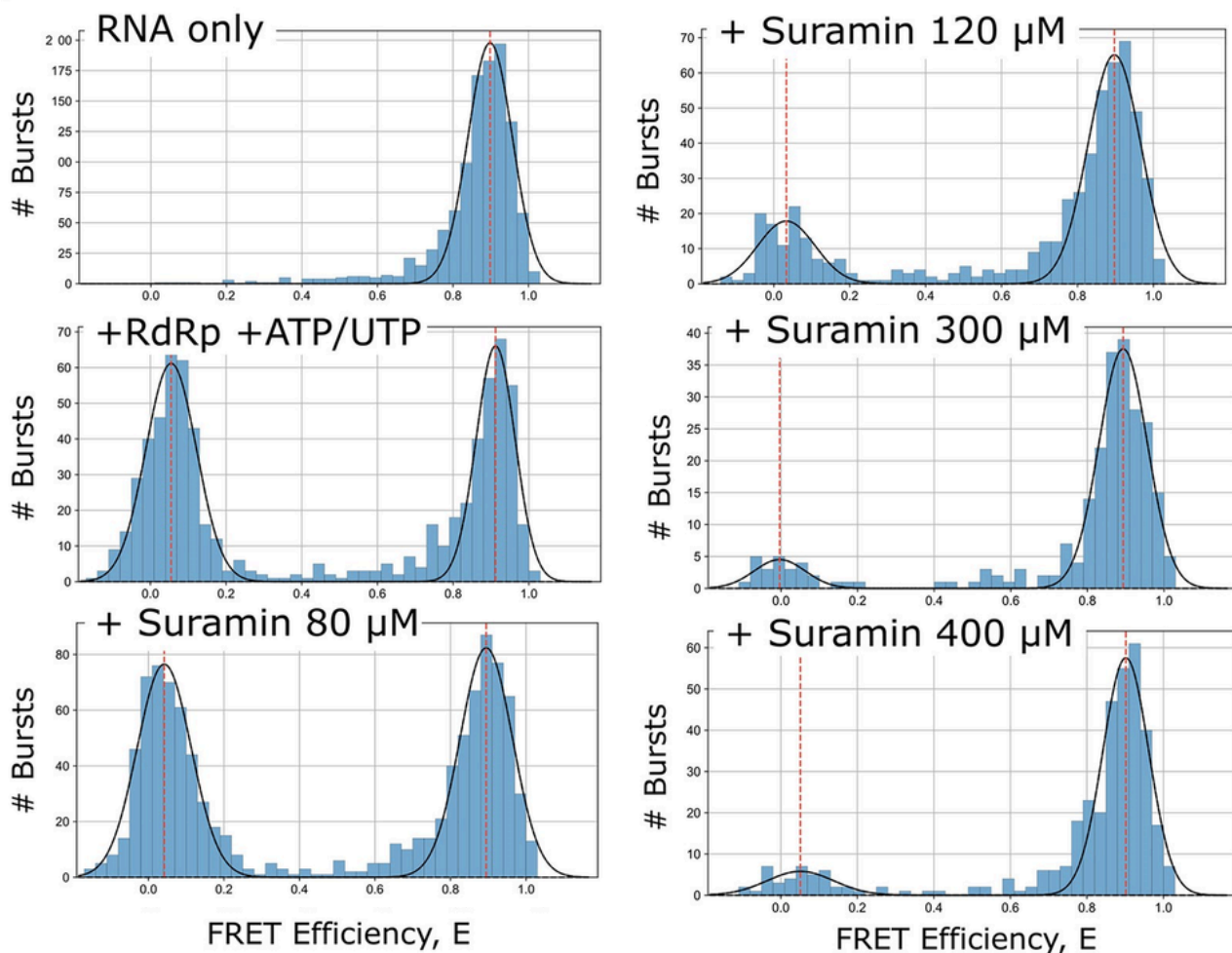


Figure 2 - smFRET data showing suramin inhibition of RdRp extension of labelled RNA
 FRET efficiency histograms across increasing suramin concentrations, ranging from 0 to 400 μM .

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.

