Dynamic two-dimensional fluorescence correlation spectroscopy. Generalized correlation and experimental factors

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Received 2nd April 2001, Accepted 9th July 2001
First published as an Advance Article on the web 30th July 2001

Dynamic two-dimensional fluorescence correlation spectroscopy (2D FCS) is presented in the general form. Dynamic 2D FCS evaluates the time correlation function between two wavelength axes when an external perturbation is applied to the sample. It displays the vibronic features with similar time response functions in the synchronous correlation spectrum and the features with different time responses in the asynchronous correlation spectrum. The correlation analysis allows detailed assignments of the vibronic spectra of multicomponent samples. The emission–emission 2D FCS has proven to be able to resolve spectra with substantial overlaps, of species in equilibrium with each other, and of reacting species whose kinetic constants are linked and multiexponential. Similarly, the correlation analysis between excitation wavelengths allows the assignment of the excitation bands to fluorescent components. When a sinusoidal light source is used to excite the sample, the excitation–emission correlation requires the collection of only four spectra, two in-phase and two quadrature. The two-dimensional excitation–emission correlation analysis uncovers the association between the excitation and the emission vibronic features, enabling the complete assignment of the component spectra. The band assignments and spectral assignments are facilitated by the two-dimensional phase map that is constructed from the synchronous and asynchronous correlation spectra. Spectral resolution can be optimized by varying the frequency of excitation and is not influenced by the detector phase angle used to collect the spectra. The resolution power of the 2D FCS is demonstrated with the retrieval of the anthracene emission spectrum from a pyrene–anthracene mixture when it contributes only 4% to the total fluorescence intensity.

Introduction

Two-dimensional fluorescence correlation spectroscopy has been shown to significantly improve the resolution in fluorescence spectroscopy1–5 and laser-induced fluorescence detection of capillary electrophoresis.6 The correlation between peaks enables spectral assignment of the vibronic transitions in the fluorescence emission spectra and peak identification in CE. Through two-dimensional correlation analysis, component vibronic bands or CE peaks buried in the heavily overlapped overall spectrum or electropherogram have been resolved. The advantage of the two-dimensional fluorescence correlation is that it does not require any a priori information of the sample.

Two-dimensional correlation spectroscopy was first introduced by Noda in the field of infrared spectroscopy.7,8 A modulated mechanical field was employed to perturb the polymer system and its time response in the IR was monitored to construct the 2D correlation spectra. The cross correlation between frequencies contained information on the dynamics of the polymer. IR bands that relax at similar rates and thus show little asynchronous correlation between them were assigned to the same side group of the polymer. The side group and the backbone of the polymer were shown to have different reorientational dynamics. The generalized 2D correlation spectroscopy later proposed by Noda9 suggested the extension of the two-dimensional correlation principles to other spectroscopic techniques. An ensemble of spectra collected at various levels of a selected experimental parameter, such as the concentration, the pressure, and the temperature are used to construct the generalized two-dimensional correlation maps. The inherent dependence in the sample that is not obvious in the original one-dimensional spectra is thus revealed. Since its introduction, 2D vibrational (infrared and Raman) correlation spectroscopy has been widely applied for spectral assignments and dynamic studies of molecular systems.10–20

Fluorescence spectroscopy is an excellent tool for probing molecular structure and dynamics,21 and for environmental22 and biomedical sensing,23 due to its high sensitivity and selectivity. Generally, the fluorescence excitation spectrum, the emission spectrum, and the fluorescence decay rates are the basis of fluorescence probe studies, sensing, and detection. A tensor of fluorescence intensity in these three independent, orthogonal axes contains rich information of the sample. The excitation–emission matrices (EEMs) introduced by Warner et al. greatly enhanced the capability of fluorescence in the analysis of multicomponent samples by combining the information on both the ground and the excited state energy levels.24 Knorr and Harris devised a method to retrieve lifetime-associated component spectra from time-resolved fluorescence emission spectra, a data matrix of the emission wavelengths and decay times.25 Knutson et al.25 recovered these decay-associated spectra (DAS) by globally linking the decays at various emission wavelengths in the nonlinear least squares fitting.26 DAS has proven to be a powerful method for probing the dynamics of biomolecules.27–29 More recently, Neal discussed a multivariate method to analyze intensity matrices collected along the axes of emission wavelength and modulation frequency.30 The photokinetics and the relative concentrations are calculated in this methodology. All three axes have been utilized by Millican and McGown in the frequency domain multiarray analysis where each array is an excitation–emission matrix obtained at a certain modulation frequency.31 The three-axis tensor of data contains most fluorescence parameters generally measured in chemical analysis. The experimental collection of intensity along all three axes, however, can be very time consuming. The statistical analysis of the data tensor to

DOI: 10.1039/b102976m

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recover the decay-associated excitation and emission spectra is computationally demanding. For example, in order to identify the component spectra with factor analysis, target transformation requires substantial knowledge of the sample.

In this paper, we will present the framework of the generalized dynamic 2D FCS, and its special cases of excitation–excitation and excitation–emission correlation. The time correlation intensity is evaluated from the experimentally measured in-phase and the quadrature spectra. All three axes of fluorescence information are collected, with a much-simplified methodology that minimizes the data collection time. The correlation is directly displayed in the time-correlation function. Statistical analysis of the data is not required, eliminating the necessity of a priori information on the sample for target transformation. The vibronic structure in the fluorescence excitation and emission can be assigned reliably from the correlation patterns. The influence of experimental parameters, such as the modulation frequency, the detector phase angle, and the concentration ratios, on the 2D FCS are investigated.

