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Förster-type energy transfer

Simultaneous 'forward' and 'reverse' transfer between unlike fluorophores

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The general case of Förster-type energy transfer is that in which energy is exchanged in both directions between two unlike fluorophores. In such cases, energy is transferred from the conventionally defined donor to the conventionally defined acceptor (forward transfer) and at the same time from the acceptor to the donor (reverse transfer). Expressions are derived to describe the fluorescence intensities and lifetimes of fluorophores undergoing simultaneous forward and reverse transfer; these are compared with corresponding quantities for the case more usually considered, in which only forward transfer is significant. It is shown that the presence of reverse transfer removes the distinction between donor and acceptor, and allows such anomalous effects as 'acceptor quenching'. A confirmatory example is described. It is shown that the equations generally used in distance determination by steady-state fluorescence spectroscopy can also be applied in the presence of reverse transfer, if a correction term is included; however, for lifetime spectroscopy the correction is more complex.

1. Introduction

Singlet-singlet energy transfer from an electronically excited donor chromophore D* to a ground-state acceptor chromophore A, resulting in the 'radiationless' decay of D* and the excitation of A (eq. 1), was first described theoretically by

$$D^* + A \xrightarrow{k_f} D + A^* \tag{1}$$

Förster [1] and has since been the subject of rigorous experimental confirmation (reviewed by Stryer [2]). It has frequently been employed as a technique for measuring inter- and intramolecular distances in proteins, nucleic acids, ribosomes and other biological structures [2–4]. In biology, it is an important elementary step in photosynthesis [5].

The rate constant for energy transfer from donor to acceptor, k^f, is measured indirectly (i) by comparing the fluorescence of the donor with and without the acceptor present (*donor quenching*) and/or (ii) by comparing the fluorescence of the acceptor with and without the donor present (*acceptor sensitisation* or *enhancement*). Donor quenching and acceptor enhancement are in many cases most easily and accurately measured by separation of the fluorophores in situ, e.g., by enzymic digestion [6,7], which allows a direct comparison to be made.

One parameter which is decisive for Förster-type energy transfer is the overlap integral between the emission spectrum $F_D(\lambda)$ of the donor and the absorption (excitation) spectrum $\epsilon_A(\lambda)$ of

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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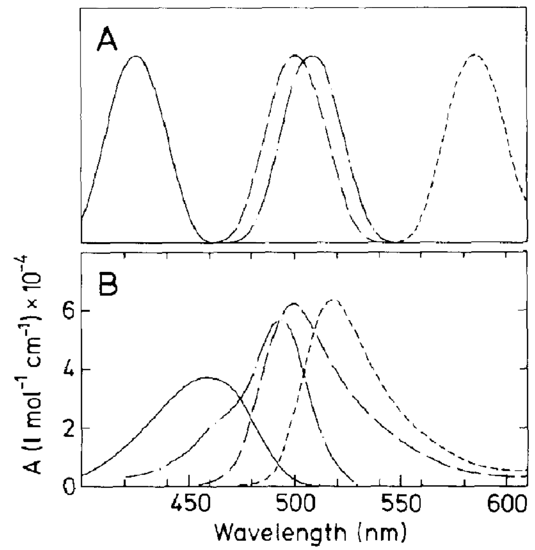

Fig. 1. Excitation and emission spectra of donor and acceptor fluorophores. (———) Donor excitation, (— — —) donor emission, (· — · — ·) acceptor excitation, (- - - - - -) acceptor emission. (A) Ideal case, where only donor emission and acceptor excitation overlap; (B) a typical real case (coumarin donor and fluorescein acceptor), in which all four spectra overlap. In B, the overlap of acceptor emission and donor excitation opens the way for reverse energy transfer.

the acceptor, defined by

$$J_f = \frac{\int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F_D(\lambda) \, d\lambda} \qquad (2)$$

where λ is wavelength. (Other determinanda are given below, cf. eq. 5, and methods for their measurement are discussed elsewhere, e.g., in refs. 2, 4, 7 and 8.) The integral J_f expresses the condition that the energy given up by the donor must equal the energy taken up by the acceptor. Ideally, the donor will absorb energy at a wavelength shorter than that of the region of overlap, and the acceptor will emit at a wavelength longer (fig. 1A). It is usually assumed that the acceptor's emission spectrum does not overlap with the excitation spectrum of the donor. This assumption is clearly physically reasonable, but a finite degree of overlap of this kind is not ruled out a priori, and fig. 1B shows an example of such overlap. This overlap leads to a finite value for a 'reverse' integral J_r, which we define as

$$J_r = \frac{\int_0^\infty F_A(\lambda)\epsilon_D(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F_A(\lambda) \, d\lambda} \qquad (3)$$

