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Identifiability via the Method of Similarity Transformation of Models for Reversible Intermolecular Two-State Excited-State Processes with Species-Dependent Rotational Diffusion or with Added Quencher

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Abstract

Deterministic identifiability analyses via similarity transformation 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 (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 $I_{\parallel}(t)$ 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 $S(t) = I_{\parallel}(t) + 2 I_{\perp}(t)$ and the difference $D(t) = I_{\parallel}(t) - I_{\perp}(t)$ 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 (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. The explicit relationships between the true and alternative model parameters are obtained.

1. Introduction

Compartmental modeling has been and is being used extensively in biology, physiology, pharmacokinetics, ecology and engineering, and a large number of applications has been reported (see, for example, references 1, 2, 3). In view of this general interest, it is rather amazing that compartmental modeling of excited-state processes in photophysics has started relatively late.^{4, 5, 6} Since the relaxation of excited-state processes can in many instances be described by a system of coupled linear differential equations, excited-state systems are *formally* equivalent with compartmental systems.

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 photo-excitation. 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 photo-excitation, it suffices to consider the excited-state compartments.

Deterministic identifiability deals with the determination of the parameters of a given model assuming error-free observations.¹⁻³ There are three possible outcomes to the identifiability analysis. (1) The parameters of an assumed model can be estimated

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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 linear, time-invariant models with two or three excited-state compartments commonly encountered in photophysics, the parameters to be identified are the rate constants and spectral parameters related to excitation and emission.

Since the first identifiability analysis of an intermolecular two-state excited-state process, 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 reference 7 for literature data). The identifiability analyses of reversible intermolecular two-state excited-state processes in the absence^{7, 8, 9} and presence¹⁰ 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).^{2, 3} The initial approach⁸⁻¹⁰ 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 used similarity transformations.^{2, 3, 11, 12} The method of similarity transformation offers a direct way of determining if 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 intermolecular 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 complex¹³ 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 14 and references therein). Chuang and Eisenthal 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.¹⁵ and by Limpouchová and Procházka.¹⁶ Based on the theory reported in these papers,^{13, 15, 16} a compartmental description was derived for the fluorescence anisotropy decay of intermolecular twostate excited-state processes together with species-dependent rotational diffusion.

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 speciesdependent rotational diffusion is presented for cylindrically symmetrical ellipsoids. The δ -response functions, $I_{\parallel}(t)$ 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 $S(t) = I_{\parallel}(t) + 2 I_{\perp}(t)$ and the difference $D(t) = I_{\parallel}(t) - I_{\perp}(t)$ 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.

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:⁸

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

where **b** is a column vector of dimension *N* whose elements are the initial concentrations of each excited-state compartment ("input"); **c** is a $1 \times N$ vector related to the contribution

of each compartment to the emission ("output" or "observation"); **A** is a $N \times N$ matrix ("compartmental matrix") containing the kinetic information ("transfer coefficients") of all processes. In other words, 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 deterministic identification (or identifiability) study investigates whether it is possible to find different realizations of the fluorescence δ -response function f(t), say (**A**, **b**, **c**) and (**A**⁺, **b**⁺, **c**⁺), so that

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

In other words, the fluorescence δ -response function should be the same for the true (**A**, **b**, **c**) and the alternative (**A**⁺, **b**⁺, **c**⁺) model parameter set.^{2, 3} Global identifiability is achieved when **A**⁺ = **A**, **b**⁺ = **b**, and **c**⁺ = **c** (i.e., a unique set of model parameters is obtained). The model is locally identifiable when there is a limited set of alternative **A**⁺, **b**⁺, and **c**⁺. An unidentifiable model is found when there are an infinite number of alternative **A**⁺, **b**⁺, and **c**⁺. The specific definitions of the compartmental matrix **A**, the excitation coefficients **b**, and the emission coefficients **c** are given in Section 3. This formulation (eq 2) is appropriate for most systems found in biomedicine, pharmacokinetics, ecosystem modeling, and engineering,¹⁻³ but is not suitable for photophysical systems where absolute values for **b** and **c** cannot be obtained. Normalized "input" and "output" vectors will be used instead, as discussed in Section 4.

