

Two-photon fluorescence: Resonance energy transfer

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(Received 24 June 1997; accepted 11 November 1997)

Fluorescence resonance energy transfer (FRET) is a technique now widely applied to probe biological and other complex systems for the determination of fluorophore separation and structure. Recently the theory behind the anisotropy of fluorescence has been extended to include the case of a residual polarization following energy transfer between fluorophores, and here the theory is further extended to accommodate two-photon excitation. This reveals not only novel polarization characteristics but also a distance dependence whose analysis does not require *a priori* knowledge of the donor-acceptor spectral overlap. The two-photon FRET anisotropy results mirror their one-photon counterparts, in terms of fluorophore separation characteristics and also their relationship to the anisotropies for isolated fluorophores. Moreover, the two-photon results are not restricted to a plane polarized input, results being given for both plane and circularly polarized pump radiation. © 1998 American Institute of Physics. [S0021-9606(98)50707-6]

I. INTRODUCTION

Progress in the fundamental theory of resonance energy transfer¹⁻⁶ has, in the last few years, rekindled interest in the anisotropic polarization of acceptor fluorescence. The detailed results for the residual anisotropy, first derived by Galanin,² have now been extended to fluorescence resonance energy transfer (FRET) beyond the Förster limit, including long-range (wave-zone) transfer.^{7,8} Here we consider the two-photon analogue of this now rather well understood process. Interest in such a process stems from a number of considerations, not least the fact that the states excited by two-photon absorption, often high in energy, will generally display an even greater tendency to shed their energy to suitable acceptors before luminescent emission occurs. From the device point of view, there is interest in the possibility of using this process to create new and more efficient types of energy trap.

Multiphoton microscopy is now becoming a commercially available technique, used *inter alia* to probe deep into biological systems. Very recently there have been reports of three-photon excitation of serotonin in leukaemic rat cells.⁹ Here the use of multiphoton near infrared excitation circumvents the otherwise destructive use of high-energy ultraviolet photons and allows the technique to be used for *in vivo* studies. Other multiphoton fluorescence studies that have been reported relate directly to biological and other systems featuring both single center chromophores and energy trapping sites.¹⁰⁻²⁰ These, together with the well-known use of FRET as a spectroscopic ruler,²¹⁻²⁵ reflect the practical utility of the theory to be developed.

In this paper results are first derived for the anisotropy of two-photon induced fluorescence without energy transfer. The limiting results have previously been derived by others,^{11,12,26,27} but are included here for the initial development of the more general theory. The results are then extended to a two-chromophore system in which the two-photon energy at the donor site is transferred via resonant

energy transfer, subject to nonradiative losses, to an acceptor species from which the signal radiation then emerges. The energetics of this process are illustrated by the schematic diagram of Fig. 1. A number of novel features emerge. For example it transpires that the fluorescence displays a residual polarization anisotropy that increases by a factor of precisely 7 as the transfer distance increases from the near-zone to wave-zone range.

A. Two-photon fluorescence from a single center

We consider first the anisotropy of fluorescence from a molecule *A*, detected after its single beam two-photon excitation and subsequent relaxation. In the most general case, without exploitation of any single-photon resonance, the quantum probability amplitude (matrix element) for the overall process is proportional to the outer product of a second rank molecular tensor **S** and an emission transition dipole moment μ ,²⁸ coupled with three polarization vectors

$$M \sim \bar{e}_{k'}^{-\lambda'} e_{k'}^{\lambda} e_{k_m}^{\lambda} \mu_k S_{lm}, \quad (1)$$

utilizing the Einstein convention of implied summation over repeated indices. The incident field is denoted by the unit vector \mathbf{e}_k^{λ} , associated with polarization λ and wave vector **k**. The signal is denoted by the complex unit vector $\bar{\mathbf{e}}_{k'}^{\lambda'}$, the overbar allowing for a general case where the light may be other than plane polarized. The primes denote a change in wave vector and polarization as befits the signal, and the constants of proportionality in Eq. (1) are omitted since they drop out of the final results for fluorescence anisotropy. The second rank tensor, representing two-photon absorption to a molecular state $|\alpha\rangle$, is derived using time-dependent perturbation theory and is explicitly given as

$$S_{(lm)} = \frac{1}{2} \sum_r \left\{ \frac{\mu_l^{ar} \mu_m^{r0}}{(E_{r0} - \eta\omega - i\Gamma_r)} + \frac{\mu_m^{ar} \mu_l^{r0}}{(E_{r0} - \eta\omega - i\Gamma_r)} \right\}, \quad (2)$$

