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TIME RESOLVED FLUORESCENCE SPECTROSCOPY: TRES AND TRANES

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Time resolved area normalized emission spectroscopy (TRANES) is one-step forward from TRES which is the current textbook standard for presenting fluorescence emission spectra during the lifetime of the excited state. TRANES helps to identify the number of emissive species in a sample. TRES and TRANES together make the interpretation of the fluorescence spectra unambiguous. Most importantly, some pitfalls in the use of TRES to interpret the excited state phenomena in complex chemical and biological systems are overcome if the analysis is extended to TRANES. This paper reviews TRES and TRANES with many examples to demonstrate the usefulness of TRANES for the interpretation of fluorescence data in complex systems.

Key Words: TRES; TRANES; Fluorescence; Solvation Dynamics; Heterogeneity; Micelle; Membrane

Introduction

Fluorescence spectroscopy has now been widely used in the study of structure and/or dynamics of molecules in complex systems, which are ubiquitous in biological, chemical and materials sciences[1,2]. The recent surge in the use of fluorescence in these studies is because of the ability to detect individual photons with space resolution using microscopy and time resolution using ultrafast lasers. Analysis of fluorescence data from complex systems poses a great challenge. The methods that are routinely used for the analysis and interpretation of data from simple systems (e.g. a fluorophore dissolved in a solvent) may not be useful for the analysis of data from complex systems. For example, time resolved emission spectroscopy (TRES) is frequently used to study the excited state dynamics and kinetics of fluorescent molecules in solutions. The standard interpretation of TRES assumes a prior knowledge of the number of fluorescent species in the ground state usually a single species. This assumption may fail if the fluorophore is present in a complex environment; e.g. microheterogeneous media with aqueous and nonaqueous components. Time resolved area normali-zed emission spectroscopy (TRANES) is a step forward and one is able to determine the number of 'species' in the sample that contribute to the observed fluorescence emission. This article reviews the important aspects of TRANES and its practical usefulness. The examples given here are discussed in more detail in a series of publications on TRANES[3-8].

Methods and Materials

Construction of TRANES Spectra

The sample is excited at a particular wavelength and steady state emission spectrum and fluorescence decays are obtained by standard methods such as TCSPC described elsewhere[3]. The four steps involved in the construction of TRANES spectra are as follows:

I. Fluorescence intensity decays are collected at all emission wavelengths/frequencies in small intervals (e.g. 5 nm).
II. Each fluorescence intensity decay, $I(\nu,t)$, is fitted to a multi-exponential function. Usually four exponentials (eq.(1)) are adequate. This step is solely for the purpose of deconvolution of the measured fluorescence from the excitation pulse and removal of noise. The criteria of good fit are random distribution of weighted residuals and chi-square value close to unity[9]. The value of the lifetimes and the sign and value of the pre-exponential factors are not given any physical significance. The average lifetime, τ_{av},

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is calculated as defined in eq. (2). It may be noted that the sum in the denominator of eq. (2) must contain only positive values of the pre-exponential factors.

$$I(\nu,t)=\sum_{i=1}^{4}\alpha_i(\nu)e^{\frac{-t}{\tau_i(\nu)}} \qquad \ldots(1)$$

$$\tau_{av}(\nu)=\frac{\sum_{i=1}^{4}\alpha_i(\nu)\tau_i(\nu)}{\sum_{i=1}^{4}\alpha_i(\nu)} \qquad \ldots(2)$$

τ_{av} is proportional to the area under the fluorescence decay and thus proportional to the quantum yield. τ_{av} varies smoothly (two or more emissive species) or unchanged (single species) with the the emission wavelength.

III. Time resolved emission spectra (TRES) are calculated at different times (t_k) using eq. (3).

$$I(\nu,t_k)=I_{ss}(\nu)\frac{\sum_{i=1}^{4}\alpha_i(\nu)e^{\frac{-t_k}{\tau_i(\nu)}}}{\sum_{i=1}^{4}\alpha_i(\nu)\tau_i(\nu)} \qquad \ldots(3)$$

In eq. (3), $I_{ss}(\nu)$ is the steady state fluorescence intensity taken from the spectrum that was corrected for the quantum efficiency of the photomultiplier.

IV. TRANES spectra are calculated by equating the area of each spectrum in TRES to a constant value; e.g. the area at time, t = 0.

It may be noted that TRES spectra may also be obtained by other methods in which case the TRANES spectra are obtained by step IV.

The experimental details and source of materials are described in detail in refs.[3]-[7].

