Supplementary Materials for

A FLIM microscopy based on acceptor-detected Förster Resonance Energy Transfer.†

Roberto F. Delgadillo1, 2, 3, †, *, Katie A. Carnes4, Kathia Zaleta-Rivera5, Omar Olmos2 and Lawrence J. Parkhurst1,*

Correspondence to delgadillo@tec.mx and LJP1@unl.edu

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Supplementary Text: FLIM-trADFRET mathematical analysis.

**Traditional trDDFRET.** The energy transfer process arises from a resonant dipole-dipole interaction between appropriately paired fluorescent D and A, which results in energy transfer of excited-state D* to A (Fig. 1C). In the case of the traditional trDDFRET (Fig. 1D), the inter-dye distance calculations require the collection of two experimental intensities: 1) the D decay intensity, $i_D^{\text{Exc}/\text{Emi}}(t)$, and 2) the D in the presence of the A, $i_D^{\text{Exc}/\text{Emi}}(t_A)$. After deconvolution, they yield the fluorescence lifetimes, $\tau_D$ and $\tau_D(A)$, respectively. The superscripts “Exc” denoting excitation wavelength and “Emi” corresponds to collected emission. The D decay deconvolution yields $\tau_D$ or $\langle \tau_D(i) \rangle$ in the case of mono- or multiphasic decays, respectively (Eqn 1). In the latter case, the energy is lost by $\langle \tau_D(i) \rangle = 1/k_{F(D)} = 1/(k_{D^0} + \Sigma k_{i(D)})$ which is the reciprocal of the sum of the natural fluorescence rate ($k_{D^0}$ =1/τ$^0$) and the inactivation pathways ($\Sigma k_{i(D)}$), where $\langle \tau_D(i) \rangle = \Sigma \alpha_D i$ and it is the sum of the area under the curve for each of the $i^{th}$ lifetimes with its respective fractional contribution ($\alpha_{D(i)}$) so that $\Sigma \alpha_{D(i)} = 1$.

$$I_D^{\text{Exc}/\text{Emi}}(t) = \gamma_D k_{F(D)} (1 - S_D) D^*(0) \cdot \Sigma_{i=1}^n \alpha_{D(i)} \cdot \exp \left(-\frac{t}{\langle \tau_D(i) \rangle} \right)$$  
Eqn 1

The $\gamma_D$ term incorporates optical and instrumental considerations, such as the photomultiplier response and the filter (or monochromator) transmittance of the D emission, $S_D$ is the statically quenched D fraction, (1-$S_D$) is the fractional population of emitting D molecules, $D^*(0)$ is the concentration of excited state D molecules at time zero. Further, $D^*(0)=I_{0}^{\text{Abs}}$, where $I_{0}^{\text{Abs}}$ is the integrated excitation intensity for a non-saturating delta (δ) pulse excitation, and $Abs=\varepsilon_{F} \cdot c_{F} \cdot l$ is the D absorbance at the excitation wavelength.

When an A molecule is present nearby, the D* energy is transferred with an efficiency (Fig 1G, 1H) dependent on the Förster distance $R_0$ (Eqn 2) where $\tau^0_D$ is the D natural lifetime on the
duplex when $\Sigma k_i$ is zero $^{15,42}$; the numerical value (9790) is a collection of universal constants for
distance ($R$) calculations in angstroms (Å); $J(\lambda)$ is the spectroscopic overlap of the normalized
donor emission, $F_D(\lambda)$, and the acceptor absorbance, $\varepsilon_A(\lambda)$, with $\lambda$ in cm; $\kappa^2$ is the square of the
angular part of the dipole-dipole interaction $^{43}$, and $\bar{n}$ is the refractive index $^{44}$ (DatabaseS2).

$$R_0 = 9790 \left( \tau_D \cdot J(\lambda) \cdot \kappa^2 \cdot \bar{n}^{-4} / \tau_D^0 \right)^{1/6} \text{Å}$$

Eqn 2

In traditional trDDFRET, $\tau_D(\lambda)$ is affected by the inter-dye $R$ (Eqn 3, Fig. 1I and 1J) where
the energy transfer rate constant ($k_t$) is a function of $R$ (Eqn 4, Fig. 1E and 1F).

$$I_{D(A)}^{520 \text{nm}}(t) = \gamma_{520 \text{nm}} \cdot F_{D\text{exc}} \cdot (1 - S_D) \cdot D'(0) \cdot J_{520}^\infty P(R) \sum_{i=1}^n \alpha_{Di} \exp\left[-\left(\tau_{Di}^{-1} + k_t\right) \cdot t\right] dR$$

