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I. S. Osad'ko

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Dependence of FRET efficiency on distance in single donor-acceptor pairs

I. S. Osad’ko
Institute for Spectroscopy, RAS, 142190 Moscow, Russia

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Possibility to create single donor-acceptor (D-A) pairs by attaching dye molecules to various sites of DNA strands with control of the inter-dye distance \( R \) enables one to measure average Förster resonance energy transfer (FRET) efficiency \( E \) as a function of \( R \). Triplet states of the dyes influence the dependence \( E(R) \) considerably. Two types of FRET efficiency are considered: \( E = E_A \) and \( E = E_D \). The efficiency \( E_A(R) = J_A(R) / [J_A(R) + J_D(R)] \) depends on the donor and the acceptor average intensities \( J_D(R) \) and \( J_A(R) \) measured in D- and A-fluorescence, whereas the efficiency \( E_D(R) = 1 - J_D(R) / J_D(\infty) \) depends only on the intensity of D-fluorescence, so-called the donor quenching method. The shape of the functions \( E_D(R) \) and \( E_A(R) \) depends strongly on whether the dyes have blinking fluorescence. FRET efficiencies \( E_D(R) \) and \( E_A(R) \) undergo the influence of many experimental factors and therefore, differ considerably from pure FRET efficiencies \( E_D^s(R) \) and \( E_A^s(R) \). Pure FRET efficiencies \( E_{D,A}^s(R) \) are calculated with the help of rate equations for D-A pairs, whose molecules have triplet states. It is shown how the calculated efficiencies \( E_{D,A}^s(R) \) can be compared to FRET efficiencies measured with the help of the intensities \( I_{D,A}(R) \) corrected by cross talk and background light. © 2015 AIP Publishing LLC. [http://dx.doi.org/10.1063/1.4915279]

I. INTRODUCTION

Experiments with fluorescence of single donor-acceptor (D-A) pairs are a very effective tool for solving a variety of physical problems. As a rule, the efficiency of Förster resonance energy transfer (FRET) serves as a main source of physical information.\(^{1-5}\)

At present, energy transfer in single D-A pairs is studied with the help of pairs attached to macromolecules such as proteins,\(^{6,8} \) dendrimers,\(^{9} \) DNA strands,\(^{10-17} \) and even cells.\(^{18} \) Specially prepared D-A pairs with a chemical link and controlled inter-dye distance \( R \) have been investigated as well.\(^{19-21} \) In such an experimental scheme, we are able to fix inter-dye distance \( R \) and to control this distance in an experiment.

A very interesting example of the experiments of such a type was demonstrated by the Meller group.\(^{12,13,17} \) Detailed scheme of their experiment is depicted in Fig. 1 of Ref. 17. Experimental scheme used by the Meller group is shown in the simplified form in Fig. 1.

Single acceptor molecule (A) is attached in a definite site of single DNA strand. Single donor molecule (Dn) is attached on the distance of \( n \) nucleotides from the acceptor molecule. Efficiency of the energy transfer was studied with the help of fluorescence time traces. The shape of time traces of D- and A-fluorescence depends considerably on the value of the inter-dye distance \( R \).\(^{12,13,17} \)

The Förster rate \( F_n \) for the energy transfer from the donor molecule Dn separated from the acceptor molecule A by \( n \) nucleotides in DNA chain is given by the following expression: \( F_n = \frac{S_{Dn}}{R_n^6} \). Here, \( S \) includes the overlap of the donor emission and acceptor absorption spectra, \( a_n \) is an orientation factor resulting from the dipolar interaction, \( R_n \) is the inter-dye distance between the donor Dn molecule and the acceptor molecule.

If the orientation factor changes, for instance, 2 times, the Förster rate \( F_n \) also changes 2 times. However, if distance \( R_n \) changes 2 times, rate \( F_n \) changes 64 times. Orientations usually do not differ too much in the experimental scheme shown in Fig. 1. Therefore, in the first approximation, we may neglect the influence of variations of the orientation factor on the Förster rate and we will take the Förster rate in the following form:

\[
F(R) = \frac{S_A}{R^6}.
\]

As a rule, the distribution of FRET efficiency is measured in experiments.\(^{22,23} \) However, the longer the time scale of the measurement is, the narrower the distribution of the intensities, and the easier it is to find the average FRET efficiency expressed via the average intensities of fluorescence. The average FRET efficiency can be calculated with the help of rate equations as well.