Results and Discussions

Interpretation of TRES and TRANES Spectra

The TRES and TRANES spectra for many samples are shown below. In TRES, the intensity decreases with time in most cases with the exception of a few where excited state kinetics is transparently seen (e.g. Fig.7 for 2-naphthol) by the rise and fall of intensity at long emission wavelengths. Subtle differences in the shape of the spectrum at different times are not visible because of continuously decreasing intensity. In contrast, TRANES spectra bring out the differences in the shapes of time resolved spectra, if any, more sharply.

The pattern of TRES and TRANES spectra may belong to one of several types. The interpretation of the different patterns in the TRANES spectra is straight forward. For example, an identical spectrum at all time (type 'a' pattern) indicates a single emissive species. Presence of one isoemissive point in the TRANES spectra (type 'b' pattern) indicates that there are two emissive species in the sample. An isoemissive point is the wavelength at which the intensity does not vary with time. Presence of N (>1) isoemissive points at different times (type 'c' pattern) indicates N+1 emissive species. Continuous shift of the emission peak for $0<t<t_k$ followed by an identical spectrum at $t> t_k$ (type 'd' pattern) indicates multiple or 'continuum' of species (e.g. solvation dynamics) during $t< t_k$ and a single species after t_k. Continuous shift of the emission peak for $0<t< t_k$ followed by an isoemissive point at $t> t_k$ (type 'e' pattern) indicates multiple or 'continuum' of species during $t< t_k$ and two species after t_k. Continuous shift of the emission peak with time (type 'f' pattern) indicates a multiple or 'continuum' of emissive species. There may also be other patterns in the TRANES spectra.

Continuous shift of the emission peak in the TRANES spectra (types d, e and f) necessarily implies that there is a continuous shift of emission peak in TRES also but not *vice versa*. This is made clear in some of the examples given below.

The proof that an isoemissive point in TRANES unambiguously indicates two emissive species is given in ref.[3]. Extension of the proof to associate N isoemissive points, occurring successively, to N+1 species is trivial.

Isoemissive point in TRANES and the Origin of the two Species

An isoemissive point in TRANES has the same significance as an isosbestic point or wavelength in time resolved absorption spectra (TRAS) in chemical kinetics. The presence of an isosbestic wavelength in TRAS, the wavelength at which the absorbance is unchanged with time, indicates that there is no intermediate species between the reactant and product species (A→B). In contrast, an isoemissive point in TRANES indicates that there are two emissive species. The origin of the two species may either be excited state kinetics (A*→B*) or simply, that the sample is a mixture of two species

A and B which were simultaneously excited to A* and B*. The observation of isoemissive point in TRANES must therefore be followed by other analysis that will help to distinguish between the different origins of the two emissive species. Here, the mathematical function to which the wavelength-dependent fluorescence decays are fitted provides the clue. For example, a negative amplitude for a decay constant (lifetime) at any emission wavelength (usually at long wavelengths) indicates that A* and B* are kinetically coupled. Absence of negative amplitude for the decay components at all emission wavelengths indicates that the sample is a mixture of A and B.

TRES and TRANES spectra for different samples are given below. The pattern for each sample is identified with one of the types described above and interpreted.

Nilered in Methanol

Fig. 1 shows the TRES and TRANES spectra of the dye nilered in methanol. The TRES spectra decay with time and the peak position is unchanged. The TRANES spectra are identical (within noise level) at all time. The pattern of TRES and TRANES spectra belongs to type 'a'. The fluorescence emission from this sample is ascribed to one emissive species. This sample may be termed as the simplest system as far as fluorescence emission is concerned in the time scale of t>10 ps. In this time scale fluorescence is due to one species only. The lower limit of 10 ps indicates that time resolved spectra below this time were not possible to obtain in the TCSPC set up.

Nilered in Methanol and Propanol

The fluorescence spectrum and lifetime of nilered in methanol are different from that in propanol. This is so because nilered is a probe that is sensitive to its local environment. The TRANES analysis of the fluorescence nilered in 2-propanol indicated that the emission is due to one species in this solvent also. Fluorescence from both samples is collected in a two-compartment cuvette by exciting both the samples simultaneously. The fluorescence spectrum and decay are thus intermediate between the two solvents. Fig. 2 shows the TRES and TRANES spectra of this 'mixture'. The pattern belongs to type 'b'. The TRES spectra show a time dependent spectral shift of the peak. The TRANES spectra reveal an isoemissive point leading to the unambiguous conclusion that the emission is due to two species.

