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Time-Resolved Area-Normalized Emission Spectroscopy (TRANES): A Novel Method for Confirming Emission from Two Excited States

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A model-free method is described for constructing time-resolved area-normalized emission spectra (TRANES) using luminescence decays at all emission wavelengths. An isoemissive point in TRANES indicates that the observed emission from the sample is due to two species only, irrespective of the origin of the two species or the excited-state kinetics. Proof for the existence of an isoemissive point in TRANES is given for various cases involving two emissive species. The isoemissive point in TRANES is qualitatively similar to the isosbestic point in time-resolved absorption spectra (TRAS) in kinetic spectrophotometry involving two species.

Introduction

Kinetics and mechanisms involving electronic excited states of molecules are being studied by a number of spectroscopic methods. Time-resolved fluorescence emission from the excited state of fluorescent molecules is a convenient and frequently used method for investigating the mechanisms of excited-state reactions.[1,2] Some of the important chemical and physical processes of excited states in solutions/condensed phases are electron transfer, proton transfer, exciplex/excimer formation, and solvent relaxation (pure or mixed solvents). In the study of excited-state kinetics, it is important to identify the formation of intermediates between the initial and final state. One of the standard methods in these studies, introduced by DeToma and Brand,[3] is to obtain a set of time-resolved emission spectra (TRES) of the sample and test different models of excited-state kinetics against them. In this paper, we report a novel and new method for the construction of a set of time-resolved area-normalized emission spectra (TRANES), which is a modified version of time-resolved emission spectra. TRANES are obtained without assumption of ground- or excited-state kinetics. A useful feature of TRANES is that an isoemissive point in the spectra supports any model that involves two (and only two) emitting species in the sample. Proof is given for the existence of an isoemissive point in TRANES for different cases in which two emissive species are kinetically coupled either irreversibly or reversibly or not coupled at all. The method is tested using a standard sample and applied to the excited-state relaxation kinetics in a viscous solvent and solvent mixture.

Experimental Section

2-Naphthol (B.D.H., Poole, U.K.) and 2-[2-(4-*N*,*N*,-diethylaminophenyl)ethenyl] thiazolo[4,5-b]quinoxaline (STQ) (gift of Prof. D. W. Rangnekar, see ref 4 for structure and synthesis) were tested to be pure by TLC. 2-Octanol (Aldrich Chemicals, Milwaukee, WI) dichloromethane, methanol, and deionized water (for buffered solutions) were used as solvents in this study. Steady-state fluorescence spectra were recorded using a spectrofluorimeter (SPEX model 1681T) and were corrected for the spectral sensitivity of the photomultiplier (Hamamatsu R928A). Time-resolved fluorescence decays were obtained by the time-correlated single-photon-counting (TCSPC) method.[5] The sample was excited in the absorption band by vertically polarized picosecond laser pulses (800 kHz) (304 nm for 2-naphthol and 380 nm for STQ dye). Fluorescence emission at the magic angle (54.7°) was dispersed in a monochromator (f/4, slit width = 2.5 nm), counted [$\leq 4 \times 10^3$ s^{-1}] by a MCP PMT (R2809), and processed through CFD, TAC, and MCA. The instrument response function is ~100 ps. The experimental setup has been described before.[6,7]

Results and Discussion

The time-resolved area-normalized emission spectra (TRANES) of 2-naphthol in water (buffered to pH 6.6 by 10 mM NaH_2PO_4 and 10 mM CH_3COONa) were obtained in four steps as follows.

Step 1: Fluorescence decays of 2-naphthol in water were obtained over the entire emission spectrum (325−525 nm) at 10-nm intervals. The peak count in the fluorescence decay was 1×10^4 for all wavelengths, except in the wings of the spectrum where the peak count was 5×10^3. Figure 1a shows the fluorescence decays at a few selected wavelengths.

