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INSTRUMENTS

**How to link accurate  
FRET efficiency  
measurements to atomic  
resolution structures**

**Technical Note**



# How to link accurate FRET efficiency measurements to atomic resolution structures

Single-molecule Förster Resonance Energy Transfer (smFRET) is a biophysical technique that measures distances on a molecular scale, whereby an excited donor fluorophore transfers energy to a nearby acceptor fluorophore, causing it to emit photons. The efficiency of this transfer depends on the distance between the donor and the acceptor, and their properties, typically occurring over a distance of ~3–10 nm. To ensure calculated distances are accurate and relate to the physical positions of the labelled biomolecules of interest, we discuss the importance of data correction and additional modelling.

## Overview of this application note:

- Accurate FRET correction of raw FRET efficiencies identifies photophysical artefacts
- High agreement between international laboratories with different instrumental setups can be achieved for determining FRET efficiencies on DNA and protein (but accurate FRET correction is crucial)
- Determination of Förster radii in individual experimental contexts is important for accurate calculation of inter-dye distances, but may not be necessary when measuring relative changes
- Accessible volume modelling captures the range of possible positions of both dyes, enabling understanding of how FRET efficiency relates to biomolecular structures

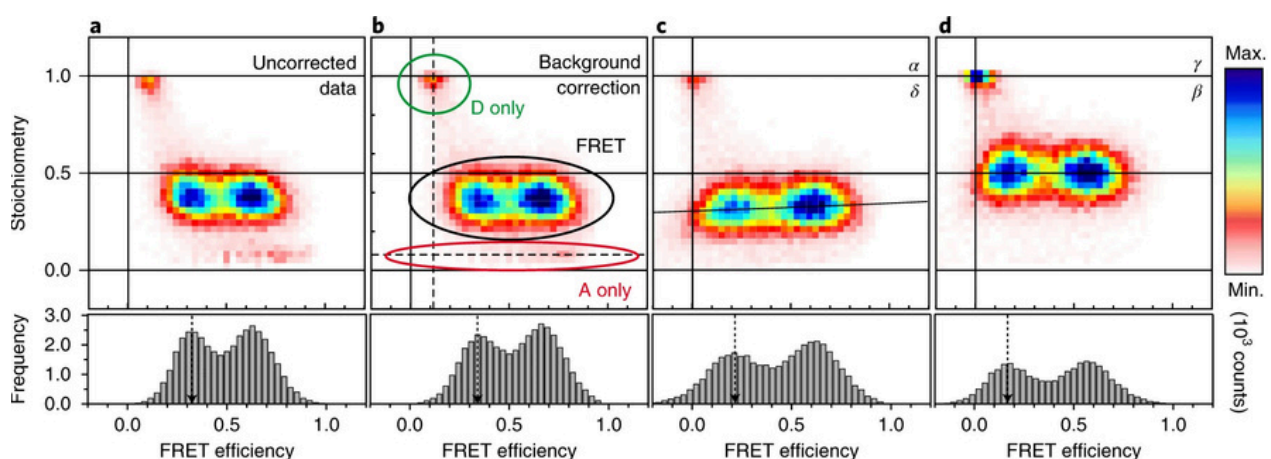
## Accurate FRET correction of raw FRET efficiencies

A typical workflow for using smFRET to calculate nanoscale distances and link these to atomic resolution structures has four main steps: generation of raw FRET efficiencies, accurate FRET correction, calculation of distances (and Förster radii), and accessible volume (AV) modelling.

Firstly, raw FRET efficiencies can be captured by smFRET using instruments such as the EI-FLEX. These efficiencies cannot be used for accurate distance calculation of intra-dye distances in their raw form, as they contain photophysical artefacts that obscure absolute distance. Four parameters are used for accurate FRET correction:

- Donor fluorescence leakage into the acceptor channel ( $\alpha$ )
- Direct excitation of the acceptor by the donor laser ( $\delta$ )
- Detection correction factor accounting for differences in detection efficiency between donor and acceptor channels ( $\gamma$ )
- Excitation correction factor reflecting the relative excitation efficiencies of the fluorophores ( $\beta$ )

An example of accurate FRET correction is shown in Figure 1, whereby donor-only and acceptor-only molecules are removed, and  $\gamma$  and  $\beta$  correction factors are applied. In this example, the E value changes from  $\sim 0.25$  to  $\sim 0.15$  following accurate FRET correction.



