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Linear spectral unmixing analysis in single-molecule FRET spectroscopy for fluorophores with large spectral overlap†

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Fluorescence resonance energy transfer (FRET) is a highly useful tool to investigate biomolecular interactions and dynamics in single-molecule spectroscopy and nanoscopy. However, the use of spectrally overlapping dye pairs results in various artifact signals that prevent accurate determination of FRET values. In this paper, an algorithmic method of spectral unmixing was devised to extract FRET values of spectrally overlapping dye pairs at the single molecule level. Application of this method allows the determination of both the donor–acceptor composition and the FRET efficiency of the samples labelled with spectrally overlapping dye pairs.

Introduction

Fluorescence resonance energy transfer (FRET)[1] has been widely used in single-molecule spectroscopy and microscopy to investigate biomolecular interactions and dynamics in the last few decades.[2–5] Since the FRET efficiency (E) strongly depends on the distance between the two fluorophores ($E \propto r^{-6}$),[6] precise measurement of inter-fluorophore distance in the 1 to 10 nm range is possible with fluorophores having a FRET radius in the nm range. Various methods have been developed for calculating E that involve measurements of the sensitized emission of the acceptor and the fluorescence emission or lifetime of the donor before and after acceptor bleaching.[7–10] The ratiometric intensity calculation that employs two channels, one for the donor emission and the other for the sensitized emission of the acceptor, stands out as the simplest method to determine E and has been widely used in FRET spectroscopy and microscopy.[7,11] However, when there exists spectral overlap in the absorption of the donor and the acceptor, direct (not sensitized) excitation of the acceptor is possible when the donor is intended to be excited. In addition, spectral overlap can cause the leakage of donor fluorescence into the acceptor-detecting channel. These factors introduce errors in FRET calculations,[12] prompting researchers to explore methods for correcting direct excitation and fluorescence leakage.[7,13]

In fluorescence microscopy, the challenge of fluorescence spectral overlap has been addressed through linear unmixing analysis.[14–16] Assuming that the total detection signal from all channels is a linear combination of signals from contributing fluorophores, signals from the donor and acceptor dyes can be distinguished using reference fluorescence spectral information.[7,17,18] Linear spectral unmixing is also effective in removing background signals, such as autofluorescence,[19,20] and has recently enabled 5-color super-resolution imaging based on single-molecule localization.[21] Successful application of linear unmixing in fluorescence microscopy suggests that it may also be applied to single-molecule spectroscopy in the presence of excessive spectral overlap.

One of the most versatile and powerful FRET methods is the alternating laser excitation FRET (ALEX-FRET)[22] technique that employs alternating laser pulses to separately excite the donor and the acceptor, which allows extraction of not only spatial information through the FRET efficiency (E) but also compositional information through the stoichiometry parameter (S) for the chemical ratio of the donor and acceptor dyes in the sample. Correction of direct acceptor excitation and fluorescence leakage from the donor-only sample has been implemented to improve the accuracy of the FRET efficiency,[23] but this correction strategy is restricted to FRET pairs with negligible spectral overlap, and therefore severe limitations still exist in fluorophore selection and multiplexed measurements.

This paper reports application of linear spectral unmixing analysis to single-molecule spectroscopy using a FRET dye pair with significant spectral overlap. We demonstrate simultaneous differentiation of different sample compositions (*i.e.*, donor-only, acceptor-only, and donor–acceptor pair) and measurement of accurate FRET efficiency using a simplified single-laser optical system.

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Experimental methods

Calculation of the FRET efficiency using linear unmixing

If a FRET pair happens to be a donor–acceptor dye pair exhibiting absorption spectral overlap, as illustrated in Fig. 1(a), both dyes can be simultaneously excited when a single excitation source is used. When a mixture of samples, either well-labelled with the donor–acceptor dye pair or mislabelled with either the donor or acceptor dye alone, are passed through the focal volume of the confocal ALEX-FRET optical system, fluorescence is emitted following alternating laser excitations. For the hypothetical fluorescence spectra shown in Fig. 1(b), we measure the fluorescence signal from the donor and acceptor dyes in two different wavelength regions, *i.e.*, detection channel 1 (ch1) and channel 2 (ch2). The average photon counts from the donor dye in ch1 and ch2 during the average diffusion time (τ) in the observation volume are respectively denoted as $R_{ch1,D}$ and $R_{ch2,D}$, while the same from the acceptor dye are likewise denoted as $R_{ch1,A}$ and $R_{ch2,A}$. These averaged photon counts are used to form a reference matrix for linear unmixing (R_{LU})

