

Supplementary Information

Incorporation of STED technique into single-molecule spectroscopy to break the concentration limit of diffusing molecules in single-molecule detection

Namdoo Kim,^{†a} Jiwoong Kwon,^{†b} Youngbin Lim,^b Jooyoun Kang,^a Sohyeon Bae^a and Seong Keun Kim^{*ab}

^a Department of Chemistry, Seoul National University, Seoul 08826, Korea.

^b Department of Biophysics and Chemical Biology, Seoul National University, Seoul 08826, Korea.

[†] These authors equally contributed to this work.

* Correspondence and requests for materials should be addressed to S.K.K. (seongkim@snu.ac.kr)

Experimental Setup

Our ALFRED system is schematically shown in Fig. S1. A super-continuum light was generated from the 780 nm output of a Ti:Sapphire laser (<100 fs, 80 MHz, Mai Tai HP, Spectra-Physics) by a photonic crystal fiber (FemtoWhite 800, NKT Photonics). An acousto-optic tunable filter (AOTF_{nC-VIS}, AA Opto-Electronic) was used to alternately pass the two excitation beams (635 nm and 510 nm) with a 50 μ s alternation period. Possible temporal cross-talks of the two excitation lasers were eliminated by fixing the on-time of the AOTF to 46 μ s with a 4- μ s off-time. The 780-nm output of the Ti: Sapphire laser was passed through two glass rods and stretched to \sim 5 ps, and further stretched to \sim 280 ps by a 100-m long polarization-maintaining single mode fiber (PMJ-A3HPC, 3S-633-4/125-3-100-1-SP, OZ Optics). The Gaussian-shaped STED beam underwent phase modulation by a phase mask (VPP-1a, RPC Photonics) and became a doughnut-shaped beam at the focal plane. The three beams were circularly polarized by half- and quarter-wave plates (RAC 3.4.15, RAC 3.2.15 and RAC 4.2.15, Bernhard Halle Nachfl.) to effectively excite and deplete the fluorescence from randomly oriented fluorophores. The STED beam was fired 160 ps after the excitation beam to achieve the maximum depletion efficiency, while co-localization between the two beams was monitored by collecting scattered light from an Au bead with an 80-nm diameter. Fluorescence signal from the sample was collected by an oil immersion type objective (PL APO, 100X, 1.4 NA, Leica) through a home-built inverted confocal fluorescence microscope and detected by two perpendicularly-lying avalanche photodiode detectors (SPCM-AQR-14, Perkin Elmer). Fluorescence from the fluorophores were separated by a dichroic mirror (630dcxr, Chroma) and further filtered by an emission filter (DY510XL: FF01-582-75-25, Semrock; Atto647N: ET655LP, Chroma). The intense STED beam was blocked by a short-pass filter (FF01-720/SP-25, Semrock). Fluorescence correlation spectroscopy (FCS) data was obtained with the correlator (Flex02-01D, Correlator.com), and the raw data was analyzed with LabVIEW program (National Instruments). Fitting of the FCS curve with the autocorrelation function is described later.

Sample Preparation

30-mers of single stranded poly-A and poly-T DNA oligomers were purchased from Integrated DNA Technologies, whose bases at the end were amino-modified for fluorophore labeling (5'-modified poly-T for DY510XL; 5'-modified poly-A for Atto647N (low-FRET); 3'-modified poly-A for Atto647N (high-FRET)). DY510XL (510XL-01, Dyomics GmbH) and Atto647N (AD647N-31, Atto-Tec GmbH) were labeled at the end of oligomers via simple click reaction in sodium borate buffer (pH 8.3, 2 h, room temperature). Unreacted oligomers and fluorophores were removed by high performance liquid chromatography (1100 series, Agilent Technologies) using the C18 reverse phase column (RPC C2/C18 ST 4.6/100, GE Healthcare). Each labeled single strand was annealed with its complementary strand by gently cooling the mixture from > 90 $^{\circ}$ C to the room temperature. Finally, 30 base-paired double stranded DNAs for the high- and low-FRET had following compositions:

High-FRET

```
5'  DY510XL TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT 3'
3'  Atto647N AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA 5'
```

Low-FRET

```
5'  DY510XL TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT 3'
3'  AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA Atto647N 5'
```

ALEX-FRET and ALFRED measurements were carried out in the presence of 30% of poly(ethylene glycol) (PEG), triplet quencher (β -mercaptoethylamine, 1 mM), and salt (NaCl, 50 mM) in Tris buffer (pH 8.0). The prepared sample was placed on a cover glass sealed by silicon spacer to prevent sample evaporation during the measurement.