**Figure 1 - Accurate FRET correction of smFRET data with low and mid-FRET populations**

A) Uncorrected data

B) Identification of leakage ( $\alpha$ ) and direct excitation ( $\delta$ ), alongside donor-only or acceptor-only labelled species

C) Identification of  $\gamma$  and  $\beta$  correction factors

D) Fully corrected FRET data

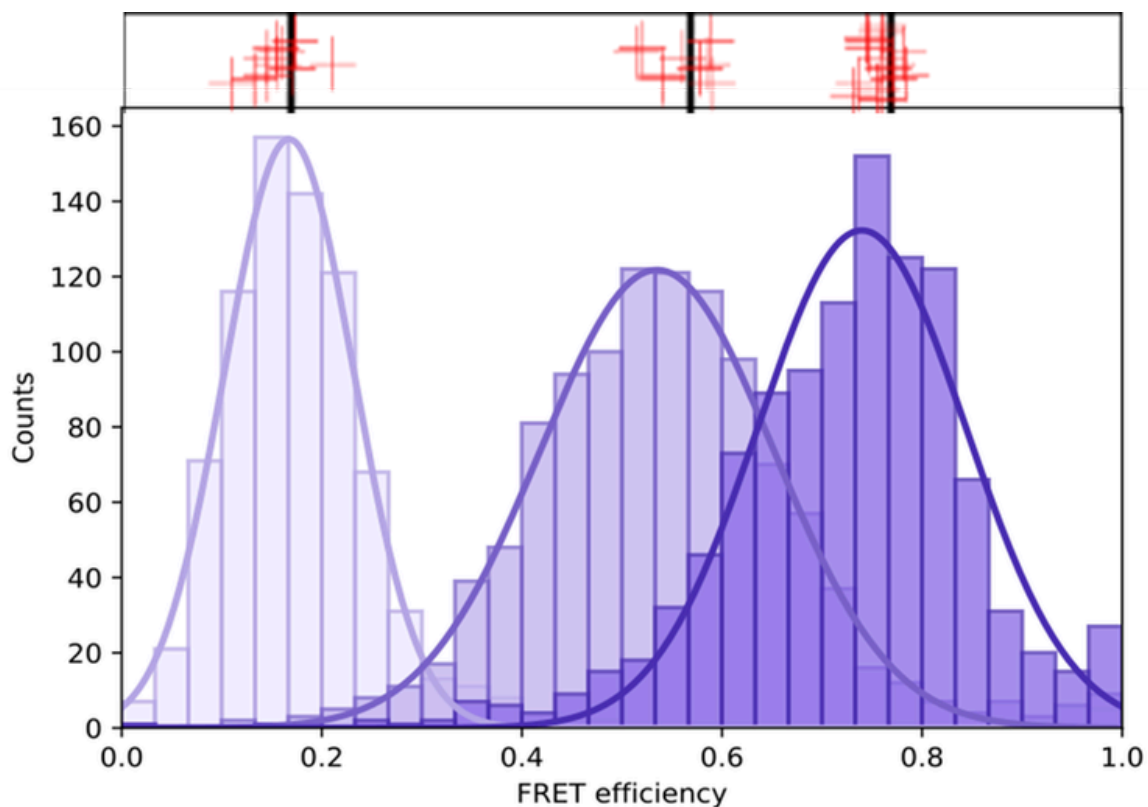
Arrows indicate how the FRET efficiency value for the low-FRET population changes with correction

Figure taken from Hellenkamp et al., 2018<sup>1</sup>

## Accurate FRET correction of raw FRET efficiencies

If accurate FRET correction is performed, FRET efficiencies can be highly reproducible between smFRET setups and methodologies, as shown by Hellenkamp et al. in an international study involving twenty laboratories<sup>1</sup>. Participants were sent samples that contained heterogeneously labelled DNA duplexes, with fluorophore pairs placed in three locations that would generate low, medium and high FRET efficiencies.

Data were in good agreement between the participating laboratories and data generated in-house by Exciting Instruments on the EI-FLEX (Figure 2). The variation between reported FRET efficiencies in this study ranged from  $\pm 0.015$  to 0.06, demonstrating that smFRET is reproducible between confocal and surface-immobilised FRET methods, even when different FRET correction methods were used.



