Application of time resolved area normalized emission spectroscopy to multicomponent systems

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Time resolved emission spectroscopy (TRES) provides information on the excited state kinetics and heterogeneity of emissive species in a system. Time resolved area normalized emission spectroscopy (TRANES), an extension to TRES, is a novel, model-free method for the analysis of intrinsic or extrinsic fluorescence probes in complex chemical and biophysical systems [Koti, Krishna, and Periasamy, J. Phys. Chem. A 105, 1767 (2001)]. Observation of a single isoemissive point in TRANES analysis of fluorescence is an unambiguous indication of the presence of two emissive species in the system. The presence of multiple isoemissive points in TRANES spectra is confirmed using simulation and experimental data of multicomponent systems. © 2001 American Institute of Physics. [DOI: 10.1063/1.1405017]

I. INTRODUCTION

Time resolved fluorescence studies have proven to be extremely useful for understanding the structure and dynamics in complex systems such as biological and model membranes using intrinsic or extrinsic fluorescence probes.1 Time resolved emission spectra (TRES) of fluorescence probes have been widely used in biophysical studies. In most cases, the fluorescence emission is likely due to “many” species and the fluorescence decay is generally multiexponential. In complex systems the fluorescence decay is often wavelength dependent and the TRES is a useful method to obtain information regarding the probe and the system. It is important to know the number of emissive species for the interpretation of fluorescence results in such systems. Recently, time resolved area normalized emission spectroscopy (TRANES), a model-free method, has been proposed for the analysis of wavelength-dependent fluorescence decays.2,3 TRANES shows an isoemissive point when two emissive species are present in the system, irrespective of their origin.

In many complex systems it is likely that there are more than two emissive species. For example, a fluorescence probe may be distributed between aqueous phase and membrane phase, and in the membrane phase itself, the probe may be distributed between various locations such as surface and interior regions. This constitutes a multicomponent system, though a single dye molecule is used as a probe. Another important situation is that the emission from two emissive species is preceded by a fast relaxation. In this case, the observation of an isoemissive point is preceded by a fast relaxation of one or both species. The present report shows that TRANES analysis could be applied to multicomponent systems, comprising three (or more) emissive species. In these cases, multiple isoemissive points are observed in different time intervals.

II. MATERIALS AND METHODS

Nile red (Nile blue A Oxazine) was purchased from Sigma Chemicals (USA), Oxonol(VI) (bis-(-3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol) and DiOC12(5) (Dodci; 3,3'-diethyloxacarbocyanine iodide) were purchased from Molecular Probes Inc., USA. Purity of all the three dyes was tested by thin layer chromatography. The fluorescence decay of each dye in methanol is single exponential. Methanol (Spectroscopy grade, S. D. Fine-Chemicals, Mumbai, India) was used as a solvent in this study. Steady state fluorescence spectra were recorded using a spectrofluorimeter (SPEX model 1681T) and corrected for the spectral sensitivity of the photomultiplier (Hamamatsu R928A). Time resolved fluorescence decays were obtained by the time correlated single photon counting method.4 The sample was excited by “vertically” polarized picosecond laser pulses (pulse width ~2 ps) from a high-repetition rate (4 MHz) Nd-Yag pumped dye laser. The wavelength of excitation was chosen at 570 nm for all experimental studies. Fluorescence emission at magic angle (54.7°) was dispersed in a monochromator (f/4, spectral width 2.5 nm), counted (4–5 × 10^3 s^-1) by a microchannel plate photomultiplier (R2809), and processed through a constant fraction discriminator, time-to-amplitude converter, and multichannel analyzer. Fluorescence decays were collected at the interval of 20 ps/channel. The instrument response function is ~50 ps.

The sample for the experiment was chosen as a mixture of three dyes in methanol as mentioned previously. Concentrations of the dyes Dodci, Nile red, and Oxonol are 1.4, 1.5, and 7.2 μM, respectively. All the measurements were carried out in an air saturated solution and the temperature of the sample was maintained constant at 25 °C throughout the experiment.