This example illustrates that two species whose emission spectra differ by as small as ~10 nm can be identified in TRANES analysis using data collected in 5 nm intervals. Probes like nilered in microheterogeneous media could exist in different environments. The fluorescence from each environment may be considered as different species if the interconversion among them is slow compared to the fluorescence time scale. Thus, TRANES analysis should be able to identify the existence of

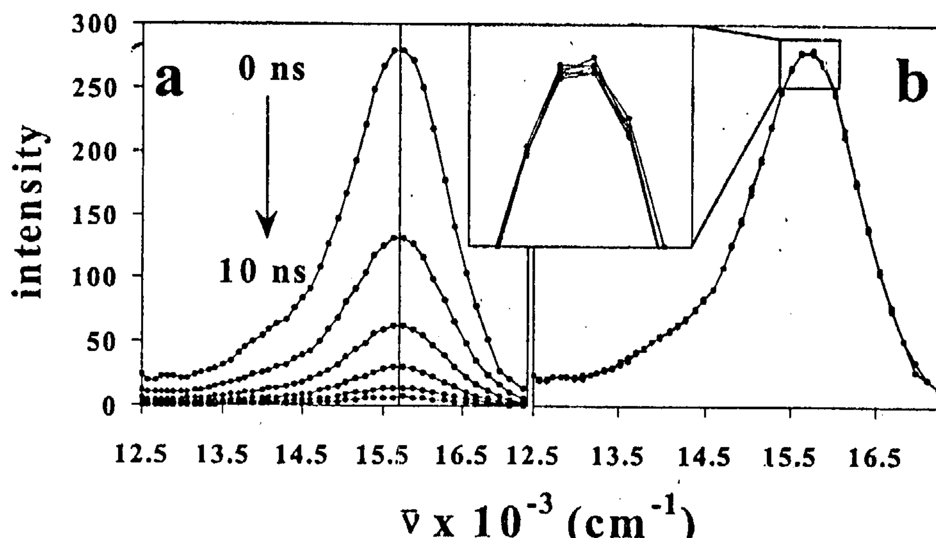

Fig. 1 (a) Time resolved emission spectra (TRES) and (b) Time resolved area normalized emission spectra (TRANES) for nile red in methanol. Inset shows the peak region in greater detail. The spectra are identical at all time. The pattern belongs to type 'a'. [nilered]=2 μM. λ_{ex}=571 nm. The spectra are shown for times, 0, 2, 4, 6, 8 and 10 ns.

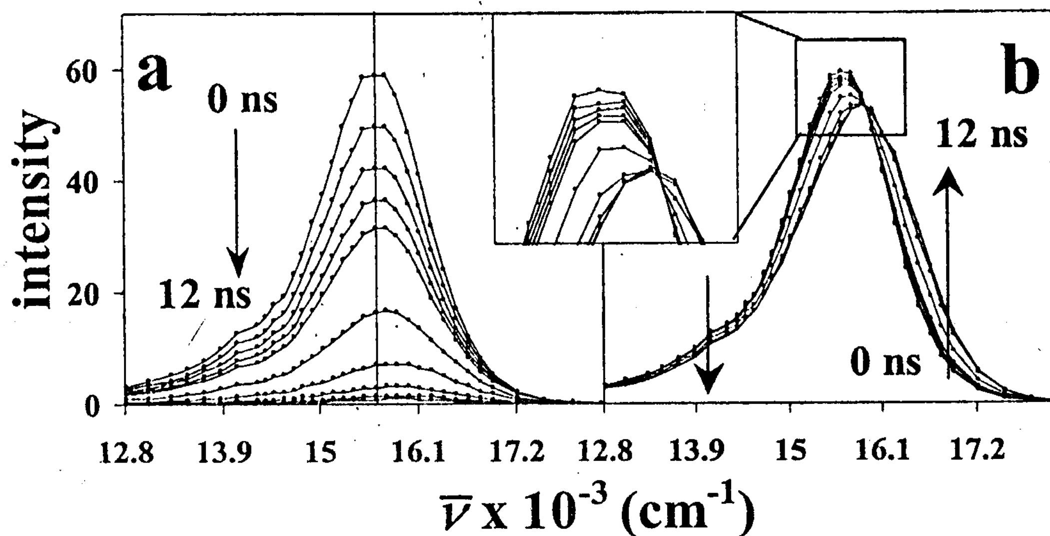

Fig. 2 (a) TRES and (b) TRANES spectra for two nilered samples, one in methanol quenched by KI (0.2M) and the other in 2-propanol, simultaneously excited in a two-compartment cuvette. The spectra are shown for times, 0, 0.2, 0.4, 0.6, 0.8, 1, 4, 7, 10, and 12 ns. TRES spectra show blue shift of spectral maximum with time. TRANES spectra show isoemissive point. The pattern belongs to type 'b'. Inset shows the peak region in greater detail.

multiple species of the same dye in microheterogeneous media.