An excellent method of constructing another (alternative) realization (\mathbf{A}^+ , \mathbf{b}^+ , \mathbf{c}^+) of f(t) is via similarity transformation,^{2, 3, 7, 11, 12} giving

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

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

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 co-reactant 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 **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} , **T** should be independent of $[X]_{k}$, $[Q]_{l}$, and λ_{i}^{em} , **T** should be independent of $[X]_{k}$, $[Q]_{l}$, and λ_{i}^{em} .

3. Kinetics

A. Reversible intermolecular two-state excited-state process with speciesdependent rotational diffusion

Insert Figure 1

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. Photo-

excitation of the system produces the excited-state species A^{*} and B^{*} which can decay by fluorescence (k_F) and non-radiative (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 co-reactant 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_{\parallel} 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 $I_{\parallel ijk}(t)$ 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 $I_{\perp iik}(t)$ for the perpendicularly polarized component can be expressed, in the case of pure transitions and *isotropic* solutions, as:¹⁷

$$I_{\parallel_{ikj}}(t) = \frac{1}{3} S_{ikj}(t) \left[1 + 2r_{ikj}(t) \right] = \frac{1}{3} S_{ikj}(t) + \frac{2}{3} D_{ikj}(t)$$
(6a)

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

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

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

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

The subscripts *i*, *j*, and *k* in $I_{\parallel ijk}(t)$ 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 co-reactant concentration [X]_k, respectively.

Matrix $A_{k,00}$ in eq 7 is given by eq 9:

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

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

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

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

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

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

$$D_{l,2M} = 6 D_{\perp l} + M^2 \left(D_{\parallel l} - D_{\perp l} \right)$$
(12)

Note the invariance of eq 11 and eq 12 to the sign of M

 $D_{\perp l}$ and $D_{\parallel l}$ (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,¹⁴ reducing to the unique component D_l (= $D_{\perp l} = D_{\parallel l}$) in the case of the *spherically symmetric* rotor *l*.

For a *spherically symmetric* rotor ($D_l = D_{\perp l} = D_{\parallel l}$), 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:

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

Vector $\mathbf{b}_{ik,LM}$ [with L = M = 0 (eq 7), or L = 2 and $M = \pm 2, \pm 1, 0$ (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 co-reactant concentration [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 $Y_L^M(\hat{\mathbf{a}}_l)^{18}$ 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 :

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

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

For L = M = 0, we have $Y_0^0(\hat{\mathbf{a}}_l) = \frac{1}{\sqrt{4\pi}}$ and $b_{lik,00} = \frac{b_{lik}}{24\pi^2}$.

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

$$\mathbf{b}_{ik,00} = \begin{bmatrix} b_{\mathrm{A}ik,00} & b_{\mathrm{B}ik,00} \end{bmatrix}^{\mathrm{T}}$$
(15)

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

$$\mathbf{b}_{ik,2M} = \begin{bmatrix} b_{Aik,2-2} & b_{Bik,2-2} & b_{Aik,2-1} & b_{Bik,2-1} & b_{Aik,20} & b_{Bik,20} & b_{Aik,21} & b_{Bik,21} & b_{Aik,22} & b_{Bik,22} \end{bmatrix}^{\mathrm{T}}$$
(16)

Vector $\mathbf{c}_{j,LM}$ [with L = M = 0 (eq 7), or L = 2 and $M = \pm 2, \pm 1, 0$ (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:

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

where $C_0 = \frac{16}{3}\sqrt{\pi^5}$, $C_2 = \frac{16}{15}\sqrt{\frac{\pi^5}{5}}$, and $Y_L^{M^*}(\hat{\mathbf{e}}_m)$ is the complex conjugate of the

appropriate spherical harmonic for the orientation of the emission transition moment $\hat{\mathbf{e}}_m$ in the molecular frame.