$$R_{LU} = \begin{pmatrix} R_{ch1,A} & R_{ch1,D} \\ R_{ch2,A} & R_{ch2,D} \end{pmatrix} \quad (1)$$

$$\begin{pmatrix} R_{ch1,A} & R_{ch1,D} \\ R_{ch2,A} & R_{ch2,D} \end{pmatrix} \begin{pmatrix} A \\ D \end{pmatrix} = \begin{pmatrix} P_{ch1} \\ P_{ch2} \end{pmatrix} \quad (2)$$

where P_{ch1} and P_{ch2} are photon counts detected in ch1 and ch2, respectively.

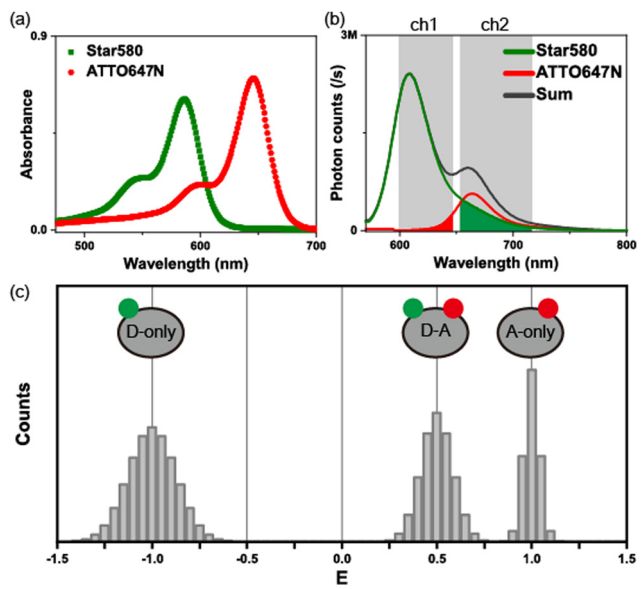

Fig. 1 (a) Absorption spectra of 5 μM Star580 and 5 μM ATTO647N. (b) Fluorescence spectra of 5 μM Star580 and 5 μM ATTO647N. Fluorescence signals are detected in two distinct channels (ch1 and ch2) of wavelengths. (c) Schematic histogram of E analyzed by linear unmixing matrix. In the case of γ= 1, the D-only sample is mainly located around E = −1, whereas the A-only sample is around E = 1. The D–A FRET pair is mainly found between E = 0 and E = 1 (*i.e.*, $0 < E < 1$).

From eqn (2), it is possible to determine the composition ratio between the acceptor (A) and donor (D) by multiplying the inverse of the linear unmixing matrix R_{LU} to the photon counts collected in each channel (P_{ch1} and P_{ch2}). When analyzing a photon burst from a D-only sample, the composition ratio would amount to D = 1 and A = 0. Likewise for an A-only sample, the composition ratio would be D = 0 and A = 1. However, due to the differing time taken for each molecule to pass through the focal volume, the photon counts of the bursts are proportional to t_D/τ (or t_A/τ), where t_D (or t_A) is the dwell time of the D (or A)-only molecule. As a result, for the D-only sample, D = t_D/τ, whereas A = t_A/τ for the A-only sample. For a D–A FRET pair, the composition ratio can be calculated from a burst, as in the following way:

$$D = \frac{t_D}{\tau}(1 - E) \quad (3)$$

$$A = \frac{t_A}{\tau}(1 + \gamma E) \quad (4)$$

In the case of the donor, the population of the excited donor is transferred as much as E to the acceptor, resulting in a reduction in the number of photons emitted from the donor. Therefore, in an ideal condition, D would be proportional to 1 − E as well as to t_D/τ due to the diffusion time, as represented by eqn (3). On the other hand, in the case of the acceptor, it can be either directly excited by light or a given population of the excited donor can be transferred to the acceptor, leading to the enhanced fluorescence represented by the two terms in eqn (4). Due to differences in the quantum yield and detection efficiency between the fluorophores, the increase in the acceptor fluorescence does not perfectly correspond to the decrease in the donor fluorescence, which is represented by the correction factor γ in eqn (4).[23–25] The experimental value of γ is determined from

$$\gamma = \frac{\langle A_{FRET} \rangle - \langle A_{acceptor} \rangle}{\langle D_{donor} \rangle - \langle D_{FRET} \rangle} \quad (5)$$

where $\langle A_{FRET} \rangle$ and $\langle A_{acceptor} \rangle$ are, respectively, the average acceptor fluorescence signal from the FRET pair and from the A-containing sample. Likewise, $\langle D_{FRET} \rangle$ and $\langle D_{donor} \rangle$ are the average donor fluorescence signal from the FRET pair and the D-containing sample, respectively.

When analysing a specific photon burst, t_D and t_A are the same, because the FRET pair labels the same single molecule. Therefore, from eqn (3) and (4), the FRET efficiency E can be expressed by

$$E = \frac{A - D}{A + \gamma D} \quad (6)$$

Since D-only, A-only, and D–A FRET pair would be distinctly located around $E = -1/\gamma$, E = 1, and $0 < E < 1$, respectively, molecular sorting is possible from the E distribution as shown in the schematic histogram of Fig. 1(c).

Sample preparation

To test the feasibility of linear spectral unmixing analysis, the FRET-pair chosen for experimentation in Fig. 1(a) and (b) comprises Star580 and ATTO647N, known for their substantial fluorescence leakage and direct excitation issues. Despite the large spectral overlap between the two dyes, the Förster radius measures 6.2 nm, comparable to that of other commonly used FRET pairs.[7,11] Five distinct double-stranded DNA (dsDNA) configurations were devised, as depicted in Fig. S1 (ESI†), each representing a specific category: A-only, D-only, low D–A FRET pair (LF), mid D–A FRET pair (MF), and high D–A FRET pair (HF). Since one complete turn of the DNA double helix is 3.4-nm long for 10 base pairs, the distance from the donor to the acceptor is 4.76 nm, 6.46 nm, and 8.16 nm for HF (14 bp), MF (19 bp), and LF (24 bp), respectively.

Experimental setup

An experimental setup was constructed to assess FRET measurements using linear spectral unmixing with a confocal microscope. Initially, a 592 nm fibre laser was employed as the excitation source to simultaneously excite both the donor and acceptor dyes, as illustrated in Fig. 1(a). Additional assessment was carried out across various excitation wavelength ranges by filtering white light with excitation filters. For detection, the proportion of leakage signals was fine-tuned using different types of dichroic mirrors. To assess the feasibility of linear unmixing analysis, an ALEX-FRET experiment was also performed by incorporating an additional light source into the same optical system. The detailed setup is shown in Fig. S2 (ESI†).

Determination of the linear unmixing matrix

The linear unmixing matrix was constructed using photon counts detected in ch1 and ch2 from a 5 nM dye solution for each dye with 150 μW 592 nm excitation in the buffer solution with a composition of 10 mM Tris pH 8.0, 50 mM NaCl, 1 mM Trolox, 5% v/v glycerol, and 20 μg ml^{-1} BSA, which remained identical in the single-molecule experiments. For the D-only sample, 30% (=130/(307 + 130)) of fluorescence leaked into ch2, as shown in Fig. 2(a). When the concentration was lowered to 40 pM to satisfy the condition for single molecule detection, the D-only sample showed sudden photon bursts typical of single-molecule signals, and their leakage fraction into ch2 was 29% on average, which is nearly the same as the leakage fraction at 5 nM. On the other hand, direct excitation of the A-only sample under the same excitation conditions was 34% (=(136 + 12)/(307 + 130)) of the total emission of the D-only sample, with a weak leakage (8% = 12/(12 + 136)) into ch1 at 5 nM as well as 40 pM (Fig. 2(b)).