Experimental section

All chemicals were obtained at the highest purity and used as received. The fluorescence spectra were collected with a frequency domain fluorescence spectrometer (SLM48000MIF, Jobin Yvon, NJ). The excitation was provided by a HeCd laser, operating at 325 nm, or a Xe lamp. For the fluorescence emission spectra, in both the steady state and the phase-sensitive modes, the laser was the excitation source unless stated otherwise. The fluorescence emission was dispersed with a monochromator at a spectral bandwidth of 2 nm. A 345 nm long-pass filter was inserted before the monochromator to effectively remove the scattered laser light. The bandwidth was 4 nm for both the steady state and phase-sensitive excitation spectra. The wider slit widths were selected to provide higher SNR for dynamic spectral measurements with lamp excitation. The fluorescence was collected with a 400 nm long-pass filter to minimize the scattering.

To measure the phase-sensitive fluorescence spectra, the detectors were modulated sinusooidally by a frequency synthesizer that also modulates the excitation source. A glycogen solution was used to calibrate the detector phase angle. The detector was 90° delayed in phase with respect to the excitation under these conditions. In most experiments, the fluorescence detector phase angle was set at 0 and 90° for the measurements of the in-phase and the quadrature spectra, respectively. To examine its effects on the two-dimensional correlation analysis, the detector phase angle was varied. In these experiments, the two spectra used to evaluate the time correlation function were collected at detector phase angles with 90° separation. The 2D fluorescence correlation spectra were calculated in MatLab (MathWorks, Inc., Natick, MA).

Results and discussion

Dynamic 2D FCS

The two-dimensional fluorescence correlation analysis examines the dynamics of the molecular system along a chosen parameter \( t \), dispersed in variable \( \lambda \): 

\[
C(t; \lambda_1^l, \lambda_2^l) = \lim_{t \to \infty} \frac{1}{2T} \int_0^{2T} A^+(t; \lambda_1^l) A(t + \tau; \lambda_2^l) d\tau 
\]  

(1)

where the asterisk denotes the complex conjugate of the fluorescence response. To establish the correlation function, an external perturbation is introduced into the system under study, as a variation in parameter \( t \). The sample responds to the perturbation according to its characteristic relaxation rates. The experimentally measured signal \( A(t; \lambda) \) is the fluorescence intensity at parameter \( t \), dispersed in the variable axis \( \lambda \). The two-dimensional correlation function at parameter \( t \) is calculated from the intensity responses between \( \lambda_1^l \) and \( \lambda_2^l \). A two-dimensional fluorescence correlation spectrum is a plot of the correlation intensity as a function of the two axes of variable \( \lambda_1^l \) and \( \lambda_2^l \). The two axes do not have to be identical, as designated by the superscripts \( a \) and \( b \). They can be fluorescence intensities at different directions of polarization, for example. Both the parameter and the variable are defined in general terms. The parameter can be any experimental factor that perturbs the fluorescent system; the dispersion in variable \( \lambda \) is characteristic of a fluorescent species that does not change with the parameter \( t \). When the variable is the fluorescence excitation or emission wavelength, the resulted technique is two-dimensional fluorescence correlation spectroscopy. The parameter can be the time in the dynamic 2D FCS, concentration ratio (concentration perturbation), or the pH (pH perturbation). The excitation wavelength can be varied for the evaluation of the emission–emission correlation spectra (excitation perturbation), or vice versa. Two-dimensional correlation capillary electrophoresis is based on the dispersion along the elution time in an electropherogram. The elution time is the variable in this technique. The 2D correlation analysis enables resolution and identification in the variable axis based on the distinctive relaxation rates in the parameter axis for different fluorescent species in the sample. In this paper, we will focus on the dynamic two-dimensional fluorescence correlation spectroscopy where the perturbation is in the time axis and the variable is the excitation or emission wavelength. A short laser pulse provides the excitation. The response of the system is monitored as fluorescence intensity decay at time \( t \) after the impulse excitation, dispersed in wavelength \( \lambda_1^l \) and \( \lambda_2^l \). The two-dimensional time correlation function is calculated from the decays collected at these two wavelengths according to eqn. (1).

A convenient way to construct the correlation function is to use a sinusoidal perturbation. Frequency domain fluorescence spectroscopy provides such an approach where the excitation beam is sinusoidally modulated to produce a sinusoidal response from the sample that is delayed in phase and demodulated in amplitude. The fluorescence response is expressed as:

\[
A(t; \lambda^r) = A(\lambda^r) e^{i(\omega t - \phi)} 
\]  

(2)

where \( A(\lambda^r) \) is the fluorescence intensity at wavelength \( \lambda^r \). We note that eqn. (2) is a simplified expression for the fluorescence response. The dc term is not shown since the phase-sensitive fluorescence measures the ac signal that is used to evaluate the two-dimensional correlation. In addition, the constant that accounts for instrumental functions has been reduced. \( \phi \) is the angular modulation frequency of the excitation. \( \phi \) is the phase delay of the fluorescence from the excitation and is independent of \( a \). Substituting eqn. (2) into eqn. (1) and carrying out the integration, we obtain the general form of the correlation function at frequency \( \omega \):

\[
C(\omega; \lambda_1^r, \lambda_2^r) = \left[ \Phi(\omega; \lambda_1^r, \lambda_2^r) + i \Psi(\omega; \lambda_1^r, \lambda_2^r) \right] e^{i \omega t}. 
\]  

