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Identifiability of Models for Intramolecular Two-State Excited-State Processes with Added Quencher and Coupled Species-Dependent Rotational Diffusion

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The parameters describing the kinetics of excited-state processes can possibly be recovered by analysis of the fluorescence decay surface measured as a function of the experimental variables. The identifiability analysis of a photophysical model assuming errorless time-resolved fluorescence data can verify whether the model parameters can be determined and may lead to the minimal experimental conditions under which this is possible. In this work, we used the method of similarity transformation to investigate the identifiability of three kinetic models utilized to describe the time-resolved fluorescence of reversible intramolecular two-state excited-state processes in isotropic environments: (1) model without added quencher, (2) model with added quencher, (3) model with added quencher coupled with species-dependent rotational diffusion described by Brownian reorientation. Without a priori information, model 1 is not identifiable. For model 2, two sets of quenching rate constants and combinations of excited-state deactivation/exchange rate constants are possible, but they cannot be allocated to a specific excited-state species. For both sets, upper and lower limits on the excited-state deactivation/exchange rate constants can be obtained. For model 3, 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 parallel and perpendicular, respectively, to the electric vector of linearly polarized excitation, are used to define the sum $S(t) \equiv I_{\|}(t) + 2\,I_{\perp}(t)$ and the difference $D(t) \equiv I_{\|}(t) - I_{\perp}(t)$. The identifiability analysis is performed using the $S(t)$ and $D(t)$ functions. Also for model 3, two sets of kinetic parameters (i.e., quenching rate constants, combinations of deactivation/exchange rate constants, and rotational diffusion coefficients) exist, but these parameters cannot be assigned unequivocally to a specific species. For the three models, an infinite number of alternative spectroscopic parameters associated with excitation and emission are found.

1. Introduction

Time-resolved fluorescence measurements are commonly used to unravel the kinetics of excited-state processes. The time evolution of polarized fluorescence provides additional information about the molecular system as compared with total fluorescence decay. Time-resolved fluorescence depolarization experiments not only provide information about the rotational dynamics of fluorophores but also allow the study of, for example, intramolecular excitation energy migration.[1]

To decide on the most suitable model to describe a specific photophysical system, fluorescence decay traces are usually measured under various experimental conditions. For the models of *reversible* intramolecular two-state excited-state processes considered in this paper, the experimental variables are the excitation and emission wavelengths, quencher concentration, and polarization. In many instances, the fluorescence response after a short excitation pulse can be analyzed in terms of a limited number of decay times τ and their associated amplitudes α. However, the empirical parameters $\{\tau, \alpha\}$ are not the primary parameters of interest. The more fundamental underlying parameters are kinetic (rate constants for deactivation, exchange, and quenching; rotational diffusion coefficients) and spectral parameters related to excitation and emission.

When a specific model is proposed for the description of excited-state processes, one should find out first if the kinetic and spectral parameters defining the model can be determined from error-free time-resolved fluorescence data. This is the topic of the deterministic identifiability (or identification) analysis.[2−4] Such an analysis informs us which information is theoretically accessible from the fluorescence decay surface and the type of experiments that have to be performed to extract this information from the data surface.

As the time evolution of fluorescence emanating from excited-state processes can in many instances be described by a system of coupled linear differential equations of first order, modeling of excited-state processes can be done within the framework of compartmental analysis. Indeed, excited-state systems are formally equivalent to compartmental systems described in other fields of research (see, for example, refs 2−4). Considering the widespread use of compartmental analysis, it seems rather surprising that compartmental modeling of excited-state processes in photophysics has emerged relatively recently.[5−7]

Now let us define the term "compartment" in a photophysical perspective. In photophysics, a compartment is defined as a subsystem composed of a distinct type of species that acts

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kinetically in a unique way. The concentrations of the constituent species can change by the exchange of material between compartments through intramolecular or intermolecular processes. In the context of compartmental modeling of excited-state processes, compartments can be divided into two kinds, ground and excited-state, depending upon the state of the species concerned. There may be inputs from ground-state compartments into one or more of the excited-state compartments by photoexcitation. There is always output from the excited-state compartments to the ground-state compartments through deactivation. If the concentrations of the species in the ground state do not change significantly upon photoexcitation, it suffices to consider the excited-state compartments.

After the first identification of an intermolecular two-state excited-state process,[7] identifiability studies of a broad range of models of intermolecular as well as intramolecular two-state and three-state excited-state processes in isotropic solution have been reported (see ref 8 for literature data). The identifiability analyses of reversible intramolecular two-state excited-state processes, both in the absence[9,10] and in the presence[11−13] of quencher, have been confined to consideration of the whole excited-state population, as monitored by total fluorescence intensity observed at the “magic angle”. In the wide field of time-resolved fluorescence spectroscopy, only a relatively small part of the literature has been devoted to examining the problem of excited-state processes linked to species-dependent rotational diffusion. The explicit expressions describing the time-resolved fluorescence anisotropy of two-state excited-state processes coupled with species-dependent rotational diffusion are rather elaborate, even without transient effects.[14−17] The theoretical work, which formed the basis for these expressions, was first described by Chuang and Eisenthal.[18]

This paper provides the first study of the identifiability of *reversible* intramolecular two-state excited-state processes coupled with species-dependent rotational diffusion. A separate identifiability study of reversible intramolecular two-state excited-state processes coupled with species-dependent rotational diffusion is justified by the fact that, for these processes, in contrast to the previously discussed intermolecular case,[19] the co-reactant concentration is not available as an experimental variable.

There are several methods available for the analysis of the deterministic identifiability (i.e., assuming error-free data). The initial identification studies of reversible intramolecular two-state excited-state processes[9−13] made use of Markov parameters and elementary functions of the rate constants. In this report, we use the method of similarity transformation[3,4,20,21] because it offers an elegant way of determining if a model is *globally* or *locally* identifiable, or completely *unidentifiable*. A model is *uniquely* (or globally) identifiable if the parameters of the assumed model can be uniquely determined from the idealized experiment. If there are a finite number of alternative parameter estimates for some or all of the model parameters that fit the data, the model is locally identifiable. An infinite number of model parameter estimates fitting the data makes the considered model unidentifiable. An extra bonus of the similarity transformation approach is that the relationship between the true and the alternative model parameters is explicitly provided.

The paper is organized as follows. In section 2, the general concepts of identifiability via similarity transformation are presented. Section 3A deals with the identification of the model of a reversible intramolecular two-state excited-state process without transient effects (i.e., with kinetics governed by time-independent rate constants). In section 3B, we investigate what the effect is on the identifiability when quencher is added to such a photophysical system. For both models, the fluorescence δ-response is expressed in matrix form appropriate for the similarity transformation method. Finally, in section 3C, we describe the identification of the model of a reversible intramolecular two-state excited-state process, without transient effects, in the presence of quencher and coupled with species-dependent rotational diffusion. Spherically and cylindrically symmetric rotors are considered, with, in the latter case, no change in the principal axes of the diffusion tensors of the two excited-state species. The case in which the principal axes of the diffusion tensors of the interconverting excited-state species are not the same is very complex[18] and is not considered here.

Imperfect data resulting from noisy observations sampled over a limited time range affect the accuracy and precision with which model parameters can be estimated. This numerical parameter estimation and the statistical properties of the parameter estimates are the subject of the second stage of any identifiability analysis and is called *numerical identifiability*. A study of the 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, is beyond the scope of this paper.