The possibility to control inter-dye distance \( R \) enables one to find the shape of the function \( E(R) \) measured in an experiment. This dependence undergoes many experimental factors and it has not been calculated theoretically yet, although the dependence \( E(R) \) has already been measured in experiments of the Meller group.\(^{12} \)

The shape of the function \( E(R) \) depends strongly on:

1. fluorescence quantum yields \( \Phi_{D,A} \) of the donor and the acceptor molecule, efficiencies \( \eta_{D,A} \) of detection in D- and A-channels, leakage of D-photons to A-channel (cross talk), and intensity \( I_n \) of background light (noise).
2. Intra-molecular dynamics of the donor and the acceptor molecule.

Methods for finding information concerning factors of the type 1 have already been discussed in many papers.\(^{6,12,13,15-17} \) In order to take into account intra-molecular dynamics, we must solve rate equations for the D-A pair of the given type. It is presented here.
The purpose of the present paper is to study influence of factors 1 and 2 on the shape of the function, $E(R)$, for the average FRET efficiency taking into account the existence of triplet levels in dyes of D-A pair.

Triplet states of D-A pair influence the value of FRET efficiency considerably. Therefore, this influence deserves careful investigation with taking into account possible T-T absorption. Such analysis will be done in the present paper. Triplet states create pauses (off periods) in fluorescence of single dye molecules excited by continuous wave (CW) laser light and, therefore, fluorescence of such molecules consists of on/off periods. These off periods can manifest themselves, for instance, in autocorrelation function.24

Many dyes, for instance, Cy5 molecule used in many works as an acceptor molecule or TMR molecule used as a donor molecule,25,12,16 have blinking fluorescence. Analysis of such blinking fluorescence enables one to find rates of singlet-triplet transitions in single molecules. For instance, complex blinking fluorescence time traces measured by Barbara group25 in fluorescence of single polymer molecule that underwent conformational changes has been treated in Ref. 26 on the basis of a theoretical model. The model allowed for both triplet states and conformational changes of the polymer molecule. All rate constants of the model have been found after treatment of experimental data.

Fluorescence of the molecules with short living triplet state will have too short off periods and therefore, in fact, such molecule can be considered as a two-level molecule with two singlet states: the ground and excited states. Blinking will emerge in fluorescence of the molecules with long lived triplet states. Such molecules should be described as a three-level molecule whose triplet level is situated between two singlet levels.

Recently, Osad’ko and Shchukina27 have calculated fluorescence time traces of 2D-2A, 3D-2A, 2D-3A, and 3D-3A pairs consisting of two- and three-level dyes with the help of Monte Carlo technique. It was shown that a qualitative shape of the fluorescence time trace enables one to understand which type of the D-A pair is able to create a time trace of the given type. Triplet states in the acceptor molecule reduce FRET efficiency considerably and full energy transfer cannot be possible in 2D-3A and 3D-3A pairs. Earlier, this important result for FRET efficiency was found in Ref. 28 as well. However, Refs. 27 and 28 ignored the above mentioned experimental factors of type 1.

FRET efficiency is measured either for freely diffusing single D-A pairs3,8,29 or for immobilized single D-A pairs.2,11,12,16 Only immobilized single D-A pairs will be considered in the present paper. Two types of the average FRET efficiency will be considered in the paper: (1) the efficiency $E_A$ for which we should measure intensities of D- and A-fluorescence in single D-A pairs, (2) the efficiency $E_D$ for which we use only D-fluorescence in single D-A pairs, i.e., the donor quenching method. We will find the dependence of both efficiencies on the inter-dye distance, i.e., the functions $E_A$ and $E_D$ which can be measured in experiments carried out in accordance with the scheme shown in Fig. 1. Since triplet levels influence considerably FRET efficiency, we will investigate also the role of possible T-T* absorption in energy transfer. It will be shown that excited triplet levels can take part in FRET as well.

II. PURE AND MEASURED EFFICIENCIES

Efficiencies of two types can be considered.12,16 The efficiency

$$E_A = \frac{J_A}{J_A + \gamma J_D},$$

in which $\gamma = \eta_A \Phi_A / \eta_D \Phi_D$ defines the ratio of fluorescence quantum yields $\Phi_{D,A}$ and efficiencies $\eta_{D,A}$ of detection in D- and A-channels. The intensities

$$J_D = I_D^s + \eta_D I_n^s,$$

$$J_A = I_A^s + \beta \eta_A I_D^s + \eta_A I_n$$

are the average intensities measured in an experiment. They take into account cross talk with the help of the coefficient $\beta$ and intensity $I_n^s$ of background light. Intensities $I_{D,A}^s$ are the intensities of pure signal. These intensities inform us about intra-molecular dynamics of the dyes excited by CW laser light. They can be calculated with the help of dynamical equations for a D-A pair. Physical model for D-A pair determines the dependence of the intensities $I_{D,A}^s(R)$ on R. Pure efficiency is expressed via these intensities as follows:

$$E_A^s(R) = \frac{I_A^s(R)}{I_A^s(R) + \gamma I_D^s(R)}.$$  

The efficiency $E_D$ of the second type is defined as follows:

$$E_D(R) = 1 - \frac{J_D(R)}{J_D(\infty)} = \frac{I_D^s(\infty) - I_D^s(R)}{I_D(\infty)} = \frac{E_D^s(R)}{1 + \eta_D I_n^s/I_D^s(\infty)}.$$  