FCS Data Analysis

FCS was employed to measure the viscosity of buffer solution and the diffusion time of dual-labeled dsDNA, the latter of which determines the binning time to be used in the analysis of ALFRED data. The fluctuations of the fluorescence signal in the small detection volume originate from various sources such as molecular diffusion, electronic transition between singlet and triplet states, and photoinduced isomerization, and are quantified by temporally autocorrelating the recorded fluorescence intensities. The autocorrelation function $G(\tau)$ is defined as

$$G(\tau) = \frac{\langle F(t) \cdot F(t + \tau) \rangle}{\langle F(t) \rangle^2} = 1 + \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2} \quad (1)$$

, where $F(t)$ and $\delta F(t)$ represent the fluorescence intensity and its fluctuation, respectively. If the fluorescence fluctuations come only from the translational diffusion of fluorescent molecules, the autocorrelation can be rewritten in the following

$$G(\tau) = 1 + \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{k^2 \tau_D}\right)^{-\frac{1}{2}}$$

form: (2)

Here, N , τ_D , and k are the average number of molecules in the detection volume, the diffusion time of the fluorescent molecule, and the aspect ratio of the observation volume, respectively. Because the diffusion time is directly related to the diffusion coefficient of the sample, we can calculate the diffusion coefficient of the dual-labeled dsDNA. Furthermore, the diffusion time also depends strongly on the viscosity of the solvent, and we can measure the viscosity of the buffer solution from Stokes-Einstein relation.

Conventional organic fluorophores usually have additional fluctuations from other sources such as the triplet state. In the case of triplet blinking, the fluorescence fluctuation is expressed as a function of kinetic constants describing the transition between the singlet and triplet states. In this case, the autocorrelation function can be expressed as

$$G(\tau) = 1 + \left[\frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{k^2 \tau_D}\right)^{-\frac{1}{2}} \right] \cdot \left[1 + \frac{F_T}{1 - F_T} \exp\left(-\frac{\tau}{\tau_T}\right) \right] \quad (3)$$

, where F_T and τ_T are the fraction of the molecules in the triplet state and the triplet state relaxation time, respectively.

Before we analyze the FCS data, we calibrated our STED-FCS setup to confirm the detection volume and to more precisely fit the results by fixing the aspect ratio k that is related to the detection volume. For this calibration process, we used a conventional organic dye, Cy5, whose diffusion coefficient has been well established from previous research ($D = 3.6 \times 10^{-6} \text{ cm}^2/\text{s}$). We measured the autocorrelation curves of the fluorescence intensity from Cy5 solution (10-s acquisition time, 10-times repeated), and fitted the results by using multi-curve and multi-parameter fitting algorithm. In this case, a slightly modified autocorrelation function in the following form was used to fit the FCS data:

$$G(\tau) = 1 + \frac{1}{N} \left(1 + \frac{4D\tau}{r^2}\right)^{-1} \left(1 + \frac{4D\tau}{k^2 r^2}\right)^{-\frac{1}{2}} \quad (4)$$

, where D represents the diffusion coefficient of the fluorescent molecule and r is the lateral $1/e^2$ -radius of the focal volume where the fluorescence intensity drops to $1/e^2$ of its peak value. Through the calibration process, we measured the effective detection volume and fixed the spatial parameters r and k to accurately analyze the autocorrelation curves.

In order to measure the viscosity and the diffusion time, we obtained the autocorrelation curve for 300 s and fitted this curve by equation (3). All measurements were repeated 5 times and each result was fitted independently, so that we can obtain the average values with the standard errors to increase the reliability.

Two-dimensional E - S Diagram from ALEX-FRET and ALFRED measurements

In both ALEX-FRET and ALFRED measurements, one obtains spatial as well as stoichiometric compositional information by measuring the FRET efficiency E and the stoichiometry parameter S , respectively, by alternating the lasers to excite the fluorescence donor and acceptor independently. From the photon counts measured at different E and S values, one can construct a two-dimensional (2D) E - S diagram.

The E and S values are calculated from the following equations of fluorescent intensities collected in each binning time:

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

$$S = F_D / (F_D + F_A), F_D = F_D^A + \gamma F_D^D \text{ and } F_A = F_A^A + F_A^D$$

, where F_X^Y ($X, Y = A$ or D ; A for acceptor and D for donor) is the fluorescence intensity of Y upon excitation of X . 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. We corrected the effects of the direct excitation and emission leakage from the photon counts, as well as the background noise, to accurately calculate the E and S values.