Eqn 3

$$k_t = \left(\frac{1}{\tau_D}\right) \left(\frac{R_0}{R}\right)^6$$

Eqn 4

The inclusion of the probability distribution function, $P(R)$ enables the determination of a
unimodal distribution $^{27}$ with a mean inter-dye distance, $\bar{R}$, and a standard deviation, $\sigma$ (Eqn 5) $^{29,45}$. However, the distribution function can be altered for a more elaborated distribution, such as a
skewed distribution.

$$P(R) = \frac{1}{\alpha \sqrt{\pi}} \exp\left[-(R - \bar{R})^2 / 2 \sigma^2\right]$$

Eqn 5

The $\bar{R}$, and $\sigma$, are fitted (Eqn 3) using nonlinear regression algorithms or a method of
moments $^{28,45}$. In such trDDFRET analyses, the initial intensity is set equal to 1 at time zero ($t_0$)
because everything to the right of $D^*(0)$ in Eqn 1 and Eqn 3, are normalized, and the optical and
instrumental factors to the left of the integral are disregarded from further consideration $^{15,43}$.

**The FLIM-trADFRET** key feature is the time course of the sensitized A emission,

$$I_{\text{trADFRET}}^{\text{Exc/Emi}}(t)$$

which derives solely from energy transferred from $D^*$ (Fig. 1K-1L) and it is
proportional to the excited A* concentration, as a function of time, yielding an A photon emission
as a function of time described by $d(h_{V(A)})/dt$. Following the $\delta$ excitation pulse, the excited $D^*$
transfers energy to A to yield excited A* whose concentration reaches a maximum at a time \( t_{\text{max}} \) and later it decays exponentially to zero following a sequential kinetic process (Fig. 1C) that is expressed in the following trADFRET equation (Eqn 6).

\[
I_{\text{trADFRET}}^{\text{Exc/Emi}}(t) = \gamma_A \cdot k_{F(A)} \cdot (1 - S_A) \cdot (1 - S_D) \cdot D^*(0) \cdot k_t \left\{ \frac{\exp[-(\tau_D^{-1} + k_t) t]}{\tau_A^{-1} - \tau_D^{-1}} \right\} \text{ Eqn 6}
\]

where \( \gamma_A \) is the A analog to \( \gamma_D \) (Eqn 3), \( k_{F(A)} \) is the rate of A fluorescence, \( S_A \) is the statically quenched population of A, (1-\( S_A \)) is the fractional population of emitting A, and the (1-\( S_D \)) and \( D^*(0) \) terms were defined previously. To include the \( P(R) \) function and multiple lifetimes, the Eqn 6 is modified resulting in Eqn 7.

\[
I_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) = \gamma_A^{620\text{ nm}} \cdot k_{F(A)} \cdot (1 - S_A) \cdot (1 - S_D) \cdot D^*(0) \cdot \int_0^\infty P(R) k_t \sum_{i=1}^n \sum_{j=1}^m \left\{ \frac{\sigma_{D1} \exp[-(\tau_D^{D1} + k_t) t] - \sigma_{A1} \exp[-(\tau_A^{A1} \cdot t)]}{\tau_A^{A1} - \tau_D^{D1}} \right\} dR \text{ Eqn 7}
\]

In practice, the primary challenge is the isolation of sensitized \( I_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) \), from the observed trADFRET intensity, \( S_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) \) (Eqn 8), which contains also two contaminating signals that are indicated below by indices (i and ii) \(^{11,46-48}\).

\[
S_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) = I_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) + (i) I_{D(A)}^{\text{Exc/620 nm}}(t) + (ii) I_A^{\text{Exc/620 nm}}(t) \text{ Eqn 8}
\]

(i) The first contaminating signal is the D intensity in the presence of the A, \( I_{D(A)}^{\text{Exc/620 nm}}(t) \) (Eqn 9), since there is a leaked signal through the 620 nm interference filter, also known as filter cross-talking. The Eqn 9 is an updated version of Eqn 3 with the \( \gamma_D^{620\text{ nm}} \) term indicating that the signal was collected at 620 nm rather than at 520 nm.

\[
I_{D(A)}^{\text{Exc/620 nm}}(t) = \gamma_D^{620\text{ nm}} \cdot k_{F(D)} \cdot (1 - S_D) \cdot D^*(0) \cdot \int_0^\infty P(R) k_t \sum_{i=1}^n \sigma_{Di} \exp[-(\tau_{Di}^{-1} + k_t) t] dR \text{ Eqn 9}
\]

