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On the Analyses of Fluorescence Depolarisation Data in the Presence of Electronic Energy Migration. I. Theory and General Description

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ABSTRACT

A new and general procedure is described for a detailed analysis of time-resolved fluorescence depolarisation data in the presence of electronic energy migration. An isotropic ensemble of bifluorophoric molecules (D_1 -R- D_2) has been studied to demonstrate its utility. Intramolecular donor-donor energy migration occurs between the two donor groups (D), which are covalently connected to a rigid linker group (R). These groups undergo restricted reorientational motions with respect to the R group. The analysis of depolarisation data basically involves the search for best-fit parameters which describe the local reorienting motions, the intermolecular D_1 - D_2 distance, as well as the mutual orientations of the donors. For this, the analysis is partly performed in the Fourier domain and the best-fit parameters are determined by using an approach based on a Genetic Algorithm. The energy migration process has been described by using Monte Carlo simulations and an extended Förster theory (EFT). It is found that the EFT provides the least time-consuming computational method. Since one-photon and two-photon excited fluorescence experiments can be applied for energy migration studies, a general and unified theoretical formulation is given.

Keywords: Extended Förster theory, Genetic algorithm, Monte Carlo simulations, two-photon excitation

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List of Abbreviations

BD = Brownian dynamics

D = donor of energy

D = director frame with its z-axis aligned along the symmetry axes of the donor orientation distribution

DDEM = donor-donor energy migration

EFT = extended Förster theory

L = laboratory frame

M = frame fixed to chromophore

MC = Monte Carlo

OP = one-photon

OPE = one-photon excited

R = linker group, biomacromolecule, host molecule

R = coordinate system fixed to the linker group

R_0 = the Förster radius

TCSPC = time-correlated single-photon counting

TP = two-photon

TPE = two-photon excited

INTRODUCTION

Since Stryer and Haugland suggested(1) that electronic energy transfer could be used as a “spectroscopic ruler”, numerous studies have been published based on this theme. The classical Förster theory(2), which neglects the influence of reorientation and spatial motions of the interacting molecules, has been applied in an overwhelming number of studies, although these approximations frequently are invalid. To overcome these limitations an extended Förster theory (EFT) has been developed(3) and applied on various chemical systems(4). The EFT provides a new tool for examining biomacromolecular structure, which can complement established structural methods. The EFT imposes complex and time-consuming data analyses, however, as compared to the conventional and crude approaches commonly used in applications of the classical Förster theory. On the other hand, also molecular information from NMR or X-ray diffraction methodologies requires extensive sample preparations and advanced data analyses. In applications of the EFT of pair-wise interaction between chromophores located at a fixed distance, the analysis involves the description of their reorientational restriction and dynamics. This demands the modelling of anisotropic potentials, which are characterised by their strength and geometrical configuration. These properties can be obtained from data measured for the donor groups in the absence of energy migration. For this, time-resolved fluorescence depolarisation studies utilising the time-correlated single-photon counting (TCSPC) technique are most useful. The obtained fluorescence decays easily cover four orders of magnitude, for which the statistical noise obeys poissonian statistics(5-6).

This paper presents a general approach for the determination of relevant molecular parameters. This is demonstrated by fitting simulated data to synthetic data that mimic TCSPC experiments. In search for the optimal parameter values, two different strategies have been implemented, namely; a Genetic Algorithm (GA)(7), and a Covariance Matrix Adaptation algorithm(8). In the procedure of fitting test data to TCSPC data, it was found convenient and time-saving to work in the Fourier transform mode.

Fluorescence depolarisation experiments can be performed by means of the standard one-photon excitation (OPE), as well as by less common two-photon excitations (TPE). Unlike OPE depolarisation experiments, linearly as well as circularly polarised two-photon excitation experiments provide additional information. Actually, OPE and TPE experiments are

independent and importantly, they provide the same molecular information about reorienting motions(9-10) and energy migration processes(4).

TPE donor-acceptor electronic energy transfer is increasingly used in life sciences(11). *E.g.* in imaging studies of living cells and bio-assays(12-15). A few applications of TPE-DDEM exist, where the aggregation of proteins have been studied(16). The theory of fluorescence depolarisation induced by TPE has been developed previously(10, 17). Recently the theory of TPE fluorescence has been extended to two-colour excitation(18). However, so far no theory of fluorescence depolarisation is available that accounts for electronic energy transport upon TPE in the presence of molecular reorientation. This motivates the following unified treatment of OPE and TPE fluorescence depolarisation using computer simulations. The approach presented accounts for DDEM and the complex motion of fluorophores in restricting potentials.

THEORETICAL PREREQUISITES

Electronic energy migration - A general formulation

Throughout this work, energy migration within pairs of fluorophores is considered. In the absence of coupling, the fluorescent groups may exhibit different or similar photophysics relaxation. Under these conditions, the fluorescence anisotropy [$r(t)$] depends on the process of electronic energy transport. The fluorescence anisotropy is defined according to(19):

$$r(t) = \frac{D(t)}{S(t)} \quad (1a)$$

where $D(t)$ and $S(t)$ are linear combinations of observed fluorescence intensities ($f_\alpha(t)$) obtained for different polariser settings (α) (6). In most experimental setups, the expression for $D(t)$ is given by the difference(6):

$$D(t) = f_{\parallel}(t) - f_{\perp}(t) \quad (1b)$$

Here light propagates along Z_L -axis of the laboratory frame with polarisations in the $X_L Y_L$ -plane, whereas the emission polariser settings are in $X_L Z_L$ -plane. The symbol $\parallel$ denotes a parallel setting of the excitation and emission polariser with respect to the X_L -axis, while $\perp$ refers to excitation and emission polariser settings parallel to the Y_L - and Z_L -axis,

respectively. A particular linear combination $S(t)$ can be constructed which is invariant to the fluorophore orientation, whereby $S(t)$ becomes directly proportional to the total intensity of emitted fluorescence(19). In experiments using linearly and circularly polarised excitation light, this can be achieved by the following combinations(6):

$$\begin{aligned} S_{\text{Lin}}(t) &= f_{\parallel}(t) + 2f_{\perp}(t) \\ S_{\text{Cir}}(t) &= 2f_{\parallel}(t) + f_{\perp}(t) \end{aligned} \quad (1c)$$

For energy migration within a donor-donor pair, the fluorescence intensities and corresponding difference and sum curves are given by:

$$\begin{aligned} f_{\alpha}(t) &= \langle \sum_{i=1,2} \tilde{\rho}_{\alpha,ii}(t) \tilde{\chi}_i^p(t) \tilde{f}_i(t) + \sum_{\substack{i,j=1,2 \\ i \neq j}} \tilde{\rho}_{\alpha,ij}(t) \tilde{\chi}_j^s(t) \tilde{f}_i(t) \rangle I_{\text{exc}}^n \\ D(t) &= \langle \sum_{i=1,2} \tilde{\rho}_{D,ii}(t) \tilde{\chi}_i^p(t) \tilde{f}_i(t) + \sum_{\substack{i,j=1,2 \\ i \neq j}} \tilde{\rho}_{D,ij}(t) \tilde{\chi}_j^s(t) \tilde{f}_i(t) \rangle I_{\text{exc}}^n \\ S(t) &= \langle \sum_{i=1,2} \rho_{S,ii} \tilde{\chi}_i^p(t) \tilde{f}_i(t) + \sum_{\substack{i,j=1,2 \\ i \neq j}} \rho_{S,ij} \tilde{\chi}_j^s(t) \tilde{f}_i(t) \rangle I_{\text{exc}}^n \end{aligned} \quad (2a)$$

where

$$\begin{aligned} \tilde{\rho}_{D,ij}(t) &= \tilde{\rho}_{\parallel,ij}(t) - \tilde{\rho}_{\perp,ij}(t) \\ \tilde{\rho}_{S \text{ Lin},ij}(t) &= \tilde{\rho}_{\parallel,ij}(t) + 2\tilde{\rho}_{\perp,ij}(t) \\ \tilde{\rho}_{S \text{ Cir},ij}(t) &= 2\tilde{\rho}_{\parallel,ij}(t) + \tilde{\rho}_{\perp,ij}(t) \end{aligned} \quad (2b)$$