Step 2: The fluorescence decay at each wavelength was deconvoluted using the instrument response function and a multiexponential function, $I(t)=\Sigma\alpha_i \exp(-t/\tau_i)$, $i = 1-4$, where α_i can be negative (excited-state kinetics), by the standard method of nonlinear least-squares and iterative reconvolution.[5,6] A three- or four-exponential function was found to be adequate for all of the decays. The sole aim of this step is to obtain a noise-free representation of the intensity decay function (for a hypothetical δ-function excitation), $I(t)$, at each wavelength. Hence, the amplitudes and lifetimes obtained in this fit do not have any physical meaning. Figure 1b shows the plot of $I(t)$ for the selected decays shown in Figure 1a.

Step 3: Time-resolved emission spectra (TRES), plotted as intensity vs wavenumber, were constructed using $\alpha_i(\nu)$ and $\tau_i(\nu)$, and steady-state emission spectrum that was corrected for the quantum efficiency of the photomultiplier. The equation used is

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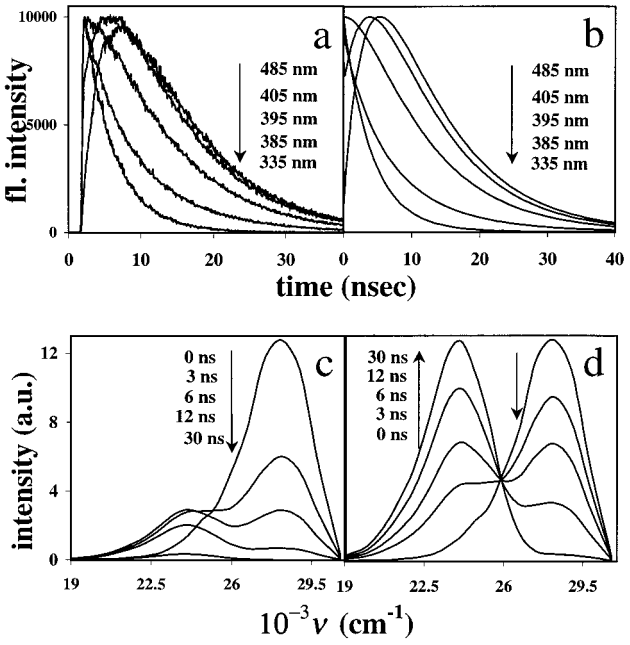

Figure 1. Step-by-step construction of TRANES for 2-naphthol in buffer (pH = 6.6). [2-naphthol] $\approx 5 \times 10^{-6}$ M. (a) Raw experimental data of fluorescence decays at 335, 385, 395, 405, and 485 nm. (b) Intensity decays at 335, 385, 395, 405, and 485 nm obtained by deconvolution of the experimental decays. (c) Time-resolved emission spectra (TRES) at 0, 3, 6, 12, and 30 ns. (d) Time-resolved area-normalized emission spectra (TRANES) at 0, 3, 6, 12, and 30 ns.

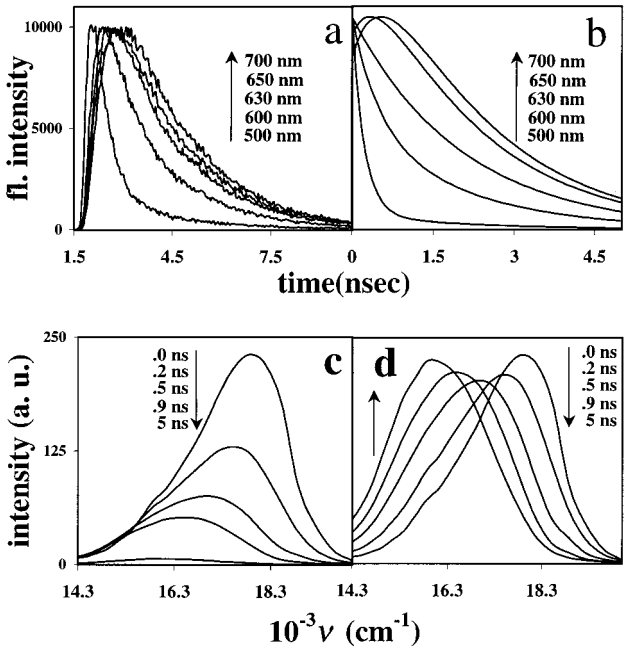

Figure 2. Step-by-step construction of TRANES for STQ dye in 2-octanol. [dye] $\approx 5 \times 10^{-6}$ M. (a) Raw experimental data of fluorescence decays at 500, 600, 630, 650, and 700 nm. (b) Intensity decays at 500, 600, 630, 650, and 700 nm obtained by deconvolution of the experimental decays. (c) Time-resolved emission spectra (TRES) at 0, 0.2, 0.5, 0.9, and 5 ns. (d) Time-resolved area-normalized emission spectra (TRANES) at 0, 0.2, 0.5, 0.9, and 5 ns.