Here,

$$E_D^s(R) = 1 - I_D^s(R)/I_D^s(\infty)$$

is pure efficiency in the donor quenching method. It can be calculated by means of rate equations for a D-A pair. Efficiency $E_D(R)$ does not depend on fluorescence quantum yields and cross talk. In accordance with Eq. (6), the measurable efficiency $E_D(R)$ is proportional to pure efficiency $E_D^s(R)$. Value of $\eta_D I_n^s/I_D^s(\infty)$ determines noise/signal ratio in D-fluorescence.
III. MODELS FOR D-A PAIRS

Rate of FRET going via the lowest triplet levels is negligible because it is proportional to the overlapping of electronic wave functions of the donor and the acceptor molecule. Therefore, we may neglect the energy transfer between the molecules via triplet levels if inter-dye distance R exceeds few nanometers. Nevertheless, triplet states play a very important role in the intra-molecular dynamics because of long life time of triplet states. Therefore, their role in FRET going via singlet levels deserves careful investigation.

The energy diagrams for a three-level donor dye with a two-level acceptor dye (3D-2A pair), for a two-level donor with a three-level acceptor (2D-3A pair), and for a three-level donor with a three-level acceptor (3D-3A pair) are depicted in Fig. 2.

Physical meaning of all the rate constants depicted in Fig. 2 is clear. A D-A pair consisting of a three-level molecule and a two-level molecule has $3 \times 2 = 6$ states. In the diagrams shown in Figs. 2(a) and 2(b), we see 4 and 5 levels. The (D*A*) level not shown in Fig. 2 relates to the electronic excitation of both molecules. This level will be populated weakly compared to the population of other levels if the excitation rate is much less compared to the fluorescence decay rate. Therefore, we excluded this state from the diagram. The (D*A*) level can be populated solely via the (D*A*) level. Therefore, we excluded this state as well. Such an approximation is correct if the excitation rate is much less than rates $\Gamma_D$ and $\Gamma_A$ of the relaxation.

We see dual donor fluorescence: 2-0 and 2-0 and 6-3 transitions in Fig. 2(b) and 2-0 and 6-3 transitions in Fig. 2(c). The state (D*A*) is absent in the scheme of Fig. 2(c). Since the acceptor molecule is not excited by the laser light, the system can arrive at the state (D*A*) only from the state (D*A*) with low population. That is why the state (D*A*) influences the dynamics of the system very weakly, and we do not take it into account either.

A. 3D-2A pairs

Let us denote the population of levels with $j = 0, \ldots, 6$ shown in Fig. 2 as $\rho_j$. Then, rate equations relating to Fig. 2(a) for 3D-2A pairs look as follows:

$$\rho_0 = -k_0 \rho_0 + \Gamma_A \rho_1 + \Gamma_D \rho_2 + g \rho_3,$$

$$\rho_1 = -\Gamma_A \rho_1 + F \rho_2,$$

$$\rho_2 = k \rho_0 - (F + G + \Gamma_D) \rho_2,$$

$$\rho_3 = G \rho_2 - g \rho_3.$$  \hspace{1cm} (8)

Consider the stationary solution of Eq. (8) at $\dot{\rho}_j = 0$. Then, we find the following relation from the second line of Eq. (8):

$$F \rho_2 = \Gamma_A \rho_1.$$  \hspace{1cm} (9)

By using the stationary solution of Eq. (8), we arrive at the following equations for the stationary intensities:

$$I_A^I / \eta_A \Phi_A = \rho_1 \Gamma_A = k F / D_0(F),$$

$$I_D^I / \eta_D \Phi_D = \rho_2 \Gamma_D = k \Gamma_D / D_0(F),$$

$$D_0(F) = \Gamma_D + G + k(1 + G / g + F / \Gamma_A).$$  \hspace{1cm} (10)

Inserting these intensities to Eq. (5) for FRET efficiency, we arrive at the following equation:

$$E_A^I = F(1 + \Gamma_D / \Gamma_A)$$

for the FRET efficiency in fluorescence of a 3D-2A pair. Although the intensities $I_{D,A}^I$ depend on the rates of singlet-triplet transitions, the efficiency described by Eq. (12) does not feel existence of the triplet state in the donor molecule. Eq. (12) coincides with that for the efficiency for a 2D-2A pair.

