Resonance Energy Transfer

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Abstract

Resonance energy transfer* is a spectroscopic process whose relevance in all major areas of science is reflected both by a wide prevalence of the effect, and through numerous technical applications. It is an optical near-field mechanism which effects a transportation of electronic excitation between physically distinct atomic or molecular components, based on transition dipole-dipole coupling. In this chapter a comprehensive survey of the process is presented, beginning with an outline of the history and highlighting the early contributions of Perrin and Förster. A review of the photophysics behind resonance energy transfer follows, and then a discussion of some prominent applications of resonance energy transfer. Particular emphasis is given to techniques used in molecular biology, ranging from the 'spectroscopic ruler' measurements of functional group separation, to fluorescence lifetime microscopy. Finally, applications to synthetic polymers and chemical sensors are examined.

1. History of RET

1.1 The first experiments

Resonance energy transfer (RET) is a process by means of which the energy of an excited atom or molecule (usually called the donor, but known historically as the 'sensitizer') is transferred non-radiatively to an acceptor molecule ('activator'), through intermolecular dipole-dipole coupling. The origins of its discovery can be traced back to 1922, when the phenomenon of resonance energy transfer ('sensitized fluorescence') was first experimentally observed by Cario and Franck[1-3] in the gas phase. This spectroscopic experiment involved illuminating a mixture of mercury and thallium vapours at a wavelength absorbed solely by the mercury; the resulting fluorescence spectra proving to include frequencies that could only be emitted from thallium. Such energy transfer in vapours was at first assumed to be uniquely associated with inter-atomic collisions, but a discovery that transfer could occur at larger separations than the collision radii showed that this was not necessarily the case. Soon RET was also being observed in solutions,[4] and over the following years in many other physical systems.

* RET is also known as Förster- or fluorescence- resonance energy transfer (FRET), or electronic energy transfer (EET)

1.2 Early developments of theory

The first theoretical explanation of the phenomenon was proposed by the Nobel laureate J. Perrin.[5] He recognized that energy could be transferred from an excited molecule to its neighbours amongst closely spaced molecules through dipole interactions; he named this process "transfert d'activation", and his paper on the subject became the earliest attempt to describe non-radiative (near-field) energy transfer. Despite its initial success, however, Perrin's model incorrectly predicted that non-radiative energy transfer should be possible between dye molecules up to an intermolecular distance of 1000 Å, deriving from an inaccurate assumption that the molecules would act as Hertzian oscillators with exactly defined resonance frequencies. Five years later,[6] Perrin's son Francis developed a corresponding quantum mechanical theory of RET, based on Kallman and London's results.[7] In this work he recognized a "spreading of absorption and emission frequency" due to the interactions of the dye with the solvent, thus reducing the probability of energy transfer. As a result, efficient transfer was calculated to occur up to 150 - 250 Å, still approximately a factor of 3 greater than experimentally observed. A detailed and readable survey of these early contributions of J. and F. Perrin can be found in a review by Berberan-Santos.[8]

1.3 Förster theory

Extending the ideas of J. and F. Perrin, Förster developed the first essentially correct theoretical treatment of RET.[9-11] Förster determined that energy transfer, through dipolar coupling between molecules, mostly depends on two important quantities: spectrum overlap and intermolecular distance. Following the observation that "the absorption and fluorescence spectra of similar molecules are far from completely overlapping", he found a means to quantify the spectral overlap integral. The dipole-dipole interaction was known to have an inverse proportionality on the cube of the molecular separation. Since the rate of energy transfer is proportional to the square of this coupling, it thus depends on the sixth power of the separation – i.e. the famous R^{-6} distance-dependence law. Moreover, the acceptor distance at which this rate equates to that of spontaneous emission by the donor, now termed the Förster radius R_0, was now calculated to be between 10 and 100 Å, in agreement with experimental observations.

Much later, the distance dependence predicted by Förster was fully verified by fluorescence studies of donor-acceptor pairs at known separations,[12,13] leading to the suggested employment of RET as a 'spectroscopic ruler' by Stryer and Haugland;[13] hence, a technique to measure the proximity relationships and conformational change in macromolecules was realized (see Section 3). With the advent of the laser, in the 1960s, the modern understanding of RET led to a raft of modern applications. An excellent in-depth review on the history of RET is given by Clegg.[14]

2. The photophysics of RET

Resonance energy transfer is a mechanism that is now known to operate across a diverse and extensive range of physical systems, encompassing not only gases and dye solutions, but also protein complexes, doped crystals and polymers, to name but a few. Nonetheless, at a fundamental level it is possible to identify numerous common features in the underlying photophysics.

2.1 Primary excitation processes

To approach the subject in detail, let us commence with the photoexcitation process that creates the conditions for RET to occur. When resonant ultraviolet or visible radiation impinges on any non-homogeneous dielectric material, the primary result of photon absorption is the population of electronic excited states in individual atomic, molecular or other nanoscale centres – henceforth, the latter are to be grouped together under the generic term 'chromophore'. Typically, such absorption is immediately followed by a rapid but partial degradation of the acquired energy, the associated losses (largely due to vibrational dissipation) ultimately to be manifest in the form of heat. This effect owes its origin to the principle that the release of electronic energy by fluorescence generally occurs from the lowest vibrational level of the excited state. However, if any nearby chromophore has a suitably disposed electronic state, of a similar or slightly lower energy, that neighbour may acquire the major part of the electronic excitation through RET – a process that takes place well before any further thermal degradation of the excited state energy occurs. The mechanism is most commonly studied through spectrometric differentiation of fluorescence emerging from the initially excited energy donor and from the energy acceptor species, as illustrated in Fig. 1. As will be shown in the following, the propensity for energy to be transferred between any two chromophores is severely restricted by distance, and if no suitable acceptor is within reach, the donor will generally shed its energy by fluorescence or local dissipation.

