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Supplementary Information

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

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Sample Preparation

Four types of ssDNA were custom-designed and purchased from IDT, as illustrated in Fig. S1. The amino modifier is positioned at the 5' end or an internal site of the ssDNA. Star580 (Abberior) and ATTO647N (Atto-Tec GmbH) were chosen as the donor and acceptor fluorophores, respectively, due to their large spectral overlap shown in Fig. 1(a) and (b). The Förster distance, R_0, for the Star580-ATTO647N pair was calculated as 6.2 nm using the following equation.

$$R_0 = 0.211(\kappa^2 n^{-4} \Phi_D J)^{\frac{1}{6}}, \qquad J = \varepsilon_A \frac{\int_0^\infty f_D(\lambda) f_A(\lambda) \lambda^4 d\lambda}{\int_0^\infty f_D(\lambda)\, d\lambda}$$

, where κ^2 is the orientation factor between the dipoles of the donor emission and the acceptor absorption, with its average value being 2/3 in the dynamic isotropic limit. n is the refractive index of the medium and Φ_D is the quantum yield of the donor. J is the overlap integral between the donor emission spectrum ($f_D(\lambda)$) and acceptor absorption spectrum ($f_A(\lambda)$) in the unit of $M^{-1}\,cm^{-1}\,nm^4$. ε_A is the extinction coefficient of the acceptor at its peak absorption wavelength in the unit of $M^{-1}\,cm^{-1}$. The quantum yield of the donor, emission spectrum of the donor, and absorption spectrum of the acceptor were measured under the same buffer conditions as our single-molecule experiment. The labeling reaction with Star580 and ATTO647N was conducted in a sodium borate buffer at pH 8.3 at room temperature for 3 hours. Labeled ssDNA strands were purified through ethanol precipitation and high-performance liquid chromatography (1100 series, Agilent Technologies) using a C18 reverse-phase column (RPC C2/C18 ST 4.6/100, GE Healthcare). Subsequently, ssDNA samples were annealed with their complementary ssDNA samples while being cooled from 90 ºC to room temperature. Finally, dsDNA samples representing donor only (D-only), acceptor only (A-only), low FRET (LF), mid FRET (MF), and high FRET (HF) cases were prepared as illustrated in Fig. S1.

Experimental Setup

The setup for sorting single molecules using the linear spectral unmixing method is based on a confocal microscope, as depicted in Fig. S2. A 592 nm fibre laser (MPB communications) served as the excitation light source, chosen for its compatibility with both the donor and acceptor dyes that absorb light at 592 nm. An 80-MHz femtosecond laser generated excitation wavelengths ranging from 532 to 600 nm using a photonic crystal fibre (FemtoWhite) and excitation filters (FB600-10, FF01-591/6, FB580-10, FB570-10 FB550-10, FB532-10). A dichroic mirror (590dcrb, Chroma, or 593lp, Chroma) positioned in front of the sample stage reflected the excitation beam, which was then focused by the objective lens (UplanApo 60x/1.20w, Olympus). To effectively excite randomly oriented fluorophores, the excitation beam was circularly polarized using half-and quarter-wave plates (RAC 3.4.15, RAC 3.2.15, Bernhard Halle Nachfl.). Discrimination of fluorescence signals between the donor and acceptor dyes was achieved through a dichroic mirror (zt633rdc, Chroma), with fluorescence above 633 nm transmitted through the dichroic and that below 633 nm reflected. Various dichroic mirrors (zt647rdc, Chroma, ff660, Semrock, and ff685, Semrock) were also employed. A lens (f = 300, Linos) was used to focus the fluorescence signal onto apertures of multimode fibres. These multimode fibres (Ø105 µm, 0.22 NA, FC/PC-FC/PC Fiber Patch Cable 1 m, Thorlabs) conveyed fluorescence signals to each APD (SPCM-AQR-14-FC, Perkin Elmer). A long-pass filter (FF01-593/LP-25, Semrock) blocked the 592 nm light and transmitted the fluorescence signal. Raw data were acquired using a LabVIEW program (NI6602, National Instruments) and analyzed with MATLAB. Fluorescence correlation spectroscopy (FCS) data were obtained with the correlator (Flex02-01D, Correlator.com).