J_r is the converse of the 'forward' integral J_f defined in eq. 2. If J_r is finite then energy can be transferred not only from the donor to the acceptor, but also from the acceptor to the donor – in fact, the terms 'donor' and 'acceptor' lose much of their meaning. The following treatment examines the effects which reverse energy transfer can have on the occurrence and the measurement of 'normal' energy transfer, and investigates the circumstances under which a significant degree of reverse transfer can be expected.

2. Theory

2.1. Definitions

We consider a macromolecule which carries a donor fluorophore D and an acceptor A, at a distance R from one another. The first-order rate constant for radiative decay (fluorescence emission) of the donor is k_D^* and that for non-radiative decay (internal conversion, etc.) is k_D^-. For time-independent R, k^f is also a first-order rate constant. The average fluorescence lifetime of a donor is

$$\tau_D = \left(k_D^* + k_D^- + k^f\right)^{-1} \qquad (4)$$

and in the case of zero energy transfer ($k^f = 0$) the lifetime is τ_D^0. Corresponding constants k_A^*, k_A^-, τ_A and τ_A^0 are defined for the acceptor, and the rate constant for reverse energy transfer is k^r (eq. 1, right to left).

The value of k^f is given by the relation [1,9]:

$$k^f = \frac{9 \ln 10}{128\pi^5 N_A/1000} \cdot R^{-6} J_f \kappa^2 n^{-4} k_D^* \qquad (5)$$

where n is the refractive index of the medium around the fluorophores, J_f is defined in eq. 2, N_A is Avogadro's number and κ^2 is an orientation factor.

The probability of energy transfer to the acceptor from an excited donor is denoted E_f, and that of the corresponding reverse transfer E_r. It follows that

$$E_f = k^f/(k_D^* + k_D^{\sim} + k^f) = k^f\tau_D = 1 - (\tau_D/\tau_D^o) \tag{6}$$

and

$$E_r = k^r/(k_A^* + k_A^{\sim} + k^r) = k^r\tau_A = 1 - (\tau_A/\tau_A^o) \tag{7}$$

It is usual to define a characteristic Förster distance R_o between the fluorophores, for which the probability of energy transfer is 50%, and it is convenient to express R as a fraction or multiple of this. Here we have two Förster distances, one for forward (D → A) and one for reverse (A → D) transfer. We denote these by R_f and R_r, respectively, and then obtain by rearrangement of eqs. 5 and 6, with 50% transfer ($E_f = 0.5$), the standard expression

$$R_f^6 = \frac{9 \ln 10}{128\pi^5 N_A/1000} \cdot J_f \kappa^2 n^{-4} k_D^* \tau_D^o \tag{8}$$

and analogously, for reverse transfer,

$$R_r^6 = \frac{9 \ln 10}{128\pi^5 N_A/1000} \cdot J_r \kappa^2 n^{-4} k_A^* \tau_A^o \tag{9}$$

Defining $x_f = R/R_f$ and $x_r = R/R_r$ and employing eqs. 6–9, we obtain

$$E_f = (1 + x_f^6)^{-1} \text{ and } E_r = (1 + x_r^6)^{-1} \tag{10}$$

Finally, we define the experimentally accessible quantities which are used to measure energy transfer. The sample is irradiated with light at a particular wavelength and the intensities of fluorescence emission from the donor and the acceptor are measured. These are then divided by the corresponding intensities in a system which is identical in all respects except that no energy transfer occurs (methods for doing this are reviewed by Fairclough and Cantor [10], and a new method is described by Epe et al. [6,7]). The intensity quotient is here denoted G for the donor and H for the acceptor. If only normal, forward transfer occurs, $G < 1$ (whence the term 'donor quenching') and $H > 1$ (whence 'acceptor enhancement'). The corresponding quotient for the donor lifetime is τ_D/τ_D^o. It is easily shown (see, e.g., ref. 4) that for normal energy transfer

$$E = 1 - G = (1 - G_{exp})/\chi_a \tag{11}$$

$$E = (H - 1)\epsilon_A/\epsilon_D = (H_{exp} - 1)(\epsilon_A/\epsilon_D)/\chi_d \tag{12}$$

and

$$E = 1 - \tau_D/\tau_D^o \tag{13}$$

where the values of ϵ_A and ϵ_D are appropriate to the excitation wavelength used. For macromolecules of which only a certain fraction is labelled with the fluorophores, the respective degrees of labelling are denoted χ_d and χ_a, respectively, where $0 < \chi_d,\ \chi_a \leq 1$ (for treatment of values above 1, see ref. 6). We define G_{exp} and H_{exp} as the apparent values of G and H observed when $\chi_a < 1$ and $\chi_d < 1$, respectively.