Nilered in 2-Octanol

The fluorescence of nilered in 2-octanol is not as simple as that in methanol or propanol. The time scale of the interaction of nilered with the solvent, that is, the solvation dynamics, becomes slowed down by the viscosity of the solvent. This is seen clearly in the pattern of TRES and TRANES spectra for this sample shown in Fig. 3, which belongs to type 'f'. The TRES spectra show a time dependent peak shift to the red. The red shift of the peak with time is also observed in the TRANES spectra. A detailed analysis of the multiexponential fluorescence decays at all emission wavelengths showed excited state kinetics attributable to solvation dynamics. Thus, the type 'f' pattern of TRES and TRANES for this sample is associated with solvation dynamics.

Nilered in Micelle

Aqueous solution of surfactant micelle is a microheterogeneous system. At least three different solvent environments exist: water, hydrophobic region of the micelle and the interface region. It is very common to observe multiexponential decay for most fluorescent probes in micelles. TRANES

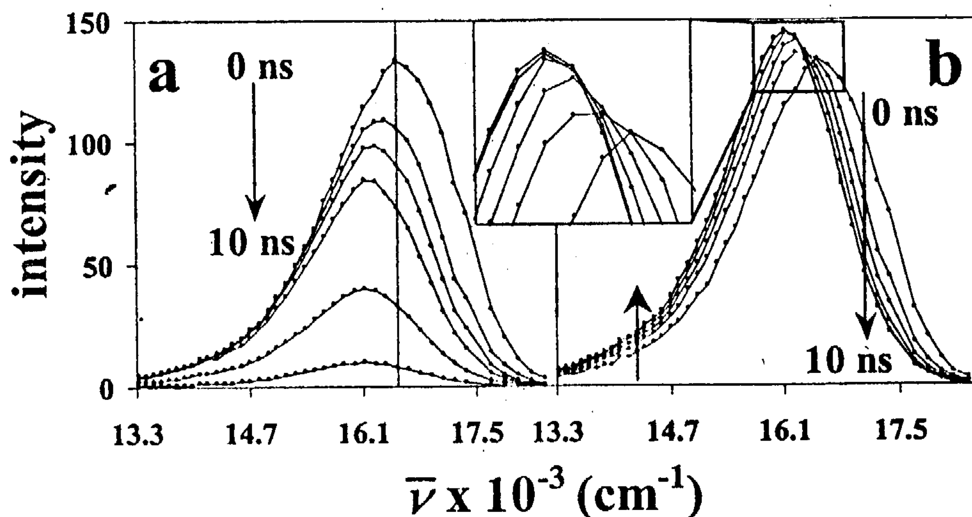

Fig. 3 (a) TRES and (b) TRANES spectra for nile red in 2-octanol. [nilered] = 2 μM. The spectra are shown for times, 0, 0.25, 0.5, 1, 4, and 10 ns. The pattern belongs to type 'f'. Inset shows the peak region in greater detail.

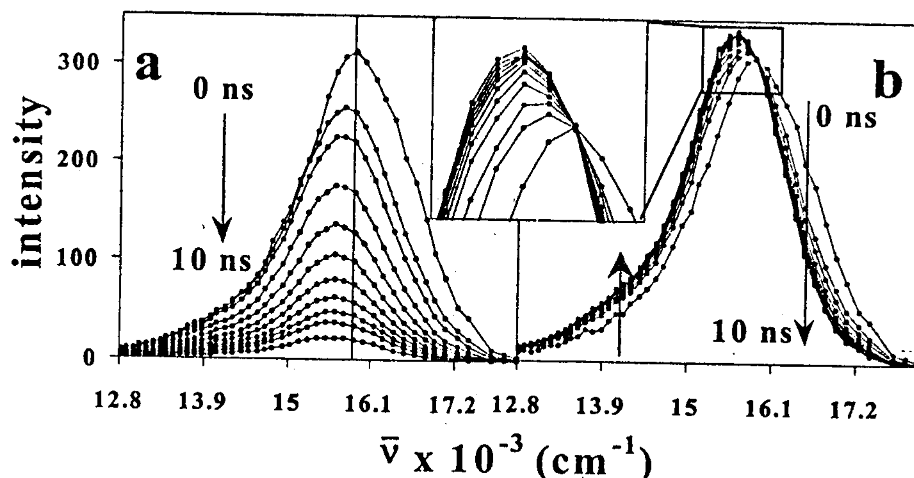

Fig. 4 (a) TRES and (b) TRANES spectra for nilered in TX-100 micelle. The pattern belongs to type 'b'. Inset shows the region of isoemissive point at 630 nm in greater detail. [dye]=2 μM. [TX]=20 mg/mL. λ_{ex}=571 nm. The spectra are shown for times, 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 10 ns.