(3)

in which

\[
\Phi(\omega; \lambda_1^r, \lambda_2^r) = A(\lambda_1^r) A(\lambda_2^r) \cos \Delta \phi 
\]  

(4)

and

\[
\Psi(\omega; \lambda_1^r, \lambda_2^r) = A(\lambda_1^r) A(\lambda_2^r) \sin \Delta \phi 
\]  

(5)
are the synchronous and asynchronous correlation intensities, respectively. \( \Delta \phi \) is the phase difference between the two wavelengths,

\[
\Delta \phi = \phi(\omega, \lambda_1^\text{ex}) - \phi(\omega, \lambda_2^\text{ex})
\]

and will be referred to as the phase differential. When both \( a \) and \( b \) denote the fluorescence emission, \( \lambda_1^\text{em} \) and \( \lambda_2^\text{em} \) are the identical axis of emission wavelength. This special case of dynamic 2D FCS—emission–emission correlation—has been treated by He et al.\(^1\). We will explore the excitation–excitation and excitation–emission correlation in this paper.

**Excitation–emission 2D FCS.** When \( a \) denotes the excitation and \( b \) the emission wavelength, the synchronous and asynchronous correlation intensities are reduced to the special form for the excitation–emission 2D FCS:

\[
\Phi(\omega, \lambda_1^\text{ex}, \lambda_2^\text{em}) = A(\lambda_1^\text{ex}) A(\lambda_2^\text{em}) \cos(\phi(\omega, \lambda_1^\text{ex}) - \phi(\omega, \lambda_2^\text{em}))
\]

(7)

\[
\Psi(\omega, \lambda_1^\text{ex}, \lambda_2^\text{em}) = A(\lambda_1^\text{ex}) A(\lambda_2^\text{em}) \sin(\phi(\omega, \lambda_1^\text{ex}) - \phi(\omega, \lambda_2^\text{em}))
\]

(8)

In practice, the real, \( A_0(\omega, \lambda) \propto A(\lambda) \cos(\phi(\omega, \lambda)) \), and the imaginary, \( A_1(\omega, \lambda) \propto A(\lambda) \sin(\phi(\omega, \lambda)) \), components of the fluorescence response, or the in-phase and the quadrature spectra, are collected experimentally. The correlation spectra are then calculated with:

\[
\Phi(\omega, \lambda_1^\text{ex}, \lambda_2^\text{em}) =
\]

\[
A_0(\omega, \lambda_1^\text{ex}) A_0(\omega, \lambda_2^\text{em}) + A_1(\omega, \lambda_1^\text{ex}) A_1(\omega, \lambda_2^\text{em})
\]

(9)

\[
\Psi(\omega, \lambda_1^\text{ex}, \lambda_2^\text{em}) =
\]

\[
A_0(\omega, \lambda_1^\text{ex}) A_1(\omega, \lambda_2^\text{em}) - A_1(\omega, \lambda_1^\text{ex}) A_0(\omega, \lambda_2^\text{em})
\]

(10)

To construct the excitation–emission correlation map, four phase-sensitive fluorescence spectra, the excitation and emission at 0° and 90° detector phase angles, are experimentally collected. The synchronous and asynchronous correlation intensities at frequency \( \omega \) are calculated according to eqn. (9) and (10).

The excitation–emission correlation spectra are shown in Fig. 1 for a sample containing pyrene and anthracene in an ethanolic solution. Pyrene and anthracene were chosen because of the strong overlap of their vibronic bands in the one-dimensional emission spectra.\(^1-5\) The synchronous emission–correlation spectrum (Fig. 1A) shows cross peaks between excitation wavelengths of 310, 326, 338, 358, and 376 nm with emission wavelengths of 374, 380, 384, 400, 416, 422, and 448 nm. A one-dimensional cross section of the 2D map at a certain fluorescence emission wavelength yields the corresponding excitation spectrum that this emission wavelength correlates with. After normalization, all excitation spectra overlap each other well and are consistent with the steady state excitation spectrum of the mixture, displaying five peaks (Fig. 2A). The recovered excitation slices are identical in the spectral region above 340 nm, while a gradual shift is observed below 340 nm in the peak maxima. This indicates that the fluorescence from one species dominates the signal above 340 nm. Overlap between component spectra is responsible for the shift below this wavelength. The small magnitude of the shift shows that either one of the components is predominant, or that the peak locations of the components are very close. The emission spectra recovered as the one-dimensional sections across the excitation axis exhibit similar characteristics (Fig. 2B). They closely resemble the steady state emission spectrum of the sample; the shifting of the spectra, especially in the spectral region between 370 and 420 nm, reveals the overlap between the component fluorescence. In brief, the spectral information contained in the synchronous correlation map is similar to the one-dimensional steady state spectrum but the 2D plot shows the signature of spectral overlap.

The assignment of all the component spectra requires (i) resolving all vibronic bands that may be buried in the strongly overlapped sample spectrum, (ii) linking bands into component spectra in each dimension (excitation and emission), and (iii) determining the association between the excitation and emission spectra. The existence of a cross peak in the synchronous correlation spectrum points to the possibility of association. A nonzero cosine in eqn. (7) can lead to such a cross peak. The association of a certain excitation peak and an emission peak, or the assignment of the peaks to a fluorescent molecule in the mixture, requires a cosine value of 1, since the phase angle \( \phi \) is independent of the excitation and the emission wavelengths. For example, the correlation peak between excitation wavelength of 376 nm and emission wavelength of 378 nm does not indicate that the excitation band and the emission band belong to the same fluorescent component, but only that the phase difference between the 376 nm excitation and 378 nm emission is not 90°. In fact, the phase difference will always be smaller than 90° for real fluorescent molecules due to their finite lifetimes.