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[22]

$$f(t) = \mathbf{c}\exp(t\mathbf{A})\mathbf{b}, \quad t \geq 0 \tag{1}$$

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 containing the kinetic information for all processes.

The set ($\mathbf{A}$, $\mathbf{b}$, $\mathbf{c}$) is called a realization of the fluorescence δ-response function $f(t)$. In the identification study, one 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 must be the same for the true ($\mathbf{A}$, $\mathbf{b}$, $\mathbf{c}$) and the alternative ($\mathbf{A}^+$, $\mathbf{b}^+$, $\mathbf{c}^+$) model parameter set.[3,4]

Unique identifiability is attained 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 one for which there is 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.

A different (alternative) realization ($\mathbf{A}^+$, $\mathbf{b}^+$, $\mathbf{c}^+$) of $f(t)$ is related to the true realization ($\mathbf{A}$, $\mathbf{b}$, $\mathbf{c}$) via similarity transformation,[3,4,20,21]

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

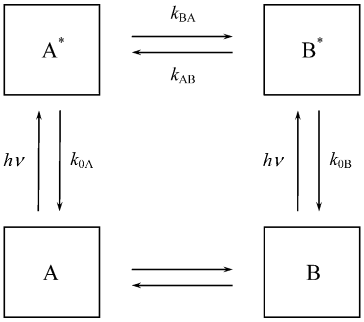
where $\mathbf{T}$ is a nonsingular matrix (i.e., det $\mathbf{T} \neq 0$).

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

$$\mathbf{b}^+ = \mathbf{T}^{-1}\,\mathbf{b} \tag{4}$$

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

SCHEME 1. Representation of the Model of a Reversible Intramolecular Two-State Excited-State Process[a]



[a] 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}

Equations 3−5 clearly illustrate that the similarity transformation elucidates the relation between the true and the alternative parameters. Equations 3−5 must be satisfied for each experimental condition. For the intramolecular models considered here, possible experimental variables are quencher concentration $[\mathrm{Q}]_k$, excitation wavelength λ_i^{ex}, and emission wavelength λ_j^{em}. This implies that the matrix **T** should be independent of $[\mathrm{Q}]_k$, λ_i^{ex}, and λ_j^{em}.[8]

3. Identifiability Analysis

A. Reversible Intramolecular Two-State Excited-State Process. Consider the causal, linear, time-invariant photophysical system consisting of two different species A and B interchanging via intramolecular processes, as depicted in Scheme 1. The two ground-state species are assumed to be in equilibrium. Photoexcitation 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} ($= k_{FB} + k_{NRB}$) stand for the composite deactivation rate constants of A* and B*, respectively. The rate constant describing the intramolecular transformation of A* into B* is represented by k_{BA}, while the reverse process is described by k_{AB}. The rate constants are obviously nonnegative.

When the photophysical system shown in Scheme 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 $f_{ij}(t)$ at emission wavelength λ_j^{em} due to excitation at λ_i^{ex} is given by[9]

$$f_{ij}(t) = \mathbf{c}_j \exp(t\,\mathbf{A})\mathbf{b}_i, \quad t \geq 0 \tag{6}$$

with the matrix **A** given by[9]

$$\mathbf{A} = \begin{bmatrix} -(k_{0A} + k_{BA}) & k_{AB} \\ k_{BA} & -(k_{0B} + k_{AB}) \end{bmatrix} \tag{7}$$

$\mathbf{b}_i$ is a 2 × 1 vector with elements $b_{li} = [l^*]_{t=0}$ (l stands for A or B), specifying the initial concentrations of excited species l^*, which depend on the excitation wavelength λ_i^{ex}.[9]

$\mathbf{c}_j$ is the 1 × 2 vector of the emission weighting factors c_{mj} of species m^* (m represents A or B) at emission wavelength λ_j^{em}.[22] The coefficient c_{mj} is defined as[22]

$$c_{mj} = k_{Fm}\int_{\Delta\lambda_j^{\mathrm{em}}} \rho_m(\lambda_j^{\mathrm{em}})d\lambda^{\mathrm{em}} \tag{8}$$

where k_{Fm} is the fluorescence rate constant of species m^*, the subscript j refers to the observation wavelength range, $\Delta\lambda_j^{\mathrm{em}}$, and $\rho_m(\lambda_j^{\mathrm{em}})$ is the spectral emission density of species m^*.[9]

Matrix **T** used in the similarity transformations is expressed by eq 9:

$$\mathbf{T} = \begin{bmatrix} t_1 & t_2 \\ t_3 & t_4 \end{bmatrix} \tag{9}$$

Performing the matrix multiplication of eq 3 yields a set of four simultaneous equations:

$$-t_1(k_{0A}^{+} + k_{BA}^{+}) + t_2k_{BA}^{+} = -t_1(k_{0A} + k_{BA}) + t_3k_{AB} \tag{10a}$$

$$t_1k_{AB}^{+} - t_2(k_{0B}^{+} + k_{AB}^{+}) = -t_2(k_{0A} + k_{BA}) + t_4k_{AB} \tag{10b}$$

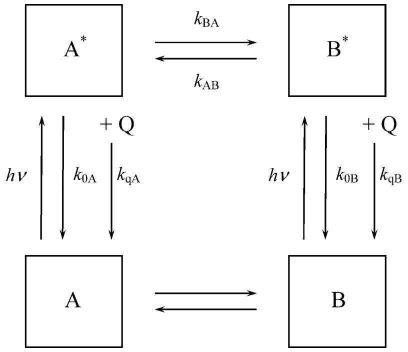
$$-t_3(k_{0A}^{+} + k_{BA}^{+}) + t_4k_{BA}^{+} = t_1k_{BA} - t_3(k_{0B} + k_{AB}) \tag{10c}$$

$$t_3k_{AB}^{+} - t_4(k_{0B}^{+} + k_{AB}^{+}) = t_2k_{BA} - t_4(k_{0B} + k_{AB}) \tag{10d}$$

There are infinite numbers of alternative k_{0A}^{+}, k_{BA}^{+}, k_{0B}^{+}, and k_{AB}^{+} values that satisfy eq 10, and the model cannot be identified in terms of rate constants. There are also unlimited numbers of alternative b_A^{+} and b_B^{+}, calculated according to eq 4. The same is true for the c_A^{+} and c_B^{+} values calculated via eq 5. To summarize, in the absence of a priori information, the model of reversible intramolecular two-state excited-state events is unidentifiable, in accordance with the deterministic identification analysis based on Markov parameters and elementary functions of the rate constants.[9]

