Compartmental Modeling of Reversible Intermolecular Two-State Excited-State Processes Coupled with Rotational Diffusion or with Added Quencher

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Analysis of related time-resolved fluorescence measurements can possibly lead to the determination of the kinetic parameters of excited-state processes. A deterministic identifiability analysis on an error-free fluorescence decay data surface has to be executed to verify whether the parameters of a particular model can be determined and may point to the minimal experimental conditions under which this will become possible. In this work, similarity transformation is chosen as an identifiability analysis approach because it also gives the explicit relationships between the true and alternative model parameters. Results are presented for two kinetic models of a reversible intermolecular two-state excited-state process in isotropic environments: (a) with coupled species-dependent rotational diffusion described by Brownian reorientation and (b) with added quencher. For model a, both spherically and cylindrically symmetric rotors, with no change in the principal axes of rotation in the latter, are considered. The fluorescence δ-response functions $I_{\|}(t)$ and $I_{\perp}(t)$, for fluorescence polarized respectively parallel and perpendicular to the electric vector of linearly polarized excitation, are used to define the sum $S(t) = I_{\|}(t) + 2\, I_{\perp}(t)$ and the difference $D(t) = I_{\|}(t) - I_{\perp}(t)$ function. The identifiability analysis is carried out using the $S(t)$ and $D(t)$ functions. The analysis involving $S(t)$ shows that two physically acceptable possible solutions for the overall rate constants of the excited-state process exist. Inclusion of information from polarized fluorescence measurements on the rotational kinetic behavior contained in $D(t)$ results in the unique set of rate constants and rotational diffusion coefficients when the rotational diffusion coefficients are different. For model b, it is shown that addition of quencher plays formally the same role as rotational diffusion as far as the identification is concerned. When the quenching rate constants are different, the rate constants of a reversible intermolecular two-state excited-state process with added quencher can be uniquely determined.

1. Introduction

Time-resolved fluorescence measurements are essential tools for investigating the dynamics of excited-state processes. To decide on the appropriate model to describe a photophysical system, fluorescence decay traces are measured under different experimental conditions, so that a multidimensional fluorescence decay data surface is generated. For the models of reversible intermolecular two-state excited-state processes—considered in this paper—the experimental axes that can be varied are the excitation and emission wavelengths, coreactant concentration, quencher concentration, and polarization. In many cases, the fluorescence response after a short excitation pulse can be described by a sum of exponentially decaying functions. In the single-curve analysis, each fluorescence decay curve is analyzed separately in terms of the decay times τ and their associated amplitudes α. A proposed model is then tested by the consistency of the recovered decay parameters. A subsequent analysis of the parameter estimates provides the relevant parameters of interest, e.g., the rate constants of the excited-state process. This conventional approach—though adequate in many cases—fails to take full advantage of relations that may exist between individual decay traces. The simultaneous or global analysis[1,2] of multiple decays uses (and tests) those relationships by keeping some model parameters in common (i.e., linked in global curve-fitting analysis) between various related experiments. For the models discussed in this paper, the decay times τ ought to be independent of the excitation and emission wavelengths and, hence, can be linked between experiments measured at different excitation and emission wavelengths. The advantages of the global analysis method are the improved model-discriminating capability and the increased accuracy and precision of the parameter estimates in comparison to the single-curve analysis.[1,2] However, in many instances, the decay times τ will vary between related experiments and hence cannot be linked. This is, for example, the case where changing the coreactant and/or quencher concentrations leads to changes in τ values. In this case, it is more appropriate to fit directly for the more fundamental underlying parameters, namely the rate constants and spectral parameters related to excitation and emission, instead of empirical parameters such as the decay times τ. By estimating directly the primary parameters of interest in a single-step analysis of the entire fluorescence decay data surface, the

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model is directly imposed on the data, and the model-testing capability is improved.

Whenever a particular model is proposed for the description of excited-state processes, one should start with a study to verify whether the kinetic and spectral parameters defining the model can be determined from error-free fluorescence decay data. This is the subject matter of the deterministic identifiability analysis.[3−5] Such an investigation may also point to the minimal set of experimental conditions under which the model parameters will be uniquely determined. The identifiability of various models of reversible intermolecular two-state excited-state processes constitutes the topic of this paper.

Since time-resolved fluorescence can in many instances be described by a set of coupled first-order differential equations, modeling of excited-state processes in photophysics can conveniently be done within the framework of compartmental analysis. Considering the extensive use of compartmental modeling in biomedicine, pharmacokinetics, analysis of ecosystems, etc. (see, for example, refs 3−5), it is rather surprising that its use in photophysics started relatively late.[6−10]

Let us define first the term “compartment” in a photophysical context. In photophysics, a compartment is defined as a subsystem composed of a distinct type of species that acts kinetically in a unique way. The concentration of the constituting species can change when the compartments exchange material through intramolecular or intermolecular processes. In the context of compartmental modeling of excited-state processes, compartments can be divided into ground and excited-state compartments depending upon the state of the composing species. There may be inputs from ground-state compartments into one or more of the excited-state compartments by photoexcitation. Since there is always output from the excited-state compartments to the ground-state compartments through (radiative and radiationless) deactivation, a photophysical system involving excited-state compartments is said to be open. If the concentrations of the species in the ground state do not significantly change upon photoexcitation, it suffices to consider the excited-state compartments.

There are three possible outcomes to the deterministic identifiability analysis.

(1) The parameters of an assumed model can be estimated uniquely and the model is *uniquely* (*globally*) identifiable from the idealized experiment.

(2) There are a finite number of alternative estimates for some or all of the model parameters that fit the data and the model is *locally* identifiable.

(3) An infinite number of model parameter estimates fit the data and the model is *unidentifiable* from the experiment.

For the models considered in this paper, the parameters to be identified are rate constants, spectral parameters related to excitation and emission, rotational diffusion coefficients, and the relative orientation of absorption and emission dipoles.

A deterministic identifiability analysis is a prerequisite before attempting to estimate the model parameters from real experimental observations. Large uncertainties and high correlations obtained in the parameter recovery from an experimental data surface might erroneously be ascribed to numerical ill-conditioning. However, these may be rather indicative of the fact that the model parameters cannot be recovered at all (i.e., the model is unidentifiable). Indeed, parameter estimates with large errors also may be due to the fact that extra time-resolved fluorescence data along additional experimental axes are required or that the used experimental technique can never generate the parameters of the assumed model.

Since the first identifiability analysis of an intermolecular two-state excited-state process,[8] identifiability studies of a large range of compartmental models of intermolecular as well as intramolecular two-state and three-state excited-state processes have been reported (see ref 11 for literature data). The identifiability analyses of reversible intermolecular two-state excited-state processes in the absence[11−13] and presence[14] of quencher have been confined to consideration of the whole excited-state population, as monitored by total (or “magic angle”-selected) fluorescence.

There are several methods available for the analysis of the deterministic identifiability (i.e., identifiability with perfect data).[4,5] The initial approach[12−14] used to investigate the identification of reversible intermolecular two-state excited-state processes involved Markov parameters and elementary functions of the rate constants. The more recent work[11] used similarity transformations.[4,5,15,16] The method of similarity transformation offers a direct way of determining whether a model is globally or locally identifiable or not identifiable at all. Moreover, similarity transformation provides the explicit relationship between the true and alternative model parameters.

This report focuses primarily on the identifiability via similarity transformation of a model of reversible *inter*molecular two-state excited-state processes, without transient effects (i.e., with kinetics governed by time-independent rate constants), accompanied by species-dependent rotational diffusion, as detected by time-resolved fluorescence anisotropy. Spherically and cylindrically symmetric rotors are considered, with in the latter case, no change in the principal axes of diffusion tensors of both excited-state species. The case where the principal axes of the diffusion tensors of both interconverting excited-state species are not the same is very complex[17] and is not considered here. In the extensive field of time-resolved fluorescence spectroscopy, only a relatively small literature has been devoted to the problem of excited-state processes coupled with species-dependent rotational diffusion (see reference 18 and references therein). Chuang and Eisenthal[17] provided the basis for the derivation of explicit expressions describing the time-resolved fluorescence anisotropy of two-state excited-state processes coupled with species-dependent rotational diffusion without transient effects. Further extensions relevant for the present study were presented by Cross et al.[19] and by Limpouchová and Procházka.[20] On the basis of the theory reported in these papers,[17,19,20] a compartmental description was derived for the fluorescence anisotropy decay of intermolecular two-state excited-state processes together with species-dependent rotational diffusion.[18]

A second issue addressed in this report is the identification of a model of reversible intermolecular two-state excited-state processes in the presence of added quencher. It will be shown that for the identification, quenching is *formally* equivalent to rotational diffusion.