analysis is extremely useful to interpret the fluorescence in such cases. Fig. 4 shows the TRES and TRANES spectra for nilered in TX-micelle. Time dependent red shift was observed in TRES whereas TRANES spectra showed an isoemissive point. The pattern belongs to type 'b' and two emissive species are inferred. The nature of the two emissive species and their location can be understood only by other experiments such as fluorescence anisotropy. Without going into the details, it was inferred that the two species in this case are the dye molecules solubilized in two different sites in the interface region, which differ in their exposure to water.

Nilered in EggPC Membrane

Lipid bilayer membranes are another class of microheterogeneous media. Fig. 5 shows the TRES and TRANES spectra of nilered in EggPC membrane. The Pattern here is similar to that observed in TX-micelle, namely, type 'b'. We infer that the fluorescence emission is due to two species.

DPH in EggPC Membrane

Diphenyl hexatriene (DPH) and its derivatives are widely used in probing the structure of bilayer membranes. The TRES and TRANES spectra (Fig. 6) for this sample showed a pattern that belongs to type 'a'. That is, the emission is attributable to one species only. What makes this example interesting (e.g. compared to nilered in methanol which is also type 'a') is that the fluorescence decay is multiexponential. The origin of multiexponential

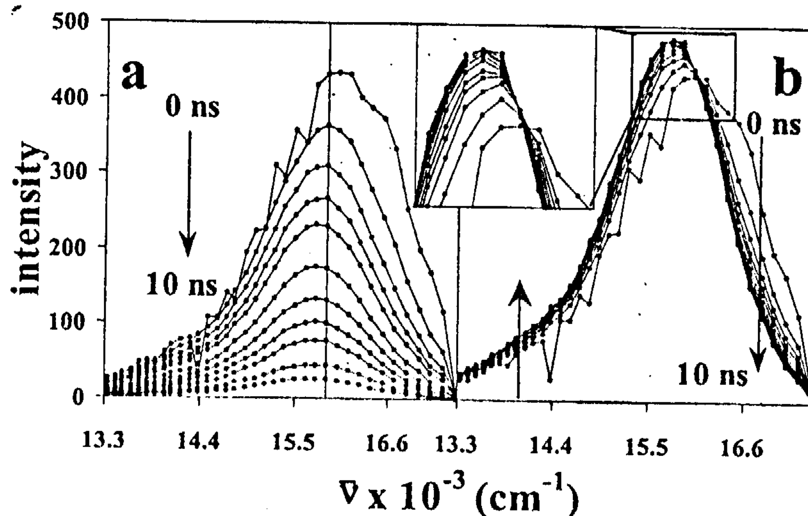

Fig. 5 (a) TRES and (b) TRANES spectra for nilered in eggPC membrane. The pattern belongs to type 'b'. Inset shows the region of isoemissive point observed at 625 nm in greater detail. [nilrred]=1 μM.; [eggPC]=0.2 mg/mL; λ_{ex}=571 nm. The spectra are shown for times, 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 ns.

