EXCITING INSTRUMENTS

Capturing accurate single-molecule FRET measurements on the EI-FLEX platform

Technical Note

Capturing accurate single-molecule FRET measurements on the EI-FLEX platform

In this technical note, we explore several key components of a confocal FRET experiment that ensure single-molecule sensitivity and accurate determination of FRET efficiencies, taking into account the influence of inefficient labelling of target molecules and photophysical artefacts. Ensuring these factors are corrected during data acquisition and analysis allows resolution of complex subpopulations, conformational changes and nanoscale measurements that can be reported with confidence.

Overview of this application note:

- How confocal volumes can achieve single-molecule sensitivity
- The use of alternating laser excitation (ALEX) and burst searching to identify doubly labelled molecules and photophysical artefacts
- How accurate FRET correction is performed using ALEX

Using confocal volumes to achieve single-molecule sensitivity

Ensemble FRET methods give information about the average status of sample populations, potentially masking diverse conformations and dynamic species. By contrast, single-molecule FRET (smFRET), which can be performed on the EI-FLEX, captures FRET efficiencies for individual molecules, resolving heterogeneous and dynamic populations to provide greater insight into complex biological processes at the fundamental level.

To achieve this, the EI-FLEX uses a femtolitre-scale confocal volume, through which freely diffusing molecules can be excited by a laser. The confocal volume is defined by the intersection of the focused excitation beam and the spatial filtering of emission imposed by the pinhole. The pinhole plays a critical role in blocking out-of-focus fluorescence, allowing only emission from the focal plane to reach the detectors (Figure 1).

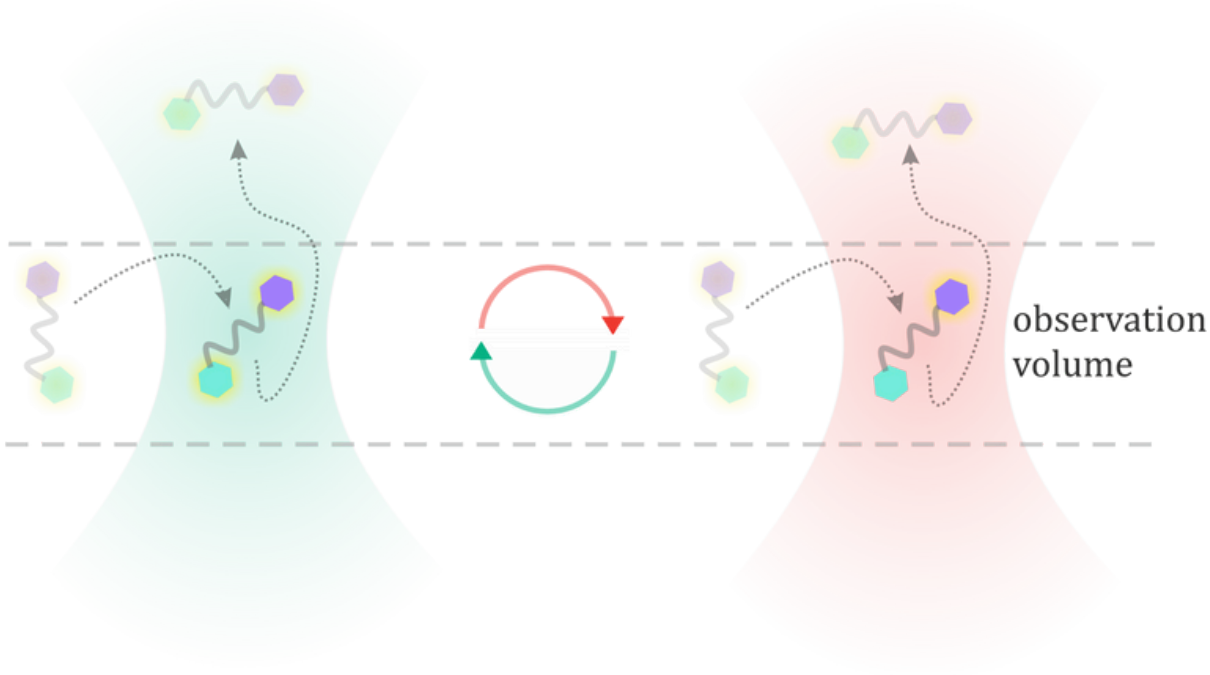

Figure 1 - Confocal volume for smFRET
Single molecules diffuse through a confocal volume formed by focusing lasers to a near diffraction-limited spot. Lasers are alternated rapidly (20 KHz) to ensure each molecule experiences multiple periods of donor and acceptor excitation, respectively.