**Figure 2 - Agreement for FRET efficiencies of three labelled DNA duplexes**

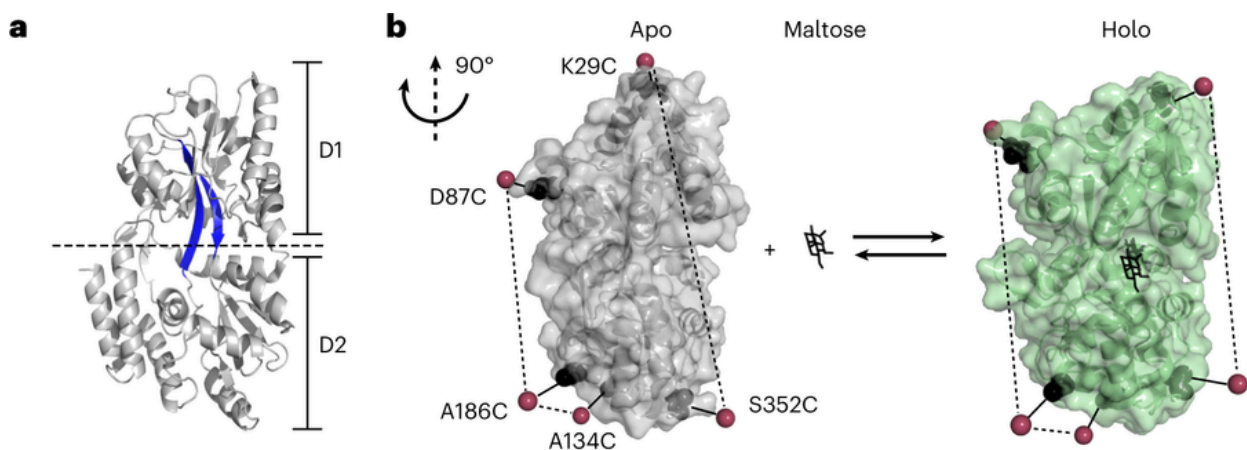
*Gaussian-fitted FRET histograms of smFRET data generated on the EI-FLEX for three DNA duplexes with dye pairs at three distinct locations. Vertical lines indicate mean FRET efficiencies for each duplex. Red crosses indicate reported FRET efficiencies for the same duplex and dye pair from participating laboratories in the Hellenkamp et al. study<sup>1</sup>.*

## Accurate FRET correction of raw FRET efficiencies

A similar study was performed by Agam et al. to measure the precision and accuracy of smFRET in proteins, which have increased flexibility and complexity of local environments compared to nucleic acids<sup>2</sup>. Here, participants were provided with three versions of the maltose-binding protein (MalE), each with dye pairs on different locations designed to provide distinct FRET efficiencies (Figure 3a and 3b).

Good agreement was seen for sixteen laboratories for reported FRET efficiencies, particularly when FRET efficiency values were high (Figure 3c). The standard deviation across all participants was found to be  $\pm 0.06$ , which was in line with what was reported in the Hellenkamp et al. study.

To investigate the potential impact of systematic error, the FRET efficiencies for maltose-bound MalE were subtracted from unbound MalE for each participant (Figure 3d). Here, the distribution of data was reduced by two-fold, indicating that the observed error was indeed likely due to systematic error. The authors attributed the major source of error to the calculation of the  $\gamma$  correction factor, which accounted for 23% of the FRET efficiency uncertainty. This highlights the importance of accurate FRET correction.