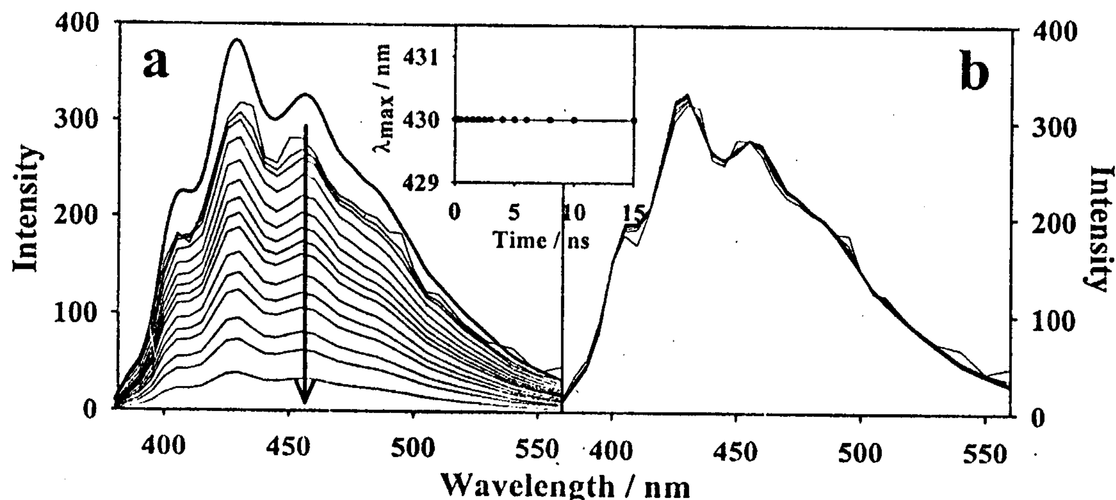

Fig. 6 (a) TRES and (b) TRANES spectra for diphenyl hexatriene (DPH) in eggPC bilayer membrane. The pattern belongs to type 'a'. The spectra are shown for time 0 to 15 ns. The inset shows the peak wavelength as a function of time.

decay of DPH is due to the orientational distribution of the dye which modifies the radiative rate and thus the lifetime[10,11].

2-Naphthol in Water

The photophysics of 2-naphthol in water is well known. It is a text book example of excited state kinetics, namely, deprotonation in the excited state. The fluorescence emission is due to two species, naphthol and naptholate ion. Fig. 7 shows the TRES and TRANES spectra for this sample (*pH* 6.6). The pattern belongs to type 'b'. The TRES spectra do show the evidence for the presence of two species, unlike the above examples with type 'b' pattern, because the spectral peaks of the two species are well separated by ~60 nm. The emission from two species is transparently evident in the TRANES spectra which show the isoemissive point.

DMABN in Acetonitrile

The photophysics of 4-*N,N*-dimethylamino-benzonitrile (DMABN) in organic solvents has been studied extensively. The origin of the 'dual' fluorescence was the focus of much debate assuming that there are only two emissive species. Fig. 8 shows the TRES and TRANES spectra of DMABN in acetonitrile. The pattern belongs to type 'b' and hence the assumption of two emissive species is confirmed by TRANES analysis.

Mixture of Three Dyes

Fig. 9 shows the TRES and TRANES spectra for a sample which is a mixture of three dyes

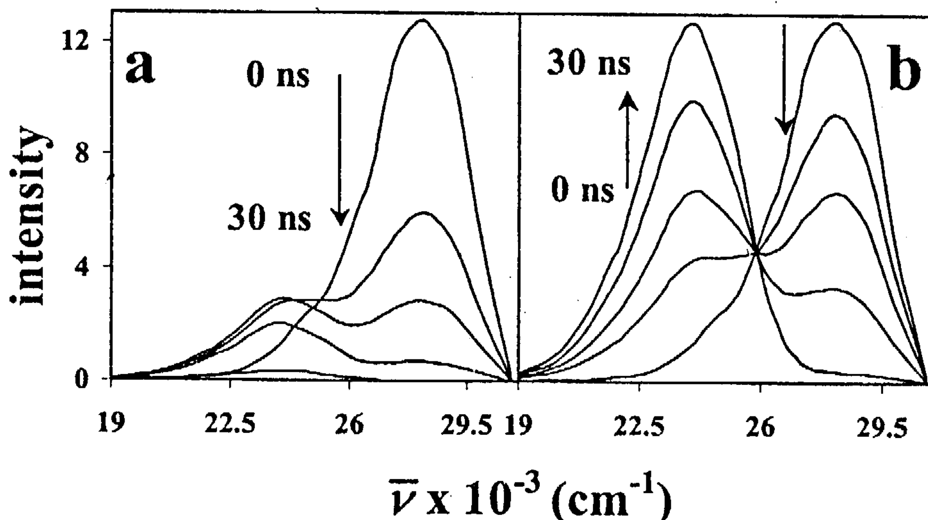

Fig. 7 (a) TRES and (b) TRANES spectra for 2-naphthol in buffer (pH 6.6). [2-naphthol] ≈ 5 μM. The pattern belongs to type 'b'. The spectra are shown for times, at 0, 3, 6, 12, and 30 ns.

