Supplementary Material

"Three-Color Alternating-Laser Excitation of Single Molecules: Monitoring Multiple Interactions and Distances"

Nam Ki Lee, Achillefs N. Kapanidis, Hye Ran Koh, You Korlann, Sam On Ho, Younggyu Kim, Natalie Gassman, Seong Keun Kim, and Shimon Weiss

1. Theory of 3c-ALEX

1-A. Theoretical relations between 2-color and 3-color FRET.

A three-probe system creates three donor-acceptor pairs (*B*-*G*, *G*-*R*, and *B*-*R*; Fig. 1 *A*) characterized by three FRET efficiencies:

$$E_{BG} = \frac{k_{FRET}^{BG}}{k_r^B + k_{nr}^B + k_{FRET}^{BG}} \tag{S1}$$

$$E_{BR} = \frac{k_{FRET}^{BR}}{k_r^B + k_{nr}^B + k_{FRET}^{BR}} \tag{S2}$$

$$E_{GR} = \frac{k_{FRET}^{GR}}{k_r^G + k_{nr}^G + k_{FRET}^{GR}} \tag{S3}$$

where E_{XY} is the FRET efficiency between *X* (donor) and *Y* (acceptor), k_{FRET}^{XY} the rate constant for FRET between *X* and *Y*, k_r^X the radiative decay rate constant of *X*, and k_{nr}^X the non-radiative decay rate constant of *X*, respectively. Similarly, for a three-color system where all probes are involved in FRET, the FRET efficiencies are:

$$E_{BG}^3 = \frac{k_{FRET}^{BG}}{k_r^B + k_{nr}^B + k_{FRET}^{BG} + k_{FRET}^{BR}} \tag{S4}$$

$$E_{BR}^3 = \frac{k_{FRET}^{BR}}{k_r^B + k_{nr}^B + k_{FRET}^{BG} + k_{FRET}^{BR}} \tag{S5}$$

$$E_{GR}^3 = \frac{k_{FRET}^{GR}}{k_r^G + k_{nr}^G + k_{FRET}^{GR}} \tag{S6}$$

where E_{XY}^3 is the FRET efficiency between *X* (donor) and *Y* (acceptor) in a triply-labeled species. In 2c-FRET, the measured E_{XY} values are used to calculate distances (R_{XY}) between two probes using Eqs. S7-S8;

$$E_{XY} = \frac{1}{1 + (R_{XY} / R_{0,XY})^6} \quad \text{(S7)}$$

$$R_0^6 = (8.785 \times 10^{23}) \phi_D \kappa^2 n^{-4} J(\nu)^6 \quad \text{Å}^6 \quad \text{(S8)}$$

where ϕ_D is the quantum yield of donor, κ^2 the orientation factor, *n* the refractive index of the media, and *J(ν)* the integral of the spectral overlap (1). When R_{XY} equals $R_{0,XY}$ (Förster radius of a *X-Y* pair), E_{XY} is 50%. In 2c-FRET, since $R_{0,XY}$ is a constant independent of R_{XY}, E_{XY} directly reports on the distance. In 3c-FRET, however, E_{XY}^3 does not *directly* report on the distance, because $R_{0,XY}$ is not necessarily constant for a three-probe system (2). For example, when *R* is close to *B*-*G* pair, it quenches *B* via FRET (from *B* to *R*), reduces the quantum yield of *B*, and changes $R_{0,BG}$ (because $R_{0,BG}$ is a function of the quantum yield of *B*; Eq. S8). As a result, $R_{0,BG}$ depends on R_{BR} (*B-R* distance). Similarly, $R_{0,BR}$ (*B-R* pair) can become a function of R_{BG}. In contrast, $R_{0,GR}$ is a constant, independent of the presence of *B*.

Therefore, we convert E_{XY}^3 to E_{XY} using Eqs. S9-S11 in order to calculate three interprobe distances:

$$E_{BG}^3 = \frac{E_{BG}(1 - E_{BR})}{1 - E_{BG}E_{BR}} \quad \text{(S9)}$$

$$E_{BR}^3 = \frac{E_{BR}(1 - E_{BG})}{1 - E_{BG}E_{BR}} \quad \text{(S10)}$$

$$E_{GR}^3 = E_{GR}\,. \quad \text{(S11)}$$

1-B. 3-color ALEX

Detailed theory for 2c-ALEX was described previously (3). Here we describe the theory for 3c-ALEX measurement of three FRET efficiencies and three probe-stoichiometries values.