Using alternating laser excitation (ALEX) to identify doubly labelled molecules

Alternating Laser EXcitation (ALEX) is a technique used in smFRET experiments to enhance measurement accuracy and data quality. In ALEX, two lasers are alternated so that only one is on at any given time, exciting either the donor or the acceptor fluorophore. This is done rapidly to ensure that each molecule experiences multiple excitation cycles by both lasers during its observation period, enabling the collection of fluorescence under strictly donor-only or acceptor-only excitation.

Labelling and purification of biomolecules is not completely efficient, resulting in the presence of donor-only and acceptor-only species, alongside the target doubly labelled molecules. In order to identify molecules with both fluorophores, ALEX can be used to calculate the stoichiometry (S), whereby:

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

$D_{ex}A_{em}$ = 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)

Donor-only molecules have an S value of ~1, while acceptor-only molecules have an S value of ~0. Doubly labelled molecules have an S value of ~0.5. Stoichiometry is typically plotted against FRET efficiency (E), enabling analysis to be performed only on the FRET efficiencies of the intended doubly labelled molecules of interest.

Burst Searching

ALEX is also used in burst searching methods in order to refine S versus E plots. Burst searching is a crucial step in analysing smFRET data. Bursts correspond to the time intervals when a fluorescent molecule is diffusing through the observation volume. To identify bursts, a chosen number of photons (M) are processed sequentially by a sliding window.
To qualify as a burst, a group of photons in the photon stream needs to meet two threshold criteria:

- Their count rate exceeds the background count rate by a chosen multiplicative factor (F)
- A minimum number of photons (L) are acquired

Beginning with the first (M) photons, a count rate is determined and if it is not (F) times greater than the background count rate, the window slides along to process subsequent photons. Once the (F) threshold is exceeded by a group of (M) photons, the start time of a potential burst is determined by the arrival time of the first photon within the window. The algorithm then slides the window along until the (F) threshold versus background is no longer satisfied, thereby determining the potential end time. If >(L) photons have been detected within that time window, those photons are classified as being part of a burst.

The burst searching algorithm processes the entire duration of the experiment in this way, after which the FRET efficiency and stoichiometry of bursts can then be calculated.
Burst searching can be performed using the All-Photon Burst Search (APBS) method, which detects bursts by simultaneously processing photons collected from both channels. While useful to assess various aspects of sample quality, an APBS does not differentiate between photons originating under donor or acceptor excitation and therefore may identify bursts from donor-only or acceptor-only molecules.

A Dual-Channel Burst Search (DCBS) leverages the power of ALEX, whereby the burst searching algorithm independently processes photons detected during donor excitation (DD and DA photons), and photons detected during acceptor excitation (AA photons), applying the thresholding criteria on a per-channel basis. Resultant bursts are the combined output of this processing in either channel. This enables the identification of bursts where both donor and acceptor dyes are active, permitting filtering for correctly doubly labelled molecules, and reducing the effects of blinking, bleaching, and coincident events, leading to cleaner and more reliable burst detection and more accurate FRET analysis.

Burst Searching

To illustrate the use of these two methods, Figure 2 shows the identification of bursts and their resultant S versus E plots for a low-FRET sample. To assess the general quality of the data, an APBS is performed first to investigate all of the bursts that were captured. The DCBS method is then used to refine this data by attributing bursts to doubly or singly-labelled molecules, and the S versus E plot is filtered to only include the population of interest (S ~0.5).