By inserting the stationary solution of Eq. (8) into Eq. (7) we arrive at the following expression for pure FRET efficiency $E_D^I(F)$ in 3D-2A pairs:

$$E_D^I(F) = 1 - \frac{\rho_3(F)}{\rho_3(0)} = \frac{F(1 + k / \Gamma_A)}{F(1 + k / \Gamma_A) + \Gamma_D + G + k (G / g + 1)}.$$  \hspace{1cm} (13)

Equation (13) in contrast to Eq. (12) depends on rates $G$ and $g$ of singlet-triplet transitions. The value of efficiency $E_D^I(F)$ will be reduced at a small value of rate $g$ of triplet relaxation, i.e., at long off-intervals in D-fluorescence.

Why does the triplet state manifest itself in the FRET efficiency $E_D^I$, though it is inconspicuous in the FRET efficiency
FIG. 3. Time instants for emission of D-photons (blue) and A-photons (red) in 3D-2A fluorescence calculated at $k = 10^5$ s$^{-1}$, $\Gamma_D = 2 \times 10^3$ s$^{-1}$, $\Gamma_A = 10^3$ s$^{-1}$, $G = 10^8$ s$^{-1}$, $g = 10^5$ s$^{-1}$, and $F = 10^6$ s$^{-1}$.

$E_A^s$? In order to answer this question, we consider photon sequences in D- and A-fluorescence of a 3D-2A pair. By applying the Monte Carlo technique to Eq. (8), we find random instants of photon emission in D- and A-fluorescence of 3D-2A pair. They are shown in Fig. 3.

The triplet state in the donor molecule influences considerably the sequence of both D- and A-photons. A-fluorescence of a 2-level acceptor molecule without dark states exhibits off-intervals because of the absence of the energy transfer when the donor molecule occupies the triplet state.

By measuring efficiency $E_A^s(F)$, we collect all D-photons during full time of the measurement. Therefore, FRET efficiency $E_A^s$ described by Eq. (7) feels the existence of off intervals. Therefore, Eq. (13) depends on rates of triplet states. Efficiency $E_A^s(F)$ takes into account solely photons of D and A-fluorescence. Off intervals in D- and A-fluorescence will equally diminish number of collected D- and A-photons. Therefore, $E_A^s(F)$ does not fill presence of off intervals.

B. 2D-3A pairs

Consider now, fluorescence of a 2D-3A pair described by the energy scheme shown in Fig. 2(b). Rate equations for this scheme look as follows:

\[
\begin{align*}
\dot{\rho}_0 &= -k\rho_0 + \Gamma_A \rho_1 + \Gamma_D \rho_2 + g \rho_3, \\
\dot{\rho}_1 &= -(\Gamma_A + G)\rho_1 + F \rho_2, \\
\dot{\rho}_2 &= k\rho_0 - (F + \Gamma_D)\rho_2 + g \rho_4, \\
\dot{\rho}_3 &= G\rho_1 - (k + g)\rho_3 + \Gamma_D \rho_4, \\
\dot{\rho}_4 &= k\rho_3 - (\Gamma_D + g)\rho_4.
\end{align*}
\]

A triplet state in the acceptor molecule changes D-fluorescence dramatically. In fact, we are facing two types of D-fluorescence. Indeed, the donor molecule is able to emit light in two situations: at the acceptor molecule occupying the ground singlet state or the triplet state. This dual D-fluorescence is shown in Fig. 2(b) by two bold blue arrows.

The intensities $I_D^s/\Phi_D \eta_D = (\rho_2 + \rho_3)\Gamma_D$ and $I_A^s/\Phi_A \eta_A = \rho_1 \Gamma_A$ can be found after solution of Eq. (14) for the stationary regime

\[
\begin{align*}
I_A^s(F)/\Phi_A \eta_A &= \frac{k\Gamma_A F}{(\Gamma_A + G)\Gamma_0}, \\
I_D^s(F)/\Phi_D \eta_D &= \frac{k\Gamma_D}{\Gamma_0} \left[1 + \frac{G}{(\Gamma_A + G)(k + \Gamma_D + g)} + \frac{kF}{G\Gamma_A + \Gamma_D + g + k + \left(1 + \frac{G}{g}\right)} \right],
\end{align*}
\]

If we insert Eqs. (15) and (16) into Eq. (5), we arrive at the following expression for FRET efficiency:

\[
E_A^s(F) = \frac{\Gamma_A + G}{\Gamma_D + F} \frac{\Gamma_A}{\Gamma_A + G} \left[1 + \frac{Gk}{g\Gamma_A (k + g + 1 + F)}\right].
\]

We find $E_A^s(\infty) < 1$, i.e., the donor molecule cannot transfer its whole energy to the acceptor molecule with a triplet state.