2.2 Coupling of electronic transitions

In systems where resonance energy transfer occurs, the donors and acceptors are usually fluorophores, i.e. chromophores that have the capacity to exhibit fluorescence decay. Moreover, in RET, the transitions of donor decay and acceptor excitation are generally electric dipole-allowed – although other possibilities occasionally arise. Accordingly the theory of energy transfer, for donor-acceptor displacements beyond the region of significant wavefunction overlap, is traditionally conceived in terms of an electrodynamical coupling between transition dipoles.

Consider the pairwise transfer of excitation between two chromophores *D* and *A*. In the context of this elementary mechanism, *D* is designated the donor and *A* the acceptor. Specifically, let it be assumed that prior excitation of the donor generates an electronically excited species *D**. Forward progress of the energy is then accompanied by donor decay to the ground electronic state. Acquiring the energy, *D* undergoes a transition from its ground to its excited state. The complete RET process is generally a singlet-singlet coupling mechanism[†], due to the constraints of spin conservation (although this does not apply to Dexter exchange, *vide infra*), and its entirety may be expressed by the following chemical equation:

$$^1D^* + {}^1A \xrightarrow{\text{RET}} {}^1D + {}^1A^* . \qquad (1)$$

The excited acceptor, *A**, subsequently decays either in a further transfer event, or by another means such as fluorescence. Since the *D** and *A** excited states are real, with measurable lifetimes, the core process of energy transfer itself is fundamentally separable from the initial electronic excitation of *D* and the eventual decay of *A*; the latter processes do not, therefore, enter into the theory of the pair transfer.

2.3 Dissipation and line-broadening

To delve more deeply into the nature of the process, it needs to be recognized that equation (1) tells only part of the story, dealing as it does with only electronic excitations. In general other, dissipative processes are also engaged. In a solid, the linewidth of optical transitions manifests the influence of local electronic environments which, in the case of strong coupling, may lead to the production of phonon side-bands. Similar effects in solutions or disordered solids represent inhomogeneous interactions with a solvent or host, while the broad bands exhibited by chromophores in complex molecular systems signify extensively overlapped vibrational levels, including those associated with skeletal modes of the superstructure. In each case, energy level broadening can allow pair transfer to occur at any point within the region of overlap between the donor emission and acceptor absorption bands, as illustrated in Fig. 2.

2.4 The Förster equation

The Förster theory delivers an expression for the rate of pairwise energy transfer, w_F, valid for any donor-acceptor separation, *R*, that is substantially smaller than the wavelengths of visible radiation. For systems where the common host material for the donor and acceptor has refractive index *n*, at the optical frequency corresponding to the mean transferred energy, the Förster result is as follows:[15]

[†] Triplet-triplet energy transfer is also allowed by the Förster mechanism.

$$w_{\mathrm{F}} = \frac{9\kappa^2 c^4}{8\pi\tau_{D^*} n^4 R^6} \int F_D(\omega)\sigma_A(\omega)\frac{\mathrm{d}\omega}{\omega^4} \quad . \tag{2}$$

In this expression, $F_D(\omega)$ denotes the normalized fluorescence spectrum of the donor, $\sigma_A(\omega)$ represents the linear absorption cross-section of the acceptor, and ω is an optical frequency in radians per unit time; the specific form of the integral within which they appear is known as the spectral overlap – one of the key determinants of energy transfer efficiency.[16] Also in equation (2), c is the speed of light and τ_{D^*} is the associated radiative decay lifetime in the absence of transfer. The latter is related to the measured fluorescence lifetime τ_{fl} through the fluorescence quantum yield $\eta = \tau_{\mathrm{fl}}/\tau_{D^*}$, where τ_{fl}^{-1} is explicitly expressible as;

$$\frac{1}{\tau_{\mathrm{fl}}} = \frac{1}{\tau_{D^*}} + \frac{1}{\tau_{D^*}}\left(\frac{R_0}{R}\right)^6 \quad . \tag{3}$$

The last term on the right-hand side of equation (3) is expressed with reference to the Förster radius R_0 – the distance at which the rates of donor deactivation by RET and by spontaneous fluorescence become equal. As is evident from Fig. 2, the propensity for forward transfer is usually significantly greater than that for backward transfer, due to a sizeable difference in the spectral overlaps for the two processes.

2.5 Orientation dependence

The κ factor in equation (2) depends on the orientations of the donor and acceptor, both with respect to each other, and with respect to their mutual displacement unit vector $\hat{\boldsymbol{R}}$, as follows:

$$\kappa = (\hat{\boldsymbol{\mu}}_D \cdot \hat{\boldsymbol{\mu}}_A) - 3(\hat{\boldsymbol{R}} \cdot \hat{\boldsymbol{\mu}}_D)(\hat{\boldsymbol{R}} \cdot \hat{\boldsymbol{\mu}}_A) \ . \tag{4}$$

For each chromophore, $\hat{\boldsymbol{\mu}}$ designates a unit vector in the direction of the appropriate transition dipole moment. The possible values of κ^2, as featured in equation (2), lie in the range (0, 4). It is evident that in the case of fixed chromophore positions and orientations the result delivered by (4) is a function of three independent angles, as shown and defined in Fig. 3:

$$\kappa = \cos\theta_T - 3\cos\theta_D \cos\theta_A \quad . \tag{5}$$

Unfavorable orientations can thus reduce the rate of energy transfer to zero; other configurations, including many of those found in photobiological systems, optimize the transfer rate. The angular disposition of chromophores is therefore a very important facet of energy transfer. It is important to note that

transfer is not *necessarily* precluded when the transition moments lie in perpendicular directions – provided that neither is also disposed orthogonally to $\boldsymbol{R}$ (= $R\,\hat{\boldsymbol{R}}$).