In the wide-ranging deterministic identifiability study presented in ref 9, we have demonstrated under which conditions the current model becomes identifiable. It is worthwhile to recollect the results of that study. For the model to be globally identifiable, unique values must be found for the rate constants $\{k_{0A}, k_{BA}, k_{0B}, k_{AB}\}$ and the spectral parameters related to excitation $\{b_A, b_B\}$ and emission $\{c_A, c_B\}$. In photophysics, values for $\{b_A, b_B\}$ and $\{c_A, c_B\}$ cannot be obtained.[8] Therefore, it is more appropriate to use the normalized parameters $\{\tilde{b}_A, \tilde{b}_B\}$ and $\{\tilde{c}_A, \tilde{c}_B\}$$[\tilde{b}_A = b_A/(b_A + b_B), \tilde{b}_B = 1 - \tilde{b}_A, \tilde{c}_A = c_A/(c_A + c_B)$, and $\tilde{c}_B = 1 - \tilde{c}_A]$. A more suitable way of formulating the problem of identifiability is to use the normalized $\tilde{b}_A$ and $\tilde{c}_A$ together with $\{k_{0A}, k_{BA}, k_{0B}, k_{AB}\}$. The results of the deterministic identifiability study accessible in ref 9 can be summarized as follows: in all cases at least three (kinetic or spectroscopic) parameters of the intramolecular two-state excited-state process must be known for the model to be identifiable. These three parameters can be (i) two rate constants and one normalized spectroscopic parameter [(1) two k_{ij} and one $\tilde{b}_A$, (2) two k_{ij} and one $\tilde{c}_A$]; (ii) one rate constant and two spectroscopic parameters [(3) one k_{ij}, one $\tilde{b}_A$, and one $\tilde{c}_A$, (4) one k_{ij} and two $\tilde{b}_A$, (5) one k_{ij} and two $\tilde{c}_A$]; (iii) three spectroscopic parameters [(6) two $\tilde{b}_A$ and one $\tilde{c}_A$, (7) one $\tilde{b}_A$ and two $\tilde{c}_A$, (8) three $\tilde{b}_A$, (9) three $\tilde{c}_A$]. Note that the conditions are symmetrical in $\tilde{b}_A$ and $\tilde{c}_A$. For conditions 1−3, one decay trace is necessary and sufficient, for conditions 4−7 two decay traces are necessary and sufficient, while for conditions 8−9 three are needed and suffice. The conditions 3, 6, and 7 require that the known $\{\tilde{b}_A, \tilde{c}_A\}$ values are different from {1, 0} or {0, 1}. That means that if one species is excited exclusively, one should NOT observe the fluorescence in the wavelength region where only the other species emits [that is, $\tilde{b}_A = 1$ and $\tilde{c}_A = 0$ or $\tilde{b}_A = 0$ and $\tilde{c}_A = 1$ are NOT valid spectroscopic parameter combinations for conditions 3, 6, and 7]. Conditions 6−9 indicate that, theoretically, spectroscopic information alone can suffice for the model to be identifiable. The identifiability study clearly shows that

SCHEME 2: Representation of the Model of a Reversible Intramolecular Two-State Excited-State Process with Added Quencher[a]



[a] It is assumed that the quencher Q only has an effect on the excited species and does not affect the ground-state equilibrium. 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. The rate constants k_{0A}, k_{0B}, k_{BA}, and k_{AB} are defined as in Scheme 1.

exciting only one species ($\tilde{b}_A = 1$ or $\tilde{b}_A = 0$), either through different absorption profiles or a large difference in equilibrium concentration, can never lead to a uniquely identifiable model. In this case, the preexponentials (α_1, α_2) of the fluorescence decay have the same absolute values but opposite signs ($\alpha_1/\alpha_2 = -1$), and such decay is not useful for attaining global identifiability. Furthermore, knowledge of the values of only one or two rate constants, based on model compounds, is not sufficient to uniquely identify the model. Condition 3 is perhaps the situation that is used most often for obtaining unique values for the relevant model parameters. In most cases in which condition 3 is applied, one assumes (i) that the deactivation rate constant k_{0A} in the intramolecular model can be equated with the deactivation rate constant of a suitable reference compound and (ii) that species A is excited exclusively ($\tilde{b}_A = 1$), and (iii) that only the emission from A* is monitored ($\tilde{c}_A = 1$). Because of symmetry between $A^{(*)}$ and $B^{(*)}$ in the considered model, A can be replaced by B in the latter sentence. It must be emphasized that the values obtained in this manner are based on assumptions (one presumes that the value of k_{0A} measured for the reference compound is transferable to the intramolecular excited-state process and that $\tilde{b}_A = 1$ and $\tilde{c}_A = 1$), which must be verified (see section 3B).[23] The results of the deterministic identifiability study[9] have been confirmed by the curve-fitting of computer-generated fluorescence decay traces. For more details, we refer to ref 9.

B. Reversible Intramolecular Two-State Excited-State Process with Quenching. Addition of an external quencher Q at concentration $[Q]_k$ to the intramolecular system shown in Scheme 1 accelerates the depopulation of the excited-state species A* and B*. The kinetic model of such an intramolecular system with added quencher is depicted in Scheme 2. It is assumed that the intermolecular quenching of A* and B* can be described by the time-invariant rate constants k_{qA} and k_{qB}, respectively. It is further presumed that the added quencher affects only the excited-state species deactivation and does not in any way change the ground-state equilibrium.

The fluorescence δ-response function $f_{ijk}(t)$ for quencher concentration $[Q]_k$, monitored at emission wavelength λ_j^{em} and due to excitation at λ_i^{ex} is given by[11,12]

$$f_{ijk}(t) = \mathbf{c}_j \exp(t\mathbf{A}_k)\mathbf{b}_i, \quad t \geq 0 \tag{11}$$

with the matrix $\mathbf{A}_k$ expressed as[11,12]

$$\mathbf{A}_k = \begin{bmatrix} -(k_{0A} + k_{BA} + k_{qA}[Q]_k) & k_{AB} \\ k_{BA} & -(k_{0B} + k_{AB} + k_{qB}[Q]_k) \end{bmatrix} \tag{12}$$

The 2 × 1 vector $\mathbf{b}_i$ and the 1 × 2 vector $\mathbf{c}_j$ are defined as in section 3A.[11,12]

The matrix multiplication of eq 3 with $\mathbf{A} = \mathbf{A}_k$ given by eq 12 and $\mathbf{T}$ expressed by eq 9 yields the following four equations:

$$-t_1(k_{0A}^+ + k_{BA}^+ - k_{0A} - k_{BA}) + t_2 k_{BA}^+ - t_3 k_{AB} = t_1(k_{qA}^+ - k_{qA})[Q]_k \tag{13a}$$

$$t_1 k_{AB}^+ - t_2(k_{0B}^+ + k_{AB}^+ - k_{0A} - k_{BA}) - t_4 k_{AB} = t_2(k_{qB}^+ - k_{qA})[Q]_k \tag{13b}$$

$$-t_3(k_{0A}^+ + k_{BA}^+ - k_{0B} - k_{AB}) + t_4 k_{BA}^+ - t_1 k_{BA} = t_3(k_{qA}^+ - k_{qB})[Q]_k \tag{13c}$$

$$t_3 k_{AB}^+ - t_4(k_{0B}^+ + k_{AB}^+ - k_{0B} - k_{AB}) - t_2 k_{BA} = t_4(k_{qB}^+ - k_{qB})[Q]_k \tag{13d}$$

The elements t_i (i = 1, ..., 4) of $\mathbf{T}$ must be independent of $[Q]_k$ so that the rhs of eq 13 have to be zero [$t_1(k_{qA}^+ - k_{qA}) = 0$, $t_2(k_{qB}^+ - k_{qA}) = 0$, $t_3(k_{qA}^+ - k_{qB}) = 0$, $t_4(k_{qB}^+ - k_{qB}) = 0$].

One can envisage that several combinations of t_i (i = 1, ..., 4), k_{qA}^+, and k_{qB}^+ are possible to satisfy the latter four equations. However, assuming $k_{qA} \neq k_{qB}$ only *two* solutions are possible. Case 1: $t_1 \neq 0$, $t_4 \neq 0$, $t_2 = t_3 = 0$, $k_{qA}^+ = k_{qA}$, $k_{qB}^+ = k_{qB}$ and Case 2: $t_1 = t_4 = 0$, $t_2 \neq 0$, $t_3 \neq 0$, $k_{qA}^+ = k_{qB}$, $k_{qB}^+ = k_{qA}$.