Equation for the efficiency $E_D^s(F)$ looks as follows:

\[
E_D^s(F) = 1 - \frac{\rho_2(F) + \rho_4(F)}{\rho_2(0) + \rho_4(0)}.
\]

By inserting the stationary solution of Eq. (14) into Eq. (19), we arrive at the following expression for FRET efficiency:

\[
E_D^s(F) = \frac{F(\Gamma_A + k + G)/(\Gamma_A + G)}{\Gamma_D + k + \frac{\Gamma_D \Gamma_A}{\Gamma_A + G} \left(\Gamma_A + k + G \frac{k}{g} + \frac{\Gamma_D \Gamma_A}{\Gamma_A + G}\right)}. \tag{20}
\]

In accordance with Eq. (20), the donor molecule cannot transfer its whole energy even at $F = \infty$ because

\[
E_D^s(\infty) = \frac{(\Gamma_A + k + G)}{\Gamma_A + k + G \frac{k}{g} + \frac{\Gamma_D \Gamma_A}{\Gamma_A + G}} < 1. \tag{21}
\]

Why is full energy transfer not possible in 2D-3A pairs? Consider a sequence of D- and A-photons calculated with the help of Eq. (14). By using the Monte Carlo technique, we arrive at the photon sequences shown in Fig. 4.

Distribution of photons in time scale presented in Fig. 4 relates to large value of $F$, i.e., to small donor-acceptor separation. Fig. 4 shows that significant donor intensity can exist even at a large value of $F$ (small $R$) if we deal with a 2D-3A pair. Indeed, Fig. 4 shows that energy transfer in 2D-3A pair does not interrupt D-fluorescence and D-fluorescence exists forever. Bright D-fluorescence exists at the acceptor molecule occupying the triplet level. Bright D-fluorescence coincides in time scale with off-intervals in A-fluorescence. D-fluorescence observed at the acceptor molecule in its ground state is weaker because the intensity of such D-fluorescence is diminished due to the energy transfer to the acceptor molecule.

C. 3D-3A pairs

Additional energy transfer via triplet levels of a donor and an acceptor can be possible in 3D-3A pairs. However, the rate of this energy transfer depends on the distance $R$ between the donor and the acceptor molecule exponentially: $\exp(-2R/L)$, where $L \sim 1$ Å. The energy transfer via triplet levels is negligible if donor and acceptor molecules are not in direct contact.
with each other. Therefore, energy transfer 4-3 in the energy scheme shown in Fig. 2(c) is omitted. Physical peculiarities of 3D-3A fluorescence have been considered in Refs. 27 and 28. In fact, they are a combination of the peculiarities of 3D-2A and 2D-3A fluorescence as it was shown in Ref. 27.

We can write seven rate equations relating to the energy diagram shown in Fig. 2(c). Applying the Monte Carlo method to these equations, we find time instants of photon emission in D- and A-fluorescence of 3D-3A pair shown in Fig. 5.

Comparison of Fig. 5 with Fig. 4 for 2D-3A fluorescence reveals off intervals in D-fluorescence of 3D-3A pair. In fact, panels of Fig. 5 look like a combination of panels for 2D-3A and 3D-2A pairs.

The expression for FRET efficiency $E_A^s$ found for 3D-3A pair looks as follows:

$$E_A^s = \frac{\bar{F}}{\bar{F} + F},$$

$$\bar{F} = \frac{F}{\left(1 + \frac{G_A}{\bar{G}_A} \right)} \left(1 + \frac{\bar{G}_A}{\bar{G}_D} \right),$$

$$Q = \frac{\bar{G}_A}{\bar{G}_D} + \frac{G_A}{G_D} \frac{1}{1 + \frac{G_A}{\bar{G}_A} \frac{G_D}{\bar{G}_D}},$$

and expression for $I_D^{\ast}$ looks as follows:

$$I_D^{\ast}(F) = \frac{1}{1 + \frac{G_D}{\bar{G}_D} \left(1 + \frac{\bar{G}_D}{\bar{G}_A} \right) + \frac{\bar{G}_D}{\bar{G}_A} \left(1 + \frac{\bar{G}_D}{\bar{G}_A} \right)}.$$ 

By inserting Eq. (25) for $I_D^{\ast}$ into equation $E_A^s = 1 - I_D^{\ast}(F)/I_D^{\ast}(0)$, we can find the expression for FRET efficiency $E_A^s$ for 3D-3A fluorescence.