In Eq. 2a $\langle \dots \rangle$ denotes an ensemble averaging over fluorophore pairs, I_{exc} is the excitation light intensity, and $n = 1, 2$ for OPE and TPE, respectively. The binary function $\tilde{f}_i(t)$ describes whether the i^{th} fluorophore, initially excited at a time $t = 0$, is still excited at a time t later in the absence of energy migration. The functions $\tilde{\chi}_i^p(t)$ and $\tilde{\chi}_i^s(t)$ describe whether the i^{th} fluorophore is primary (p) or secondary (s) excited. Taken together the set of functions $\{\tilde{\chi}_i^p(t), \tilde{\chi}_i^s(t), \tilde{f}_i(t); i = \{1, 2\}\}$ accounts for the history of excitation for a particular pair of fluorophores. The *ensemble averages*, which are denoted $\chi_i^p(t) = \langle \tilde{\chi}_i^p(t) \tilde{f}_i(t) \rangle$, $\chi_i^s(t) = \langle \tilde{\chi}_i^s(t) \tilde{f}_i(t) \rangle$ and $f_i(t) = \langle \tilde{f}_i(t) \rangle$ [$i = \{1, 2\}$] refer to the primary and secondary excitation probability, as well as the photophysics decay, respectively. The functions $\tilde{\rho}_{\alpha,ij}(t)$ accounts for the probability that a photon with a given polarisation is absorbed by the i^{th} fluorophore and then followed by the emission of a photon with the polarisation α from the j^{th}

fluorophore. Here $\tilde{\rho}_{\alpha,ij}(t)$, $\tilde{\chi}_i^p(t)$ and $\tilde{\chi}_i^s(t)$ depend on the orientations $\mathcal{Q}_i(t)$, $\mathcal{Q}_j(t)$ of the i^{th} and j^{th} fluorophore at the times of absorption and emission.

Under the assumption of independent photophysics and energy migration processes, the fluorescence anisotropy of pairs of *identical* molecules can be written as:

$$r(t) = \langle \sum_{i=1,2} \tilde{r}_{ii}(t) \tilde{\chi}_i^p(t) + \sum_{\substack{i,j=1,2 \\ i \neq j}} \tilde{r}_{ij}(t) \tilde{\chi}_j^s(t) \rangle$$

$$\tilde{r}_{ij}(t) = \tilde{\rho}_{D,ij}(t) / \rho_S(t) \quad (3)$$

By introducing new functions $\tilde{\chi}_i(t)$, which are equal to unity only when the i^{th} fluorophore is initially excited, and moreover accounting for the fact that $\tilde{\chi}_1(t) + \tilde{\chi}_2(t) = 1$, Eq. 3 can be rewritten as follows:

$$r(t) = \frac{1}{2} \sum_{i=1,2} \langle \tilde{r}_{ii}(t) \tilde{\chi}(t) \rangle + \frac{1}{2} \sum_{\substack{i,j=1,2 \\ i \neq j}} \langle \tilde{r}_{ij}(t) (1 - \tilde{\chi}(t)) \rangle \quad (4)$$

In Eq. 4, $\tilde{\chi}(t)$ depends on the energy migration rate $\omega(t)$, which in the point dipole-dipole approximation of interaction (2) is given by

$$\omega(t) = \frac{3}{2} \frac{\kappa_D^2(t)}{\tau_D} \left(\frac{R_0}{R} \right)^6 \quad (5)$$

where $\kappa_D^2(t) = \left[\hat{\mu}_1(t) \cdot \hat{\mu}_2(t) - 3 \left(\hat{\mu}_1(t) \cdot \hat{R} \right) \left(\hat{\mu}_2(t) \cdot \hat{R} \right) \right]^2$ defines the orientation factor of the energy migration rate, and τ_D is the donor fluorescence lifetime. Here $\hat{\mu}_1$ and $\hat{\mu}_2$ denote unit vectors along the electronic transition dipole moments of donors, whereas $\hat{R}$ is a unit vector connecting the donor groups. By using Monte Carlo (MC) simulations, $\tilde{\chi}(t)$ can be determined. The algorithm for this procedure, as well as the EFT approach is outlined in Appendix I.

The EFT provides a more general approach for the evaluation of the fluorescence anisotropy. According to the EFT of donor-donor energy migration (DDEM)(3), the expression for $r(t)$ has the same analytical shape as that given by Eq. 4. However, the excitation probability of the primary excited donor is then given by the following averaged stochastic function(3):

$$\tilde{\chi}_{\text{EFT}}(t) = \frac{1}{2} \left[1 + \exp \left(- 2 \int_0^t \omega(t') dt' \right) \right] \quad (6)$$

In the presence of DDEM, the expressions for the anisotropy are equal in OPE and in TPE fluorescence depolarisation experiments (*cf.* Eq. 4). In both cases the expressions for the orientation factors of absorption-emission probabilities for isotropically oriented donor-pairs, $\tilde{\rho}_{ij}(t)$, exhibit the same analytical structure (*cf.* Appendix II):

$$\begin{aligned}\tilde{\rho}_{ij}(t) &= \sum_{l=0}^2 \sum_{p_A, p_E=-l}^l C_{p_A, p_E}^{(l)} V_{p_A, p_E}^{ij(l)}(t) \\ V_{p_A, p_E}^{ij(l)}(t) &= D_{p_A, p_E}^{(l)*}(\Omega_{M_i M_j}(t))\end{aligned}\quad (7a)$$

The indices i and j refer to energy migration within a pair of donors, *i.e.* $i, j = \{1, 2\}$. The Wigner rotation matrix elements $D_{p_A, p_E}^{(l)}(\Omega_{M_i M_j})$ (20), transform the irreducible tensor components between the M-frames, which are linked to the donor groups (*cf.* Fig. 1). The time-dependent anisotropy is described by a set of functions denoted by $V_{p_A, p_E}^{ij(l)}(t)$. The time-independent functions $C_{p_A, p_E}^{(l)}$ account for molecular absorption/emission transition tensors, as well as the polarisers settings used in OPE and TPE experiments. The number of non-vanishing $C_{p_A, p_E}^{(l)}$ -functions can be considerably reduced by aligning M-frames along the principal axes of the transition moment tensors. These tensors are defined by three non-vanishing diagonal components and the orientation of the principal axes relative to the fluorophore. In the case of OPE, this is achieved by aligning the z-axis of M-frame along transition moment.

