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Measuring nanoscale distances with single-molecule FRET

Application note

In this application note, we demonstrate how single-molecule Förster Resonance Energy Transfer (smFRET) can be used to measure nanoscale distances on the EI-FLEX. smFRET was performed on a model DNA duplex labelled with donor and acceptor fluorophores at defined positions, to measure the FRET efficiency and calculate the distance between the two dyes. These data showcase the application of smFRET in probing nanoscale distances on single molecules with Ångström-level precision.

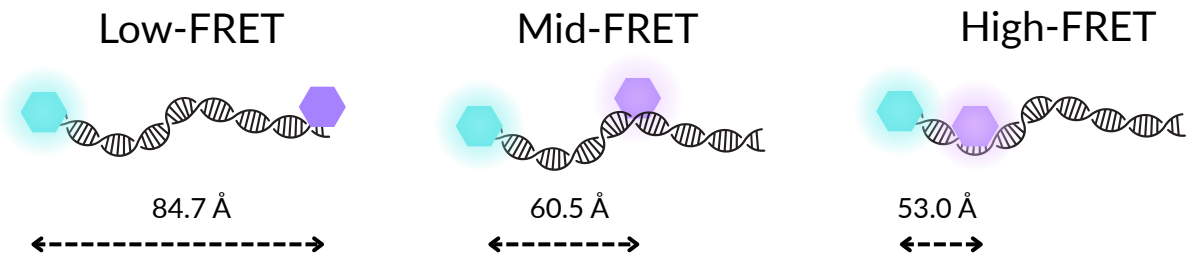


Figure 1 - Illustration of low-FRET, mid-FRET and high-FRET DNA standards
The donor fluorophore is conjugated on one strand, with an acceptor fluorophore conjugated at various distances that produce low, mid or high-FRET efficiencies. Calculated interdye distances are given in Å.

Overview of this application note:

- Three DNA duplex standards that have low, mid and high FRET efficiencies are used to demonstrate the smFRET technique
- Alternating laser excitation is used to identify doubly-labelled DNA complexes and to determine their FRET efficiency and stoichiometry
- FRET efficiencies and known Förster radii are used to calculate nanoscale distances between fluorophore pairs

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.

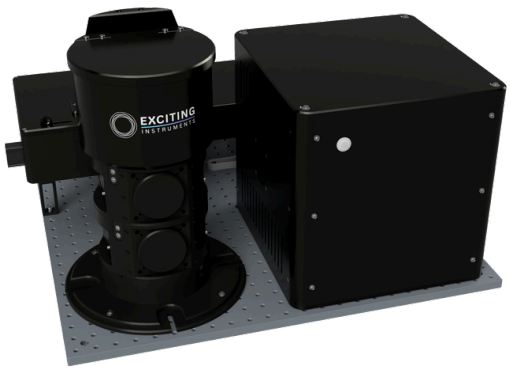
Alternating Laser EXcitation (ALEX): A measurement scheme in which a donor and an acceptor laser are switched on and off in rapid sequence, hitting each molecule multiple times with either laser. This allows emission arising from donor and acceptor excitation to be distinguished. This enables the calculation of stoichiometry, which is used to identify and separate doubly labelled molecules from donor-only and acceptor-only species.

Stoichiometry: A measure of the balance between donor and acceptor emission from a single molecule. It is determined from the ratio of emission detected under donor excitation to the total detected under both donor and acceptor excitation. Stoichiometry values near 0.5 indicate doubly labelled molecules, while values closer to 0 or 1 indicate acceptor-only or donor-only species, respectively.

Förster radius (R_0): The distance between a pair of fluorophores that gives a 50% FRET efficiency; usually given in Ångströms (Å). It is used to determine distances between fluorophores using measured FRET efficiencies. The Förster radii for fluorophore pairs can be determined experimentally or found online.

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

Identifying FRET states on DNA duplexes

Figure 2 presents FRET efficiency (E) versus stoichiometry (S) plots for the low-, mid-, and high-FRET DNA duplexes, following the removal of donor-only and acceptor-only populations and correction of experimental artefacts (see next section for more information). A stoichiometry value near 0.5 indicates bursts from doubly labelled DNA species.