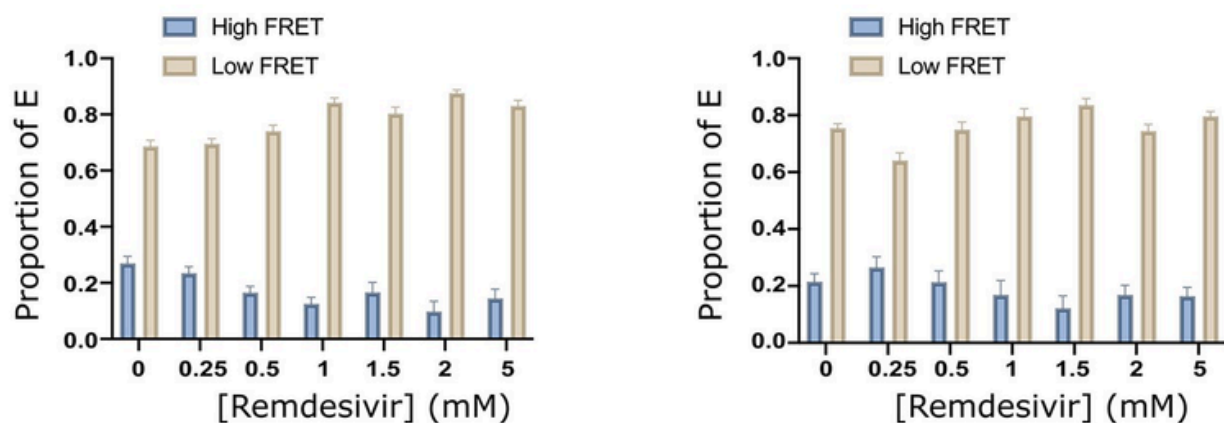


Figure 3 - Effect of increasing remdesivir concentration on the proportion of high and low-FRET efficiencies (E)
 (Left) 500 μM ATP/UTP, (Right) 5 μM ATP/UTP
 Error bars represent the standard error of the Gaussian fit of FRET efficiency histograms.

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.

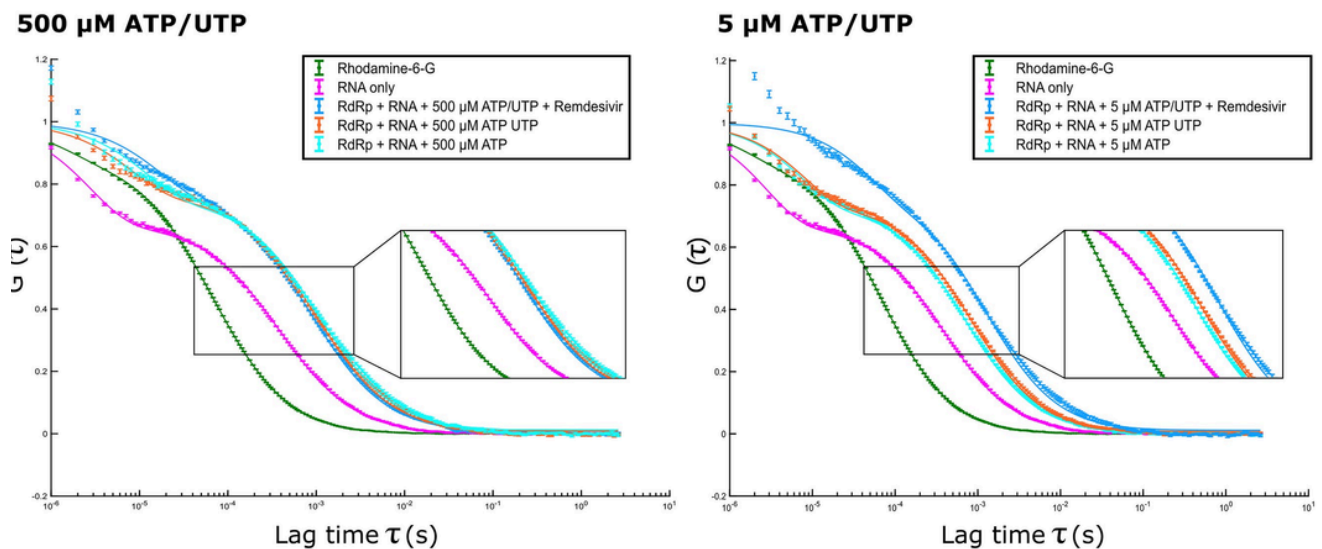


Figure 4 – FCS data demonstrates that RdRp stalls on RNA in low NTP conditions

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.

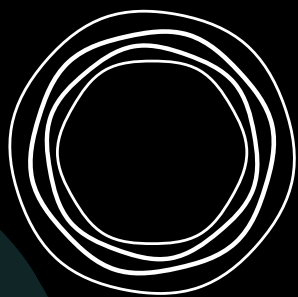
Complementary gel and FCS data highlighted that limiting NTP conditions cause RdRp stalling on partially extended RNA molecules. Therefore, the precise method of action for remdesivir depends on both its own concentration and that of available NTPs. In contrast, the mechanism of action for the non-nucleoside 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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