The dwell time of a dye-labelled dsDNA molecule in the 1 fL observation volume was estimated to be 0.5 ms by fluorescence correlation spectroscopy analysis in Fig. S3(a) (ESI†), and the average number of molecules in the observation volume is 3 at 5 nM. Since the photon counts are proportional to the concentration of the sample, as shown in Fig. S3(b) (ESI†), the detected photon counts in ch1 and ch2 at 5 nM are reduced to the number of molecules dwelling for 0.5 ms, which yields the reference matrix for linear unmixing, R_{LU}.

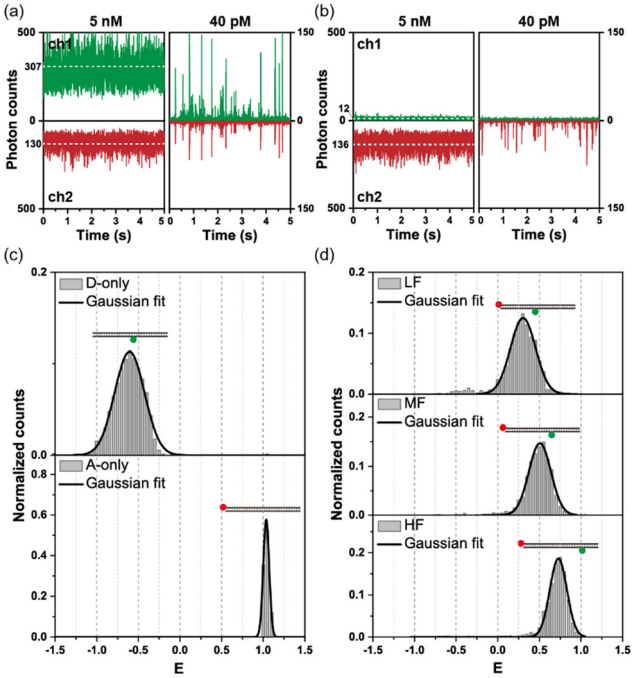

Fig. 2 The photon time traces detected in ch1 and ch2 of 5 nM and 40 pM: (a) D-only and (b) A-only samples. The binning time of each trace is 0.5 ms. Average photon counts of each sample are shown by white dotted lines. (c) and (d) *E* histograms for the D-only, A-only, and LF, MF, and HF samples. All histograms are normalized by the sum of the frequency counts. Black lines are Gaussian fits.

Results and discussion

Comparison of FRET efficiency *E* measured by linear spectral unmixing *vs.* ALEX-FRET

Under the single-molecule condition (40 pM), the photon bursts from each of the A-only, D-only, LF, MF, and HF samples were analysed using R_{LU}. They were clearly differentiated in the *E* histogram, with all distinctive *E* values, as shown in Fig. 2(c) and (d), and Table 1. We conducted a *Z*-test to determine whether each sample group was distinct from one another

Table 1 *E* values for the D-only, A-only, and HF, MF, and LF samples with comparison against ALEX-FRET results. The *E* value was taken from the central position of the Gaussian distribution, whose standard deviation σ is determined from Fig. 2(c), (d) and Fig. S6(a), (b) (ESI)

	E, (mean ± 1σ)		
	Linear unmixing under 592 nm excitation	532 nm/635 nm ALEX-FRET	592 nm/635 nm ALEX-FRET
D-only	−0.60 ± 0.17	—	—
A-only	1.04 ± 0.04	—	—
D–A, high FRET	0.73 ± 0.10	0.75 ± 0.12	0.57 ± 0.14
D–A, mid FRET	0.51 ± 0.13	0.50 ± 0.16	0.35 ± 0.18
D–A, low FRET	0.30 ± 0.15	0.26 ± 0.17	0.18 ± 0.18

and found that there was a statistically significant difference between LF, MF, HF, A-only, and D-only samples, with a p-value of less than 0.05. The E values of the LF, MF, and HF samples are nearly identical to those obtained by ALEX-FRET using 532 nm/635 nm that features correction of direct excitation and fluorescence leakage (Table 1, Fig. 3(a) and Fig. S4 (ESI†)). On the other hand, when a set of different excitation wavelengths was chosen (592 nm/635 nm) for ALEX-FRET, inaccurate FRET values were obtained due to significant direct excitation and fluorescence leakage (Fig. 3(b)). These results underscore the clear advantage of the linear unmixing method, demonstrating its capability to successfully perform FRET and stoichiometric analysis just like ALEX-FRET, even when ALEX-FRET fails due to pronounced spectral overlap between the FRET pair.

