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RESEARCH PAPER

Efficiency of Resonance Energy Transfer in Homo-Oligomeric Complexes of Proteins

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Abstract A theoretical model is proposed for the apparent efficiency of fluorescence (Förster) resonance energy transfer (FRET) in mixtures of free monomers and homo-oligomeric protein complexes of uniform size. The model takes into account possible pathways for transfer of optical excitations from single donors to multiple acceptors and from multiple donors (non-simultaneously) to single acceptors. This necessary departure from the standard theory has been suggested in the literature, but it has only been successfully implemented for a few particular cases, such as for particular geometries of the oligomers. The predictions of the present theoretical model differ significantly from those of the standard theory, with the exception of the case of dimers, for which agreement is observed. This model therefore provides new insights into the FRET behavior of oligomers comprising more than two monomers, and also suggests means for determining the size of oligomeric protein complexes as well as the proportion of associated and unassociated monomers.

Keywords Förster (fluorescence) resonance energy transfer · FRET · Fluorescence theory · Protein – protein interaction · Protein association · Protein self-association · Interaction stoichiometry

1 Introduction

Many biological processes rely on and are regulated by protein–protein interactions. For instance, protein–protein interactions play essential roles in signal transduction pathways [1], while many higher cognitive functions of the brain such as learning and memory are believed to be encoded by changes in synapses between neuronal axons and dendrites [2]. Also, a large class of membrane receptor proteins, called G-protein-coupled receptors

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(GPCRs) [3, 4], are involved in cellular signaling in various organisms, and are often used as targets for drugs. The mechanisms underlying protein–protein interactions as well as their kinetics are varied and often poorly understood.

Fully quantitative studies of protein–protein interactions in vivo have emerged in recent years, aided by combinations of protein tagging strategies involving biologically synthesizable probes (e.g., GFP) [5, 6] and refinements in the characterization and use of a short-range process of non-radiative transfer of optical excitations, called Förster (or fluorescence) resonance energy transfer (FRET) [7–9], as a sensor of proximity. The mechanism underlying FRET is well understood. When a fluorescent molecule lies within a few nanometers from an optically excited molecule, the excitation energy of the former can be transferred to the latter nonradiatively. The first molecule is called an "acceptor" of energy (A), while the second molecule is a "donor" (D). By using FRET, it is possible to study protein association in living cells [10–22]. Two avenues have been usually explored in FRET studies in living cells, as follows. (Note that much of the following terminology will be introduced more rigorously in the next section.)

(1) *Estimation of the intermolecular distances within a protein complex.* This is based on knowledge of the efficiency of energy transfer, E, and of the Förster distance, R_0, (defined as the distance at which E falls to half of its maximum value) [8, 11]. E can be conveniently determined from fluorescence lifetime measurements [7, 23], whose results are independent of local concentrations of A and D molecules, and also from intensity-based measurements [21], which are concentration dependent.
(2) *Evaluation of protein interaction stoichiometry.* The proportion of interacting proteins and the size of the oligomers formed by them can be determined, in principle, using intensity-based FRET methods, which are uniquely suited to detect concentrations of fluorescent molecules. It has been possible to determine certain indices that are proportional to the concentration of interacting and noninteracting molecules [11, 14, 20], the ratio between donor-tagged to acceptor-tagged molecules [24] or the fraction of interacting molecules out of a total population of molecules [21].

Studies concerning the determination of oligomer size from FRET measurements have advanced steadily over the past few decades. Due to the complexity of the problem, however, theoretical models must resort to certain approximations. For instance, Adair and Engelman (AE) [25] proposed a simple model for the apparent FRET efficiency in ensembles of homo-oligomeric protein complexes of uniform size, which was inspired by a site-directed cross-linking method used by Milligan and Koshland [26] for establishing the dimeric nature of the aspartate chemoreceptor. Raicu et al. [21] have extended the AE model to include free monomers, and then used an approximate form of this model to determine the fraction of oligomers of a G-protein coupled receptor, the Sterile 2 α factor in yeast, and the average number of monomers, n, in an oligomer (which turned out to be equal to 2). This theory makes the simplifying assumption that the energy transfer efficiency is the same for all donor-acceptor pairs, and that transfer of energy always occurs from single acceptors to single donors, for any oligomer size. Alternative models have considered possible energy transfer between more than one acceptor and one neighboring donor, but assumed that FRET efficiency to distant monomers is negligible [27].

In oligomeric complexes other than dimers, the number of pathways for de-excitation of donors through FRET is greater than one and depends on the number of donors and acceptors in the complex. Conversely, acceptors can be excited by several donors through *quasi-parallel* processes, in which the excitation of each donor can be transferred with a non-zero probability to the same acceptor. Note that truly parallel (i.e., simultaneous)

transfer of excitation from two or more donors to an acceptor or from one donor to two or more acceptors is forbidden by quantum mechanics. Multimeric complexes can also be tagged so as to transfer the energy *serially* from one molecule to the second, from there to the third, and so on.

Recent studies of multiple-pathway FRET included: distance measurements between one or more donors and multiple identical acceptors [28–31], photobleaching kinetics of *D–A* complexes involving five FRET steps (i.e., serial FRET) [32], and FRET efficiency determination (in a quasi-parallel process) for an oligomer with a symmetrical ring structure – the pentamer of phospholamban [11]. In oligomers with varying proportions of Ds and As, the existence of multiple quasi-parallel pathways for excitation/de-excitation can lead to FRET efficiencies that are different from those of dimers, in which a one-to-one correspondence exists between D and A. Thus, the "true" FRET efficiency in multimeric complexes differs from the "true" FRET efficiency of a single *D–A* pair, which we will also call the "pair-wise" FRET efficiency hereafter. When FRET-productive oligomers are mixed with free monomers or with oligomers that contain only As or only Ds (which are not FRET-productive), the term "apparent" FRET efficiency is used instead.

Here we introduce a general theoretical model for the apparent FRET efficiency in mixtures of multimeric complexes. The theory incorporates multiple pathways of energy transfer between donors and acceptors situated at arbitrary distances from one another within the complex. The protein complexes are assumed to play functional roles in the cell, and therefore to be stable over the entire period of time necessary for FRET measurements. Possible contributions to FRET due to random encounters between acceptors and donors at high concentrations are not considered at this stage [33, 34].

Our theoretical model provides new insights into the FRET behavior of oligomers with $n>2$, and suggests means for determination of the protein interaction stoichiometry in vivo. Special cases of the proposed theory are analyzed analytically and numerically, and are compared to the standard model.