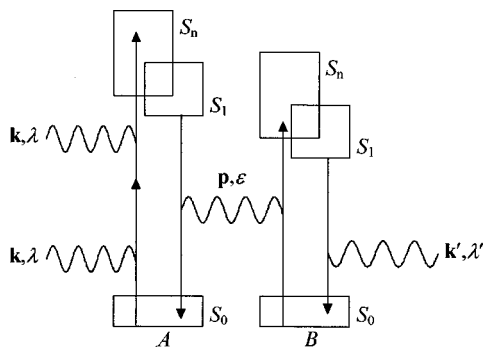


FIG. 1. Modified Jablonski diagram showing the essential energetics of two-photon fluorescence with energy transfer; S_0 represents the ground state and its associated manifold and S_1 , S_n denote higher electronic states of the same spin multiplicity. Single-center two-photon fluorescence may be considered as relating to the above diagram, by removal of molecule B , the fluorescence signal then being the radiation that emerges from A .

where the imaginary addenda in the energy denominators properly reflect finite lifetimes $1/\Gamma_r$, for each excited state $|r\rangle$, with signs as determined by time-reversal considerations.²⁹ Since the absorbed photons are from a single laser beam the tensor is represented by an index-symmetric expression, as denoted by the parentheses around the subscripts. The rate of two-photon fluorescence is then expressible as

$$\Gamma \sim \bar{e}_{k'_k}^{-\lambda'} e_{k'_l}^{\lambda} e_{k'_m}^{\lambda} e_{k'_p}^{\lambda'} \bar{e}_{k'_q}^{-\lambda} \bar{e}_{k'_r}^{-\lambda} \mu_k S_{(lm)} \bar{\mu}_p \bar{S}_{(qr)}. \quad (3)$$

If the molecules are randomly oriented or free to rotate (as, for example, in a molecular fluid) it is necessary for the isotropic average of Eq. (3) to be calculated. Then the result is given by

$$\langle \Gamma \rangle \sim \bar{e}_{k'_k}^{-\lambda'} e_{k'_l}^{\lambda} e_{k'_m}^{\lambda} e_{k'_p}^{\lambda'} \bar{e}_{k'_q}^{-\lambda} \bar{e}_{k'_r}^{-\lambda} \mu_\lambda S_{(\mu\nu)} \bar{\mu}_0 \bar{S}_{(\pi\rho)} \\ \times \langle \lambda_{k\lambda} \lambda_{l\mu} \lambda_{m\nu} \lambda_{p0} \lambda_{q\pi} \lambda_{r\rho} \rangle, \quad (4)$$

where the angular brackets indicate averaging over the direction cosines, $\lambda_{k\lambda} \dots$, representing the relative orientation of the laboratory (radiation) and molecular frames of reference. The result of the average is a sixth rank dual tensor comprising a linear combination of 225 isotropic tensor isomers, expressed in the terms of Kronecker deltas.^{28,30} Contraction with the radiation parameters in Eq. (4) leads to scalar products between the polarization vectors, which can readily be calculated for signal polarizations parallel and perpendicular to that of the input. However, unlike the one-photon fluorescence anisotropy ratios, the two-photon absorption process offers the prospect of measuring two different and independent fluorescence anisotropy ratios, corresponding to an initial excitation using either a plane or circularly polarized pump. The general expression defining the anisotropy using a plane polarized pump is the familiar $r_0 = (\langle \Gamma \rangle_{\text{lin}\parallel} - \langle \Gamma \rangle_{\text{lin}\perp}) / (\langle \Gamma \rangle_{\text{lin}\parallel} + 2\langle \Gamma \rangle_{\text{lin}\perp})$, which after calculation of the rotational average of Eq. (4), gives

$$r_0^{\text{lin}} = \frac{1}{7} \left\{ \frac{3s_1 + 6s_2 - 2s_3 - 4s_4 + 3s_5 + 6s_6}{s_3 + 2s_4} \right\}, \quad (5)$$

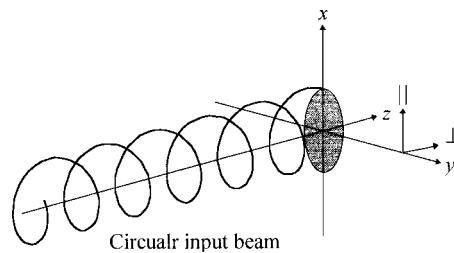


FIG. 2. For circularly polarized excitation the pump radiation propagates along the z axis and the electric vector sweeps around the x - y plane. For fluorescence along the y axis the "parallel" signal is defined as polarized along the x axis and the "perpendicular" signal along the z axis.