Reproducibility under different optical conditions

To demonstrate its reproducibility, linear unmixing analysis was conducted across various optical conditions, including different signal ratios in ch1 and ch2 or varied excitation light sources. The wavelength ranges detected in ch1 and ch2 were adjusted by replacing a dichroic mirror in the detector, as shown in Fig. S5(a) (ESI†). This resulted in considerably different matrix elements for the linear unmixing matrix, as shown in Fig. S5(b) and (c) (ESI†). However, this led to no significant alteration in the FRET value, as demonstrated in Fig. 4(a)–(h) and Table S1 (ESI†).

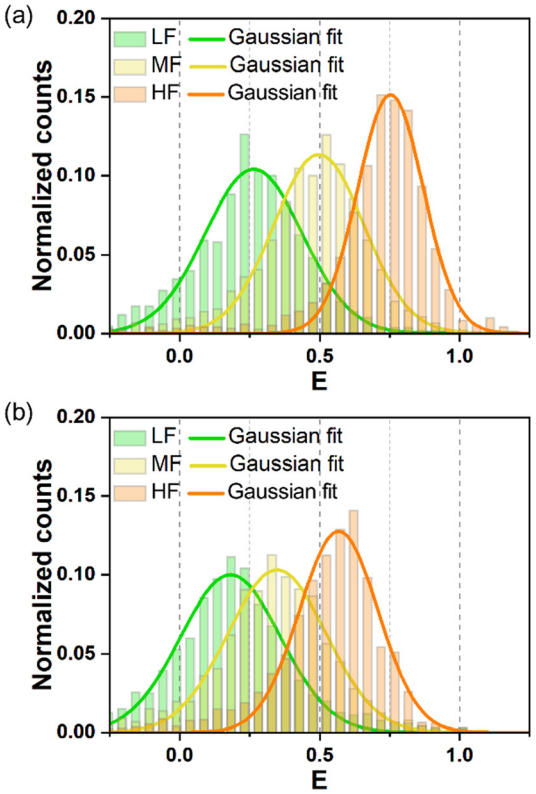

Fig. 3 The E histograms of the LF, MF, and HF samples obtained on the setup of 532 nm/635 nm ALEX-FRET (a) and 592 nm/635 nm ALEX-FRET (b).