2 FRET Efficiency for Dimeric Complexes

To introduce the general terms and concepts, in this section we consider the simpler case of a population of proteins that form stable homo-dimeric complexes. Fluorescent tags, which can act as acceptors (A) and donors (D) of energy through FRET, are attached to each of the proteins of interest. The aim is to relate the apparent FRET efficiency of a mixture of free monomers and homo-dimers to the spectroscopic properties of D and A (e.g., quantum yields in the presence and absence of FRET), on one hand, and to experimentally measurable parameters (e.g., fluorescence intensities or lifetimes), on the other hand. Some of the equations obtained in this section will be used in the next section in deriving the apparent FRET efficiency for the case of multimeric complexes. Throughout this paper, FRET is defined as the nonradiative transfer of energy from excited donors to unexcited acceptors, and no donor–donor transfer (or homo-FRET) is taken into account.

To begin with, we define the quantum yields of acceptors and donors as the rate of emission of photons following excitation, namely:

$$Q^D = \frac{\Gamma^{r,D}}{\Gamma^{r,D} + \Gamma^{nr,D}}, \text{ and} \tag{1a}$$

$$Q^A = \frac{\Gamma^{r,A}}{\Gamma^{r,A} + \Gamma^{nr,A}}, \tag{1b}$$

where $\Gamma^{r,X}$ and $\Gamma^{nr,X}$ (X=D, A) are the rate constants for de-excitation through radiative (i.e., photons) and nonradiative (e.g., internal conversion) processes, and $\left(\Gamma^{r,X} + \Gamma^{nr,X}\right)^{-1} = \tau_X$ is the lifetime of the excited state of X (or the fluorescence lifetime).

When conditions exist for FRET, the additional pathways for donor de-excitation (in addition to radiative and nonradiative de-excitation) lead to a different quantum yield for the donor, which is given by:

$$Q^{DA} = \frac{\Gamma^{r,D}}{\Gamma^{r,D} + \Gamma^{nr,D} + \Gamma^{FRET}}, \tag{2}$$

where $\left(\Gamma^{r,D} + \Gamma^{nr,D} + \Gamma^{FRET}\right)^{-1} = \tau_{DA}$ is the fluorescence lifetime of D in the presence of A (or FRET). If the mechanism of energy transfer is of a dipolar or Förster type, the rate constant of nonradiative transfer from D to A is given by $\Gamma^{FRET} = \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)(R_0/r)^6$ where r is the D–A separation, and R_0 is the well-known Förster distance [7–9]. On the other hand, the quantum yield of the acceptor remains unchanged, since FRET only introduces a new pathway for de-excitation of the donor, which only affects the excitation of the acceptor.

The proportion of photons dissipated through FRET by the excited donor, called the FRET efficiency, is:

$$E = \frac{\Gamma^{FRET}}{\Gamma^{r,D} + \Gamma^{nr,D} + \Gamma^{FRET}} = 1 - \frac{\tau_{DA}}{\tau_D} = \frac{R_0^6}{R_0^6 + r^6}. \tag{3}$$

The extra term in the sum of de-excitation rate constants (in the denominator) modifies the lifetime of the donor such that $\tau_{DA}<\tau_D$, while τ_A remains unchanged. The second part of (3) provides a sensitive means for detecting FRET from *fluorescence lifetime measurements*, while the third relates the FRET efficiency to the distance between D and A.

By combining (1a) with (2) and (3), we obtain a relation,

$$Q^{DA} = Q^D(1 - E), \tag{4}$$

which indicates that the donor emission is reduced through FRET. This reduction, known as *donor quenching*, can be used to quantify the interaction between D and A using measurements of donor fluorescence intensity in the presence and absence of acceptor (in fact, it is donor de-quenching that is easier to realize practically – see below).

Finally, the excitation rate constants of A and D in the absence of FRET are, respectively [35]:

$$\Gamma^{ex,A} = I_0(\lambda_{ex})/(hcN_A)\varepsilon^A(\lambda_{ex}), \text{and} \tag{5a}$$

$$\Gamma^{ex,D} = I_0(\lambda_{ex})/(hcN_A)\varepsilon^D(\lambda_{ex}), \tag{5b}$$

where $I_0(\lambda_{ex})$, $\varepsilon^A(\lambda_{ex})$ and $\varepsilon^D(\lambda_{ex})$ are the intensity of the incident radiation and the absorption cross-sections at excitation wavelength λ_{ex}, h is Planck's constant, c is the speed of light, and N_A is Avogadro's number. In the presence of FRET, the excitation rate constant of the donor remains unchanged, while the excitation rate constant of the acceptor increases according to:

$$\Gamma^{ex,AD} = \Gamma^{ex,A} + \Gamma^{ex,D}E. \tag{6}$$

This increased acceptor excitation rate, called *acceptor sensitized emission*, can be used to detect FRET from acceptor emission intensity measurements.

When two populations of D and A molecules that can form dimeric complexes are mixed together, several species of dimers may be formed: AA, AD, DD as well as free A and D monomers, each with their own excitation and emission efficiencies. Using the above definitions and relations, one can write the expressions for emission intensities of such mixtures as:

$$\begin{aligned} F^{DA}(\lambda_{ex}) &= \Gamma^{ex,D}\left\{[D]Q^D + [D]_D Q^D + [D]_A Q^{DA}\right\} \\ &= \Gamma^{ex,D}[D]_T Q^D - \Gamma^{ex,D}[D]_A Q^D E, \text{and} \end{aligned} \tag{7a}$$

$$\begin{aligned} F^{AD}(\lambda_{ex}) &= \Gamma^{ex,A}\left\{[A]Q^A + [A]_A Q^A\right\} + \Gamma^{ex,AD}[A]_D Q^A \\ &= \Gamma^{ex,A}[A]_T Q^A + \Gamma^{ex,D}[A]_D Q^A E, \end{aligned} \tag{7b}$$

where $[D]$ and $[A]$ are the concentrations of free D and A molecules, $[D]_A$ is the concentration of D molecules that form complexes with A molecules, $[A]_D$ is the concentration of A molecules that form complexes with D molecules, $[D]_D$ is the concentration of D in D-only complexes, $[A]_A$ is the concentration of A in A-only complexes, while $[D]_T = [D] + [D]_D + [D]_A$ and $[A]_T = [A] + [A]_A + [A]_D$ are the total concentrations of D and A molecules.