### IV. ROLE OF T-T* ABSORPTION IN ENERGY TRANSFER

Consider the influence of T-T* absorption in D-molecules on A-fluorescence. Time instants presented in Fig. 3 have been simulated on the basis of Eq. (8), relating to the energy diagram displayed in Fig. 2(a). White intervals in Fig. 3 result from strict forbidding for A-fluorescence while the D-molecule occupies its triplet state. However, a strong restriction for the energy transfer from the donor molecule occupying a triplet state ($D_T$) exists only if $D_T$-$D_T^*$ transitions (T-T* absorption) with the excitation of upper triplet levels is not effective. It is realized often because the overlapping of the singlet-singlet band with the triplet-triplet band, as a rule, is absent or small. However, the frequency of laser light can coincide with the wing of the T-T* absorption band in some D-A pairs. In such pairs, the energy transfer from the donor molecule occupying the triplet state $D_T$ to the acceptor molecule occupying the ground singlet state is possible if T-T* absorption exists. Then, A-photons will fill white intervals in Fig. 3. Rate $\tilde{k}$ in the diagram shown in Fig. 6 determines the intensity of T-T* absorption.

Due to T-T* absorption, the donor molecule can get to the excited triplet state ($D_T^* A$). Transition from ($D_T^* A$) state to ($D_T A^*$) state, i.e., the energy transfer shown by fine dashed line is possible and state ($D_T A^*$) can emit light as the bold red arrow shows. Rate of transitions between states ($D_T^* A$) and ($D_T A^*$) is described by the following equation:

$$f = \frac{2\pi}{\hbar} w_{D_T^* A} \left| \langle D_T^* A \vert V_{2B} \vert D_T A^* \rangle \right|^2 \Delta(E_{D_T^* A} - E_{D_T A^*}).$$

Here, $V_{DA}$ is the operator of the dipole-dipole interaction between the donor and the acceptor molecule, $w_{D_T^* A}$ is the probability of finding a D-A pair in the initial electron-vibron-phonon state ($D_T^* A$), $\Delta(E)$ is the density of quantum states. The transition described by Eq. (27) is not forbidden. Therefore, the value of the matrix element in Eq. (27) is comparable with the value of the matrix element for singlet-singlet transitions in the Förster formula for rate $F$. However, the probability $w_{D_T^* A}$ is less at least two orders of magnitude compared to the similar probability in the Förster formula for $F$. Indeed, relaxation $\Gamma_D$ from upper triplet level goes with the rate of the order of $10^{12}$ s$^{-1}$. This rate constant is few orders of magnitude larger compared to rate constant $\Gamma_D$ of the decay from the first excited singlet level, D*. Therefore, rate constant $f$ of transitions to luminescent state ($D_T A$) is two or three orders of the magnitude less than rate $F$. Hence, A-fluorescence during off-intervals will be two-three orders of magnitude weaker compared to A-fluorescence in on-intervals. Such weak A-fluorescence will be even less than background fluorescence in off-intervals.

Consider how possible T-T* absorption in A-molecule can change the results obtained for fluorescence of a 2D-3A pair. The energy diagram with the rate $\tilde{k}$ of T-T* absorption is shown in Fig. 7.

Two blue bold arrows in Fig. 7 show dual D-fluorescence. Since the acceptor molecule gets to triplet state ($D_T A^*$), A-fluorescence stops. It is true if we neglect possible T-T* absorption. If we allow for such absorption, quantum state ($DA^*_T$) will be populated. This state will be depopulated with large

![Fig. 5](image-url) Time instants for photon emission in 3D-3A fluorescence calculated at $k = 10^3$ s$^{-1}$, $1/T_D = 2 \times 10^8$ s$^{-1}$, $1/T_A = 10^8$ s$^{-1}$, $G_D = 10^6$ s$^{-1}$, $G_A = 5 \times 10^5$ s$^{-1}$, $g_A = 500$ s$^{-1}$, $F = 10^8$ s$^{-1}$.

![Fig. 6](image-url) Energy diagram of 3D-2A pair with rate $\tilde{k}$ of T-T* absorption.
FIG. 7. Energy diagram for 2D-3A pair with rate $k$ of T-T* absorption.

rate, $\Gamma_A \approx 10^{12}$ s$^{-1}$. This depopulation will not be accompanied by fluorescence because of very low quantum yield of such emission. The main influence of T-T* absorption will manifest itself in the appearance of the probability transition from state $(DA_T^*)$ to state $(D^* A_T)$. Fine dashed line in Fig. 7 shows such transition. Rate of such transition is given by the following formula:

$$f = \frac{2\pi}{\hbar} w_{D A_T^*} |\langle DA_T^* | V_{DA} | D^* A_T \rangle|^2 \Delta (E_{DA_T^*} - E_{D^* A_T}).$$  

(28)

The matrix element in Eq. (28) has a value comparable to the value for a similar matrix element in the Förster formula for $F$. However, probability $w_{D A_T^*}$ of finding the system in state $(D^* A_T)$ is very small because rate $\Gamma_A \approx 10^{12}$ s$^{-1}$ is few orders of magnitude larger than rate $\Gamma_D$ playing similar role in the Förster theory. Therefore, transition $(DA_T^*) \rightarrow (D^* A_T)$ going with small rate $f$ results in weak additional emission to bright D-fluorescence. Hence, although energy transfer via excited triplet levels is possible, we may neglect this channel of transfer because of weak population of the excited triplet levels.