The possibility of spectral assignment lies in the sine factor of the asynchronous correlation intensity. A sine value of 0 in eqn. (8) is a good basis for band association. The asynchronous correlation intensity can be positive or negative in sign, depending upon the phase relationship between the two wavelengths. If a component correlates with the rest of the spectrum positively in the excitation axis, its emission correlation peaks would be in the negative direction.

The positive and negative sides of the asynchronous correlation spectrum are depicted in Fig. 1B and 1C, respectively. The positive cross peaks between the 310, 326, 342,
358, and 376 nm excitation and 368, 374, 380, 384, 390, 394, and 420 nm emission indicate that these five excitation bands are not associated with the seven vibronic features in the emission. We will refer to these groups of excitation and emission bands as the excitation group 1 and emission group 1. Similar conclusions can be drawn for the negative cross peaks. The excitation spectrum encompassing bands at 306, 320, and 336 nm (excitation group 2) and the emission spectrum composed of bands at 378, 400, 424, and 450 nm (emission group 2) do not belong to the same fluorescent molecule. The excitation group 1 and emission group 2 thus comprise the fluorescence excitation and emission signatures of one fluorescent species in the sample, anthracene. The other component pyrene’s fluorescence characteristics are described by excitation group 2 and emission group 1.

The recovery of the excitation and emission spectra of pyrene and anthracene from the mixture is demonstrated in Fig. 3. The cross sections of the asynchronous correlation spectra along the emission peaks clearly recovered the five vibronic features of anthracene excitation. The three negative peaks in Fig. 3A are assigned to pyrene excitation. One-dimensional slices of the correlation spectrum along the excitation peaks, on the other hand, reveal the emission spectra of pyrene in the positive direction and anthracene in the negative direction (Fig. 3B). The spectra between the pure pyrene and anthracene features are the "transitional regions" where both pyrene and anthracene contribute to the fluorescence intensity.

At peak locations of the correlation map, one of the fluorescent components contributes predominantly. The spec-
tum of the other component can thus be recovered at these peak locations. This protocol of recovering 1D spectra is illustrated in Fig. 3C. Plotted are the cross sections in the emission axis labeled with the corresponding excitation wavelengths. At wavelengths of excitation group 2 (306, 320, and 336 nm), pyrene is predominantly excited, and the cross sections display emission transitions at 378, 400, 424, and 450 nm for anthracene. Fluorescence emission spectrum of pyrene can be extracted at excitation group 1 wavelengths of 310, 326, 342, 358, and 376 nm, where anthracene is excited. In fact, at excitation wavelength longer than 340 nm, anthracene is the main contributor to fluorescence. All the cross sections in this spectral region of the 2D correlation map represent the emission spectrum of pyrene. Thus the asynchronous correlation spectrum, by using the fluorescence decay kinetics, allows the resolution of the sample spectrum into pyrene and anthracene component spectra. All vibronic bands in the excitation and emission are retrieved for pyrene and anthracene. For example, the excitation peaks around 322 and 336 nm in the one-dimensional spectrum of the mixture are resolved into two pairs of bands: 320 nm pyrene/326 nm anthracene and 336 nm pyrene/340 nm anthracene. The emission bands of pyrene and anthracene buried in the 378 nm peak of the mixture are clearly resolved although their wavelength separation is only 2 nm. Furthermore, the asynchronous correlation analysis enables the association between the excitation and emission spectra for each fluorescent component.

Several applications can be envisioned of the dynamic two-dimensional fluorescence correlation spectroscopy: (i) after the excitation and emission spectra are recovered through correlation analysis, the unknown fluorophore can be identified by matching with a spectral library; (ii) for a known molecule, its fluorescence characteristics in a specific microenvironment can be retrieved from a sample mixture; (iii) the 2D correlation spectrum of the mixture is resolved into two pairs of bands, associated with pyrene and anthracene, respectively. The asynchronous correlation spectrum, when the two wavelength axes are identical, is antisymmetric7 $\Psi(\alpha, \lambda_1^e, \lambda_2^s) = -\Psi(\alpha, \lambda_2^e, \lambda_1^s)$, and contains similar spectral information to the steady state spectrum of the mixture.

The diagonal of the asynchronous map (Fig. 4B) displays zero correlation intensity, as predicted by eqn. (8) for autocorrelation where the phase difference is zero. The synchronous correlation spectrum splits the 332 nm and 336 nm peaks of the sample spectrum into two pairs of bands, associated with pyrene and anthracene, respectively. The asynchronous correlation spectrum, when the two wavelength axes are identical, is antisymmetric7 $\Psi(\alpha, \lambda_1^e, \lambda_2^s) = -\Psi(\alpha, \lambda_2^e, \lambda_1^s)$. A negative correlation peak exists for every positive peak, at the location symmetric to the diagonal. Through similar procedures as discussed in the excitation–emission 2D FCS, the excitation–excitation correlation resolves the overall sample excitation into component spectra. (Fig. 5).