In any, at least partially, fluid or disordered system the relative orientation of all donor-acceptor pairs may not be identical, and it is then the distributional average of κ^2 that determines the overall measured response. In the isotropic case (completely uncorrelated orientations) the κ^2 factor averages to $\frac{2}{3}$; departures from this value provide the quantitative signature of a degree of orientational correlation. In molecules of sufficiently high symmetry it can also happen that either the donor or the acceptor transition moment is not unambiguously identifiable with a particular direction in the corresponding chromophore reference frame. Specifically, the electronic transition may then relate to a transition involving a degenerate state – as can occur with square planar complexes, for example.[17] Alternatively, the same observational features might indicate rapid but orientationally confined motions. The considerable complication which each of these effects brings into the trigonometric analysis of RET has been extensively researched and reported by van der Meer.[18]

2.6 Förster radius

In applications of RET to spectroscopy, as noted above, it is usually significant that the electronically excited donor can in principle release its energy by spontaneous decay, the ensuing fluorescence also being amenable to detection by any suitably placed photodetector. Since the alternative possibility (that of energy being transferred to another chromophore within the system) has such a sharp decline in efficiency as the distance to the acceptor increases, it is commonplace to invoke the critical distance R_0. The Förster rate equation is itself often cast in an alternative form, exactly equivalent to equation (2), explicitly exhibiting this critical distance:[19]

$$w_{\mathrm{F}} = \frac{3\kappa^2}{2}\frac{1}{\tau_{D^*}}\left(\frac{\bar{R}_0}{R}\right)^6 \quad . \tag{6}$$

Here the symbol with its over-bar, $\bar{R}_0$, is defined as the Förster radius for which the orientation factor κ^2 would assume its isotropic average value, $\frac{2}{3}$.[20] For complex systems the angular dependence is quite commonly disregarded and the following, simpler expression employed;

$$w_{\mathrm{F}} = \frac{1}{\tau_{D^*}}\left(\frac{R_0}{R}\right)^6, \tag{7}$$

leading to a transfer efficiency Φ_{T} expressible as:

$$\Phi_{\mathrm{T}} = \frac{1}{1+\left(R/R_0\right)^6} = 1-\frac{\tau_{\mathrm{fl}}}{\tau_{D^*}} = 1-\frac{I_{\mathrm{fl}}}{I_{D^*}} \quad , \tag{8}$$

where, I_{fl} and I_{D^*} are the intensities of the donor fluorescence with the acceptor present and excluded, respectively, and τ_{fl} specifically denotes the fluorescence lifetime of the donor measured within its RET environment. As graphically depicted in Fig. 4, a donor-acceptor displacement equal to R_0 corresponds to a transfer efficiency of 50%.

The final equality on the right of equation (8), which holds provided decay processes follow single-exponential decay kinetics, provides a formula cast in terms of easily measurable quantities. This is particularly useful since it allows energy transfer efficiencies to be calculated simply on the basis of intensity measurements (for example using a fluorimeter), obviating the separate time-resolved measurements that are otherwise generally necessary for evaluation of the characteristic decay lifetimes τ_{fl} and τ_{D^*}. When a given electronically excited chromophore is within a distance R_0 of a suitable acceptor, RET will generally be the dominant decay mechanism; conversely, for distances beyond R_0, spontaneous decay (usually fluorescence) will be the primary means of donor deactivation.

2.7 Polarization features

When linearly polarised laser light is used to excite any specific species within a complex disordered solid or liquid system, the probability for excitation of any particular molecule is proportional to $\cos^2\theta$, where θ is the angle between the appropriate excitation transition moment and the electric polarisation vector of the input radiation. Consequently the population of excited molecules has a markedly anisotropic distribution, a phenomenon associated with the term *photoselection*. If radiative decay were to ensue instantaneously, i.e. from precisely the initially populated excited level, then the fluorescence would carry the full imprint of that anisotropy and itself exhibit a degree of polarization – the highest value possible. Accounting for the necessary three-dimensional rotational average,[21] it is readily shown that the fluorescence intensity components polarized parallel to and perpendicular to the polarization of the excitation beam, $I_{||}$ and $I_{\perp}$ respectively, would then lie in the ratio 3:1. Commonly observed departures from this result thus signify the extent to which the orientation of the emission dipole differs from that of the prior, initial excitation – which may be due to intervening decay, molecular motion or intermolecular energy transfer.

The two most widely used quantitative expressions of polarization retention are the *fluorescence anisotropy*, *r*, or the *degree of polarization*, *P*. Both convey the same information; they are defined and related as follows:

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}, \qquad P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \quad \Rightarrow \quad r = \frac{2P}{3 - P} \quad . \tag{9}$$

The denominator of the expression for r designates the net fluorescence intensity. In a specific situation where the donor and acceptor have transition dipole moments oriented in parallel, then $r = 0.4$ and $P = 0.5$.

A key molecular factor determining any loss in polarization is the angle θ between the directions of the absorption and emission transition dipole moments. In terms of this parameter and its influence on the measured fluorescence anisotropy, the case where internal decay intervenes between excitation and fluorescence decay within a single molecule is no different from that of a donor-acceptor pair where the absorption and emission processes are spatially separated – provided the donor and acceptor in the latter case have a fixed mutual orientation (the orientation of the pair being random). The following result, derived by Levshin[22] and Perrin[23] can be applied in both situations:

$$P = \frac{3\cos^2\theta - 1}{3 + \cos^2\theta} \quad . \tag{10}$$

In the case of a donor-acceptor pair, θ is to be interpreted as the angle θ_T shown in Fig. 3. Equation (10) thus allows direct calculation of this microscopic parameter, through measurement of the macroscopic quantity P. Moreover when P proves to exhibit a time-dependent decay, a study of the kinetics provides information on the extent of rotational motion intervening between the absorption and emission events.