V. CALCULATION OF EFFICIENCIES AND DISCUSSION

Expressions for the efficiencies found in Sec. III are rather complicated. However, they can be simplified considerably if we take into account the following hierarchy of rate constants: $G_{D,A}/\Gamma_{D,A} \ll \frac{k}{\Gamma_{D,A}} \ll \frac{\tau_{on}}{\Gamma_{D,A}} \ll \frac{\tau_{off}}{\Gamma_{D,A}} \ll 1$. Then, Eq. (13) for 3D-2A pair takes the following form:

$$E_{D}^s(F) = \frac{F}{F + \Gamma_D \left( \frac{k_g}{\tau_{off}} + 1 \right)}$$  

(13a)

and Eqs. (18) and (20) for a 2D-3A pair look as follows:

$$E_{A}^s(F) = \frac{F}{\Gamma_D + \frac{F}{\Gamma_{A}} \left( \frac{k_g}{\tau_{off}} + 1 \right)},$$  

(18a)

$$E_{D}^s(F) = \frac{F}{\Gamma_D + \frac{F}{\Gamma_{A}} \left( \frac{k_g}{\tau_{off}} + 1 \right)}.$$  

(20a)

Equations (23) and (24) take the following form:

$$\bar{F} = \frac{F}{1 + \frac{F}{\Gamma_D} \frac{kQ}{\Gamma_A}},$$  

(23a)

$$Q = \frac{\frac{G_A}{\tau_{on}}}{1 + \frac{G_A}{\tau_{off}} \frac{k_g}{\tau_{off}}}.$$  

(24a)

The combination of rate constants,

$$kG_{D,A}/\Gamma_{D,A} \ll \frac{\tau_{off}}{\tau_{on}} \approx \frac{\tau_{off}^{D,A}}{\tau_{on}^{D,A}} = r_{D,A},$$  

(29)

is expressed via the average duration $\tau_{off}^{D,A}$ and $\tau_{on}^{D,A}$ of off/on intervals in blinking fluorescence of the donor and the acceptor molecule isolated from each other. Making substitution $F/\Gamma_D = (R_F/R)^{1/6}$ in all formulas for the efficiencies and taking into account Eq. (29), we arrive at the following expressions for FRET efficiencies.

For 3D-2A pair,

$$E_A^s = \left[ 1 + (R/R_F)^{6} \right]^{-1},$$  

(30)

$$E_D^s = \left[ 1 + (R/R_F)^{6}(1 + r_D) \right]^{-1}.$$

Here, $R_F = (S_A/\Gamma_D)^{1/6}$ is the Förster radius.

For 2D-3A pair,

$$E_{A,D}^s = \left[ 1 + r_A + (R/R_F)^{6} \right]^{-1}.$$  

(32)

For 3D-3A pair,

$$E_A^s = \left[ \Lambda_A + (R/R_F)^{6} \right]^{-1},$$  

(33)

$$\Lambda_A = 1 + kQ/\Gamma_A,$$  

(34)

$$E_D^s = \left[ \Lambda_D + (R/R_F)^{6}(1 + r_D) \right]^{-1},$$  

(35)

$$\Lambda_D = 1 + (1 + r_D)kQ/\Gamma_A.$$  

(36)

Combination of constants $kQ/\Gamma_A$ looks as follows:

$$kQ/\Gamma_A = \frac{r_A}{1 + r_D \frac{\tau_{off}}{\tau_{on}}}.$$  

(37)

If the transition to triplet state of A-molecule is absent, we have $r_A = Q = 0$. In this case, we find that Eq. (33) for a 3D-3A pair coincides with Eq. (30) for a 3D-2A pair and Eq. (35) is transformed to Eq. (31). If the transition to triplet state of D-molecule is absent, we have $r_D = 0$ and $kQ/\Gamma_A = r_A$. In this case, Eqs. (33) and (35) are transformed to Eq. (32) for a 2D-3A pair.

