

# Pitfalls and limitations in the practical use of Förster's theory of resonance energy transfer†

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Disparate presentations in the literature of the basic equations of Förster's theory of resonance energy transfer are clarified and the limitations of these equations are discussed.

## Introduction

Theodor Förster's theory of resonance energy transfer<sup>1–6</sup> has found numerous applications in photophysics, photochemistry, photobiology, and many other fields of science, because it allows one to determine the distance between two suitable chromophores, an energy donor, D, and an acceptor, A, separated in the 1–10 nm range. Förster Resonance Energy Transfer (FRET) is used as a “spectroscopic ruler”<sup>7</sup> in bioanalytical applications,<sup>8–10</sup> for instance, to determine the distance between two markers attached to a biopolymer whose tertiary structure is not known. Moreover, FRET permits monitoring of the approach or separation of two species, one bearing D, and the other A. FRET is used in clinical tests and even to track the motion of single molecules.<sup>11</sup>

We were struck by the fact that the basic relationships of Förster's theory are

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presented in substantially disparate forms in current reviews, textbooks and papers, and even in Förster's original publications. This may lead to confusion if not to erroneous determinations of interchromophoric distances. Following extensive discussion of these issues, we have decided to publish the present article in order to help researchers to avoid such problems and pitfalls when using Förster's theory.

Let us recall that, in Förster's theory, the rate constant for energy transfer  $k_{ET}$  is given by eqn (1), where  $k_D = 1/\tau_D^0$  is the decay rate constant of the excited donor in the absence of ET,  $R$  is the distance between donor and acceptor, and  $R_0$  is the *critical quenching radius* or *Förster radius*, *i.e.*, the distance at which the rate constants  $k_{ET}$  and  $k_D$  are equal, such that ET and spontaneous decay of the excited donor are equally probable.<sup>12,13</sup>

$$k_{ET} = k_D \left[ \frac{R_0}{R} \right]^6 = \frac{1}{\tau_D^0} \left[ \frac{R_0}{R} \right]^6 \quad (1)$$

The transfer efficiency  $\Phi_{ET}$  is defined by eqn (2) and can be related to the ratio  $R/R_0$  by eqn (3), which provides a straightforward way to estimate the interchromophoric distance  $R$ , provided that  $R_0$  is known. The Förster radius  $R_0$  can indeed be determined from experimentally accessible spectroscopic quantities (*vide infra*). In those cases where distances can be calculated or measured by other methods, it has been amply demonstrated that remarkably consistent values of  $R$  are obtained from FRET measurements.

$$\Phi_{ET} = \frac{k_{ET}}{k_D + k_{ET}} \quad (2)$$

$$\Phi_{ET} = \frac{1}{1 + (R/R_0)^6} \quad (3)$$

## “Fluorescence Resonance Energy Transfer”: a misnomer

The term FRET first appeared in papers relevant to life sciences, as the acronym of “Fluorescence Resonance Energy Transfer”. Another interpretation of FRET was given by van der Meer as “Fluorescence *with* Resonance Energy Transfer”.<sup>14,15</sup> However, fluorescence is *not* involved in resonance ET, which is non-radiative. Moreover, under suitable conditions, Förster's theory may be applied even to donor–acceptor pairs undergoing triplet–singlet ET.<sup>16,17</sup> The acronym FRET is now so widely used that the solution to overcome this situation—and a way to acknowledge the author for his important contribution—is to consider that F in FRET means “Förster” or “Förster-type”, the interpretation recommended in the IUPAC Glossary of terms used in photochemistry, rather than “fluorescence”.<sup>12</sup>

We agree with R. Clegg, who published a comprehensive article on the history of FRET:<sup>18</sup> “*there are other modes of energy transfer, and circumstances where Förster transfer is not valid; these require different theoretical foundations. However, reserving “Förster” for the “F” in FRET, whenever we mean Förster transfer, gives credit to the person who made it possible for us to gain valuable, quantitative insight into so many processes at the molecular scale, through relatively easy experiments.*” Limitations of the validity of Förster's theory are mentioned below.