Very different behaviour is observed for RET systems in which the donor and acceptor are orientationally uncorrelated, i.e. where they are both, independently, randomly oriented. In such cases there is a very rapid loss of polarization 'memory', and it transpires that the associated degree of anisotropy is precisely 1/25, i.e. $r = 0.04$;[24] two or more energy transfer jumps will therefore usually, to all intents and purposes, totally destroy any polarization in any ensuing fluorescence. However it should be noted that there is a surprising recovery in the anisotropy at distances approaching the transfer wavelength. The effect is sufficiently strong to warrant attention in dilute solution studies.

2.8 Diffusion effects

So far only RET between a donor-acceptor pair has been considered. The discussion is now extended to an ensemble of donors *D* and acceptors *A*, all units of which are distributed randomly within an *n*-dimensional volume. For

systems in which translational diffusion is extremely slow compared to the rate of energy transfer, the time-dependence of the donor intensity decay at time *t*, $I_{D^*}(t)$, as obtained by Förster,[11] is given by the following expression:

$$I_{D^*}(t) = I_{D^*}(0)\exp\left[-\frac{t}{\tau_{D^*}} - 2\gamma\left(\frac{t}{\tau_{D^*}}\right)^{\frac{n}{6}}\right] \quad . \tag{11}$$

The most commonly applied form of this expression is when *n* equals 3, i.e. RET in three dimensions. In equation (11), the parameter γ is explicitly written as;

$$\gamma = \frac{2}{3}\pi^{\frac{3}{2}} C_A R_0^3 \quad , \tag{12}$$

in which C_A is the concentration of acceptors (number per unit volume) and $(4/3)\pi R_0^3 C_A$ represents the average number of acceptor chromophores in a sphere of radius R_0; the orientational factor is again set as $\frac{2}{3}$.

The case where diffusion is comparable to the transfer rate is very complicated, and calculations by Butler and Pilling[25] have shown that large errors arise on using Förster theory for systems with diffusion coefficients in excess of 10^{-5} cm^2 s^{-1}. To address such systems, a successful approximation was developed by Gösele *et al.*[26] This approach involves the insertion of a multiplier *G* within the second term in the exponential of equation (11). With *n* = 3, the parameter *G* is given by;

$$G = \left(\frac{1 + 5.47x + 4.00x^2}{1 + 3.34x}\right)^{\frac{3}{4}} \quad , \tag{13}$$

in which $x = D\left(R_0^6/\tau_{D^*}\right)^{-1/3} t^{2/3}$, where *D* is the mutual diffusion coefficient. In contrast to the Förster theory, the above method provides an excellent approximation – as was fully verified by the authors of ref. 25.

2.9 Long-range transfer

Förster theory is found to be increasingly inaccurate for RET as donor-acceptor distances extend over and beyond 100 Å. It was originally assumed that a 'radiative' process accounted for energy transfer over such donor-acceptor separations, and some recent literature on the subject still perpetuate this initial over-statement. Certain sources wrongly treat Förster 'radiationless' energy transfer as exact, distinct and separable from 'radiative' energy transfer – the

latter signifying successive but independent processes of fluorescence emission by a donor, and capture of the ensuing photon by an acceptor.

Although that certainly is the observed character of resonance energy transfer over very long distances – as for example between donor and acceptor components in a dilute solution – it is now known that both 'radiative' and Förster transfer are simply the long- and short-range limits of one powerful, all-pervasive mechanism. The latter, determined from quantum electrodynamical calculations, is the outcome of the unified theory of RET.[27] This theory not only embraces Förster and 'radiative' energy transfer, but also addresses the intermediate range in which neither of these mechanisms are fully valid. An expression for the total pairwise energy transfer rate, ranging from molecular dimensions up to interstellar distances, is written as;

$$w = w_{\mathrm{F}} + w_{\mathrm{I}} + w_{\mathrm{rad}} \quad , \tag{14}$$

where w_{F} represents the Förster rate of equation (2), w_{rad} is the rate of 'radiative' energy transfer – explicitly given by;

$$w_{\mathrm{rad}} = \frac{9\kappa'^2}{8\pi\tau_{\mathrm{D}}R^2}\int F_{\mathrm{D}}(\omega)\sigma_{\mathrm{A}}(\omega)\,\mathrm{d}\omega \quad , \tag{15}$$

and w_{I} is the intermediate term that is expressed as:

$$w_{\mathrm{I}} = \frac{9c^2}{8\pi\tau_{\mathrm{D}}n^2R^4}(\kappa^2 - 2\kappa\kappa')\int F_{\mathrm{D}}(\omega)\sigma_{\mathrm{A}}(\omega)\frac{d\omega}{\omega^2} \quad . \tag{16}$$

In both equation (15) and (16), the symbol κ' denotes an orientation factor identical to (4) but with the '3' omitted from the second term. In summary, the unified theory of RET contains not only the R^{-6} term of Förster theory and the R^{-2} term denoting the inverse-square law of 'radiative' transfer, but also a previously unidentified R^{-4} intermediate term.

2.10 Dexter transfer

Before concluding this section, it is worth observing that other forms of donor-acceptor coupling are also possible, although considerably less relevant to the systems of interest in the following focused account on applications. For example, the transfer of energy between atomic or molecular components with significantly overlapped wavefunctions is usually described in terms of Dexter theory[28] – where the coupling involves electron exchange and carries an exponential decay with distance, directly reflecting the radial form of overlapping wavefunctions and electron distributions. Unlike Förster transfer, singlet-triplet energy exchange ($^3D^* + {}^1A \rightarrow {}^1D + {}^3A^*$) may also be allowed by the Dexter mechanism. This is because Dexter transfer does not involve

transition dipole moments and, thus, is unaffected by the dipole-forbidden character of the transitions $T_1 \rightarrow S_0$ and $S_0 \rightarrow T_1$ within chromophores *D* and *A*, respectively. Compared to materials in which the donor and acceptor orbitals do not spatially overlap, such systems are of less use for either device or analytical applications. This is largely because the coupled chromophores lose their electronic and optical integrity, the Dexter mechanism being operational only at very short distances (< 10 Å).