Now we shall consider Case 1. The rhs of eq 13a is zero if $t_1 \neq 0$ and $k_{qA}^+ = k_{qA}$. Then the rhs of eq 13c is zero if $t_3 = 0$ (because $k_{qA}^+ - k_{qB} \neq 0$). Since $\mathbf{T}$ must be nonsingular (det $\mathbf{T} \neq 0$) we have $t_4 \neq 0$. The requirement that the rhs of eq 13d be zero leads to $k_{qB}^+ = k_{qB}$. The rhs of eq 13b is zero if $t_2 = 0$ (because $k_{qB}^+ - k_{qA} \neq 0$).

Under the conditions that $t_1 \neq 0$, $t_4 \neq 0$, $t_2 = t_3 = 0$ we can proceed as follows. From eq 13a we have $k_{0A}^+ + k_{BA}^+ = k_{0A} + k_{BA}$; from eq 13d it follows that $k_{0B}^+ + k_{AB}^+ = k_{0B} + k_{AB}$. Equations 13b and 13c now reduce to, respectively, eqs 14a and 14b:

$$t_1 k_{AB}^+ = t_4 k_{AB} \tag{14a}$$

$$t_4 k_{BA}^+ = t_1 k_{BA} \tag{14b}$$

This leads to $k_{AB}^+ k_{BA}^+ = k_{AB} k_{BA}$. To conclude, when $\mathbf{T}$ is given by

$$\mathbf{T} = \begin{bmatrix} t_1 & 0 \\ 0 & t_4 \end{bmatrix} \tag{15}$$

the individual deactivation and exchange rate constants cannot be determined. Only the sums $S_A \equiv k_{0A} + k_{BA}$ and $S_B \equiv k_{0B} + k_{AB}$ and the product $P \equiv k_{AB}\, k_{BA}$ can be recovered. The alternative set is identical to the original one: S_A^+ ($\equiv k_{0A}^+ + k_{BA}^+$) $= S_A$, S_B^+ ($\equiv k_{0B}^+ + k_{AB}^+$) $= S_B$, and P^+ ($\equiv k_{AB}^+\, k_{BA}^+$) $= P$. So, given that there are three equations and four unknown rate constants (k_{0A}^+, k_{BA}^+, k_{0B}^+, k_{AB}^+), unique values for the four rate constants cannot be found. However, lower and upper limits on these rate constants can be set:[12,13]

$$0 < k_{0A}^{+} < S_A - P/S_B \tag{16a}$$

$$P/S_B < k_{BA}^{+} < S_A \tag{16b}$$

$$0 < k_{0B}^{+} < S_B - P/S_A \tag{16c}$$

$$P/S_A < k_{AB}^{+} < S_B \tag{16d}$$

If one of the deactivation (e.g., $k_{0A}^{+} = k_{0A}$) or exchange rate constants, together with one of the quenching rate constants (e.g. $k_{qA}^{+} = k_{qA}$), is known, the remaining rate constants are uniquely determined from eq 13: $k_{qB}^{+} = k_{qB}$, $k_{BA}^{+} = k_{BA}$, $k_{0B}^{+} = k_{0B}$, $k_{AB}^{+} = k_{AB}$, and $t_1 = t_4$, $t_2 = t_3 = 0$ (i.e., $\mathbf{T} = t_1\, \mathbf{I}_2$ with $\mathbf{I}_2$ the 2 × 2 identity matrix). In this case, the alternative rate constants are identical with the true ones, and all deactivation/exchange and quenching rate constants are uniquely determined.

There are an unlimited number of sets of alternative $\mathbf{b}^{+}$ (eq 4) and $\mathbf{c}^{+}$ (eq 5) for Case 1:

$$b_1^{+} = b_1/t_1 \text{ and } b_2^{+} = b_2/t_4 \tag{17a}$$

$$c_1^{+} = c_1 t_1 \text{ and } c_2^{+} = c_2 t_4 \tag{17b}$$

so that

$$c_1^{+}\, b_1^{+} = c_1 b_1 \text{ and } c_2^{+}\, b_2^{+} = c_2 b_2 \tag{17c}$$

Note that eqs 4 and 5 always lead to $\mathbf{c}^{+}\, \mathbf{b}^{+} = \mathbf{c}\, \mathbf{b}$, independent of the form of the nonsingular $\mathbf{T}$. This is to be expected because this equality is equivalent to $f^{+}(0) = f(0)$; that is, the fluorescence δ-response function at time zero is the same for the true and alternative spectral parameters. (Since Markov parameter m_0 is given by $m_0 = \mathbf{c}\, \mathbf{b}$, the above-mentioned equality also can be written as $m_0^{+} = m_0$). Equation 17c satisfies this equality.

Now we consider Case 2. The rhs of eq 13a is zero if, alternatively, $t_1 = 0$ and $k_{qA}^{+} \neq k_{qA}$. The nonsingularity requirement of $\mathbf{T}$ (det $\mathbf{T} \neq 0$) leads to $t_2 \neq 0$ and $t_3 \neq 0$. The requirement that the rhs of eqs 13b and 13c be zero leads to $k_{qB}^{+} = k_{qA}$ and $k_{qA}^{+} = k_{qB}$, respectively. The rhs of eq 13d is zero if $t_4 = 0$ (because $k_{qB}^{+} - k_{qA} \neq 0$).

Under the conditions that $t_2 \neq 0$, $t_3 \neq 0$, $t_1 = t_4 = 0$ we can proceed as follows. From eq 13b we have $k_{0B}^{+} + k_{AB}^{+} = k_{0A} + k_{BA}$; from eq 13c it follows that $k_{0A}^{+} + k_{BA}^{+} = k_{0B} + k_{AB}$. Equations 13a and 13d now reduce, respectively, to eqs 18a and 18b:

$$t_2 k_{BA}^{+} = t_3 k_{AB} \tag{18a}$$

$$t_3 k_{AB}^{+} = t_2 k_{BA} \tag{18b}$$

which lead to $k_{AB}^{+} k_{BA}^{+} = k_{AB} k_{BA}$. To conclude, when $\mathbf{T}$ is given by

$$\mathbf{T} = \begin{bmatrix} 0 & t_2 \\ t_3 & 0 \end{bmatrix} \tag{19}$$

the alternative set of rate constant combinations is $S_A^{+} = S_B$, $S_B^{+} = S_A$, and $P^{+} = P$. Hence, the alternative set contains the original rate constant combinations but with switched labeling (that is, all rate constants of A* are now those of B* and vice versa). In other words, two sets of rate constant combinations are possible, but they cannot be assigned unambiguously to a specific species. Note that S_A^{+} and S_B^{+} are always correctly associated with the rate constants of quenching.