2.2. *Kinetics of simultaneous forward and reverse energy transfer*

Illumination at any wavelength will excite both fluorophores in proportion to their extinction coefficients at that wavelength. With the usual approximations, e.g., that solvent relaxation is fast, the loss and gain of populations of D* and A* are summarised in fig. 2. ζ is a constant of proportionality involving concentration, photon flux, etc. We note that the system is symmetric in D and A,

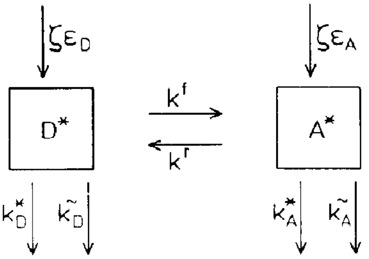

Fig. 2. Loss and gain of populations of excited donors D* and acceptors A*. The $\zeta\epsilon$ rate constants are zero order in D* and A*, and the others are first order in D* or A*, respectively.

a symmetry not usually considered because $k^r \approx 0$. This symmetry makes the distinction between donor and acceptor a purely formal one.

The concentrations of D* and A* change with time according to the kinetic equations

$$\frac{\mathrm{d}[\mathrm{D}^*]}{\mathrm{d}t} = \zeta\epsilon_\mathrm{D} + k^\mathrm{r}[\mathrm{A}^*] - \left(k_\mathrm{D}^* + k_\mathrm{D}^- + k^\mathrm{f}\right)[\mathrm{D}^*] \tag{14}$$

$$\frac{\mathrm{d}[\mathrm{A}^*]}{\mathrm{d}t} = \zeta\epsilon_\mathrm{A} + k^\mathrm{f}[\mathrm{D}^*] - \left(k_\mathrm{A}^* + k_\mathrm{A}^- + k^\mathrm{r}\right)[\mathrm{A}^*] \tag{15}$$

in which it has implicitly been assumed that all macromolecules carry both a donor and an acceptor ($\chi_\mathrm{d} = \chi_\mathrm{a} = 1$). This assumption will be discarded later.

The consequences of eqs. 14 and 15 will now be examined, first for steady-state fluorescence intensity and then for fluorescence lifetime.

2.3. Steady-state fluorescence intensity

Under constant illumination, the kinetic system reaches a stationary state, so $\mathrm{d}[\mathrm{D}^*]/\mathrm{d}t$ and $\mathrm{d}[\mathrm{A}^*]/\mathrm{d}t$ are zero. The solutions of eqs. 14 and 15 are then:

$$[\mathrm{D}^*] = \zeta \cdot \frac{\epsilon_\mathrm{D} + \epsilon_\mathrm{A} E_\mathrm{r}}{\tau_\mathrm{D}^{-1} - k^\mathrm{f} E_\mathrm{r}} \tag{16}$$

and

$$[\mathrm{A}^*] = \zeta \cdot \frac{\epsilon_\mathrm{A} + \epsilon_\mathrm{D} E_\mathrm{f}}{\tau_\mathrm{A}^{-1} - k^\mathrm{r} E_\mathrm{f}} \tag{17}$$

The fluorescence signals from donor and acceptor are $\xi_\mathrm{D}[\mathrm{D}^*]$ and $\xi_\mathrm{A}[\mathrm{A}^*]$, respectively, where the constants of proportionality $\xi_{\mathrm{A,D}}$ contain the respective quantum yields along with instrumental factors. To relate the signals to energy transfer we consider two cases: (i) the general case, and (ii) the special case of zero energy transfer, in which $k^\mathrm{f} = k^\mathrm{r} = E^\mathrm{f} = E^\mathrm{r} = 0$ and τ_D and τ_A become $\tau_\mathrm{D}^\mathrm{o}$ and $\tau_\mathrm{A}^\mathrm{o}$, respectively.