Interesting correlation patterns appeared in the 340–390 nm region of the asynchronous correlation map (Fig. 4B). In this spectral region, anthracene fluorescence is predominant; the sample essentially contains a single component. All vibronic bands should thus be in phase with each other, predicting a zero

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**Excitation–excitation 2D FCS.** Similar to the excitation–emission correlation,1 the excitation–excitation correlation is a special case of the 2D FCS when the correlation intensities are calculated as a function of two axes of excitation wavelengths. The correlation intensities are simplified versions of the generalized correlation in eqn. (4) and (5).

$$
\Phi(\alpha, \lambda_1^e, \lambda_2^e) = \mathcal{A}(\lambda_1^e) \mathcal{A}(\lambda_2^e) \cos\left[\phi(\alpha, \lambda_1^e) - \phi(\alpha, \lambda_2^e)\right] 
$$

$$
\Psi(\alpha, \lambda_1^e, \lambda_2^s) = \mathcal{A}(\lambda_1^e) \mathcal{A}(\lambda_2^s) \sin\left[\phi(\alpha, \lambda_1^e) - \phi(\alpha, \lambda_2^s)\right] 
$$

The correlation spectra are calculated with the phase-sensitive excitation spectra measured experimentally:

$$
\Phi(\alpha, \lambda_1^e, \lambda_2^e) = 
\left[ A_k \left(\alpha, \lambda_1^e\right) A_k \left(\alpha, \lambda_2^e\right) + A_k \left(\alpha, \lambda_1^e\right) A_k \left(\alpha, \lambda_2^s\right) \right] 
$$

$$
\Psi(\alpha, \lambda_1^e, \lambda_2^s) = 
\left[ A_k \left(\alpha, \lambda_1^e\right) A_k \left(\alpha, \lambda_2^s\right) - A_k \left(\alpha, \lambda_1^s\right) A_k \left(\alpha, \lambda_2^s\right) \right] 
$$

The construction of the correlation spectra thus requires the collection of two phase-sensitive excitation spectra, one at $0^\circ$, and the other at $90^\circ$ of detector phase angle.

The excitation–excitation correlation spectra are shown in Fig. 4 for the pyrene–anthracene mixture. Plotted on the sidebars of the 2D correlation spectra is the one-dimensional fluorescence excitation spectrum of the mixture. In the synchronous correlation (Fig. 4A), four fluorescence excitation peaks at 326, 338, 358, and 376 nm show strong autocorrelation on the diagonal. When two excitation wavelengths have identical time response functions, as expected in the autocorrelation, they correlate strongly. In addition, off-diagonal cross correlation peaks are observed between the four excitation peaks. A component excitation spectrum that spans the entire excitation wavelength region, such as anthracene in this sample, or similar time response functions of different components can cause such off-diagonal correlation. The 2D synchronous correlation spectrum is symmetric to the diagonal,7 $\Phi(\alpha, \lambda_1^e, \lambda_2^e) = \Phi(\alpha, \lambda_2^e, \lambda_1^e)$, and contains similar spectral information to the steady state spectrum of the mixture.
asynchronous correlation intensity throughout this spectral range. The (340–390 nm, 340–390 nm) area of the asynchronous correlation spectrum thus should show a flat square with zero intensity value. Instead, 358 and 378 nm peaks are clearly split into a few peaks that correlate with each other with a complex pattern. Close examination of the cross sections of the 2D map (Fig. 5) reveals four additional bands at 346, 354, 366, and 374 nm. These bands are attributed to the Raman scattering of the solvent OH stretch at 3332 cm\(^{-1}\). The phase-sensitive excitation spectra were collected with a 400 nm long pass filter in the emission channel, allowing the Raman scattering of the 392, 400, 418 and 424 nm wavelengths to enter the detector. The 392 and 418 nm bands are attributed to pyrene fluorescence; 400 and 424 nm bands are from anthracene vibronic transitions.

**Emission–emission 2D FCS.** The general form of 2D FCS reduces to the emission–emission correlation when both wavelength axes are the fluorescence emission wavelength. He et al. have discussed the general features of this special mode of 2D FCS.\(^1\) Fig. 6 illustrates the one-dimensional cross sections of the 2D asynchronous correlation map. The emission spectra of pyrene (top traces of the plot) and anthracene (bottom traces of the plot) are extracted from these cross sections.

It is of interest to compare the spectral recovery in Fig. 5 and 6 with Fig. 3. The one-dimensional excitation spectra of the fluorescent components obtained in the excitation–excitation 2D FCS (Fig. 5) are very similar to those from the excitation–emission correlation (Fig. 3A). The resolutions of the emission spectra are feasible in both the emission–emission 2D FCS (Fig. 6) and excitation–emission correlation (Fig. 3B). The additional information carried in the complementary axis in excitation–emission 2D FCS, however, accentuates the spectral features. For example, very few cross sections of the emission–emission correlation map displayed the one-dimensional fluorescence spectrum of pyrene (Fig. 6). This is a direct consequence of the heavy overlap between pyrene and anthracene emission bands. At any wavelength where both components contribute to the fluorescence substantially, the cross section will be a weighted sum of the component emission spectra. Aided with the excitation axis in the excitation–emission 2D FCS, the pyrene spectrum can be retrieved in many cross sections (top curves in Fig. 3B). The 340–390 nm spectral range of the excitation, where anthracene fluorescence is predominant, enables this improved resolution. In Fig. 3A, the correlation with the emission axis accentuates the 340–390 nm region compared to the excitation–excitation correlation.