with the subscript denoting an anisotropy of fluorescence without energy transfer, to avoid confusion later on. The component parameters are

$$\left. \begin{aligned} s_1 &= \mu_\lambda S_{(\lambda\mu)} \bar{\mu}_\mu \bar{S}_{(\nu\nu)} \\ s_2 &= \mu_\lambda S_{(\lambda\mu)} \bar{\mu}_\nu \bar{S}_{(\mu\nu)} \\ s_3 &= \mu_\lambda S_{(\mu\mu)} \bar{\mu}_\lambda \bar{S}_{(\nu\nu)} \\ s_4 &= \mu_\lambda S_{(\mu\nu)} \bar{\mu}_\lambda \bar{S}_{(\mu\nu)} \\ s_5 &= \mu_\lambda S_{(\mu\mu)} \bar{\mu}_\nu \bar{S}_{(\lambda\nu)} \\ s_6 &= \mu_\lambda S_{(\mu\nu)} \bar{\mu}_\mu \bar{S}_{(\lambda\nu)} \end{aligned} \right\}. \quad (6)$$

If the fluorescence is from a nondegenerate singlet state, and providing intermediate state resonances are avoided, both the transition dipole moment and the second-rank molecular tensor are real: These conditions are henceforth assumed throughout. Taking account of index symmetry allows simplification of Eq. (5) as the parameters s_1 and s_5 , and also s_2 and s_6 , become equal. It is then apparent that in its simplest form the linear anisotropy can be expressed in terms of just two parameters $p_1 = (s_1 + s_5) + 2(s_2 + s_6)$ and $p_2 = (s_3 + 2s_4)$, such that Eq. (5) can be represented in the compact form

$$r_0^{\text{lin}} = \frac{1}{7} \left\{ \frac{3p_1 - 2p_2}{p_2} \right\}. \quad (7)$$

The anisotropy ratio applicable when the pump is circularly polarized is defined as $r_0 = (\langle \Gamma \rangle_{\text{cir}\parallel} - \langle \Gamma \rangle_{\text{cir}\perp}) / (\langle \Gamma \rangle_{\text{cir}\perp} + 2\langle \Gamma \rangle_{\text{cir}\parallel})$, following the notation of Wan and Johnson.^{10,11} Specifically, for fluorescence collected at right angles to the input beam, $\Gamma_{\text{cir}\parallel}$ represents a signal linearly polarized in the plane of the circular input field, and $\Gamma_{\text{cir}\perp}$, represents a signal linearly polarized perpendicular to this, i.e., parallel to the input direction of propagation, as illustrated in Fig. 2. The anisotropy calculated using the appropriate complex form for the radiation vectors, $\mathbf{e}_{L/R} = 1/\sqrt{2}(\mathbf{e}_x \pm i\mathbf{e}_y)$, is in its most general form given by

$$r_0^{\text{cir}} = \frac{1}{7} \left\{ \frac{-6s_1 + 9s_2 + 4s_3 - 6s_4 - 6s_5 + 9s_6}{-s_3 + 3s_4} \right\}. \quad (8)$$

Equation (8) involves three linearly independent combinations of the molecular parameters $s_1 - s_6$, because of the differing combinations of s_3 and s_4 in the numerator and denominator. Thus we can write

$$r_0^{\text{cir}} = \frac{1}{7} \left\{ \frac{3p'_1 - 2p'_2}{p'_3} \right\}, \quad (9)$$

where the primed parameters are defined as; $p'_1 = -2(s_1 + s_5) + 3(s_2 + s_6)$, $p'_2 = (3s_4 - 2s_3)$, and $p'_3 = (3s_4 - s_3)$.

The above general results can be directly employed if the parameters in terms of which they are cast are available, either through computational molecular modeling, or by experiments of the kind detailed in Appendix B. However where such information is not readily obtainable, further simplifications leading to tractable results are still possible if the molecular tensor \mathbf{S} is dominated by the terms in which the absorption dipole moments μ^{ar} and μ^{r0} are parallel, and against which the fluorescence transition dipole makes an angle θ . Then we obtain the following limits for two-photon fluorescence anisotropy:

$$r_0^{\text{lin}} = \frac{2}{7}(3 \cos^2 \theta - 1), \quad -\frac{2}{7} \leq r_0^{\text{lin}} \leq \frac{4}{7}, \quad (10)$$

$$r_0^{\text{cir}} = \frac{1}{7}(3 \cos^2 \theta - 1), \quad -\frac{1}{7} \leq r_0^{\text{lin}} \leq \frac{2}{7}, \quad (11)$$

and in this special case the information content is clearly the same with either input polarization. These results, which reproduce those derived elsewhere,^{10-13,26,27} are also based on the assumption that rotational relaxation is slow compared to the fluorescence lifetime, as commonly applies for large chromophores in viscous media, and also as justified by many experimental results.¹⁰⁻¹³ Thus Eqs. (5), (7)–(11) model the incoherent ultrafast molecular response of the system. If rotational dynamics are on a timescale comparable to the fluorescence, then the results acquire a time dependence that can be modeled using appropriate weighting factors.³¹⁻³⁴