The paper is organized as follows. In section 2, the general concepts of identifiability via similarity transformation are presented. In section 3A, the polarization-selected kinetics of a reversible intermolecular two-state excited-state process coupled with species-dependent rotational diffusion is presented for cylindrically symmetrical ellipsoids. The δ-response functions, $I_{\|}(t)$ and $I_{\perp}(t)$, for fluorescence polarized respectively parallel and perpendicular to the electric vector of linearly polarized excitation, are used to define the sum $S(t) = I_{\|}(t) + 2\,I_{\perp}(t)$ and the difference $D(t) = I_{\|}(t) - I_{\perp}(t)$ function. The sum, $S(t)$, and difference, $D(t)$, functions are expressed in matrix form, suitable for the identifiability analysis. Section 3B gives the matrix formulation of the fluorescence δ-response $Q(t)$ of a model of reversible intermolecular two-state excited-state processes in the presence of added quencher. Section 4 deals with the deterministic identifiability analysis of these two kinetic models. In section 4A we show how the information from polarized measurements—expressed in $S(t)$ and $D(t)$—is used for the determination of the rate constants and rotational diffusion coefficients. Section 4B describes the identification analysis involving $Q(t)$ for the model with added quencher.

Numerical identifiability (curve-fitting) which takes into account the noise level on the experimental data, the sampling and the sensitivity of the algorithms used in the estimation of the parameters are not considered in this study.

2. Identifiability Analysis via Similarity Transformation: General Concepts

For a linear, time-invariant compartmental system with N excited-state compartments, the fluorescence δ-response function $f(t)$ can be expressed as[12]

$$f(t) = \mathbf{c}\,\exp(\mathbf{A}t)\mathbf{b} \tag{1}$$

where $\mathbf{b}$ is a column vector of dimension N whose elements are the initial concentrations of each excited-state compartment; $\mathbf{c}$ is a $1 \times N$ vector related to the contribution of each compartment to the emission; $\mathbf{A}$ is a $N \times N$ matrix ("compartmental matrix") containing the kinetic information ("transfer coefficients") of all processes. Equation 1 shows that the response of a linear, time-invariant compartmental system to an impulsive perturbation consists of a sum of exponentials (usually with as many exponentials as compartments).

The set ($\mathbf{A}$, $\mathbf{b}$, $\mathbf{c}$) is called a realization of the fluorescence δ-response function $f(t)$. The deterministic identification (or identifiability) study investigates whether it is possible to find different realizations of $f(t)$, e.g., ($\mathbf{A}^+$, $\mathbf{b}^+$, $\mathbf{c}^+$), so that

$$f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = f(t, \mathbf{A}^+, \mathbf{b}^+, \mathbf{c}^+) \tag{2}$$

In other words, the fluorescence δ-response function $f(t)$ should be the same for the true ($\mathbf{A}$, $\mathbf{b}$, $\mathbf{c}$) and the alternative ($\mathbf{A}^+$, $\mathbf{b}^+$, $\mathbf{c}^+$) model parameter set.[4,5]

Global identifiability is achieved when $\mathbf{A}^+ = \mathbf{A}$, $\mathbf{b}^+ = \mathbf{b}$, and $\mathbf{c}^+ = \mathbf{c}$ (i.e., a unique set of model parameters is obtained). The model is locally identifiable when there is a limited set of alternative $\mathbf{A}^+$, $\mathbf{b}^+$, and $\mathbf{c}^+$. An unidentifiable model is found when there are an infinite number of alternative $\mathbf{A}^+$, $\mathbf{b}^+$, and $\mathbf{c}^+$.

The specific definitions of the compartmental matrix $\mathbf{A}$, the excitation coefficients $\mathbf{b}$, and the emission coefficients $\mathbf{c}$ are given in section 3. The formulation given in eq 2 is appropriate for most systems found in biomedicine, pharmacokinetics, ecosystem modeling, and engineering,[3–5] but it is not suitable for photophysical systems where absolute values for $\mathbf{b}$ and $\mathbf{c}$ cannot be obtained, as will be discussed in section 4.

Any other (alternative) realization ($\mathbf{A}^+$, $\mathbf{b}^+$, $\mathbf{c}^+$) of $f(t)$ is related to the true set ($\mathbf{A}$, $\mathbf{b}$, $\mathbf{c}$) via similarity transformation,[4,5,11,15,16]

$$\mathbf{A}^+ = \mathbf{T}^{-1}\mathbf{A}\mathbf{T} \tag{3}$$

where $\mathbf{T}$ is a constant invertible (or nonsingular) matrix (i.e., det $\mathbf{T} \neq 0$) having the same dimension as $\mathbf{A}$.

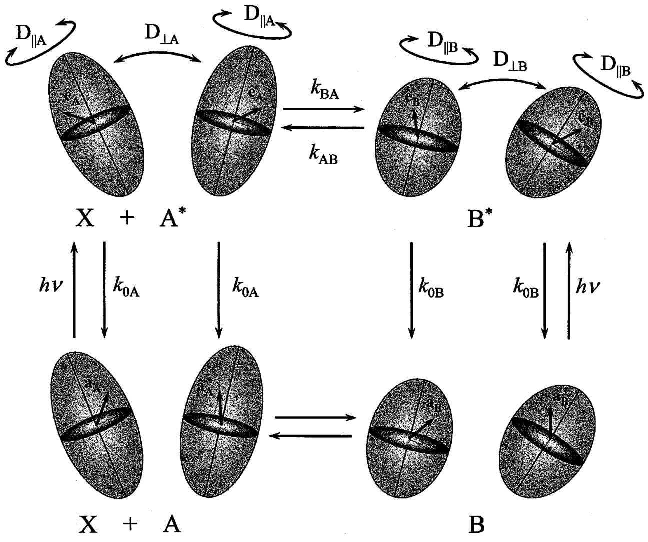


Figure 1. Graphic representation of a reversible intermolecular two-state excited-state process, including rotation. Species A* and B* are pictured as being initially excited from their ground states A and B by an infinitely short linearly polarized light pulse at wavelength λ_i^{ex} in a unique absorption band. The excited-state processes are described by the deactivation rate constants k_{0A} and k_{0B}, and the excited-state exchange rate constants k_{BA} and k_{AB}. The transformation of species A* into B* is mediated by coreactant X with concentration $[X]_k$. Simultaneously the species rotate with rate constants determined by the corresponding rotational diffusion tensors D_A and D_B, which may differ between the species. The polarized emission of each species depends on the relative orientation of its emission transition moment (with unit vector $\hat{\mathbf{e}}_A$ or $\hat{\mathbf{e}}_B$) at the instance of emission with respect to the absorption moment (with unit vector $\hat{\mathbf{a}}_A$ or $\hat{\mathbf{a}}_B$) in the species initially excited.

One can rewrite eq 3 in the form

$$\mathbf{T}\mathbf{A}^+ = \mathbf{A}\mathbf{T} \tag{4}$$

The alternative $\mathbf{b}^+$ and $\mathbf{c}^+$ are given by

$$\mathbf{b}^+ = \mathbf{T}^{-1}\mathbf{b} \tag{5a}$$

$$\mathbf{c}^+ = \mathbf{c}\mathbf{T} \tag{5b}$$

Equations 3 (or 4) and 5 should be satisfied for each experimental condition. For the models considered, the possible experimental variables are coreactant concentration $[X]_k$, quencher concentration $[Q]_l$, excitation wavelength λ_i^{ex}, and emission wavelength λ_j^{em} (and in principle also the orientations of the polarizers in the excitation and emission paths). This implies that the matrix $\mathbf{T}$ should be independent of $[X]_k$, $[Q]_l$, λ_i^{ex}, and λ_j^{em}. Indeed, since $\mathbf{c}^+$ should not depend on $[X]_k$, $[Q]_l$, and λ_i^{ex}, $\mathbf{T}$ should be independent of $[X]_k$, $[Q]_l$, and λ_i^{ex}. Similarly, because of the independence of $\mathbf{b}^+$ (and $\mathbf{A}^+$) of λ_j^{em}, $\mathbf{T}$ should be independent of λ_j^{em}.