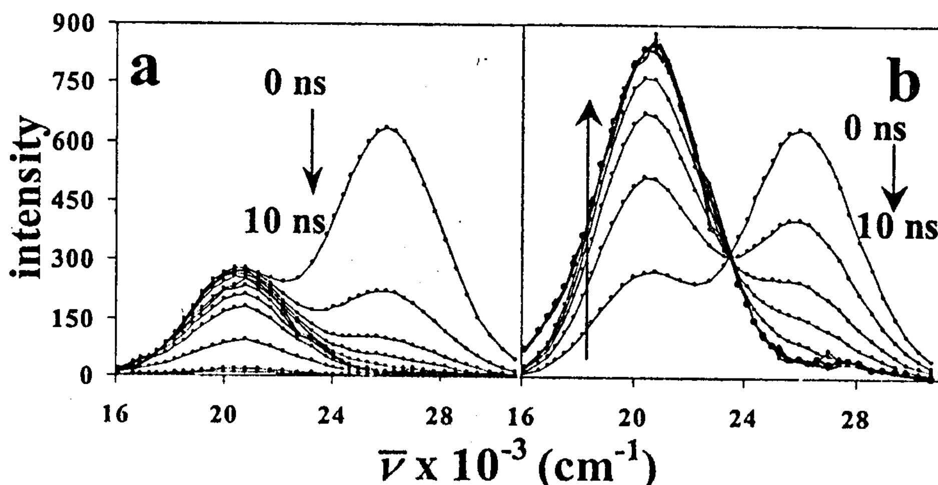

Fig. 8 (a) TRES and (b) TRANES spectra for DMABN in acetonitrile. The spectra belong to type 'b'. Excitation wavelength = 295 nm. [DMABN] = 20 μM. The spectra are shown for t=0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5, 1, 3, 8 and 10 ns.

(oxonol, nilered and DODCI) in methanol. The fluorescence spectra and lifetimes of these dyes differed from each other. The concentrations of the dye were adjusted such that the contribution of each dye to the total fluorescence was significant. The TRES spectra are not helpful to identify that the sample is a mixture of three emissive species. At first glance, the TRANES spectra (Fig. 9b) do not reveal much. However, careful examination of the TRANES spectra reveal that there are two isoemissive points which at occur at different time intervals (c and e). The pattern of TRES and TRANES belongs to type 'c'. An example of TRANES analysis of emission from four species is described in ref. [5]. Thus, multiple emissive species in a sample can be identified if the lifetimes are sufficiently distinct from each other.

TRANES and Solvation Dynamics

The pattern of the TRANES spectra can be used to confirm if solvation dynamics of a fluorescent probe does or does not occur in the time scale of the experiment. Proof of existence of a 'continuum' of species or multiple emissive species with energy levels ranging from the initially excited state to a final relaxed one is the prerequisite for the solvation model. Time dependent red shift of the emission peak in TRES and TRANES is the necessary (but not sufficient, see below) condition. The TRES and TRANES spectra for nilered in octanol (Fig. 3) showed such a time dependent spectral shift in both indicating solvation dynamics. However, for nilered in methanol and propanol 'mixture' (Fig. 2), TRES (or peak normalized TRES) spectra showed time dependent spectral shift (a blue shift!) that would be misleading because TRANES spectra showed an isoemissive point which is the expected pattern for this sample. Similarly, for Nile red in TX-100 micelle and egg PC membrane,the TRES spectra in Figs. 4 and 5 showed a continuous red shift with time but the TRANES spectra revealed an isoemissive point, leading to the unambiguous conclusion that there are two emissive species. If one stops the analysis at the TRES level then one is led to the wrong conclusion that the red shift in TRES is due to solvation dynamics of the fluorophore. Extending the analysis to TRANES level, one concludes that there are two emissive species of different lifetimes and different spectral maxima, and not a continuum of species. Solvation dynamics for the dye in this micelle is probably over (<50 ps) before the emergence of the isoemissive point. Inclusion of TRANES spectra of the dye at shorter time scale may reveal a type 'e' pattern. TRANES spectra belonging to type 'd' or 'e' patterns have been observed for a few dyes in EggPC membranes[8] and in micelles (unpublished results), which require a more detailed study.

As remarked above, the pattern of time dependent spectral shift in TRANES spectra is a necessary but not sufficient condition to confirm solvation dynamics. A sample in which the fluorophore is extensively heterogeneous in the ground state may also show apparent time dependent spectral shift in TRES and TRANES. Thus, it is essential to rule out ground state heterogeneity of the fluorophore for studying solvation dynamics of probes in microheterogeneous media.

