11-2008

2d Regional Correlation Analysis Of Single-molecule Time Trajectories

Xuefei Wang

H. Peter Lu

Bowling Green State University, hplu@bgsu.edu

2D Regional Correlation Analysis of Single-Molecule Time Trajectories

Xuefei Wang and H. Peter Lu*

Bowling Green State University, Center for Photochemical Sciences, Department of Chemistry, Bowling Green, Ohio 43403

Received: May 20, 2008; Revised Manuscript Received: September 16, 2008

We report a new approach of 2D regional correlation analysis capable of analyzing fluctuation dynamics of complex multiple correlated and anticorrelated fluctuations under a noncorrelated noise background. Using this new method, by changing and scanning the start time and end time along a pair of fluctuation trajectories, we are able to map out any defined segments along the fluctuation trajectories and determine whether they are correlated, anticorrelated, or noncorrelated; after which, a cross-correlation analysis can be applied for each specific segment to obtain a detailed fluctuation dynamics analysis. We specifically discuss an application of this approach to analyze single-molecule fluorescence resonance energy transfer (FRET) fluctuation dynamics where the fluctuations are often complex, although this approach can be useful for analyzing other types of fluctuation dynamics of various physical variables as well.

Introduction

A deviation of a physical variable from its mean value is a fluctuation, and time-dependent fluctuations in biological and chemical systems are critical and widely observed.[1−28] In a conventional ensemble-averaged measurement, most of the single-molecule fluctuations are averaged out and only a mean of the physical variable is measured.[19,24−27,29−36] It has been extensively demonstrated that fluctuations contain critical information on the molecular-level mechanism and dynamics of chemical and biological systems, especially for the inhomogeneous molecular systems, such as proteins,[1,2,19,20,24−28,37−43] DNA/RNA,[1,20,44−52] living cells,[53−56] cell membranes,[40,57−60] and chemical reactive interfaces and surfaces.[11,61−63] Time-correlation function analysis has been a typical approach to study fluctuation dynamics, and the algorithm and computational approaches of correlation analyses have been developed and established.[64−67] In recent years, the extensive developments of single-molecule spectroscopy and nanotechnology have made fluctuation analysis one of the most used analytical approaches.[4−17,19−22,63,66−70] Especially, in single-molecular optical spectral analyses, correlation function is a useful method to measure molecular properties from the fluctuation time trajectories, which may contain detailed information of molecular conformational changes, reactivity, molecular interactions, and also the intrinsic and measurement noises.[2,11,58,65,71] These rather complex applications on analyzing single-molecule fluctuations have encountered some limitations for conventional correlation analyses. For example, fluctuations of molecular variables can be either correlated or anticorrelated or noncorrelated depending on the nature of the variables and the temporal fluctuation of the molecular local environment.[1,4−22,24−28,63,72,73] A conventional correlation analysis typically calculates a correlation function based on the whole time trajectory of a physical variable. Presumably, the longer the trajectory is accounted for the calculation, the higher the signal-to-noise ratio of the analysis may have for the fluctuation dynamics. However, this conventional approach is often incapable of revealing some critical fluctuation dynamics that intermittently appear in a complex fluctuation system under a noise background. For example, when the correlated and anticorrelated fluctuations appear intermittently in a long fluctuation time trajectory,[74] to calculate a correlation function indiscriminately, accounting for the whole trajectory may detect neither of the fluctuation dynamics due to the cancelation between the correlated and anticorrelated fluctuations in such a calculation approach.

A commonly observed signal fluctuation in single-molecule spectroscopy is protein conformational fluctuation probed by fluorescence resonant energy transfer (FRET) between a single pair of donor and acceptor dye molecules tethered to a protein molecule.[19,75−77] Protein conformational changes probed by the changes of FRET donor−acceptor (D−A) distance result in both donor and acceptor fluorescence signal fluctuations. If the fluctuations are entirely originated from FRET, the fluorescence intensity fluctuations of donor and acceptor are anticorrelated due to the fact that the donor signal goes up and the acceptor signal goes down when the D−A distance increases, and the donor signal goes down and the acceptor signal goes up when the D−A distance decreases. However, under real experimental conditions, other sources of fluctuations may also exist, for example, the thermal fluctuation of the overall local environment that statistically makes both the donor and acceptor fluorescence signals go up and down together and fluctuate correlatively. Typically, measurement noise and fast fluctuation beyond the measurement time responses fluctuate noncorrelatively. Often, fluctuations from different sources may dominate intermittently the overall signal fluctuation at different time segments of a fluctuation trajectory. Conventional correlation function calculation treats the whole fluctuation trajectory equally and does not identify the type changes of intermittent fluctuations, which often averages out the nondominate types of fluctuations that can be important for understanding the complex protein conformational dynamics. We have developed a new approach of 2D regional correlation function analysis of single-molecule time trajectories to identify and differentiate the time segments of correlated, anticorrelated, and noncorrelated fluctuations in a long fluctuation trajectory so that the different types of fluctuations can be exclusively mapped out in a detailed analysis without averaging

* To whom correspondence should be addressed. E-mail: hplu@bgsu.edu.