$$I(\nu,t) = I_{ss}(\nu)\frac{\Sigma_j \alpha_j(\nu)\, e^{-t/\tau_j(\nu)}}{\Sigma_j \alpha_j(\nu)\, \tau_j(\nu)} \quad (1)$$

where $I_{ss}(\nu)$ is the steady-state fluorescence intensity at ν and $\alpha_j(\nu)$ and $\tau_j(\nu)$ are the values of the fit parameters. Figure 1c shows the TRES for a few selected times: $t = 0-30$ ns.

Step 4: TRANES were constructed by normalizing the area of each spectrum in TRES such that the area of the spectrum at time t is equal to the area of the spectrum at $t = 0$. Figure 1d shows TRANES for 2-naphthol in buffer.

As seen in Figure 1d, TRANES of 2-naphthol in water (pH 6.6) show an isoemissive point at 389 nm. The excited-state kinetics of 2-naphthol in water is a two-state process involving deprotonation.[8] The pK_a of 2-naphthol is 9.5 in the ground state and 2.8 in the excited state.[8] 2-Naphthol exists as a protonated species in the ground state at pH 6.6 and is deprotonated in the excited state. Thus, one observes emission from both protonated and deprotonated species.

$$R-OH^* \rightarrow R-O^-{}^* + H^+ \quad (2)$$

The observation of an isoemissive point in TRANES, Figure 1d, is in total agreement with the above mechanism of excited-state kinetics of 2-naphthol and with the idea that the emission comes from two species. It can be noted that, if one were to record time-resolved absorption spectra of the two excited species (assuming that both species have sufficiently long lifetimes), then an isosbestic point would be observed at the wavelength where the molar absorption coefficients of the two species are equal. Such absorption experiments are possible in laser flash photolysis experiments. The isoemissive point in TRANES can therefore be considered as a fluorescence equivalent to the isosbestic point in absorption spectra for two kinetically coupled species. A mathematical proof for the existence of an isoemissive point in TRANES is given in the Appendix. TRANES is therefore a very useful practical method for mechanistic analysis of fluorescence spectra and decays.

The method of TRANES was applied to the case of fluorescence photophysics of a dye molecule (STQ) in a viscous solvent (2-octanol) and solvent mixture (dichloromethane/methanol, 9:1 v/v). The excited-state kinetics is due to solvent relaxation because of a change in dipole moment upon excitation.[9] The fluorescence emission spectrum of STQ dye occurs in the region of 500−700 nm in organic solvents. TRANES is therefore useful in this case to determine whether the kinetics involves two emitting species or not. The fluorescence decays were obtained at 5-nm (2-octanol) or 10-nm (solvent mixture) intervals in the range 500−700 nm. The fluorescence decays were analyzed to obtain TRANES by the method described above. The results for the dye in 2-octanol are shown in Figure 2a−d. An isoemissive point is not observed in the TRANES of STQ in 2-octanol, indicating the absence of two-state kinetics in the solvent relaxation in octanol. The resuls of TRES and TRANES for the dye in solvent mixture are shown in Figure 3. An isoemissive point in TRANES is observed in the solvent mixture, which supports a model that the emission occurs from two species.