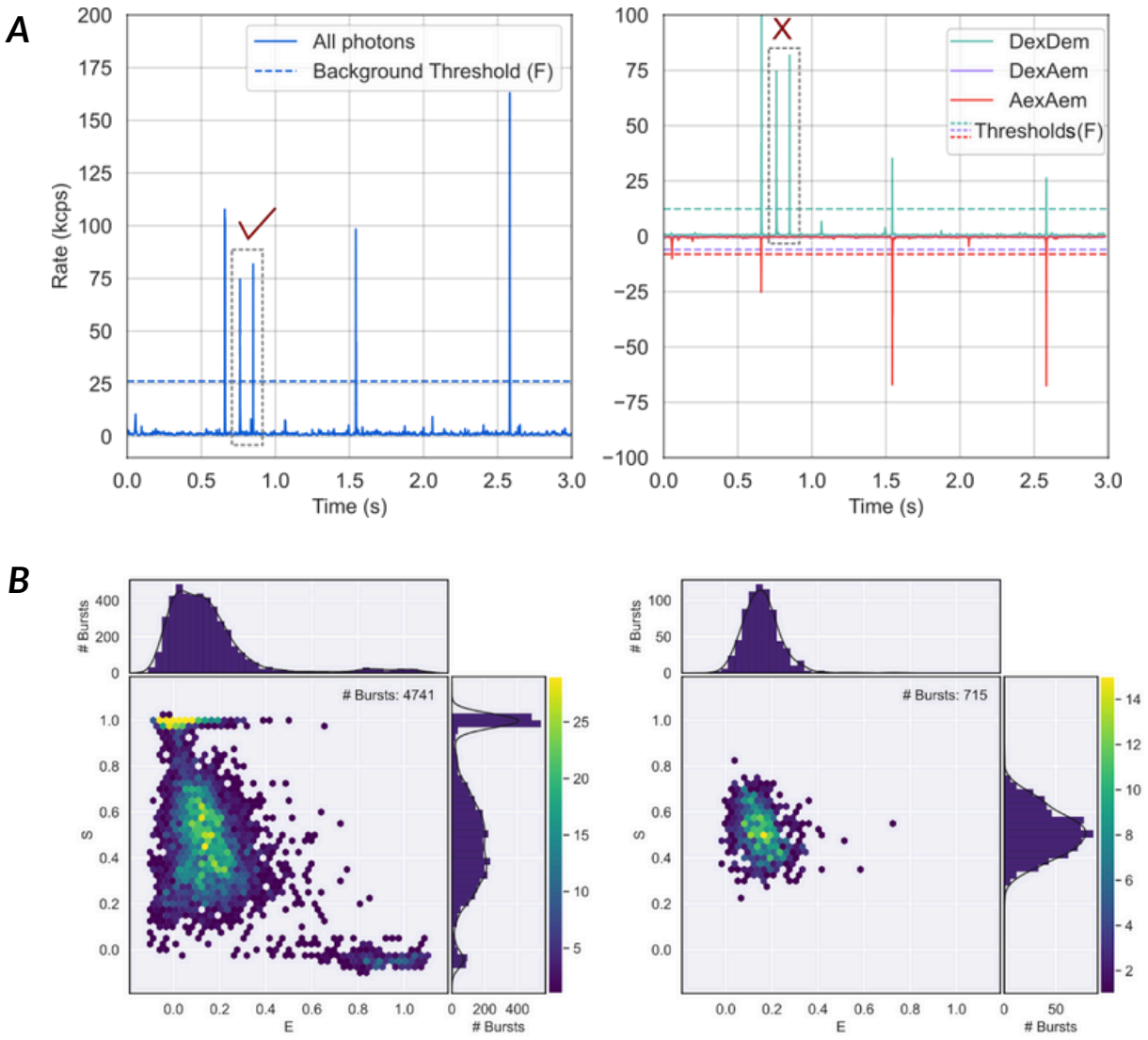

Figure 2 - Comparison of photon burst identification between APBS and DCBS methods
A) Identified bursts for APBS (left) and DCBS (right) methods
B) E vs S plots for the bursts identified by an APBS (left) and a DCBS (right)

Performing accurate FRET correction

Another benefit of performing ALEX is the ability to correct for photophysical artefacts. The identification of distinct molecular sub-populations enables the calculation of inter-dye distances from accurate FRET corrected efficiencies. There are four key correction factors that ALEX can be used to determine (Figure 3)[1]:

- Donor fluorescence leakage into the acceptor channel (α)
- Direct excitation of the acceptor by the donor laser (δ)
- Detection correction factor accounting for differences in detection efficiency between donor and acceptor channels (γ)
- Excitation correction factor reflecting the relative excitation efficiencies of the fluorophores (β)

By quantifying these parameters, ALEX not only distinguishes doubly labelled species from donor-only or acceptor-only species but also corrects for photophysical artefacts.