3. Applications of RET to molecular biology

The field in which measurements of resonance energy transfer have without doubt had the greatest impact is molecular biology. The importance of RET to this subject, especially in application to biological macromolecules, was first realised following the construction of spectroscopic equipment for routine fluorescence measurements.[29-32] Towards the turn of the twenty-first century, RET underwent a period of significant redevelopment as a spectroscopic technique.[33] This resurgence arose mainly due to the advent of new experimentation methods, for example single-pair RET,[34] and further advances in instrumentation. The key advantage of RET techniques over others is that fluorescence measurements are highly sensitive, being made against a zero background; moreover the uv/visible signals are relatively easy to detect, they are specific and the required instrumentation is non-invasive.

3.1 Spectroscopic ruler

A major use of RET, based on its strong distance dependence, exploits its capacity to supply accurate spatial information about molecular structures. This derives from a quantitative assessment of the inter-chromophore separations, based on comparisons between the corresponding RET efficiencies.[35-38] Such a technique is popularly known as a 'spectroscopic ruler'. The elucidations of molecular structure by such means usually lack information on the relative *orientations* of the groups involved, and as an expedient the calculations usually ignore the kappa parameter (4). The apparent crudeness of this approach becomes more defensible on realizing that, even if it were to introduce a factor of two inaccuracy, the deduced group spacing would still be in error by only 12% (since $2^{1/6} = 1.12$). Refinements to the theory to accommodate the effect of fluctuations in position or orientation of the participant groups introduce considerable complexity, although progress is being made in several areas.[39-43]

3.2 Conformational change

Through identification of motions in macromolecules, i.e. the variation in proximity of one chromophore with respect to another, a number of valuable RET applications arise; including the detection of conformational changes and

folding in proteins,[36,44-46] and the inspection of intracellular protein-protein[47-50] and protein-DNA[51,52] interactions (see for example Figs 5 and 6). These and other such processes can be registered by selectively exciting one chromophore using laser light, and monitoring either the decrease in fluorescence from that chromophore, or the rise in the generally longer-wavelength fluorescence from the other chromophore as it adopts the role of acceptor. The judicious use of optical dichroic filters can make this RET technique perfectly straightforward – see Fig. 1. In cases where the two material components of interest do not display suitably overlapped absorption and fluorescence features in an optically accessible wavelength range, molecular tagging with site-specific 'extrinsic' (i.e. artificially attached) chromophores can solve the problem. Located at a molecular site of interest, and being selected on the basis of a significant spectral overlap with the counterpart component, such tags can act either in the capacity of donor or acceptor. Lanthanide ions, with their characteristically prominent and line-like absorption features, prove particularly valuable in this connection.[53] Also useful in this respect are the semiconductor nanocrystals known as quantum dots. These crystalline nanoparticles offer several unique traits, including size- and composition-tunable emission from visible to infrared wavelengths, the possibility of a single light source simultaneous exciting different-sized dots, large absorption coefficients across a wide spectral range, and very high level of photostability.[54,55]

3.3 Intensity-based imaging

In the last two decades there has been burgeoning interest in microscopy based on RET,[56-60] typical instrumentation for which is illustrated in Fig. 7. There are three specific types of RET method routinely used in the production of biological images. The principles of sensitized-emission RET have already been described (Fig. 1). For microscopy purposes this method is fairly inaccurate; no RET donor-acceptor pair is ideal, i.e. there will almost always be some overlap between the donor and acceptor absorbance bands and also the donor and acceptor emission spectra. Therefore, filters that completely separate these kinds of spectrum are difficult to design. Various calculational algorithms[61-63] have been proposed to compensate for this problem, although the methods are complex and no single procedure has received universal acceptance.

A widely used alternative, experimental approach[64,65] involves deliberately photobleaching the acceptor, the result of which is complete exclusion of RET. In this method, the donor emission is analysed before and after the acceptor is bleached by the input of an intense laser beam (at a suitable wavelength). The difference between the donor intensities, with and without the laser input, enables a determination of the transfer efficiency by employing equation (8). Here, account is taken of spectral bleed-through between the two absorbance bands, and equally between the two emission bands. Signal contamination is still not entirely eliminated, due to a small

amount of back-transfer through donor excitation by acceptor emission. Often the main disadvantage in prolonged illumination of the acceptor is the possibility of damage to the sample. Therefore, in practice, photobleaching is seldom appropriate for *in vivo* studies.

3.4 Lifetime-based imaging

Fluorescence need not be characterized from excitation and emission spectra alone; highly significant information can also be secured from lifetime measurements. Thus, when suitable time-resolved instrumentation is available, the determination of decay kinetics (usually on the nanosecond timescale) enables analysis through RET-based fluorescence lifetime imaging microscopy (FRET-FLIM).[66-69] In this method, spectral bleed-through is no longer an issue since measurements are made only for the determination of donor lifetimes; back-transfer is usually extremely low and within the noise level. The presence of the acceptor within the local environment of the donor influences the fluorescence lifetime of the donor. By measuring the donor lifetime in the presence and absence of the acceptor one can accurately calculate the transfer efficiency by use of equation (8). Drawbacks to FRET-FLIM are the technical challenges the technique presents, and the expense of the equipment. Nonetheless, in optical systems that are equipped to provide both intensity and lifetime measurements, a comparison of the two types of image affords a particularly rich source of information, as illustrated by the cancer cell images of Fig. 8.