Since there are three equations and four unknown deactivation and exchange rate constants (k_{0A}^{+}, k_{BA}^{+}, k_{0B}^{+}, k_{AB}^{+}), unique values for these four rate constants cannot be found. However, the rate constants have to satisfy the following inequalities:[12,13]

$$0 < k_{0A}^{+} < S_B - P/S_A \tag{20a}$$

$$P/S_A < k_{BA}^{+} < S_B \tag{20b}$$

$$0 < k_{0B}^{+} < S_A - P/S_B \tag{20c}$$

$$P/S_B < k_{AB}^{+} < S_A \tag{20d}$$

The number of alternative sets of $\mathbf{b}^{+}$ (eq 21a) and $\mathbf{c}^{+}$ (eq 21b) for Case 2 is limitless too:

$$b_1^{+} = b_2/t_3 \text{ and } b_2^{+} = b_1/t_2 \tag{21a}$$

$$c_1^{+} = c_2 t_3 \text{ and } c_2^{+} = c_1 t_2 \tag{21b}$$

so that

$$c_1^{+} b_1^{+} = c_2 b_2 \text{ and } c_2^{+} b_2^{+} = c_1 b_1 \tag{21c}$$

Equation 21c satisfies the condition $\mathbf{c}^{+}\, \mathbf{b}^{+} = \mathbf{c}\mathbf{b}$.

If one of the exchange or deactivation rate constants (e.g., $k_{0A}^{+} = k_{0A}$) is known, and one of the quenching rate constants is switched ($k_{qA}^{+} = k_{qB}$), the remaining rate constants are uniquely determined from eq 13: $k_{qB}^{+} = k_{qA}$, $k_{BA}^{+} = k_{0B} + k_{AB} - k_{0A}$, $k_{0B}^{+} = k_{0A} + k_{BA} - k_{AB}k_{BA}/(k_{0B} + k_{AB} - k_{0A})$, $k_{AB}^{+} = k_{AB}k_{BA}/(k_{0B} + k_{AB} - k_{0A})$ and $t_2/t_3 = k_{AB}/(k_{0B} + k_{AB} - k_{0A})$.

All cases in which the rhs of one of the eqs 13 is set to zero by simultaneously setting t_i ($i = 1, ..., 4$) and the difference in quenching rate constants ($k_{qx}^{+} = k_{qy}$, $x, y = $ A, B) equal to zero leads to contradictions. We consider one case to illustrate this. The rhs of eq 13a is zero if simultaneously $t_1 = 0$ and $k_{qA}^{+} = k_{qA}$. The condition $t_1 = 0$ and the requirement det $\mathbf{T} \neq 0$ lead to $t_2 \neq 0$ and $t_3 \neq 0$. The requirement that the rhs of eq 13c be zero produces $k_{qA}^{+} = k_{qB}$. This equality in combination with $k_{qA}^{+} = k_{qA}$ leads to $k_{qA} = k_{qB}$, which is in contradiction with the assumption that $k_{qA} \neq k_{qB}$. A similar reasoning can be followed starting with the rhs of eqs 13b, 13c, or 13d. The same conclusions as just described will be obtained.

Now we consider the case in which $k_{qA} = k_{qB}$ ($= k_q$). The requirement that the rhs of eq 13 should be zero leads to four equations [$t_i(k_q^{+} - k_q) = 0$, $i = 1, ..., 4$]. If one assumes $k_q^{+} = k_q$, the matrix $\mathbf{T}$ can be given by eqs 15 or 19 or other possible forms of $\mathbf{T}$ containing three or four nonzero elements t_i. The only condition for the rate constants that can be deduced from eq 13 (with the rhs set to zero) is that the determinant of the coefficients of the system of the four homogeneous equations in four unknowns $\{k_{0A}^{+}, k_{BA}^{+}, k_{0B}^{+}, k_{AB}^{+}\}$ is equal to zero. An infinite number of solutions for the alternative rate constants $\{k_{0A}^{+}, k_{BA}^{+}, k_{0B}^{+}, k_{AB}^{+}\}$ can be obtained. Alternatively, the rhs of eq 13 can be set to zero if $t_1 = t_2 = t_3 = t_4 = 0$. This, however, leads to a null $\mathbf{T}$ matrix, which is not an acceptable transformation matrix. The only information that can be obtained for $\mathbf{b}^{+}$ and $\mathbf{c}^{+}$ is that $\mathbf{c}^{+}\, \mathbf{b}^{+} = \mathbf{c}\, \mathbf{b}$. As mentioned before, this equality is always valid, for any nonsingular $\mathbf{T}$.

To summarize, the model of a reversible intramolecular two-state excited-state process with added quencher is locally identifiable, in agreement with the deterministic identification analysis based on Markov parameters and elementary functions of the rate constants.[11,12]

In the deterministic identifiability analysis published in ref 11, we have investigated the prerequisites for obtaining the unique set of model parameters. It is instructive to recall the results of that study. The following conditions have to be fulfilled to make a reversible intramolecular two-state excited-state process with added quencher uniquely identifiable: (i) the fluorescence decay surface must include at least one set of decay traces measured for a minimum of three different quencher concentrations at the same excitation/emission wavelength setting (one of the quencher concentrations may be equal to zero); (ii) the two rate constants of quenching must be different ($k_{qA} \neq k_{qB}$); (iii) at least one model parameter (k_{ij}, $\tilde{b}_A$, or $\tilde{c}_A$) must be known. Under these conditions, four sets of model parameters are mathematically possible. If the known model parameter is a rate constant (k_{0A}, k_{BA}, k_{0B}, or k_{AB}, but not k_{qA} or k_{qB}), decay traces of a suitable reference compound measured at a minimum of two quencher concentrations must be included in the analysis to obtain the unique set of rate constant values. The unique set of ($\tilde{b}_A$, $\tilde{c}_A$) values can be recovered by including decay curves at a minimum of two quencher concentrations and at an additional excitation wavelength with a different $\tilde{b}_A$, or at another emission wavelength with a different $\tilde{c}_A$. If the known model parameter is a $\tilde{b}_A$ value different from zero and one, the fluorescence decay surface must include at least nine decay traces measured at four emission wavelengths with different $\tilde{c}_A$ (corresponding to at least three quencher concentrations at the first emission wavelength and at least two quencher concentrations at the other three emission wavelengths), to uniquely determine the set of model parameters. If the known model parameter is a $\tilde{c}_A$ value different from zero and one, the fluorescence decay surface must include at least nine decay traces measured at four excitation wavelengths with different $\tilde{b}_A$ (corresponding to at least three quencher concentrations at the first excitation wavelength and at least two quencher concentrations at the other three excitation wavelengths), to uniquely determine the set of model parameters. A decay trace with $\tilde{b}_A = 1$ and $\tilde{c}_A = 0$ or $\tilde{b}_A = 0$ and $\tilde{c}_A = 1$ never provides useful information. Full details can be found in ref 11.

In the absence of a priori information, upper and lower bounds can be specified for the rate constants k_{ij} if (i) the fluorescence decay surface includes at least one set of decay traces measured at a minimum of three different quencher concentrations and (ii) the rate constants of quenching are different ($k_{qA} \neq k_{qB}$).[12] This analysis method allows one to distinguish reversible from irreversible intramolecular two-state excited-state processes.

In a numerical identifiability study,[13] computer-generated fluorescence decay surfaces have been used to investigate the criteria under which reliable estimates of the bounds on the rate constants k_{ij} can be obtained. If the values of k_{qA} and k_{qB} are substantially different, reliable estimates are obtained. If k_{qA} and k_{qB} are nearly equal in value, the quality of the estimates of the bounds depends on the combinations of the values of the rate constants k_{ij}. It may happen that no reliable limits for the rate constants can be obtained so that another quencher is required.

