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## Supplemental Information

# Ab Initio Derivation of the FRET Equations Resolves Old Puzzles and Suggests Measurement Strategies

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## Supplementary Results

### SR1. Derivation of $E_{app}$ and concentrations relations for two excitation wavelengths

In the case of two excitation wavelengths, one may write the following variants of Eqs. 34 from the main text for the donor and acceptor emission in the presence of FRET:

$$F_1^{Da} = F_1^{D\prime} - F_{FRET,1}^{D}, \tag{S1a}$$

$$F_1^{Ad} = F_1^{A\prime} + \frac{Q^A}{Q^D} F_{FRET,1}^{D}, \tag{S1b}$$

$$F_2^{Da} = F_2^{D\prime} - F_{FRET,2}^{D}, \tag{S1c}$$

$$F_2^{Ad} = F_2^{A\prime} + \frac{Q^A}{Q^D} F_{FRET,2}^{D}, \tag{S1d}$$

where the subscripts "1" and "2" stand for the first and second excitation wavelength, respectively. In addition, using the notations given by Eqs. 33 in the main text, we introduce the following notations for the ratios of the various terms in Eqs. S1:

$$F_1^{D\prime}/F_2^{D\prime} = \frac{\varepsilon_1^D}{\varepsilon_2^D} \frac{\{[D]+[D]_d\}\Pi_{D1}+[D]_a\Pi_{Da1}}{\{[D]+[D]_d\}\Pi_{D2}+[D]_a\Pi_{Da2}} \equiv \rho^{ex,D}, \tag{S2a}$$

$$F_1^{A\prime}/F_2^{A\prime} = \frac{\varepsilon_1^A}{\varepsilon_2^A} \frac{\{[A]+[A]_a\}\Pi_{A1}+[A]_d\Pi_{Ad1}}{\{[A]+[A]_a\}\Pi_{A2}+[A]_d\Pi_{Ad2}} \equiv \rho^{ex,A}. \tag{S2b}$$

After dividing Eq. S1a by $Q^D$ and S1b by $Q^A$ and adding up the resulting expressions, we have:

$$\frac{F_1^{Da}}{Q^D} + \frac{F_1^{Ad}}{Q^A} = \frac{F_1^{D\prime}}{Q^D} + \frac{F_1^{A\prime}}{Q^A}. \tag{S3a}$$

Similarly, we obtain the following expression by combining Eqs. S1c and S1d:

$$\frac{F_2^{Da}}{Q^D} + \frac{F_2^{Ad}}{Q^A} = \frac{F_2^{D\prime}}{Q^D} + \frac{F_2^{A\prime}}{Q^A}. \tag{S3b}$$

Then, substituting $F_1^{D\prime}$ and $F_1^{A\prime}$ from Eqs. S2a and S2b, respectively, into Eq. S3a, and dividing the resulting equation by $\rho^{ex,D}$ we obtain

$$\frac{F_1^{Da}}{Q^D}\frac{1}{\rho^{ex,D}} + \frac{F_1^{Ad}}{Q^A}\frac{1}{\rho^{ex,D}} = \frac{F_2^{D\prime}}{Q^D} + \frac{F_2^{A\prime}}{Q^A}\frac{\rho^{ex,A}}{\rho^{ex,D}}. \tag{S4}$$

Subtracting Eq. S4 from Eq. S3b and rearranging the terms, we obtain:

$$F_2^{A\prime} = \left(F_2^{Ad} - F_1^{Ad}\frac{1}{\rho^{ex,D}} + F_2^{Da}\frac{Q^A}{Q^D} - F_1^{Da}\frac{1}{\rho^{ex,D}}\frac{Q^A}{Q^D}\right)(1-\rho^{ex,A}/\rho^{ex,D})^{-1}. \qquad \text{(S5)}$$

For pulsed excitation, $\Pi_D = \Pi_{Da} = 1$ and therefore Eqs. 32a and S2a provide that $F_1^{Da}/F_2^{Da} \equiv \rho^{ex,D}$, in which case Eq. S5 becomes

$$F_2^{A\prime} = \left(F_2^{Ad} - F_1^{Ad}\frac{1}{\rho^{ex,D}}\right)(1-\rho^{ex,A}/\rho^{ex,D})^{-1}. \qquad \text{(S5')}$$

Further, by solving Eq. S3a for $F_1^{D\prime}$ and using Eq. S2b to substitute for $F_1^{A\prime}$, we obtain

$$F_1^{D\prime} = F_1^{Da} + F_1^{Ad}\frac{Q^D}{Q^A} - F_2^{A\prime}\rho^{ex,A}\frac{Q^D}{Q^A}, \qquad \text{(S6)}$$

where $F_2^{A\prime}$ is determined from experiments via Eq. S5.

Finally, by inserting $F_1^{D\prime}$ from Eq. S6 into Eq. 36, we obtain:

$$E'_{app} = \left(1 + \frac{F_1^{Da}}{F_1^{Ad} - F_2^{A\prime}\rho^{ex,A}}\frac{Q^A}{Q^D}\right)^{-1}, \qquad \text{(S7)}$$

where $F_2^{A\prime}$ is connected to experiment via Eq. S5. For pulsed excitation, we may substitute $F_2^{A\prime}$ from Eq. S5' and obtain:

$$E'_{app} = \left[1 + \frac{F_1^{Da}(1-\rho^{ex,A}/\rho^{ex,D})}{F_1^{Ad} - F_2^{Ad}\rho^{ex,A}}\frac{Q^A}{Q^D}\right]^{-1}. \qquad \text{(S7')}$$