By introducing a set of convenient notations,

$$F^D(\lambda_{ex}) = \Gamma^{ex,D}[D]_T Q^D, \tag{8a}$$

$$F^A(\lambda_{ex}) = \Gamma^{ex,A}[A]_T Q^A, \tag{8b}$$

$$F^D(FRET) = \Gamma^{ex,D}[D]_A Q^D E, \text{and} \tag{8c}$$

$$F^A(FRET) = \Gamma^{ex,D}[A]_D Q^A E, \tag{8d}$$

equations (7a) and (7b) can be recast as:

$$F^{DA}(\lambda_{ex}) = F^D(\lambda_{ex}) - F^D(FRET), \text{and} \tag{9a}$$

$$F^{AD}(\lambda_{ex}) = F^A(\lambda_{ex}) + F^A(FRET). \tag{9b}$$

Two similarly defined but quantitatively different apparent FRET efficiencies can be introduced:

$$E_{app}^{Dq} \equiv \frac{F^D(FRET)}{F^D(\lambda_{ex})}, \text{and} \tag{10a}$$

$$E_{app}^{Ase} \equiv \frac{F^A(FRET)}{F^A(\lambda_{ex})} \tag{10b}$$

where the superscripts "*Dq*" and "*Ase*" stand for "donor quenching" and "acceptor sensitized emission." By using (9a) and (9b) to solve for F^D(FRET) and F^A(FRET), (10a) and (10b) can be rewritten in terms of only the experimentally measurable parameters F^{DA}, F^D, F^{AD} and F^A, as:

$$E_{app}^{Dq} = 1 - \frac{F^{DA}(\lambda_{ex})}{F^{D}(\lambda_{ex})}, \text{and} \tag{11a}$$

$$E_{app}^{Ase} = \frac{F^{AD}(\lambda_{ex})}{F^{A}(\lambda_{ex})} - 1. \tag{11b}$$

We note that, while $F^{DA}(\lambda_{ex})$ and $F^{AD}(\lambda_{ex})$ can be easily determined experimentally by measuring the fluorescence intensity of the system of interest containing both species, determinations of $F^D(\lambda_{ex})$ and $F^A(\lambda_{ex})$ require further consideration. First, although it is rarely possible to physically separate a natural oligomer into donor-tagged and acceptor-tagged components and thus to determine the fluorescence intensity of the donor in the absence of energy transfer, it is almost always possible to inactivate the acceptor through, e.g., photobleaching [35, 36], and then to measure $F^D(\lambda_{ex})$. Secondly, it is usually possible to excite the acceptor at a wavelength $\lambda \neq \lambda_{ex}$ at which the donor is not excited. Then, by knowing the excitation spectrum of the acceptor in the absence of the donor, it is possible to infer the level of acceptor emission upon excitation at λ_{ex} in the absence of FRET, $F^A(\lambda_{ex})$.

Further, by using (8a), (8b), (8c) and (8d) to relate the fluorescence intensities to the properties of the four fluorescent species (DD, DA, AD, AA), the apparent FRET efficiencies for dimers [(10a) and (10b)] become:

$$E_{app}^{Dq} = \alpha_D E, \text{and} \tag{12a}$$

$$E_{app}^{Ase} = \alpha_A \frac{\varepsilon^D}{\varepsilon^A} E, \tag{12b}$$

where $\alpha_D = [D]_A / [D]_T$ and $\alpha_A = [A]_D / [A]_T$.

Equations (11a) and (11b) permit the determination of the apparent FRET efficiencies from measurable parameters in both sensitized emission and donor de-quenching experiments, while (12a) and (12b), together with knowledge of the true FRET efficiency, E, give the possibility to determine the stoichiometry of the protein complexes through determination of the fractions of interacting donors, α_D, and acceptors, α_A, in the case of dimeric complexes [21, 25].

A complete theory of FRET should permit determination of apparent FRET efficiency for any size of the oligomer (in terms of the number of monomers, n). We will introduce such a theory in the next section.

3 Ensembles of Multimeric Complexes of Uniform Size

In this section we will derive general expressions for the apparent FRET efficiency for mixtures of free monomers and multimeric complexes of uniform size. Then, we will relate them to pair-wise FRET efficiency, as defined above for dimers [(3)]. We assume that donor and acceptor excitations are rare occurrences, i.e., there always is a single D or A in an

excited state in a complex). We will first consider arbitrary distances between acceptors and donors within complexes (for which Förster mechanism is still valid), and will then investigate the particular case of equal distances between every donor-acceptor pair in the complex; the latter approximation can be rigorously correct for dimers and trimers (in the case of membrane proteins), and should provide a good approximation for larger oligomers, as long as the distances between all acceptors and donors are of the same order of magnitude as their Förster distance. For three-dimensional oligomers, this approximation might hold for even larger numbers of monomers in the complex.

3.1 The General Case: Arbitrary *D–A* Distances

To relate the fluorescence intensities to the spectral properties of the fluorescent molecules, one needs to refine the definitions of FRET efficiency and quantum yield of donors in complexes by taking into account that, for an oligomer of size n, there are different possible configurations, q (see Fig. 1), k ways (equal to the number of donors) for the initial excitation energy of D to be lost through emission of photons or internal conversion, and $n–k$ ways (equal to the number of acceptors) for losing excitation energy through FRET (see Fig. 2). All donors are identical, and therefore all have the same rate constants of excitation, $\Gamma^{ex,D}$, as well as radiative, $\Gamma^{r,D}$, and nonradiative, $\Gamma^{nr,D}$, de-excitation; the same is true of the acceptors. The quantum yield of the i-th donor in a complex having a particular configuration, q, is therefore:

$$Q^{DA}_{i,k,n,q} = \frac{Q^D}{1 + \sum_{j=1}^{n-k} \Gamma^{FRET}_{i,j,q} \Big/ \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)}, \tag{13}$$

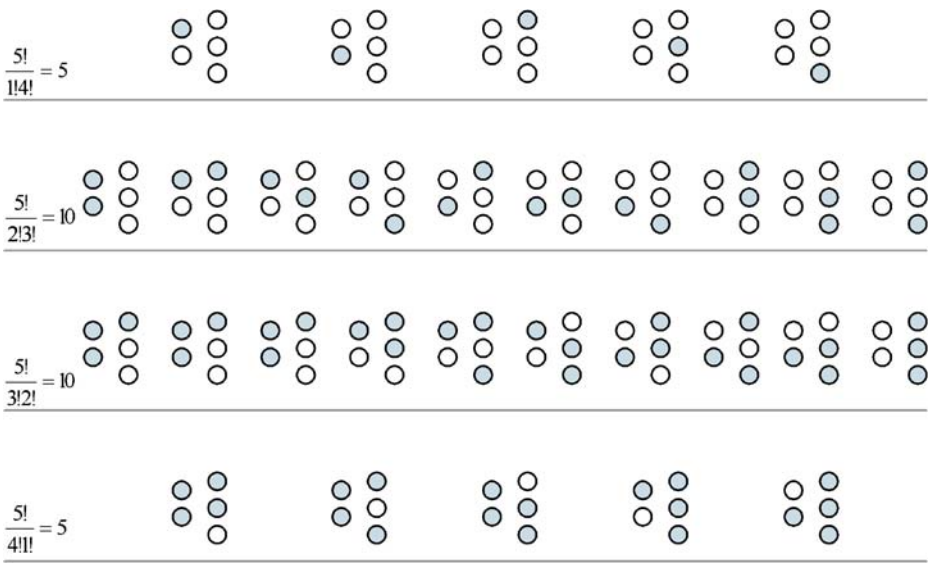

Fig. 1 All possible configurations assumed by donors and acceptors within an oligomer (a pentamer in this example)