(ii) The second contaminating signal is the A intensity due to direct excitation, \( I_A^{\text{Exc/620 nm}}(t) \) (Eqn 10) collected through the 620 nm filter where \( \tau_{ij} \) is equivalent to \( \tau_{Di} \) (Eqn 1). Experimentally, it can be obtained for each duplex series, e.g. Xr*DNA\(^{481}\) nm/620 nm for the
FluoTime and in the case of the LaserStrobe with Xr*DNA_{ds}\textsuperscript{585 nm/620 nm}, and double-labeled duplex, Xr*DNA\textsubscript{ds} *Fl\textsuperscript{585 nm/620 nm}, since D is not excited at that 585 nm.  

\[ I_\text{Exc/620 nm}^A(t) = \gamma_\text{Exc/620 nm} A_0 \cdot k_{F(A)} \cdot (1 - S_A) \cdot A^*(0) \cdot \sum_{j=1}^n \alpha_{Aj} \exp \left( -\frac{t}{\tau_{Aj}} \right) \]  

Eqn 10

The second issue is the extent of \( i \) and \( ii \) to the observed \( S_{\text{ADFRET}}^{\text{Exc/620 nm}}(t) \) in Eqn 8, as they depend on the experimental variables and optical terms. Consequently, our trADFRET strategy is centered on knowing the “r” ratio intensity at time zero \( (t_0) \) of \( ii \) over \( i \) for the double-labeled duplexes at 620 nm for a given excitation wavelength. To obtain the “r” value, we normalize \( S_{\text{ADFRET}}^{\text{Exc/620 nm}} \) to 1 (this normalization is indicated by a line over the term; e.g., \( \bar{I} \) or \( \bar{S} \)) at \( t_0 \) when the energy transfer has not yet occurred, and the sensitized trADFRET is zero \( (I_{\text{ADFRET}}^{\text{Exc/620 nm}}(t_0) = 0) \); therefore, the initial intensity derives exclusively from the sum of leaked D(A) and direct A intensity \( (i + ii) \), thus yielding the “r” value (Eqn 11).

\[ r = \frac{ii}{i} = \frac{I_{\text{ADFRET}}^{\text{Exc/620 nm}}(t_0)}{I_{D(A)}^{\text{Exc/620 nm}}(t_0)} \]  

Eqn 11

The “r” value yields sufficient information to accurately scale these two contributions to attain the sensitized \( I_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) \) (Eqn 12). The deconvoluted intensities \( S_{\text{ADFRET}}^{\text{Exc/620 nm}}, I_{D(A)}^{\text{Exc/620 nm}} \) and \( I_{A}^{\text{Exc/620 nm}} \) can be also expressed with the corresponding derived lifetimes, \( \tau_{\text{trADFRET}}, \langle \tau_{D(A)} \rangle \) and \( \langle \tau_A \rangle \), respectively.

\[ I_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) = S_{\text{trADFRET}}^{\text{Exc/620 nm}}(t) - \left[ \left( \frac{1}{1+r} \right) \cdot I_{D(A)}^{\text{Exc/620 nm}}(t) + \left( \frac{r}{1+r} \right) \cdot I_{A}^{\text{Exc/620 nm}}(t) \right] = \tau_{\text{trADFRET}} - \left( \frac{1}{1+r} \right) \langle \tau_{D(A)} \rangle + \left( \frac{r}{1+r} \right) \langle \tau_A \rangle \]  

Eqn 12

We propose three methods to evaluate the “r” value:

a) Background lifetime. The procedure utilizes a standard solution of A (Xr*DNA_{ds}) and D (N*Fl or N*Flint) duplexes that are prepared in the reference cuvette of a double-cuvette spectrophotometer with the exact dye absorbance as the double-labeled duplex of interest.
(TableS1) which is placed in the sample compartment. Successive aliquots of A and D duplexes are added until a flat line in the differential spectra appears (Fig. S3C, red line). The lifetime of the standard solution, $\langle \tau_{std} \rangle$, does not have a FRET component, however, it can be used to fit for the pre-exponential parameters (brackets, Eqn 13) of the well-known D and A lifetimes, whose ratio yields the “r” value (Fig S2, DatabaseS7). In the general case of multiple lifetimes, the $\langle \tau_D \rangle$ and $\langle \tau_A \rangle$ terms correspond to $\Sigma \alpha_i \tau_i$, with $\Sigma \alpha_i$ always equal to 1.

$$\langle \tau_{std} \rangle = \left[ \frac{1}{(1+r)} \right] \langle \tau_D \rangle + \left[ \frac{r}{(1+r)} \right] \langle \tau_A \rangle$$  