3. Kinetics

A. Reversible Intermolecular Two-State Excited-State Process with Species-Dependent Rotational Diffusion. The linear, time-invariant photophysical system consisting of two different interchanging species A and B, each with distinct rotational characteristics—as depicted in Figure 1—is considered. The two ground-state species are assumed to be in equilibrium. Photoexcitation of the system produces the excited-state species A* and B* which can decay by fluorescence (k_F) and nonradiative (k_{NR}) processes. k_{0A} ($=k_{FA} + k_{NRA}$) and k_{0B} ($=k_{FB} + k_{NRB}$) denote the composite deactivation rate constants of A* and B*, respectively. The rate constant describing the intermolecular (with coreactant X) transformation of A* into B* is represented by k_{BA}. The reverse process, described by k_{AB}, is concentration independent. All the rate constants are assumed independent of the instantaneous orientation of the species. The physical requirement restricts the rate constants to be nonnegative. The rotational relaxation of each excited-state species is governed by its principal rotational diffusion constants, here $D_{\perp}$ and $D_{\|}$ for rotation, respectively, of and about the symmetry axis of each of the cylindrically symmetric rotors depicted in Figure 1. When the photophysical system shown in Figure 1 is excited with a δ-pulse of low intensity at time zero, so that the ground-state species population is not appreciably depleted, the fluorescence δ-response function $I_{\|ijk}(t)$ for the plane-polarized component of emission of the two excited states (A* and B*), having its electric vector polarized parallel to the electric vector of the plane-polarized excitation light, and the fluorescence δ-response function $I_{\perp ijk}(t)$ for the perpendicularly polarized component can be expressed, in the case of pure transitions and *isotropic* solutions, as[21]

$$I_{\|ijk}(t) = \frac{1}{3}S_{ijk}(t)[1 + 2r_{ijk}(t)] = \frac{1}{3}S_{ijk}(t) + \frac{2}{3}D_{ijk}(t) \quad (6a)$$

$$I_{\perp ijk}(t) = \frac{1}{3}S_{ijk}(t)[1 - r_{ijk}(t)] = \frac{1}{3}S_{ijk}(t) - \frac{1}{3}D_{ijk}(t) \quad (6b)$$

where $r_{ijk}(t)$ denotes the fluorescence emission anisotropy and where[18]

$$S_{ijk}(t) = 3\mathbf{c}_{j,00}\exp(\mathbf{A}_{k,00}t)\mathbf{b}_{ik,00} \quad (7)$$

$$D_{ijk}(t) = 3\mathbf{c}_{j,2M}\exp(\mathbf{A}_{Dk}t)\mathbf{b}_{ik,2M} \quad (8)$$

The subscripts i, j, and k in $I_{\|ijk}(t)$ and $I_{\perp ijk}(t)$ (eq 6), in $S_{ijk}(t)$ (eq 7) and in $D_{ijk}(t)$ (eq 8) refer to the excitation wavelength λ_i^{ex}, the emission wavelength λ_j^{em}, and the coreactant concentration $[\mathrm{X}]_k$, respectively.

Matrix $\mathbf{A}_{k,00}$ in eq 7 is given by eq 9:

$$\mathbf{A}_{k,00} = \begin{bmatrix} -(k_{0\mathrm{A}} + k_{\mathrm{BA}}[\mathrm{X}]_k) & k_{\mathrm{AB}} \\ k_{\mathrm{BA}}[\mathrm{X}]_k & -(k_{0\mathrm{B}} + k_{\mathrm{AB}}) \end{bmatrix} \quad (9)$$

$\mathbf{A}_{Dk}$ in eq 8 is defined as

$$\mathbf{A}_{Dk} = \begin{bmatrix} \mathrm{A}_{Dk,2-2} & 0 & 0 & 0 & 0 \\ 0 & \mathrm{A}_{Dk,2-1} & 0 & 0 & 0 \\ 0 & 0 & \mathrm{A}_{Dk,20} & 0 & 0 \\ 0 & 0 & 0 & \mathrm{A}_{Dk,21} & 0 \\ 0 & 0 & 0 & 0 & \mathrm{A}_{Dk,22} \end{bmatrix} \quad (10)$$

with blocks $\mathbf{A}_{Dk,2M}$ given by eq 11:

$$\mathbf{A}_{Dk,2M} = \begin{bmatrix} -(D_{\mathrm{A},2M} + k_{0\mathrm{A}} + k_{\mathrm{BA}}[\mathrm{X}]_k) & k_{\mathrm{AB}} \\ k_{\mathrm{BA}}[\mathrm{X}]_k & -(D_{\mathrm{B},2M} + k_{0\mathrm{B}} + k_{\mathrm{AB}}) \end{bmatrix} \quad (11)$$

with $M = -2, -1, 0, 1, 2$. $D_{l,2M}$ (l denotes either A or B) is given by

$$D_{l,2M} = 6D_{\perp l} + M^2(D_{\|l} - D_{\perp l}) \quad (12)$$

Note the invariance of eqs 11 and 12 to the sign of M.

$D_{\perp l}$ and $D_{\|l}$ (see Figure 1) are the components of the rotational diffusion tensor of the *cylindrically* symmetric species l in its molecular reference frame (x, y, z), chosen such that the rotational diffusion tensor is diagonal,[18] reducing to the unique component D_l ($= D_{\perp l} = D_{\|l}$) in the case of the *spherically symmetric* rotor l.

For a *spherically symmetric* rotor ($D_l = D_{\perp l} = D_{\|l}$), the matrices $\mathbf{A}_{Dk,2M}$ (eq 11) are all identical and independent of M. Now each matrix block $\mathbf{A}_{Dk,2M}$ can be written as

$$\mathbf{A}_{Dk,2M} = \begin{bmatrix} -(6D_{\mathrm{A}} + k_{0\mathrm{A}} + k_{\mathrm{BA}}[\mathrm{X}]_k) & k_{\mathrm{AB}} \\ k_{\mathrm{BA}}[\mathrm{X}]_k & -(6D_{\mathrm{B}} + k_{0\mathrm{B}} + k_{\mathrm{AB}}) \end{bmatrix} \quad (13)$$

Vector $\mathbf{b}_{ik,LM}$ [with $L = M = 0$ (eq 7), or $L = 2$ and $M = \pm 2$, ± 1, 0 (eq 8)] contains the excitation coefficients $b_{lik,LM}$ (l denotes either species A or B). As before, the subscripts i and k in $\mathbf{b}_{ik,LM}$ refer to the excitation wavelength λ_i^{ex} and coreactant concentration $[\mathrm{X}]_k$, respectively. The subscripts L and M of the $b_{lik,LM}$ coefficients refer to the orientation of the absorption transitions. The elements $b_{lik,LM}$ can be expressed as the product of the initial concentration of l*, b_{lik}, the appropriate spherical harmonic $Y_L^M(\hat{\mathbf{a}}_l)$[22] for the orientation of the absorption transition moment $\hat{\mathbf{a}}_l$ in the molecular frame of species l, and a scaling factor B_L:[18]

$$b_{lik,LM} = B_L b_{lik} Y_L^M(\hat{\mathbf{a}}_l) \quad (14)$$

with

$$B_0 = \frac{1}{12}\sqrt{\frac{1}{\pi^3}} \quad \text{and} \quad B_2 = \frac{1}{30}\sqrt{\frac{5}{\pi^3}}$$

For $L = M = 0$, we have

$$Y_0^0(\hat{\mathbf{a}}_l) = \frac{1}{\sqrt{4\pi}} \quad \text{and} \quad b_{lik,00} = \frac{b_{lik}}{24\pi^2}$$

The 2 × 1 vector $\mathbf{b}_{ik,00}$ in eq 7 is explicitly given by eq 15:

$$\mathbf{b}_{ik,00} = [b_{\mathrm{A}ik,00} \;\; b_{\mathrm{B}ik,00}]^{\mathrm{T}} \quad (15)$$

while the 10 × 1 vector $\mathbf{b}_{ik,2M}$ in eq 8 is expressed as

$$\mathbf{b}_{ik,2M} = [b_{\mathrm{A}ik,2-2} b_{\mathrm{B}ik,2-2} b_{\mathrm{A}ik,2-1} b_{\mathrm{B}ik,2-1} b_{\mathrm{A}ik,20} b_{\mathrm{B}ik,20} b_{\mathrm{A}ik,21} b_{\mathrm{B}ik,21} b_{\mathrm{A}ik,22} b_{\mathrm{B}ik,22}]^{\mathrm{T}} \quad (16)$$

Vector $\mathbf{c}_{j,LM}$ [with $L = M = 0$ (eq 7), or $L = 2$ and $M = \pm 2$, ± 1, 0 (eq 8)] contains the corresponding emission coefficients $c_{mj,LM}$ (m represents either species A* or B*). As before, the subscript j in $\mathbf{c}_{j,LM}$ refers to the emission wavelength λ_j^{em}. The emission coefficients $c_{mj,LM}$ are given by[18]

$$c_{mj,LM} = C_L c_{mj} Y_L^{M*}(\hat{\mathbf{e}}_m) \quad (17)$$

where $C_0 = 16/3\sqrt{\pi^5}$, $C_2 = 16/15\sqrt{\pi^5/5}$, and $Y_L^{M*}(\hat{\mathbf{e}}_{\mathrm{m}})$ is the complex conjugate of the appropriate spherical harmonic for the orientation of the emission transition moment $\hat{\mathbf{e}}_{\mathrm{m}}$ in the molecular frame.