From the definitions of G and H (above) we have for the donor, using eq. 6:

$$G = \frac{[\mathrm{D}^*]\text{ in case (i)}}{[\mathrm{D}^*]\text{ in case (ii)}} = \frac{\epsilon_\mathrm{D} + \epsilon_\mathrm{A} E_\mathrm{r}}{\tau_\mathrm{D}^{-1} - k^\mathrm{f} E_\mathrm{r}} \Bigg/ \frac{{\tau_\mathrm{D}^\mathrm{o}}^{-1}}{\epsilon_\mathrm{D}}$$

$$= \frac{(1 + (\epsilon_\mathrm{A}/\epsilon_\mathrm{D}) E_\mathrm{r})(1 - E_\mathrm{f})}{1 - E_\mathrm{f} E_\mathrm{r}} \tag{18}$$

Likewise, for the acceptor, using eq. 7,

$$H = \frac{(1 + (\epsilon_\mathrm{D}/\epsilon_\mathrm{A}) E_\mathrm{f})(1 - E_\mathrm{r})}{1 - E_\mathrm{r} E_\mathrm{f}} \tag{19}$$

Before solving these, we note that they have the properties expected, in that:

(i) They are symmetric in D and A, mutatis mutandis.

(ii) For the special case of no transfer, $E_\mathrm{f} = E_\mathrm{r} = 0$, whence $G = 1 = H$.

(iii) For the special case of normal transfer they reduce to eqs. 11 and 12, respectively (with $\chi_\mathrm{d} = 1 = \chi_\mathrm{a}$).

(iv) For the special case of homotransfer, where the fluorophores are identical, so that $E_\mathrm{f} = E_\mathrm{r} > 0$ and $\epsilon_\mathrm{A} = \epsilon_\mathrm{D}$, they give $G = 1 = H$, implying, as expected, that homotransfer does not affect signal intensities.

(v) For excitation at a very long wavelength, ϵ_D vanishes and consequently G becomes meaningless (eq. 18). However, eq. 19 gives

$$H = \frac{1 - E_\mathrm{r}}{1 - E_\mathrm{f} E_\mathrm{r}} \leq 1 \tag{20}$$

so that the expected acceptor enhancement turns out to be a quenching of the acceptor by reverse transfer of energy from A to D. The unambiguity of this effect makes this situation ideal for the direct observation of reverse energy transfer, as discussed below.

2.4. Modification for χ_d, χ_a less than unity

For fluorophore pairs with forward transfer only, eq. 11 states that

$$(1 - G_\mathrm{exp})/\chi_\mathrm{a} = E = 1 - G \tag{21}$$

whence

$$G = (G_\mathrm{exp} - 1)/\chi_\mathrm{a} + 1 \tag{22}$$

Similarly, eq. 12 leads to

$$H = (H_{\mathrm{exp}} - 1)/\chi_{\mathrm{d}} + 1 \tag{23}$$

Eqs. 22 and 23 belong to standard theory. They provide the means of correcting G_{exp} and H_{exp} when χ_{d} and/or χ_{a} is less than 1.0, a situation that is encountered frequently owing to incomplete labelling.

In the presence of both forward and reverse energy transfer, the intensity of the donor signal when energy transfer is restricted to a fraction χ_{a} of the donors is obtained by a weighted sum of eq. 16 with and without acceptor present:

$$\mathrm{Intensity} = \xi_{\mathrm{D}}\zeta\left(\chi_{\mathrm{a}} \cdot \frac{\epsilon_{\mathrm{D}} + \epsilon_{\mathrm{A}}E_{\mathrm{r}}}{\tau_{\mathrm{D}}^{-1} - k^{\mathrm{f}}E_{\mathrm{r}}} + (1 - \chi_{\mathrm{a}})\epsilon_{\mathrm{D}}\tau_{\mathrm{D}}^{\mathrm{o}}\right) \tag{24}$$

Without energy transfer, this reduces as before to

$$\mathrm{Intensity} = \xi_{\mathrm{D}}\zeta\epsilon_{\mathrm{D}}\tau_{\mathrm{D}}^{\mathrm{o}} \tag{25}$$

The ratio of these expressions gives G_{exp}:

$$G_{\mathrm{exp}} = \chi_{\mathrm{a}}\frac{\epsilon_{\mathrm{D}} + \epsilon_{\mathrm{A}}E_{\mathrm{r}}}{\tau_{\mathrm{D}}^{-1} - k^{\mathrm{f}}E_{\mathrm{r}}} \cdot \frac{\tau_{\mathrm{D}}^{\mathrm{o}^{-1}}}{\epsilon_{\mathrm{D}}} + (1 - \chi_{\mathrm{a}}) \tag{26}$$

Combination of eqs. 26 and 18 followed by rearrangement gives eq. 22. For acceptor enhancement, an expression analogous to eq. 26 is obtained, which reduces to eq. 23.