B. Two-photon fluorescence with resonance energy transfer

The theory is now developed to include energy transfer between the initially excited species and a second fluorophore species. Thus it is assumed that there is an energy transfer route between the donor A initially excited by the two-photon process, and an acceptor B , from which the fluorescence signal is detected. This naturally depends on suitable overlap between the emission and absorption spectra of the donor and acceptor molecules.^{1,2,5,6} A perfectly general expression for the probability amplitude of the complete process is here expressible as

$$M \sim e^{-\lambda'} e_{k'_i}^{\lambda'} e_{k'_m}^{\lambda} e_{k'_n}^{\lambda} \mu_i^{\uparrow B} \mu_j^{\uparrow B} V_{jk} \mu_k^A S_{(lm)}, \quad (12)$$

where transition dipoles etc. associated with the molecules A and B are given superscript labels and the arrows identify the upward and downward transitions in B to remove ambiguity. The second-rank tensor \mathbf{S} is as defined in Eq. (2) and is again index symmetric if, as usual, a single pump beam is employed. The tensor V_{jk} is the fully retarded dipole-dipole interaction tensor, which holds the link between Förster energy transfer and the noncompeting radiative transfer.^{3,4} In cases where A and B are sufficiently close for wave function overlap, a means of appropriately generalizing the form of V_{ij} has been discussed by Scholes and Ghiggino.³⁵

From Eq. (12) an expression for the rate is obtained in the usual manner

$$\Gamma \sim e^{-\lambda'} e_{k'_i}^{\lambda'} e_{k'_m}^{\lambda} e_{k'_n}^{\lambda} e^{-\lambda} e_{k'_q}^{-\lambda} e_{k'_r}^{-\lambda} \mu_i^{\uparrow B} \mu_j^{\uparrow B} V_{jk} \mu_k^A \times S_{(lm)} \bar{\mu}_n^{\downarrow B} \bar{\mu}_o^{\downarrow B} \bar{V}_{op} \bar{\mu}_p^A \bar{S}_{(qr)}. \quad (13)$$

The rotationally averaged result, appropriate if the molecules or chromophores are orientationally uncorrelated, is significantly more complex than the single-center results. In allowing for random orientation of the respective molecules two independent orientational averages are first required to decouple each molecule from the radiation vector frame. In stages, assuming a linearly polarized pump, this procedure is as follows: First the sixth-rank molecular average with respect to molecule A is performed yielding

$$\Gamma \sim \frac{e^{-\lambda'} e_{k'_i}^{\lambda'} e_{k'_n}^{\lambda} \mu_i^{\downarrow B} \mu_j^{\uparrow B} \bar{\mu}_n^{\downarrow B} \bar{\mu}_o^{\uparrow B}}{105} \times [(3\Xi_{jo} - \Omega_{jo})p_1 + (3\Omega_{jo} - 2\Xi_{jo})p_2], \quad (14)$$

where the molecular parameters p_1 and p_2 relevant to the donor molecule A are as previously defined and the two new parameters Ξ and Ω are represented by

$$\left. \begin{aligned} \Xi_{jo} &= V_{jk} e_{k'_k}^{\lambda} e_{k'_m}^{-\lambda} \bar{V}_{om} \\ \Omega_{jo} &= V_{jk} \bar{V}_{ok} \end{aligned} \right\}. \quad (15)$$

The second stage of calculation is to perform a fourth-rank average to decouple molecule B from the laboratory frame. Assuming fluorescence occurs between nondegenerate states we have

$$\Gamma \sim \frac{e^{-\lambda'} e_{k'_i}^{\lambda'} e_{k'_n}^{\lambda}}{3150} [\{(3\delta_{ij}\delta_{no} - 2\delta_{in}\delta_{jo} + 3\delta_{io}\delta_{jn}) \times (\mu^{B\uparrow} \cdot \mu^{B\downarrow})^2\} + \{(-\delta_{ij}\delta_{no} + 4\delta_{in}\delta_{jo} - \delta_{io}\delta_{jn}) \times |\mu^{B\uparrow}|^2 |\mu^{B\downarrow}|^2\}] [(3\Xi_{jo} - \Omega_{jo})p_1 + (3\Omega_{jo} - 2\Xi_{jo})p_2]. \quad (16)$$