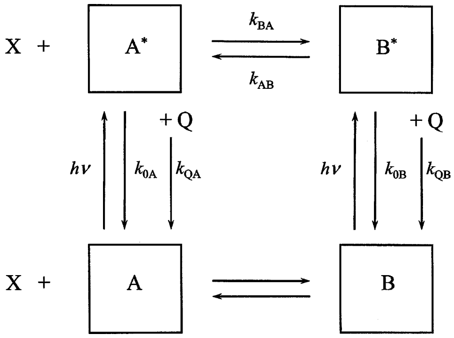
For $L = M = 0$, we have $c_{mj,00} = 8\pi^2 c_{mj}/3$.

The coefficient c_{mj} is defined as[12]

$$c_{mj} = k_{\mathrm{F}m}\int_{\Delta\lambda_j^{\mathrm{em}}} \rho_m(\lambda_j^{\mathrm{em}})\, \mathrm{d}\lambda^{\mathrm{em}} \quad (18)$$

where $k_{\mathrm{F}m}$ is the fluorescence rate constant of species m*, the

SCHEME 1: Scheme Representing a Reversible Intermolecular Two-State Excited-State Process with Added Quencher[a]



[a] It is assumed that the quencher Q has only an effect on the excited species and does not affect the ground-state equilibrium. The excited-state processes are described by the deactivation rate constants k_{0A} and k_{0B}, and the excited-state exchange rate constants k_{BA} and k_{AB}. The additional quenching of A* and B* due to the external quencher Q is described by the rate constants k_{QA} and k_{QB}, respectively.

subscript j refers to the observation wavelength range, $\Delta\lambda_j^{em}$, and $\rho_m(\lambda_j^{em})$ is the spectral emission density of species m*.

Vector $\mathbf{c}_{j,00}$ in eq 7 is explicitly given by eq 19:

$$\mathbf{c}_{j,00} = [c_{Aj,00} c_{Bj,00}] \tag{19}$$

while vector $\mathbf{c}_{j,2M}$ in eq 8 is expressed as

$$\mathbf{c}_{j,2M} = [c_{Aj,2-2} c_{Bj,2-2} c_{Aj,2-1} c_{Bj,2-1} c_{Aj,20} c_{Bj,20} c_{Aj,21} c_{Bj,21} c_{Aj,22} c_{Bj,22}] \tag{20}$$

The matrix and vector formulations of **A** (eqs 9, 10), **b** (eqs 15, 16), and **c** (eqs 19, 20) will prove particularly convenient in addressing the identifiability analysis to the considered models.

The identification analysis is simpler if one uses the "sum" $S_{ijk}(t) = I_{\parallel ijk}(t) + 2I_{\perp ijk}(t)$ and "difference" $D_{ijk}(t) = I_{\parallel ijk}(t) - I_{\perp ijk}(t)$ functions of the *polarized* fluorescence δ-response functions $I_{\parallel ijk}(t)$ and $I_{\perp ijk}(t)$. $S_{ijk}(t)$ corresponds to the total time-resolved emission of the photophysical system, is independent of rotational diffusion, and does not contain any information about the orientations of the transition moments. Information about rotational diffusion is contained in $D_{ijk}(t)$.

B. Reversible Intermolecular Two-state Excited-state Process with Added Quencher. Consider the molecular system (see Scheme 1) with an equilibrium between two different species A and B in the ground state which form upon photoexcitation the excited-state species A* and B*, respectively. The deactivation of these excited-state species via fluorescence and nonradiative processes is described by the combined rate constants k_{0A} for A* and k_{0B} for B*. By addition of an external quencher, Q, with concentration $[Q]_l$ to the photophysical system, the depletion of the excited states is enhanced by k_{QA} $[Q]_l$ for A* and k_{QB} $[Q]_l$ for B*. It is assumed that the quencher Q has only an effect on the excited species and does not affect the ground-state equilibrium. The transformation of A* into B* is labeled with the rate constant k_{BA}, while the reverse process is described by k_{AB}. When the system of Scheme 1 is excited at time zero with a δ-pulse of low intensity, which does not significantly deplete the ground-state species, the fluorescence δ-response function $Q_{ijkl}(t)$ at coreactant concentration $[X]_k$ and quencher concentration $[Q]_l$, monitored at emission wavelength λ_j^{em} due to excitation at λ_i^{ex} can be expressed in matrix notation:[14]

$$Q_{ijkl}(t) = \mathbf{c}_j \exp(\mathbf{A}_{kl}t)\mathbf{b}_{ik} \tag{21}$$

with $\mathbf{A}_{kl}$ given by eq 22:[14]

$$\mathbf{A}_{kl} = \begin{bmatrix} -(k_{QA}[Q]_l + k_{0A} + k_{BA}[X]_k) & k_{AB} \\ k_{BA}[X]_k & -(k_{QB}[Q]_l + k_{0B} + k_{AB}) \end{bmatrix} \tag{22}$$

$\mathbf{b}_{ik}$ and $\mathbf{c}_j$ are given by eqs 23 and 24, respectively:[14]

$$\mathbf{b}_{ik} = [b_{Aik} \;\; b_{Bik}]^T \tag{23}$$

$$\mathbf{c}_j = [c_{Aj} \;\; c_{Bj}] \tag{24}$$

4. Identifiability Analysis

A. Reversible Intermolecular Two-State Excited-State Process with Species-Dependent Rotational Diffusion. Since both $S_{ijk}(t)$ (eq 7) and $D_{ijk}(t)$ (eq 8) can be expressed in matrix form, the identification analysis via similarity transformation is carried out using the $S_{ijk}(t)$ and $D_{ijk}(t)$ functions.

Let us start with the identification involving $S_{ijk}(t)$. For $f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = S_{ijk}(t)$ (eq 7), we have that $\mathbf{A} = \mathbf{A}_{k,00}$, (eq 9), $\mathbf{b} = \mathbf{b}_{ik,00}$ (eq 15), $\mathbf{c} = \mathbf{c}_{j,00}$ (eq 19). Matrix **T** is then given by eq 25:

$$\mathbf{T} = \begin{bmatrix} t_1 & t_2 \\ t_3 & t_4 \end{bmatrix} \tag{25}$$

As $S_{ijk}(t)$ reflects the time dependence of the total fluorescence and contains information only on the excited states, we can expect that the identifiability analysis will be the same as that reported for a reversible intermolecular two-state excited-state process.[11] Therefore, we refer to ref 11 for more mathematical details. As the results of the identifiability analysis involving $S_{ijk}(t)$ will be used in the analysis with $D_{ijk}(t)$, we will sketch the identifiability procedure.

Performing the matrix multiplication in eq 4 with $\mathbf{A} = \mathbf{A}_{k,00}$ yields a set of four simultaneous equations. Since the elements t_i ($i = 1, ..., 4$) of **T** are independent of $[X]_k$ and since $k_{BA} \neq 0$, we have $t_2 = 0$ and $t_1(k_{BA}^+ - k_{BA}) = 0$. If $t_1 = 0$, then also t_3 and t_4 have to be zero and **T** becomes the null matrix, which is not a valid transformation matrix. From the alternative, $k_{BA}^+ = k_{BA}$, we have $t_4 = t_1 + t_3$, so that the matrix multiplication in eq 4 yields a set of four equations as a function of t_1 and t_3 (eq 26):

$$-t_1 k_{0A}^+ = -t_1 k_{0A} + t_3 k_{AB} \tag{26a}$$

$$t_1 k_{AB}^+ = (t_1 + t_3) k_{AB} \tag{26b}$$

$$t_3(k_{0B} + k_{AB} - k_{0A}^+) = 0 \tag{26c}$$

$$t_3 k_{AB}^+ = (t_1 + t_3)(k_{0B}^+ + k_{AB}^+ - k_{0B} - k_{AB}) \tag{26d}$$

From eq 26c one concludes that either $t_3 = 0$ or $k_{0A}^+ = k_{0B} + k_{AB}$.