The ALEX-FRET experiment was performed using two light sources on the aforementioned setup. For the 532 nm/635 nm ALEX-FRET configuration, a 532 nm laser (Cobalt Samba™, Cobolt) and a 635 nm laser (TECRL-25G-635-TTL,

World Star Tech) were employed to excite the donor and acceptor dyes, respectively. Similarly, in the 592 nm/635 nm ALEX-FRET experiment, a 592 nm fibre laser and a 635 nm laser were used to excite the donor and acceptor dyes, respectively. An acousto-optic tunable filter (AOTFnC-VIS, AA Opto-Electronic) alternated between 532 nm (or 592 nm) and 635 nm laser excitations. A dichroic mirror (zt532/635rdc, Chroma) situated in front of the sample stage reflected both laser beams. In front of the APDs, another dichroic mirror (zt633rdc, Chroma) divided the wavelength detection range into those below 633 nm and above 633 nm. Bandpass filters (ET655LP, Chroma, and FF01-582-75, Semrock) were utilized to filter fluorescence.

FCS Data Analysis

The diffusion time of a freely diffusing dsDNA pair passing through the focal volume was determined through FCS analysis. For calibration, we employed the conventional organic dye Cy5 in a 10 mM Tris pH 8.0 and 50 mM NaCl buffer. The diffusion coefficient of Cy5 has been extensively studied and is well known ($D = 3.7\times10^{-6}$ cm^2/s).[1] FCS experiment was carried out with ATTO647N-labeled dsDNA in 10 mM Tris pH 8.0, 50 mM NaCl, 1 mM Trolox, and 5% v/v glycerol, which is identical to the buffer condition of our single-molecule experiment. The diffusion time was estimated using the autocorrelation function below, taking into account triplet state kinetics.[2]

$$G(t) = \left[\frac{1}{N}(1+\frac{t}{\tau})^{-1}(1+\frac{t}{k^2\tau})^{-\frac{1}{2}}\right]\cdot\left[1+\frac{F_T}{1-F_T}\exp\left(-\frac{t}{\tau_T}\right)\right]$$

Here, N, τ, and k represent the average number of molecules in the observation volume, the diffusion time of the fluorescent molecule, and the aspect ratio of the observation volume, respectively. F_T and τ_T denote the fraction of molecules in the triplet state and the decay time from the triplet state, respectively. As depicted in Fig. S3 (a), the diffusion time of ATTO647N-labeled dsDNA is determined to be 0.5 ms.

Measurement and data analysis for linear unmixing method and ALEX-FRET

In the linear spectral unmixing method, samples were exposed to a 150 µW 592 nm laser, and data were acquired for 1000 s, which yields signal traces exhibiting photon bursts. Burst selection involved choosing photon bursts with a sum of photons measured in ch1 and ch2 equal to or greater than 20, which were then multiplied by the inverse of the reference matrix to calculate the compositions of *D* and *A*. Subsequently, bursts with *A*-composition, *D*-composition, or the sum of them exceeding 1.2 were further selected to ensure accurate calculation results. The γ value and *E* histograms were derived from the burst dataset.

In ALEX-FRET measurements, the donor and acceptor were alternatively excited using 240 µW 532 nm (or 100 µW 592 nm) and 21 µW 635 nm (or 60 µW 635 nm) lights. The FRET efficiency *E* and stoichiometry parameter *S* were simultaneously obtained from the photon counts $F_X{}^Y$, the fluorescence of *Y* when *X* is excited (*X*, *Y* = *A* or *D*) from the following equations:

$$E = F_D^A / (F_D^A + \gamma F_D^D)$$
$$S = F_D^A + \gamma F_D^D / (F_D^A + \gamma F_D^D + F_A^A + F_A^D).$$

Here, the detection correction factor, γ, is defined as ($\Phi_A\eta_A/\Phi_D\eta_D$), where Φ is the quantum yield of the fluorophore and η is the detection efficiency of the photodetector. All data presented in this paper were corrected

for direct excitation and γ correction was also applied, following the approach outlined in the work of N. Lee et al.[3]