III. RESULTS AND DISCUSSION

Experiment and theory have shown that one isoemissive point is observed in TRANES when there are only two emis-
Transient area normalized emission spectroscopy (TRANES) was introduced as a tool to distinguish between different fluorescent species present in the system. It was also shown that TRANES could distinguish between the solvent environments of Nile red in methanol and ethanol though the emission maxima differ by only 7 nm. In heterogeneous and biophysical systems, there exists a possibility of many fluorescent species simply because of segregation of a fluorescent probe in aqueous and other nonaqueous (or protein) phases. The ability of TRANES to distinguish between multiple fluorescent species in such complex systems is investigated.

TRANES were obtained in four steps as described before. (a) Collection of fluorescence decays at 5 nm intervals in the entire range of emission spectrum. (b) Fitting the experimental fluorescence decays to a multieponential function and obtain the wavelength-dependent parameters (lifetimes and their amplitudes). (c) Construction of TRES using steady state fluorescence spectrum (corrected for the photomultiplier response), and the wavelength-dependent decay parameters. (d) Normalizing the area of TRES to a constant value (usually to the area of spectrum at time \( t = 0 \) ns) to obtain TRANES.

### A. Three-component system

A three-component system consisting of a mixture of Nile red, Doddi, and Oxonol in methanol was chosen for the experimental studies. Figure 1(a) shows the steady state spectra of the individual dyes that contributed to the total intensity and the steady state spectrum of the mixture. Table I gives the details of the fluorescence data for the three dyes. Fluorescence decays were obtained in the entire range of emission spectrum at the intervals of 5 nm. TRES and TRANES are constructed after the analysis of fluorescence decays. TRES is shown in Fig. 1(b) and TRANES in Fig. 1(c) for various times between 0 and 10 ns. Figures 1(d)–1(f) show the TRANES between the time intervals 0 and 0.1 ns, 0.1 and 1 ns, and 1 and 10 ns, respectively. TRANES during the time interval of 0–0.1 ns shows an isoemissive point at \( 16320 \text{ cm}^{-1} \). Another isoemissive point at \( 16025 \text{ cm}^{-1} \) was observed in the interval of 1–10 ns. These isoemissive points are interpreted as follows.

Oxonol has a very short fluorescence lifetime (\(<0.07\) ns) compared to Doddi (0.88 ns) and Nile red (2.8 ns).

### Table I. Fluorescence parameters for the three dyes in the mixture.

<table>
<thead>
<tr>
<th>Dye</th>
<th>( \lambda_{\text{em}} ) (peak) (nm)</th>
<th>FWHM ( \Delta \nu ) (cm(^{-1}))</th>
<th>Fluorescence lifetime ( \tau ) (ns)</th>
<th>Steady state (area) intensity (a.u.)</th>
<th>Fractional intensity at time ( t = 0 ) ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxonol</td>
<td>633</td>
<td>959</td>
<td>0.07</td>
<td>100</td>
<td>0.59</td>
</tr>
<tr>
<td>Doddi</td>
<td>601</td>
<td>857</td>
<td>0.88</td>
<td>558</td>
<td>0.26</td>
</tr>
<tr>
<td>Nile red</td>
<td>636</td>
<td>1406</td>
<td>2.8</td>
<td>1115</td>
<td>0.15</td>
</tr>
</tbody>
</table>