3.5 Other applications

Beyond the realm of molecular biology, RET has value in a number of more specifically chemical applications. Two prominent examples are to be found in the fields of synthetic macromolecules and chemical sensors. In polymer science, building on the pioneering principles of Morawetz,[70] RET is now used to determine morphological information on polymer interfaces. Such studies have, for instance, enabled the quantitative characterisation of interfacial thickness in polymers of various structures.[71] Moreover, RET has been utilized in the study of polymer conformational dynamics. One especially interesting application is the effective differentiation between various collapsed and/or ordered homopolymer chain conformations (spherical, rod and toroidal; as depicted in Fig. 9) through the associated distribution of transfer effiencies.[72,73]

The fabrication of RET-based, analyte-specific sensors has enabled detection of a variety of species, including dimers of functionalized calixarenes in organic solutions,[74] copper(II) in aqueous solution,[75] hydrogen peroxide,[76] phosgene[77] and many others. These chemical sensors usually work on the principle of a donor-acceptor system designed such that the presence of the analyte causes the acceptor chromophore to move within closer proximity to a donor, enabling the implementation of an RET process that is not observed in

the analyte's absence. Therefore, on irradiation of the system with the relevant chemical present, a strong emission from the acceptor signals the presence of the analyte.

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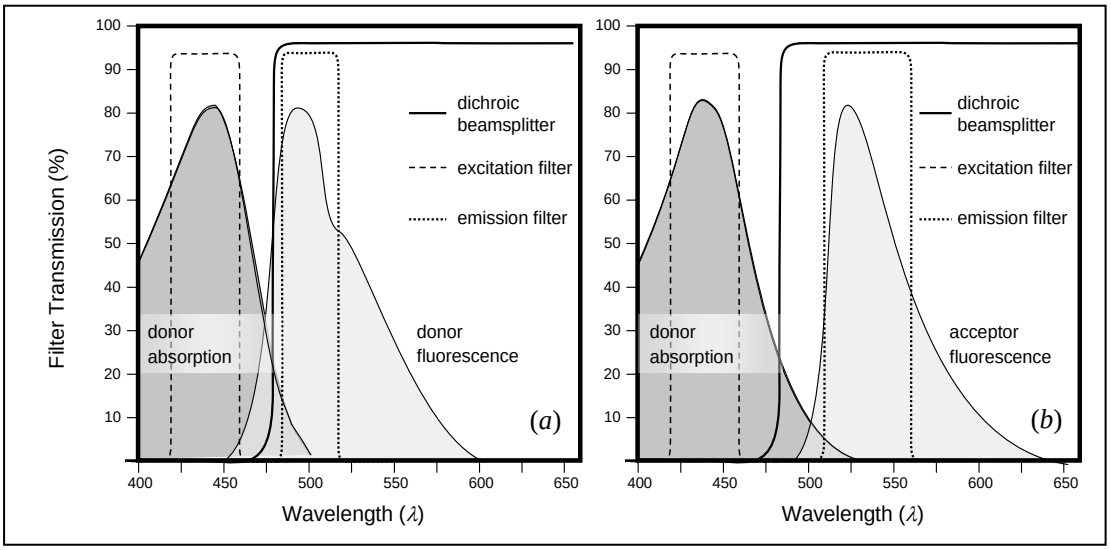

Figure 1. Typical spectral discrimination between the fluorescence from donor and acceptor species (here notionally based on a cyan fluorescent protein donor and a yellow fluorescent protein acceptor): (*a*) the transmission characteristics of a short-wavelength filter ensure initial excitation of only the donor; a dichroic beam-splitter and another narrow emission filter ensuring that only the (Stokes-shifted) fluorescence from the donor reaches a detector; (*b*) in the same system a longer-wavelength emission filter ensures capture of only the acceptor fluorescence, following RET.

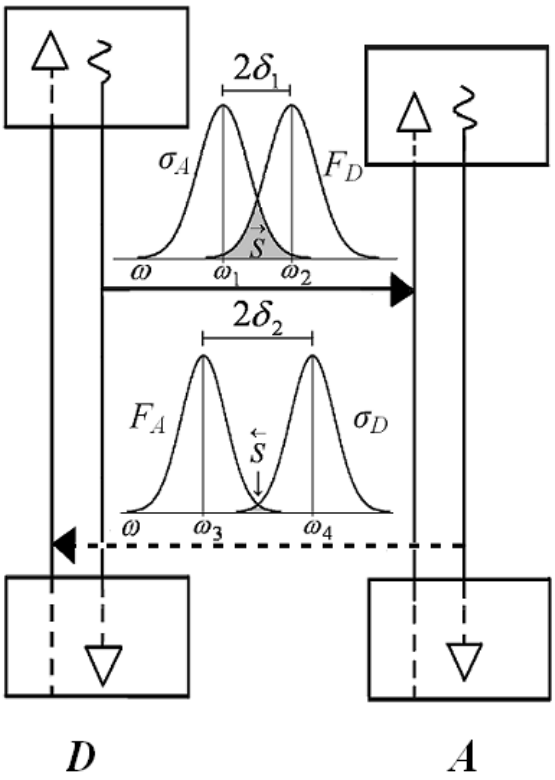

Figure 2. Energetics and spectral overlap features (top) for energy transfer from *D* to *A* (and below, potentially backward transfer from *A* to *D*). For each chromophore *F* denotes the fluorescence spectrum and σ the absorption. Wavy downward lines denote vibrational dissipation.