If $t_3 = 0$, the original rate constants are obtained: $k_{0A}^+ = k_{0A}$, $k_{AB}^+ = k_{AB}$, $k_{BA}^+ = k_{BA}$, $k_{0B}^+ = k_{0B}$. This set corresponds to $\mathbf{T} = t_1\mathbf{I}_2$, with $\mathbf{I}_2$ the 2 × 2 identity matrix.

If alternatively $k_{0A}^+ = k_{0B} + k_{AB}$ ($t_3 \neq 0$), then from eqs 26a and 26b we have $k_{AB}^+ = k_{0A} - k_{0B}$ and substituting eq 26b into eq 26d yields $k_{0B}^+ = k_{0B}$.

Now matrix **T** takes the form

$$\mathbf{T} = \begin{bmatrix} t_1 & 0 \\ t_3 & t_1 + t_3 \end{bmatrix} \qquad (27)$$

with $t_3 \neq 0$.

To summarize, we obtain two sets of rate constant values: set S1 (the original or "true" set):

$$k_{0\mathrm{A}}^{+} = k_{0\mathrm{A}} \qquad (28a)$$

$$k_{\mathrm{AB}}^{+} = k_{\mathrm{AB}} \qquad (28b)$$

$$k_{\mathrm{BA}}^{+} = k_{\mathrm{BA}} \qquad (28c)$$

$$k_{0\mathrm{B}}^{+} = k_{0\mathrm{B}} \qquad (28d)$$

with $\mathbf{T} = t_1\mathbf{I}_2$ and set S2 (the alternative set) given by eq 29:

$$k_{0\mathrm{A}}^{+} = k_{0\mathrm{B}} + k_{\mathrm{AB}} \qquad (29a)$$

$$k_{\mathrm{AB}}^{+} = k_{0\mathrm{A}} - k_{0\mathrm{B}} \qquad (29b)$$

$$k_{\mathrm{BA}}^{+} = k_{\mathrm{BA}} \qquad (29c)$$

$$k_{0\mathrm{B}}^{+} = k_{0\mathrm{B}} \qquad (29d)$$

with **T** given by eq 27 with $t_3 \neq 0$. Equation 29b requires that $k_{0\mathrm{A}} > k_{0\mathrm{B}}$. For set S2 we have from eq 26b that $t_3/t_1 = (k_{0\mathrm{A}} - k_{0\mathrm{B}} - k_{\mathrm{AB}})/k_{\mathrm{AB}}$.

Now we will show that the ambiguity in the rate constants (i.e., two possible sets) can be resolved by a monoexponential $f(t)$ at $[\mathrm{X}]_k = 0$. Indeed, the fluorescence δ-response function $f(t)$ becomes monoexponential for $[\mathrm{X}]_k = 0$, with decay rate constant $k_{0\mathrm{A}}$. From this monoexponential $f(t)$, we have $k_{0\mathrm{A}}^{+} = k_{0\mathrm{A}}$. Equation 26a leads then to $t_3 = 0$ and from eq 26b we have $k_{\mathrm{AB}}^{+} = k_{\mathrm{AB}}$. From eq 26d, we obtain $k_{0\mathrm{B}}^{+} = k_{0\mathrm{B}}$, so that the original set is obtained ($\mathbf{T} = t_1\mathbf{I}_2$). To conclude, if the monoexponential δ-response function $f(t)$ at $[\mathrm{X}]_k = 0$ can be recorded, the model of the reversible intermolecular two-state excited-state process becomes uniquely identifiable in terms of rate constants.

Next we will demonstrate that, for $\mathbf{T} = t_1\mathbf{I}_2$ (set S1), the normalized $\tilde{b}_{\mathrm{A}ik}$ ($\tilde{b}_{\mathrm{B}ik} = 1 - \tilde{b}_{\mathrm{A}ik}$) and $\tilde{c}_{\mathrm{A}j}$ ($\tilde{c}_{\mathrm{B}j} = 1 - \tilde{c}_{\mathrm{A}j}$) are unique.

For $S_{ijk}(t)$ the alternative $\mathbf{b}^{+}$ (eq 5a) and $\mathbf{c}^{+}$ (eq 5b) for $\mathbf{T} = t_1\,\mathbf{I}_2$ are given by

$$\mathbf{b}_{ik,00}^{+} = \mathbf{b}_{ik,00}/t_1 \qquad (30)$$

$$\mathbf{c}_{mj,00}^{+} = t_1\mathbf{c}_{mj,00} \qquad (31)$$

We define the normalized $\tilde{b}_{\mathrm{A}ik,00}^{+}$, $\tilde{b}_{\mathrm{A}ik,00}$, $\tilde{c}_{\mathrm{A}j,00}^{+}$, and $\tilde{c}_{\mathrm{A}j,00}$ as

$$\tilde{b}_{\mathrm{A}ik,00}^{+} = b_{\mathrm{A}ik,00}^{+}/(b_{\mathrm{A}ik,00}^{+} + b_{\mathrm{B}ik,00}^{+}) \qquad (32a)$$

$$\tilde{b}_{\mathrm{A}ik,00} = b_{\mathrm{A}ik,00}/(b_{\mathrm{A}ik,00} + b_{\mathrm{B}ik,00}) \qquad (32b)$$

$$\tilde{c}_{\mathrm{A}j,00}^{+} = c_{\mathrm{A}j,00}^{+}/(c_{\mathrm{A}j,00}^{+} + c_{\mathrm{B}j,00}^{+}) \qquad (32c)$$

$$\tilde{c}_{\mathrm{A}j,00} = c_{\mathrm{A}j,00}/(c_{\mathrm{A}j,00} + c_{\mathrm{B}j,00}) \qquad (32d)$$

Use of these normalized elements in eq 30 leads to

$$\tilde{b}_{\mathrm{A}ik,00}^{+} = \tilde{b}_{\mathrm{A}ik} \quad \text{and} \quad \tilde{b}_{\mathrm{B}ik,00}^{+} = \tilde{b}_{\mathrm{B}ik} \qquad (33a)$$

Analogously, eq 31 gives

$$\tilde{c}_{\mathrm{A}j,00}^{+} = \tilde{c}_{\mathrm{A}j} \quad \text{and} \quad \tilde{c}_{\mathrm{B}j,00}^{+} = \tilde{c}_{\mathrm{B}j} \qquad (33b)$$

Equation 33 shows that the normalized $\tilde{b}_{\mathrm{A}ik,00} = \tilde{b}_{\mathrm{A}ik}$ and $\tilde{c}_{\mathrm{A}j,00} = \tilde{c}_{\mathrm{A}j}$ are unique. The use of normalized $\tilde{b}_{\mathrm{A}ik}$ and $\tilde{c}_{\mathrm{A}j}$ in global compartmental analysis[6,7,12] allows $\tilde{b}_{\mathrm{A}ik}$ to be linked at the same coreactant concentration $[\mathrm{X}]_k$ and excitation wavelength λ_i^{em}, whereas $\tilde{c}_{\mathrm{A}j}$ can be linked at the same emission wavelength λ_j^{em}.[12]

Now we consider the case where $f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = D_{ijk}(t)$ (eq 8) in which we will use the results of the identifiability analysis involving $S_{ijk}(t)$. We assume that the similarity transformations for $S_{ijk}(t)$ and $D_{ijk}(t)$ are independent. Also the transformations of the various blocks $\mathbf{A}_{Dk,2M}$ in $\mathbf{A}_{Dk}$ are independent. For a *cylindrically symmetric* rotor, $\mathbf{A} = \mathbf{A}_{Dk}$ (eq 10) with blocks $\mathbf{A}_{Dk,2M}$ given by eq 11, $\mathbf{b} = \mathbf{b}_{ik,2M}$ (eq 16), $\mathbf{c} = \mathbf{c}_{j,2M}$ (eq 20). Matrix **T** is a block-diagonal matrix (eq 34, see Appendix):

$$\mathbf{T} = \begin{bmatrix} T_{-2} & 0 & 0 & 0 & 0 \\ 0 & T_{-1} & 0 & 0 & 0 \\ 0 & 0 & T_0 & 0 & 0 \\ 0 & 0 & 0 & T_1 & 0 \\ 0 & 0 & 0 & 0 & T_2 \end{bmatrix} \qquad (34)$$

with the matrices $\mathbf{T}_M$ ($M = -2, -1, 0, 1, 2$) expressed as

$$\mathbf{T}_M = \begin{bmatrix} t_{M,1} & t_{M,2} \\ t_{M,3} & t_{M,4} \end{bmatrix} \qquad (35)$$