The previously presented identifiability analyses for models of intramolecular two-state excited-state processes[11−13] have been experimentally investigated, using bis(2-pyrenecarboxylic acid) 1,6-hexanediyl ester with iodomethane as quencher.[23] Two different data analysis approaches were examined. In the first approach, where no information was known beforehand, it was possible to obtain upper and lower limits on the rate constants $\{k_{0A}, k_{BA}, k_{0B}, k_{AB}\}$ by analyzing at different preset values of the rate constant k_{0A}. For the other approach, where the time-resolved emission of the reference compound hexyl 2-pyrenecarboxylate was used in the fittings, the parameter values of k_{0A} and k_{qA} were defined by linking these parameters with the corresponding rate constants of the reference compound.[23]

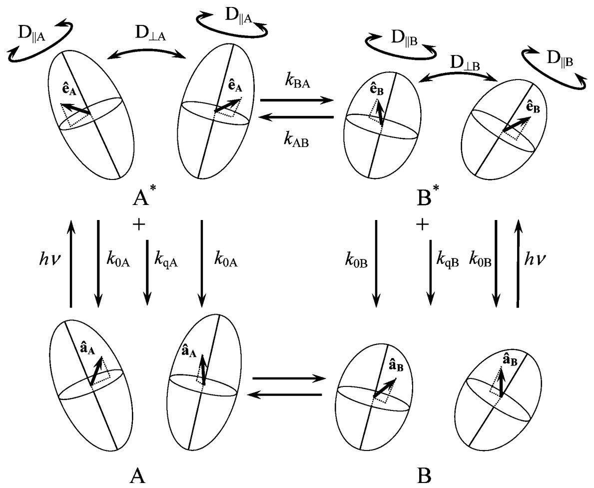


Figure 1. Graphic representation of a reversible intramolecular two-state excited-state process, including rotation. Species A* and B* are pictured as being initially excited from their ground states A and B by an infinitely short linearly polarized light pulse at wavelength λ_i^{ex} in a unique absorption band for each species. The rate constants k_{0A}, k_{0B}, k_{BA}, k_{AB}, k_{qA}, and k_{qB} are defined as in Schemes 1 and 2. The species rotate with rate constants determined by their corresponding rotational diffusion tensors, which 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 time of emission with respect to the absorption moment (with unit vector $\hat{\mathbf{a}}_A$ or $\hat{\mathbf{a}}_B$) at the time of excitation of the species initially excited.

The identifiability of models of irreversible intramolecular two-state excited-state processes with added quencher is beyond the scope of the current study. Details on how to distinguish the two possible competing models (one with a unidirectional excited-state process and one without excited-state process) can be found in ref 24.

C. Reversible Intramolecular Two-State Excited-State Process with Species-Dependent Rotational Diffusion and Quenching. The linear and 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 deactivation rate constants k_{0A} and k_{0B}, the excited-state exchange rate constants k_{BA} and k_{AB}, and the quenching rate constants k_{qA} and k_{qB} are defined as in Schemes 1 and 2. All the rate constants are assumed independent of the instantaneous orientation of the species. The rotational relaxation of each excited-state species is governed by its principal rotational diffusion constants, here $D_\|$ and $D_\perp$ for rotation, respectively, of and about the principal 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 combined in "sum" $S_{ijk}(t) \equiv I_{\|ijk}(t) + 2I_{\perp ijk}(t)$ and "difference" $D_{ijk}(t) \equiv I_{\|ijk}(t) - I_{\perp ijk}(t)$ functions.

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

The identification is straightforward if one uses $S_{ijk}(t)$ and $D_{ijk}(t)$ instead of $I_{\|ijk}(t)$ and $I_{\perp ijk}(t)$. $S_{ijk}(t)$ corresponds to the total time-resolved emission of the system and can be expressed in matrix form by[16]

$$S_{ijk}(t) = 3\mathbf{c}_{j,00} \exp(t\,\mathbf{A}_{k,00})\mathbf{b}_{ik,00}, \quad t \geq 0 \tag{22}$$

$S_{ijk}(t)$ is independent of the rotational diffusion and does not contain any information about the orientations of the transition moments.

$D_{ijk}(t)$ contains information about rotational diffusion and can be expressed as[16]

$$D_{ijk}(t) = 3\mathbf{c}_{j,2M} \exp(t\,\mathbf{A}_{Dk})\mathbf{b}_{ik,2M}, \quad t \geq 0 \tag{23}$$

Now we will define the vectors and matrices in eqs 22 and 23.

Matrix $\mathbf{A}_{k,00}$ in eq 22 is given by eq 24:

$$\mathbf{A}_{k,00} = \begin{bmatrix} -(k_{0A} + k_{BA} + k_{qA}[Q]_k) & k_{AB} \\ k_{BA} & -(k_{0B} + k_{AB} + k_{qB}[Q]_k) \end{bmatrix} \tag{24}$$

$\mathbf{A}_{Dk}$ in eq 23 is defined as

$$\mathbf{A}_{Dk} = \begin{bmatrix} \mathbf{A}_{Dk,2-2} & 0 & 0 & 0 & 0 \\ 0 & \mathbf{A}_{Dk,2-1} & 0 & 0 & 0 \\ 0 & 0 & \mathbf{A}_{Dk,20} & 0 & 0 \\ 0 & 0 & 0 & \mathbf{A}_{Dk,21} & 0 \\ 0 & 0 & 0 & 0 & \mathbf{A}_{Dk,22} \end{bmatrix} \tag{25}$$

with blocks $\mathbf{A}_{Dk,2M}$ given by eq 26:

$$\mathbf{A}_{Dk,2M} = \begin{bmatrix} -(D_{A,2M} + k_{0A} + k_{BA} + k_{qA}[Q]_k) & k_{AB} \\ k_{BA} & -(D_{B,2M} + k_{0B} + k_{AB} + k_{qB}[Q]_k) \end{bmatrix} \tag{26}$$

with $M = -2, -1, 0, 1, 2$.

$D_{l,2M}$ (l symbolizes either A or B) is given by

$$D_{l,2M} = 6D_{\perp l} + M^2(D_{\| l} - D_{\perp l}) \tag{27}$$

Note the invariance of eqs 26 and 27 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,[16] reducing to the unique component D_l ($= D_{\perp l} = D_{\| l}$) in the case of the *spherically* symmetric rotor l.

Vector $\mathbf{b}_{ik,LM}$ [with $L = M = 0$ (in eq 22), or $L = 2$ and $M = \pm 2, \pm 1, 0$ (in eq 23)] contains the excitation coefficients $b_{lik,LM}$ (l stands for 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 quencher concentration $[Q]_k$, respectively. The subscripts L and M of the $b_{lik,LM}$ coefficients refer to the orientation of the absorption transition moments. The elements $b_{lik,LM}$ can be expressed as the product of the initial concentration of l*, b_{lik}, the appropriate spherical harmonic $Y_L^M(\hat{\mathbf{a}}_l)$[25] 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:[16]

$$b_{lik,LM} = B_L b_{lik} Y_L^M(\hat{\mathbf{a}}_l) \tag{28}$$

with $B_0 = 1/12\sqrt{1/\pi^3}$ and $B_2 = 1/30\sqrt{5/\pi^3}$.[16]

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

The 2×1 vector $\mathbf{b}_{ik,00}$ in eq 22 is explicitly given by eq 29:

$$\mathbf{b}_{ik,00} = [b_{Aik,00} \;\; b_{Bik,00}]^{\mathrm{T}} \tag{29}$$

while the 10×1 vector $\mathbf{b}_{ik,2M}$ in eq 23 is expressed as

$$\mathbf{b}_{ik,2M} = [b_{Aik,2-2}\, b_{Bik,2-2}\, b_{Aik,2-1}\, b_{Bik,2-1}\, b_{Aik,20}\, b_{Bik,20}\, b_{Aik,21}\, b_{Bik,21}\, b_{Aik,22}\, b_{Bik,22}]^{\mathrm{T}} \tag{30}$$

Vector $\mathbf{c}_{j,LM}$ [with $L = M = 0$ (in eq 22), or $L = 2$ and $M = \pm 2, \pm 1, 0$ (in eq 23)] 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:[16]

$$c_{mj,LM} = C_L c_{mj} Y_L^{M}{*}(\hat{\mathbf{e}}_m) \tag{31}$$

with $C_0 = 16/3\sqrt{\pi^5}$, $C_2 = 16/15\sqrt{\pi^5/5}$, 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 $c_{mj,00} = 8\pi^2 c_{mj}/3$.