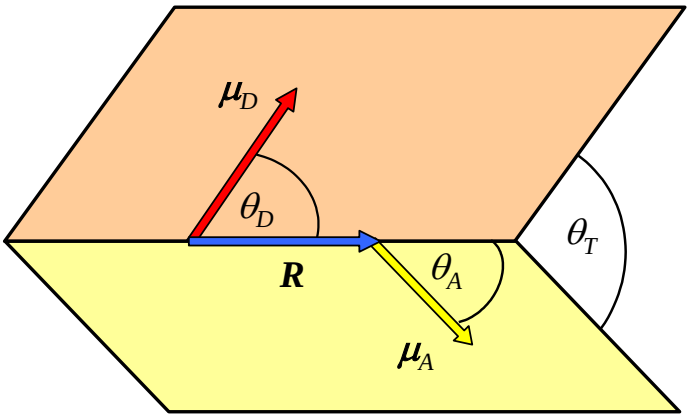

Figure 3. Relative orientations and positions of the donor and acceptor and their transition moments: Here, angles θ_D and θ_A subtended by donor and acceptor transition moments ($\boldsymbol{\mu}_D$ and $\boldsymbol{\mu}_A$, respectively) against the inter-chromophore displacement vector, **R**; the symbol θ_T is the angle between the transition moments.

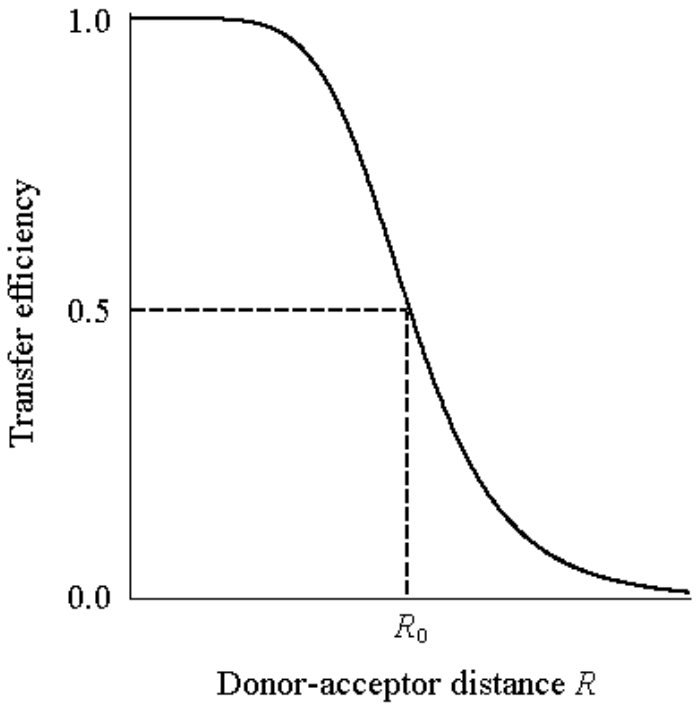

Figure 4. Distance dependence of the transfer efficiency between a pair of chromophores, calculated according to equation (8).

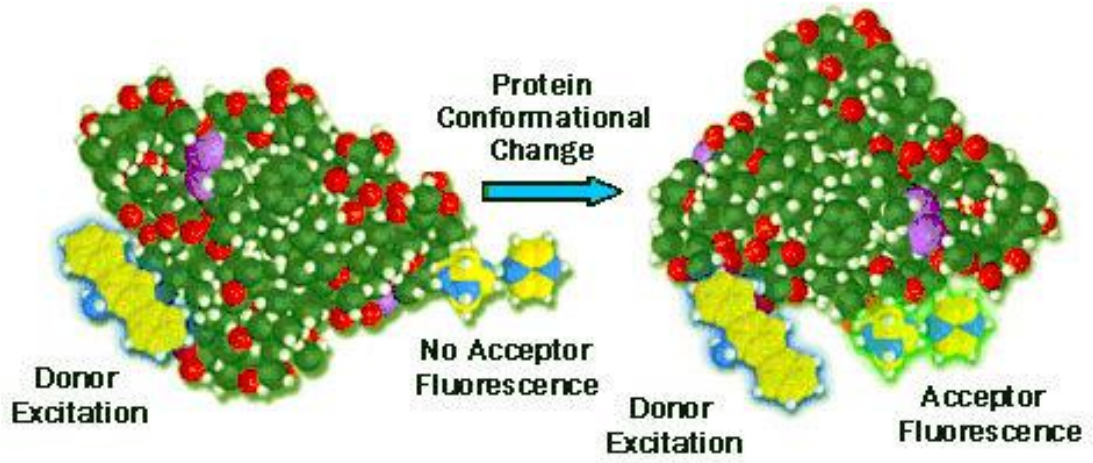

Figure 5. Graphical depiction of RET detection of protein conformational change. Adapted from Olympus Corporation website.

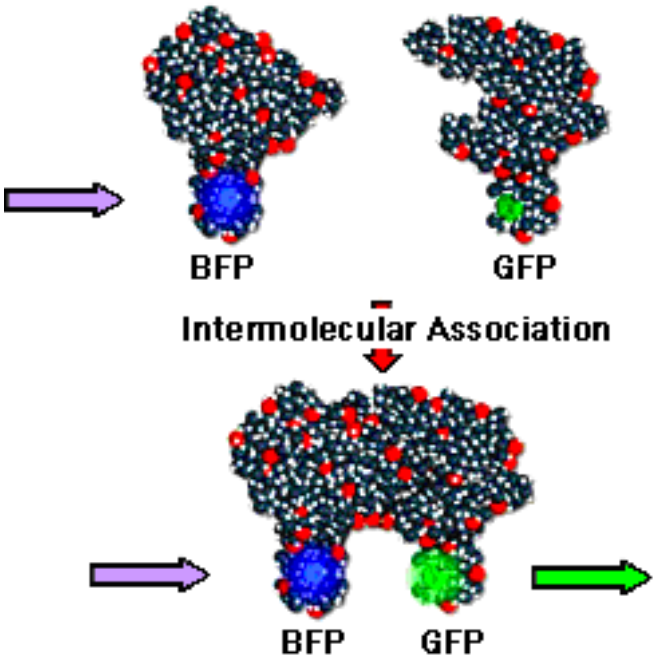

Figure 6. Graphical depiction of RET detection of *in vivo* protein-protein interactions. Purple arrow denotes input laser of wavelength 380 nm and green arrow indicates protein emission at 510 nm. BFP and GFP are acronyms for blue and green fluorescent proteins, respectively. Adapted from Olympus Corporation website.