Because **T** and $\mathbf{A} = \mathbf{A}_{Dk}$ are both block-diagonal matrices, the matrix multiplication of eq 4 is split into five separate matrix multiplications (two of those are identical; $M = -2$ and $M = +2$; $M = -1$ and $M = +1$). It is straightforward to show that the matrix multiplication involving $\mathbf{A}_{Dk,2M}^{+}$ and $\mathbf{A}_{Dk,2M}$ [for $M = \pm 2, \pm 1, 0$ (eq 36)]

$$\mathbf{T}_M\mathbf{A}_{Dk,2M}^{+} = \mathbf{A}_{Dk,2M}\mathbf{T}_M \qquad (36)$$

also leads to two sets of alternative parameters: set D1 (corresponding to $\mathbf{T}_M = t_{M,1}\mathbf{I}_2$) given by eq 37

$$k_{0\mathrm{A}}^{+} + D_{\mathrm{A},2M}^{+} = k_{0\mathrm{A}} + D_{\mathrm{A},2M} \qquad (37a)$$

$$k_{\mathrm{AB}}^{+} = k_{\mathrm{AB}} \qquad (37b)$$

$$k_{\mathrm{BA}}^{+} = k_{\mathrm{BA}} \qquad (37c)$$

$$k_{0\mathrm{B}}^{+} + D_{\mathrm{B},2M}^{+} = k_{0\mathrm{B}} + D_{\mathrm{B},2M} \qquad (37d)$$

and set D2 (eq 38):

$$k_{0\mathrm{A}}^{+} + D_{\mathrm{A},2M}^{+} = k_{0\mathrm{B}} + k_{\mathrm{AB}} + D_{\mathrm{B},2M} \qquad (38a)$$

$$k_{\mathrm{AB}}^{+} = k_{0\mathrm{A}} - k_{0\mathrm{B}} + D_{\mathrm{A},2M} - D_{\mathrm{B},2M} \qquad (38b)$$

$$k_{\mathrm{BA}}^{+} = k_{\mathrm{BA}} \qquad (38c)$$

$$k_{0\mathrm{B}}^{+} + D_{\mathrm{B},2M}^{+} = k_{0\mathrm{B}} + D_{\mathrm{B},2M} \qquad (38d)$$

For set D2 matrix $\mathbf{T}_M$ takes the form

$$\mathbf{T}_M = \begin{bmatrix} t_{M,1} & 0 \\ t_{M,3} & t_{M,1} + t_{M,3} \end{bmatrix} \qquad (39)$$

with $t_{M,3}/t_{M,1} = (k_{0\mathrm{A}} - k_{0\mathrm{B}} - k_{\mathrm{AB}})/k_{\mathrm{AB}}$.

To solve for the individual $k_{0\mathrm{A}}^{+}$, k_{AB}^{+}, k_{BA}^{+}, $k_{0\mathrm{B}}^{+}$, $D_{\perp\mathrm{A}}^{+}$, $D_{\parallel\mathrm{A}}^{+}$, $D_{\perp\mathrm{B}}^{+}$, and $D_{\parallel\mathrm{B}}^{+}$, one should combine the equations describing sets S1 (eq 28) and S2 (eq 29) with the equations describing sets D1 (eq 37 with $M = \pm2, \pm1, 0$) and D2 (eq 38 with $M = \pm2, \pm1, 0$). $k_{BA}{}^{+} = k_{BA}$ in all four sets. The equations describing sets D1 and D2 are indeed not sufficient to lead to unique solutions for the unknown rate constants and diffusion coefficients. In principle 16 combinations of eqs 28, 29, 37, and 38 are possible (e.g., S1, D1 for $M = 0, \pm2$, D2 for $M = \pm1$; S2, D2 for $M = 0$, D1 for $M = \pm1, \pm2$; etc.). However, of the 16 possible combinations, only two will lead to a solution for the rate constants and diffusion coefficients. Indeed, S1 (eq 28) can only be combined with D1 (eq 37 with $M = \pm2, \pm1, 0$). The combination of set S1 (eq 28) with set D2 (eq 38) is not valid because eq 28b and eq 38b expressing $k_{AB}{}^{+}$ are incompatible, and hence no solution is possible. Equivalently, S2 (eq 29) can only be combined with D2 (eq 38 with $M = \pm2, \pm1, 0$), because the combination of S2 with D1 does not lead to a solution (eq 29b and eq 37b expressing $k_{AB}{}^{+}$ are contradictory).

Combining eq 28 describing S1 with eq 37 describing D1 leads to a set of 10 simultaneous equations in 8 unknowns $k_{0A}{}^{+}$, $k_{AB}{}^{+}$, $k_{BA}{}^{+}$, $k_{0B}{}^{+}$, $D_{\perp A}{}^{+}$, $D_{\|A}{}^{+}$, $D_{\perp B}{}^{+}$, and $D_{\|B}{}^{+}$. Solution of this overdetermined set of equations yields the original set of rotational diffusion coefficients:

$$D_{\perp A}{}^{+} = D_{\perp A} \tag{40a}$$

$$D_{\|A}{}^{+} = D_{\|A} \tag{40b}$$

$$D_{\perp B}{}^{+} = D_{\perp B} \tag{40c}$$

$$D_{\|B}{}^{+} = D_{\|B} \tag{40d}$$

Hence, by combining set S1 and D1 the original rate constants and rotational diffusion coefficients are obtained.

Now we examine the second possible combination (S2 and D2). Equations 29b and 38b lead to $D_{A,2M} = D_{B,2M}$ for $M = \pm2, \pm1, 0$, yielding

$$D_{\perp A} = D_{\perp B} \tag{41a}$$

$$D_{\|A} = D_{\|B} \tag{41b}$$

From eqs 29a and 38a we have $D_{A,2M}{}^{+} = D_{B,2M}$ for $M = \pm2, \pm1, 0$, yielding

$$D_{\perp A}{}^{+} = D_{\perp B} \tag{42a}$$

$$D_{\|A}{}^{+} = D_{\|B} \tag{42b}$$

From eqs 29d and 38d we have $D_{B,2M}{}^{+} = D_{B,2M}$ for $M = \pm2, \pm1, 0$, yielding

$$D_{\perp B}{}^{+} = D_{\perp B} \tag{43a}$$

$$D_{\|B}{}^{+} = D_{\|B} \tag{43b}$$

If the rotational diffusion coefficients of both species are equal ($D_{\perp A} = D_{\perp B}$ and $D_{\|A} = D_{\|B}$), the alternative rate constants are given by set S2 (eq 29) and the alternative diffusion coefficients are the original ones ($D_{\perp A}{}^{+} = D_{\perp B}{}^{+} = D_{\perp A} = D_{\perp B}$ and $D_{\|A}{}^{+} = D_{\|B}{}^{+} = D_{\|A} = D_{\|B}$).

To summarize, the identifiability analysis involving both $S_{ijk}(t)$ and $D_{ijk}(t)$ shows that the model for reversible intermolecular two-state excited-state processes with coupled rotational diffusion for a *cylindrically symmetric* ellipsoid is uniquely identifiable in terms of rate constants and rotational diffusion coefficients when the rotational diffusion of the two species is different. If the rotational characteristics of the two species are identical, a second set of rate constants (S2) is possible.

Now we consider the case where $f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = D_{ijk}(t)$ for a *spherically symmetric* rotor. As $D_l = D_{\|l} = D_{\perp l}$, the expression for $D_{l,2M}$ becomes independent of M and reduces to $D_{l,2M} = 6D_l$.

An identification analysis similar to that for the *cylindrically symmetric* ellipsoid also gives two solutions: (i) the set of alternative rate constants is the original set (S1, eq 28) and the alternative rotational diffusion coefficients are the original ones;

$$D_A{}^{+} = D_A \tag{44a}$$

$$D_B{}^{+} = D_B \tag{44b}$$

(ii) when the rotational diffusion coefficients of both species are the same ($D_A = D_B$), the set of alternative rate constants is described by eq 29 (S2) and the alternative rotational diffusion coefficients are the original ones ($D_A{}^{+} = D_B{}^{+} = D_A = D_B$).