It is here convenient to introduce two more expressions for $V_{p_A, p_E}^{ij(l)}(t)$:

$$V_{p_A, p_E}^{ij(l)}(t) = \sum_q D_{p_A, q}^{(l)*}(\Omega_{M_i R}(0)) D_{p_E, q}^{(l)}(\Omega_{M_j R}(t)) \quad (7b)$$

$$V_{p_A, p_E}^{ij(l)}(t) = \sum_{q_A, q_E} \left[D_{p_A, q_A}^{(l)*}(\Omega_{M_i D_i}(0)) D_{p_E, q_E}^{(l)}(\Omega_{M_j D_j}(t)) \right] D_{q_A, q_E}^{(l)*}(\Omega_{D_i D_j}) \quad (7c)$$

Eq. 7b describes the orientation of the absorbing and emitting fluorophores ($\Omega_{M_i R}$, $i = \{1, 2\}$), relative to linker frame R. This form is useful in computing the correlation functions, because the trajectories of fluorophore motions are conveniently computed in the linker frame. In Eq. 7c, $V_{p_A, p_E}^{ij(l)}(t)$ describes transformations between the donor frames and the corresponding local director frame ($\Omega_{M_i D_i}$, $i = \{1, 2\}$), as well as an inter-director frame transformation ($\Omega_{D_i D_j}$).

The mutual orientation of the D-frames defines *the configuration angles* for a bifluorophoric

molecule D_1 -R- D_2 . Eq. 7c is of particular importance for evaluating residual anisotropies, which are discussed below. For uniaxially symmetric orientation distributions, it is most convenient to align the Z_{D_i} -axis of each director frame along an effective symmetry axis.

The residual fluorescence anisotropy

After sufficiently long times following excitation (denoted $t \rightarrow \infty$), the anisotropy reaches a stationary level (r_∞), which is commonly referred to as the residual anisotropy. In this time limit, the excitation probability functions converge to the value of $\tilde{\chi}_{\text{EFT}}(t \rightarrow \infty) = \frac{1}{2}$. Moreover, the initially created orientation distribution reaches the equilibrium distribution, *i.e.* the angles $\Omega_{M_i D_i}(0)$ and $\Omega_{M_i D_i}(t \rightarrow \infty)$ become uncorrelated. Thus, the orientation correlation functions $\langle V_{p_A p_E}^{ij(l)}(t \rightarrow \infty) \rangle$ are given by:

$$\langle V_{p_A p_E}^{ij(l)}(t \rightarrow \infty) \rangle = \sum_{q_A, q_E} \langle D_{p_A q_A}^{(l)*}(\Omega_{M_i D_i}(0)) \rangle \langle D_{p_E q_E}^{(l)}(\Omega_{M_j D_j}(t)) \rangle D_{q_A q_E}^{(l)*}(\Omega_{D_i D_j}) \quad (8)$$

For equal orientation distributions of both donor groups, the indices $i, j = \{1, 2\}$ can be omitted and the averaged matrix elements in Eq. 8 become order parameters(21), denoted $\langle D_{p,q}^{(l)}(\Omega_{M_i D_i}) \rangle = S_{p,q}^{(l)}$. Thus, Eq. 8 can be written:

$$\langle V_{p_A p_E}^{ij(l)}(t \rightarrow \infty) \rangle = \sum_{q_A, q_E} S_{p_A q_A}^{(l)*} S_{p_E q_E}^{(l)} D_{q_A q_E}^{(l)*}(\Omega_{D_i D_j}) \quad (9)$$

Taken together, the Eqs. 4, 7c and 9 lead to the following residual anisotropy:

$$r_\infty = \frac{1}{2} \sum_{l=0} \sum_{p_A, p_E} C_{p_A p_E}^{(l)} \sum_{q_A, q_E} S_{p_A q_A}^{(l)*} S_{p_E q_E}^{(l)} \left(1 + \frac{1}{2} \sum_{i \neq j} D_{q_A q_E}^{(l)*}(\Omega_{D_i D_j}) \right) \quad (10)$$

Special cases

For standard OPE depolarisation experiments(6), it is possible to show that:

$$\tilde{r}_{ij}^{(1)} = \frac{3}{5} \sum_{p_A, p_E} M_{A p_A}^{(2)} M_{E p_E}^{(2)*} V_{p_A p_E}^{ij(2)} \quad (11)$$

by using Eq. A2.8 and a unit norm for the transition moment vectors. Here, $M_{A p_A}^{(2)}$ and $M_{E p_E}^{(2)}$ are irreducible tensor components of the absorption (A) and the emission (E) tensors in a fluorophore-fixed frame. These tensors are generated as the direct product between of corresponding transition dipole moments (*cf.* Appendix II). If both electronic transition

moments are aligned along the molecular z-axis, and in the absence of energy migration, Eq. 11 is transformed into the following frequently cited formula;

$$r(t) = \langle \tilde{r}_{ii}^{(1)}(t) \rangle = \frac{2}{5} \langle V_{00}^{ii(2)}(t) \rangle \quad (12)$$

From Eq. 7c and the eulerian angular representation it then follows that $r(t) = \frac{2}{5} \langle P_2(\cos \delta_M(t)) \rangle$, which is another frequently cited equation. Here P_2 denotes the second rank Legendre polynomial and $\delta_M(t)$ stands for the angle between the absorption and emission transition moments.

Actually in TPE one obtains the same time-dependence as for OPE, *i.e.* $r^{(2)}(t) = r_0^{(2)} \langle P_2(\cos \delta_M(t)) \rangle$, for fluorophores with only the component $T_{ZZ} \neq 0$ and the emission transition dipole moment aligned parallel to molecular z-axis. Here the fundamental anisotropy(19) $r_0^{(2)} = 4/7$. If one assumes a uniaxial orientation distribution of each donor group, the residual anisotropy value (*cf.* Eq. 10) is given by $r_\infty = r_0 \frac{1}{2} \left(S_{0,0}^{(2)} \right)^2 (1 + P_2(\cos \delta))$. Here $S_{0,0}^{(2)}$ denotes an order parameter and δ is angle between the z-axes of local director frames.

One should notice that previously published equations for the TPE anisotropy in the presence of energy migration (4, 22) are *only* justified for this particular case. The general expressions for $\tilde{r}_{ij}^{(2)}(t)$ are more complex (*cf.* Appendix II) and the corresponding anisotropies are considerably different from the OPE anisotropy.

SIMULATIONS AND ANALYSES OF FLUORESCENCE DEPOLARISATION DATA

To illustrate the use of MC simulations and the EFT for the analyses of fluorescence depolarisation experiments, a simulation approach has been used. Test data were then fitted to synthetic data that mimic time-correlated single photon counting experiments. Since data were generated with known modelling parameters, a critical examination of the MC and EFT approach could be performed. The generation of the fitting test data and synthetic experimental data follows the same computational procedures, except for the addition of Poissonian noise to the synthetic data, which is needed to mimic the statistics of the TCSPC

technique. Hereby the molecular parameters can be fully controlled, which enables an objective comparison between different strategies of analysis.

Since the analyses involve the determination of several parameters, two different procedures have been used. To start parameters that concern reorienting motions, transition dipoles and TP absorption tensor components were determined. For this, data corresponding to a particular system in the absence of energy migration were analysed. With knowledge of these parameters, the analysis was focussed on the bifluorophoric system, and the parameters involved in the energy migration process.

Different strategies for fitting parameters have been tested. Because multiple solutions could be consistent with experimental data, a method is required which efficiently searches for all local solutions. In practise, most optimisation methods tend to converge into *one* single local optimum. However, this inconvenience can be overcome by utilising a two-step optimisation procedure. First a coarse grained and slowly converging search is performed to reveal regions of the parameter space that contain local optima. Next a fast converging search is performed for each region of potential interest. For this and at both stages, it was found convenient to use a GA, since this algorithm is easily tuned for a global scanning, or for finding the exact value of a local optimum. In the present work a slightly modified PIKAIA GA routine(7) was used. Another route for the optimisation is a Covariance Matrix Adaptation algorithm(8), which was tested. Details concerning the modelling of molecular structures and dynamics, the calculation of observable intensities, as well as the examined strategies for the fitting of modelling parameters, are summarised in Appendix I.