3c-ALEX produces nine distinct photon-emission streams (Fig. S1B), corresponding to nine photon-counts for a single burst of fluorescence in solution. To describe photon-counts, we used the simple notation of $F_{X_{exc}}^{Y_{em}}$ where *X* denotes excitation laser, *B* = 477 nm, *G* = 532 nm, and *R* = 633 nm (each laser excites dominantly probes of *B*, *G*, and *R*, respectively), and *Y* denotes photon detector, *B* for 495-525 nm, *G* for 580-620 nm, and *R* for 665-750 nm emission range (the emission wavelengths were selected by the combination of wavelength filters and dichroic mirrors, and each detector detects dominantly the emission of *B*, *G*, and *R*, respectively). For typical FRET pairs, photon-counts induced by FRET for donor-acceptor species contains two types of crosstalk: one is "leakage", *i.e.*, donor-emission detected at acceptor detection channel (Fig. S1A, "*Lk*"), and the other is "direct-excitation", *i.e.*, direct excitation of acceptor by donor-excitation laser (Fig. S1A, "*Dir*"). Accounting for these two crosstalks is significant for accurate FRET measurements (3). In case of 3c-FRET, additional crosstalks are present (Fig. S1B); we describe these crosstalks using the advanced notation ${}^{Z}F_{X_{exc}}^{Y_{em}}$, which represents the sub-photon-counts emitted by fluorophore Z upon X laser-excitation and detected by Y detector. In the case of the photons originating from FRET, we denote $Z = X \rightarrow Y$ to signify their FRET-induced origin. 2c-FRET contains three types of photon origin of D (donor), A (acceptor) and $D \rightarrow A$, while 3c-ALEX contains seven types of photon origins as B, G, R, $B \rightarrow G$, $G \rightarrow R$, $B \rightarrow R$, and $B \rightarrow G \rightarrow R$.

Definitions for photon-counts of a fluorescence burst generated by 3c-ALEX. The first laser, exciting B dominantly, generates three types of fluorescence intensities (Fig. S1B "0-36 μs"), and each of them is represented by the sum of the sub-photon-counts from the seven types of photon origin. For example, the photon count ${}^{*}F_{B_{exc}}^{R_{em}}$ can be written as follows:

$$\begin{aligned} {}^{*}F_{B_{exc}}^{R_{em}} &= {}^{B}F_{B_{exc}}^{R_{em}} + {}^{G}F_{B_{exc}}^{R_{em}} + {}^{R}F_{B_{exc}}^{R_{em}} + {}^{B\rightarrow G}F_{B_{exc}}^{R_{em}} + {}^{G\rightarrow R}F_{B_{exc}}^{R_{em}} + {}^{B\rightarrow R}F_{B_{exc}}^{R_{em}} + {}^{B\rightarrow G\rightarrow R}F_{B_{exc}}^{R_{em}} \\ &= Lk_2 + (Dir_1 \circ Lk_3) + Dir_2 + (Lk_3 \circ E_{BG}^{3}) + (Dir_1 \circ E_{GR}^{3}) + {}^{B\rightarrow R}F_{B_{exc}}^{R_{em}} + {}^{B\rightarrow G\rightarrow R}F_{B_{exc}}^{R_{em}} \end{aligned} \quad \text{(S12)}$$

where ${}^{*}F_{X_{exc}}^{Y_{em}}$ denotes the photon-counts that contain crosstalks to be corrected afterward ($F_{X_{exc}}^{Y_{em}}$ is the photon-counts that do not contain crosstalks or crosstalks corrected one). The two main types of crosstalks are written as Lk_n and Dir_n, (Fig. S1B). Crosstalks due to a combination of two crosstalks, or a combination of crosstalk and FRET are denoted by ($A \circ B$). Similarly:

$$F_{B_{exc}}^{B_{em}} = {}^{B}F_{B_{exc}}^{B_{em}} \quad \text{(S13)}$$

$${}^{*}F_{B_{exc}}^{G_{em}} = Lk_1 + Dir_1 + {}^{B\rightarrow G}F_{B_{exc}}^{G_{em}} \quad \text{(S14)}$$

$$F_{G_{exc}}^{B_{em}} = 0 \quad \text{(S15)}$$

$$F_{G_{exc}}^{G_{em}} = {}^{G}F_{G_{exc}}^{G_{em}} \quad \text{(S16)}$$

$${}^{*}F_{G_{exc}}^{R_{em}} = Lk_4 + Dir_3 + {}^{G\rightarrow R}F_{G_{exc}}^{R_{em}} \quad \text{(S17)}$$

$$F_{R_{exc}}^{B_{em}} = 0 \quad \text{(S18)}$$

$$F_{R_{exc}}^{G_{em}} = 0 \quad \text{(S19)}$$

$$F_{R_{exc}}^{R_{em}} = {}^{R}F_{R_{exc}}^{R_{em}} \, . \quad \text{(S20)}$$

Several of the photon-counts in Eq. S12-S20 are zero, because B- and G-excitations by the R-excitation laser and B-excitation by the G-excitation laser are negligible, and because the emission of G is not detected at B-detector and the emission of R is not detected at B- and G-detectors. The non-zero photon-counts in Eqs. S12−S20 can be written as a function of excitation and emission properties, and FRET efficiencies:

$$F_{B_{exc}}^{B_{em}} = I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_B\eta_{B_{em}}^{B_{dec}}(1-E_{BG}^3-E_{BR}^3) \quad \text{(S21)}$$

$$\begin{aligned} {}^{*}F_{B_{exc}}^{G_{em}} &= [I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_B\eta_{B_{em}}^{G_{dec}}(1-E_{BG}^3-E_{BR}^3)] + [I_{B_{exc}}\sigma_{B_{exc}}^{G}\phi_G\eta_{G_{em}}^{G_{dec}}(1-E_{GR}^3)] \\ &+ I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_G\eta_{G_{em}}^{G_{dec}}E_{BG}^3(1-E_{GR}^3) \end{aligned} \quad \text{(S22)}$$