For $D_{ijk}(t)$ the alternative $\mathbf{b}^{+}$ (eq 5a) and $\mathbf{c}^{+}$ (eq 5b) for $\mathbf{T}_M = t_{M,1}\mathbf{I}_2$ are given by

$$b_{lik,2M}{}^{+} = b_{lik,2M}/t_{M,1} \tag{45a}$$

$$c_{mj,2M}{}^{+} = t_{M,1}c_{mj,2M} \tag{45b}$$

with $l, m = $ A, B and $M = \pm2, \pm1, 0$.

Therefore, one has

$$b_{lik,2M}{}^{+}c_{mj,2M}{}^{+} = b_{lik,2M}c_{mj,2M} \tag{46}$$

The products of the spherical harmonics implicitly contained in eq 46 can simply be summed via the addition theorem,[22] yielding the second-order Legendre polynomial $P_2(\hat{\mathbf{a}}_l\cdot\hat{\mathbf{e}}_m)$ of the cosine of the angle between transition moments $\hat{\mathbf{a}}_l$ and $\hat{\mathbf{e}}_m$

$$\sum_{M=-2}^{2} b_{lik,2M}c_{mj,2M} = \frac{5}{4\pi}B_2C_2b_{lik}c_{mj}P_2(\hat{\mathbf{a}}_l\cdot\hat{\mathbf{e}}_m) \tag{47}$$

This theorem in combination with eq 46 and $b_{lik}{}^{+}c_{mj}{}^{+} = b_{lik}c_{mj}$ yield

$$P_2(\hat{\mathbf{a}}_l{}^{+}\cdot\hat{\mathbf{e}}_m{}^{+}) = P_2(\hat{\mathbf{a}}_l\cdot\hat{\mathbf{e}}_m) \tag{48}$$

with $l, m = $ A, B and where $\hat{\mathbf{a}}_l{}^{+}$ and $\hat{\mathbf{e}}_m{}^{+}$ denote alternative transition moments. This implies that all $P_2(\hat{\mathbf{a}}_l\cdot\hat{\mathbf{e}}_m)$ can be uniquely determined.

The normalized $\tilde{b}_{Aik,2M}{}^{+}$, $\tilde{b}_{Aik,2M}$, $\tilde{c}_{Aj,2M}{}^{+}$, and $\tilde{c}_{Aj,2M}$ are defined as

$$\tilde{b}_{Aik,2M}{}^{+} = b_{Aik,2M}{}^{+}/(b_{Aik,2M}{}^{+} + b_{Bik,2M}{}^{+}) \tag{49a}$$

$$\tilde{b}_{Aik,2M} = b_{Aik,2M}/(b_{Aik,2M} + b_{Bik,2M}) \tag{49b}$$

$$\tilde{c}_{Aj,2M}{}^{+} = c_{Aj,2M}{}^{+}/(c_{Aj,2M}{}^{+} + c_{Bj,2M}{}^{+}) \tag{49c}$$

$$\tilde{c}_{Aj,2M} = c_{Aj,2M}/(c_{Aj,2M} + c_{Bj,2M}) \tag{49d}$$

Use of these normalized elements in eqs 45a and 45b leads to

$$\tilde{b}_{Aik,2M}{}^{+} = \tilde{b}_{Aik,2M} \tag{50a}$$

$$\tilde{c}_{Aj,2M}{}^{+} = \tilde{c}_{Aj,2M} \tag{50b}$$

Substitution of eq 14 in eq 50a gives

$$\frac{Y_2^{M}(\hat{\mathbf{a}}_A^{+})}{Y_2^{M}(\hat{\mathbf{a}}_B^{+})} = \frac{Y_2^{M}(\hat{\mathbf{a}}_A)}{Y_2^{M}(\hat{\mathbf{a}}_B)} \tag{51}$$

so that the *ratio* of the spherical harmonics for the orientation of the absorption transition moments $\hat{\mathbf{a}}_A$ and $\hat{\mathbf{a}}_B$ is uniquely identified.

Similarly, substitution of eq 17 in eq 50b yields

$$\frac{Y_2^{M*}(\hat{\mathbf{e}}_A^{+})}{Y_2^{M*}(\hat{\mathbf{e}}_B^{+})} = \frac{Y_2^{M*}(\hat{\mathbf{e}}_A)}{Y_2^{M*}(\hat{\mathbf{e}}_B)} \tag{52}$$

implying that the *ratio* of the spherical harmonics for the orientation of the emission transition moments $\hat{\mathbf{e}}_A$ and $\hat{\mathbf{e}}_B$ is uniquely determined.

In conclusion, if the rotational diffusion coefficients of the two species are different, rotational diffusion joined with an intermolecular two-state excited-state process makes this model uniquely identifiable in terms of rate constants k, rotational diffusion constants D and normalized $\tilde{b}_{Aik,2M}$ and $\tilde{c}_{Aj,2M}$.

B. Reversible Intermolecular Two-State Excited-State Process with Added Quencher. The expressions for $\mathbf{A}_{Dk,2M}$ (eq 11 for polarized fluorescence) and $\mathbf{A}_{kl}$ (eq 22 for quenching) are *formally* equivalent. Therefore, we want to investigate if the role played by rotational diffusion in the polarized fluorescence measurements can be taken up by quenching. For $f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = Q_{ijkl}(t)$ we have that $\mathbf{A} = \mathbf{A}_{kl}$ (eq 22), $\mathbf{b} = \mathbf{b}_{ik}$ (eq 23), and $\mathbf{c} = \mathbf{c}_j$ (eq 24). Matrix $\mathbf{T}$ is given by eq 25.

The matrix multiplication of eq 4 yields

$$-t_1(k_{0A}^{+} + k_{BA}^{+}[X]_k + k_{QA}^{+}[Q]_l) + t_2 k_{BA}^{+}[X]_k = -t_1(k_{0A} + k_{BA}[X]_k + k_{QA}[Q]_l) + t_3 k_{AB} \tag{53a}$$

$$t_1 k_{AB}^{+} - t_2(k_{0B}^{+} + k_{AB}^{+} + k_{QB}^{+}[Q]_l) = -t_2(k_{0A} + k_{BA}[X]_k + k_{QA}[Q]_l) + t_4 k_{AB} \tag{53b}$$

$$-t_3(k_{0A}^{+} + k_{BA}^{+}[X]_k + k_{QA}^{+}[Q]_l) + t_4 k_{BA}^{+}[X]_k = t_1 k_{BA}[X]_k - t_3(k_{0B} + k_{AB} + k_{QB}[Q]_l) \tag{53c}$$

$$t_3 k_{AB}^{+} - t_4(k_{0B}^{+} + k_{AB}^{+} - k_{0B} - k_{AB}) = t_2 k_{BA}[X]_k + t_4(k_{QB}^{+} - k_{QB})[Q]_l \tag{53d}$$

Since the elements t_i ($i = 1, ..., 4$) of $\mathbf{T}$ are independent of $[X]_k$ and since $k_{BA} \neq 0$, we have from eq 53d that $t_2 = 0$. Furthermore, since the elements t_i also are independent of $[Q]_l$, we have from eq 53d that $k_{QB}^{+} = k_{QB}$ (the alternative, $t_4 = 0$, would lead to the null $\mathbf{T}$ matrix). Thus, the set of equations in eq 53 is simplified to the following set (eq 54):

$$-t_1(k_{0A}^{+} - k_{0A}) - t_3 k_{AB} = t_1(k_{BA}^{+} - k_{BA})[X]_k + t_1(k_{QA}^{+} - k_{QA})[Q]_l \tag{54a}$$

$$t_1 k_{AB}^{+} = t_4 k_{AB} \tag{54b}$$

$$-t_3(k_{0A}^{+} - k_{0B} - k_{AB}) = [t_1 k_{BA} + (t_3 - t_4) k_{BA}^{+}][X]_k + t_3(k_{QA}^{+} - k_{QB})[Q]_l \tag{54c}$$