## The Förster radius: a “critical” parameter!

Förster's expression for the critical transfer distance  $R_0$ , written using the currently accepted symbols, is given in eqn (4), where  $\Phi_D$  is the luminescence quantum yield of

the donor in the absence of the acceptor,  $\kappa$  is an orientation factor,  $n$  is the average refractive index of the medium in the region of spectral overlap,  $N_A$  is the Avogadro constant, and  $J$  is a spectral overlap integral. Eqn (4) was misprinted ( $\pi^6$  instead of  $\pi^5$ ) in some papers by Förster<sup>5,6</sup> and corrected later by himself.<sup>19,20</sup>

$$R_0^6 = \frac{9(\ln 10) \kappa^2 \Phi_D}{128\pi^5 N_A n^4} J \quad (4)$$

The integral  $J$  can be defined in either the wavenumber or the wavelength scale, as given by eqn (5) and (6) for  $J^{\tilde{\nu}}$  and  $J^\lambda$ , respectively. The quantities  $\bar{I}_{\tilde{\nu}}^{D^*}$  and  $\bar{I}_\lambda^{D^*}$  represent the normalized spectral radiant intensity of the excited donor, defined as the derivative of the emission intensity  $I$  with respect to wavenumber or wavelength, respectively. The integrals  $J^{\tilde{\nu}}$  and  $J^\lambda$  are identical because  $\bar{I}_{\tilde{\nu}}^{D^*}$  and  $\bar{I}_\lambda^{D^*}$  are normalized to unit area and  $\varepsilon_A(\tilde{\nu}) = \varepsilon_A(\lambda)$ . However, it should be recalled that the spectral intensities are not equal but  $I_{\tilde{\nu}}^{D^*} = \lambda^2 I_\lambda^{D^*}$ . Current spectrofluorometers based on grating monochromators have a constant wavelength bandpass and the natural integral to be calculated is (6), though calculation of integral (5) is equally valid. Care has to be taken to calculate the integrals after the emission spectrum has been corrected for the spectrofluorometer exit channel response function. A reference compound with known emission spectrum can be used for this purpose as quoted in ref. 21. Notice, however, that  $\bar{I}_\lambda^{D^*}$  is incorrectly defined in that reference as the peak-normalized fluorescence spectrum. Normalization has to be carried out to unit area as stated in eqn (5) and (6).

$$J^{\tilde{\nu}} = \int \bar{I}_{\tilde{\nu}}^{D^*} \varepsilon_A(\tilde{\nu}) \frac{d\tilde{\nu}}{\tilde{\nu}^4}; \int \bar{I}_{\tilde{\nu}}^{D^*} d\tilde{\nu} = 1 \quad (5)$$

$$J^\lambda = \int \bar{I}_\lambda^{D^*} \varepsilon_A(\lambda) \lambda^4 d\lambda; \int \bar{I}_\lambda^{D^*} d\lambda = 1 \quad (6)$$

In eqn (4), the units of the physical quantities involved can be chosen arbitrarily, as long as their product is the same on both sides. The common units of the quantities appearing in the spectral overlap integral  $J^\lambda$  are  $M^{-1} \text{ cm}^{-1} = \text{mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$  for the decadic molar absorption coefficient,  $\varepsilon_A$ , and nm for wavelength,  $\lambda$ . Collecting the numerical constants and length conversion factors, the practical expression (7) follows. Other equally valid expressions containing different numerical factors are

obtained when different units are chosen (see ref. 8, p. 16).

$$\frac{R_0}{\text{nm}} = 0.02108 \left[ \frac{\kappa^2 \Phi_D}{n^4} \left( \frac{J^\lambda}{\text{mol}^{-1} \text{ dm}^3 \text{ cm}^{-1} \text{ nm}^4} \right) \right]^{1/6} \quad (7)$$