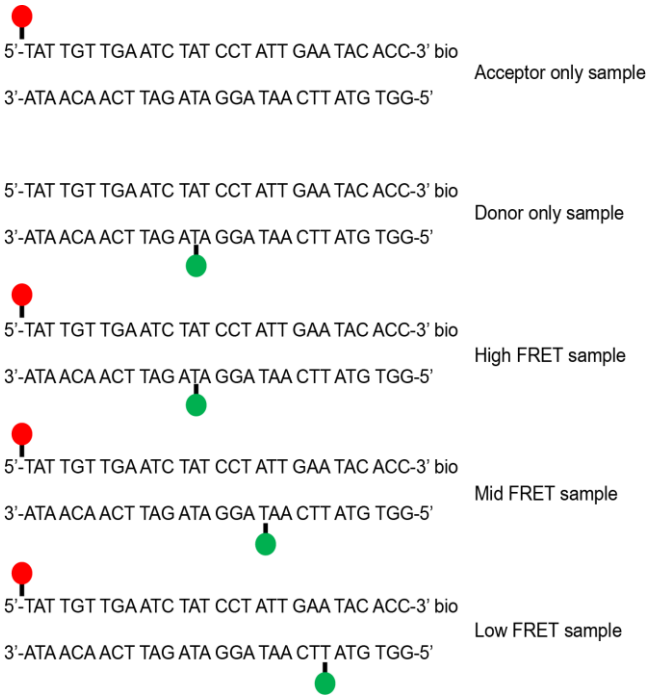

Figure S1 Five types of dsDNA samples used in this experiment. The top two types of dsDNA samples represent *A*-only and *D*-only cases, while the bottom three types represent high, mid, and low FRET *D*-*A* pair, respectively, according to their relative distance between *D* and *A*.

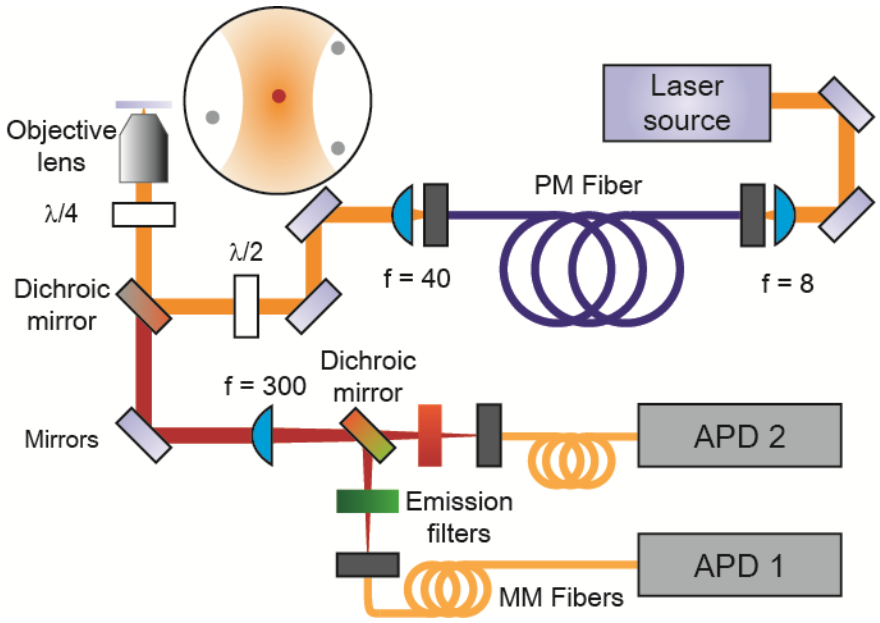

Figure S2 Schematic illustration of the confocal setup for linear spectral unmixing method and ALEX-FRET experiment.