$$\begin{aligned} {}^{*}F_{B_{exc}}^{R_{em}} &= [I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_B\eta_{B_{em}}^{R_{dec}}(1-E_{BG}^3-E_{BR}^3)] + [I_{B_{exc}}\sigma_{B_{exc}}^{G}\phi_G\eta_{G_{em}}^{R_{dec}}(1-E_{GR}^3)] + I_{B_{exc}}\sigma_{B_{exc}}^{R}\phi_R\eta_{R_{em}}^{R_{dec}} \\ &+ [I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_G\eta_{G_{em}}^{R_{dec}}E_{BG}^3(1-E_{GR}^3)] + I_{B_{exc}}\sigma_{B_{exc}}^{G}\phi_R\eta_{R_{em}}^{R_{dec}}E_{GR}^3 + I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_R\eta_{R_{em}}^{R_{dec}}E_{BR}^3 \\ &+ I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_R\eta_{R_{em}}^{R_{dec}}E_{BG}^3E_{GR}^3 \end{aligned} \quad \text{(S23)}$$

$$F_{G_{exc}}^{G_{em}} = I_{G_{exc}}\sigma_{G_{exc}}^{G}\phi_G\eta_{G_{em}}^{G_{dec}}(1-E_{GR}^3) \quad \text{(S24)}$$

$${}^{*}F_{G_{exc}}^{R_{em}} = [I_{G_{exc}}\sigma_{G_{exc}}^{G}\phi_G\eta_{G_{em}}^{R_{dec}}(1-E_{GR}^3)] + I_{G_{exc}}\sigma_{G_{exc}}^{R}\phi_R\eta_{R_{em}}^{R_{dec}} + I_{G_{exc}}\sigma_{G_{exc}}^{G}\phi_R\eta_{R_{em}}^{R_{dec}}E_{GR}^3 \quad \text{(S25)}$$

$$F_{R_{exc}}^{R_{em}} = I_{R_{exc}}\sigma_{R_{exc}}^{R}\phi_R\eta_{R_{em}}^{R_{dec}} \quad \text{(S26)}$$

where $I_{B_{exc}}$, $I_{G_{exc}}$ and $I_{R_{exc}}$ are *B*-, *G*- and *R*-excitation laser intensities, respectively; $\sigma_{X_{exc}}^{Y}$ is the absorption cross-section of *Y* (probe) upon *X* laser-excitation; ϕ_B, ϕ_G and ϕ_R are quantum yields of *B*, *G* and *R*, respectively; $\eta_{X_{em}}^{Y_{dec}}$ denotes the detection efficiency of *X* (probe)-emission by the *Y* detector; and E_{XY}^3 denotes the FRET efficiency between *X* (donor) and *Y* (acceptor) of triply-labeled species.

Crosstalk corrections. It is essential to account for crosstalks for accurate FRET measurements (3). As in 2c-ALEX, crosstalks can be corrected in 3c-ALEX using the singly-labeled species. Among the six photon-counts, ${}^{*}F_{B_{exc}}^{G_{em}}$, ${}^{*}F_{B_{exc}}^{R_{em}}$ and ${}^{*}F_{G_{exc}}^{R_{em}}$ require crosstalk corrections.

i) Crosstalk corrections for ${}^{*}F_{B_{exc}}^{G_{em}}$. ${}^{*}F_{B_{exc}}^{G_{em}}$ contains two crosstalks, Lk_1 and Dir_1 (Eq. S14). The Lk_1 contribution can be defined on the basis of $F_{B_{exc}}^{B_{em}}$ as:

$$Lk_1 = I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_B\eta_{B_{em}}^{G_{dec}}(1-E_{BG}^3-E_{BR}^3) = (\eta_{B_{em}}^{G_{dec}}/\eta_{B_{em}}^{B_{dec}})F_{B_{exc}}^{B_{em}} = l_1F_{B_{exc}}^{B_{em}} \quad \text{(S27)}$$

where l_1 is the leakage coefficient for *B*-emission into *G*-detection channel; l_1 can be determined using the ratio ${}^{*}F_{B_{exc}}^{G_{em}}/F_{B_{exc}}^{B_{em}}$ for *B*-only species. The Dir_1 contribution can be defined on the basis of $F_{G_{exc}}^{G_{em}}$ as:

$$Dir_1 = I_{B_{exc}}\sigma_{B_{exc}}^{G}\phi_G\eta_{G_{em}}^{G_{dec}}(1-E_{GR}^3) = [I_{B_{exc}}\sigma_{B_{exc}}^{G}/I_{G_{exc}}\sigma_{G_{exc}}^{G}]F_{G_{exc}}^{G_{em}} = d_1F_{G_{exc}}^{G_{em}} \quad \text{(S28)}$$

where d_1 is the direct-excitation coefficient for *G*-excitation by *B*-excitation laser; d_1 can be determined using the ratio ${}^{*}F_{B_{exc}}^{G_{em}}/F_{G_{exc}}^{G_{em}}$ for *G*-only species. After the subtractions of Lk_1 and Dir_1, ${}^{*}F_{B_{exc}}^{G_{em}}$ contains only the photon-counts induced by the FRET from *B* to *G*:

$$F_{B_{exc}}^{G_{em}} = {}^{B\to G}F_{B_{exc}}^{G_{em}} = {}^{*}F_{B_{exc}}^{G_{em}} - Lk_1 - Dir_1 = I_{B_{exc}}\sigma_{B_{exc}}^{B}\phi_G\eta_{G_{em}}^{G_{dec}}E_{BG}^3(1-E_{GR}^3)\,. \quad \text{(S29)}$$

ii) Using similar crosstalk corrections for ${}^{*}F_{B_{exc}}^{R_{em}}$, we obtain:

$$F_{B_{exc}}^{R_{em}} = I_{B_{exc}} \sigma_{B_{exc}}^{B} \phi_R \eta_{R_{em}}^{R_{dec}} (E_{BR}^3 + E_{BG}^3 E_{GR}^3) \quad \text{(S30)}$$

iii) Using similar crosstalk corrections for ${}^{*}F_{G_{exc}}^{R_{em}}$, we obtain:

$$F_{G_{exc}}^{R_{em}} = I_{G_{exc}} \sigma_{G_{exc}}^{G} \phi_R \eta_{R_{em}}^{R_{dec}} E_{GR}^3 \quad \text{(S31)}$$

E (FRET efficiency) determination. After the corrections for crosstalk terms, we obtain six photon-counts that are used (along with Eqs. S9–S11) for determining the three FRET efficiencies of 3c-FRET, for example:

$$F_{B_{exc}}^{B_{em}} = I_{B_{exc}} \sigma_{B_{exc}}^{B} \phi_B \eta_{B_{em}}^{B_{dec}} (1 - E_{BG}^3 - E_{BR}^3) = I_{B_{exc}} \sigma_{B_{exc}}^{B} \phi_B \eta_{B_{em}}^{B_{dec}} \frac{(1-E_{BG})(1-E_{BR})}{1 - E_{BG}E_{BR}} \quad \text{(S32)}$$

Similarly,

$$F_{B_{exc}}^{G_{em}} = I_{B_{exc}} \sigma_{B_{exc}}^{B} \phi_G \eta_{G_{em}}^{G_{dec}} \frac{E_{BG}(1-E_{BR})(1-E_{GR})}{1 - E_{BG}E_{BR}} \quad \text{(S33)}$$

$$F_{B_{exc}}^{R_{em}} = I_{B_{exc}} \sigma_{B_{exc}}^{B} \phi_R \eta_{R_{em}}^{R_{dec}} \frac{[E_{BR}(1-E_{BG}) + E_{BG}(1-E_{BR})E_{GR}]}{1 - E_{BG}E_{BR}} \quad \text{(S34)}$$

$$F_{G_{exc}}^{G_{em}} = I_{G_{exc}} \sigma_{G_{exc}}^{G} \phi_G \eta_{G_{em}}^{G_{dec}} (1 - E_{GR}) \quad \text{(S35)}$$

$$F_{G_{exc}}^{R_{em}} = I_{G_{exc}} \sigma_{G_{exc}}^{G} \phi_R \eta_{R_{em}}^{R_{dec}} E_{GR} \quad \text{(S36)}$$

$$F_{R_{exc}}^{R_{em}} = I_{R_{exc}} \sigma_{R_{exc}}^{R} \phi_R \eta_{R_{em}}^{R_{dec}} . \quad \text{(S37)}$$

The three photon-counts induced by the excitation of *B* (Eqs. S32–S34) can be reduced to two FRET-related ratios:

$$E_{BG}^{*} = \frac{F_{B_{exc}}^{G_{em}}}{F_{B_{exc}}^{B_{em}} + F_{B_{exc}}^{G_{em}}} = \frac{\gamma_{BG} E_{BG}(1-E_{GR})}{1 - E_{BG} + \gamma_{BG} E_{BG}(1-E_{GR})} \quad \text{(S38; Eq. 1 in text)}$$

$$E_{BR}^{*} = \frac{F_{B_{exc}}^{R_{em}}}{F_{B_{exc}}^{B_{em}} + F_{B_{exc}}^{R_{em}}} = \frac{\gamma_{BR}\{E_{BG}E_{GR}(1-E_{BR}) + E_{BR}(1-E_{BG})\}}{(1-E_{BG})(1-E_{BR}) + \gamma_{BR}\{E_{BG}E_{GR}(1-E_{BR}) + E_{BR}(1-E_{BG})\}} \quad \text{(S39; Eq. 2 in text)}$$

where E_{XY}^{*} represents a proximity–ratio (an approximate value of *E*) and γ_{XY} denotes the detection-correction factor (4) [$\gamma_{XY} = (\phi_Y \eta_{Y_{em}}^{Y_{dec}})/(\phi_X \eta_{X_{em}}^{X_{dec}})$]. Since all γ-factors for 3c-FRET can be measured using 2c-ALEX scheme(3), they can be treated here as measurable constants. With all γ-factors known, however, these two ratiometric-*E* equations (Eqs. S38-S39) are insufficient to determine all three FRET efficiencies. For this reason, single-molecule FRET using single-laser excitation cannot determine three *FRET efficiencies* from a single burst (5). In 3c-ALEX, the second laser excites *G* that generates two types of photon-counts of $F_{G_{exc}}^{G_{em}}$ and $F_{G_{exc}}^{R_{em}}$ (Eqs. S35-S36); the ratio of these two photon-counts reports on E_{GR} as:

$$E_{GR}^{*} = \frac{F_{G_{exc}}^{R_{em}}}{F_{G_{exc}}^{G_{em}} + F_{G_{exc}}^{R_{em}}} = \frac{\gamma_{GR} E_{GR}}{(1 - E_{GR}) + \gamma_{GR} E_{GR}} \qquad \text{(S40; Eq. 3 in text)}$$