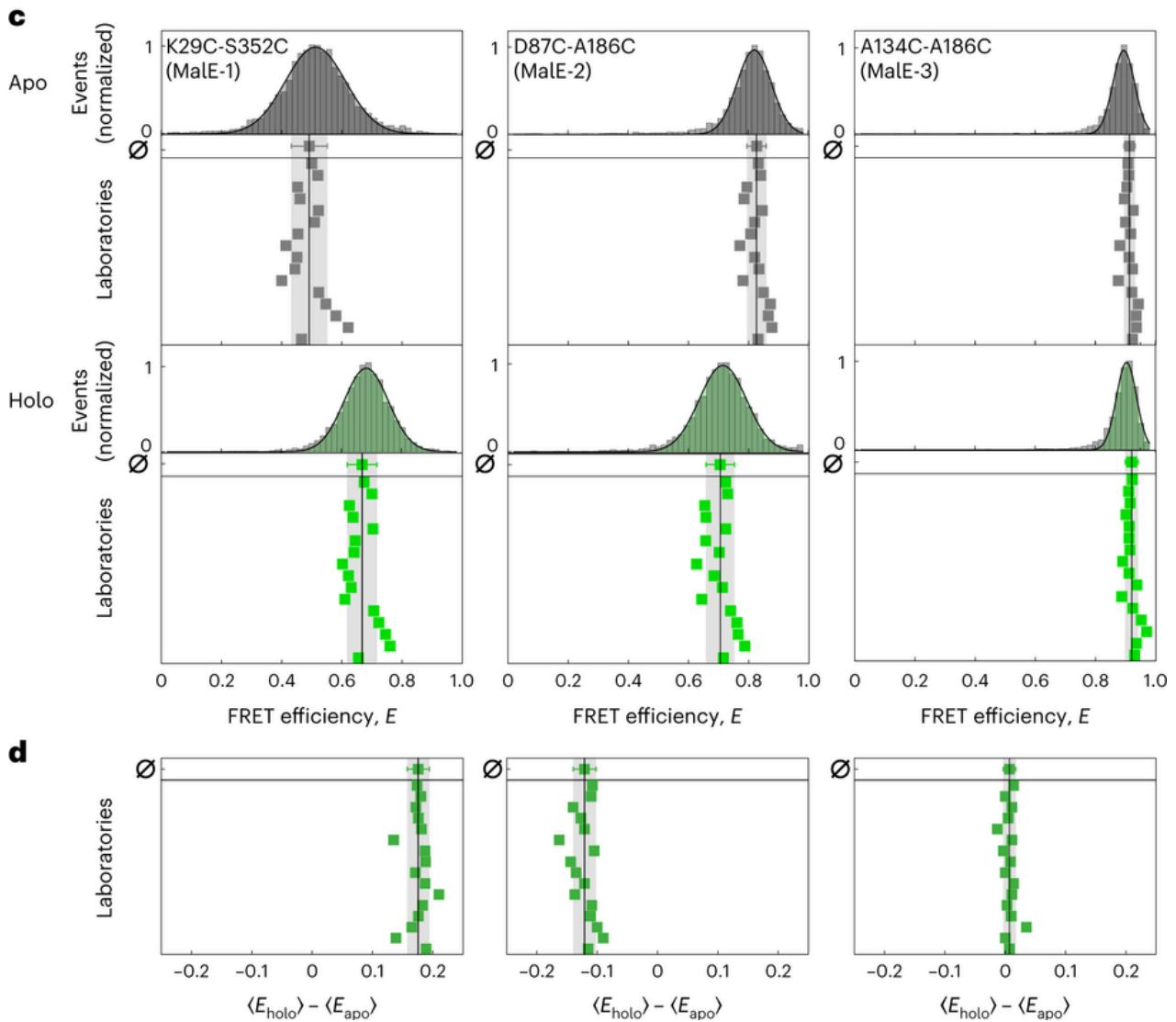


**Figure 3a and b- FRET efficiencies for three dye pairs on unbound and maltose-bound MalE across sixteen laboratories**

A) Structure of MalE

B) Positions of dyes for both unbound and maltose-bound MalE (dotted lines indicate dye pairs and their separation)

Figure taken from Agam et al. 2023<sup>2</sup>



**Figure 3c and d - FRET efficiencies for three dye pairs on unbound and maltose-bound MalE across sixteen laboratories**

C) FRET efficiency data for sixteen laboratories for unbound (grey) and bound (green) MalE, showing FRET efficiency histograms and spread of individual data

D) Normalised FRET efficiency values (unbound subtracted from bound for each participating laboratory)

Figure taken from Agam et al. 2023<sup>2</sup>



## Calculation of nanoscale distances using accurate FRET efficiencies

Once accurate FRET efficiencies have been calculated, the distance between dye pairs can be determined using the following equation, which links FRET efficiency to inter-dye distance:

$$R = R_0 \times \sqrt[6]{\frac{1 - E}{E}}$$

whereby:

R = distance between the fluorophores

R<sub>0</sub> = Förster radius (distance at which the FRET efficiency is 50 % for a given dye pair)