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

The coefficient c_{mj} is defined as:

$$c_{mj} = k_{\rm Fm} \int_{\Delta\lambda_j^{\rm em}} \rho_m \left(\lambda_j^{\rm em}\right) d\lambda^{\rm em}$$
(18)

where k_{Fm} is the fluorescence rate constant of species m^* , the subscript *j* refers to the observation wavelength range, $\Delta \lambda_j^{\text{em}}$, and $\rho_m(\lambda_j^{\text{em}})$ 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:

$$\mathbf{c}_{j,2M} = \begin{bmatrix} c_{Aj,2-2} & c_{Bj,2-2} & c_{Aj,2-1} & c_{Bj,2-1} & c_{Aj,20} & c_{Bj,20} & c_{Aj,21} & c_{Bj,21} & c_{Aj,22} & c_{Bj,22} \end{bmatrix}$$
(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

Insert Scheme 1

Consider the molecular system (see Scheme 1) with an equilibrium between two different species A and B in the ground state which form upon photo-excitation the excited-state species A^{*} and B^{*}, respectively. The deactivation of these excited-state species via fluorescence and non-radiative 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 co-reactant concentration $[X]_k$ and quencher concentration $[Q]_l$, monitored at emission wavelength λ_i^{em} due to excitation at λ_i^{ex} can be expressed in matrix notation:

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

with \mathbf{A}_{kl} given by eq 22:

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

 \mathbf{b}_{ik} and \mathbf{c}_i are given by eqs 23 and 24, respectively:

$$\mathbf{b}_{ik} = \begin{bmatrix} b_{\mathrm{A}ik} & b_{\mathrm{B}ik} \end{bmatrix}^{\mathrm{T}}$$
(23)

$$\mathbf{c}_{j} = \begin{bmatrix} c_{Aj} & c_{Bj} \end{bmatrix}$$
(24)

4. Identifiability analysis

A. Reversible intermolecular two-state excited-state process with speciesdependent 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's 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:

$$\mathbf{T} = \begin{bmatrix} t_1 & t_2 \\ t_3 & t_4 \end{bmatrix}$$
(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. Therefore, we refer to reference 7 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 \mathbf{T} are independent of $[\mathbf{X}]_k$ and since $k_{BA} \neq 0$, we have that $t_2 = 0$ and $t_1 \left(k_{BA}^+ - k_{BA} \right) = 0$. If $t_1 = 0$, then also t_3 and t_4 have to be zero and \mathbf{T} becomes the null matrix, which is not a valid transformation matrix. From the alternative, $k_{BA}^+ = k_{BA}$, we have that $t_4 = t_1 + t_3$, so that the matrix multiplication in eq 4 yields a set of four equations as a function of t_1 and t_3 (eqs 26):

$$-t_1 k_{0A}^+ = -t_1 k_{0A} + t_3 k_{AB}$$
(26a)

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

$$t_{3}\left(k_{0B}+k_{AB}-k_{0A}^{+}\right)=0$$
(26c)

$$t_{3}k_{AB}^{+} = (t_{1} + t_{3})(k_{0B}^{+} + k_{AB}^{+} - k_{0B} - k_{AB})$$
(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

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

with $t_3 \neq 0$.