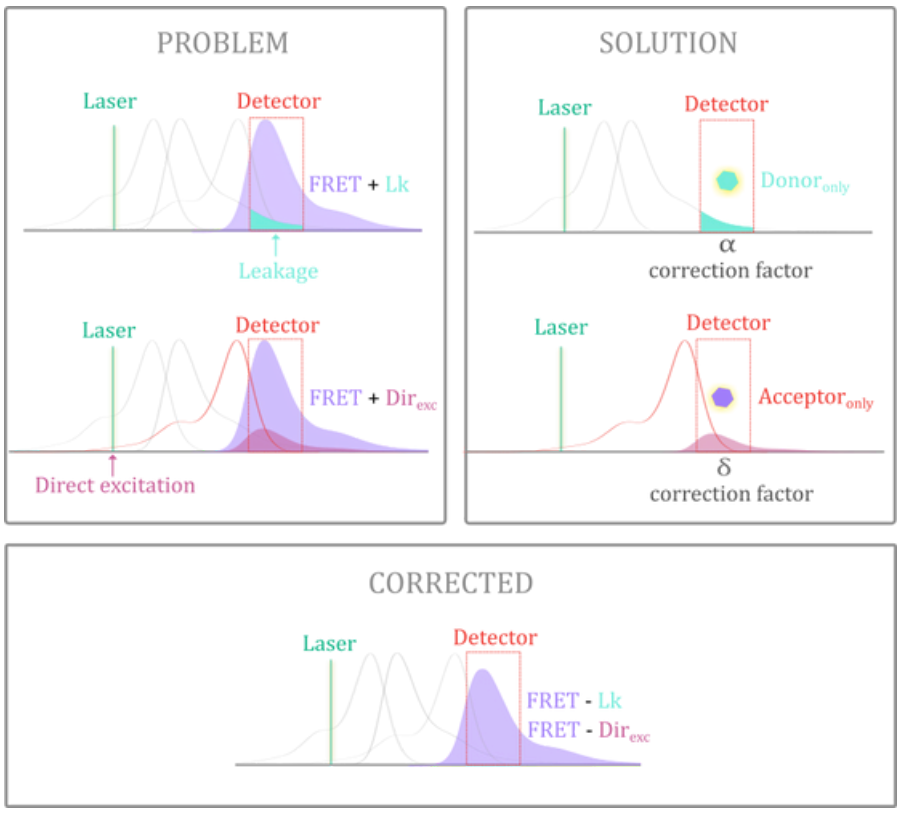

Figure 3 - Accurate FRET correction (α and δ)
Donor fluorescence leakage into the acceptor channel (α) and direct excitation of the acceptor by the donor laser (δ) can be corrected to ensure accurate FRET measurements

Performing accurate FRET correction

Leakage factor (α)

The leakage factor (α) is determined from donor-only bursts, which appear at high stoichiometry (S~1). Because no acceptor dye is present, these bursts should produce no photons in the acceptor channel, and the true FRET efficiency of these bursts should therefore be zero. Any photons detected in the acceptor channel under donor excitation must therefore originate from donor fluorescence leaking onto the acceptor detector. The apparent non-zero FRET values of donor-only bursts directly report this leakage. By measuring the mean E of this donor-only population, the leakage contribution can be quantified and used to correct all bursts.

Direct excitation factor (δ)

The direct excitation factor (δ) is determined from acceptor-only bursts, which appear at low stoichiometry (S~0). Because no donor dye is present, the photons detected under donor excitation should theoretically be zero, and the stoichiometry of these bursts should be zero. However, the stoichiometry of acceptor-only bursts often is not zero, so any photons arising under donor excitation must have been emitted by the acceptor, and their contribution can be isolated from the apparent stoichiometry of acceptor-only species; deviation from zero is taken as the direct excitation value.

Detection efficiency factor (γ) and excitation efficiency factor (β)

The detection efficiency factor (γ) and excitation efficiency factor (β) are dependent on the local/global fluorophore environment, quantum yields of the fluorophores and some instrument/hardware-specific parameters. They arise from an imbalance in how well the donor and acceptor dyes are excited and detected by the system.

The detection and excitation efficiency correction factors (γ and β) are calculated from doubly labelled FRET populations. ALEX enables isolation of those bursts which have an intermediate S value (~0.5) and variable E value (values between 0 and 1). In the theoretical ideally balanced case, the S value of doubly labelled species (irrespective of FRET efficiency) would be 0.5. Deviation from this value arises due to the imbalance in excitation and/or detection of the donor or acceptor dyes, which produces a systematic tilt of the doubly labelled populations in the E–S plot.

Performing accurate FRET correction

One method of resolving these factors involves determining the population centre via Gaussian fitting of individual E and S histograms. The extracted E and S population centres are then fitted to the equation shown below to obtain γ and β parameters for a particular dye-pair and set-up[1].

$$S = \frac{1}{1 + \beta\gamma + (1 - \gamma) \times \beta \times E}$$

Specifically, E is plotted against 1/S with a linear fit; γ and β are then determined from the slope and the intercept of the line, respectively. After applying the appropriate correction, both populations should align at S~0.5 on this graph (Figure 4).