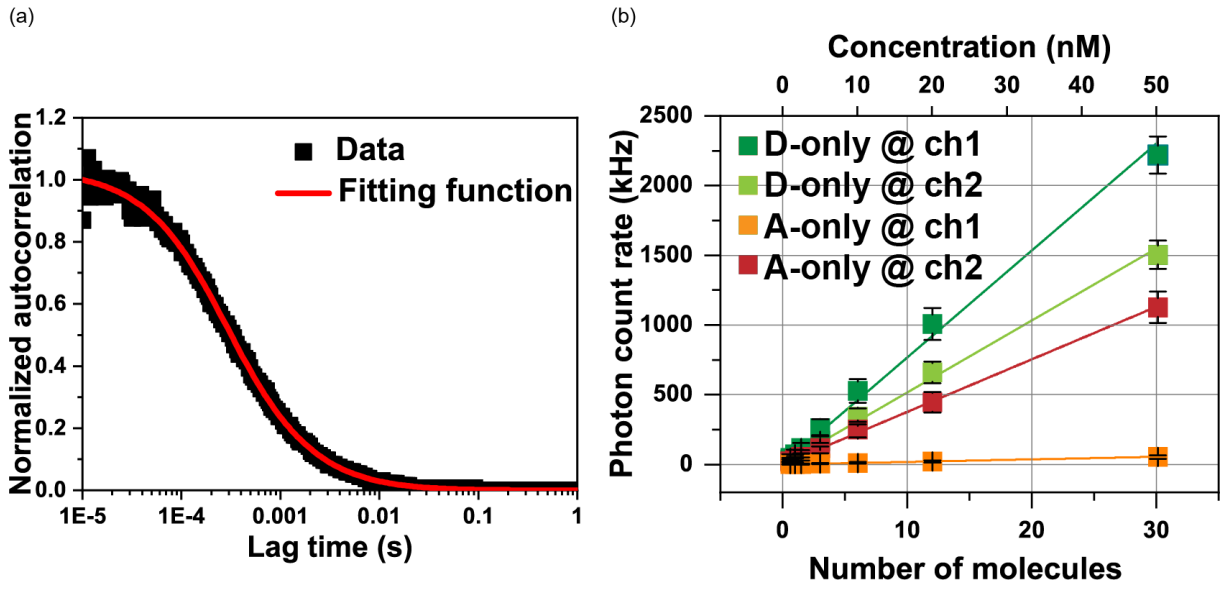

Figure S3 (a) Autocorrelation function from FCS measurements of Atto647N-labeled dsDNA under the same buffer conditions as with single-molecule experiment. The diffusion time τ is 0.5 ms. (b) The photon count rate of channel 1 (615~645 nm) and channel 2 (> 655 nm) under 100 µW 592 nm excitation vs. sample concentration (or the average number of molecules in the focal volume).