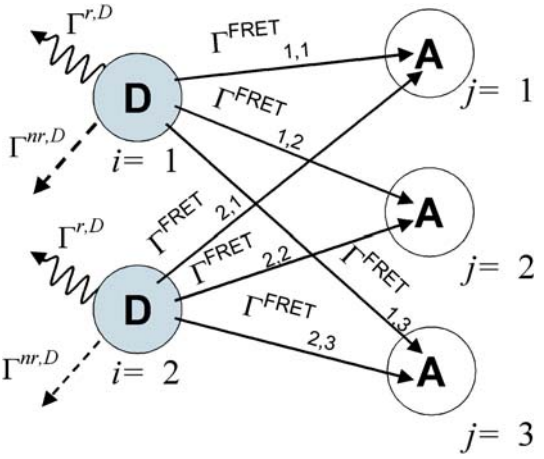

Fig. 2 Schematic diagram showing the various pathways through which excited donors in a pentamer (with two donors and three acceptors) can lose energy. Energy flows are indicated by *arrows*, together with their respective rate constants (see text for definition of symbols). This picture can be easily extended to incorporate oligomers of any size. *Solid*, *dash* and *wavy lines* represent respectively FRET, non-radiative de-excitation and radiative de-excitation

where j is a summation index for acceptors, and $\Gamma^{FRET}_{i,j,q} = \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)\left(R^0_{i,j,q} \Big/ r_{i,j,q}\right)^6$ is the rate constant for FRET between single pairs of D and A. Note that, in the general case considered above, the Förster radius depends on the *D–A* pair, due to different orientation factors [7, 8]. The FRET efficiency reads:

$$E_{i,k,n,q} = \sum_{j=1}^{n-k} \frac{\Gamma^{FRET}_{i,j,q} \Big/ \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)}{1 + \sum_{j=1}^{n-k} \Gamma^{FRET}_{i,j,q} \Big/ \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)} \equiv \sum_{j=1}^{n-k} E_{i,j,q}, \tag{14}$$

where the notation $E_{i,j,q} = \frac{\Gamma^{FRET}_{i,j,q} \Big/ \left(\Gamma^{r,D}+\Gamma^{nr,D}\right)}{1+\sum_{j=1}^{n-k} \Gamma^{FRET}_{i,j,q} \Big/ \left(\Gamma^{r,D}+\Gamma^{nr,D}\right)} = \frac{\left(R^0_{i,j,q} \Big/ r_{i,j,q}\right)^6}{1+\sum_{j=1}^{n-k} \left(R^0_{i,j,q} \Big/ r^0_{i,j,q}\right)^6}$ has been used.

Substituting for $\sum_{j=1}^{n-k} \Gamma^{FRET}_{i,j,q} \Big/ \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)$ between (13) and (14) an equation for the quantum yield of D is obtained,

$$Q^{DA}_{i,k,n,q} = Q^D\left(1 - E_{i,k,n,q}\right), \tag{15}$$

which is similar to (4). The quantum yield of each acceptor in complexes with donors remains the same as in the absence of FRET, while the excitation rate constant is modified, to take into account excitation through FRET, as:

$$\Gamma^{ex,AD}_{j,k,q} = \Gamma^{ex,A} + \Gamma^{ex,D} \sum_{i=1}^{k} E_{i,j,q}, \tag{16}$$

where the sum with respect to i includes the pair-wise FRET efficiency between each donor and the acceptor j.

The emission intensities of donors, $F_n^{DA}(\lambda_{ex})$, and acceptors, $F_n^{AD}(\lambda_{ex})$, for a mixture of oligomers of size n and free D and A monomers excited with a wavelength λ_{ex} can be immediately written, by analogy to (7a) and (7b), as:

$$F_n^{DA}(\lambda_{ex}) = \Gamma^{ex,D}\left\{[D]Q^D + \mu_{oligo}\sum_{k=1}^{n}\sum_{config,q} P_D^k P_A^{n-k}\sum_{i=1}^{k} Q_{i,k,n,q}^{DA}\right\}, \tag{17a}$$

$$F_n^{AD}(\lambda_{ex}) = Q^A\left\{\Gamma^{ex,A}[A] + \mu_{oligo}\sum_{k=0}^{n-1}\sum_{config,q} P_D^k P_A^{n-k}\sum_{j=1}^{n-k} \Gamma_{j,k,q}^{ex,AD}\right\}, \tag{17b}$$

where the summation over k takes into account mixed oligomers, i.e., oligomers that contain at least $k=1$ and at most $k=n-1$ donors (with $k=0$ corresponding to acceptors-only complexes and $k=n$ corresponding to donors-only); μ_{oligo} is the total concentration of oligomers, and P_D and P_A are the fractions of donor and acceptor concentrations in oligomers, as given respectively by $\{[D]_D + [D]_A\}/\{[D]_D + [D]_A + [A]_A + [A]_D\}$ and $\{[A]_A + [A]_D\}/\{[D]_D + [D]_A + [A]_A + [A]_D\}$. Note that, after replacing $\sum_{config,q}$ with $\binom{n}{k} = \frac{n!}{k!(n-k)!}$, $[D]_T$ and $[A]_T$ may be expressed in terms of the binomial distribution, as:

$$[D]_T \equiv [D] + [D]_A + [D]_D = [D] + \mu_{oligo} n P_D = [D] + \mu_{oligo}\sum_{k=1}^{n} k\binom{n}{k} P_D^k P_A^{n-k}, \text{and} \tag{18a}$$

$$[A]_T \equiv [A] + [A]_D + [A]_A = [A] + \mu_{oligo} n P_A = [A] + \mu_{oligo}\sum_{k=0}^{n-1} (n-k)\binom{n}{k} P_D^k P_A^{n-k}. \tag{18b}$$

With these equations, and by taking (13) and (16) into account, (17a) and (17b) become:

$$F_n^{DA}(\lambda_{ex}) = \Gamma^{ex,D} Q^D\left\{[D]_T - \mu_{oligo}\sum_{k=1}^{n-1}\sum_{config,q} P_D^k P_A^{n-k}\sum_{i=1}^{k} E_{i,k,n,q}\right\}, \tag{19a}$$

$$F_n^{AD}(\lambda_{ex}) = Q^A\left\{\Gamma^{ex,A}[A]_T + \Gamma^{ex,D}\mu_{oligo}\sum_{k=1}^{n-1}\sum_{config,q} P_D^k P_A^{n-k}\sum_{i=1}^{k} E_{i,k,n,q}\right\}. \tag{19b}$$