Using Eqs. S38–S40, three accurate FRET efficiencies can be derived:

$$E_{BG} = \frac{E_{BG}^{*}}{\gamma_{BG}(1 - E_{GR})(1 - E_{BG}^{*}) + E_{BG}^{*}} \qquad \text{(S41)}$$

$$E_{GR} = \frac{E_{GR}^{*}}{\gamma_{GR} + E_{GR}^{*}(1 - \gamma_{GR})} \qquad \text{(S42)}$$

$$E_{BR} = \frac{E_{BR}^{*}(1 - E_{BG} + E_{BG} E_{GR} \gamma_{BR}) - \gamma_{BR} E_{BG} E_{GR}}{\gamma_{BR}(1 - E_{BG} - E_{BG} E_{GR}) + E_{BR}^{*}[1 - E_{BG} - \gamma_{BR}(1 - E_{BG} - E_{BG} E_{GR})]} . \qquad \text{(S43)}$$

The three E_{XY} are subsequently used for the calculation of three distances using Eq. S7.

2. Ensemble measurement.

The quantum yields for Alexa 488, TMR and Alexa 647 were measured as described (6) using singly-labeled DNA in single-molecule buffer (10 mM HEPES-NaOH pH 7, 500 mM NaCl, 100 µg/ml BSA, 1 mM mercaptoethylamine, and 5% glycerol); the values were 0.90, 0.35 (or 0.25), and 0.32 for Alexa 488, TMR and Alexa 647, respectively (see Table below). The quantum yield of TMR was sensitive to the labeling positions, while those of Alexa 488 and Alexa 647 are not. The steady-state fluorescence anisotropy values of Alexa 488, TMR and Alexa 647 in dsDNA were measured to be 0.05, 0.12 (or 0.18) and 0.19, respectively (see Table); these values are low compared to the anisotropies of the immobile probes (0.36-0.4), indicating substantial rotational freedom of the probes, and justifying the assumption κ^2 =2/3. Using the κ^2 approximation, the Förster radius R_0 for the Alexa 488-TMR, TMR-Alexa 647, and Alexa 488-Alexa 647 pairs were calculated to be 62 Å, 66 Å (62 Å for ϕ_{TMR} =0.25), and 56 Å, respectively.

Dye	Position	QY[a]	r^b
Alexa 488	*C1* bot	0.90	0.05
	C2 top	0.90	0.05
TAMRA	*C1* top	0.25	0.18
	C2 bot	0.25	0.18
	C3-a bot	0.35	0.12
Alexa 647	*C1* bot	0.32	0.19
	C3-a bot	0.32	0.19

[a] Quantum yield

[b] Steady-state fluorescence Anisotropy

3. γ-determination.

For calculation of accurate-FRET (S41-S43), measurements of γ-factors (detection-correction factors (4)) are necessary (3). γ-factors for 3c-FRET can be defined (using the notation of "*Theory of 3c-ALEX*") as:

$$\gamma_{BG} = \frac{\phi_G \eta_{G_{em}}^{G_{dec}}}{\phi_B \eta_{B_{em}}^{B_{dec}}}, \ \gamma_{GR} = \frac{\phi_R \eta_{R_{em}}^{R_{dec}}}{\phi_G \eta_{G_{em}}^{G_{dec}}}, \ \gamma_{BR} = \frac{\phi_R \eta_{R_{em}}^{R_{dec}}}{\phi_B \eta_{B_{em}}^{B_{dec}}}. \qquad \text{(S44)}$$

Since by definition:

$$\gamma_{BR} = \gamma_{BG} \times \gamma_{GR}, \qquad \text{(S45)}$$

two γ-factors are sufficient for accurate-FRET measurements. Since TMR has a different quantum yield depending on the labeling positions in dsDNA (see "2. Ensemble measurement"), we divided the five dsDNA constructs into two groups: group 1 (*C3-a* and *C3-c*, ϕ_{TMR} = 0.35) and group 2 (*C1*, *C2* and *C3-b*, ϕ_{TMR} = 0.25); for group 1, we experimentally determined the γ-factors using two sets of FRET pairs, and for group 2 we used the "standard pair" method (ref.3).

i) Group 1: using the two sets of FRET pairs (for γ_{BR}, *B-R* of *C3-a* and *C3-c*; for γ_{GR}, *G-R* of *C3-a* and *C3-c*), and the method described in ref. 3 (in section of "Calculation of γ"), we obtained γ_{BR} = 0.91±0.07 and γ_{GR} = 0.74±0.04 (mean ± standard deviation of 4 individual experiments), and using Eq. S45, γ_{BG} = 1.23.

ii) Group 2: when identical probes are used for the different *D-A* pairs, γ-factors can be determined using known γ-factor as standard (see section of "Determination of γ for various *D-A* pairs using a standard pair" in ref 3). Employing this method, γ-factors of group 2 were determined using the γ-factors for group 1 as standard. Since the difference between group 1 and group 2 is only the quantum yield of TMR, we could obtain the three values as γ_{BG} = 0.88, γ_{GR} = 1.04, and γ_{BR} = 0.91.