However, most papers and textbooks show a factor 9000 instead of 9 in the numerator of eqn (4).<sup>8</sup> Apparently, this is done to account for the conversion of  $\text{dm}^3$  (appearing in  $\varepsilon_A$ ) to  $\text{cm}^3$ . Such an equation no longer has equal units on both sides (*caveat emptor!*). The innocent user is led to the erroneous conclusion that the numerical constant in eqn (7) should be 0.0667 instead of 0.02108, yielding more than three times larger  $R_0$  values! Another way of forcing equations to accommodate ill-defined units was given by Förster: while a factor 9000 is found in ref. 5, a factor of 9 appears in ref. 20 together with  $N'$ , “the number of molecules per millimole”, instead of  $N_A$  in the denominator of eqn (4). Naturally,  $N' = 6.022 \times 10^{20} \text{ mmol}^{-1} = 6.022 \times 10^{23} \text{ mol}^{-1}$  is nothing but the Avogadro constant expressed in different units. These confusing and unnecessary practices should be avoided.<sup>22</sup> On doing such conversions, the units in eqn (4) must be given explicitly, as in the scaled eqn (4a) and (4b).

$$\left( \frac{R_0}{\text{cm}} \right)^6 = \frac{9000(\ln 10)}{128\pi^5} \left( \frac{\text{mol}^{-1}}{N_A} \right) \times \frac{\kappa^2 \Phi_D}{n^4} \left( \frac{J^\lambda}{\text{mol}^{-1} \text{ dm}^3 \text{ cm}^3} \right) \quad (4a)$$

$$\left( \frac{R_0}{\text{cm}} \right)^6 = \frac{9(\ln 10)}{128\pi^5} \left( \frac{\text{mmol}^{-1}}{N_A} \right) \times \frac{\kappa^2 \Phi_D}{n^4} \left( \frac{J^\lambda}{\text{mol}^{-1} \text{ dm}^3 \text{ cm}^3} \right) \quad (4b)$$

The evaluation of  $R_0$  requires great care. It should be emphasized that severe errors may be introduced by applying  $R_0$  values for couples of commercial dyes as given by the manufacturer or in the literature without regard of the microenvironment pertinent to the actual experiment. The fluorescence overlap integral and the donor fluorescence quantum yield must be determined and the refractive index of the microenvironment estimated in each case.

## About the orientation factor

The orientation factor,  $\kappa^2$ , shown in eqn (4) poses some difficulties when actual calculations are performed. Frequently, fast isotropical rotation of donor and acceptor is assumed, yielding  $\kappa^2 = 2/3$ .<sup>23</sup> This assumption seems to be justified in cases of practical interest, particularly when dealing with donor and acceptor chromophores bound to polypeptides and proteins.<sup>24</sup> Complete depolarization of fluorescence in simple donor–acceptor systems can be used to check randomization of the transition dipoles during the excited state lifetime. However, care must be taken in multichromophoric systems, because partial depolarization can be the result of energy migration (an example is given later). For a full account of  $\kappa^2$  values in different practical situations, see ref. 14 and 25.

In certain cases, transition moments have fixed orientations and distances may be calculated with a great degree of confidence. The general expression for  $\kappa$ , given usually in angular notation,<sup>23</sup> is shown in vectorial form in eqn (8), where  $\vec{\mu}_D$  and  $\vec{\mu}_A$  are the donor and acceptor transition moment vectors, respectively, and  $\vec{r}$  is the unit vector in the direction of  $R$ . A similar expression can be found in ref. 26 (eqn (8) therein; notice that the transition moments and distance are considered as unit vectors in that equation). It is common practice to state that  $\kappa^2 = 0$  when  $\vec{\mu}_D$  and  $\vec{\mu}_A$  are perpendicular. However, eqn (8) requires in addition that at least one of them is perpendicular to  $\vec{r}$ . A typical example can be found in ref. 27. In addition to this special case, there are situations where  $\kappa^2$  equals 0 without requiring that both terms on the right-hand side of eqn (8) vanish. The actual condition is that the electric field generated by the donor dipole is perpendicular to the transition moment of the acceptor.

$$\kappa = \frac{\vec{\mu}_D \cdot \vec{\mu}_A - 3(\vec{\mu}_D \cdot \vec{r})(\vec{\mu}_A \cdot \vec{r})}{\mu_D \mu_A} \quad (8)$$