Eqn 13

b) The filter ratio method. It finds the leaked D(A) intensity, $I_{D(A)}^{Exc/620 \text{ nm}}(t_0)$, of the Xr*N ds*Fl or Xr*N ds*Fl int by adjusting the intensity collected with the 520 nm interference filters, $I_{D(A)}^{Exc/520 \text{ nm}}(t_0)$ (Eqn 14). It is carried out by obtaining the intensity ratio of the single labeled D (e.g. N ds*Fl or N ds*Fl int) acquired with both interference filters at the same DNA ds concentration and the same excitation intensity (Eqn 14, in parenthesis). The resulted $I_{D(A)}^{Exc/620 \text{ nm}}(t_0)$ is set to be equal to the $I_{D(A)}^{Exc/620 \text{ nm}}(t_0)$, in Eqn 12 and consequently, the remaining signal is the contribution of $I_{D(A)}^{Exc/620 \text{ nm}}(t_0) = \left[ \frac{1}{(1+r)} \right] \cdot I_{D(A)}^{Exc/620 \text{ nm}}(t_0)$, in Eqn 12 and consequently, the remaining signal is the contribution

$$I_{D(A)}^{Exc/620 \text{ nm}}(t_0) = \left[ \frac{1}{(1+r)} \right] \cdot I_{D(A)}^{Exc/520 \text{ nm}}(t_0)$$  

Eqn 14

c) The spectroscopy method. It independently evaluates “r” using the dye spectroscopic parameters on the duplexes, (Eqn 15, DatabaseS9).

$$r = \frac{\mathcal{E}_{Xr} \cdot QY_{Xr} \cdot \tau_{Fl} \cdot \int Xr \cdot \Phi_{Fl} \cdot T(\lambda) \cdot P(\lambda) \cdot d\lambda}{\mathcal{E}_{Fl} \cdot QY_{Fl} \cdot \tau_{Fl} \cdot \int Fl \cdot \Phi_{Fl} \cdot T(\lambda) \cdot P(\lambda) \cdot d\lambda} = \frac{\mathcal{E}_{A} \cdot (1-S_A) \cdot \Phi_D \cdot \tau_D \cdot \int A \cdot \Phi_D \cdot T(\lambda) \cdot P(\lambda) \cdot d\lambda}{\mathcal{E}_{D} \cdot (1-S_D) \cdot \Phi_D \cdot \tau_D \cdot \int D \cdot \Phi_D \cdot T(\lambda) \cdot P(\lambda) \cdot d\lambda}$$  

Eqn 15

Where $\mathcal{F}_{Xr}$ (or $\mathcal{F}_{A}$) and $\mathcal{F}_{Fl}$ (or $\mathcal{F}_{D}$) integrals are of the form $\int \mathcal{F}(\lambda) \cdot T(\lambda) \cdot P(\lambda) \cdot d\lambda$ where I is the corrected emission spectrum normalized to an area of 1, $P(\lambda)$ is the PMT response as a function of wavelength. The $T(\lambda)$ term is the transmission factor for the filter or monochromator, and it is the
most strongly dependent on wavelength. The $\varepsilon$ term is the absorbance at the excitation wavelength, and $QY$ is the quantum yield so that $QY = (1-S) \cdot \Phi$, where $S$ is the fraction of molecules that are statically quenched, $(1-S)$ is the fluorescence emitting fraction, and $\Phi$ is the dynamic lifetime, which is the ratio of the observed lifetime and the natural lifetime ($\Phi = \Sigma \alpha_i \tau_i / \tau^0 = \langle \tau \rangle / \tau^0$) \(^{27}\).

**The trADFRET distance calculation below 120 Å.** To simplify calculations, we grouped the optical terms and intrinsic dye properties within a $Gain$ term that arises from the normalization procedure to make calculations independent of laser intensity (Eqn 16).

$$Gain = \left[ \frac{\gamma_A^{620\text{ nm}} k_F(A)(1-S_A)(1-S_D) \cdot D^*(0)}{\gamma_D^{620\text{ nm}} k_F(D)(1-S_D) \cdot D^*(0)} \right] = \frac{\tau_D(1-S_A)}{\tau_A}$$

Eqn 16

Since the optical train is the same for both dyes, the $\gamma_A/\gamma_D$ ratio can be eliminated. Later, we substituted $\frac{\tau_D}{\tau_A} = r \cdot \frac{\varepsilon_D(1-S_D) \cdot \phi_D \cdot \int D}{\varepsilon_A(1-S_A) \cdot \phi_A \cdot \int A}$ from the second equality in Eqn 15, to express $Gain$ in terms of more accessible experimental quantities (Eqn 17).