Generating fluorescence data

Generated reorientation trajectories of the donor groups in a pair were used for calculating the absorption-emission probabilities ($\tilde{\rho}(t)$) and excitation probabilities ($\tilde{\chi}_{\text{EFT}}(t)$). The latter property was calculated from the EFT (*cf.* Eq. 6) (23-24). The simulated time-resolved anisotropy (*cf.* Fig. 2A) was constructed from $\tilde{\rho}(t)$ and $\tilde{\chi}_{\text{EFT}}(t)$ by means of Eq. 4. Then the fluorescence anisotropy was used to calculate the intensity decay curves, $f_{\parallel}(t)$ and $f_{\perp}(t)$. In principle these decays are obtained from time-resolved fluorescence depolarisation experiments(6) under idealised conditions, *e.g.* an infinitely short excitation pulse and an ideal

detector. In order to mimic real experimental intensities $[F_\alpha(t), \alpha \in \{\parallel, \perp\}]$, $f_\alpha(t)$ needs to be convoluted with an experimental instrumental response function ($I(t)$), *i.e.* $F_\alpha(t) = I(t) * f_\alpha(t)$ (6, 25). The synthetic intensity decay curves thus obtained are illustrated in Fig. 2B.

A common technique for fast convolution utilises the fast Fourier transform of data sets, which are further convoluted with the instrumental response function. Data are first transformed into the Fourier domain, thereafter multiplied and finally transformed back to the time domain. The Fourier transform of $F_\parallel(t)$ is exemplified in Fig. 3, where the final inverse Fourier transform was avoided by performing the data analysis in the Fourier domain. Likely, this approach can be developed further by treating the depolarisation process, as a whole, in the frequency domain.

Simulated OPE and TPE fluorescence depolarisation in the absence of DDEM

The fluorescence depolarisation decays $[F_\parallel(t)$ and $F_\perp(t)]$ corresponding to one- and two-photon excitation of a hypothetical bifluorophoric molecule, D₁-R-D₂, were generated in the absence of energy migration. In the OPE experiment, the absorption and emission transition dipoles are assumed to be parallel and directed along the molecular z-axis. The two-photon absorption tensor is defined with non-zero components in the molecular YZ-plane, *i.e.* $T_{xx} = T_{xy} = T_{xz} = 0$. The depolarisation decays were convoluted with a real instrumental response function for which the full width at half maximum is 100 ps. Moreover, poissonian noise was added in order to mimic the statistics of TCSPC experiments,. Typical simulated decay curves are shown in Fig. 4A-B. Here, the intensity decay rate is dictated by the *intrinsic* rotational correlation times ($\tau_{cx} = 3.0$ ns, $\tau_{cy} = 12.5$ ns, $\tau_{cz} = 9.0$ ns) and orientational restrictions of the donor groups with respect to the linker group. These restrictions define an anisotropic distribution of donor orientations, which are revealed by the stationary residual anisotropies (r_∞ , *cf.* Fig. 4A-B). The value of the residual anisotropy(26) are related to the local order parameters(22). In OPE depolarisation experiments, this relation is: $r_\infty^{(1)} / r_0^{(1)} = S_{0,0}^{(2)2} = S_{zz}^2$, where S_{zz} is the order parameter referring to the molecular Z-axis. The rather high value of $S_{zz} = 0.7$, implies that the Z-axis is preferentially oriented parallel to the $C_{\infty v}$ -axis of the orientation distribution.

Simulated OPE and TPE fluorescence depolarisation in the presence of DDEM

Qualitatively, the anisotropy data generated in the presence of energy migration and reorienting motions are similar to those discussed in the previous subsection (*cf.* Fig. 4A-B). The mimicked experimental data refer to the configuration parameters (*cf.* Appendix I) and the fluorophore-fluorophore distance given by $\beta_{D_1R,\text{exp}} = \pi - \beta_{D_2R,\text{exp}} = \beta_{DR,\text{exp}} = 33^\circ$, $\delta_{\text{exp}} = 123^\circ$ and $R_{\text{exp}}/R_0 = 0.95$, respectively. The TP absorption tensor components were chosen to be; $T_{ZZ} = 1$, $T_{YY} = 0.5$, and $T_{YZ} = 0.707$. As compared to data shown in absence of DDEM, the differences concern a faster relaxation and lowered residual anisotropies. The former is explained by fact that excitation jumps cause an apparent increase of the reorientation of the excited donors. The lowered plateau values are expected because the angular distribution of excited donors becomes broader as compared to that in the absence of DDEM, provided that the orienting donor distributions are not collinear. Furthermore, the residual anisotropy values depend on the order parameters of the donors D_1 and D_2 , as well as by the angle δ (*cf.* Fig. 1) between the symmetry axes of corresponding orientation distributions (*cf.* Eq. 10).

Reanalyses of synthetic data

A modified genetic algorithm has been used to examine whether the correct distance and configuration angles can be recovered. For this test data were fitted to the synthetic experimental OPE and TPE depolarisation data. Here the test data were synthesised in the same way as the synthesised experimental data. In order to speed up the analysis, poissonian noise was not added, and tenfold fewer rotational trajectories were used. As a goodness of fit parameter the reciprocal value of the reduced χ_r^2 -value was used. As is illustrated in Fig. 4 C-D the obtained maps of χ_r^2 -values are very similar from the analyses of OPE and TPE data. Clearly two regions of acceptable solutions (s1 and s2) are revealed. In each region the best values of χ_r^2 are close to unity, implying solutions that perfectly fit to the experimental data. The known correct values of configuration parameters are centred at s1, while the solution s2 provides a somewhat shorter inter-fluorophore distance, *i.e.* $R_{s2} \approx 0.88/R_0$. The configuration angles $\beta_{DR,s2} \approx 63^\circ$ and $\delta_{s2} \approx 56^\circ$ differ considerably from the experimental

values $\beta_{\text{DR,exp}} = 33^\circ$ and $\delta_{\text{exp}} = 123^\circ$. The two solutions obtained for the configuration angle δ can be ascribed to the $\cos^2 \delta$ -dependence of the residual anisotropy (*cf.* Eq. 10), since $\delta_{s1} \approx 123^\circ$ and $\delta_{s2} \approx \pi - \delta_{s1} \approx 56^\circ$ correspond the same r_∞ -value. The behaviour of the initial part of the anisotropy decay depends on the history of energy transfer rates within the ensemble of donor-donor pairs. By considering the special case of a narrow orientation distribution of the donors for which ω is almost static, a qualitative explanation can be given. In this limit configuration parameters that provide the same anisotropy decay are related by equation (*cf.* Eq.5):

$$(\cos \delta + 3 \cos^2 \beta_{\text{DR}})^2 / R^6 = \omega_{\text{const}} \quad (13)$$

This equation exhibits two branches of an *infinite number* of solutions for β_{DR} and R , all of which correspond to two different values of δ . The situation is however similar for dynamic cases, but the time-dependence of the DDEM rate ω then shifts the solutions relative to the static case, and an infinite set of solutions converge to the observed couple of discrete solutions.