Introducing the notations

$$F^D(\lambda_{ex}) = \Gamma^{ex,D} Q^D\left\{[D] + \mu_{oligo} n P_D\right\} = \Gamma^{ex,D} Q^D [D]_T, \tag{20a}$$

$$F^A(\lambda_{ex}) = \Gamma^{ex,A} Q^A\left\{[A] + \mu_{oligo} n P_A\right\} = \Gamma^{ex,A} Q^A [A]_T, \tag{20b}$$

$$F^D(FRET) = \Gamma^{ex,D} Q^D \mu_{oligo}\sum_{k=1}^{n-1}\sum_{config,q} P_D^k P_A^{n-k}\sum_{i=1}^{k} E_{i,k,n,q}, \text{and} \tag{20c}$$

$$F^A(FRET) = \Gamma^{ex,D} Q^A \mu_{oligo} \sum_{k=1}^{n-1} \sum_{config,q} P_D^k P_A^{n-k} \sum_{i=1}^{k} E_{i,k,n,q}, \tag{20d}$$

and using again the definitions of the apparent FRET efficiencies given by (10a) and (10b), the following equations are obtained:

$$E_{app}^{Dq} = \frac{\mu_{oligo} \sum_{k=1}^{n-1} \sum_{config,q} P_D^k P_A^{n-k} \sum_{i=1}^{k} E_{i,k,n,q}}{[D] + \mu_{oligo} n P_D} = \frac{\mu_{oligo}}{[D]_T} \sum_{k=1}^{n-1} \sum_{config,q} P_D^k P_A^{n-k} \sum_{i=1}^{k} E_{i,k,n,q}, \tag{21a}$$

$$E_{app}^{Ase} = \frac{\mu_{oligo} \sum_{k=1}^{n-1} \sum_{config,q} P_D^k P_A^{n-k} \sum_{i=1}^{k} E_{i,k,n,q}}{[A] + \mu_{oligo} n P_A} \frac{\varepsilon^D}{\varepsilon^A} = \frac{\mu_{oligo}}{[A]_T} \frac{\varepsilon^D}{\varepsilon^A} \sum_{k=1}^{n-1} \sum_{config,q} P_D^k P_A^{n-k} \sum_{i=1}^{k} E_{i,k,n,q}. \tag{21b}$$

For large numbers of monomers in complexes, it is increasingly difficult to explicitly write down all the terms of the sums in (21a) and (21b). A similar difficulty may arise if nothing is known about the relative disposition of monomers in complexes, which is often the case with newly investigated oligomeric systems. In both of these cases, it is useful to make reasonable assumptions regarding FRET. Previous works have considered either equal distances between *D–A* pairs within the complex [21, 25] or that no FRET occurs between distant neighbors [27]. Since choosing the threshold for k above which no significant FRET occurs requires some knowledge of the particular geometry of the oligomer, we will take the latter approximation no further in this paper. However, some insight can be gained by looking closely at the first type of approximation, which we will discuss next.

3.2 Approximations for Equal *D–A* Distances

If the distances between all donor-acceptor pairs within the complex can be approximated as equal, then $\Gamma_{i,j,q}^{FRET} \equiv \Gamma^{FRET}$ for any *D–A* FRET pair. The quantum yield and the FRET efficiencies for each oligomer can be related to the FRET efficiency of a single *D–A* pair (i.e., the true FRET efficiency, E, for dimers). By solving for $\Gamma^{FRET} / \left(\Gamma^{r,D} + \Gamma^{nr,D}\right)$ from (3) and substituting into (14), $E_{i,k,n,q}$ and $Q_{i,k,n,q}^{DA}$ can be related, for all configurations, q (which become indistinguishable), and donors, i, to the FRET efficiency of a single *D–A* pair, E, as:

$$E_{i,k,n} = \frac{(n-k)E}{1 + (n-k-1)E}, \tag{22a}$$

$$Q_{i,k,n}^{DA} = Q^D \left(1 - E_{i,k,n}\right). \tag{22b}$$

which lead to significant simplifications of the notations (20a), (20b), (20c), (20d):

$$F^D(\lambda_{ex}) = \Gamma^{ex,D} Q^D \left\{ [D] + \mu_{oligo} n P_D \right\} = \Gamma^{ex,D} Q^D [D]_T, \tag{23a}$$

$$F^A(\lambda_{ex}) = \Gamma^{ex,A} Q^A \left\{ [A] + \mu_{oligo} n P_A \right\} = \Gamma^{ex,A} Q^A [A]_T, \tag{23b}$$

$$F^D(FRET) = \Gamma^{ex,D} Q^D \mu_{oligo} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}, \tag{23c}$$

$$F^A(FRET) = \Gamma^{ex,D} Q^A \mu_{oligo} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}. \tag{23d}$$

In this case, the apparent FRET efficiencies [(21a) and (21b)] become:

$$\begin{aligned} E_{app}^{Dq} &= \frac{\mu_{oligo} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}}{[D] + \mu_{oligo} n P_D} \\ &= \frac{\mu_{oligo}}{[D]_T} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}, \end{aligned} \tag{24a}$$

$$E_{app}^{Ase} = \frac{\mu_{oligo} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}}{[A] + \mu_{oligo} n P_A} \frac{\varepsilon^D}{\varepsilon^A}$$

$$= \frac{\mu_{oligo}}{[A]_T} \frac{\varepsilon^D}{\varepsilon^A} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}. \tag{24b}$$

Further, if *no free monomers* are present in the system, (24a) and (24b) become:

$$E_{app}^{Dq} = \frac{\sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}}{n P_D}, \text{and} \tag{25a}$$

$$E_{app}^{Ase} = \frac{\sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}}{n P_A} \frac{\varepsilon^D}{\varepsilon^A}. \tag{25b}$$

3.3 Comparison with Other Models in the Literature

Equations (24a) and (24b) and (25a) and (25b) differ markedly from (12a) and (12b) – and, thereby, from other results in the literature – in that E and the summation over k in the

numerator cannot be decoupled. For large E values (i.e., $E \cong 1$), $1+(n-k-1)E$ approaches $(n-k)$ and (24a) and (24b) become:

$$E_{app}^{Dq} \cong \frac{\mu_{oligo} \sum_{k=1}^{n-1} k \binom{n}{k} P_D^k P_A^{n-k}}{[D] + \mu_{oligo} n P_D} E = \frac{\mu_{oligo} n P_D \left(1 - P_D^{n-1}\right)}{[D] + \mu_{oligo} n P_D} E \equiv \alpha_D E, \tag{26a}$$

$$E_{app}^{Ase} \cong \frac{\mu_{oligo} \sum_{k=1}^{n-1} k \binom{n}{k} P_D^k P_A^{n-k}}{[A] + \mu_{oligo} n P_A} \frac{\varepsilon^D}{\varepsilon^A} E = \frac{\mu_{oligo} n P_D \left(1 - P_D^{n-1}\right)}{[A] + \mu_{oligo} n P_A} \frac{\varepsilon^D}{\varepsilon^A} E \equiv \alpha_A \frac{\varepsilon^D}{\varepsilon^A} E, \tag{26b}$$

where α_D and α_A are the fractions of the total population of donors and acceptors that form oligomeric complexes. Notably, (26a) is identical to the equation obtained by Raicu et al. [21], and reduces further, for $[D]=0$, to the result obtained by Adair and Engelman [25], whereas (26b) differs significantly from the previous model [21].