$$Gain = r \cdot \frac{\varepsilon_D(1-S_D) \cdot \phi_D \cdot \int D}{\varepsilon_A \cdot \phi_A \cdot \int A}$$

Eqn 17

The non-linear fitting of the sensitized $I_{trADFRET}^{Exc/620\text{ nm}}(t)$ (Eqn 18) yields the $Gain$ that establishes the curve peak, and the $\bar{R}$ and $\sigma$ values which are equivalent to those obtained by trDDFRET (Eqn 7) \(^{29}\).

$$I_{trADFRET}^{Exc/620\text{ nm}}(t) = Gain \cdot \int_0^{\infty} P(R) \cdot k_t \cdot \sum_{i=1}^{n} \sum_{j=1}^{m} \left( \frac{\alpha_{D_i} \exp(-((\tau_{D_i}^{-1}+k_t)\cdot t)-\alpha_{A_j} \exp(-\tau_{A_j}^{-1} \cdot t))}{\tau_{A_j}^{-1}-(\tau_{D_i}^{-1}+k_t)} \right) dR$$

Eqn 18

**The trADFRET distance calculations above 120 Å.** The $\bar{R}$ and $\sigma$ parameters are highly correlated in the range 100-120 Å (Fig. S2), and it is not possible to separate them, therefore, we simplified our trADFRET analysis by defining a new term, the transfer quantum yield ($\Phi_t$) which is the energy fraction of $D^*$ transfer to the A shown in Eqn 19 for a single donor or multiphase decays, $\tau_D$ and $\langle \tau_{D_i} \rangle$, respectively.
\[
\Phi_t = \sum \frac{\alpha_i k_t}{(k_t + 1/\langle\tau_D\rangle)} = \sum \frac{\alpha_i k_t}{(k_t + (\tau_{Di} + 1)/\langle\tau_{Di}\rangle)} \]

\[
= \sum \frac{\alpha_i k_t \langle\tau_{Di}\rangle}{(k_t (\tau_{Di}) + 1)} = \frac{k_t \tau_D}{(k_t \tau_D + 1)}
\]

Eqn 19

Accordingly, the trADFRET calculation in Eqn 18 can be simplified but it requires that the integration over the distance being neglected by setting \(P(R)\) equal to 1, yielding a single average distance \(\langle r \rangle_{Rs}\). Consequently, the area under the \(I_{trADFRET}^{Exc/620\ nm}(t)\) in terms of lifetimes (Eqn 12) is proportional to the steady-state intensity at the detected wavelength, revealing a key relationship between \(\Phi_t\) and \(\tau_A\) (Eqn 20).

\[
I_{ADFRET}^{Exc/620\ nm}(t) = \tau_{ADFRET}^{Obs} - \left[ \frac{1}{(1+r)} \right] \langle \tau_{D(A)} \rangle + \left[ \frac{r}{(1+r)} \right] \langle \tau_A \rangle = Gain \cdot \Phi_t \langle \tau_A \rangle
\]

Eqn 20

The last equality in Eqn 20 is consistent with the insight that sensitized \(I_{trADFRET}^{Exc/Em}(t)\) (Eqn 6) must be proportional to the product of \(\Phi_t\) and \(QY_A\) which is defined in Eqn 21.

\[
QY_A = \Phi_A (1 - S_A) = \langle (\tau_A)/d_A \rangle \cdot (1 - S_A) = \langle \tau_A \rangle \cdot k_F(A) \cdot (1 - S_A)
\]

Eqn 21

When FRET is present the \(\tau_{Di(A)}\) can be expressed also in terms of \(\Phi_t\) (Eqn 22).

\[
\langle \tau_{D(A)} \rangle = \langle \tau_{Di} \rangle + \langle \tau_{Di} \cdot \Phi_t \rangle
\]

Eqn 22

Then, we substitute Eqn 22 into Eqn 20 resulting in Eqn 23.

\[
I_{trADFRET}^{Exc/620\ nm}(t) = \tau_{trADFRET}^{Obs} - \left[ \frac{1}{(1+r)} \right] \langle \tau_{Di} \rangle + \left[ \frac{r}{(1+r)} \right] \langle \tau_A \rangle = Gain \cdot \Phi_t \langle \tau_A \rangle
\]