References for Supporting Information

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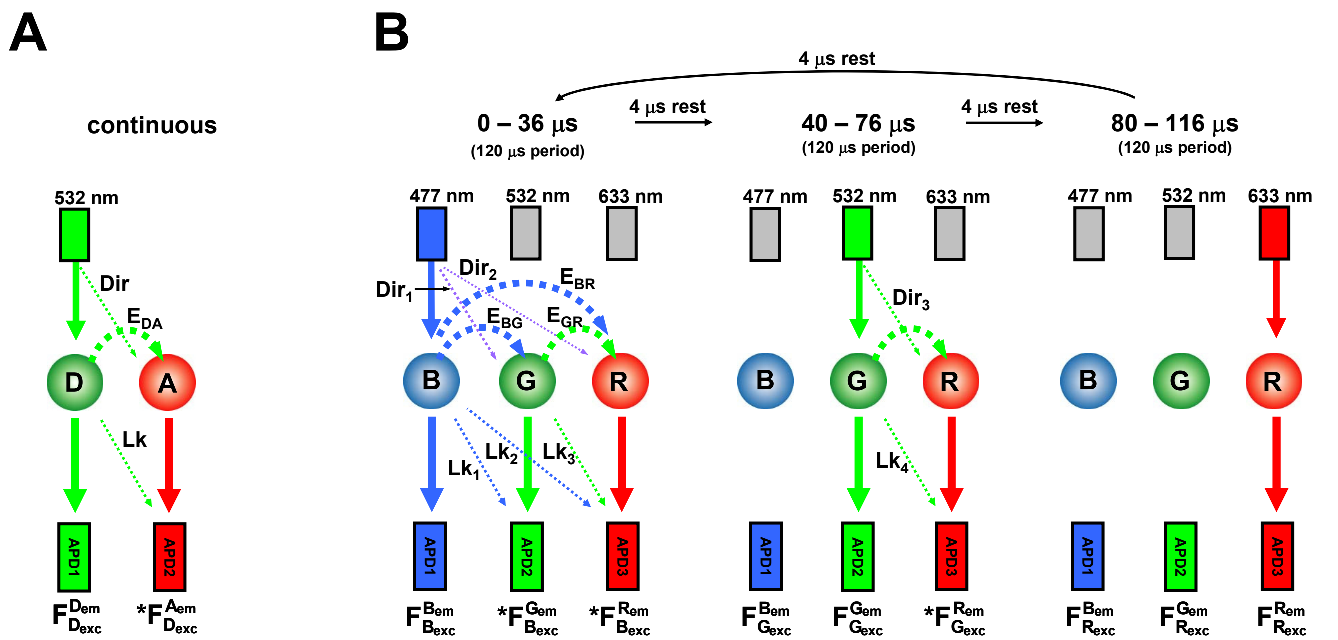

FIGURE S1. Schematic of alternating excitation, observable photon emissions, and crosstalks of 3c-ALEX. (***A***) Conventional single-molecule 2-color FRET (2c-FRET) using single-laser excitation. 532-nm laser excites *D* directly, and *A* indirectly via FRET, results in photon emissions $F_{B_{exc}}^{B_{em}}$ and $^{*}F_{B_{exc}}^{G_{em}}$. Two crosstalks are present in 2c-FRET system; *Dir* denotes "direct excitation", *i.e.*, the direct excitation of *A* by *D* excitation laser, and *Lk* denotes "leakage", *i.e.*, the emission of *D* detected by APD2 (*A* emission detection channel). (***B***) 3c-ALEX. The 477-nm laser mainly excites *B* and gives rise to non-zero photon-counts $F_{B_{exc}}^{B_{em}}$, $^{*}F_{B_{exc}}^{G_{em}}$, and $^{*}F_{B_{exc}}^{R_{em}}$; the 532-nm laser mainly excites *G* and gives rise to $F_{G_{exc}}^{G_{em}}$, $^{*}F_{G_{exc}}^{R_{em}}$; and the 633-nm laser mainly excites *R* and gives rise to $F_{R_{exc}}^{R_{em}}$. The ratios based on the six photon-counts report on three proximity-ratios (E_{XY}) and three probe-stoichiometries (S_{XY}). 3c-ALEX results in three *Dir* crosstalks and four *Lk* crosstalks.