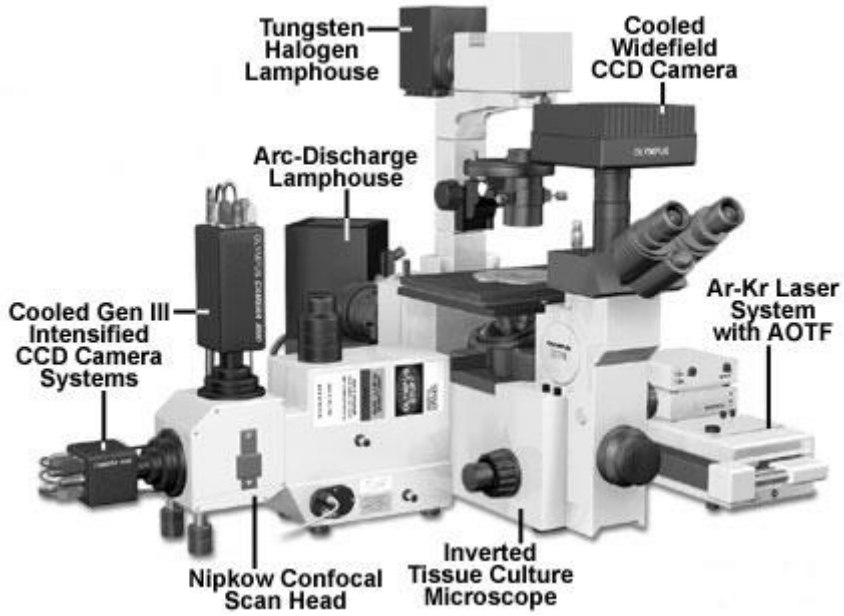

Figure 7. Typical commercial set-up of a microscope based on RET. Adapted from Olympus Corporation website.

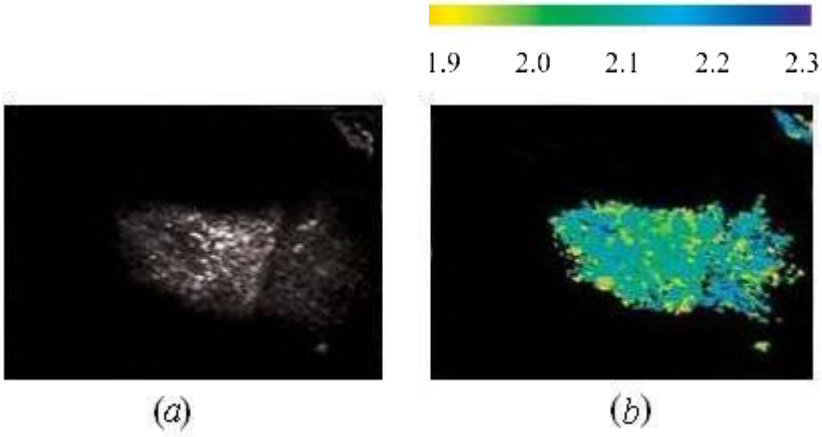

Figure 8. MDA-MB-231 cancer cell images recorded with argon laser two-photon excitation, RET microscope based on; (*a*) intensity and (*b*) fluorescence lifetime (ns). In the latter image, areas of locally reduced lifetime signify clustered intracellular vesicles. Adapted from ref. 69.

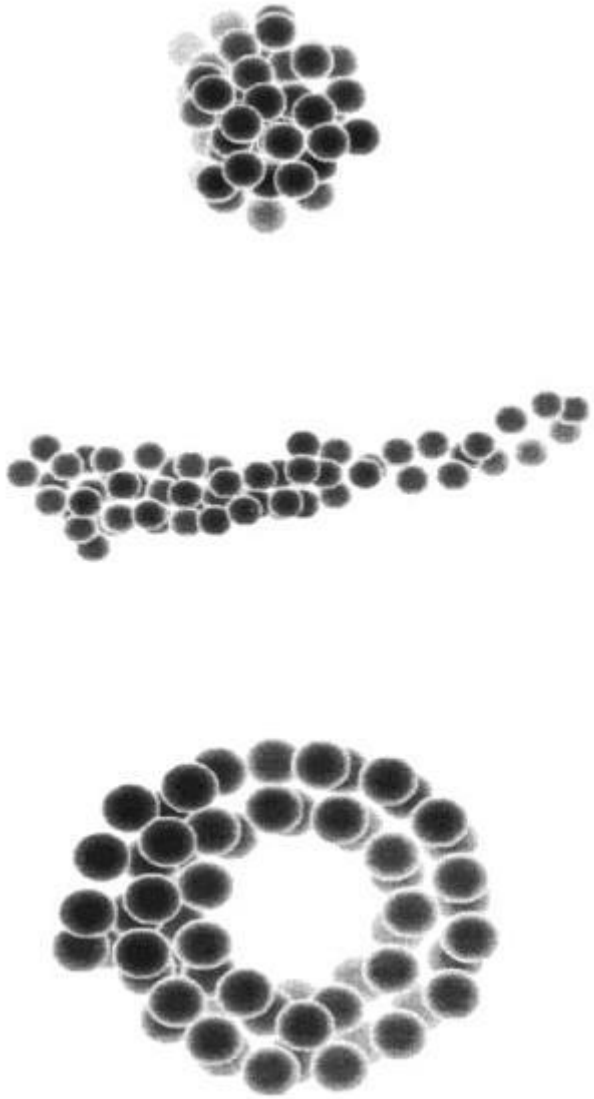

Figure 9. Snapshots of various morphological constructions of a homopolymer chain. The structures from top to bottom are spherical, rod and toroidal. Adapted from ref. 72.