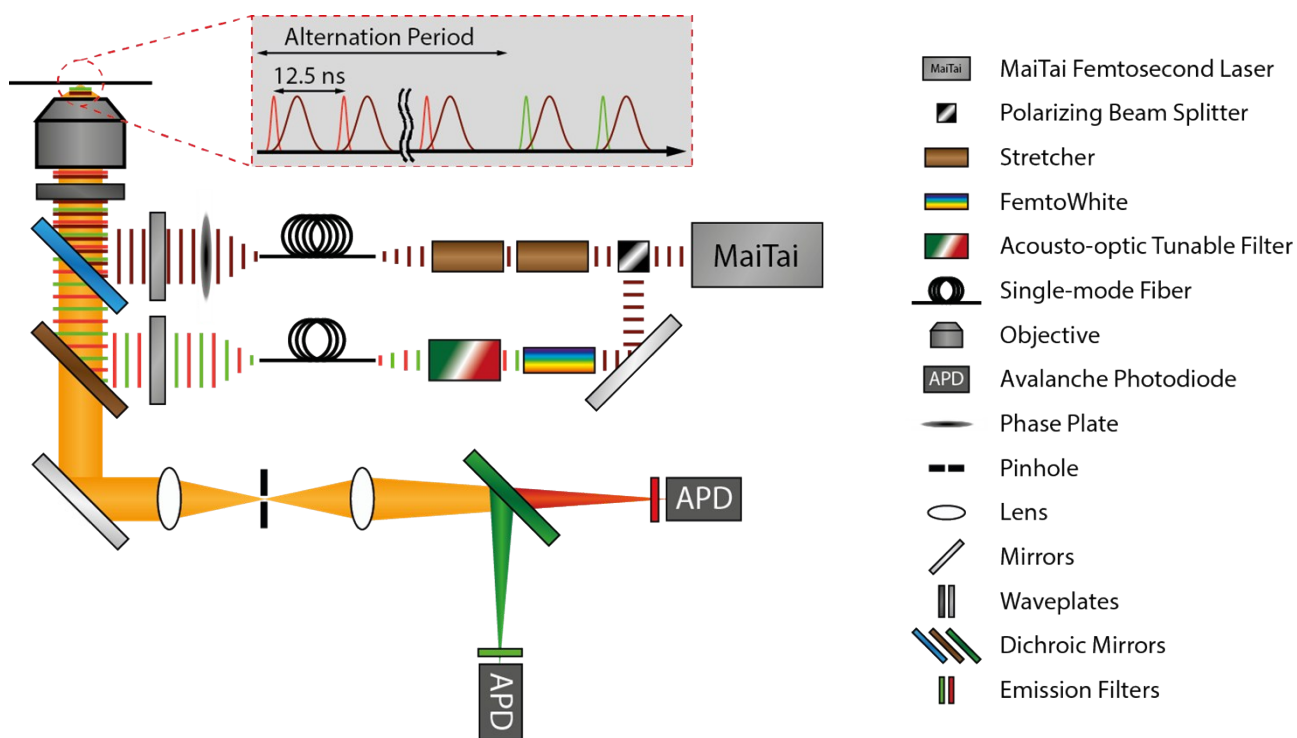


Fig. S1 Schematic illustration of the ALFRED setup. An 80-MHz femtosecond laser generates two excitation beams (510-nm green and 635-nm red) by using a photonic crystal fiber (FemtoWhite) and excitation filters (not shown), in addition to the doughnut-shaped depletion beam (780 nm). An AOTF is used to alternately pass the two excitation beams with a 50 μ s alternation period. Each beam is spatially filtered by passing through a single-mode fiber, and circularly polarized by waveplates. The excitation and depletion beams are precisely focused together at the focal point to confine the effective excitation volume. As a result, the final detection volume is 100-times smaller than the diffraction-limited one. The fluorescence from the confined excitation volume is filtered by a pinhole, dichroic mirrors, and emission filters before it is detected by the APDs.