It then still remains to decouple the intermolecular displacement vector $\mathbf{R}(=\mathbf{r}_B - \mathbf{r}_A)$, from the radiation frame, as discussed in detail elsewhere.^{8,36} This involves the expansion of Eq. (16) and contraction of indices as determined by the Kronecker deltas. The tensorial part of the energy transfer function now takes the forms represented explicitly by Eqs. (A1.8)–(A1.10) of Appendix A, and applying the appropriate averages Eq. (16) then generates a result for the observable rate. For a linearly polarized pump, the completely general rate of two-photon fluorescence is thus

$$\Gamma \sim \frac{1}{1575} [\{p_1(9a - 6b + c)(\mu^{B\uparrow} \cdot \mu^{B\downarrow})^2\} + \{p_1(-3a + 7b - 2c)|\mu^{B\uparrow}|^2 |\mu^{B\downarrow}|^2\} + \{p_2(-6a + 11b - 3c)(\mu^{B\uparrow} \cdot \mu^{B\downarrow})^2\} + \{p_2(4a - 7b + 6c)|\mu^{B\uparrow}|^2 |\mu^{B\downarrow}|^2\}], \quad (17)$$

where we have introduced the notation

$$\begin{aligned}
 a &= \left\{ \cos^2 \theta \left(1 + \frac{2\text{Re}(y)}{3} + \frac{2}{15} |y|^2 \right) + \frac{1}{15} |y|^2 \right\} \\
 b &= \left(1 + \frac{2\text{Re}(y)}{3} + \frac{1}{3} |y|^2 \right). \\
 c &= (3 + 2\text{Re}(y) + |y|^2)
 \end{aligned} \quad (18)$$

In Eq. (18) θ as before is the angle between the absorption and fluorescence dipole moments of the donor, and y is a function of the product $k|\mathbf{R}|$, with $\hbar ck$ defining the energy transferred from the donor to the acceptor and $R = |\mathbf{R}|$ being the chromophore separation;

$$y(kR) = \frac{-3 + (kR)^2 - i2(kR)^3 - (kR)^4}{1 - (kR)^2 + (kR)^4}. \quad (19)$$

With the calculations for the isotropically averaged signals complete it is then possible to derive the anisotropy in its most general form. It is noted that because energy transfer takes place between two independently mobile molecules the donor-acceptor distance R typically falls between two asymptotic limits, as determined by the value of k . For $k|\mathbf{R}| \ll 1$ we obtain short-range behavior and for $k|\mathbf{R}| \gg 1$ the long-range counterpart. However, it is convenient to form a single general expression for the anisotropy, valid for all R , which thereby also embraces both the former nonradiative and the latter radiative limits

$$r_1^{\text{lin}}(y) = h(y) \frac{(3p_1 - 2p_2) \{3(\mu_B^\uparrow \cdot \mu_B^\downarrow)^2 - |\mu_B^\uparrow|^2 |\mu_B^\downarrow|^2\}}{175p_2 |\mu_B^\uparrow|^2 |\mu_B^\downarrow|^2}, \quad (20)$$

where the asymptotes are determined by the limiting values held within the separation function $h(y)$ defined as

$$h(y) = \frac{(15 + 10\text{Re}(y) + 2|y|^2)}{(3 + 2\text{Re}(y) + |y|^2)}. \quad (21)$$

The limits for short- and long-range behavior are readily calculated using the appropriate values of y

$$\text{Short-range } \text{Re}(y) = -3, \quad \text{Im}(y) = 0, \quad (22)$$

$$\text{Long-range } \text{Re}(y) = -1, \quad \text{Im}(y) = 0. \quad (23)$$

In the case where a circularly polarized pump induces the two-photon absorption, following a similar sequence of calculations we obtain the general rate as

$$\begin{aligned}
 \Gamma \sim & \frac{1}{1575} [\{2p_1'(9a - 6b + c)(\mu^{B\uparrow} \cdot \mu^{B\downarrow})^2\} + \{p_1'(-3a \\
 & + 7b - 2c)|\mu^{B\uparrow}|^2 |\mu^{B\downarrow}|^2\} + \{s_3(24a - 23b + 5c) \\
 & \times (\mu^{B\uparrow} \cdot \mu^{B\downarrow})^2\} + \{s_3(-8a + 21b - 10c) \\
 & \times |\mu^{B\uparrow}|^2 |\mu^{B\downarrow}|^2\} + \{s_4(-36a + 45b - 11c)(\mu^{B\uparrow} \cdot \mu^{B\downarrow})^2\} \\
 & + \{s_4(12a - 35b + 22c)|\mu^{B\uparrow}|^2 |\mu^{B\downarrow}|^2\}], \quad (24)
 \end{aligned}$$

and the anisotropy is similarly defined as

$$r_1^{\text{cir}}(y) = h(y) \frac{(3p_1' - 2p_2') \{3(\mu_B^\uparrow \cdot \mu_B^\downarrow)^2 - |\mu_B^\uparrow|^2 |\mu_B^\downarrow|^2\}}{175p_3' |\mu_B^\uparrow|^2 |\mu_B^\downarrow|^2}, \quad (25)$$