FIG. 1. (a) Steady state fluorescence spectra of dyes, Doddi (d), Nile red (n), and Oxonol (o) and their sum in the mixture (m) (see Table I for quantitative details). (b) TRES of the mixture of three dyes from 0 to 10 ns. (c) TRANES of the mixture of three dyes from 0 to 10 ns. (d) TRANES of the mixture between 0 and 0.1 ns, and the first isoemissive point at \( 16320 \text{ cm}^{-1} \). (e) TRANES of the mixture between 0.1 and 1 ns, intermediate region. (f) TRANES of the mixture between 1 and 10 ns, and the second isoemissive point at \( 16025 \text{ cm}^{-1} \).
The conditions for the observation of an isoemissive point in the TRANES of multicomponent systems [with short (τ₁), medium (τ₂), and long (τ₃) lifetime components] during various time intervals are derived in the Appendix. The conditions for the observation of an isoemissive point in the short time interval (t < Δtⱼ − τⱼ) are (i) τᵢ ≪ τⱼ and (ii) the fluorescence intensity contribution of the longest lifetime component (Nile red) is negligible compared to the other components (Oxonol and Dodci) in this interval. These conditions are satisfied for the sample mixture as follows. (i) Fluorescence lifetime of Oxonol is very small compared to that of Nile red and (ii) the fractional intensity at t = 0 due to Nile red (0.15) is small compared to the sum of intensities Oxonol (0.59) and Dodci (0.26). Thus, an isoemissive point is observed in the time interval of 0–0.1 ns.

During the time interval of 0.1–1.0 ns, the fluorescence from each of the three dyes is significant and hence TRANES does not show any isoemissive point [see Fig. 1(e)]. After 1 ns, emission from Oxonol is practically absent. Emission after 1 ns is mainly due to Dodci and Nile red. As expected, TRANES shows an isoemissive point at 16 025 cm⁻¹ (624 nm) after 1 ns as shown in Fig. 1(f). In a later section, we show that one or more isoemissive points in TRANES can be easily identified by a plot of wavelength of crossing (λₓₓₓₓ) versus time (Fig. 4). Thus, it is experimentally shown that TRANES analysis can give two isoemissive points in different time intervals for a three-component system. Observation of two or more isoemissive points in TRANES confirms the multicomponent character of the system. A necessary condition for multiple isoemissive points is that the decay time constants and spectral peaks of the components are significantly different from each other.

### B. Computer simulations for multicomponent systems

In computer simulations, the fluorescence data for a three-component system (species 1, species 2, and species 3) were simulated using the steady state spectral and lifetime parameters as given in Table II. The parameters were chosen such that the three components have the same half width [full width at half maximum (FWHM)], same shape, and equal height but different peak positions and lifetimes. The peak positions were chosen to be close to each other as 600, 620, and 650 nm in visible region.

<table>
<thead>
<tr>
<th>Species No.</th>
<th>λₑₑₑₑ (peak) (nm)</th>
<th>FWHM Δν (cm⁻¹)</th>
<th>Fluorescence lifetime τ_f (ns)</th>
<th>Asymmetry Factor (b)</th>
<th>Peak height (g₀)</th>
<th>Fractional intensity at time t = 0 ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>600</td>
<td>1200</td>
<td>0.05</td>
<td>−0.2</td>
<td>100</td>
<td>0.97</td>
</tr>
<tr>
<td>2</td>
<td>620</td>
<td>1200</td>
<td>2.00</td>
<td>−0.2</td>
<td>100</td>
<td>0.02</td>
</tr>
<tr>
<td>3</td>
<td>650</td>
<td>1200</td>
<td>5.00</td>
<td>−0.2</td>
<td>100</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**TABLE II. Fluorescence parameters for the three species in the three-component system.**

![Graph](image-url)
The spectra were calculated using lognormal line shape function. Lognormal line shape function can be written as

\[ g(\nu) = \begin{cases} 
  g_0 \exp \left( -\ln(2) \left( \frac{\ln(1 + \alpha)}{\beta} \right)^2 \right) & \text{for } \alpha > -1 \\
  0 & \text{for } \alpha \leqslant -1
\end{cases} \]

(1)

where \( \alpha = \frac{2b(\nu - \nu_p)}{\Delta} \).