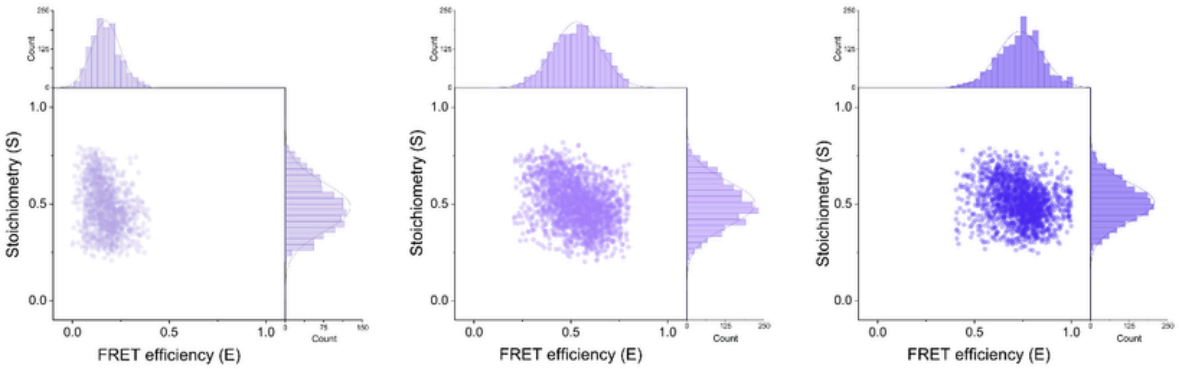


Figure 2 - E vs S plot for low FRET, mid-FRET and high-FRET DNA standards labelled with donor and acceptor fluorophores

The histograms revealed the measured FRET efficiencies were 0.16 ± 0.06 for low-FRET species, 0.54 ± 0.13 for mid-FRET species, and 0.75 ± 0.11 for high-FRET species, as shown in Figure 3.

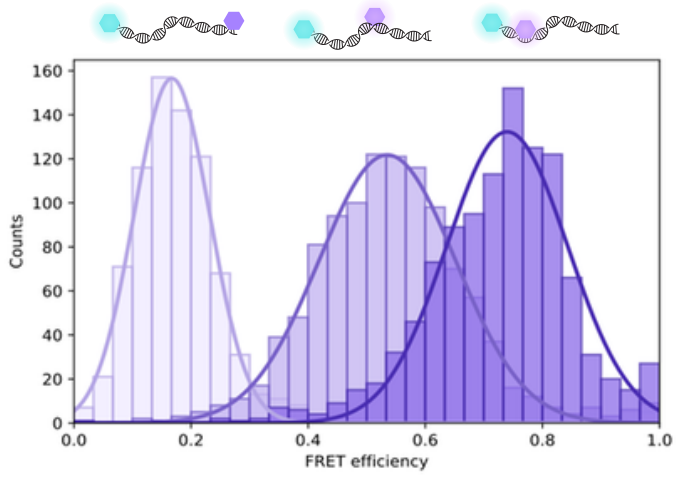


Figure 3 - FRET efficiency histogram for low-FRET, mid-FRET and high-FRET DNA constructs.

The same constructs were used to estimate FRET efficiency in an international, blind, multilaboratory study by Hellenkamp et al., who reported E values of 0.15 ± 0.02 for low-FRET constructs, 0.56 ± 0.03 for mid-FRET constructs, and 0.76 ± 0.015 for high-FRET constructs[1]. The close agreement between our measurements and those reported by Hellenkamp et al. highlights the reproducibility of FRET efficiency determination across independent experiments, confirming the robust performance of our instrument.

Using Alternating Laser EXcitation (ALEX) to analyse FRET data

Alternating Laser EXcitation (ALEX) enables the calculation of the two fluorescence ratios that were used in the data shown in Figures 1 and 2: FRET efficiency (E), which reflects the distance between the donor and acceptor, and stoichiometry (S), which indicates the donor–acceptor labelling status and enables isolation of doubly-labelled species for analysis.