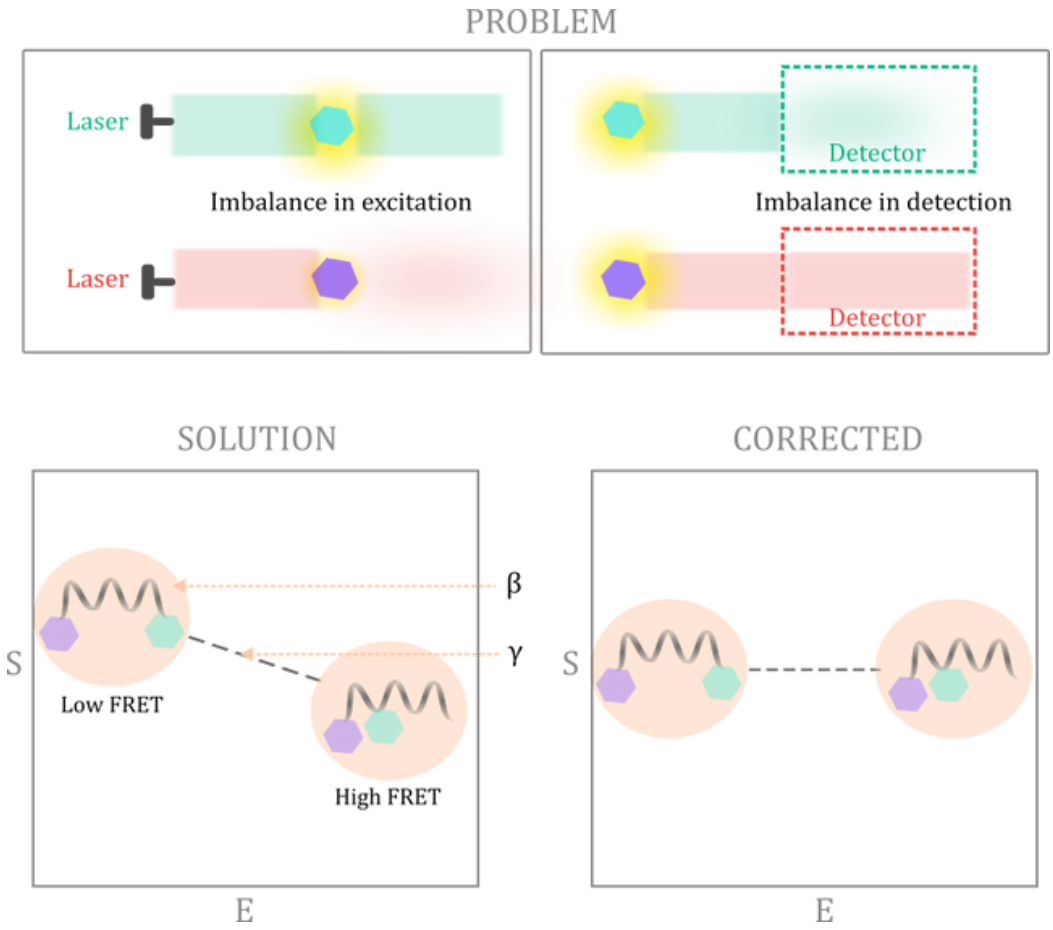

Figure 4 - Accurate FRET correction (γ and β)
Upper) Imbalances in excitation and emission detection require correction
Lower) FRET efficiency (E) plotted against stoichiometry (S) for low and high-FRET populations allows the values for γ (line slope) and β (line intercept) to be solved; application of these correction values aligns both populations at S~0.5.

Summary

Single-molecule sensitivity is achieved through the measurement of dilute labelled samples in confocal volumes, which ensures data is only collected on molecules that diffuse through the designated femtolitre volume. Accurate distance determination relies on the appropriate determination of four correction factors (α, δ, γ and β) and applying them to measured FRET efficiencies. ALEX can be used to calculate these correction factors, alongside identifying donor-only and acceptor-only populations. Together, these methods ensure that FRET efficiencies for doubly labelled species can be isolated from the rest of the signal for highly sensitive resolution of sub-populations, intermediate conformational states, and accurate determination of nanoscale distances between fluorophores.

For more information on the theory of smFRET and the capabilities of the EI-FLEX, see our technical notes page. Discover a range of applications for smFRET and the EI-FLEX platform on our website.

References

1. Lee, N. K. et al. Accurate FRET Measurements within Single Diffusing Biomolecules Using Alternating-Laser Excitation. Biophys J 88, 2939–2953 (2005).

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