Here \( g_0 \) and \( b \) represent the peak height and asymmetry factor, respectively. \( \nu_p \) is the frequency of emission maximum. The FWHM, \( \Gamma \), of this function and the width parameter \( \Delta \) are related by

\[ \Gamma = \Delta \left( \frac{\sinh(b)}{b} \right). \]

Fluorescence spectra of the three components (species 1, 2, and 3) and their total fluorescence (steady state fluorescence of the resultant three-component system) are shown in Fig. 2(a). Fluorescence lifetimes of the three components were chosen as 50 ps, 2 ns, and 5 ns. Fluorescence decays were calculated at 5 nm intervals to be consistent with the contributions of the three species at different wavelengths. As described before, TRES spectra were constructed in the interval between 0 and 10 ns, which are shown in Fig. 2(b). TRANES is constructed from TRES by area normalization and the spectra between 0 and 10 ns are shown in Fig. 2(c). It is not easy to identify the isoemissive points in Fig. 2(c). [Isoemissive points are clearly seen in the plot of \( \lambda_{\text{cross}} \) versus time, Fig. 4(b).] TRANES shows two distinct isoemissive points, one in the interval of 0–0.2 ns at 16 240 cm\(^{-1}\) (616 nm) [Fig. 2(d)] and another in the interval of 1–10 ns at 15 725 cm\(^{-1}\) (636 nm) [Fig. 2(f)].

Computer simulation studies were also done on a four-component system. The fluorescence parameters used for the four-component system are given in Table III. Simulated fluorescence spectra for the four components (species 1, 2, 3, and 4) and the sum of their individual fluorescence (steady state fluorescence) are shown in Fig. 3(a). Following the same procedure used for three-component case, TRES was calculated using lognormal line shape function.

![Fig. 3](https://example.com/fig3.png)

**FIG. 3.** (a) Simulated steady state fluorescence spectra of the four species, species 1 (1), species 2 (2), species 3 (3), and species 4 (4) and their sum in the mixture (m) (see Table III for quantitative details). (b) TRES of the mixture of the three species from 0 to 20 ns. (c) TRANES of the mixture of three species from 0 to 20 ns. (d) TRANES of the mixture between 0 and 0.2 ns, and the first isoemissive point at 17 560 cm\(^{-1}\) (616 nm) [Fig. 2(d)] and another in the interval of 1–10 ns at 15 725 cm\(^{-1}\) (636 nm) [Fig. 2(f)].

<table>
<thead>
<tr>
<th>Species No.</th>
<th>( \lambda_{\text{em}} ) (nm)</th>
<th>FWHM ( \Delta \nu ) (cm(^{-1}))</th>
<th>Fluorescence lifetime ( \tau_f ) (ns)</th>
<th>Asymmetry Factor (b)</th>
<th>Peak height ( g_0 )</th>
<th>Fractional intensity at time ( t = 0 ) ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>550</td>
<td>1500</td>
<td>0.05</td>
<td>-0.2</td>
<td>100</td>
<td>0.932</td>
</tr>
<tr>
<td>2</td>
<td>570</td>
<td>1500</td>
<td>1.00</td>
<td>-0.2</td>
<td>100</td>
<td>0.046</td>
</tr>
<tr>
<td>3</td>
<td>610</td>
<td>1500</td>
<td>3.00</td>
<td>-0.2</td>
<td>100</td>
<td>0.016</td>
</tr>
<tr>
<td>4</td>
<td>650</td>
<td>1500</td>
<td>8.00</td>
<td>-0.2</td>
<td>100</td>
<td>0.006</td>
</tr>
</tbody>
</table>

![Table III](https://example.com/table3.png)

**TABLE III.** Fluorescence parameters for the four species in the four-component system.
constructed in the interval of 0–20 ns, which is shown in Fig. 3(b). TRANES spectra in the same interval are shown in Fig. 3(c). Three isoemissive points are hidden in Fig. 3(c). The three isoemissive points are distinctively seen when TRANES spectra are plotted in different time intervals as shown in Figs. 3(d)–3(f). The first isoemissive point occurs in the interval between 0 and 0.2 ns at 17 560 cm\(^{-1}\) (569 nm) as shown in Fig. 3(d). The second and third isoemissive points occur in the intervals of 0.5–2 ns [at 16 720 cm\(^{-1}\) (598 nm)] and 8–20 ns [at 15 850 cm\(^{-1}\) (631 nm)] are shown in Figs. 3(e) and 3(f), respectively.