E = FRET efficiency

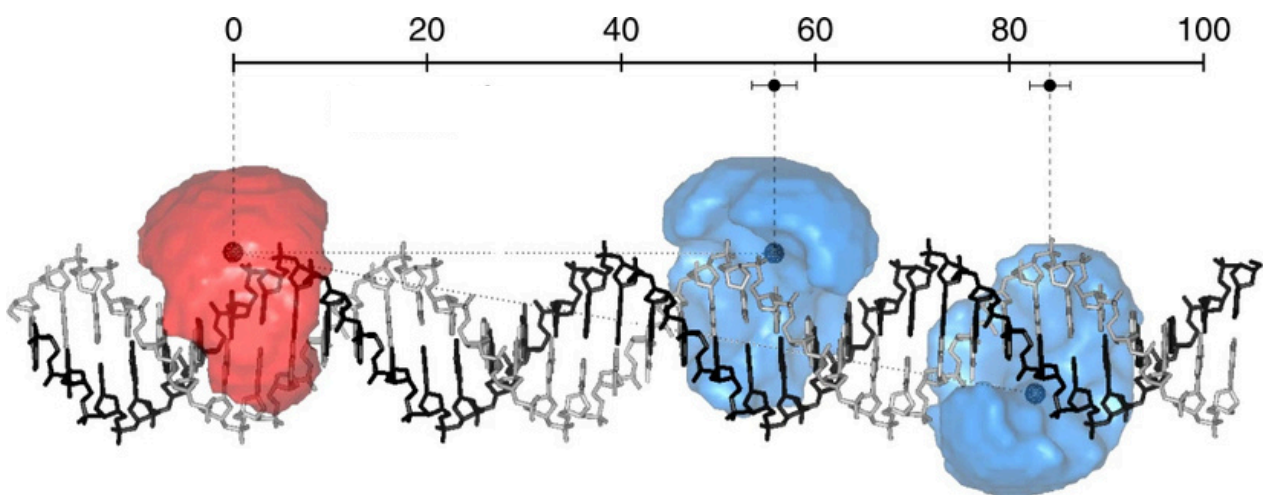
The Förster radius has been published for many dye pairs, and these values can be used to determine approximate distances where high accuracy is not necessary, such as when comparing relative distance changes. If determining highly accurate distances is a priority, Förster radii must be calculated for dye pairs in each individual use case. This is because several factors can influence the distance at which FRET efficiency is at 50%, including the local environment (effect of nearby residues or bases that may impact fluorescence or linker movement) and the buffer in which the reaction is occurring.

Since the Förster radius is directly proportional to the inter-dye distance, any error in Förster radius is then carried forward into calculated distances. This was demonstrated by Craggs et al., who used smFRET to calculate the distance between two dyes placed on a DNA molecule when it was bound to DNA polymerase<sup>3</sup>. The authors calculated their own Förster radii from free dye and that of a doubly labelled DNA molecule bound to DNA polymerase, observing a 4% difference in Förster radius and the resultant calculated inter-dye distance.

## Accessible volume modelling links inter-dye distance to biomolecular structures

The final step for linking accurate FRET efficiency to atomic resolution structures requires modelling of the accessible volume (AV) of the dyes. The dyes used in smFRET are formed of fluorophores that are conjugated to flexible linkers of approximately 20 Å that can move around their anchored position. This will impact the resultant FRET efficiency depending on the position of each dye when measurements are taken. For this reason, the inter-dye distance does not directly map onto the biomolecule that is being investigated.

Therefore, in order to accurately relate the inter-dye distances to atomic resolution structures, AV modelling is used. AV modelling is a geometric method that uses an algorithm to simulate all of the possible positions that dyes could take around a fixed point, forming 'density clouds' around their anchored points. An example of this is shown in Figure 4, illustrating the bound positions and predicted AVs for one donor and two acceptor dyes used in the Hellenkamp et al. study<sup>1</sup>.



**Figure 4 - Dye positions and AV modelling clouds on a double-stranded DNA molecule**

Positions of a donor dye (red) and two acceptor dyes (blue) on a DNA duplex showing 'clouds' of all possible dye positions from AV modelling. Distances between dyes are given in Ångströms above. Figure taken from Hellenkamp et al., 2018<sup>1</sup>



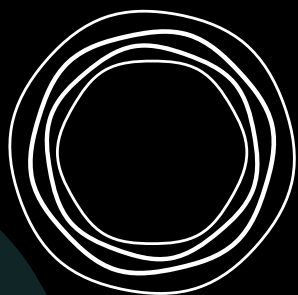
## Summary

In summary, smFRET is an excellent method for determining nanoscale distances on single molecules with high reproducibility, even across different instrument set-ups and analysis methods. Accurate FRET correction is crucial for identifying and removing photophysical artefacts that can cause large deviations in reported distances. Likewise, when highly accurate inter-dye distances are required, additional measurements to calculate Förster radii in individual experimental contexts are necessary. Accurately relating inter-dye distance to atomic resolution structure can be achieved through accessible volume modelling that takes into account the flexibility of the dye and its linker relative to the biomolecule it is bound to.

For a deeper dive on the techniques discussed in this section, see our [Resource Library](#) for more information.

## References

1. Hellenkamp, B. et al. Precision and accuracy of single-molecule FRET measurements –a multi-laboratory benchmark study. *Nat Methods* 15, 669–676 (2018).
2. Agam, G. et al. Reliability and accuracy of single-molecule FRET studies for characterization of structural dynamics and distances in proteins. *Nat Methods* 20, 523–535 (2023).
3. Craggs, T. D. et al. Substrate conformational dynamics facilitate structure-specific recognition of gapped DNA by DNA polymerase. *Nucleic Acids Res.* 47, 10788–10800 (2019).



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