Consequently, the usual degree-of-labelling corrections may be applied in situations where both forward and reverse energy transfer occur simultaneously.

2.5. *Determination of distance*

We define a new quantity E_{app}. This is the value obtained for E if it is evaluated by inserting experimental values for G, H, χ_{d} and χ_{a} into eqs. 11 and 12, which only cover the case of normal transfer. In the absence of reverse energy transfer, $E_{\mathrm{app}} = E$. In its presence, E_{app} is an incorrect estimate of E_{f} and E_{r}, and it can be corrected as follows.

From eqs. 11 and 18,

$$E_{\mathrm{exp}} = 1 - G = \frac{E_{\mathrm{f}} - (\epsilon_{\mathrm{A}}/\epsilon_{\mathrm{D}})E_{\mathrm{r}} + (\epsilon_{\mathrm{A}}/\epsilon_{\mathrm{D}} - 1)E_{\mathrm{r}}E_{\mathrm{f}}}{1 - E_{\mathrm{r}}E_{\mathrm{f}}} \tag{27}$$

(The same relation follows from eqs. 12 and 19. Therefore, the same value of E_{app} is obtained from donor quenching and from acceptor enhancement.) Multiplication of eq. 27 by $(1 - E_{\mathrm{f}}E_{\mathrm{r}})$, followed by substitution using eqs. 10 and the definition $a = (R_{\mathrm{f}}/R_{\mathrm{r}})^6$, leads to

$$E_{\mathrm{exp}} = \frac{1}{1 + x_{\mathrm{f}}^6} - \frac{\epsilon_{\mathrm{A}}/\epsilon_{\mathrm{D}}}{1 + ax_{\mathrm{f}}^6} + \left(\frac{\epsilon_{\mathrm{A}}}{\epsilon_{\mathrm{D}}} - 1 + E_{\mathrm{exp}}\right)\frac{1}{1 + x_{\mathrm{f}}^6} \cdot \frac{1}{1 + ax_{\mathrm{f}}^6} \tag{28}$$

which upon simplification and division by x_{f}^6 ($x_{\mathrm{f}} \neq 0$) yields

$$x_{\mathrm{f}}^6 = 1/E_{\mathrm{exp}} - 1/a - (\epsilon_{\mathrm{A}}/\epsilon_{\mathrm{D}})/aE_{\mathrm{exp}} - 1 \tag{29}$$

or, in terms of distances,

$$R^6 = R_{\mathrm{f}}^6\left(\frac{1}{E_{\mathrm{exp}}} - 1\right) - R_{\mathrm{r}}^6\left(\frac{\epsilon_{\mathrm{A}}/\epsilon_{\mathrm{D}}}{E_{\mathrm{exp}}} + 1\right) \tag{30}$$

The influence of reverse transfer on a distance determination is expressed clearly in eq. 30. If there is no overlap of acceptor emission and donor excitation, then J_{r} and thus R_{r} are zero. Alternatively, there may be overlap between these, but with $R_{\mathrm{r}} \ll R_{\mathrm{f}}$. In either case, there will be negligible reverse transfer, E_{app} will be equal to E_{f}, and eq. 30 will collapse to give the usual Förster formula (cf. eq. 10):

$$R = R_{\mathrm{o}}\left(\frac{1}{E} - 1\right)^{1/6} \tag{31}$$

We conclude that, for distance measurements made by the steady-state method, the third term in eq. 30 may be regarded as a correction term whose effect on a particular measurement can easily be checked and, if necessary, included in the evaluation of experimental data. Its negative sign means that distances uncorrected for reverse transfer look greater than they actually are.