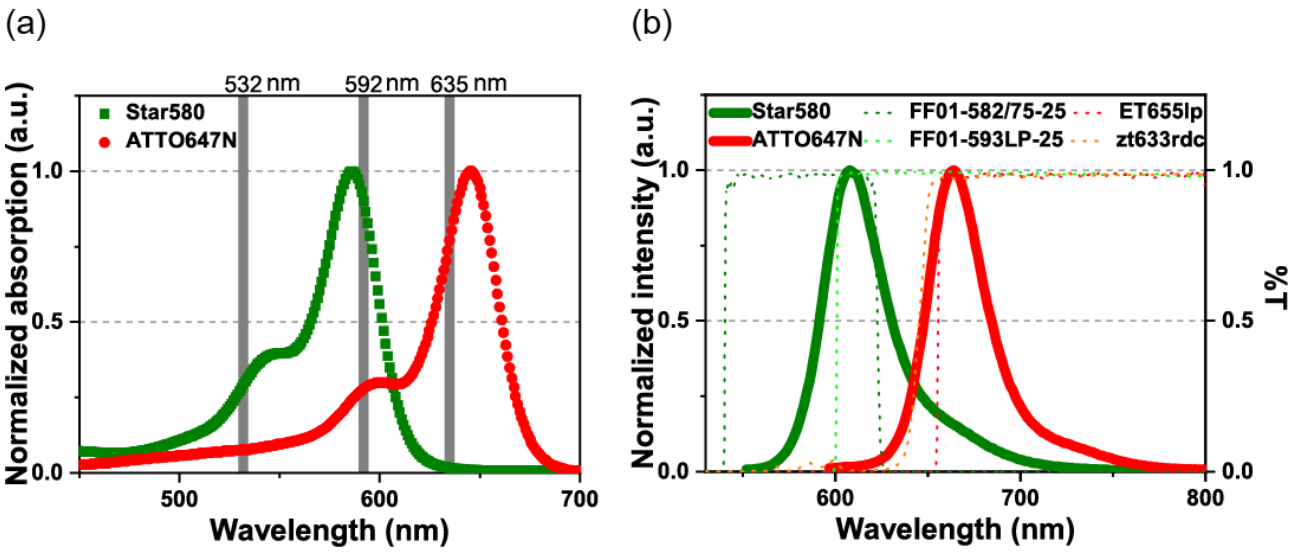

Figure S4 (a) The absorption spectra of Star580 (donor dye) and ATTO647N (acceptor dye) (b) The fluorescence spectra of the donor and the acceptor dyes. The filter set and the dichroic mirror are aslo illustrated. The dichroic mirror zt 633rdc divided detected wavalength range into channel 1 and channel 2. In the ALEX-FRET experiment using 532 nm/635nm lights, the 532 nm light mainly excited the donor, while the 635 nm light excited the acceptor. The direct excitation coefficient and the leakage coefficient were both 0.24. In the ALEX-FRET experiment using 592 nm/635 nm lights, the 592 nm light mainly excited the donor, and FF01-593LP-25 filter was additionally used to block the scattering of the 592 nm light. The direct excitation coefficient and the leakage coefficient were both 0.45.