In constructing TRANES, in step 2, we used a readily available multiexponential fitting program to obtain a noise-free representation of the intensity decay, $I(\nu,t)$. In principle, one could use any arbitrary mathematical decay function (for example, a sum of rational polynomials, a sum of exponentials where the exponent is nonlinear in time, etc.) for fitting the decay. Using a multiexponential function has an advantage in fluorescence analysis because exponential decay is the natural law for the excited state. After the TRANES analysis, one might or might not be able to use the lifetime and amplitude values of the multiexponential fit as physically meaningful parameters. For example, in diffusion-controlled fluorescence-quenching reactions (excimer, exciplex formation, energy transfer, etc.),

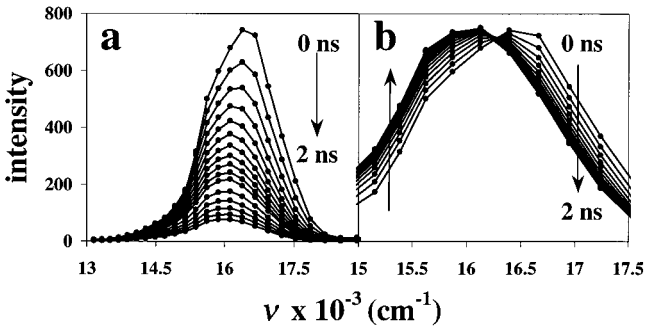

Figure 3. (a) Time-resolved emission spectra (TRES) and (b) time-resolved area-normalized emission spectra (TRANES) of STQ dye in the solvent mixture (dichloromethane/methanol, 9:1 v/v). [dye] $\approx 5 \times 10^{-6}$ M.

the fluorescence decay is nonexponential.[12] The TRANES analysis would support a two-species model (see Appendix), but the lifetimes and amplitudes of multiexponential fits have no physical significance. On the other hand, in the case of 2-naphthol, the TRANES analysis indicated that the sample is a case of two emissive species. In this case, the mechanism is also known, and one expects the fluorescence decay to consist of only two exponentials. Fitting a two-exponential decay (with noise) to a four-exponential function in step 2 would generate results such that the net result is essentially two exponentials. That is, either the two additional lifetimes have values that are close to the true values or the amplitudes of the extra lifetimes are negligibly small. More importantly, the average lifetime, defined as $\tau_{av} = \Sigma\alpha_i\tau_i/\Sigma\alpha_i$, is independent of any fit, which satisfies the usual criteria of a good fit. One must, of course, exercise caution that the noise in the early data is not fitted to an "ultrashort" exponential decay.

In the case of STQ in 2-octanol, the TRANES analysis indicated that this sample does not have two emissive species. On the other hand, the fluorescence decays at several wavelengths could indeed be fitted adequately by two or three exponentials. There was, however, no global consistency in the values of these lifetimes. This is expected because a continuum of excited states is observed during solvent relaxation in viscous solvents. On the other hand, the TRANES analysis indicated that two emissive species is adequate for the solvent relaxation in the solvent mixture of dichloromethane−methanol. In the latter case, it appears that there is an energy barrier between the initially excited state and the relaxed state. Solvent exchange in the excited state, that is, a change of solvent composition around the excited state, is likely to be an activated process. However, there is no experimental or theoretical proof for this proposal.

As shown in the Appendix, theory predicts that an isoemissive point exists if there are two and only two emissive species, irrespective of the origin of the two species or their excited state kinetics. The wavelength of the isoemissive point is the one where the ratio of the radiative rates at that wavelength is equal to the ratio of total radiative rates of the two species. In practice, one uses experimental data subject to error bars. Observation of an isoemissive point in TRANES cannot be interpreted as "proof" for the existence of two emissive species. It must be interpreted as supporting all hypotheses that predict two emissive species.

We now consider instrumental or analysis artifacts and other factors that affect the TRANES analysis. Artifacts can cause a shift in or loss of the isoemissive point. The shift in the isoemissive point from its theoretical position might not be serious for the mechanistic analysis. On the other hand, a loss of the isoemissive point is a serious concern. TRANES uses spectral data obtained in steady-state and time-resolved fluorimeters. The steady-state emission spectrum ought to be a proper representation of the intensity (eq A1 in the Appendix). Therefore, emission spectra will have to be corrected for the variation in the sensitivity of the spectrometer (for example, the response of the photomultiplier) with wavelength. Using an uncorrected steady-state emission spectrum in the TRANES analysis will have the effect of shifting the isoemissive point. In time-resolved fluorimetry, it is not necessary to correct fluorescence decays for the spectral sensitivity of the spectrometer. On the other hand, it is necessary to obtain fluorescence decays over the entire range of the steady-state spectrum. Each spectrum in TRES (Figure 1c) must be complete. If one or more spectra in TRES is incomplete, the isoemissive point might be lost, depending on the extent of the missing spectral area, which might otherwise be present. In actual practice, it can be time-consuming to collect decays at extreme ends of the emission spectrum. Spectral incompleteness to the extent of 5−10% of the area can be completed by using a spectral fit with an empirical but well-tested function such as a log-normal spectral function.[10]