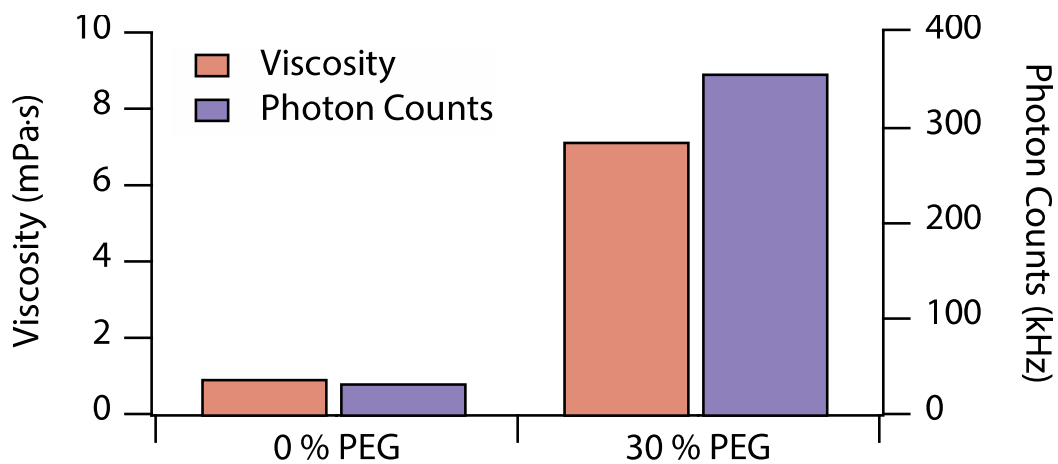


Fig. S2 Use of PEG to increase the viscosity of solution and the photon counts of DY510XL. 30% of PEG in the solution increases the viscosity 7 times to >7 mPa·s, which allows slower diffusion of the sample molecules. It is also noted that the photon counts of DY510XL in 30% PEG solution are ~10 times larger than in the normal solution with 0% PEG. The fluorescence lifetime of DY510XL is also increased to 1.51 ns with 30% PEG from 0.5 ns without PEG (not shown).

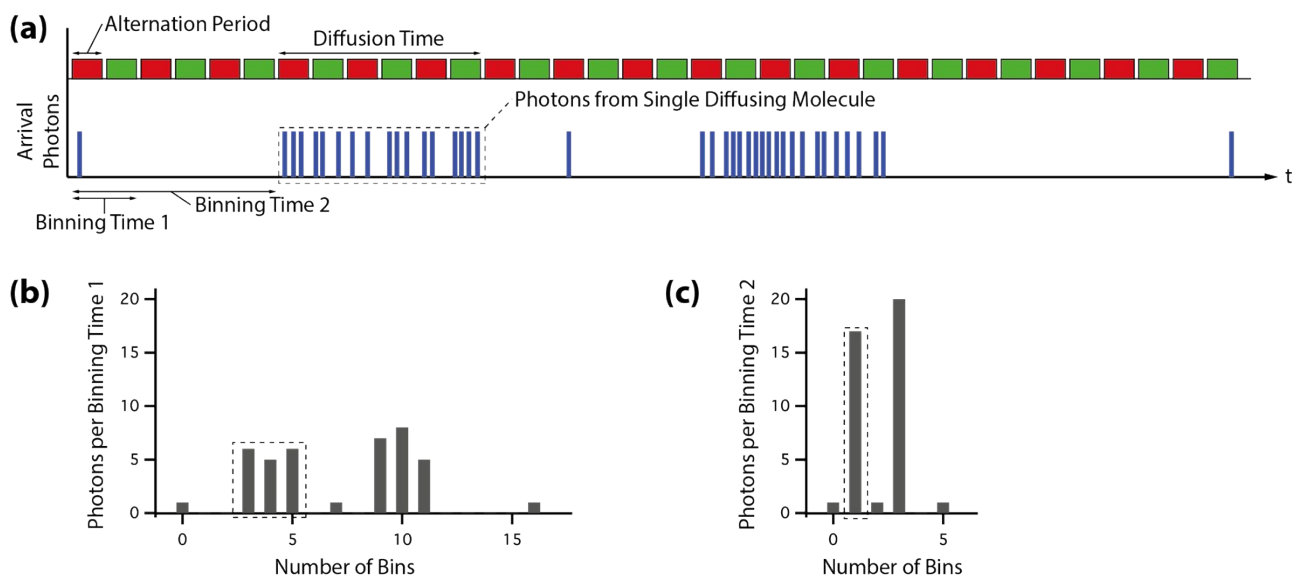


Fig. S3 (a) Schematic model for the alternation period, diffusion time, and binning time against the arrival photons. To measure the FRET efficiency and the stoichiometry parameter of the sample, the alternation period has to be much shorter than the diffusion time or the binning time. (b and c) Criteria for selecting a proper binning time. When we set the binning time shorter than the diffusion time ((b), binning time 1), the photons from single diffusing molecule are not properly binned into a single burst (dashed box in (a) and (b)). In this case, the smaller photon counts lead to a larger standard deviation in the FRET efficiency E and the stoichiometry parameter S . On the other hand, when the binning time is comparable to or longer than the diffusion time ((c), binning time 2), all of the photons from single diffusing molecule are added to constitute a single bin (dashed box in (a) and (c)).