FRET efficiency, E can be calculated by the following equation:

$$E = \frac{D_{ex}A_{em}}{D_{ex}D_{em} + D_{ex}A_{em}}$$

$D_{ex}A_{em}$ = Acceptor emission under donor excitation (red emission under green excitation)
$D_{ex}D_{em}$ = Donor emission under donor excitation (green emission under green excitation)

The stoichiometry ratio, S is calculated by the following equation:

$$S = \frac{D_{ex}D_{em} + D_{ex}A_{em}}{D_{ex}D_{em} + D_{ex}A_{em} + A_{ex}A_{em}}$$

$D_{ex}A_{em}$ = Acceptor emission under donor excitation (red emission under green excitation)
$D_{ex}D_{em}$ = Donor emission under donor excitation (green emission under green excitation)
$A_{ex}A_{em}$ = Acceptor emission under acceptor excitation (red emission under red excitation)

Using Alternating Laser EXcitation (ALEX) to analyse FRET data

Together, they enable identification of doubly-labelled species, distinct from donor-only or acceptor-only species (Figure 4), and correction for artefacts such as leakage, direct excitation of the acceptor by the donor laser, and photobleaching (**for more information on these artefacts and their correction, please see our technical note on accurate FRET correction**). Doubly labelled FRET pairs have a stoichiometry of ~0.5, while donor-only and acceptor-only molecules have stoichiometry values of ~1 and ~0, respectively.

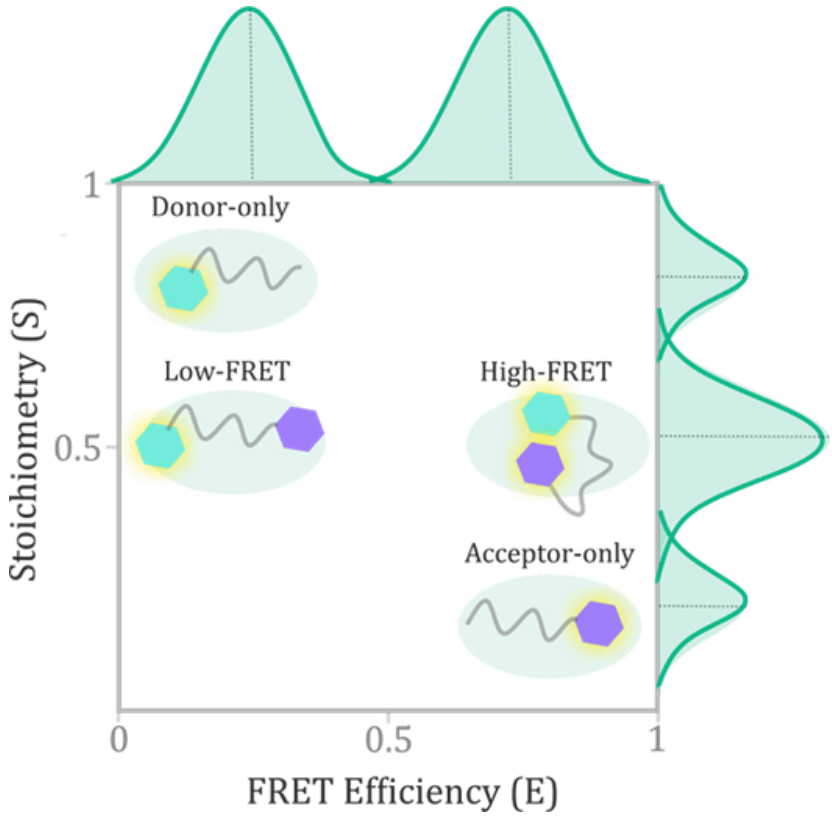


Figure 4 - A representative plot displaying FRET efficiency against stoichiometry

Using FRET efficiency to calculate distances between fluorophore pairs

Next, nanoscale distances between the fluorophore pairs for each of the three DNA constructs were calculated. FRET efficiency is related to the distance between fluorophores in the following manner:

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

whereby
R = distance between the fluorophores
R_0 = Förster radius (distance at which the FRET efficiency is 50%)
E = FRET efficiency

The Förster radius for the two fluorophores used in this study (ATTO 550 and ATTO 647N) was reported to be 62.6Å[1]. Therefore, the distances between the donor and acceptor fluorophores were calculated to be 84.7 Å for the low-FRET pair, 60.5 Å for the mid-FRET pair, and 53.0 Å for the high-FRET pair.

In summary, the EI-FLEX enables accurate calculation of FRET efficiency and inter-fluorophore distances on DNA duplexes. By integrating the strengths of ALEX and smFRET, the EI-FLEX can take into account photophysical artefacts to provide reliable FRET efficiency measurements. Notably, this approach operates at picomolar concentrations, eliminates the need for complex biomolecule immobilisation, and yields real-time, single-molecule insights into biomolecular interactions.

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. Hellenkamp, B. et al. Precision and accuracy of single-molecule FRET measurements—a multi-laboratory benchmark study. Nat Methods 15, 669–676 (2018).

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