It can be noted that the loss of the isoemissive point in Figure 2d is so glaring that correcting the missing spectral region (<5%) below 14300 cm^{-1} will not result in an isoemissive point.

Conclusion

Time-resolved area-normalized emission spectra (TRANES), which are constructed by the method described in this paper, represent a very useful extension to the well-known and frequently used time-resolved emission spectra (TRES) for interpreting fluorescence spectra and decays and for understanding the excited-state processes involved. An isoemissive point must be observed if there are two emissive species, irrespective of the origin of the species or their kinetics. The condition for the observation of an isoemissive point in TRANES is similar to the condition for the observation of an isosbestic point in the time-dependent absorption spectra of two kinetically coupled species. An important difference in TRANES is that an isoemissive point will also be observed for a mixture of A and B even if there is no kinetic coupling between A* and B* in the excited state. Thus, the isoemissive point in TRANES is a practical test for the presence of two emitting species in the sample.

Appendix

Proof for Isoemissive Point in the TRANES of a Sample with Two Emissive Species. Irreversible Kinetics. Consider the case of emission from two species A* and B*. Consider the case where A* and B* are formed by excitation of A and B, and A* and B* are kinetically coupled. For simplicity, let us consider that the excited-state coupling is irreversible: A* → B*. Let τ_a and τ_b be the fluorescence lifetimes of A* and B*, respectively. The time-dependent emission spectrum is the sum of the emission spectra of A* and B*[11]

$$I(\nu, t) = k_{ra}(\nu)A_0e^{-t/\tau_a} + k_{rb}(\nu)B_0e^{-t/\tau_b} + k_{rb}(\nu)A_0\frac{k_{AB}\tau_a\tau_b}{\tau_b - \tau_a}(e^{-t/\tau_b} - e^{-t/\tau_a}) \quad \text{(A1)}$$

where $I(\nu,t)$ is the total emission (photons/s) at the frequency ν at time t; A_0 and B_0 are the initial concentrations of A* and B*, respectively, upon excitation; k_{AB} is the rate constant of the

irreversible conversion in the excited state; and $k_{ra}(\nu)$ and $k_{rb}(\nu)$ are the frequency-dependent (wavelength-dependent) radiative rates of A* and B*, respectively. The third term in eq A1 is due to the emission of the B* formed from A*. The rate constant of the irreversible kinetics is included in the definition τ_a. That is, $1/\tau_a = k_{ra} + k_{nra} + k_{AB}$, where k_{nra} is the nonradiative rate constant.

Let us assume that the spectra of the two species overlap and that TRANES can be constructed by normalizing the areas of the time-dependent spectra using the area of spectrum at $t = 0$. The areas of the spectra at $t = 0$ and $t = t$ are given by

$$S_0 = k_{ra}A_0 + k_{rb}B_0 \tag{A2}$$

$$S_t = k_{ra}A_0^{-t/\tau_a} + k_{rb}B_0 e^{-t/\tau_b} + k_{rb}A_0\frac{k_{AB}\tau_a\tau_b}{\tau_b - \tau_a}(e^{-t/\tau_b} - e^{-t/\tau_a}) \tag{A3}$$