Taken together, this demonstrates that the determination of configuration parameters (β_{DR} and R) can *only* be achieved if one accounts for the dynamics of energy migration.

CONCLUDING REMARKS

This paper describes new procedures and a general theoretical description and analysis of OPE and TPE depolarisation experiments, which simultaneously accounts for reorienting motions and electronic energy migration within pairs of donor molecules. The approach for analysing synthetic depolarisation data yields values on physical parameters, which are consistent with known values on the corresponding parameters. It is shown that configuration parameters can be determined only by considering the dynamics of energy migration. **An overall aim of combining DDEM and depolarisation experiments is to determine configuration parameters. While the OPE and TPE experiments are independent, the obtained configuration parameters from the OPE and TPE data are the same (*cf.* Fig. 4). This can be expected since the molecular configuration is invariant to the excitation used. Actually, performing independent experiments strengthens the probability of finding reliable values on the configuration parameters. Moreover, TP experiments introduce new features that can be**

exploited. In particular, using circularly polarised excitation also provides independent information about the configuration. Finally, longer excitation wavelength can be selected, which could be useful in studies of biological systems.

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APPENDIX I: Simulations of depolarisation experiments

Modelling molecular structure and dynamics

A general bifluorophoric molecule denoted by D_1 -R- D_2 has been considered in which planar donor groups are covalently connected to a rigid linker group (*cf.* Fig. 1). The studied ensemble D_1 -R- D_2 molecules isotropically oriented. The donor groups undergo local independent reorienting motions, which are described by Brownian dynamics simulation using a restricting potential, $U_i(\Omega_{M_i D_i})$ $i = \{1, 2\}$. Hence the donors undertake a series of instant random reorientations in time. At each moment the reorientation of a donor depends only on its current position. Thus, the displacement is independent of the history of previous motions (*i.e.* in accordance with a Markov process). The orientation of the fluorescent groups has been encoded by quaternions(27). If at time t the orientation is $q(t)$, then at time $t + \Delta t$ later it is given by the quaternionic product $\Delta q(t)q(t)$. The **increment** $\Delta q(t)$ is related to the angular displacements of a donor according to the following relation(27)

$$\begin{pmatrix} \Delta q_0(t) \\ \Delta q_1(t) \\ \Delta q_2(t) \\ \Delta q_3(t) \end{pmatrix} = \frac{1}{2} \begin{pmatrix} 0 & -\Delta\Omega_x & -\Delta\Omega_y & -\Delta\Omega_z \\ \Delta\Omega_x & 0 & \Delta\Omega_z & -\Delta\Omega_y \\ \Delta\Omega_y & -\Delta\Omega_z & 0 & \Delta\Omega_x \\ \Delta\Omega_z & \Delta\Omega_y & -\Delta\Omega_x & 0 \end{pmatrix} \begin{pmatrix} q_0(t) \\ q_1(t) \\ q_2(t) \\ q_3(t) \end{pmatrix} \quad (A1.1)$$

In Eq. A1.1, $\Delta q_i(t)$ $i=\{0...3\}$ denote **increment** components in the immobile frame, and $\Delta\Omega_i$ $i=\{x, y, z\}$ are components of angular displacement in the molecular frame. The angular displacement can be calculated by using the Euler-Langevin equation(28):

$$I\ddot{\Omega} = -\zeta\dot{\Omega} + \mathbf{F}(\Omega) + \mathcal{R}'(t) \quad (A1.2)$$

Here, I is the moment of inertia of a fluorophore group, $\mathbf{F}(\Omega) = -\nabla_{\mathbf{R}} U(\Omega)$ is the torque caused by the restricting potential, $\mathcal{R}'(t)$ is a random torque imposed on fluorophore group by a stochastic surrounding, and ζ is a friction matrix. From the integration of Eq. A1.2 over the time-interval $(t, t + \Delta t)$ and by choosing $\Delta t \gg \zeta^{-1} I$ one obtains that(28):

$$\Delta\Omega(\Delta t) = \zeta^{-1} \mathbf{F}(\Omega) \Delta t + \mathcal{R}(\Delta t) \quad (\text{A1.3})$$

Here the stochastic displacement $\mathcal{R}(\Delta t)$ is a random vector with a zero mean-value and the covariance matrix $\Sigma_f = 2kT\Delta t\zeta^{-1}$. The distribution of $\mathcal{R}(\Delta t)$ has no principal meaning and is conveniently normalised. Hereby it is given by $\mathcal{R}(\Delta t) = \sqrt{2kT\Delta t\zeta^{-1}} \mathcal{N}^{(3)}(0,1)$, where $\mathcal{N}^{(3)}(0,1)$ is a random vector with a standard normal distribution. In practice it is convenient to diagonalise the friction matrix by aligning the molecular frame along its eigenvectors. Hereby the components of angular displacements are given by:

$$\Delta\Omega_i = \frac{1}{2} U_i F_{\Omega i}(\Omega) \frac{\Delta t}{3\tau_{ci}} + \mathcal{N}_i(0,1) \sqrt{\frac{\Delta t}{3\tau_{ci}}} \quad i = \{x, y, z\} \quad (\text{A1.4})$$

In Eq. A1.4, ζ_i stand for eigenvalues of the friction matrix, U_i are coefficients that characterise the magnitude of the orienting torque (in units of $k_B T$). The force $F_{\Omega i}(\Omega)$ depends on components of the angular torque. The rotational correlation coefficients $\tau_{ci} = \zeta_i / (6k_B T)$ characterise the rotational motion of the donor about its molecular axes in the absence of the restricting potential. For the simulating the motions of a planar fluorophore the shape of the orienting potential has been used:

$$U = \varphi k_B T (1 - \cos \beta_{\text{MD}}) (1 - \varepsilon \cos 2\gamma_{\text{MD}}), \quad (\text{A1.5})$$

In Eq. A1.5, φ is the force of the potential, whereas ε characterises its anisotropy. For $\varepsilon > 0$ this potential shifts the maximum of the fluorophore angular distribution to $\beta_{\text{MD}} = 0, \gamma_{\text{MD}} = 0 \text{ or } \pi$, thus reflecting main features of orientational restrictions imposed by host molecule on a planar donor group.

The reorientation trajectories for the two donor groups were generated relative to the **D**-frames, for which the z-axes were aligned along the symmetry axes of these distributions. The initial positions of fluorophores $\Omega(t_0)$ were calculated by the inversion/rejection method(29),

and it was assumed that the fluorophore orientation in the external potential obeys the Boltzmann equilibrium distribution.

The **positions** of the symmetry axes of the donor orientation distributions with respect to the **R** frame are described by the angles $\Omega_{D_1R}(\alpha_{D_1R}, \beta_{D_1R}, \gamma_{D_1R}), \Omega_{D_2R}(\alpha_{D_2R}, \beta_{D_2R}, \gamma_{D_2R})$ (cf. Fig. 1).

Accounting for the fact that we consider axially symmetrical donor orientation distributions

this set of Euler angles can be replaced by angles δ, β_{D_1R} and β_{D_2R} . These are referred to as the *configuration* angles. Furthermore, a two-fold symmetry is assumed for the symmetry axes of the distributions, *i.e.* $\beta_{D_1R} = \pi - \beta_{D_2R} = \beta_{DR}$. The hypothetical donor groups are assumed to be planar, as is also true for most fluorescent probes. In this case two-photon absorption tensor with respect to frame fixed to a donor (**M**-frame or molecular frame) consists of three independent components. The direction of the emission transition moment (and absorption transition moment in case of OPE) was chosen to coincide with the z-axis of the **M**-frame.