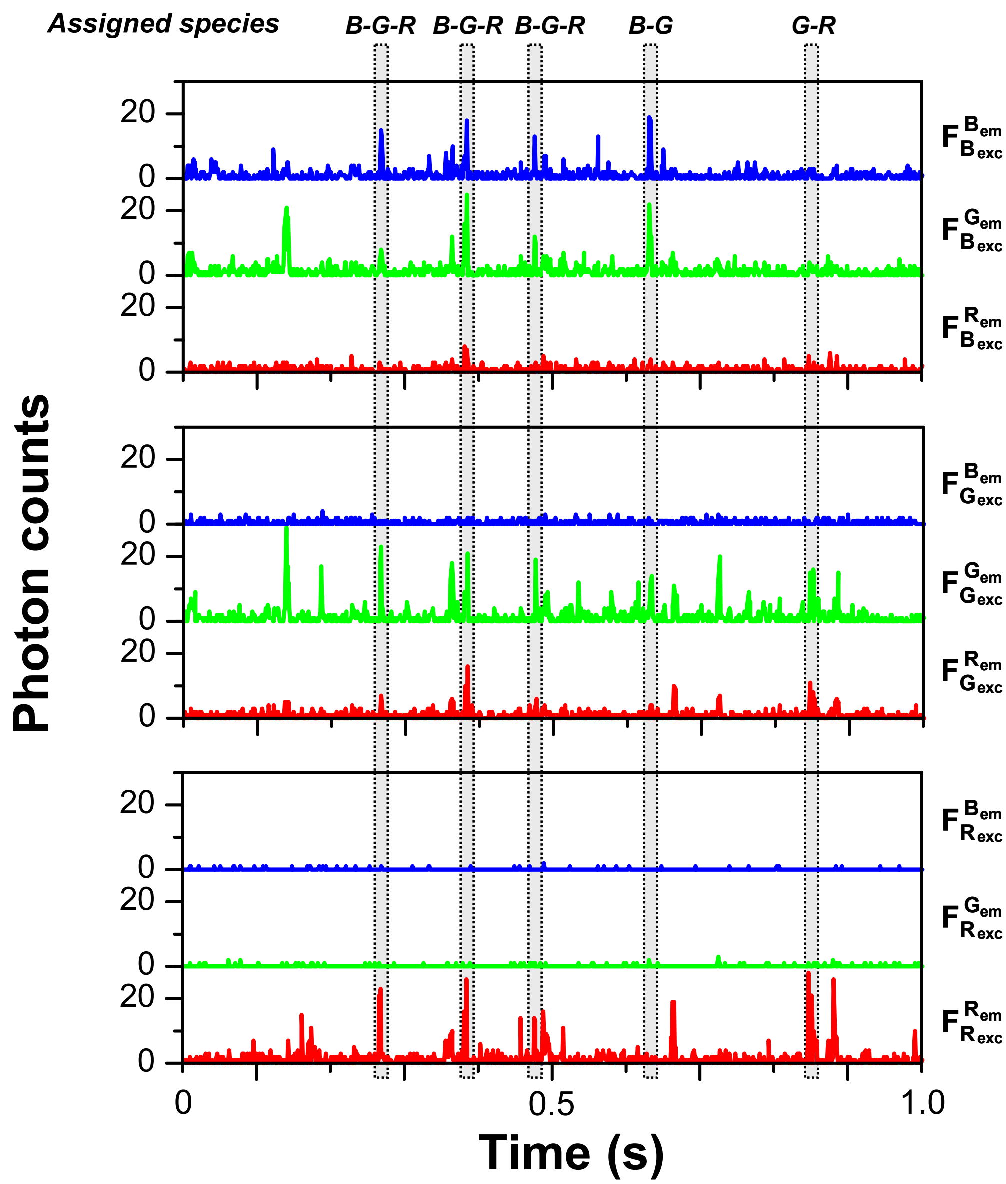

FIGURE S2. Time-traces generated by 3c-ALEX (1 ms binning; *C3-c* of Fig 2 *A*). $F_{X_{exc}}^{Y_{em}}$ represents photon counts, where *X* denotes excitation laser, *B* = 477 nm, *G* = 532 nm, and *R* = 633 nm, and *Y* denotes photon detector, *B* for APD1, *G* for APD2, and *R* for APD3. APD1 detects dominantly the emission of *B*, APD2 of *G*, and APD3 of *R*, respectively. The background noises are < 0.7 kHz for each detection channel. FRET efficiencies between *B* and *G*, *G* and *R*, and *B* and *R* of *C3-c*, are approximately 42%, 14%, and 24%, respectively. Five bursts are assigned representatively in the time-traces; *B-G-R* denotes triply-labeled species, while *B-G* and *G-R* denote doubly-labeled species.