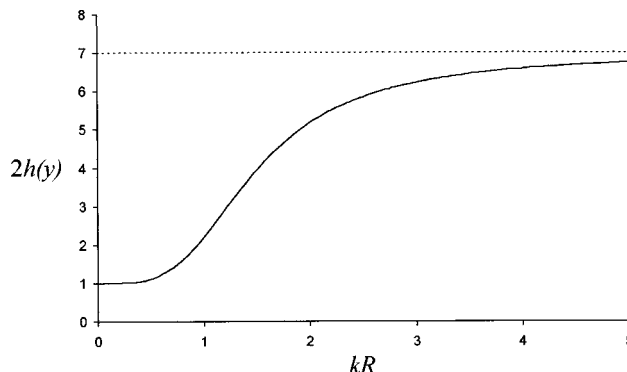


FIG. 3. Plot of the function $h(y)$ (inherent in all FRET anisotropic signals) with increasing donor-acceptor separation. The asymptotic values at the short-range, $k|\mathbf{R}| \ll 1$, and long-range, $k|\mathbf{R}| \gg 1$, limits are $1/2$ and $7/2$, respectively.

where p_1', p_2', p_3', s_3 , and s_4 are as defined in Sec. I A. Once more we notice that Eqs. (24) and (25) are both dependent upon three individual molecular parameters. As alluded to above, it is the $h(y)$ dependent terms in Eqs. (20) and (25) which entirely embody the distance dependence of these energy transfer results. A plot of this function with increasing chromophore separation is illustrated in Fig. 3, clearly showing its behavior between its two asymptotic limits ($1/2$ nonradiative or $7/2$ radiative).

The new and fully general results given above for the two-photon fluorescence anisotropy are directly applicable *per se*, if the molecular parameters in terms of which they are cast are determinable. It may be expedient where this is not the case to reduce the complexity of the expressions through the assumption that the two-photon tensor is dominated by terms with the absorption dipole moments μ^{ar} and μ^{r0} parallel. Then, substantially simpler results of a form once again resembling Eqs. (10) and (11) ensue, and with arbitrary angles θ and ϕ between the absorption and fluorescence dipole moments for the donor and acceptor molecules respectively, we obtain

$$r_1^{\text{lin}}(y) = h(y) \frac{2}{175} \{(3 \cos^2 \phi - 1)(3 \cos^2 \theta - 1)\}, \quad (26)$$

$$r_1^{\text{cir}}(y) = h(y) \frac{1}{175} \{(3 \cos^2 \phi - 1)(3 \cos^2 \theta - 1)\}. \quad (27)$$

Once again in this reduced form we have $r_1^{\text{cir}} = \frac{1}{2} r_1^{\text{lin}}$, mirroring the single-center result, removing any utility for separate measurement under different input polarization conditions. Although these results alone do not make it possible to determine the angles θ or ϕ experimentally, the angle θ for the donor molecule may be determined from the corresponding single center results, obtainable when the acceptor species is absent or derivatized. Then it becomes possible to determine the orientation of the fluorescence dipole moment of the acceptor molecule with respect to its absorption moment. Utilizing the results for both the single- and the two-center fluorescence anisotropy, key indicators of molecular symmetry are thereby obtainable.

Interestingly the two-photon residual anisotropy results mirror two features of their single-photon absorption

counterpart.^{7,8} Firstly the same factor of 7 appears as the difference between the radiative and Förster-type energy transfer results; secondly a similar relationship is observed with the corresponding single center results, enabling the anisotropy of two-photon energy transfer to be expressed in terms of its single centre result

$$r_1(y) = h(y) \frac{r_0}{25} (3 \cos^2 \phi - 1), \quad (28)$$

which can legitimately be applied to all the results obtained from either linearly or circularly polarized input.

II. DISCUSSION

General expressions for two-photon fluorescence anisotropy have been derived for a system comprising two chemical species *A* and *B*. Experimentally verifiable ratios have been determined explicitly for (i) two-photon excited fluorescence by a donor *A*, (ii) resonance energy transfer and fluorescence from an acceptor *B*. Results have been derived without necessary assumption of any physical restrictions on the participant molecules, enabling the fluorescence anisotropy to be expressed in terms of a comprehensive set of molecular parameters amenable to experimental or computational determination. In anticipation of cases where these parameters are not readily obtained, we have also given simplified results, applicable under specified conditions with proven utility in the analysis of fluorescence experiments.

It has been shown that the relationships between the single-center and the short- and long-range residual two-photon fluorescence anisotropies mirror the counterpart relationships for one-photon fluorescence migration.^{7,8} In general, the limiting FRET residual anisotropy ratios for *n*-photon excitation processes can be calculated from the corresponding single-center *n*-photon fluorescence anisotropy r_0 , using Eq. (28) and $r_0(n) = (n/2n + 3)(3 \cos^2 \theta - 1)$, always subject to stringent conditions on the relative directions of the transition dipoles involved.¹⁶ Nonetheless our work has also led to general results of more universal application, not necessarily subject to those conditions.