Simulation of energy transfer

The energy migration rates, $\omega(\Omega_i(t), \Omega_j(t))$ within each donor-donor pair were calculated (Eq. 5) by using generated reorientation trajectories. The fluorescence lifetime of the donors was chosen to be $\tau_D = 11$ ns. The obtained migration rates were used to compute the excitation functions $\tilde{\chi}(\omega(t))$ for the ensemble of pairs. Two approaches have been used for the computation of $\tilde{\chi}(\omega(t))$, namely; MC simulations and the EFT (Eq. 6). Details concerning the MC simulations are given elsewhere(30). The MC approach relies on the simulation of random events of energy migration. This procedure introduces an additional randomness in computing $\tilde{\chi}(\omega(t))$. To achieve the same accuracy as in the EFT approach, the MC simulations require the averaging over larger ensembles. In general the EFT approach is faster, especially at high rates of energy transfer. The results presented were obtained by utilising the EFT.

Calculation of anisotropy decays

The orientation functions $\tilde{\rho}_{ij}(t) = \tilde{\rho}(\Omega_i(0), \Omega_j(t))$ were calculated according to Eq. 7 by using generated trajectories. This involves the computation of $V_{PA,PE}^{ij(l)}(t)$, for which two

strategies have been used. One utilises Eq. 7b, whereby the orientation trajectories $\Omega_{M_i D_i}(t)$ generated in **D**-frame were used to calculate $V_{P_A, P_E}^{ij(l)}(t)$. Alternatively, Eq. 7c was calculated after transforming the trajectories of $\Omega_{M_1 D_1}(t)$, $\Omega_{M_2 D_2}(t)$ into $\Omega_{M_1 M_2}(t)$. The former expressions for $V_{P_A, P_E}^{ij(l)}(t)$ are more complex, and the calculations therefore more time-consuming. The latter approach, however, requires more time for angular transformations. It turned out to be more effective to use Eq. 7c. The time needed for calculating $\tilde{\rho}_{ij}(t)$ can be reduced two-fold, since it is sufficient to calculate the correlations only for currently excited fluorophore within a pair. Terms in Eq. 4 corresponding to the unexcited fluorophore will vanish, due to multiplication by the excitation probability function. The final anisotropy decays were calculated by using Eqs. 3 and 4. To simulate decays that mimic real experiments, 10^6 trajectories were used, whereas 10^4 trajectories were sufficient for the generation of test curves.

Transformation to observable intensities

In practise the fluorescence anisotropy cannot be measured, but constructed from the observables $f_{\parallel}(t)$ and $f_{\perp}(t)$. To facilitate the analysis of real depolarisation experiments by the methods described here, the simulated anisotropy $[r(t)]$ was transformed into $f_{\parallel}(t)$ and $f_{\perp}(t)$. For instance, in depolarisation experiments using linearly polarised excitation (*cf.* Eq. 1) one obtains that:

$$\begin{aligned} f_{\parallel}(t) &= \frac{1}{3} f(t) (1 + 2r(t)) \\ f_{\perp}(t) &= \frac{1}{3} f(t) (1 - r(t)) \end{aligned} \tag{A1.6}$$

, where $f(t)$ denotes the photophysics' decay. For the analysis of true experimental data, the measured photophysics' decay, or its smoothed fitting can be used. For the synthetic results the photophysics was calculated by using a definite τ_D -value.

APPENDIX II: Orientation factors of absorption-emission probabilities ($\tilde{\rho}$) in OPE and TPE experiments

For a fluorophore with the transition moment $\vec{\mu}$, the orientational part of the probability ($\mathcal{P}_{OP}$) of the absorption or emission of *one photon* with the polarisation $\vec{\alpha}$ is given by:

$$\mathcal{P}_{\text{OP}} = |\vec{\mu} \cdot \vec{\alpha}|^2 = \left| \sum_{\text{p}} \bar{\alpha}_{\text{p}}^{(1)*} \mu_{\text{p}}^{(1)} \right|^2 \quad (\text{A2.1a})$$

, where the vector components are given in the **spherical** irreducible representation. Here and in the following text, the bar above a vector/tensor property x (written as $\bar{x}$) denotes the irreducible representation of the complex conjugate of the corresponding vector/tensor. Thus, $\bar{\alpha}_{\text{p}}^{(l)}$ stands for an irreducible representation of the complex conjugation of the polarisation vector $\vec{\alpha}^*$.

The orientational part for the absorption of *two photons* with the polarisations α and β is given by:

$$\mathcal{P}_{\text{TP}} = |\vec{\alpha} \cdot \mathbb{T} \cdot \vec{\beta}|^2 = |\mathbb{A} \cdot \mathbb{T}|^2 = \left| \sum_l \sum_{\text{p}} \bar{\mathbb{A}}_{\text{p}}^{(l)*} \mathbb{T}_{\text{p}}^{(l)} \right|^2 \quad (\text{A2.1b})$$

In Eq. A2.1b, $\mathbb{T}$ stands for the TP absorption tensor and $\mathbb{A} = (\alpha \otimes \beta)$. Obviously, this expression differs from Eq. A2.1a, only by the rank of the tensors involved. Thus, one could use a general expression (see below, Eq. A2.1c), while remembering that this equation provides the one-photon probability if the absorption and emission tensors consist only of first rank components, $\mathbb{T}_{\text{p}}^{(1)} = \mu_{\text{p}}^{(1)}$.

General expressions

$$\mathcal{P} = \left| \sum_l \sum_{\text{p}} \bar{\mathbb{A}}_{\text{p}}^{(l)*} \mathbb{T}_{\text{p}}^{(l)} \right|^2 = \sum_{l_1, l_2} \sum_{\text{p}_1, \text{p}_2} \left(\bar{\mathbb{A}}_{\text{p}_1}^{(l_1)*} \bar{\mathbb{A}}_{\text{p}_2}^{(l_2)} \right) \left(\mathbb{T}_{\text{p}_1}^{(l_1)} \mathbb{T}_{\text{p}_2}^{(l_2)*} \right) = \sum_{l_1, l_2, l} \sum_{\text{p}} \mathbb{A}_{\text{p}}^{(l_2, l_1, l)*} \mathbb{T}_{\text{p}}^{(l_1, l_2, l)} \quad (\text{A2.1c})$$

Here the following tensor products $\mathbb{A}^{(l_1, l_2, l)}$ and $\mathbb{T}^{(l_1, l_2, l)}$ have been introduced:

$$\mathbb{A}_{\text{p}}^{(l_1, l_2, l)} = \sum_{\text{p}_1, \text{p}_2} \left[\begin{matrix} l_1 & l_2 & l \\ \text{p}_1 & \text{p}_2 & \text{p} \end{matrix} \right] \mathbb{A}_{\text{p}_1}^{(l_1)} \bar{\mathbb{A}}_{\text{p}_2}^{(l_2)}, \quad \mathbb{T}_{\text{p}}^{(l_1, l_2, l)} = \sum_{\text{p}_1, \text{p}_2} \left[\begin{matrix} l_1 & l_2 & l \\ \text{p}_1 & \text{p}_2 & \text{p} \end{matrix} \right] \mathbb{T}_{\text{p}_1}^{(l_1)} \bar{\mathbb{T}}_{\text{p}_2}^{(l_2)}.$$