**Two-dimensional phase map**

The time correlation function in 2D FCS provides dynamic information about the sample. Two fluorescent species that have very similar fluorescence lifetimes strongly correlate with each other in the synchronous spectrum while the asynchronous correlation shows negligible intensity. In contrast, two components with significantly differing lifetimes have high asynchronous correlation intensity and small synchronous correlation. In the frequency domain fluorescence spectroscopy, each fluorescent species is represented by a vector in a complex plane.\(^32,33\) The phase shift \(\phi\) between the fluorescence emission and the excitation wave determines the direction of the vector, or the angle between the vector and the real axis. The synchronous and asynchronous correlation intensities are proportional to the sine and cosine between the two vectors representing the fluorescence decays at wavelengths \(\lambda_1\) and \(\lambda_2\) (eqn. (4) and (5)). The ratio of the asynchronous intensity to the synchronous intensity defines the angle differential between the two wavelengths for which the correlation function is evaluated.

\[
\Delta \phi = \phi(\lambda_1) - \phi(\lambda_2) = \tan^{-1}\left[\frac{\gamma(\alpha; \lambda_1^\alpha, \lambda_2^\alpha)}{\phi(\alpha; \lambda_1^\alpha, \lambda_2^\alpha)}\right]
\]

(15)

The two-dimensional phase map, a plot of the angle differential as a function of the two wavelength axes, provides a direct visualization of the dynamic mapping in the sample.

The phase map for a mixture of glycogen and benzo[a]pyrene is shown in Fig. 7. In the one-dimensional fluorescence correlation spectrum of pyrene (Fig. 6), the 340–390 nm region compared to the emission–emission 2D FCS, however, accentuates the spectral features. For example, very few cross sections of the emission–emission correlation map displayed the one-dimensional fluorescence spectrum of pyrene (Fig. 6). This is a direct consequence of the heavy overlap between pyrene and anthracene emission bands. At any wavelength where both components contribute to the fluorescence substantially, the cross section will be a weighted sum of the component emission spectra. Aided with the excitation axis in the excitation–emission 2D FCS, the pyrene spectrum can be retrieved in many cross sections (top curves in Fig. 3B). The 340–390 nm spectral range of the excitation, where anthracene fluorescence is predominant, enables this improved resolution. In Fig. 3A, the correlation with the emission axis accentuates the 340–390 nm region compared to the excitation–excitation correlation.

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\[
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The phase map for a mixture of glycogen and benzo[a]pyrene is shown in Fig. 7. In the one-dimensional fluorescence
spectrum plotted on the wavelength axes, the peak at 364 nm is the scattering from glycogen. Benzo[a]pyrene has five vibronic bands in the spectral range of 370–500 nm. The phase differential is zero on the diagonal of Fig. 7, as expected for autocorrelation. Zero phase differentials are clearly observed between the glycogen scattering from 355 to 370 nm, i.e., in the (355–370 nm, 355–370 nm) square of the plot. The second region of zero phase difference appears between all vibronic bands of benzo[a]pyrene, from 370 nm to 500 nm, as all vibronic bands have the same phase angle. The correlation of glycogen with benzo[a]pyrene produces a flat high plain at 60° of phase differential on one side of the diagonal and a flat valley on the other side, resulted from the antisymmetric properties of the asynchronous correlation. With the modulation frequency known, a two-dimensional map of fluorescence lifetime difference can be calculated from this phase map. At the modulation frequency of 20 MHz, the lifetime difference between benzo[a]pyrene and glycogen is estimated to be 13.8 ns from the phase difference of 60°, in good agreement with the fluorescence lifetime of 13.7 ns for benzo[a]pyrene. This illustrates an interesting use of the scattered light in 2D FCS: the peaks that correlate with the scattered light at a constant intensity are vibronic transitions of a single fluorescent species, whose lifetime can be retrieved from the phase map.

With increasing number of components and increasing spectral overlap in the sample, the phase map quickly becomes very complex. The phase map for the excitation–excitation correlation of a pyrene-anthracene mixture is shown in Fig. 8. In the spectral range above 340 nm, anthracene is the only molecule contributing to the fluorescence intensity. The phase map thus shows an angle differential of zero throughout the area of (340–390 nm, 340–390 nm). Small grooves that have nonzero phases are caused by the Raman scattering. In the (300–340 nm, 300–340 nm) region, the phase differential displays a complex pattern, reflecting the strong overlap between the pyrene and anthracene excitation. In the corner of (300–340 nm, 340–390 nm), three ridges are observed at a maximum of 27°, corresponding to the phase difference between pyrene and anthracene. Based on the fluorescence lifetimes of 18.3 and 4.2 ns for pyrene and anthracene, respectively, the difference between their phase angles at 20 MHz is estimated to be 34.2°. The deviation of the observed phase difference from the predicted value is caused by the additional contribution of anthracene in the 300–340 nm spectral range, which lowers the observed phase. The excitation peaks of anthracene that are slightly shifted from those of pyrene create valleys of smaller phase differential, separating the ridges. The 2D phase map clearly supports the correlation pattern analysis and the spectral assignment.