2.6. *Fluorescence lifetime*

After excitation of the fluorophores D and A with a non-saturating pulse of light, the concentrations of D* and A* are, respectively,

$$[\mathrm{D}^*](0) = \zeta\epsilon_{\mathrm{D}} \text{ and } [\mathrm{A}^*](0) = \zeta\epsilon_{\mathrm{A}} \tag{32}$$

where ζ is a constant of proportionality as in eqs. (14 and 15) (cf. fig. 2). The subsequent, time-dependent behaviour of the system is given by the solutions of eqs. 14 and 15 with $\zeta = 0$ and initial values as in eq. 32. Standard methods show that each solution is the sum of two exponentially decaying functions of the form

$$[\mathrm{D}^*] = d_1 \exp(-\lambda_1 t) + d_2 \exp(-\lambda_2 t) \tag{33}$$

and

$$[\mathrm{A}^*] = a_1 \exp(-\lambda_1 t) + a_2 \exp(-\lambda_2 t) \tag{34}$$

where

$$\lambda_{1,2} = (k_\mathrm{D} + k_\mathrm{A})/2 \pm \sqrt{(k_\mathrm{D} - k_\mathrm{A})^2/4 + k^\mathrm{f} k^\mathrm{r}} \tag{35}$$

and $(k_\mathrm{D}^* + k_\mathrm{D}^\sim + k^\mathrm{f})$ is abbreviated to k_D and $(k_\mathrm{A}^* + k_\mathrm{A}^\sim + k^\mathrm{r})$ to k_A. From now on λ_1 will contain the positive root and λ_2 the negative.

The coefficients are

$$\left.\begin{aligned}
d_1 &= (\lambda_1 - \lambda_2)^{-1}(\zeta\epsilon_\mathrm{D}(k_\mathrm{D} - \lambda_2) - \zeta\epsilon_\mathrm{A} k^\mathrm{r})\\
d_2 &= (\lambda_1 - \lambda_2)^{-1}(\zeta\epsilon_\mathrm{D}(\lambda_1 - k_\mathrm{D}) + \zeta\epsilon_\mathrm{A} k^\mathrm{r})\\
a_1 &= (\lambda_1 - \lambda_2)^{-1}(\zeta\epsilon_\mathrm{A}(k_\mathrm{A} - \lambda_2) - \zeta\epsilon_\mathrm{D} k^\mathrm{f})\\
a_2 &= (\lambda_1 - \lambda_2)^{-1}(\zeta\epsilon_\mathrm{A}(\lambda_1 - k_\mathrm{A}) + \zeta\epsilon_\mathrm{D} k^\mathrm{f})
\end{aligned}\right\} \tag{36}$$

The signal strength at the wavelength of observation, i.e., the observed fluorescence decay curve, is therefore

$$\begin{aligned}
F(t) &= \xi_\mathrm{D}[\mathrm{D}^*](\mathrm{t}) + \xi_\mathrm{A}[\mathrm{A}^*](t)\\
&= \zeta(\lambda_1 - \lambda_2)^{-1}\{[\xi_\mathrm{D}(\epsilon_\mathrm{D}(k_\mathrm{D} - \lambda_2) - k^\mathrm{r}\epsilon_\mathrm{A})\\
&\quad + \xi_\mathrm{A}(\epsilon_\mathrm{A}(k_\mathrm{A} - \lambda_2) - k^\mathrm{f}\epsilon_\mathrm{D})] \exp(-\lambda_1 t)\\
&\quad + [\xi_\mathrm{D}(\epsilon_\mathrm{D}(\lambda_1 - k_\mathrm{D}) + k^\mathrm{r}\epsilon_\mathrm{A})\\
&\quad + \xi_\mathrm{A}(\epsilon_\mathrm{A}(\lambda_1 - k_\mathrm{A}) + k^\mathrm{f}\epsilon_\mathrm{D})] \exp(-\lambda_2 t)\}
\end{aligned} \tag{37}$$

Although this equation is cumbrous, its consequences are easily seen:

(i) If reverse transfer does not take place, then $k^\mathrm{r} = 0$ and so, from eq. 35,

$$\lambda_1 = k_\mathrm{D} = \tau_\mathrm{D}^{-1} \text{ and } \lambda_2 = k_\mathrm{A} = \tau_\mathrm{A}^{\circ\,-1} \tag{38}$$

That is, the separate curves of acceptor and quenched donor are observed; as expected, only the donor's lifetime is affected by the energy transfer.

(ii) If, as is usually the case, wavelengths are chosen so that only the emission from the donor is observed ($\xi_\mathrm{A} = 0$), then eq. 37 reduces to a single-exponential decay only as long as there is no reverse transfer ($k^\mathrm{r} = 0$). However, if reverse energy transfer occurs ($k^\mathrm{r} > 0$), then even the 'pure donor' decay signal will be double exponential. This fact may call for correction or re-interpretation of data in the literature.