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

$$k_{0A}^{+} = k_{0A} \tag{28a}$$

$$k_{\rm AB}^+ = k_{\rm AB} \tag{28b}$$

$$k_{\rm BA}^+ = k_{\rm BA} \tag{28c}$$

$$k_{0\mathrm{B}}^{+} = k_{0\mathrm{B}} \tag{28d}$$

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

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

$$k_{\rm AB}^{+} = k_{\rm 0A} - k_{\rm 0B} \tag{29b}$$

$$k_{\rm BA}^+ = k_{\rm BA} \tag{29c}$$

$$k_{0\mathrm{B}}^{+} = k_{0\mathrm{B}} \tag{29d}$$

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

Now we will show that the ambiguity in the rate constants (i.e., two possible sets) can be resolved by a mono-exponential f(t) at $[X]_k = 0$. Indeed, the fluorescence δ -response function f(t) becomes mono-exponential for $[X]_k = 0$, with decay rate constant k_{0A} . From this mono-exponential f(t), we have that $k_{0A}^+ = k_{0A}$. Equation 26a leads then to $t_3 = 0$ and from eq 26b we have $k_{AB}^+ = k_{AB}$. From eq 26d we obtain $k_{0B}^+ = k_{0B}$, so that the original set is obtained ($\mathbf{T} = t_1 \mathbf{I}_2$). To conclude, if the mono-exponential δ -response function f(t) at $[X]_k = 0$ 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}_{Aik} ($\tilde{b}_{Bik} = 1 - \tilde{b}_{Aik}$) and \tilde{c}_{Aj} ($\tilde{c}_{Bj} = 1 - \tilde{c}_{Aj}$) 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

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

$$\mathbf{c}_{mj,00}^{+} = t_1 \, \mathbf{c}_{mj,00} \tag{31}$$

Let's define the normalized $\tilde{b}_{Aik,00}^+$, $\tilde{b}_{Aik,00}^-$, $\tilde{c}_{Aj,00}^+$, and $\tilde{c}_{Aj,00}^-$:

$$\widetilde{b}_{Aik,00}^{+} = b_{Aik,00}^{+} / \left(b_{Aik,00}^{+} + b_{Bik,00}^{+} \right)$$
(32a)

$$\tilde{b}_{Aik,00} = b_{Aik,00} / (b_{Aik,00} + b_{Bik,00})$$
(32b)

$$\widetilde{c}_{Aj,00}^{+} = c_{Aj,00}^{+} / \left(c_{Aj,00}^{+} + c_{Bj,00}^{+} \right)$$
(32c)

$$\widetilde{c}_{Aj,00} = c_{Aj,00} / (c_{Aj,00} + c_{Bj,00})$$
(32d)

Use of these normalized elements in eq 30 leads to

$$\widetilde{b}_{Aik,00}^{+} = \widetilde{b}_{Aik}$$
 and $\widetilde{b}_{Bik,00}^{+} = \widetilde{b}_{Bik}$ (33a)

Analogously, eq 31 gives

$$\widetilde{c}_{Aj,00}^{+} = \widetilde{c}_{Aj}$$
 and $\widetilde{c}_{Bj,00}^{+} = \widetilde{c}_{Bj}$ (33b)

Equation 33 shows that the normalized $\tilde{b}_{Aik,00} = \tilde{b}_{Aik}$ and $\tilde{c}_{Aj,00} = \tilde{c}_{Aj}$ are unique. The use of normalized \tilde{b}_{Aik} and \tilde{c}_{Aj} in global compartmental analysis^{4, 5, 8} allows \tilde{b}_{Aik} to be linked at the same co-reactant concentration [X]_k and excitation wavelength λ_i^{em} , whereas \tilde{c}_{Aj} can be linked at the same emission wavelength λ_j^{em} .

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):

$$\mathbf{T} = \begin{bmatrix} \mathbf{T}_{.2} & 0 & 0 & 0 & 0 \\ 0 & \mathbf{T}_{.1} & 0 & 0 & 0 \\ 0 & 0 & \mathbf{T}_{0} & 0 & 0 \\ 0 & 0 & 0 & \mathbf{T}_{1} & 0 \\ 0 & 0 & 0 & 0 & \mathbf{T}_{2} \end{bmatrix}$$
(34)

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

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

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

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

$$k_{\rm AB}^+ = k_{\rm AB} \tag{37b}$$

$$k_{\rm BA}^+ = k_{\rm BA} \tag{37c}$$

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

and set D2 (eq 38):