C. Identification of multiple isoemissive points in complex systems

The examples of experimental and simulated data for multicomponent systems described previously indicate that multiple isoemissive points are observed during different time intervals. The time interval in which an isoemissive point will be observed cannot be determined \textit{a priori}. A simple procedure that is described in the following is useful to identify the time intervals.

We define \(\lambda_{\text{cross}}\) as the wavelength (or wavelengths) of intersection of two spectra at times \(t\) and \(t+\Delta t\) in the TRANES where \(\Delta t\) is sufficiently small. The plots of \(\lambda_{\text{cross}}\) vs \(t\) for the three cases described previously are shown in Fig. 4. An isoemissive point is identified when \(\lambda_{\text{cross}}\) is constant in a given time interval. Figure 4(a) shows the plot of \(\lambda_{\text{cross}}\) versus time plot for the TRANES spectra shown in Fig. 1(c), for the experimental data of dye mixture. Two isoemissive points, which are hidden in Fig. 1(c), are clearly identified in Fig. 4(a). The first isoemissive point occurs for \(t<0.1\) ns and the second isoemissive point occurs for \(t>1\) ns. Similarly, Figs. 4(b) and 4(c) reveal transparently the isoemissive points that are hidden in Figs. 2(c) and 3(c), respectively. Thus, it is useful to plot \(\lambda_{\text{cross}}\) vs \(t\) for identifying isoemissive points in multicomponent systems.

The TRANES method uses the time resolved fluorescence data from a sample and the procedure described in this paper is able to identify the number of emissive species without assuming the spectral shape or lifetime of the species. The method is unique and applicable only for emission decay data. To our knowledge, there is no other model-free method that deals with emission decay analysis for the determination of the number of emission species.

IV. CONCLUSIONS

Experimental and simulated fluorescence data of multicomponent (fluorophore) systems are subjected to TRANES analysis. Multiple isoemissive points in the TRANES spectra at different time intervals are observed in these systems if the lifetimes and spectra of emissive species are distinctly separate. Observation of \(N\) isoemissive points in the TRANES confirms the presence of \(N+1\) emissive species. The method of TRANES analysis is recommended for complex heterogeneous and biological systems to determine the number of emissive species.

ACKNOWLEDGMENT

One of the authors (A.S.R.K.) would like to thank G. S. Lakshmikanth for many useful comments.

APPENDIX: PROOF OF ISOEMISSIVE POINT IN THE TRANES FOR A SAMPLE WITH THREE EMISSIVE SPECIES

Consider the case of emission from three independent species \(A^*, B^*,\) and \(C^*\) formed by the excitation of \(A, B,\) and \(C,\) respectively. Let \(\tau_a, \tau_b,\) and \(\tau_c (\tau_a<\tau_b<\tau_c)\) be the fluorescence lifetimes of \(A^*, B^*,\) and \(C^*,\) respectively. The time-dependent emission spectrum is the sum of the emission spectra of \(A^*, B^*,\) and \(C^*\) and is given as

\[ I(t) = I_{A^*}(t) + I_{B^*}(t) + I_{C^*}(t) \]
\[ I(v,t) = k_{ra}(v)A_0 e^{-t/\tau_a} + k_{rb}(v)B_0 e^{-t/\tau_b} + k_{rc}(v)C_0 e^{-t/\tau_c}. \]  

(A1)

Here \( I(v,t) \) is the total emission (photons/s) at the frequency \( v \) at time \( t \); \( A_0, B_0, \) and \( C_0 \) are the initial concentrations of \( A^*, B^*, \) and \( C^* \), respectively; \( k_{ra}(v), k_{rb}(v), \) and \( k_{rc}(v) \) are the frequency-dependent (wavelength-dependent) radiative rates of \( A^*, B^*, \) and \( C^* \), respectively.