To simply illustrate a conceivable experimental implementation of our results we consider again the special case where the two-photon molecular tensor is dominated by terms with parallel absorption dipoles. Here, to gain maximum information it may be envisaged that the single centre anisotropy r_0 should first be obtained from the donor in dilute form. This yields the necessary information on the angle between absorption and fluorescence transition dipole moments to carry into the residual (energy transfer) anisotropy expressions. Introducing the acceptor molecule and measuring its fluorescence anisotropy will determine the mechanism of excitation.

In designing an experiment to observe the two-photon fluorescence residual anisotropy the molecular pair must be chosen with care. The donor molecule must be selected such that no single-photon absorption at the fundamental pump frequency takes place. Likewise a suitable acceptor molecule must remain transparent to both the input and its harmonic, to ensure that the emergent signal is solely due to resonance

energy transfer. Despite the need for a degree of overlap between the relevant emission and the absorption bands of the pair, the emission spectra of the two species must be well separated in order to determine the signal without any ambiguity. These conditions are as illustrated schematically in Fig. 1.

It is noted that the increasing tunability of laser light, through both the advent of vibronic solid state lasers and the refining of nonlinear optical materials, ensures that obtaining the necessary range of frequencies should not present a problem. A comprehensive listing of donor-acceptor pairs with relevant spectral overlap data, suitable for detailed validation of the theory, is tabulated in reference.⁵ Our theory is increasingly applicable now that high-order multiphoton absorption processes are becoming more commonplace. Most interestingly a number of studies on excited state fluorophores within biological complexes have been observed via multiphoton fluorescence imaging.^{9,14,17} Moreover, the use of FRET as a spectroscopic ruler^{21,25} in the determination of fluorophore separations suggests that recording the polarization characteristics of the fluorescence signal may not only determine the excited-state molecular symmetry but also prove a simpler way of measuring fluorophore separation.

ACKNOWLEDGMENTS

We are pleased to acknowledge most helpful communications with Dr. A. A. Demidov on this manuscript during its formative preparation. One of us (P.A.) would also like to acknowledge funding from the EPSRC for a studentship during which much of this work was undertaken.

APPENDIX A: THEORETICAL FRAMEWORK FOR BIMOLECULAR EXCITATION

The intermolecular coupling is accommodated within the probability amplitudes by a retarded dipole-dipole interaction expressible through the following index-symmetric tensor V_{jk} ^{3,5}

$$V_{jk}(k, \mathbf{R}) = \frac{\exp(ikR)}{4\pi\epsilon_0 R^3} [(1-ikR)(\delta_{jk} - 3\hat{R}_j\hat{R}_k) - (kR)^2(\delta_{jk} - \hat{R}_j\hat{R}_k)]. \quad (A1.1)$$

It is expedient to write Eq. (A1.1) as a product of two separate functions

$$V_{jk} = f(k, R) g_{jk}(kR, \hat{\mathbf{R}}). \quad (A1.2)$$

One of these functions, g_{jk} , is dimensionless and embodies the tensorial behavior

$$g_{jk}(kR, \hat{\mathbf{R}}) = \{\delta_{jk} + y(kR)\hat{R}_j\hat{R}_k\}, \quad (A1.3)$$

with the real and imaginary parts of y given by

$$\text{Re}(y) = \frac{-3 + k^2 R^2 - k^4 R^4}{1 - k^2 R^2 + k^4 R^4}, \quad \text{Im}(y) = \frac{-2k^3 R^3}{1 - k^2 R^2 + k^4 R^4}. \quad (A1.4)$$

For the other function featuring in Eq. (A1.2) we have

$$f(k, R) = \frac{z(kR)\exp(ikR)}{4\pi\epsilon_0 R^3}, \quad (A1.5)$$

likewise with the real and imaginary parts of z given by

$$\text{Re}(z) = (1 - k^2 R^2), \quad \text{Im}(z) = -kR. \quad (\text{A1.6})$$

These results hold over an unrestricted range of intermolecular distances (beyond the extent of wave function overlap); in the short- and long-range limits $k|\mathbf{R}| \ll 1$ and $k|\mathbf{R}| \gg 1$ we have, respectively,

Short-range;

$$f(kR) = 1/4\pi\epsilon_0 R^3, \quad \text{Re}(y) = -3, \\ \text{Im}(y) = 0, \quad g_{jk}(kR, \hat{\mathbf{R}}) = \{\delta_{jk} - 3\hat{R}_j \hat{R}_k\}.$$

Long-range;

$$f(kR) = k^2 \exp(ikR)/4\pi\epsilon_0 R, \quad \text{Re}(y) = -1, \\ \text{Im}(y) = 0, \quad g_{jk}(kR, \hat{\mathbf{R}}) = \{\delta_{jk} - \hat{R}_j \hat{R}_k\}.$$