The $\left[\begin{matrix} l_1 & l_2 & l \\ \text{p}_1 & \text{p}_2 & \text{p} \end{matrix} \right]$ symbol is a Clebch-Gordon coefficient(20). The joint probability of the absorption-emission processes is then given by:

$$\mathcal{P}_{\text{AE}} = \mathcal{P}_{\text{A}} \mathcal{P}_{\text{E}} = \sum_{\substack{l_{\text{A}1}, l_{\text{A}2}, l_{\text{A}} \\ l_{\text{E}1}, l_{\text{E}2}, l_{\text{E}}}} \sum_{\text{p}_{\text{AL}}, \text{p}_{\text{EL}}} \mathbb{A}_{\text{p}_{\text{AL}}}^{L(l_{\text{A}2}, l_{\text{A}1}, l_{\text{A}})*} \mathbb{A}_{\text{p}_{\text{EL}}}^{L(l_{\text{E}2}, l_{\text{E}1}, l_{\text{E}})} \mathbb{T}_{\text{p}_{\text{AL}}}^{L(l_{\text{A}1}, l_{\text{A}2}, l_{\text{A}})} \mathbb{T}_{\text{p}_{\text{EL}}}^{L(l_{\text{E}1}, l_{\text{E}2}, l_{\text{E}})*} \quad (\text{A2.2})$$

Here the tensor components are expressed with respect to the laboratory frame (L). The components of the transition tensors in the L-frame are time-dependent for reorienting donors. It convenient to express these components through tensor components which refer to the donor-fixed frame, $\mathbb{T}_{\text{p}_{\text{A}}}^{(l_{\text{A}1}, l_{\text{A}2}, l_{\text{A}})}$, $\mathbb{T}_{\text{p}_{\text{E}}}^{(l_{\text{E}1}, l_{\text{E}2}, l_{\text{E}})}$. This is achieved by the transformation:

$$\mathbb{T}_{\text{pL}}^{L(l_1, l_2, l)} = \sum_{\text{p}, \text{q}} D_{\text{p}, \text{q}}^{(l)*}(\Omega_{\text{MR}}) D_{\text{q}, \text{pL}}^{(l)}(\Omega_{\text{RL}}) \mathbb{T}_{\text{p}}^{(l_1, l_2, l)}, \quad (\text{A2.3})$$

where $D_{\text{p}, \text{q}}^{(l)}(\Omega)$ are Wigner rotation matrix elements. This transformation yields that:

$$\begin{aligned} \mathcal{P}_{\text{AE}} = \sum_{\substack{l_{\text{A2}}, l_{\text{A1}}, l_{\text{A}} \\ l_{\text{E1}}, l_{\text{E2}}, l_{\text{E}}}} \sum_{\substack{\text{p}_{\text{A}}, \text{q}_{\text{A}} \\ \text{p}_{\text{E}}, \text{q}_{\text{E}}}} \sum_{\text{p}_{\text{AL}}, \text{p}_{\text{EL}}} \left[\mathbb{A}_{\text{ApAL}}^{L(l_{\text{A2}}, l_{\text{A1}}, l_{\text{A}})*} \mathbb{A}_{\text{EpEL}}^{L(l_{\text{E2}}, l_{\text{E1}}, l_{\text{E}})} \mathbb{T}_{\text{ApA}}^{(l_{\text{A1}}, l_{\text{A2}}, l_{\text{A}})} \mathbb{T}_{\text{EpE}}^{(l_{\text{E1}}, l_{\text{E2}}, l_{\text{E}})*} \times \right. \\ \left. \times D_{\text{p}_{\text{A}}, \text{q}_{\text{A}}}^{(l_{\text{A}})*}(\Omega_{\text{MAR}}) D_{\text{p}_{\text{E}}, \text{q}_{\text{E}}}^{(l_{\text{E}})}(\Omega_{\text{MER}}) D_{\text{q}_{\text{A}}, \text{p}_{\text{AL}}}^{(l_{\text{A}})*}(\Omega_{\text{RL}}) D_{\text{q}_{\text{E}}, \text{p}_{\text{EL}}}^{(l_{\text{E}})}(\Omega_{\text{RL}}) \right] \end{aligned} \quad (\text{A2.4})$$

Eq. A2.4 can be simplified by averaging over an isotropic orientation distribution of the bifluorophoric molecules (Ω_{RL}) gives:

$$\langle D_{\text{q}_{\text{A}}, \text{p}_{\text{AL}}}^{(l_{\text{A}})*}(\Omega_{\text{RL}}) D_{\text{q}_{\text{E}}, \text{p}_{\text{EL}}}^{(l_{\text{E}})}(\Omega_{\text{RL}}) \rangle_{\Omega_{\text{RL}}} = \frac{1}{2l_{\text{A}} + 1} \delta_{l_{\text{A}}, l_{\text{E}}} \delta_{\text{q}_{\text{A}}, \text{q}_{\text{E}}} \delta_{\text{p}_{\text{AL}}, \text{p}_{\text{EL}}} \quad (\text{A2.5})$$

Finally, one obtains the following expression for the orientation factors of probabilities which appear in the anisotropy expressions:

$$\begin{aligned} \tilde{\rho}(t) &= \langle \mathcal{P}_{\text{AE}} \rangle_{\Omega_{\text{RL}}} = \sum_l \sum_{\text{p}_{\text{A}}, \text{p}_{\text{E}}} C_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{\rho(l)} V_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{(l)}(t) \\ V_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{(l)}(t) &= \sum_{\text{q}} D_{\text{p}_{\text{A}}, \text{q}}^{(l)*}(\Omega_{\text{MAR}}(t)) D_{\text{p}_{\text{E}}, \text{q}}^{(l)}(\Omega_{\text{MER}}(t)) = D_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{(l)*}(\Omega_{\text{MAM}_\text{E}}(t)) \\ C_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{\rho(l)} &= \frac{1}{2l+1} \sum_{\substack{l_{\text{A1}}, l_{\text{A2}} \\ l_{\text{E1}}, l_{\text{E2}}}} \sum_{\text{p}} \mathbb{A}_{\text{Ap}}^{L(l_{\text{A1}}, l_{\text{A2}}, l)} \mathbb{A}_{\text{Ep}}^{L(l_{\text{E1}}, l_{\text{E2}}, l)*} \mathbb{T}_{\text{ApA}}^{(l_{\text{A1}}, l_{\text{A2}}, l)} \mathbb{T}_{\text{EpE}}^{(l_{\text{E1}}, l_{\text{E2}}, l)*} \end{aligned} \quad (\text{A2.6})$$

Here $C_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{\rho(l)}$ are constants that characterise the experimental polarisation setup and the transition properties of the fluorophores, whereas $V_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{(l)}(t)$ include all information about their molecular reorientation.

Thus, the experimental polarisation setup for OP or TP absorption, which followed by the emission of one photon is described by:

$$\begin{aligned} C_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{\rho \text{OP}(l)} &= \frac{1}{2l+1} \sum_{\text{p}} \bar{A}_{\text{Ap}}^{L(l)} \bar{A}_{\text{Ep}}^{L(l)*} M_{\text{ApA}}^{(l)} M_{\text{EpE}}^{(l)*} \\ C_{\text{p}_{\text{A}}, \text{p}_{\text{E}}}^{\rho \text{TP}(l)} &= \frac{1}{2l+1} \sum_{l_1, l_2} \sum_{\text{p}} \mathbb{A}_{\text{Ap}}^{L(l_1, l_2, l)} \bar{A}_{\text{Ep}}^{L(l)*} \mathbb{T}_{\text{ApA}}^{(l_1, l_2, l)} M_{\text{EpE}}^{(l)*} \end{aligned} \quad (\text{A2.7})$$

In Eq. A2.7, $\bar{A}_{\text{p}}^{(l)} = \sum_{\text{p}_1, \text{p}_2} \begin{bmatrix} 1 & 1 & l \\ \text{p}_1 & \text{p}_2 & \text{p} \end{bmatrix} \alpha_{\text{p}_1}^{(1)} \bar{\alpha}_{\text{p}_2}^{(1)}$ and $M_{\text{p}}^{(l)} = \sum_{\text{p}_1, \text{p}_2} \begin{bmatrix} 1 & 1 & l \\ \text{p}_1 & \text{p}_2 & \text{p} \end{bmatrix} \mu_{\text{p}_1}^{(1)} \mu_{\text{p}_2}^{(1)}$ are components of the

irreducible representation of second rank polarisation and OP transition tensors.