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

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

$$k_{\rm BA}^+ = k_{\rm BA} \tag{38c}$$

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

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

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

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

To solve for the individual k_{0A}^+ , k_{AB}^+ , k_{BA}^+ , k_{0B}^+ , $D_{\perp A}^+$, $D_{\parallel A}^+$, $D_{\perp B}^+$, and $D_{\parallel B}^+$, one should combine the equations describing sets S1 (eq 28) and S2 (eq 29) with the equations describing sets D1 (eq 37 with $M = \pm 2, \pm 1, 0$) and D2 (eq 38 with $M = \pm 2, \pm 1, 0$). $k_{BA}^+ = k_{BA}$ 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 sixteen combinations of eqs 28, 29, 37, and 38 are possible (e.g., S1, D1 for $M = 0, \pm 2$, D2 for $M = \pm 1$; S2, D2 for M = 0, D1 for $M = \pm 1, \pm 2$; etc.). However, of the sixteen 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 $M = \pm 2, \pm 1, 0$). 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 $M = \pm 2, \pm 1, 0$), 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}^+ , $D_{\perp A}^+$, $D_{\perp B}^+$, and $D_{\parallel B}^+$. Solution of this overdetermined set of equations yields the original set of rotational diffusion coefficients:

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

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

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

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

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

Let's now examine the second possible combination (S2 and D2). Equations 29b and 38b lead to $D_{A,2M} = D_{B,2M}$ for $M = \pm 2, \pm 1, 0$, yielding

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

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

From eqs 29a and 38a we have $D_{A,2M}^+ = D_{B,2M}$ for $M = \pm 2, \pm 1, 0$, yielding

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

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

From eqs 29d and 38d we have $D_{B,2M}^+ = D_{B,2M}$ for $M = \pm 2, \pm 1, 0$, yielding

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

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

If the rotational diffusion coefficients of both species are equal $(D_{\perp A} = D_{\perp B})$ and $D_{\parallel A} = D_{\parallel B}$, 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 $D_{\parallel A}^{+} = D_{\parallel B}^{+} = D_{\parallel A} = D_{\parallel B}$).

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.

Let's now consider the case where $f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = D_{ijk}(t)$ for a *spherically symmetric* rotor. As $D_l = D_{\parallel l} = D_{\perp l}$, the expression for $D_{l,2M}$ becomes independent of M and reduces to $D_{l,2M} = 6 D_l$.

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;

$$D_{\rm A}^+ = D_{\rm A} \tag{44a}$$

$$D_{\rm B}^+ = D_{\rm B} \tag{44b}$$

(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

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

$$c_{mj,2M}^{+} = t_{M,1} c_{mj,2M}$$
(45b)

with *l*, m = A, B and $M = \pm 2, \pm 1, 0$.

Therefore one has that

$$b_{lik,2M}^{+} c_{mj,2M}^{+} = b_{lik,2M} c_{mj,2M}$$
(46)

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

$$\sum_{M=-2}^{2} b_{lik,2M} c_{mj,2M} = \frac{5}{4\pi} B_2 C_2 b_{lik} c_{mj} P_2 (\hat{\mathbf{a}}_l \cdot \hat{\mathbf{e}}_m)$$
(47)

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

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

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

$$\widetilde{b}_{Aik,2M} = b_{Aik,2M} / \left(b_{Aik,2M} + b_{Bik,2M} \right)$$
(49b)

$$\widetilde{c}_{Aj,2M}^{+} = c_{Aj,2M}^{+} / \left(c_{Aj,2M}^{+} + c_{Bj,2M}^{+} \right)$$
(49c)

$$\widetilde{c}_{Aj,2M} = c_{Aj,2M} / \left(c_{Aj,2M} + c_{Bj,2M} \right)$$
(49d)

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

$$\widetilde{b}_{Aik,2M}^{+} = \widetilde{b}_{Aik,2M}$$
(50a)

$$\widetilde{c}_{Aj,2M}^{+} = \widetilde{c}_{Aj,2M}$$
(50b)