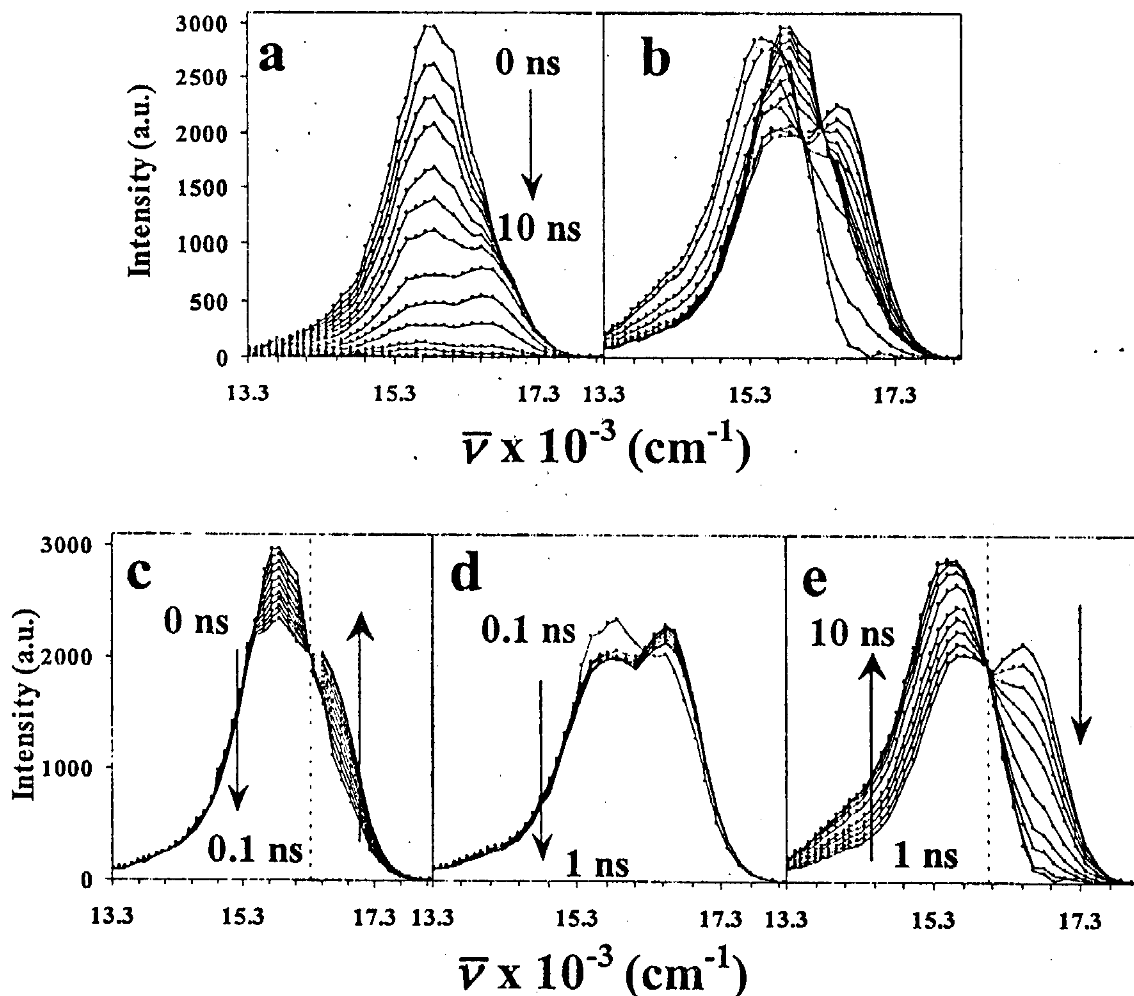

Fig. 9 (a) TRES (b) TRANES spectra for the mixture of nilered, oxonol and DODCI in methanol from 0 – 10 ns. (c) TRANES spectra between 0 and 0.1 ns and the first isoemissive point at 16320 cm^{-1}. (d) TRANES spectra between 0.1 ns and 1 ns intermediate region. (e) TRANES spectra between 1 ns and 10 ns and the second isoemissive point at 16025 cm^{-1}.

Conclusions

TRANES analysis can distinguish fluorophores that are in different environments. If the equilibrium kinetics between molecules in different environments is slow, then the fluorophore may be considered a 'mixture' of two species of the same molecule. It is shown that TRANES spectra of Nile red can distinguish two environments as 'different' as propanol and methanol (Fig. 2) or even ethanol and methanol[4].

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