Absorption-emission probabilities for common OPE/TPE experiments

A frequently used OPE polariser setup is $\alpha_{\text{A}\parallel}^{\text{L}} = (1, 0, 0)$; $\alpha_{\text{E}\parallel}^{\text{L}} = (1, 0, 0)$; $\alpha_{\text{E}\perp}^{\text{L}} = (0, 0, 1)$ (in a Cartesian representation) for which one obtains that:

$$\begin{aligned}
 \tilde{\rho}_{\text{Lin,D}}^{(1)}(t) &= \tilde{\rho}_{\parallel,\parallel}^{(1)}(t) - \tilde{\rho}_{\parallel,\perp}^{(1)}(t) = \frac{1}{5} \sum_{\text{p}_A, \text{p}_E} \left[M_{\text{Ap}_A}^{(2)} M_{\text{Ep}_E}^{(2)*} \right] V_{\text{p}_A, \text{p}_E}^{(2)}(t) \\
 \tilde{\rho}_{\text{Lin,S}}^{(1)} &= \tilde{\rho}_{\parallel,\parallel}^{(1)}(t) + 2\tilde{\rho}_{\parallel,\perp}^{(1)}(t) = \frac{1}{3} \|\mu_A\|^2 \|\mu_E\|^2 \\
 \tilde{r}_{\text{Lin}}^{(1)}(t) &= \frac{\tilde{\rho}_{\text{Lin,D}}^{(1)}(t)}{\tilde{\rho}_{\text{Lin,S}}^{(1)}} = \frac{3}{5} \sum_{\text{p}_A, \text{p}_E} M_{\text{p}_A}^{(2)} M_{\text{p}_E}^{(2)*} V_{\text{p}_A, \text{p}_E}^{(2)}(t)
 \end{aligned} \tag{A2.8}$$

Here $\|\mu_A\|$ and $\|\mu_E\|$ denote the norms of the transition moments which assumed to be unit.

For TPE experiments using the same polariser settings the corresponding equation reads:

$$\tilde{r}_{\text{Lin}}^{(2)}(t) = \frac{\sqrt{3}}{5} \sum_{\text{p}_A, \text{p}_E} \frac{\mathbb{T}_{\text{Ap}_A}^{(2,0,2)} + \mathbb{T}_{\text{Ap}_A}^{(0,2,2)} + \frac{2}{\sqrt{7}} \mathbb{T}_{\text{Ap}_A}^{(2,2,2)}}{\mathbb{T}_{\text{A0}}^{(0,0,0)} + \frac{2}{\sqrt{5}} \mathbb{T}_{\text{A0}}^{(2,2,0)}} M_{\text{Ep}_E}^{(2)*} V_{\text{p}_A, \text{p}_E}^{(2)}(t) \tag{A2.9}$$

In TPE experiments using circular polarised excitation $\alpha_{\text{A Cir}}^{\text{L}} = (-\frac{1}{\sqrt{2}}, \frac{i}{\sqrt{2}}, 0)$ the anisotropy is given by:

$$\tilde{r}_{\text{Cir}}^{(2)}(t) = \frac{\sqrt{3}}{\sqrt{35}} \sum_{\text{p}_A, \text{p}_E} \frac{\mathbb{T}_{\text{Ap}_A}^{(2,2,2)}}{\mathbb{T}_{\text{A0}}^{(2,2,0)}} M_{\text{Ep}_E}^{(2)*} V_{\text{p}_A, \text{p}_E}^{(2)}(t) \tag{A2.10}$$

Note that for experiments with circular excitation polarisation denominator of anisotropy expression $\tilde{\rho}_{\text{Cir,S}}^{(1)} = 2\tilde{\rho}_{\parallel,\parallel}^{(1)} + \tilde{\rho}_{\parallel,\perp}^{(1)}$ differs from cases based on using linearly polarised excitation. The Eqs. A2.9 and A2.10 coincide with the previously published equations 9, 11 in reference(22), substituting $\Theta_0^{(0)} = \frac{1}{\sqrt{5}} \mathbb{T}_{\text{A0}}^{(2,2,0)}$, $\Theta_{\text{p}_A}^{(2)} = -\frac{\sqrt{2}}{\sqrt{7}} \mathbb{T}_{\text{Ap}_A}^{(2,2,2)}$.

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Figure 1. The chemical structure of a hypothetical bifluorophoric molecule $[D_1\text{-}R\text{-}D_2]$ in which the linker part (R) could be a protein. The donor groups D_1 and D_2 are chemically and photophysically identical. The schematic below indicates the different coordinate systems needed to describe the orientation of the initially one-photon-excited (OPE) or two-photon excited (TPE) donor, as well as its orientation at the time of fluorescence emission. The laboratory fixed and the linker fixed frames are denoted L and R , respectively. The z -axes of the D_1 and D_2 frames are chosen to coincide with the symmetry axes of the donor orientation distributions. The z -axis of the R -frame connects the two donors. Here T_A and M_E denote the two-photon absorption tensor and the one-photon emission transition moment tensor, respectively.

Figure 2. (A) Simulated anisotropy curve (solid line), instrument response function ($I(t)$, solid line sharp peak) and photophysics ($F(t)$) curve (dashed line). Here $I(t)$ and $F(t)$ curves were normalised to maximum anisotropy value. (B) The polarised fluorescence intensity decays $F_{\parallel}(t)$ and $F_{\perp}(t)$ obtained from the data shown in (A) by using a transformation according to Eq. A1.6 and by a convolution with the instrumental response function. The decays were normalised to give value $F_{\parallel, \text{peak}} = 5 \cdot 10^4$ counts. The decays indicated by points include Poissonian noise, which is inherent in TCSPC experiments.

Figure 3. (A) Real (solid line) and imaginary (dashed line) parts of Fourier transform of $F_{\parallel}(t)$ ($\mathcal{F}(F_{\parallel}(t))$) after normalisation (to $F_{\parallel, \text{peak}} = 5 \cdot 10^4$ counts) and convolution with the instrumental response function, $I(t)$. Insert display the initial frequency region, which is indicated by dotted vertical line. (B) Magnitudes of $\mathcal{F}(F_{\parallel}(t))$: before convolution with the $I(t)$ (dashed line), after convolution with $I(t)$ (solid line) and with Poissonian noise added (dots).

Figure 4. Simulated OPE (A) and TPE (B) anisotropy curves in absence of EM (black dots) and in presence of EM (red dots). The maps C and D show the results obtained by using a GA in fitting the configuration (β_{DR}, δ) and the distance (R) parameters to the independent OPE and TPE depolarisation data. The red lines indicate values of the configuration parameters used for synthesising depolarisation data that mimic TCSPC-experiments.