where

$$k_{ra} = \int k_{ra}(\nu)\, d\nu \tag{A4}$$

and

$$k_{rb} = \int k_{rb}(\nu)\, d\nu \tag{A5}$$

The normalized spectrum at $t = t$ in TRANES is therefore

$$I_N(\nu,t) = \frac{S_0}{S_t} I(\nu,t) \tag{A6}$$

Clearly, the condition for the existence of an isoemissive point in TRANES is $\partial I_N(\nu,t)/\partial t = 0$. Differentiating eq A6 with respect to t and equating $\partial I_N(\nu,t)/\partial t$ to zero, one obtains, after rearrangement and simplification

$$\frac{k_{ra}(\nu)}{k_{rb}(\nu)} = \frac{k_{ra}}{k_{rb}} \tag{A7}$$

as the condition for an isoemissive point in TRANES. An isoemissive point in TRANES occurs at the frequency ν where the ratio of the frequency-dependent radiative rates is equal to the ratio of the total radiative rates of the two emitting species.

The same condition applies for the case where the two emitting species are not kinetically coupled ($k_{AB} = 0$), in which case the sample is a mixture of A and B. The condition is same when $k_{AB} \neq 0$ but $B_0 = 0$. That is, *A* is the only species in the ground state.

Reversible Kinetics. Consider the case where A* and B* are formed by the excitation of A and B, and A* and B* are kinetically coupled reversibly. Let k_{AB} and k_{BA} be the first-order or pseudo-first-order rate constants of conversion of A* to B* and B* to A*, respectively. The coupled differential equations for the kinetics of A* and B* can be solved by Laplace transform method, and the equations for the concentrations of A* and B* and for the time-dependent emission spectrum are reported by Laws and Brand (eqs 9−13 in ref 8). For the purpose of this paper, and for consistency with the equations given above for irreversible kinetics, we write the time-dependent emission spectrum for reversible kinetics as

$$I(\nu,t) = k_{ra}(\nu)[\alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}] + k_{rb}(\nu)[\beta_1 e^{-t/\tau_1} + \beta_2 e^{-t/\tau_2}] \tag{A8}$$

where $k_{ra}(\nu)$ and $k_{rb}(\nu)$ are the frequency-dependent radiative rate constants and

$$\alpha_1 = \frac{-1}{\gamma_1 - \gamma_2}[A_0(Y - \gamma_1) + B_0 k_{BA}] \tag{A9}$$

$$\alpha_2 = \frac{1}{\gamma_1 - \gamma_2}[A_0(Y - \gamma_2) + B_0 k_{BA}] \tag{A10}$$

$$\beta_1 = \frac{-1}{\gamma_1 - \gamma_2}[A_0 k_{AB} + B_0(\gamma_2 - Y)] \tag{A11}$$

$$\beta_2 = \frac{1}{\gamma_1 - \gamma_2}[A_0 k_{AB} + B_0(\gamma_1 - Y)] \tag{A12}$$

where

$$\gamma_1 = (\tau_1)^{-1} = {}^1/_2\{(X + Y) + [(Y - X)^2 + 4k_{AB}k_{BA}]^{1/2}\} \tag{A13}$$

$$\gamma_2 = (\tau_2)^{-1} = {}^1/_2\{(X + Y) - [(Y - X)^2 + 4k_{AB}k_{BA}]^{1/2}\} \tag{A14}$$

$$X = k_{ra} + k_{nra} + k_{AB} \tag{A15}$$

$$Y = k_{rb} + k_{nrb} + k_{BA} \tag{A16}$$

k_{ra}, k_{rb}, k_{nra}, and k_{nrb} in eqs A13 and A14 are the radiative and nonradiative rate constants of A* and B*. The areas of the emission spectra at $t = 0$ and $t = t$ are

$$S_0 = k_{ra}(\alpha_1 + \alpha_2) + k_{rb}(\beta_1 + \beta_2) \tag{A17}$$

$$S_t = k_{ra}[\alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}] + k_{rb}[\beta_1 e^{-t/\tau_1} + \beta_2 e^{-t/\tau_2}] \tag{A18}$$

where k_{ra} and k_{rb} are defined by eqs A4 and A5, respectively. The normalized spectrum at $t = t$ is defined as in eq A6. Equating $\partial I_N(\nu,t)/\partial t$ to zero, the condition for an isoemissive point is obtained as

$$S_t \frac{\partial I(\nu, t)}{\partial t} = I(\nu, t) \frac{dS_t}{dt} \tag{A19}$$

Explicit substitution in eq 19 and simplification leads eq A7.