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

\[ S_0 = k_{ra}A_0 + k_{rb}B_0 + k_{rc}C_0, \]  

(A2)

\[ S_t = k_{ra}A_0 e^{-t/\tau_a} + k_{rb}B_0 e^{-t/\tau_b} + k_{rc}C_0 e^{-t/\tau_c}, \]  

(A3)

where

\[ k_{ra} = \int k_{ra}(v) dv, \]  

(A4)

\[ k_{rb} = \int k_{rb}(v) dv, \]  

(A5)

and

\[ k_{rc} = \int k_{rc}(v) dv. \]  

(A6)

The normalized spectrum at time \( t \) in TRANES is therefore

\[ I_N(v,t) = \frac{S_0}{S_t} I(v,t). \]  

(A7)

The condition for the existence of an isoemissive point in TRANES is \( \partial I_N(v,t)/\partial t = 0 \).

We get

\[ S_t \frac{\partial I_N(v,t)}{\partial t} = I(v,t) \frac{\partial S_t}{\partial t}, \]  

(A8)

1. **Interval 0 ≤ t ≤ Δt₁ ≅ τₐ ≅ τₖ**

In this interval, as \( \Delta t_1 ≅ \tau_k \) one can approximate the intensity due to species \( C^* \) is constant since \( \exp(-\Delta t_1/\tau_k) \approx 1 \). The fluorescence emission intensity in this interval is given as

\[ I(v,t) = k_{ra}(v)A_0 e^{-t/\tau_a} + k_{rb}(v)B_0 e^{-t/\tau_b} + k_{rc}(v)C_0 e^{-t/\tau_c}. \]  

(A9)

and the area under the whole spectrum at any time is given as

\[ S_t = k_{ra}A_0 e^{-t/\tau_a} + k_{rb}B_0 e^{-t/\tau_b} + k_{rc}C_0. \]  

(A10)

An isoemissive point in this time interval can be obtained only if \( k_{rc}/C_0 \) is negligible compared to the other two terms. That is, the fast decay terms make a dominant contribution to intensity in this time interval. Neglecting the third term, and using Eq. (A8), we obtain the condition for isoemissive point at the frequency (wavelength) as

\[ \frac{k_{ra}(v)}{k_{rb}(v)} = \frac{k_{ra}}{k_{rb}}. \]  

(A11)

2. **Interval Δt₁ ≤ t ≤ Δt₂ = 4 τₐ**

In this interval the emission intensity from each of the three species is significant. Hence there will be no isoemissive point.

3. **Interval Δt₂ < t < ∞**

When \( \Delta t_2 \gg \tau_a \), the intensity from the species \( A^* \) is practically absent as \( \exp(-\Delta t_2/\tau_a) \approx 0 \). The fluorescence emission intensity in this case is given as

\[ I(v,t) = k_{rb}(v)B_0 e^{-t/\tau_b} + k_{rc}(v)C_0 e^{-t/\tau_c}. \]  

(A12)

and area under the whole spectrum at any time \( t \) in this interval is given as

\[ S_t = k_{rb}B_0 e^{-t/\tau_b} + k_{rc}C_0 e^{-t/\tau_c}. \]  

(A13)

Substituting Eqs. (A12) and (A13) in Eq. (A8), on simplification we get

\[ \frac{k_{rb}(v)}{k_{rc}(v)} = \frac{k_{rb}}{k_{rc}} \]  

(A14)

as the condition for the isoemissive point.