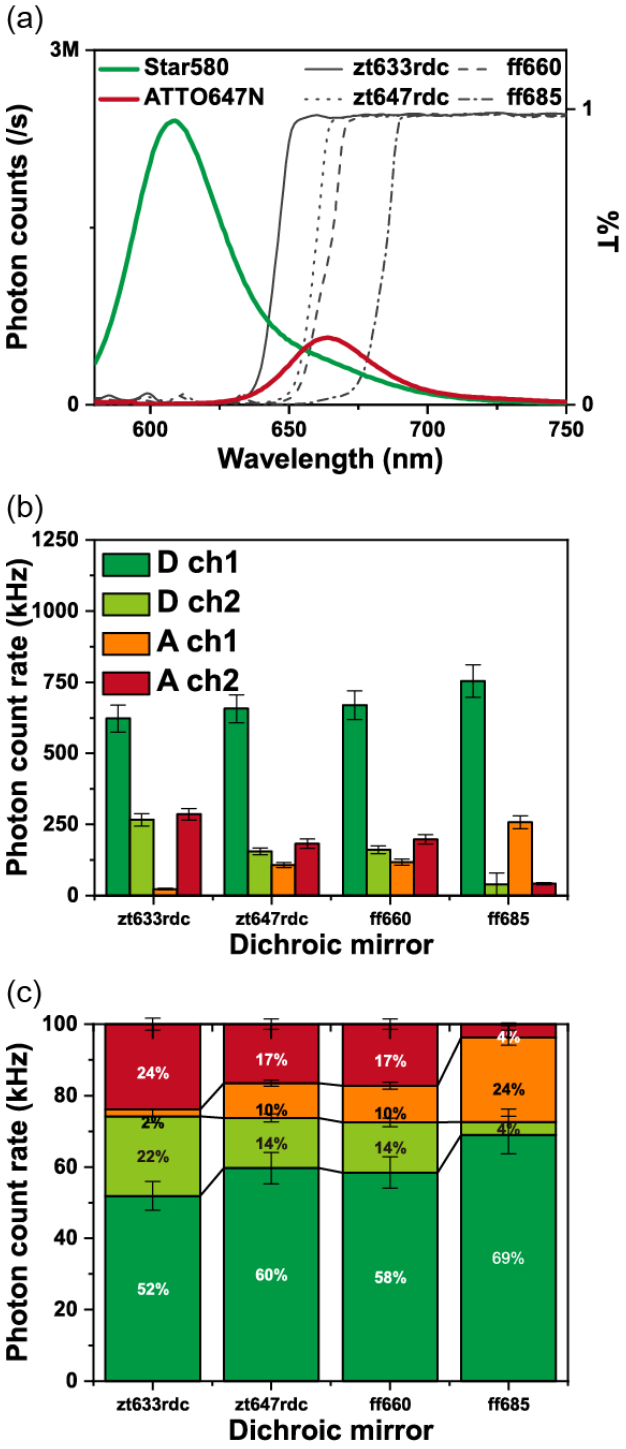

Figure S5 (a) Fluorescence spectra of Star580 and ATTO647N shown against % transmittance (% T) of dichroic mirrors. (b) The detected photon count rates of 5 nM *D*-only (*D*) and *A*-only (*A*) samples collected in ch1 and ch2. (c) 100% stacked columns of the detected photon count rates of Fig. S5(b).

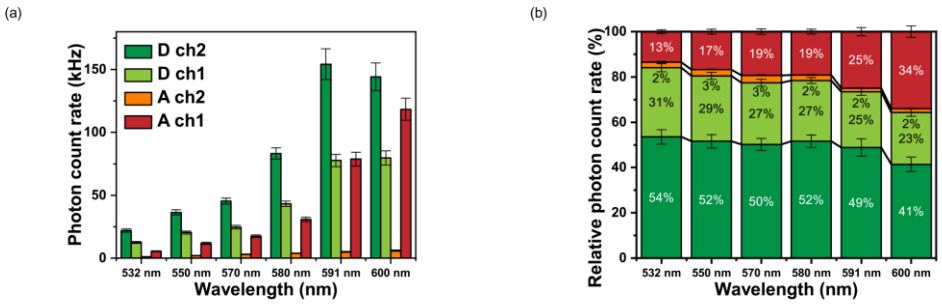

Figure S6 (a) Photon count rates of 5 nM *D*-only (*D*) and *A*-only (*A*) collected in ch1 and ch2 upon laser excitation over the range of 532 to 600 nm. (b) 100% stacked columns of the photon count rates of Fig. S6(a).

Table S1 FRET efficiency of the LF, MF, and HF samples for various dichroic mirrors. The gamma correction factor has slightly different values depending on dichroic mirrors. The center position of the Gaussian fitting function was taken to be the FRET efficiency, and σ is the standard deviation of the Gaussian fitting function.

	γ	***E*, mean±1σ**		
		LF	MF	HF
zt647rdc	1.79	0.30 ± 0.17	0.54 ± 0.16	0.77 ± 0.14
FF660	1.75	0.28 ± 0.15	0.50 ± 0.13	0.76 ± 0.13
FF685	1.93	0.32 ± 0.36	0.51 ± 0.40	0.74 ± 0.39

Table S2 FRET efficiency of the LF, MF, and HF samples at various excitation wavelengths. The gamma correction factor has different values depending on dichroic mirrors. The center position of the Gaussian fitting function was taken to be the FRET efficiency, and σ is the standard deviation of the Gaussian fitting function.

Excitation wavelength (nm)	γ	***E*, mean±1σ**		
		LF	MF	HF
580	1.89	0.26 ± 0.17	0.53 ± 0.14	0.78 ± 0.10
591	1.70	0.33 ± 0.17	0.57 ± 0.14	0.79 ± 0.10
600	1.31	0.37 ± 0.16	0.57 ± 0.13	0.78 ± 0.10

Table S3 Relative areas of Gaussian fitting functions in Fig. 3(b), which presents the relative concentration of different species (HF and LF in this case).

	30 pM LF 10 pM HF		20 pM LF 20 pM HF		10 pM LF 30 pM HF	
Relative Area (%)	77	23	47	53	29	71
Relative Area (%)*0.4	31	9	19	21	12	28

Reference

1. A. Loman, T. Dertinger, F. Koberling and J. Enderlein, Chem Phys Lett, 2008, 459, 18.
2. J. Widengren, U. Mets and R. Rigler, J Phys Chem-Us, 1995, 99, 13368.
3. N.K. Lee, A.N. Kapanidis, Y. Wang, X. Michalet, J. Mukhopadhyay, R.H. Ebright and S. Weiss, Biophys. J., 2005, 88, 2939.