## A practical example

Intramolecular Förster-type ET processes were investigated in a polyphenylene dendrimer substituted with three identical, spatially well-separated, *para*-substituted peryleneimide chromophores.<sup>28</sup> Direct excitation of the peryleneimide units results in

energy hopping among the chromophores. The critical radius  $R_0$  was calculated using the following experimental data: fluorescence quantum yield of the donor,  $\Phi_D = 0.98$ ; dipole–dipole orientation factor calculated from three-dimensional molecular mechanics modelling,  $\kappa^2 = 2.1$ ; solvent refractive index,  $n = 1.496$  (toluene); spectral overlap integral calculated using eqn (6),  $J = 2.5 \times 10^{14} \text{ M}^{-1} \text{ cm}^{-1} \text{ nm}^4$ . The acceptor absorption coefficient, calculated as one third of the absorption coefficient of the substituted dendrimer, is expressed in  $\text{M}^{-1} \text{ cm}^{-1}$  and wavelength is expressed in nm. Using eqn (7), a value  $R_0 = 4.56 \text{ nm}$  is obtained. From the donor excited state lifetime,  $\tau_D^0 = 4.0 \text{ ns}$ , obtained by time-resolved measurements, and the energy hopping rate constant,  $k_{\text{hopp}} = k_{\text{ET}} = 4.6 \text{ ns}^{-1}$ , determined by anisotropy measurements, an average interchromophoric distance  $R \approx 2.8 \text{ nm}$  is calculated, in excellent agreement with the value obtained by molecular mechanics modelling, 2.8 nm.

The individual variables involved in the calculation may have substantial uncertainties but, due to the sixth-power dependence, the uncertainty in  $R_0$  can be relatively small. Though error limits should be evaluated in each particular case, the error of  $R_0$  values calculated through eqn (4) may be conservatively estimated as 5%, as long as  $\kappa^2$  can be ascertained with confidence. The standard representation of the ET efficiency according to eqn (3) is given in Fig. 1. It may be seen from the figure that the maximum sensitivity in the calculation of  $R$  is found at  $R \approx R_0$  and any practical calculation of  $R/R_0$  is restricted to the interval 0.5–2. Since current values of  $R_0$  generally range from 2 to 5 nm, the theory is useful for the calculation of  $R$ -values between 1 and 10 nm. For a more general discussion see ref. 13, p. 249.

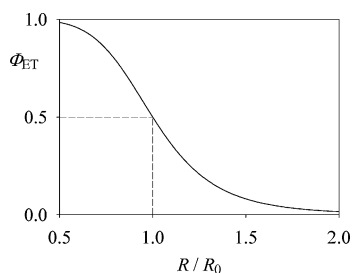


Fig. 1 Graphical representation of eqn (3).

## On the validity of Förster's theory

Judicious application of Förster's theory is very useful for the determination of intermolecular distances on the nanoscale, the typical range of the dimensions of the molecular structures regulating cellular function.<sup>29</sup> Therefore, FRET measurements can be used to determine geometries and to explore intermolecular interactions in complex systems such as membranes, proteins, nucleic acids and carbohydrates.<sup>30</sup> Together with the intrinsic sensitivity of fluorescence techniques and their relative simplicity, FRET measurements are selective, specific and non-invasive. The last property, essential when the structure of biological membranes is studied, can be achieved using chromophore-labelled isomorphs of biomolecules.<sup>31</sup> However, it should be kept in mind that application of Förster's theory, which is based on the dipolar approximation, is restricted to the weak coupling limit. During the last decades, advances were made in order to study molecular aggregates, which required extension of the theory into the intermediate and strong coupling cases and other improvements.<sup>26</sup>

The dipole approximation of transition densities is perfectly satisfactory for calculating oscillator strengths or radiative rate constants because the wavelength of light far exceeds the size of molecules. Averaging of the transition density is, however, inappropriate when a molecule interacts with another molecule that is nearby. Thus, special attention must be paid to the case where the donor–acceptor separation approaches the size of the chromophores. The Coulombic term is then strongly influenced by the shape of the molecular charge distribution because it originates from a coupling of donor and acceptor transition densities. This shape effect in the Coulombic coupling can be captured by considering the total interaction as a sum of electrostatic interactions between point monopoles located at the atoms of the chromophores.<sup>32,33</sup> Alternatively, the transition density can be calculated using quantum chemical methods enabling accurate estimation of the Coulombic coupling.<sup>34</sup> Multipole expansions of the Coulombic coupling converge slowly for molecules and truncated expansions do not necessarily improve the dipole approximation. It can thus be shown that, for allowed transitions, the dipole approxi-

mation typically works well at distances around  $R_0$  or larger. As demonstrated by the example of the previous section, the theory may hold even in cases where the separation of the chromophores  $R$  is on the order of  $R_0/2$ , as long as it exceeds their dimensions.