Eqn 23

A second key insight leads to further simplification and relies on the fact that \(\langle \tau_{std} \rangle\) exactly mimics the double-labeled duplex (Xr*N*Fl and Xr*N*Flint) background (Eqn 13) and we can obtain a lifetime difference \(\langle \tau_{Diff} \rangle\) according to Eqn 24, yielding a very useful relationship in the most right equality.

\[
\langle \tau_{Diff} \rangle = \tau_{trADFRET}^{Obs} - \langle \tau_{std} \rangle = Gain \cdot \Phi_t \cdot \langle \tau_A \rangle - \frac{1}{(1+r)} \cdot \tau_{Di} \cdot \Phi_t \langle \tau_A \rangle = \Phi_t \left[ Gain \cdot \langle \tau_A \rangle - \frac{1}{(1+r)} \langle \tau_{Di} \rangle \right]
\]

Eqn 24

Then by substituting \(\Phi_t\) (Eqn 19), we can obtain \(\langle \tau_{Diff} \rangle\) for the case of multiple \(\langle \tau_{Di} \rangle\) (Eqn 25) and a single \(\tau_D\) lifetime (Eqn 26), such as observed in the single Fl labeled oligos for the N’ and N series, respectively.
\[ \langle \tau_{\text{diff}} \rangle = \sum_{i=1}^{n} \left[ \frac{a(k_t \cdot \langle \tau_{D_i} \rangle)}{(k_t \cdot \langle \tau_{D_i} \rangle + 1)} \right] \left[ \text{Gain} \cdot \tau_A - \frac{\langle \tau_{D_i} \rangle}{(1 + r)} \right] \]  
\text{Eqn 25}

\[ \langle \tau_{\text{diff}} \rangle = \left[ \frac{k_t \cdot \tau_D}{(k_t \cdot \tau_D + 1)} \right] \left[ \text{Gain} \cdot \tau_A - \frac{\tau_D}{(1 + r)} \right] \]  
\text{Eqn 26}

At distances beyond 120 Å, further simplification can be carried out since the \( k_t \cdot \langle \tau_{D_i} \rangle \) and \( k_t \cdot \tau_D \) in the denominator are very small, so that \( k_t \cdot \tau_D \ll 1 \) and \( k_t \cdot \langle \tau_{Di} \rangle \ll 1 \) (Eqn 27) for multi- (middle equality) or monophasic (right equality) decays, respectively. Now, the \( k_t \) in Eqn 4 can be modified by changing \( R \) for \( \tau_{R_s} \) (Eqn 28).

\[ \langle \tau_{\text{diff}} \rangle = \sum_{i=1}^{n} k_t \cdot \langle \tau_{D_i} \rangle \left[ \text{Gain} \cdot \tau_A - \frac{\langle \tau_{D_i} \rangle}{(1 + r)} \right] = k_t \cdot \tau_D \left[ \text{Gain} \cdot \tau_A - \frac{\tau_D}{(1 + r)} \right] \]  
\text{Eqn 27}

\[ k_t = \left( \frac{1}{\tau_D} \right) \left( \frac{R_0}{\tau_{R_{ss}}} \right)^6 \]  
\text{Eqn 28}

At this point, we substitute \( k_t \cdot \tau_D \) from Eqn 27 into Eqn 28 to obtain \( \tau_{R_{ss}}^6 \) (Eqn 29), as our main FLIM-trADFRET for our N series that has a single donor decay.

\[ \tau_{R_{ss}}^6 = \frac{R_0^6}{k_t \cdot \tau_D} = \frac{R_0^6}{\left( \frac{\text{Gain} \cdot \tau_A - \tau_D}{(1 + r)} \right)^{\langle \tau_{\text{diff}} \rangle}} \]  
\text{Eqn 29}

Since the energy transfer depends on the reference \( \tau_D \) (Eqn 28), we can approximate the term \( \sum_{i=1}^{n} k_t \cdot \langle \tau_{Di} \rangle \sim k_t \cdot \tau_D \), and have a relationship for multiple lifetimes (Eqn 30), as seen in the case of the multiphasic donor in the N’ series.