The coefficient c_{mj} is defined by eq 8. Vector $\mathbf{c}_{j,00}$ in eq 22 is explicitly given by eq 32:

$$\mathbf{c}_{j,00} = [c_{Aj,00}\; c_{Bj,00}] \tag{32}$$

while the 1×10 vector $\mathbf{c}_{j,2M}$ in eq 23 is expressed as:

$$\mathbf{c}_{j,2M} = [c_{Aj,2-2}\, c_{Bj,2-2}\, c_{Aj,2-1}\, c_{Bj,2-1}\, c_{Aj,20}\, c_{Bj,20}\, c_{Aj,21}\, c_{Bj,21}\, c_{Aj,22}\, c_{Bj,22}] \tag{33}$$

The matrix and vector formulations of $\mathbf{A}$ (eqs 24−26), $\mathbf{b}$ (eqs 29, 30), and $\mathbf{c}$ (eqs 32, 33) will turn out to be particularly suitable in addressing the identification analysis.

As both $S_{ijk}(t)$ (eq 22) and $D_{ijk}(t)$ (eq 23) can be expressed as a function of $\mathbf{A}$, $\mathbf{b}$, and $\mathbf{c}$, the identifiability analysis via similarity transformation is carried out using the $S_{ijk}(t)$ and $D_{ijk}(t)$ functions.

In the following, it will be assumed that $k_{qA} \neq k_{qB}$. We start with the identification involving $S_{ijk}(t)$ (eq 22) and the matrix multiplication (eq 3) with $\mathbf{A}_{k,00}$ (eq 24). Since $S_{ijk}(t)$ reflects the time dependence of the total fluorescence and contains information only on the excited states, the identifiability analysis will be identical to that of a reversible intramolecular two-state excited-state process with quenching involving $f_{ijk}(t)$ (eq 11) with $\mathbf{A}_k$ defined by eq 12 (section 3B). To summarize the results, two sets of rate constant values are obtained: (1) set S1 (the original or "true" set) is found when $\mathbf{T}$ is given by eq 15:

$$k_{qA}^{+} = k_{qA} \tag{34a}$$

$$k_{qB}^{+} = k_{qB} \tag{34b}$$

$$S_A^{+} = S_A \tag{34c}$$

$$S_B^{+} = S_B \tag{34d}$$

$$P^{+} = P \tag{34e}$$

(2) Set S2 (the alternative set with switched labels) is found when $\mathbf{T}$ is given by eq 19:

$$k_{qA}^{+} = k_{qB} \tag{35a}$$

$$k_{qB}^{+} = k_{qA} \tag{35b}$$

$$S_{A}^{+} = S_{B} \tag{35c}$$

$$S_{B}^{+} = S_{A} \tag{35d}$$

$$P^{+} = P \tag{35e}$$

As for the model of reversible intramolecular two-state excited-state processes (without species-dependent rotational motions; section 3B), upper and lower limits on the rate constants $\{k_{0A}, k_{BA}, k_{0B}, k_{AB}\}$ can be set (eqs 16 for set S1 and eqs 20 for set S2). The requirements for obtaining the unique set of model parameters are the same as before. It is crucial that k_{qA} is different from k_{qB}.

Now we consider the identifiability involving $D_{ijk}(t)$ (eq 23) in which we will use the results of the identifiability analysis of $S_{ijk}(t)$. We assume that the similarity transformations for $S_{ijk}(t)$ and $D_{ijk}(t)$ are independent, as also are the transformations of the various blocks $\mathbf{A}_{Dk,2M}$ (eq 26) in $\mathbf{A}_{Dk}$ (eq 25). For a *cylindrically symmetric* rotor, $\mathbf{A} = \mathbf{A}_{Dk}$ (eq 25) with blocks $\mathbf{A}_{Dk,2M}$ given by eq 26, $\mathbf{b}_{ik,2M}$ (eq 30), $\mathbf{c}_{j,2M}$ (eq 33), $\mathbf{T}$ is a block-diagonal matrix given by eq 36:[19]

$$\mathbf{T} = \begin{bmatrix} \mathbf{T}_{-2} & 0 & 0 & 0 & 0 \\ 0 & \mathbf{T}_{-1} & 0 & 0 & 0 \\ 0 & 0 & \mathbf{T}_{0} & 0 & 0 \\ 0 & 0 & 0 & \mathbf{T}_{1} & 0 \\ 0 & 0 & 0 & 0 & \mathbf{T}_{2} \end{bmatrix} \tag{36}$$

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

$$\mathbf{T}_M = \begin{bmatrix} t_{M,1} & t_{M,2} \\ t_{M,3} & t_{M,4} \end{bmatrix} \tag{37}$$

Because $\mathbf{T}$ and $\mathbf{A} = \mathbf{A}_{Dk}$ are both block-diagonal matrices, the matrix multiplication of eq 3 splits into five separate matrix multiplications, two of which are identical: $M = -2$ and $M = +2$; $M = -1$ and $M = +1$.[19] It is straightforward to show that the matrix multiplication (eq 38) involving $\mathbf{A}_{Dk,2M}^{+}$ and $\mathbf{A}_{Dk,2M}$ (for $M = \pm2, \pm1, 0$)

$$\mathbf{T}_M\, \mathbf{A}_{Dk,2M}^{+} = \mathbf{A}_{Dk,2M}\, \mathbf{T}_M \tag{38}$$

also leads to two sets of alternative parameters: (1) set D1 (when $t_{M,1} \neq 0$, $t_{M,4} \neq 0$, $t_{M,2} = t_{M,3} = 0$ in $\mathbf{T}_M$, eq 37) with k_{qA}^{+}, k_{qB}^{+}, and P^{+} given by eqs 34a, 34b, and 34e, respectively, and

$$S_{A}^{+} + D_{A,2M}^{+} = S_{A} + D_{A,2M} \tag{39a}$$

$$S_{B}^{+} + D_{B,2M}^{+} = S_{B} + D_{B,2M} \tag{39b}$$

(2) set D2 (when $t_{M,2} \neq 0$, $t_{M,3} \neq 0$, $t_{M,1} = t_{M,4} = 0$ in $\mathbf{T}_M$, eq 37) with k_{qA}^{+}, k_{qB}^{+}, and P^{+} given by eqs 35a, 35b, and 35e (or 34e), respectively, and

$$S_{A}^{+} + D_{A,2M}^{+} = S_{B} + D_{B,2M} \tag{40a}$$

$$S_{B}^{+} + D_{B,2M}^{+} = S_{A} + D_{A,2M} \tag{40b}$$

The equations describing sets D1 and D2 are indeed not sufficient to lead to unique solutions for the unknown parameters $D_{\perp A}^{+}$, $D_{\|A}^{+}$, $D_{\perp B}^{+}$, and $D_{\|B}^{+}$. Combinations of the eqs 39a, 39b, 40a, and 40b considered for various values of M are linearly dependent and cannot be used to solve for the unknown parameters. To solve for $D_{\perp A}^{+}$, $D_{\|A}^{+}$, $D_{\perp B}^{+}$, and $D_{\|B}^{+}$, one should combine the equations describing sets S1 (eq 34) and S2 (eq 35) with the extra equations describing sets D1 (eq 39 with $M = \pm2, \pm1, 0$) and D2 (eq 40 with $M = \pm2, \pm1, 0$). $P^{+} = P$ in all four sets.