(iii) Finally, if donor and acceptor are identical ($\epsilon_\mathrm{D} = \epsilon_\mathrm{A} = \epsilon$; $\tau_\mathrm{D} = \tau_\mathrm{A} = \tau$; $k_\mathrm{D} = k_\mathrm{A}$; $k^\mathrm{f} = k^\mathrm{r}$; $\xi_\mathrm{D} = \xi_\mathrm{A} = \xi$), then eq. 37 describes the well-known phenomenon of homotransfer and reduces to

$$F(t) = 2\xi\zeta\epsilon \exp(-t/\tau^\circ) \tag{39}$$

That is, as expected, energy transfer in this case leaves the lifetimes unaffected.

It is clear that, in contrast to the evaluation of intensity data, the evaluation of fluorescence decay curves in cases where reverse transfer is present cannot be achieved by a simple correction: a double (or, if not all molecules with a donor carry an acceptor, a triple) exponential curve-fitting is needed.

3. Results and discussion

3.1. Observation of reverse energy transfer

The fluorophore pair coumarin/fluorescein has been used by us (ref. 7 and work to be published) for distance determination on ribosomal particles and by many others for similar purposes. The excitation and emission spectra of these dyes are shown in fig. 1B. The reverse overlap integral is significant: the respective numerical values of J_f and J_r are 1.55×10^{15} and 2.41×10^{13}, giving values of 51.6 Å for R_f and 26.6 Å for R_r.

Experiments were carried out on samples of transfer RNA specifically labelled with fluorescein at position 8 and coumarin at position 76 [11]. First, emission spectra were obtained by excitation at 460 nm, where $\epsilon_\mathrm{A}/\epsilon_\mathrm{D} = 0.54$; these are

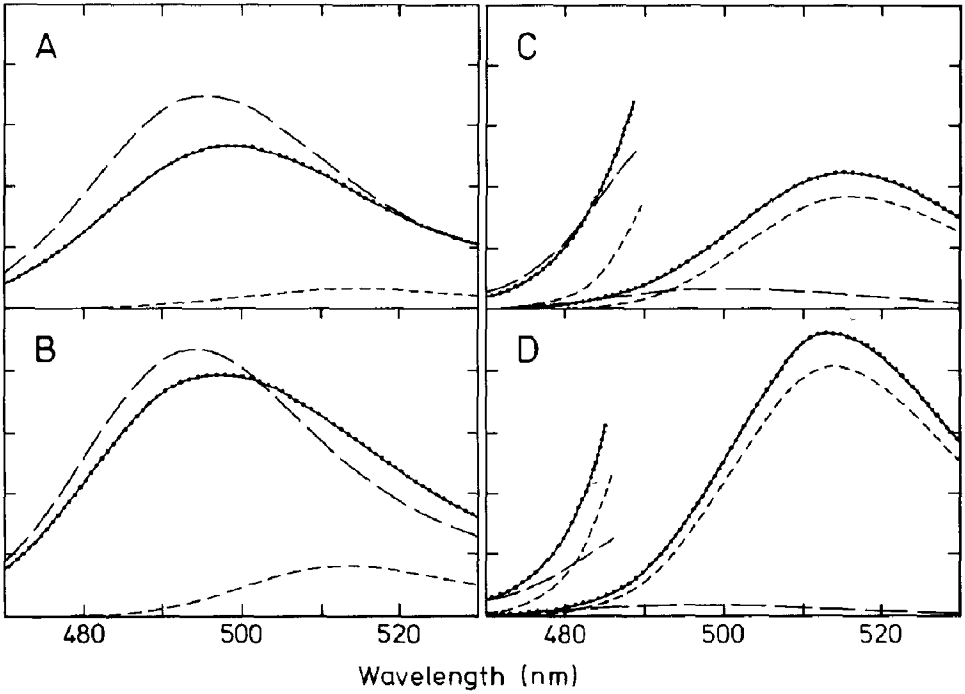

Fig. 3. Measurement of the distance between the fluorophores coumarin and fluorescein, bound to s^4U_8 and A_{76} of $tRNA^{Phe}$. (A, B) Excitation at 460 nm (above, before digestion; below, after). (C, D) As A and B but excitation at 504 nm. (———) Emission from doubly labelled tRNA, (— — —) from tRNA labelled with donor only, (- - - - -) from tRNA labelled with acceptor only, (· · · · · ·) linear combination of the latter curves giving best fit to the first. For details of the extraction of the observables G and H from the curve-fitting, see ref. 7. In C and D, the spectra are shown additionally magnified 10× in the region 470–490 nm. (Inspection of the values at 470 mm, where no acceptor emission occurs, shows qualitatively that the apparent donor quenching is smaller in C and D than in A and B. This is due to the greater weight of reverse transfer for excitation at 504 nm than at 460 mm.)