If the two possible states of the D and A molecules are free monomers and dimers (i.e., $n=2$), then the results previously described in the literature are recovered exactly from (24a) and (24b):

$$E_{app}^{Dq} = \frac{2\mu_{oligo} P_D P_A}{[D] + 2\mu_{oligo} P_D} E \equiv \alpha_D E, \tag{27a}$$

$$E_{app}^{Ase} = \frac{2\mu_{oligo} P_D P_A}{[D] + 2\mu_{oligo} P_A} \frac{\varepsilon^D}{\varepsilon^A} E \equiv \alpha_A \frac{\varepsilon^D}{\varepsilon^A} E, \tag{27b}$$

where α_D and α_A are now identical to those in (12a) and (12b) for dimers. Finally, if there are no free monomers in the system:

$$E_{app}^{Dq} = (1 - P_D) E \equiv \alpha_D E, \text{and} \tag{28a}$$

$$E_{app}^{Ase} = P_D \frac{\varepsilon_D}{\varepsilon_A} E \equiv \alpha_A \frac{\varepsilon_D}{\varepsilon_A} E. \tag{28b}$$

It appears from the above derivations that exact decoupling between E and the fraction of interacting donors (acceptors) to give $E_{app}^{Dq} = \alpha_D E$ (or $E_{app}^{Ase} = \alpha_A E \varepsilon^D / \varepsilon^A$) is only possible for the case of dimers. Previously, we have employed identities of this type for investigating the homo-oligomerization of a G-protein coupled receptor [21], and obtained, among other results, the size of the oligomer ($n=2$). Use of this identity is widespread in the literature. It is now obvious that caution needs to be exercised when using such approximate forms for FRET efficiency. We will discuss this aspect further in the next section.

4 Numerical Results

To illustrate the degree of improvement over existing theories that the current theory provides, in Fig. 3 we plotted the approximate forms of the expressions derived above for

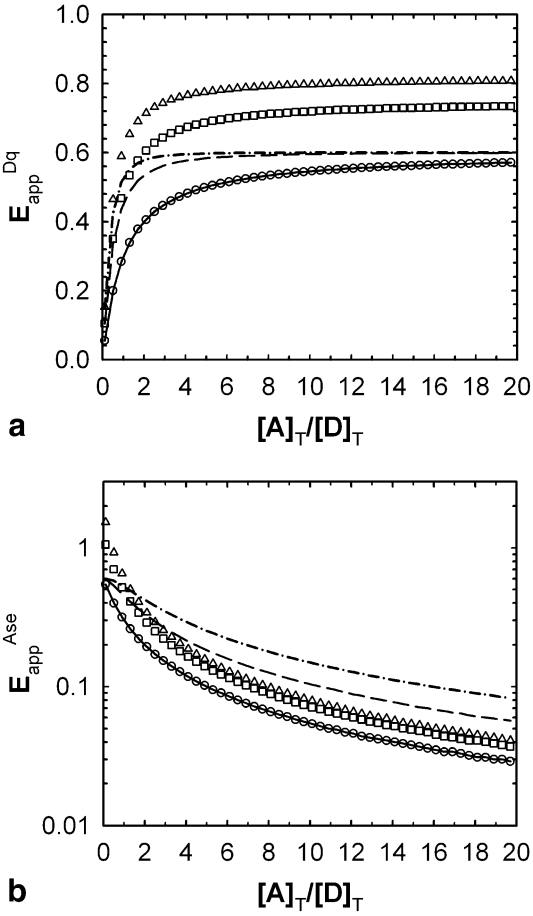

Fig. 3 Apparent FRET efficiencies vs. $[A]_T/[D]_T$ concentration ratio, as determined from (**a**) donor quenching (superscript "Dq") and (**b**) acceptor sensitized emission for oligomers (superscript "Ase") of uniform size in the absence of free monomers. *Points*, predictions by the present theory [(25a) and (25b) with E=0.6]: *circles* – dimers (n=2); *squares* – trimers (n=3); *triangles* – tetramers (n=4). *Lines*, predictions by the model of Adair and Engelman [25] for donor quenching (**a** only), and by a similar theory proposed by Raicu et al. [21] for acceptor sensitized emission (**b**): *solid lines* – dimers; *dashed lines* – trimers; *dash-dot lines* – tetramers. Notice that current and previous theories only agree for the simple case of dimers

the apparent FRET efficiencies of systems containing only oligomers [(25a) and (25b)] against the ratio of acceptor to donor concentrations, $[A]_T/[D]_T$, together with simulations using the existing models for E_{app}^{Dq} [25] and E_{app}^{Ase} [21]. In plotting these expressions, the ratio of the extinction coefficients in (25b) was omitted for simplicity, and the following expressions were used for the probability of donors and acceptors to be found in an oligomer: $P_D = \{[D]_D + [D]_A\}/\{[D]_D + [D]_A + [A]_A + [A]_D\}$ and $P_A = \{[A]_A + [A]_D\}/\{[D]_D + [D]_A + [A]_A + [A]_D\}$. Three cases were investigated: dimers (n=2), trimers (n=3) and tetramers (n=4).

As expected, Fig. 3 shows perfect agreement between the present model and the previous models in the case of dimers. Furthermore, both the published and the present theory account for the general features of the FRET dependence on the A/D ratio for any oligomer size: the apparent efficiency sensed by donors increases with the acceptor-donor concentration ratio, while the efficiency sensed by acceptors presents the opposite tendency. This is because addition of acceptors to a fixed population of donors increases the probability for the donors to lose their excitation through FRET, leading to higher transfer efficiency as sensed by each donor. On the other hand, an increased number of acceptors within complexes leads to a decreased probability of excitation for each of them, hence the lower efficiency sensed by each acceptor.

However, for all practical values of the pair-wise FRET efficiency (typically, ~0.1–0.7), large discrepancies are observed between the two theoretical models for oligomers of size larger than two. The present theory takes into account the transfer of excitation from multiple donors to single acceptors and from single donors to multiple acceptors. For that reason, although explicitly introduced in (22a) and (22b) through (25a) and (25b) for comparison to the classical theory, the pair-wise FRET efficiency, E, is not identical to the *true* FRET efficiency of an oligomer that contains both acceptors and donors (given by Eq. 22a). The latter is instead obtained as a function of the former, and is generally greater than E (except for dimers, when it is equal to E), but always subunitary. By contrast, previous modeling attempts relied on calculating the average number of *D–A* pairs in each complex and then multiplying it by the pair-wise FRET efficiency to calculate E_{app}^{Dq} and E_{app}^{Ase} [21, 25]. Therefore, for large oligomers with small numbers of acceptors (i.e., for subunitary $[A]_T/[D]_T$ ratios), previous models only account for pair-wise energy transfer, which is limited by the small number of acceptors. This leads to an underestimate of E_{app}^{Ase} in such complexes (see Fig. 3b), because more than one donor can actually transfer energy to acceptors, which make the acceptors sense a higher rate of transfer compared to the rate sensed by each donor individually. On the other hand, for high concentrations of acceptors in each complex (i.e., for supraunitary $[A]_T/[D]_T$ ratios), the previous theories imply that the energy transferred pair-wise is limited by the small number of donors; this leads to an underestimate of the rate of transfer sensed by donors, E_{app}^{Dq} (Fig. 3a), because each donor actually transfers its excitations to more than one acceptor, and to an overestimate of the rate of transfer sensed by the acceptors, E_{app}^{Ase} (Fig. 3b), because in reality an acceptor is excited by less than one donor on average. These effects therefore explain the discrepancies between previous and current theory.