\[ \tau_{R_{ss}}^6 = \frac{R_0^6}{\sum_{i=1}^{n} k_t \cdot \langle \tau_{Di} \rangle} = \sum_{i=1}^{n} \frac{R_0^6}{\left( \frac{\text{Gain} \cdot \tau_A - \langle \tau_{Di} \rangle}{(1 + r)} \right)^{\langle \tau_{\text{diff}} \rangle}} \]  
\text{Eqn 30}
**Fig. S1.** (A) The structure of the double-labeled N’ oligos labeled with 5’-Xr and 3’-Fl attached by a 6-carbon long linker to the 5’ phosphate of guanine and the 3’ phosphate of cytosine, respectively. (B) The structure of the double-labeled 29N’, the number 29 is the number of nucleotides that separate the dyes. (C) The structure of the double-labeled N oligo series labeled with Fl attached to d-thymine (dT-Fl_int) by a 12-atom linker and the 5’Xr. (D) Structure of the double-labeled 56N, with 56 nucleotide interdye separation with 5 extra nucleotides at the 3’ end.
**Fig. S2** Standard mixture preparation and “r” ratio by Method a. (A, B) The successive differential spectra of the double-labeled duplex and the standard mixture of D (Fl or Fl<int>) and A (Xr) single labeled duplexes for the N and N’, respectively; when the absorbance peaks are matched, a flat line appears (C, red line) The $\langle \tau_{\text{std}} \rangle$ is acquired with an aliquot of the standardized mixture and the fitting algorithms can find the pre-exponentials (Eqn 13) to obtain the “r” value or $I_A/I_D(A)$ cross-talking ratio (DatabaseS7). (D) The N’ series “r” values for both instruments are not the same since the excitation and detection systems are different. The “r” ratio for the 34N oligo is shown for the FluoTime and the N series values are shown in Table 3.
Fig. S3. The “r” value calculated by Method b. (A) The instrument response function (IRF, blue) and raw intensities of donor single labeled N’*Fl duplex collected by the 620-nm (orange) and 520-nm interference filter (yellow) and the respective deconvoluted curves (B) whose relation at time zero is the $I_D^{Exc/620 \text{ nm}}(t_0)/I_D^{Exc/520 \text{ nm}}(t_0)$ ratio (Eqn 14, DatabaseS8). The $S_{NADFRET}$ and $I_{D(A)}$ (trDDFRET) raw data of 14N’ (C) and 29N’ (D) duplexes collected by 620-nm and 520-nm interference filters. (E) The 14N’ $S_{NADFRET}$ (green) and $I_{D(A)}$ (trDDFRET, brown) deconvoluted decays, the latter is multiplied by the $Fl$ ratio to yield the “r” value at 620 nm filter (orange). (F)
The 14N’ sum (blue) of the leaked $I_{D(A)}$ (orange) and directly excited $I_A$ (Xr*N’, pink) is removed from the observed $S_{trADFRET}$ (green) to obtain the sensitized $I_{trADFRET}$ (red).
Fig. S4. The “r” ratio calculated by Method c. (A, B) The excitation ratio was calculated by overlapping the absorbance spectra of the N’ and N series, respectively. The donor emission spectra of N*Fl and N*Fl_int (orange) are set at the corresponding $\varepsilon_{\text{max}}$ of 75,600 M$^{-1}$cm$^{-1}$ (± 800 M$^{-1}$cm$^{-1}$) and 85,000 M$^{-1}$cm$^{-1}$ (± 1,500 M$^{-1}$cm$^{-1}$), respectively. The singly labeled A (pink) was normalized to $\varepsilon_{\text{max}}$ of 121,000 M$^{-1}$cm$^{-1}$ (± 2,000 M$^{-1}$cm$^{-1}$). The sum of the single labeled spectra (green) overlaps with the double-labeled spectrum (blue). (C) The Fl/Xr excitation ratio vs wavelength for the N’ (blue) or N (red) series. The LaserStrobe excitation was selected at 481 nm since we seek to minimize the A emission and FluoTime was fixed to 470 nm. (D) The A (pink) and D corrected emission spectra (N'*Fl, yellow and N*Fl_int, green) normalized to a total area of
1 and the transmittance of 520 nm (blue, 48.2%) and 620 nm interference filter (dark blue, 52.3%).

(E) Integrated photon intensity of $\int A$ (pink) and $\int D$ (N'*Fl, yellow and N*Fl_{int}, green) that passed the 520 nm and 620 nm interference filters to obtain the “$r$” value (Eqn 15, Table S1, DatabaseS9).
Fig. S5 The Gain, and $t_{\max}$ dependence for sensitized $I_{trADFRET}$ as a function of $\bar{R}$ and $\sigma$. The $\bar{R}$ was varied from 60 to 120 Å at 5 Å intervals and $\sigma$ from 3 to 9 Å at 2 Å intervals. The simulations were carried out with Eqn 18 and 29N’ data, stepping through $\bar{R}$ and $\sigma$ and using simple rectangular integration with a very small time step. The insert shows that, while Gain can be determined at long distances, very high precision is required to determine $\sigma$. The Gain and $t_{\max}$ are the two observable features and the former correlates well with the area under the sensitized $I_{trADFRET}$ curve (DatabaseS10).
Table S1. The “r” ratio was acquired with “Method c” for both series (Database9) a.