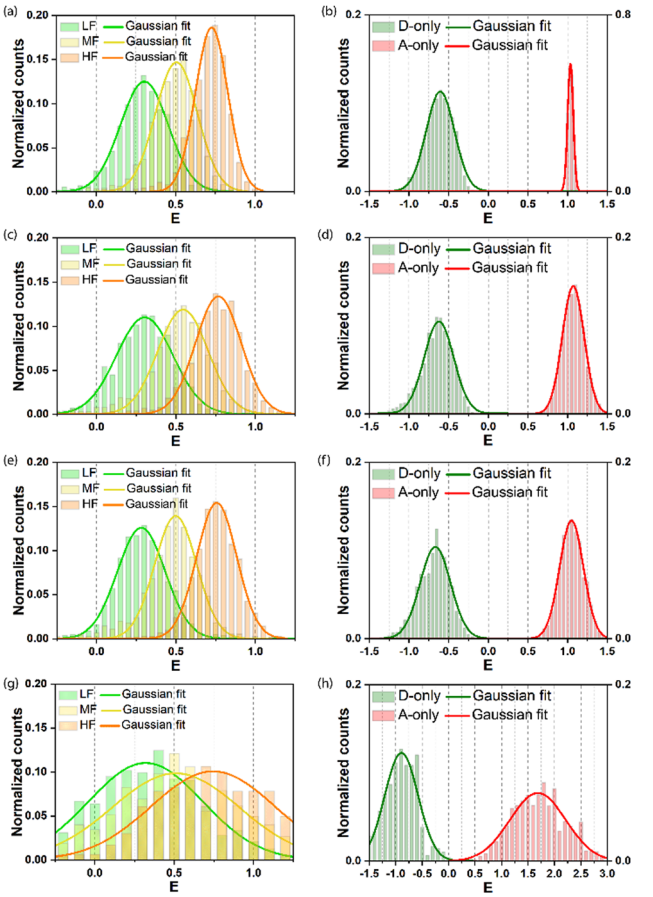

Fig. 4 The E histograms of LF, MF, HF, D-only, and A-only samples obtained by linear spectral unmixing with zt633rdc (a) and (b), zt647rdc (c) and (d), ff660 (e) and (f), and ff685 (g) and (h).

In the extreme case where the measurement wavelength range is shifted completely out of scale, however, both majority signals of the donor and acceptor are measured in ch1, and the FRET efficiency distribution becomes wider and the prediction accuracy suffers, as illustrated in Fig. 4(g) and (h) and Table S1 (ESI†).

We note that linear spectral unmixing yields consistent results across various excitation wavelengths, as illustrated in Fig. 5 and Table S2 (ESI†). When using a light source between 580 nm to 600 nm range, both the donor and acceptor are adequately excited and the photon bursts are sufficient to accurately determine the FRET efficiency of LF, MF, and HF.

With irradiation below 570 nm, however, there was insufficient fluorescence signal from both the donor and acceptor in the actual single-molecule experiment, although we were still able to obtain the matrix elements of the unmixing matrix, as shown in Fig. S6 (ESI†).

When the diffusion speed of the molecule is reduced, as in the case of a heavy molecule, the dwell time of a single molecule in the focal volume increases. This prolonged residence facilitates the acquisition of a sufficient fluorescence

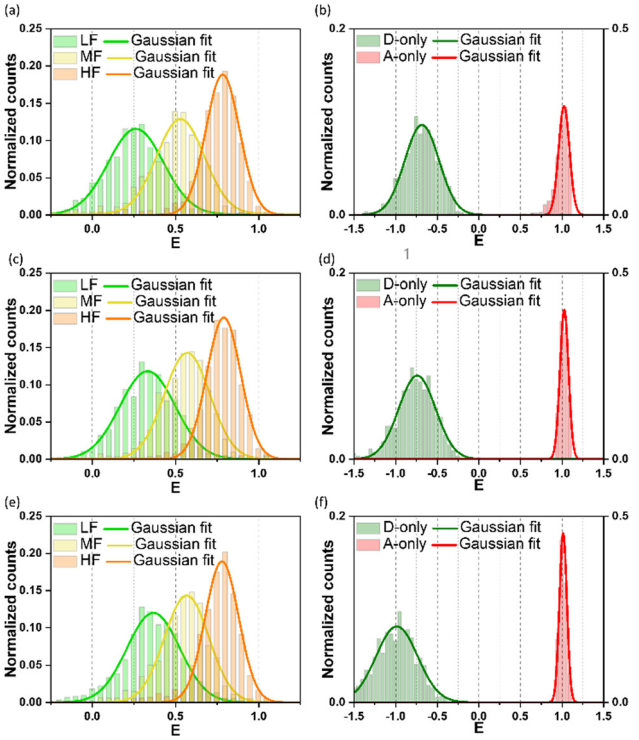

Fig. 5 The *E* histograms of LF, MF, HF, D-only, and A-only samples upon 580 nm (a) and (b), 591 nm (c) and (d), and 600 nm excitation (e) and (f).