The combination of eq 34 (set S1) with eq 39 (set D1) leads to

$$D_{\perp A}^{+} = D_{\perp A} \tag{41a}$$

$$D_{\|A}^{+} = D_{\|A} \tag{41b}$$

$$D_{\perp B}^{+} = D_{\perp B} \tag{41c}$$

$$D_{\|B}^{+} = D_{\|B} \tag{41d}$$

Hence, by combining sets S1 and D1, the alternative parameters are the original ones given by eqs 34 and 41.

The combination of eq 35 (set S2) with eq 40 (set D2) yields:

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

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

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

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

Thus, by combining sets S2 and D2 the alternative parameters are the switched ones, given by eqs 35 and 42.

The combination of set S1 (eq 34) with set D2 (eq 40) or the combination of set S2 (eq 35) with set D1 (eq 39) are only compatible if $S_A = S_B$ and $k_{qA} = k_{qB}$. The latter condition is in conflict with $k_{qA} \neq k_{qB}$, which is required to obtain local identifiability, i.e., the sets S1, S2, D1, and D2.

For all acceptable combinations, the number of alternative $\mathbf{b}_{ik,00}$ and $\mathbf{b}_{ik,2M}$, and $\mathbf{c}_{j,00}$ and $\mathbf{c}_{j,2M}$ is unlimited.

To summarize, the identifiability analysis involving both $S_{ijk}(t)$ and $D_{ijk}(t)$ shows that the model of reversible intramolecular two-state excited-state processes with quenching and coupled rotational diffusion for a *cylindrically symmetric* ellipsoid produces two sets of kinetic parameters (i.e., quenching rate constants, combinations of exchange and deactivation rate constants, rotational diffusion coefficients) with switched labels. This means that in the absence of a priori information one cannot assign these parameters to a specific species.

We now consider the case in which $f(t, \mathbf{A}, \mathbf{b}, \mathbf{c}) = D_{ijk}(t)$ for a *spherically symmetric* rotor. As $D_l = D_{\|l} = D_{\perp l}$, the expression for $D_{l,2M}$ (eq 27) 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: (1) the set of alternative quenching rate constants and combinations of deactivation/exchange rate constants is the original set (S1, eq 34), and the alternative rotational diffusion coefficients are the original ones (eq 43):

$$D_{A}^{+} = D_{A} \tag{43a}$$

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

(2) The second set of alternative quenching rate constants and combinations of deactivation/exchange rate constants is given by eq 35 (set S2) and the alternative rotational diffusion

coefficients are given by eq 44:

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

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

Hence, for a spherically symmetric rotor also, as would be expected from the result for the cylindrically symmetric one, of which it is a reduced case, two sets of kinetic parameters are possible: the original ones and those with switched labels. This model is thus only locally identifiable.

4. Conclusions

In this paper, we have examined the feasibility of obtaining information from reversible intramolecular two-state excited-state events. We have demonstrated that similarity transformations can be applied effectively to study the deterministic identifiability of three kinetic models of reversible intramolecular two-state excited-state processes in isotropic environments. The simplest model has neither quencher nor linked species-dependent rotational motions and is unidentifiable. We have specified the conditions under which this model becomes uniquely identifiable. When the initial model is expanded to include a quencher, two sets of quenching rate constants and combinations of excited-state deactivation/exchange rate constants are possible. These combinations are always associated with the proper quenching rate constants but, without extra (spectroscopic and/or kinetic) information, they cannot be allocated to a specific excited species. Upper and lower limits can be assigned to the deactivation and exchange rate constants for both sets. The requirements for obtaining unique identifiability are discussed. In the final model considered, species-dependent rotational diffusion is coupled with the reversible intramolecular two-state excited-state process in the presence of quencher. The functions $I_{\|}(t)$ and $I_{\perp}(t)$ are used to define the functions $S(t)$ and $D(t)$. 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 directly 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 identification is more straightforward if one uses $S(t)$ and $D(t)$ instead of $I_{\|}(t)$ and $I_{\perp}(t)$. The identifiability analysis involving $S(t)$ is the same as for the previous model with quencher (in the absence of species-dependent rotational motions). Coupling the rotational diffusion with the overall excited-state kinetics of a reversible intramolecular two-state excited-state process in the presence of quencher leads to two sets of kinetic parameters (i.e., quenching rate constants, combinations of deactivation/exchange rate constants, and rotational diffusion coefficients), but these parameters cannot be assigned unambiguously to a specific species. It has to be emphasized that although separate deactivation (k_{0A}, k_{0B}) and exchange (k_{AB}, k_{BA}) rate constants cannot be determined without a priori information, the quenching rate constant for species A* (respectively B*) is always correctly associated with the rotational diffusion constant of A* (respectively B*). In the absence of extra information, the number of alternative spectroscopic parameters ($\mathbf{b}^+$ and $\mathbf{c}^+$) is limitless in all three models. The similarity transformation approach used here for the identifiability analysis not only indicates if a model is identifiable or not but has the important advantage of providing explicit relationships between the true and the alternative model parameters.

The last model, which includes a quencher and species-dependent rotational motions, may well be applicable to a wide range of molecular and biomolecular systems. Possible applications, such as reversible intramolecular excimer and exciplex formation, and conformational change, are depicted schematically in Figure 2.

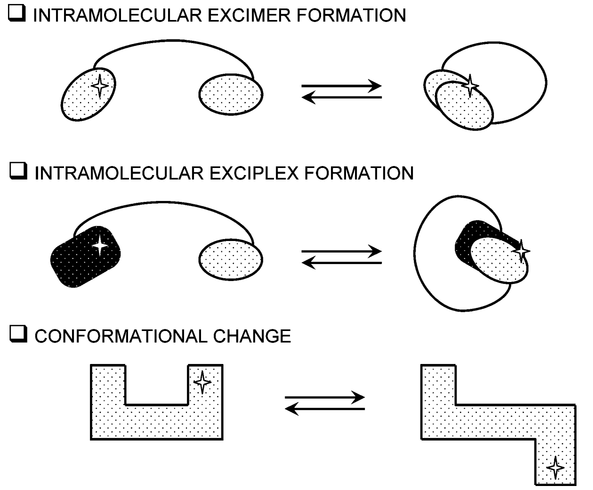


Figure 2. Schematic representation of possible reversible intramolecular two-state excited-state processes. The star is used to denote the excited species.

The deterministic identifiability study presented here constitutes the essential first stage in the analysis of intramolecular two-state excited-state events. The deterministic identification problem reduces to the question of whether a system of algebraic equations has a unique solution. The importance of identifiability analyses in photophysics is not yet fully recognized at present. Besides the intrinsic importance of the specific work reported, this study highlights the importance of identifiability applied to photophysical systems.

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