In the rotationally averaged rate equations [cf. Eq. (16)], each term proves to contain a product of components of the coupling tensor with components of its complex conjugate, of the general form $V_{jk} \bar{V}_{no} = f \bar{f} g_{jk} \bar{g}_{no}$. For the tensorial part of the coupling function we can write the result as

$$g_{jk} \bar{g}_{no} = \{\delta_{jk} + y \hat{R}_j \hat{R}_k\} \{\delta_{no} + \bar{y} \hat{R}_n \hat{R}_o\}, \quad (\text{A1.7})$$

and three forms emerge according to the different kinds of index pairing that arise

$$g_{jk} \bar{g}_{no} = \delta_{jk} \delta_{no} + \delta_{jk} \bar{y} \hat{R}_n \hat{R}_o + \delta_{no} y \hat{R}_j \hat{R}_k \\ + |y|^2 \hat{R}_j \hat{R}_k \hat{R}_n \hat{R}_o, \quad (\text{A1.8})$$

$$g_{jk} \bar{g}_{nk} = \delta_{jn} + 2\text{Re}(y) \hat{R}_j \hat{R}_n + |y|^2 \hat{R}_j \hat{R}_n, \quad (\text{A1.9})$$

$$g_{jk} \bar{g}_{jk} = 3 + 2\text{Re}(y) + |y|^2. \quad (\text{A1.10})$$

APPENDIX B

The parameters in terms of which the fluorescence anisotropy for the cases of linearly and circularly polarized input are expressed are composites of four independent parameters as first outlined by McClain.²⁸ This set of parameters is applicable under the conditions that the excitation is from a single frequency pump and that the fluorescence is between nondegenerate (singlet) states. This enables the set of four parameters ($s_1 + s_5$), ($s_2 + s_6$), s_3 and s_4 to be determined by four different polarization experiments. These parameters are applicable when the second rank tensor is real.

We now define the four polarization experiments

$$I_{\text{lin } \parallel}^{(1)} = \frac{\mathcal{D}}{105} \{4(s_1 + s_5) + 8(s_2 + s_6) + s_3 + 2s_4\}, \quad (\text{B2.1})$$

$$I_{\text{lin } \perp}^{(2)} = \frac{\mathcal{D}}{105} \{-2(s_1 + s_5) - 4(s_2 + s_6) + 3s_3 + 6s_4\}, \quad (\text{B2.2})$$

$$I_{\text{cir } \parallel}^{(1)} = \frac{\mathcal{D}}{105} \{-4(s_1 + s_5) + 6(s_2 + s_6) - s_3 + 5s_4\}, \quad (\text{B2.3})$$

$$I_{\text{cir } (L) \text{ cir } (R)}^{(1)} = \frac{\mathcal{D}}{105} \{-4(s_1 + s_5) + 20(s_2 + s_6) - s_3 + 5s_4\}, \quad (\text{B2.4})$$

where the subscripts represent the polarization of the pump and the signal radiation respectively with $I_{\text{pump sig}}^{(n)}$ representing the measured intensity; \mathcal{D} is a beam parameter $\mathcal{D} = (\pi k'^2 \bar{I}_0 g^{(2)}) / (128c^3 \epsilon_0^3 \hbar^2)$ where k' is the wave vector of the emerging signal radiation, \bar{I}_0 is the mean irradiance of the pump and $g^{(2)}$ its second-order degree of coherence. The fourth signal above, Eq. (B2.4), requires the fluorescence to be collected collinearly with the input, with reversed helicity. Eqs. (B2.1)–(B2.4) represent the intensity in terms of the molecular parameters. Thus these four experiments allow the four molecular parameters to be deduced; using Cramer's rule we obtain

$$(s_1 + s_5) = \frac{15}{2\mathcal{D}} (3I_{\text{lin } \parallel}^{(1)} - I_{\text{lin } \perp}^{(2)} + 3I_{\text{cir } \parallel}^{(3)} - 2I_{\text{cir } (L) \text{ cir } (R)}^{(4)}), \quad (\text{B2.5})$$

$$(s_2 + s_6) = \frac{15}{2\mathcal{D}} (-I_{\text{cir } \parallel}^{(3)} + I_{\text{cir } (L) \text{ cir } (R)}^{(4)}), \quad (\text{B2.6})$$

$$s_3 = \frac{15}{\mathcal{D}} (-I_{\text{lin } \parallel}^{(1)} + 2I_{\text{lin } \perp}^{(2)} - 4I_{\text{cir } \parallel}^{(3)} + I_{\text{cir } (L) \text{ cir } (R)}^{(4)}), \quad (\text{B2.7})$$

$$s_4 = \frac{15}{\mathcal{D}} (I_{\text{lin } \parallel}^{(1)} + 2I_{\text{cir } \parallel}^{(3)} - I_{\text{cir } (L) \text{ cir } (R)}^{(4)}), \quad (\text{B2.8})$$

giving the magnitudes of the molecular parameters in terms of the measured intensities.

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