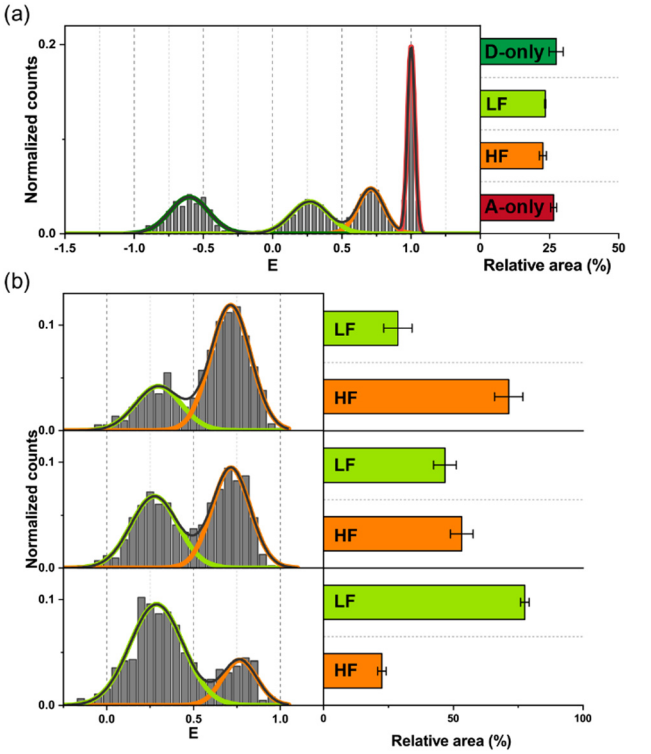

Fig. 6 (a) The *E* histogram for a mixture of D-only, A-only, HF, and LF samples of 10 pM each, constructed with γ = 1.84 using four Gaussian fits for the D-only (green), A-only (red), LF (yellow green) and HF (orange) samples. (b) *E* histograms of 1 : 3 (10 pM LF, 30 pM HF), 2 : 2 (20 pM LF, 20 pM HF) and 3 : 1 (30 pM LF, 10 pM HF) mixture samples constructed with γ = 1.84 using two Gaussian fits for the LF (yellow green) and HF (orange) samples. Black lines are the sum of Gaussian fits. The relative area under each Gaussian function is shown on the right side of the *E* histogram. The error bars of the relative areas were calculated using the standard deviations of the three experiments.

signal, providing a more favourable condition for the application of linear unmixing analysis.

We note that, in our *Z*-test on the *E* values obtained under different excitation conditions while keeping the sample conditions constant, there was a greater variance in the *p*-value (below and above 0.05). Although achieving identical FRET efficiencies can be challenging under different excitation conditions, linear spectral unmixing appears to give reliable results for quantitative prediction of the FRET distribution under the same excitation conditions.

Quantification of mixture compositions

To examine whether linear spectral unmixing can accurately measure quantitative populations of a mixed sample, mixtures with different compositions were analysed. The 1 : 1 : 1 : 1 mixture of the A-only, D-only, LF, and HF species exhibits four distinct population groups, as depicted in Fig. 6(a). Gaussian function fitting yielded peak area ratios for the A-only, D-only, LF, and HF species (representing the concentration ratio of each species) of approximately 1.1 : 1.1 : 0.9 : 0.9. We note that there are more D-only and A-only samples than the two FRET pairs, LF and HF. This is presumably because, when annealing ssDNA samples, a small amount of ssDNA labeled with a donor or an acceptor molecule persists without forming dsDNA.

We also ran another composition test on three different mixtures of HF and LF in the ratios of 3 : 1, 2 : 2, and 1 : 3, for which we obtained population ratios of 77 : 23, 47 : 53, and 29 : 71, respectively, as illustrated in Fig. 6(b) and Table S3 (ESI†).

Conclusions

We demonstrated the feasibility of linear spectral unmixing analysis in distinguishing fluorescence signals from the donor and acceptor at the single-molecule level, particularly when there exists significant spectral overlap. As with ALEX-FRET, accurate estimation of the FRET efficiency and sorting of the sample compositions were simultaneously achieved even when ALEX-FRET fails due to the spectral overlap. Quantitative analysis of populations for multi-species mixtures was shown to work as well. As the spectral overlap issue has been recognised as a major hindrance to expanding the existing single-molecule spectroscopic capabilities, we note that the linear spectral unmixing method may open doors to versatile multiplexing and multi-colour analysis for single-molecule FRET spectroscopy.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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