$$t_3 k_{AB}^{+} = t_4(k_{0B}^{+} + k_{AB}^{+} - k_{0B} - k_{AB}) \tag{54d}$$

Since $\mathbf{T}$ is independent of $[X]_k$, we have from eq 54a that $k_{BA}^{+} = k_{BA}$ (the alternative, $t_1 = 0$, would lead to the null $\mathbf{T}$ matrix), and from eq 54c, $t_1 = t_4 - t_3$. Moreover, since $\mathbf{T}$ is independent of $[Q]_l$, we have from eq 54a that $k_{QA}^{+} = k_{QA}$. From eq 54c, it is evident that two cases have to be considered to ensure that $\mathbf{T}$ is independent of $[Q]_l$: either $t_3 = 0$ or $k_{QA}^{+} = k_{QB}$. Now the set of eqs 54 is reduced to the set of eqs 26. (i) If $t_3 = 0$, from eq 54a we obtain $k_{0A}^{+} = k_{0A}$, from eq 54b $k_{AB}^{+} = k_{AB}$, and from eq 54d $k_{0B}^{+} = k_{0B}$. Hence, the alternative set of rate constants equals the original set (S1, eq 28) with $k_{QA}^{+} = k_{QA}$ and $k_{QB}^{+} = k_{QB}$. This set corresponds to $\mathbf{T} = t_1 \mathbf{I}_2$. (ii) If alternatively $t_3 \neq 0$ we have $k_{QA}^{+} = k_{QB}$. In combination with $k_{QA}^{+} = k_{QA}$, this yields $k_{QA} = k_{QB}$. Equation 54c produces $k_{0A}^{+} = k_{0B} + k_{AB}$, and from eqs 54a and 54b we have $k_{AB}^{+} = k_{0A} - k_{0B}$ and substituting eq 54b into eq 54d yields $k_{0B}^{+} = k_{0B}$. To summarize, if the quenching rate constants are identical ($k_{QA} = k_{QB}$), the alternative set of rate constants is given by set S2 (eq 29) and $k_{QA}^{+} = k_{QB}^{+} = k_{QA} = k_{QB}$. In that case, $\mathbf{T}$ is given by eq 27 with $t_3/t_1 = (k_{0A} - k_{0B} - k_{AB})/k_{AB}$. Since all rate constants should be positive, this set is only possible when $k_{0A} > k_{0B}$.

For $S_{ijk}(t)$ the alternative $\mathbf{b}^{+}$ (eq 5a) and $\mathbf{c}^{+}$ (eq 5b) for $\mathbf{T} = t_1\mathbf{I}_2$ are given by

$$\mathbf{b}^{+} = \mathbf{b}/t_1 \tag{55a}$$

$$\mathbf{c}^{+} = t_1\mathbf{c} \tag{55b}$$

Use of normalized elements in eqs 55 leads to unique normalized $\tilde{b}_{Aik}$ and $\tilde{c}_{Aj}$: $\tilde{b}_{Aik}^{+} = \tilde{b}_{Aik}$ and $\tilde{c}_{Aj}^{+} = \tilde{c}_{Aj}$. Hence, addition of quencher to a reversible intermolecular two-state excited-state process makes this model uniquely identifiable in terms of rate constants and normalized $\tilde{b}_{Aik}$ and $\tilde{c}_{Aj}$ if the quenching rate constants are different. Quenching takes up the role played by rotational diffusion in polarized measurements.

5. Discussion and Conclusions

We have demonstrated that the similarity transformation approach can be applied successfully in the identifiability study of models of reversible intermolecular two-state excited-state processes with (i) coupled species-dependent rotational diffusion described by Brownian reorientation, and with (ii) added quencher. The results obtained are in perfect agreement with the deterministic identifiability studies using Markov parameters and elementary functions of the rate and diffusion constants.[14,18] The similarity transformation approach has the additional advantage of providing the explicit relationship between the true and alternative model parameters.

In the case of coupled species-dependent rotational diffusion the sum $S(t) = I_{\|}(t) + 2\,I_{\perp}(t)$ and the difference $D(t) = I_{\|}(t) - I_{\perp}(t)$ are used in the identifiability. The sum curve $S(t)$ describes the time dependence of the total fluorescence and contains information only on the excited states as a whole. In the difference curve $D(t)$, the rotational kinetic behavior interacts closely with the overall excited-state kinetics. Because of the clear dependence of $S(t)$ and $D(t)$ on $\mathbf{A}$, $\mathbf{b}$, and $\mathbf{c}$, the identifiability analysis is simpler if one uses the $S(t)$ and $D(t)$ functions instead of $I_{\|}(t)$ and $I_{\perp}(t)$. If the rotational diffusion constants of both species are different, coupling the rotational diffusion with the overall excited-state kinetics makes the model globally identifiable in terms of the rate constants and the rotational diffusion constants. In that case, inclusion of polarization as an experimental coordinate abrogates the need for the extra experimental coordinate supplied heretofore by addition of a quenching agent. The role of quenching is taken up by the diffusion constants [compare the matrices $\mathbf{A}_{Dk,2M}$ (eq 11) and $\mathbf{A}_{kl}$ (eq 22)]. It has to be emphasized that the rotational characteristics of the individual rotors can be obtained even when they cannot be directly separated because of the excited-state reaction.

The model of reversible intermolecular two-state excited-state processes with species-dependent rotational diffusion may well be applicable to a wide range of molecular and biomolecular systems, where fast kinetics of reversible processes are of interest. The change brought about by the excited-state process involving the coreactant leads in general to a change in size and shape of the rotating unit containing the fluorophore. Relatively small changes of this kind (on the order of factors of two in the principal rotational diffusion constants) are expected for excimers and exciplexes. Another application is the reversible interaction between a ligand and a receptor. The fluorescent receptor may be (i) a fluorescent probe and of comparable size and molecular weight to the ligand or (ii) it can be a macromolecule, most commonly a protein. In case i, only relatively small changes in the effective rotational unit, either in size or shape, may be expected, even for ligands of comparable size to the receptor. In case ii, when the fluorescent moiety is the ligand and relatively small compared to the macromolecular receptor, these changes may be very large. An application in the field of biochemistry involves the binding of a small fluorescent molecule by intercalation into double-helical regions of a nucleic acid.

In the literature some systems have been described with intramolecular rearrangements of the excited-state species upon interconversion.[23] An identifiability analysis similar to the one described here can be performed for these intramolecular two-state excited-state processes and will be reported elsewhere.

To conclude, the model of reversible intermolecular two-state excited-state processes becomes uniquely identifiable when (i) a monoexponential fluorescence δ-response function $f(t)$ at $[\mathrm{X}]_k = 0$ is used together with the biexponential $S(t)$, (ii) quencher is added to this photophysical system and the quenching rate constants of both excited-state species are different, and (iii) the polarized fluorescence δ-response functions $I_{\parallel}(t)$ and $I_{\perp}(t)$ are analyzed at different coreactant concentrations $[\mathrm{X}]_k$.

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Appendix

Here we show that the transformation matrix **T** for matrix $\mathbf{A}_{Dk}$ takes the form given in eq 34.

The similarity transformation expressed in eq 3 essentially defines a transformation of basis vectors in a vector space **V**. An operator $\mathscr{A}$ defined on the vector space **V** can be represented by the matrix **A** assuming a set of basis vectors $\{e_i\}$ of the vector space **V**. If we assume another basis, e.g., $\{f_i\}$, the operator $\mathscr{A}$ is represented by another matrix, e.g., $\mathbf{A}^+$. The matrices **A** and $\mathbf{A}^+$ are related by an expression of the type given by eq 3. The matrix **T** defines the transition matrix from the basis $\{e_i\}$ to the basis $\{f_i\}$. When **A** is a block diagonal matrix, there are subspaces, e.g., W_i, in the vector space **V** which are mapped onto itself under the action of the operator $\mathscr{A}$. These subspaces W_i are said to be invariant under the operator $\mathscr{A}$.

For the matrix $\mathbf{A}_{Dk}$ there are 5 subspaces each of dimension 2. It can be shown[18] that the eigenvalues of $\mathbf{A}_{Dk}$ can be properly paired and labeled with the correct value of M. The subspaces corresponding to the paired eigenvalues can then be labeled also, so that one obtains $\{W_M | M = -2, -1, 0, 1, 2\}$. Because $\mathbf{A}_{Dk,2M} = \mathbf{A}_{Dk,2-M}$ the subspaces W_M and W_{-M} can be swapped.

When also $\mathbf{A}_{Dk}^+$ is a block diagonal matrix, the matrix **T** maps basis vectors of W_M onto W_{M^*}. Since the eigenvalues of $\mathbf{A}_{Dk}^+$ can also be properly paired and labeled, one has that $M = |M^*|$. Therefore, **T** is a block diagonal matrix.

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