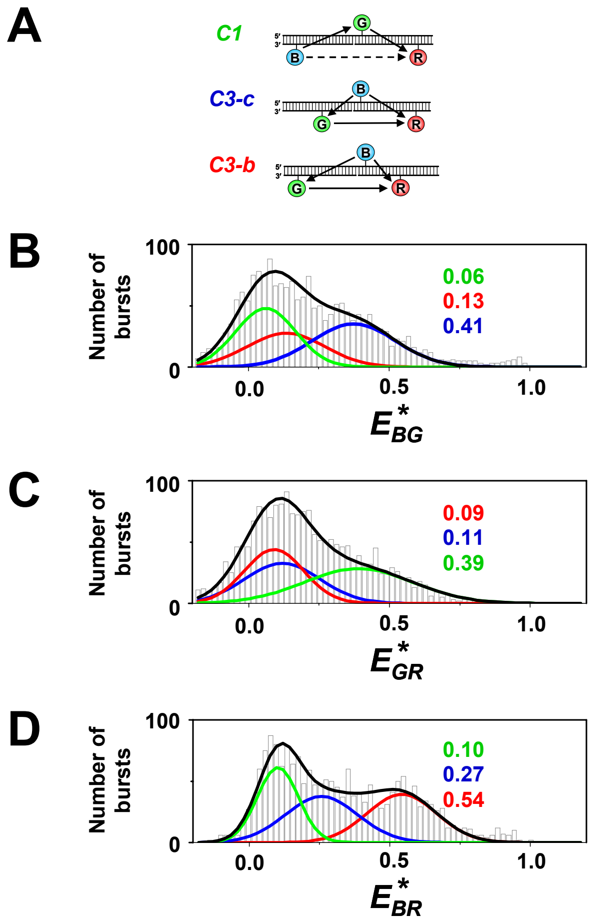

FIGURE S3. 1-D *E** histograms of 1:1:1 mixture of *C1*, *C3-c*, and *C3-b* (Fig. 7 *E*). (***A***) Schematic description of three triply-labeled species. (***B-D***) 1-D *E** histograms, obtained after collapsing the burst points of 3-D *E** histogram in Fig. 7 *E* to each axis. Green, Blue, and Red lines represent individual Gaussian fits of *C1*, *C3-c*, and *C3-b*, respectively, and Black lines, sum of the three Gaussian fits. Individual Gaussian fits of *C1*, *C3-c*, and *C3-b* were obtained from the 3-D histogram; the molecules (burst points) of *C1*, *C3-c*, and *C3-b* were selected graphically, respectively, and mean proximity-ratios for each species were obtained using Gaussian fitting. The measured proximity-ratios agree well with those measured from solutions containing a single triply-labeled species, and the ratio of molecules is 1.0:1.0:1.2, consistent with the initial mixing ratio. However, the three species are hardly discriminated in 1-D *E** histograms, and thereby the proximity-ratios of each species and the mixing ratio are not accessible in 1-D *E** histograms.

TABLE S1. Crosstalks factors: leakage and direct excitation.

Crosstalks	factors[a]	
Lk	l_1	0.19
	l_2	0.01
	l_3, l_4	0.14
Dir[b]	d_1	0.09~0.15
	d_2	0.01~0.02
	d_3	0.11~0.18

[a] For definition of each factor, see Fig. S1B and "*Theory of 3c-ALEX*".

[b] *Dir* depends on the excitation laser powers of three lasers.

TABLE S2. FRET efficiencies and distances in 2-color and 3-color ALEX[a]

			3-color ALEX			2-color ALEX[b]		
dsDNA	FRET pair	R_0 (Å)	(E^*)[c]	E	R (Å)	(E^*)[c]	E	R (Å)
C1	B-G	62	0.09 ± 0.03	0.15 ± 0.03	83 ± 2	0.13 ± 0.01	0.14 ± 0.01	84 ± 2
	G-R	62	0.44 ± 0.02	0.42 ± 0.02	68 ± 2	0.46 ± 0.01	0.45 ± 0.01	66 ± 2
	B-R	56	0.10 ± 0.03	0.04 ± 0.01	98 ± 5	*0.01* ± 0.01[d]	*0.01* ± 0.01[d]	*105* ± 6[d]
C2	B-G	62	0.10 ± 0.01	0.11 ± 0.01	88 ± 2	0.10 ± 0.01	0.12 ± 0.01	87 ± 1
	G-R	62	0.05 ± 0.01	0.05 ± 0.01	101 ± 4	0.06 ± 0.01	0.06 ± 0.01	99 ± 4
	B-R	56	0.21 ± 0.01	0.22 ± 0.01	69 ± 1	0.21 ± 0.01	0.22 ± 0.01	69 ± 1
C3-a	B-G	62	0.43 ± 0.02	0.47 ± 0.02	63 ± 1	0.47 ± 0.01	0.42 ± 0.01	65 ± 1
	G-R	66	0.26 ± 0.01	0.32 ± 0.01	75 ± 1	0.28 ± 0.01	0.34 ± 0.01	74 ± 1
	B-R	56	0.64 ± 0.01	0.63 ± 0.01	51 ± 1	0.57 ± 0.02	0.59 ± 0.02	53 ± 1
C3-b	B-G	62	0.11 ± 0.01	0.13 ± 0.01	85 ± 2	0.10 ± 0.01	0.11 ± 0.01	88 ± 2
	G-R	62	0.09 ± 0.01	0.09 ± 0.01	92 ± 2	0.09 ± 0.02	0.09 ± 0.02	92 ± 4
	B-R	56	0.58 ± 0.01	0.60 ± 0.01	52 ± 1	0.58 ± 0.01	0.60 ± 0.01	52 ± 1
C3-c	B-G	62	0.44 ± 0.02	0.42 ± 0.02	65 ± 1	0.48 ± 0.02	0.42 ± 0.02	65 ± 1
	G-R	66	0.10 ± 0.01	0.13 ± 0.01	90 ± 1	0.11 ± 0.01	0.15 ± 0.01	89 ± 1
	B-R	56	0.29 ± 0.01	0.26 ± 0.01	67 ± 1	0.20 ± 0.01	0.22 ± 0.01	69 ± 1

[a] All the averages and the standard deviations were obtained from more than four individual measurement.

[b] Three independent 2-color ALEX measurements were carried for the doubly-labeled species.

[c] E^*: simple proximity ratio

[d] *E* is too small to give meaningful distance value.