Modulation Frequency

The phase angle and demodulation factor, or the modulus of the vector representing the fluorescence response of a molecule, are both determined by the modulation frequency and the lifetime.\textsuperscript{32,33} We will analyze the effects of modulation frequency on the correlation analysis with a sample containing two fluorophores. At zero frequency, both vectors are aligned with the real axis for the steady state measurements. Since the phase differential is 0°, the synchronous correlation is at the highest intensity while the asynchronous correlation vanishes. For a fluorophore with a given lifetime, an increase in the modulation frequency will rotate the vector toward the imaginary axis, thus increasing the phase angle. The increase, however, is proportional to the arctangent of the product of frequency and lifetime and is thus nonlinear. The two vectors for the fluorophores with different lifetimes will thus rotate toward the imaginary axis at different rates, leading to an initial increase in the phase differential. An increase in the asynchronous correlation intensity and a concurrent decrease in the synchronous correlation are predicted. With further increase in frequency, the angle differential starts to become smaller as both vectors approach the imaginary axis. At a very high modulation frequency, both vectors will be aligned with the imaginary axis. The angle differential of 0° predicts a maximized synchronous and minimized asynchronous correlation intensity.

Fig. 9 illustrates the simulated intensities of the synchronous and asynchronous correlation as a function of the modulation frequency and the ratio of fluorescence lifetimes. The linear modulation frequency $f$ is related to the angular modulation frequency $\phi$ by $\phi = 2\pi f$. One lifetime was fixed at 1 ns, and the other was varied from 1 to 100 ns in the simulation. The objective in spectral resolution is to maximize the asynchronous correlation. Of interest are (i) the optimum modulation frequency for a certain fluorophore pair; and (ii) the range of frequencies that will provide reasonable signal-to-noise ratio. As shown in Fig. 9A, for each lifetime ratio, a broad range of frequencies can be used to achieve high asynchronous correlation intensity. For example, a frequency range from 25 to 51 MHz will induce asynchronous correlation intensities above the half of the maximum at a lifetime ratio of 4. This range widens to 9–49 MHz when the lifetime ratio is 40. The optimum frequency shifts to the lower range at longer lifetimes. $\cos \Delta \phi (\alpha, \lambda_1^2, \lambda_2^2)$ reaches high values at both the low and the high frequencies at all lifetime ratios (Fig. 9B).

The optimum frequency for a pair of fluorophores with lifetimes of $t_1$ and $t_2$ is derived by directly maximizing the angle differential:

$$f_{opt} = \left[2\pi \sqrt{t_1 t_2} \right]^{-1}.$$

At this frequency, the maximum angle differential is

$$\Delta \phi_{max} = \tan^{-1} \left( \sqrt{t_2/t_1} \right) - \tan^{-1} \left( \sqrt{t_1/t_2} \right).$$

where $t_2$ is the longer lifetime of the two. Under these conditions, the two lifetime vectors are exactly bisected by the 45° line.

In practice, the measured phase-sensitive spectrum is weighted by the demodulation factor, or the modulus of the lifetime vector. With this consideration, the optimum frequency range narrows down substantially and shifts to lower frequencies, as shown in Fig. 9C. For example, for the lifetime ratios of 4 and 40, the best ranges of frequency are 1–24 MHz and 13–34 MHz, respectively. The synchronous correlation intensity diminishes rapidly when the frequency increases, especially at long fluorescence lifetimes (Fig. 9D). This is the consequence of the shortened modulus of the vector at high modulation frequencies. The measured fluorescence intensity is directly proportional to this modulus.
To examine the effects of modulation frequency on 2D FCS, we measured the phase-sensitive fluorescence spectra of the pyrene–anthracene mixture at frequencies between 1 and 70 MHz. The constructed 2D asynchronous correlation spectra are shown in Fig. 10 for a few representative modulation frequencies. At 1 MHz, the 2D asynchronous correlation spectrum does not exhibit clearly defined correlation patterns. The one-dimensional emission spectrum of anthracene is well resolved from the cross sections of the correlation map, but the spectral signatures of pyrene cannot be recovered reliably. At 10 MHz, the correlation peaks have much higher intensities than the noise level and clearly show the vibronic features of both pyrene and anthracene. The component spectra are retrieved from the cross sections of the 2D map (Fig. 10B). The SNR deteriorates slightly at 20 MHz although the spectral resolution is still quite good. Above 30 MHz, the decrease in SNR leads to noisy one-dimensional slices and difficult spectral assignment. The experimental optimum frequency for the pyrene–anthracene sample is close to 10 MHz, lower than the calculated 18.2 MHz, as the result of the demodulation. A frequency range of about 20 MHz in width can be used to construct 2D correlation spectra with reasonable SNR. Such a frequency tolerance is crucial for the measurements of an unknown sample.

Detector phase angle

To calculate the correlation intensities in eqn. (9) and (10), the in-phase and quadrature spectra need to be collected at 0° and 90° detector phase angles. In phase-sensitive measurements, the detector phase angle is set with scattered light, which has a phase delay of zero. In practice, the phase angle can be slightly off exact zero, presenting a systematic error in the measurements. In some experiments, detector phase angles other than 0° and 90° may be intentionally chosen for improved SNR. For example, if the fluorescence lifetimes of both fluorophores are very short, a significantly high modulation frequency is needed to reach the optimum angle differential. The modulation of the source becomes very inefficient and results in decreased SNR. In such cases, a lower frequency has to be used for the measurements. Because both lifetime vectors are close to the real axis, the quadrature spectrum, or the projection of the lifetime vectors onto the imaginary axis, has very low intensities. To improve the SNR, it is advantageous to measure the phase-sensitive spectra at, for example, 45° and 135° instead of 0° and 90°. The fluorescence response at a detector phase angle of φD is a modified form of eqn. (2):

$$A_f(\tau, \phi_D) = A_f(\lambda) e^{i(\phi_D - \phi_f)}$$

(18)