When dealing with distances shorter than 1 nm, short-range interactions resulting from interchromophore orbital overlap<sup>35,36</sup> should also be examined. In the case of naphthalene dimers at an interchromophoric distance of 0.4 nm, the contribution of short-range interactions to the overall calculated electronic coupling is only 17%, which amounts to a 37% increase in the ET rate constant.<sup>37</sup> Such examples show that in many cases where Förster's theory is not strictly applicable, the predominant mechanism of transfer remains to be the dipole–dipole interaction.

Energy transfer processes that are usually not well described by Förster's theory are encountered in photosynthetic light harvesting.<sup>38,39</sup> These natural systems have served to enhance our understanding of energy transfer phenomena beyond the Förster equation. To achieve a high efficiency of energy migration, interchromophore separations are commonly much less than  $R_0$ . While the dipole approximation fails in these cases, it is generally found that short-range effects of orbital overlap are usually small and can be safely ignored. When the electronic coupling of chromophores is very strong, molecular exciton states<sup>40</sup> may serve as spatially extended donors or acceptors.<sup>26</sup> Departure from Förster's theory is significant in that case and cannot be ignored.<sup>41,42</sup> Even symmetry forbidden (dark) transitions can be very efficient excitation energy acceptors.<sup>43</sup>

Another ingredient of the Förster equation that has received recent scrutiny is the solvent screening factor,  $1/n^4$ . The solvent dielectric properties appear in equations for absorption and emission, but in Förster's derivation these effects cancel.<sup>44</sup> The refractive index dependence that remains in the final equation solely represents how the Coulombic interaction is influenced by the optical dielectric constant ( $n^2$ ) of the solvent surrounding donor and acceptor. The polarizability of the solvent enhances energy transfer by increasing the donor and acceptor transition dipole strengths, but that effect is counterbalanced by a screening of the interaction.<sup>45</sup> The net effect depends on

the structure of the dielectric environment around the donor and acceptor molecules. Recent work suggests that approximating the solvent screening by  $1/n^2$  is satisfactory when the dipole approximation for the electronic coupling works well, but when donor and acceptor approach each other closely, the screening becomes exponentially reduced.<sup>46</sup>

Some of these mechanisms that influence energy transfer dynamics in complex systems are more suited to the specialist. From a pragmatic viewpoint, we find that Förster's theory typically works well when the critical radius  $R_0$  exceeds the size of the chromophores, thus demonstrating its quantitative value. Furthermore, even when it is not quantitatively accurate, Förster's theory still provides the framework upon which we can elucidate the more complex ways that molecules interact with each other on short length scales.

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- 15 Comment by Prof. W. van der Meer, Kentucky: FRET is now an abbreviation that is almost as established as NMR. Soon, nobody will say Förster Resonance Energy Transfer or Fluorescence with Resonance Energy Transfer anymore. We will all call it simply FRET. Let's not forget, however, that there is a didactic aspect here: future spectroscopists must understand what FRET involves, and when they will be exposed to FRET for the first time, the explanation of the acronym helps to understand the principles involved. The only problem is that Fluorescence Resonance Energy Transfer is misleading in that it suggests that Fluorescence is transferred, and that is not correct. Fluorescence serves to detect the phenomenon. I have proposed to solve this problem by inserting a virtually silent "with". Fluorescence with Resonance Energy Transfer is, in my opinion, the best name for FRET, because it is both descriptive and correct. Braslavsky *et al.* state that Fluorescence is not involved in Resonance Energy Transfer. However, Fluorescence is almost always used to detect it. They also point out that Förster's theory is applicable to donor-acceptor pairs undergoing triplet-singlet energy transfer. Perhaps it is an idea to use the name Förster Resonance Energy Transfer in situations where it is not clear Fluorescence is used. I propose to use the name Fluorescence with Resonance Energy Transfer for all cases where there is Resonance Energy Transfer and Fluorescence is used to measure it.
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