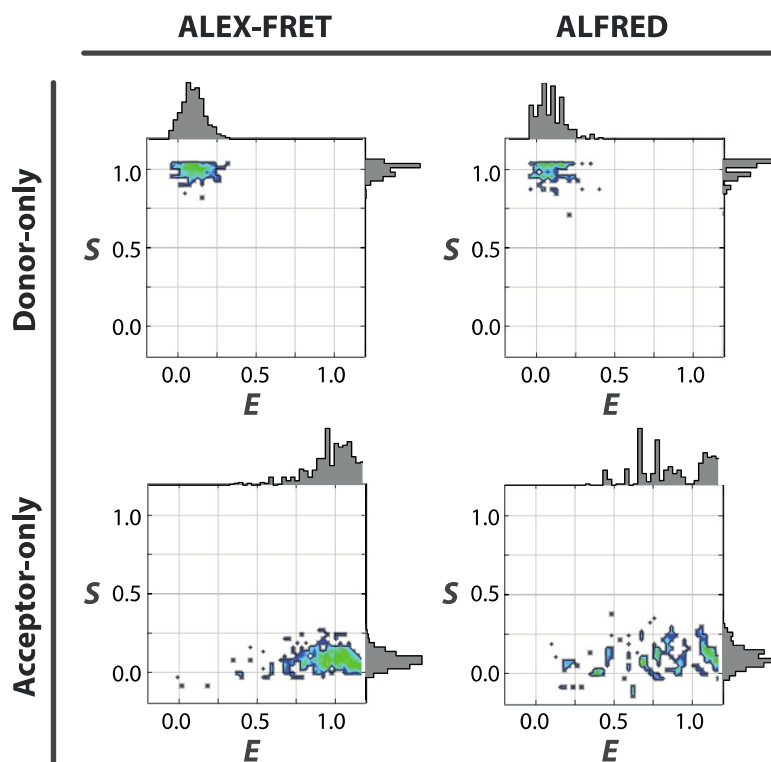


Fig. S4 (a) 2D diagrams of the “donor-only”- and “acceptor-only”-labeled species measured by ALEX-FRET vs. ALFRED at a concentration of 100 pM and 1 nM, respectively. The donor-only species appear near $S = 1$ and $E = 0$, while the acceptor-only species show up across the bottom of the diagram ($S = 0$). ALEX-FRET and ALFRED give nearly identical 2D E - S diagrams in this case, giving validity of the new ALFRED technique in reference to ALEX-FRET.

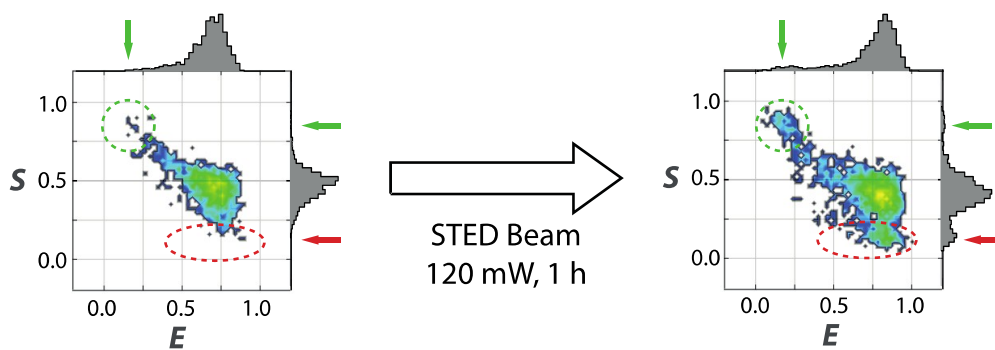


Fig. S5 Comparison of the 2D E - S diagrams of dual-labeled dsDNA sample at 5 nM before and after irradiating with the intense STED beam (780 nm, 120 mW, 1 h) measured by ALEX-FRET. Due to the strong laser power of the STED beam, more fluorophores are photobleached than without the STED beam, generating more singly-labeled species and giving rise to the higher peak intensities for the donor-only and acceptor-only species after 1 hour of the STED beam irradiation (dotted green and red circles, green and red arrows in 1D histograms).