The fluorescence spectra measured at φD and φD + 90° are $A_f(\lambda) \cos(\phi_D - \phi_f)$ and $A_f(\lambda) \sin(\phi_D - \phi_f)$, respectively, instead of $A_R(\lambda)$ and $A_I(\lambda)$. Substituting these fluorescence spectra into eqn. (9) and (10), we again arrive at the desired synchronous and asynchronous correlation intensities in eqn. (7) and (8). The same results are obtained because $\cos(\Delta \phi)$ and $\sin(\Delta \phi)$ are independent of the detector phase angle. This independence is demonstrated experimentally by measuring the phase-sensitive fluorescence spectra of the pyrene–anthracene sample at four pairs of detector phase angles: 0°–90°, 22.5°–112.5°, 45°–135°, and 67.5°–157.5° at a frequency of 10 MHz. The four asynchronous correlation spectra constructed exhibit similar SNR and identical correlation patterns. The 1D emission spectra are reliably recovered from the 2D correlation maps in all cases (data not shown). In this sample, the choice of detector phase angle is not crucial since the two lifetime vectors are well separated at the optimum frequency. Good SNR is achieved at all detector phase angles. For applications such as tissue fluorescence for cancer diagnosis, the lifetimes of tissue fluorophores are short and modulation frequencies lower than the optimum ones have to be used.23 Detector phase angles of 45° and 135° have proven to provide improved SNR for these measurements. The capability of choosing the detector phase angles lends tremendous flexibility in chemical and biomedical applications of 2D FCS.

Concentration ratios

The concentration ratios of pyrene to anthracene were varied to determine the concentration limit where the 1D spectra can be reliably recovered. Nine pyrene:anthracene concentration ratios were examined: 1000:1, 100:1, 10:1, 5:1, 1:1, 1:5, 1:10, 1:100, and 1:1000. Fig. 11 shows the recovered 1D cross sections for the 1:5 and 1:10 ratios. In these two samples, the

![Fig. 9 Simulated correlation intensities as a function of the modulation frequency and the fluorescence lifetime. A and C, asynchronous correlation intensity; B and D, synchronous correlation intensity. The correlation intensities in C and D include the demodulation factor. Fluorescence lifetime $\tau_f$ was fixed at 1 ns in the simulation.](image-url)
total intensity contributions of anthracene are about 8 and 4%, respectively. At the 8% level, both the pyrene and anthracene spectra were retrieved from the 2D correlation map with high SNR, displaying all vibronic bands. At the 4% level, the recovered pyrene spectrum has similar SNR as the other concentration ratios since it is the predominant fluorophore in the sample. The recovered anthracene spectrum exhibits significant level of noise, although the four emission peaks are still clearly observable. The ability of 2D FCS to extract the main spectral features for fluorophores that contribute only 4% to the total fluorescence signal is quite encouraging.

Two-dimensional fluorescence correlation spectroscopy has been implemented with several modes of perturbations: modulated excitation,1,4–6 wavelength2,3 and concentration perturba-

Fig. 10  Effects of the modulation frequency on 2D FCS. Plots on the left are the two-dimensional asynchronous correlation spectra. The recovered one-dimensional spectra of pyrene (the upper trace in each plot) and anthracene (the lower trace) are on the right. Modulation frequencies: 1 MHz in A, 10 MHz in B, 20 MHz in C, 30 MHz in D, and 40 MHz in E. The ethanolic solution was composed of 5 μM pyrene and 5 M anthracene.
The recovery of pyrene and anthracene spectra from mixtures. Concentrations: 1 μM anthracene–5 μM pyrene for the 1:5 concentration ratio; 1 μM anthracene–10 μM pyrene for the 1:10 concentration ratio. The modulation frequency was 10 MHz.

Fig. 11 The recovery of pyrene and anthracene spectra from mixtures.

The principles of dynamic 2D FCS can be applied to studies of the kinetic process is the natural relaxation of the excited state molecules since the time correlation function is evaluated. In this paper, the kinetic process is the natural relaxation of the excited state molecules characterized by their fluorescence lifetimes. The principles of dynamic 2D FCS can be applied to studies of lateral diffusion, rotational reorientation and chemical kinetics. Dynamic 2D FCS measurements are advantageous in in situ measurements, such as in biological cells and biomedical samples, where the variation of concentration ratios is difficult and often undesirable. The modulated excitation allows kinetic investigation without any disturbance to the system under study. Absence of disturbance is essential in fluorescence probe studies. This work has shown that dynamic 2D FCS enables complete assignment of the excitation and emission bands of fluorescent components.

The resolution limit where individual spectra can be extracted depends on the fluorescence lifetimes of the components, the signal-to-noise ratio, and the degree of spectral overlap. The effects of these parameters on spectral resolution are under study. Fluorescence decay analysis allows the resolution of two molecules whose lifetimes differ by only 20% when the total fluorescence is collected. The resolution limit is a function of fluorescence intensities of the components and the noise level. Electronic noise is the controlling factor in phase-sensitive measurements close to the detection limit. At high fluorescence intensities and weak spectral overlap, components with a small lifetime difference can be resolved. In contrast, a relatively large lifetime difference is required to resolve components with strong spectral overlap at low concentrations. The coupling of spectral dispersion in 2D FCS can potentially improve component resolution than measurements without spectral information.

Acknowledgements

We thank the National Institute of Health, National Cancer Institute, for the funding of this work. The project was also supported in part by a Central Investment Fund for Research Enhancement grant of the University of Iowa.

References