Consider how Eqs. (30)-(37) found for pure efficiencies $E_{D,A}^s$ in fluorescence of 3D-2A, 2D-3A, and 3D-3A pairs can be used in experiments similar to those carried out by Meller group.12,13,17 The scheme of the samples studied in these experiments is shown in Fig. 1. The donor molecule (TMR) or (Cy3) is attached to the DNA strands on the controlled distance $R$ from the acceptor molecule (Cy5). The cross talk and background light corrected intensities, i.e.,

$$I_D^s = J_D - \eta_D I_D,$$  

(38)

FIG. 8. Dependence of FRET efficiencies in 3D-2A pair on the inter-dye distance. $E_A^s$ (curve 1) and $E_D^s$ at $rd=1$ (curve 2) and 10 (curve 3). $R_F = 10$ nm.
Influence of triplet levels on the dependence of the energy transfer on inter-dye distance 3D-2A pairs.

\[ I'(A) = I_A - \beta \eta_D \eta_B (1 + \beta) I_n \]  

(39)
can be used to find efficiencies \( E_D'(F) \) and \( E_A'(F) \). These efficiencies can be compared with the efficiencies calculated with the help of (30)-(37).

The right hand sides in Eqs. (38) and (39) can be measured in an experiment. Inserting Eqs. (38) and (39) to Eqs. (5) and (7) for FRET efficiencies, we find pure efficiencies measured in an experiment. On the other hand, pure efficiencies \( E_{D,A}' \) can be calculated with the help of (30)-(37). Fig. 8 shows calculated dependence of the FRET efficiency in 3D-2A pair on inter-dye distance.

Efficiency \( E_A' \) calculated with the help of (30) is shown by curve 1. It does not feel the existence of triplet levels in the donor molecule. We find \( E_A' = 0.5 \) at \( R = R_F \).

Efficiency \( E_D' \) calculated with the help of Eq. (31), quite the contrary, feels the existence of triplet levels. They reduce the efficiency. The longer the off intervals are, the less the value of the efficiency is. The efficiency equals 0.4 at \( \tau_{\text{off}}/\tau_D = 1 \) and equals 0.07 at \( \tau_{\text{off}}/\tau_D = 10 \). The horizontal dashed lines show this fact in Fig. 8. Hence, solely efficiency \( E_D' \) describes real energy transfer in 3D-2A pairs correctly.

Consider the efficiency of energy transfer in 2D-3A pairs. Both efficiencies \( E_{D,A}' \) are described by Eq. (32). Dependence of \( E_{D,A}'(R) \) at various values of \( \tau_{\text{off}}/\tau_{\text{on}} \) is shown in Fig. 9.

The efficiency of energy transfer cannot reach unity in 2D-3A pairs as curves in Fig. 9 show. The longer the off intervals are, the less the value of the efficiency is.

Consider now FRET efficiency in 3D-3A pairs. The dependence of both efficiencies on inter-dye distance is shown in Fig. 10.

Dashed lines 1 describe dependence: \( 1/[1 + (R/R_F)^6] \), which does not take into account the influence of triplet levels.

Theoretical curve marked by squares in Fig. 9 is almost similar to the measured dependence. The curves with squares presented in Figs. 9 and 11 are related to a 2D-3A pair at \( \tau_{\text{off}}/\tau_{\text{on}} = 1 \) and \( R_F = 10 \) nm. Therefore, we will try to fit the experimental data with the help of Eq. (32) by varying a single parameter: \( R_F \). Curve 4 in Fig. 11 related to \( R_F = 20 \) nm fits the experimental data well.

VI. CONCLUSION

At present, the equation \( E = [1 + (R/R_F)^6]^{-1} \) for FRET efficiency is often used in many works. However, Figs. 9–10 show that this equation can be used only if the following inequality, \( k_G (D,A)/F_D,A \text{eff}_{D,A}^{D,A} = \tau_{\text{off}}^{D,A}/\tau_{\text{on}}^{D,A} = \tau_D < 1 \), is realized. This inequality means that blinking in D- and A-fluorescence is absent. If the inequality is not realized, we must use equations like Eqs. (31)–(33) and Eq. (35). They describe the influence of triplet states on FRET efficiency.

Triplets states in the dyes of D-A pairs are responsible for pauses existing in the train of fluorescence photons as Figs. 3–5 show. These very pauses are the reason for reduced energy transfer in D-A pairs with triplet levels.

Schemes in Figs. 6 and 7 show that transitions between the donor and the acceptor molecules are not forbidden in 3D-2A and 2D-3A pairs if excited triplet states are involved via T-T absorption. Although A-photon will fill pauses shown in A fluorescence presented in Figs. 3–5, however, the intensity of...
such additional A-fluorescence will be negligible because of weak population of the excited triplet level.

We express pure efficiencies \( E_{D,A}^o(R) \) via average durations \( \tau_{D,A}^{on,off} \) of on/off intervals. Values \( \tau_{D,A}^{on,off} \) can be measured in experiments with the donor and the acceptor molecules isolated from each other. Therefore, Eqs. (31)-(37) include a single adjustable parameter \( R_F = \left( \frac{S_a}{\Gamma_D} \right)^{1/6} \). By varying this parameter, we can fit the measured dependence by the theoretical curve \( E_{D,A}^o(R) \).

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