shown in fig. 3A and B. The method of separation by digestion [6,7] was employed to measure E_{exp}, the value obtained being 0.82. Use of eq. 31 along with the R_f value above led to an apparent fluorophore separation of 40.1 Å. This value was corrected by use of the complete expression (eq. 30), giving 39.1 Å.

Thus, the error introduced by ignoring reverse transfer is in this case rather less than that introduced by the usual uncertainty in the orientation factor (usually 5–10%). However, in this case the error due to ignoring reverse transfer is rather small, since the actual intramolecular distance ($\approx$ 40 Å) between the fluorophores is considerably greater than the reverse Förster distance R_r (≈ 26 Å). If the distance between the fluorophores is smaller, or one of the ratios R_r/R_f and ϵ_A/ϵ_D is larger, then a greater error must be reckoned with.

Since the correction is easily made it is obviously worth including. However, the small effect seen in fig. 3A and B does not provide evidence for the existence of reverse transfer; this is to be sought by using longer excitation wavelengths, where ϵ_A/ϵ_D is larger, as stated above.

Emission spectra were taken again, with excitation wavelengths of 504 nm, at which $\epsilon_A/\epsilon_D = 27$, and at 512 nm, where $\epsilon_A/\epsilon_D = 67$ (cf. fig. 1B). For excitation at 504 nm, the emission spectra are shown in fig. 3C and D and are qualitatively analogous to those in fig. 3A and B. The value of E_{exp} is 0.40 (cf. 0.82 expected without reverse transfer). If this is inserted into eq. 31, with re-

Table 1

Summary of experimental results for the coumarin-fluorescein pair, with $R_f = 51.6$ Å and $R_r = 26.6$ Å

The table demonstrates that eq. 30 gives correct values for R even in the presence of reverse energy transfer, while the conventional eq. 31 gives correct results only when reverse transfer is very small. The crystallographic value for R is 41 Å [12].

λ_{exc} (nm)	ϵ_A/ϵ_D	E_{exp}	R (eq. 31) (Å)	R (eq. 30) (Å)
460	0.54	0.82	40.1	39.1
504	27	0.40	55.2	39.9

verse transfer neglected, then a highly erroneous result, 55.2 Å, is obtained for the distance between the fluorophores. If the correct equation (eq. 30) is used, then the value obtained for R is correct: 39.9 Å. These data are summarised in table 1.

Measurement at an excitation wavelength of 512 nm, where $\epsilon_A/\epsilon_D = 67$, may be anticipated to give an even more extreme manifestation of reverse energy transfer. This was found qualitatively to be the case: the value obtained for the acceptor enhancement, expected to be above 1.0, was only 0.88, i.e., reverse energy transfer resulted in a paradoxical 'acceptor quenching'. The donor emission was too weak to be observed at all unless the acceptor was present, thus implying a paradoxical 'donor enhancement' due to reverse energy transfer. However, the data obtained by excitation at 512 nm were not sufficiently accurate to permit quantitative evaluation – as is frequently encountered in measurements made at the edge of an electronic transition.

3.2. Conclusions

The purpose of this paper has been to draw attention to the phenomenon of simultaneous forward and reverse transfer between unlike fluorophores and to explore situations in which it has consequences that affect significantly the usual phenomenon of one-way energy transfer. Formally, we can regard simultaneous transfer as a general effect, of which two special cases are at the one extreme normal energy transfer, i.e., forward transfer only, from a clearly defined donor to a clearly defined acceptor, and at the other extreme homotransfer, taking place between two identical fluorophores. The latter is of relatively little interest as a technique of measurement, since it leaves fluorescence intensities and lifetimes unchanged and only affects, under certain conditions, the anisotropy of emission.

When distances are measured by steady-state fluorescence, ignoring reverse transfer makes them appear too large. (In the extreme case, homotransfer would make them appear infinite.) However, by using eq. 30, a correction can easily be made without additional experimental data. In contrast, the analysis of fluorescence lifetimes is considerably complicated by reverse transfer, even if only donor emission is registered.

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