| LaserStrobe (Exc 481 nm) | \( \varepsilon \) (M\(^2\)cm\(^{-1}\)) | QY | \( \tau \) (ns) | \( f \) | “r” ratio |
|-------------------------|-----------------|-----|-------------|------|---------|
| N’ series               |                 |     |             |      |         |
| Xr*N’                  | 3247.9 (± 54.0) | 0.80 (± 0.02) | 5.28 (± 0.01) | 6.824 (± 0.100) | 1.957 (± 0.221) |
| N’*Fl                  | 43295.4 (± 459) | 0.22 (±0.01) | 2.86 (± 0.03) | 0.515 (± 0.050) |
| ratio                  | 0.075 (± 0.001) | 3.64 (± 0.19) | 0.54 (± 0.01) | 13.244 (± 1.300) |
| FluoTime (Exc 470 nm)  | \( \varepsilon \) (M\(^2\)cm\(^{-1}\)) | QY | \( \tau \) (ns) | \( f \) |
| Xr*N’                  | 2629.1 (± 43.0) | 0.80 (± 0.02) | 5.21 (± 0.01) | 6.82 (± 0.10) | 3.416 (± 0.538) |
| N’*Fl                  | 32731.4 (± 347) | 0.22 (± 0.01) | 3.05 (± 0.04) | 0.34 (± 0.05) |
| ratio                  | 0.080 (±0.002)  | 3.64 (± 0.19) | 1.71 (± 0.02) | 19.95 (± 2.93) |

| LaserStrobe (Exc 481 nm) | \( \varepsilon \) (M\(^2\)cm\(^{-1}\)) | QY | \( \tau \) (ns) | \( f \) | “r” ratio |
|-------------------------|-----------------|-----|-------------|------|---------|
| N series                |                 |     |             |      |         |
| Xr*N                   | 3535.7 (± 58.0) | 0.80 (± 0.02) | 5.28 (± 0.01) | 6.824 (± 0.100) | 0.429 (± 0.046) |
| N*Fl\(_{tot}\)         | 56692.5 (± 1000.0) | 0.83 (± 0.02) | 2.84 (± 0.03) | 0.515 (± 0.050) |
| ratio                  | 0.062 (± 0.002)  | 0.96 (± 0.03) | 1.86 (± 0.02) | 13.244 (± 1.300) |
| FluoTime (Exc 470 nm)  | \( \varepsilon \) (M\(^2\)cm\(^{-1}\)) | QY | \( \tau \) (ns) | \( f \) |
| Xr*N                   | 2908.6 (± 48.1) | 0.80 (± 0.02) | 5.21 (± 0.01) | 6.82 (± 0.10) | 0.874 (± 0.135) |
| N*Fl\(_{tot}\)         | 37741.5 (± 666.0) | 0.83 (± 0.02) | 3.05 (± 0.04) | 0.34 (± 0.05) |
| ratio                  | 0.077 (± 0.002)  | 0.96 (± 0.03) | 1.71 (± 0.02) | 20.07 (± 2.97) |

\(^{a}\)620-nm interference filter
Table S2. The “r” ratio of the three methodologies for N’ series and the respective cross-talking $I_A/I_{D(A)}$ ratio of leaked intensities.

| Method* | LS N’ series | Cross talking | $I_A/I_{D(A)}$ ratio$^b$ |
|---------|--------------|---------------|---------------------------|
| a       | 1.891 (± 0.069) | $I_{(A)} \cdot \frac{r}{1+r}$ | 0.654 (± 0.023) |
| b       | 1.825 (± 0.065) | $I_{D(A)} \cdot \frac{1}{1+r}$ | 0.346 (± 0.012) |
| c       | 1.957 (± 0.221) |               |                           |
| Average | 1.891 (± 0.066) |               |                           |

| Method | FT N’ series | Cross talking | $I_A/I_{D(A)}$ ratio |
|--------|--------------|---------------|---------------------|
| a      | 3.450 (± 0.045) | $I_{(A)} \cdot \frac{r}{1+r}$ | 0.774 (± 0.066) |
| b      | NA           | $I_{D(A)} \cdot \frac{1}{1+r}$ | 0.226 (± 0.019) |
| c      | 3.416 (± 0.538) |               |                     |
| Average | 3.433 (± 0.292) |               | 10                  |

$^a$620-nm interference filter, Database9.

$^b$Ratio at $t=0$