**SR2. Evaluating the effect of acceptor direct excitation on donor lifetime**

We may gain some understanding of the effect of acceptor direct excitation upon the donor fluorescence decay by evaluating the integral in the exponent of Eq. 14 in the main text using the approximations that the acceptor fluorescence follows the same exponential decay curve as it would in the absence of FRET, $\varphi_{A^*d,j}(t) = \exp(-t/\tau^A)$, and that all acceptors are equally excited by both laser light and via FRET. Inserting this expression into Eq. 14 of the main text and performing the integration, we obtain:

$$p_{D^*a}(t) = p_{D^*0}\exp\left[-t\left(1/\tau^D + \textstyle\sum_{j=1}^n \gamma_j^{tr}\right) + p_{A^*0}\tau^A(1-e^{-t/\tau^A})\textstyle\sum_{j=1}^n \gamma_j^{tr}\right]. \qquad \text{(S8)}$$

Figure S1 illustrates the small effect of the competition between energy transfer and direct excitation upon the fluorescence decay curves of the donors. As it can be seen, the donor fluorescence decreases slower with time for $p_{A^*0} > 0$. This deviation of the donor decay curve from the ideal situation wherein no acceptor is excited directly by laser light is smaller when the oligomer contains only one acceptor or the FRET efficiency is small.

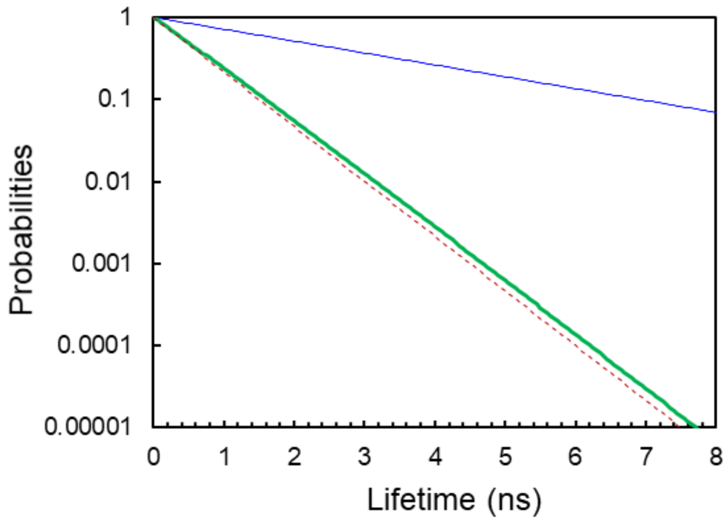


**Figure S1.** Fluorescence decay curves for donors in the absence (thin solid line) and presence of FRET with (thick solid line) and without (thin dashed line) the correction for competition between energy transfer and direct excitation of acceptors. We assumed that there are three acceptors and one donor in each molecular complex (i.e., the quaternary structure is that of a tetramer). The value of the donor lifetime used for simulating the thin solid line using equation (S8) with $\gamma_1^{tr} = \gamma_2^{tr} = \gamma_3^{tr} = 0$ and $p_{A^*0} = 0$ (i.e., in the absence of acceptors) was $\tau^D = 3 \times 10^9 s$ (36). The energy transfer rates used additionally for simulating the dashed line with equation (S5) in the presence of acceptors but absence of their direct excitation were $\gamma_1^{tr} = \gamma_2^{tr} = \gamma_3^{tr} = 4 \times 10^9 s^{-1}$. The effect of acceptor direct excitation (thick solid line) was incorporated by using the complete equation (S8) and the additional parameter values: $\tau^A = 3 \times 10^9 s$, $p_{A^*0} = 0.1$.

## SR3. Derivation of the probabilities expressions for CW excitation

For monomeric acceptors, solving equation (5a) from the main text together with $P_D = \int_{t_0}^{t}(1 - p_{D^*})dt = \delta t - P_{D^*}$ , we obtain

$$P_D = \frac{\delta t}{1 + I\mathcal{E}^D \tau^D}, \tag{S9}$$

where $I$ is the light irradiance (in W/m$^2$), $\tau^D$ is the donor lifetime in the absence of FRET, and $\mathcal{E}^D = \varepsilon^D(\lambda_{ex})\ln(10)\lambda_{ex}(hcN_A)^{-1}$, with $\lambda_{ex}$ being the excitation wavelength, $h$ Plank's constant, $c$ the speed of light, and $N_A$ Avogadro's number. Similarly, by solving equation (5a) together with $P_{Da} = \int_{t_0}^{t}(1 - p_{D^*a})dt = \delta t - P_{D^*a}$ , we obtain

$$P_{Da} = \frac{\delta t}{1 + I\mathcal{E}^D \tau^{Da}}. \tag{S10}$$

For monomeric acceptors, solving equation (5b) together with $P_A = \int_{t_0}^{t}(1 - p_{A^*})dt = \delta t - P_{A^*}$ , we obtain

$$P_A = \frac{\delta t}{1+I\mathcal{E}^A\tau^A}. \quad \text{(S11)}$$

Similarly, for dimers (i.e., one donor bound to one acceptor), by solving equation (5d) together with $P_{Ad} = \int_{t_0}^{t}(1 - p_{A^*d})dt = \delta t - P_{A^*d}$, we obtain

$$P_{Ad} = \frac{\delta t}{1+I\mathcal{E}^A\tau^{Ad}}\left(1 - \frac{EI\mathcal{E}^D\tau^A}{1+I\mathcal{E}^D\tau^{Da}}\right), \quad \text{(S12)}$$

where $\tau^{Ad} = \tau^A$, to a first approximation, and $\mathcal{E}^A = \varepsilon^A(\lambda_{ex})\ln(10)\lambda_{ex}(hcN_A)^{-1}$. The second term in the parenthesis reduces the acceptor integrated probability value, compared to that in the absence of FRET, because both FRET and laser light excite the acceptors, which spend less time in their ground state as a result.