The present theory properly takes into account all the above effects, although it still uses an approximation that the rates of direct excitation (i.e., by incident light) of both Ds and As are low. This common approximation in fluorescence spectroscopy and microscopy, which does not pertain exclusively to the theoretical model introduced here, is justified by the very short lifetimes of typical fluorescent molecules (~1 ns), compared to the rate of arrival of photons at the fluorescent molecule at usual excitation intensities: ~1 photon per 10–100 ns per entire excitation area, if we consider, for instance, the optimum excitation in two-photon microscopes [37, 38]. Nevertheless, such approximations could have unforeseen consequences when used for fluorescent tags with lifetimes well in excess of a few nanoseconds, such as in the case of Lanthanides [39] subjected to high excitation light intensities.

The effect of the free monomers on the apparent FRET efficiencies has been tested by plotting (24a) and (24b) against the ratio of acceptor to donor concentrations, $[A]_T/[D]_T$, for assumed values of the $[A]/[A]_T$ and $[D]/[D]_T$ ratios (Fig. 4). An expression was derived from the definition of $[A]_T$ and $[D]_T$,

$$\{[A]_A + [A]_D\}/\{[D]_D + [D]_A\} = \{[A]_T/[D]_T\}\{1 - [A]/[A]_T\}/\{1 - [D]/[D]_T\},$$

which relates the concentrations of bound acceptors and donors to those of free monomers, and was used to compute the probabilities P_D and P_A from $[A]_T/[D]_T$ and assumed values for $[A]/[A]_T$ and $[D]/[D]_T$. Further, by using the identities $n\mu_{oligo}P_D = [D]_D + [D]_A = [D]_T - [D]$ and $n\mu_{oligo}P_A = [A]_A + [A]_D = [A]_T - [A]$, the ratios $\mu_{oligo}/[D]_T$ and $\mu_{oligo}/[A]_T$ in (24a) and (24b) were replaced by $\{1 - [D]/[D]_T\}/nP_D$, and $\{1 - [D]/[D]_T\}/nP_D$, respectively, and the apparent FRET efficiencies were computed. As may be seen from Fig. 4, addition of free donors affected E_{app}^{Dq} more strongly than E_{app}^{Ase}, while addition of free acceptors affected E_{app}^{Ase} more markedly. It may be also inferred

that, when the experimental data are noisy, the effect of the free monomers could be misinterpreted as originating from lower values of the pair-wise FRET efficiency, E, which would have similar effects on E_{app}. In such cases, if possible, E should be determined separately, such as from lifetime measurements. Finally, depending on the peculiarities of the biological system investigated, alternative ways of quantifying the proportion of free Ds and As might need to be explored, instead of the constant $[A]/[A]_T$ and $[D]/[D]_T$ ratios used in this paper.

5 Discussion

5.1 Definition of E_{app}^{Ase} Revisited

From the widely used definition of the FRET efficiency determined from acceptor sensitized emission, $E_{app}^{Ase} \equiv F^A(FRET)/F^A(\lambda_{ex})$, which we have also adopted above [(10b)], it follows that the lower the excitation wavelength, the lower the acceptor emission

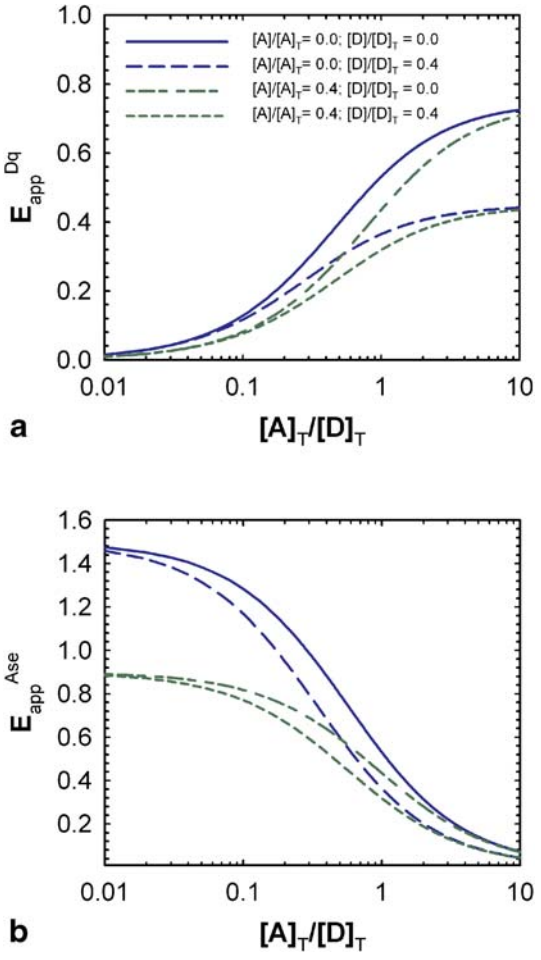

Fig. 4 Apparent FRET efficiencies vs $[A]_T/[D]_T$ concentration ratio, as predicted by the present theory [(24a) and (24b) with E=0.5] for **(a)** donor quenching (superscript "Dq") and **(b)** acceptor sensitized emission for oligomers (superscript "Ase") of uniform size in the presence of various fractions of free monomers, $[A]/[A]_T$ and $[D]/[D]_T$

under direct excitation, $F^A(\lambda_{ex})$, and the higher the efficiency E_{app}^{Ase}. A remarkable consequence of this definition, as predicted by the present model, is that, while the pair-wise efficiency remains subunitary (as it should), E_{app}^{Ase} may exceed unity for $n>2$ and $[A]_T/[D]_T < 1$. In an ideal FRET experiment, one would like to employ *D–A* pairs for which the donor excitation spectrum is well separated from the acceptor excitation spectrum, while ensuring that the donor emission overlaps perfectly with the acceptor excitation spectrum (i.e., that D and A present large Stokes shifts). In that case, however, it would be impossible to adopt the above definition for E_{app}^{Ase}, because the denominator $F^A(\lambda_{ex})$ would be zero (i.e., $E_{app}^{Ase} \to \infty$). This could occur in experiments using Lanthanides [39] as fluorescent tags, or when using two photon excitation [7, 37, 38] of a variant of the green fluorescent protein called GFP2 [40]. In the latter case, the large Stokes shift of the GFP2 coupled with the more selective character of the two-photon excitation [41] may lead to the absence of direct excitation of the acceptor at wavelengths at which the donor is optimally excited; in that case, the standard definition for E_{app}^{Ase} leads to infinite values for efficiency, which is clearly unacceptable from a physical standpoint.