Substitution of eq 14 in eq 50a gives

$$\frac{Y_2^M\left(\hat{\mathbf{a}}_{\mathrm{A}}^{+}\right)}{Y_2^M\left(\hat{\mathbf{a}}_{\mathrm{B}}^{+}\right)} = \frac{Y_2^M\left(\hat{\mathbf{a}}_{\mathrm{A}}\right)}{Y_2^M\left(\hat{\mathbf{a}}_{\mathrm{B}}\right)}$$
(51)

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

Similarly, substitution of eq 17 in eq 50b yields

$$\frac{Y_2^{M^*}(\hat{\mathbf{e}}_{\rm A}^+)}{Y_2^{M^*}(\hat{\mathbf{e}}_{\rm B}^+)} = \frac{Y_2^{M^*}(\hat{\mathbf{e}}_{\rm A})}{Y_2^{M^*}(\hat{\mathbf{e}}_{\rm B})}$$
(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 $\tilde{c}_{Ai,2M}$.

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 **T** is given by eq 25.

The matrix multiplication of eq 4 yields

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

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

$$-t_{3}\left(k_{0A}^{+}+k_{BA}^{+}[X]_{k}+k_{QA}^{+}[Q]_{l}\right)+t_{4}k_{BA}^{+}[X]_{k}=t_{1}k_{BA}[X]_{k}-t_{3}\left(k_{0B}+k_{AB}+k_{QB}[Q]_{l}\right)$$
(53c)

$$t_{3}k_{AB}^{+} - t_{4}\left(k_{0B}^{+} + k_{AB}^{+} - k_{0B} - k_{AB}\right) = t_{2}k_{BA}\left[X\right]_{k} + t_{4}\left(k_{QB}^{+} - k_{QB}\right)\left[Q\right]_{l}$$
(53d)

Since the elements t_i (i = 1, ..., 4) of **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 **T** matrix). Thus, the set of eqs 53 is simplified to the following set (eqs 54):

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

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

$$-t_{3}(k_{0A}^{+}-k_{0B}-k_{AB}) = [t_{1}k_{BA} + (t_{3}-t_{4})k_{BA}^{+}][X]_{k} + t_{3}(k_{QA}^{+}-k_{QB})[Q]_{l}$$
(54c)

$$t_{3}k_{AB}^{+} = t_{4}\left(k_{0B}^{+} + k_{AB}^{+} - k_{0B} - k_{AB}\right)$$
(54d)

Since **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 **T** matrix) and from eq 54c $t_1 = t_4 - t_3$. Moreover, since **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 **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 $k_{QA}^+ = k_{QB}^+ = k_{QB} = k_{QB}$. In that case, **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

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

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

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 twostate 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.^{10, 14} The similarity transformation approach has the additional advantage of providing the explicit relationship between the true and alternative model parameters.

We have shown via the method of similarity transformation that the model of reversible intermolecular two-state excited-state processes becomes uniquely identifiable when a mono-exponential fluorescence δ -response function f(t) at $[X]_k = 0$ is used together with the bi-exponential S(t). A second strategy to obtain a uniquely identified model is when quencher is added to this photophysical system and the quenching rate constants of both excited-state species are different. In both cases all rate constants and the normalized spectral parameters are uniquely determined. A third possibility is by using the polarized fluorescence δ -response functions $I_{\parallel}(t)$ and $I_{\perp}(t)$. These functions are used to define the sum $S(t) = I_{\parallel}(t) + 2 I_{\perp}(t)$ and the difference $D(t) = I_{\parallel}(t) - I_{\perp}(t)$ function. 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 A, b, and c, the identifiability analysis is simpler if one uses the S(t)and D(t) functions instead of $I_{\parallel}(t)$ 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 $A_{Dk,2M}$ (eq 11) and A_{kl} (eq 22)].

The model of reversible intermolecular two-state excited-state processes with speciesdependent 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 co-reactant 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.¹⁹ 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.

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Appendix

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

For the matrix \mathbf{A}_{Dk} there are 5 subspaces each of dimension 2. It can be shown¹⁴ 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 also 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.

Figure captions

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 co-reactant 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.

Scheme 1. Scheme representing a reversible intermolecular two-state excited-state process with added quencher. 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.

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