General Case of Reversible Kinetics by an Arbitrary Mechanism. The most general case of excited-state reversible kinetics involving two emissive species can be written in rate-equation form as

$$\frac{-d[A^*]}{dt} = (k_{ra} + k_a + k_{qa})[A^*] - k_{qb}[B^*] \tag{A20}$$

$$\frac{-d[B^*]}{dt} = (k_{rb} + k_b + k_{qb})[B^*] - k_{qa}[A^*] \tag{A21}$$

where k_{ra} is the radiative rate constant, k_a is the sum of all other rate coefficients of A* that do not lead to B*, and k_{qa} is the sum of rate coefficients (of quenching processes) that lead to B*. k_{rb}, k_b, and k_{qb} are defined for B* in a similar way. In the extreme case, k_a, k_{qa}, k_b, and k_{qb} are time-dependent and depend on the mechanism and complexity of the sample. For example, in diffusion-controlled reactions, the rate coefficient of quenching decreases with time to a constant value,[12] which can be verified in fluorescence experiments.[6,13] Equations A20 and A21 might not be exactly solvable. However, the existence of an isoemissive point in TRANES can be shown without solving eqs A20 and A21.

The fluorescence intensities at ν and t due to A* and B* are

$$I_A(\nu,t) = q_a(\nu,t)\,|\frac{dA^*}{dt}| \quad \text{(A22)}$$

$$I_B(\nu,t) = q_b(\nu,t)|\,\frac{dB^*}{dt}| \quad \text{(A23)}$$

$q_a(\nu,t)$ and $q_b(\nu,t)$ are the fractional quantum yields of A* and B*, respectively, at $t = t$ in the spectral region between ν and $\nu + d\nu$. $q_a(\nu,t)$ and $q_b(\nu,t)$ are time-dependent because of the time-dependent rate coefficients. Because the radiative rates of A* and B* are constants, one can write

$$q_a(\nu,t) = \frac{k_{ra}(\nu)}{k_{ra} + k_a + k_{qa}} \quad \text{(A24)}$$

$$q_b(\nu,t) = \frac{k_{rb}(\nu)}{k_{rb} + k_b + k_{qb}} \quad \text{(A25)}$$

Substituting eqs A20, A21, A24, and A25 into eqs A22 and A23 and replacing the multiplied time-dependent functions by a single function as $a_1(t)$, $a_2(t)$, $b_1(t)$, or $b_2(t)$, one can write

$$I_A(\nu,t) = k_{ra}(\nu)[A_0a_1(t) + B_0a_2(t)] \quad \text{(A26)}$$

$$I_B(\nu,t) = k_{rb}(\nu)[B_0b_1(t) + A_0b_2(t)] \quad \text{(A27)}$$

where A_0 and B_0 are the initial concentrations of A* and B*, respectively. It can be noted that, irrespective of the mechanisms and complexity of the two-state kinetics, $a_1(0) = 1$, $a_2(0) = 0$, $b_1(0) = 1$, $b_2(0) = 0$, and $a_1(\infty) = a_2(\infty) = b_1(\infty) = b_2(\infty) = 0$. The sum of the emission from A* and B* is therefore

$$I(\nu,t) = k_{ra}(\nu)[A_0a_1(t) + B_0a_2(t)] + k_{rb}(\nu)[B_0b_1(t) + A_0b_2(t)] \quad \text{(A28)}$$

The areas of the spectra at $t = 0$ and $t = t$ are

$$S_0 = k_{ra}A_0 + k_{rb}B_0 \quad \text{(A29)}$$

$$S_t = k_{ra}[A_0a_1(t) + B_0a_2(t)] + k_{rb}[B_0b_1(t) + A_0b_2(t)] \quad \text{(A30)}$$

The area-normalized spectrum $I_N(\nu,t)$ is defined by eq A6. Following arguments similar to those used in the previous cases, one obtains eq A7 as the condition for an isoemissive point in this case also.

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