In such cases, one could define E_{app}^{Ase} relative to the donor emission in the absence of the acceptor, i.e., from the ratio of (20d) and (20a), which leads to:

$$E_{app}^{Ase} = \frac{\mu_{oligo} \sum_{k=1}^{n-1} \sum_{config,q} P_D^k P_A^{n-k} \sum_{i=1}^{k} E_{i,k,n,q}}{[D] + \mu_{oligo} n P_D} \frac{Q^A}{Q^D}, \tag{29}$$

which, in the particular case of equal *D–A* distances, leads to:

$$E_{app}^{Ase} = \frac{\mu_{oligo} \sum_{k=1}^{n-1} \frac{k(n-k)E}{1+(n-k-1)E} \binom{n}{k} P_D^k P_A^{n-k}}{[D] + \mu_{oligo} n P_D} \frac{Q^A}{Q^D}. \tag{30}$$

Alternatively, one could use (20a), (20b), (20c) and (20d) [or (23a), (23b), (23c), and (23d)] directly by plotting them against $[A]_T/[D]_T$ and determine the unknown parameters, such as μ_{oligo}, and n, from fitting to the experimental data, assuming that E is known. Both methods would work best if either $[D]=[A]$ (=0 or ≠0) or if the dependence of the concentration of oligomers on the concentration of free monomers is known (from, e.g., the law of mass action, if applicable).

5.2 The Case of Large Oligomers

In the above sections, we have discussed in general terms the relationship between apparent FRET efficiency and oligomer size, but made no explicit reference to possible limitations imposed by the finite Förster distance, R_0. One could argue that R_0 effectively sets an upper limit on the oligomer size that is still detectable through FRET, since *D–A* pairs situated at large distances within large oligomers would not sense each other through FRET. However, in these cases, it might still be possible to use the general form of the present theory, which assumes arbitrary distances [i.e., (21a) and (21b)], to relate the apparent FRET efficiency to the size and geometry of the oligomer even for large complexes. This is because, even if two given *D* and *A* molecules might be too widely separated to exchange energy with one another, they could still participate in FRET separately with their nearest neighbors within the oligomer, which leads to sizeable FRET efficiencies for the whole oligomer. Nevertheless, before a definite conclusion can be drawn in this regard, more in depth

analysis is required of comparative FRET efficiencies for oligomers of various geometries and sizes, which is beyond the scope of the present paper.

5.3 Connecting the Theory to Experiment

An important aspect regarding applications of the present theory concerns the determination of the apparent FRET efficiencies and the ratios of total acceptor to total donor concentrations. Determination of the latter is easily done from knowledge of the former, by noticing that $E_{app}^{Dq}\big/E_{app}^{Ase} = (\varepsilon_A/\varepsilon_D)[A]_T\big/[D]_T$ [from (21a) and (21b)], while determination of apparent efficiencies requires knowledge of the baselines for acceptor and donor fluorescence, i.e., $F^A(\lambda_{ex})$ and $F^D(\lambda_{ex})$ in (11a) and (11b). As mentioned above, it is usually possible to excite the acceptor at a wavelength $\lambda \neq \lambda_{ex}$ at which the donor is not excited, and by knowing the excitation spectrum of the acceptor in the absence of the donor, to infer the level of acceptor emission upon excitation at λ_{ex} in the absence of FRET, $F^A(\lambda_{ex})$. On the other hand, $F^D(\lambda_{ex})$ can be measured after inactivating the acceptor through photobleaching [35, 36].

However, acceptor inactivation poses difficulties in experiments on live cells, since bleached acceptors may diffuse out of the investigated area, while unbleached acceptors may diffuse into it. The errors thus introduced may be estimated from measurements of fluorescence recovery after photobleaching (FRAP) [18], and/or reduced by decreasing the acceptor bleaching time. The time reduction may be achieved, e.g., by using acceptors that are very sensitive to photobleaching, or by using multiphoton excitation, which is known to be more photobleaching efficient than single photon excitation. In addition, the theory suggests an alternative way of determining $[D]_T$, and, thereby the $[A]_T\big/[D]_T$ ratios, without recourse to complete acceptor bleaching. The method relies on the observation that photobleaching is an "all-or-nothing" process causing the bleached molecules to become totally inactive, and effectively reducing the concentrations of optically detectable acceptors. If the concentration of acceptors before and after bleaching is probed by wavelengths long enough to efficiently excite the acceptors but not the donors, it is possible to determine the ratio of the concentrations of active acceptors after and before bleaching, η_A, from the ratio of their emission intensities. This ratio in turn modifies the effective values of the three types of acceptor concentrations to $\eta_A[A]_D$, $\eta_A[A]_A$, and $\eta_A[A]_T$, which further modify the probabilities P_D and P_A in equations (21a) and (21b) or their approximate forms. The ratio of (21a) and (21b) for the incomplete bleaching gives $\eta_A(\varepsilon_A/\varepsilon_D)[A]_T\big/[D]_T$, which replaces the simpler expression given above for $E_{app}^{Dq}\big/E_{app}^{Ase}$, and allows one to determine the concentration ratio, since both the efficiencies and η_A are known experimentally. Subsequently, E_{app}^{Ase}, determined before any acceptor photobleaching occurs, can be plotted against $[A]_T\big/[D]_T$, and then the analysis proceeds as described above, this time only for E_{app}^{Ase}. The gain in speed using the partial photobleaching method proposed here could be dramatic, since a reduction in acceptor intensity of, let us say, 24% is usually achieved in much less than a quarter of the time necessary to bleach four times (i.e., 96%) more acceptors [21], due to the fact that the concentration of unbleached molecules decays exponentially. This advantage may be offset by loss of information regarding E_{app}^{Dq}, which is, however, somewhat redundant with that from E_{app}^{Ase}.

In conclusion, we have shown that a detailed account of the multiple pathways for energy transfer between optically excited donors and unexcited acceptors provides important new insight into the behavior of homo-oligomeric complexes of proteins investigated through FRET. Most notably, by using the present model it should become possible not only to

discriminate between dimers and larger oligomers, but also to determine the size of the oligomers by studying the variation of the FRET efficiency with the acceptor–donor concentration ratio. This may be achieved by using sensitized emission and donor de-quenching (through, e.g., acceptor photobleaching). In addition, it should also be possible to use the theoretical model developed herein for extracting information regarding the relative disposition of the monomers within an oligomer, by assuming particular geometries and then evaluating the accuracy with which the model fits the data.

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