10.1021/jp804453j CCC: $40.75
Published on Web 10/25/2008

them out or losing the critical identification of some important fluctuation behaviors.

Correlation Functions

There are second-order (correlation between two variables) and higher-order time-correlation functions,[13,58,64,65,67] and here, we only focus our discussion on second-order cross-correlation functions calculated from two-band fluctuation trajectories. The cross-correlation evaluates the time-dependent strength between two fluctuating variables.[58,65−67,73] The cross-correlation ($C_{cross}(t)$) functions are defined by eqs 1 and 2.

$$C_{cross}(t) = \langle \Delta A(0)\, \Delta B(t)\rangle / \langle \Delta A(0)\, \Delta B(0)\rangle = \langle (A(0) - \langle A\rangle)(B(t) - \langle B\rangle)\rangle / \langle (A(0) - \langle A\rangle)(B(0) - \langle B\rangle)\rangle \quad (1)$$

When $A = B$, we have an autocorrelation function

$$C_{auto}(t) = \langle \Delta A(0)\, \Delta A(t)\rangle / \langle \Delta A(0)^2\rangle = \langle (A(0) - \langle A\rangle)(A(t) - \langle A\rangle)\rangle / \langle (A(0) - \langle A\rangle)^2\rangle \quad (2)$$

where $A(t)$ and $B(t)$ are the signal variables measured in time trajectories $\{A(t)\}$ and $\{B(t)\}$. $\langle A\rangle$ and $\langle B\rangle$ are the means of the fluctuation trajectories of $\{A(t)\}$ and $\{B(t)\}$, respectively. In spectroscopic fluctuation analyses, $\{A(t)\}$ and $\{B(t)\}$ can be the time trajectories of spectral intensity, spectral mean, fluorescence polarization, photon counts, and other physical parameters. Mathematically, a time-correlation function for continuous or discrete fluctuation trajectory $\{A(t)\}$ is calculated by

$$\begin{aligned} C_{auto}(t) &= \langle \Delta A(0)\, \Delta A(t)\rangle / \langle \Delta A(0)^2\rangle \\ &= \int d\tau (A(\tau) - \langle A\rangle)(A(\tau - t) - \langle A\rangle) / \int d\tau (A(\tau) - \langle A\rangle)^2 \\ &= \sum (A(\tau) - \langle A\rangle)(A(\tau - t) - \langle A\rangle) / \sum (A(\tau) - \langle A\rangle)^2 \end{aligned} \quad (3)$$

The cross-correlation analyses are methods to study the time-dependent behaviors of fluctuating signals. In a measurement, other fluctuation factors often exist to contribute to the measured signal trajectory, which may have a great influence on the correlation function in terms of its decay rate, dynamic behavior, amplitude, and signal-to-noise ratio. For example, in a single-molecular fluorescence measurement, a weak signal can be easily affected not only by the background of noise but also by the fluctuation of overall system and environment. For these reasons, a time trajectory of a variable is not necessarily monotonically dominated by a single-type signal fluctuation due to a single physical origin. For example, in a single-molecule FRET measurement of protein conformational dynamics, the measured donor and acceptor (D−A) fluorescence intensity trajectories should show the anticorrelated D−A intensity fluctuation associated with the D−A distance fluctuation. Environment thermal fluctuations typically give correlated or noncorrelated D−A florescence intensity fluctuations. Specifically, the measurement noise, the fluctuations beyond the measurement time resolution, and the thermal fluctuations of the local environment often intermittently dominate segments of a trajectory to show correlated or noncorrelated fluctuations.[74,78] Practically, an overall calculation of time-correlation function may not reveal the intermittently appearing anticorrelated FRET fluctuation or even correlated thermal fluctuations. To provide a more effective and precise fluctuation analysis, we have developed a new and unique 2D regional correlation analysis method to enable a detailed correlation analysis for complex fluctuation trajectories, such as the single-molecule FRET D−A fluorescence fluctuation trajectories. This method is powerful to identify the correlation signal from any time segments of an experimental fluctuation time trajectory of a physical variable, which is not possible by a conventional correlation analysis. Using the 2D regional correlation analysis, by changing and scanning the start time and end time along a fluctuation trajectory or a pair of fluctuation trajectories, we are able to identify whether a specific segment fluctuation is correlated, anticorrelated, or noncorrelated; after which, a correlation analysis can be applied for each specific segment to obtain a detailed fluctuation dynamics analysis.

2D Regional Correlation Analysis

The primary analytical approach of the 2D regional correlation analysis is to calculate a two-dimensional cross-correlation function amplitude distribution (TCAD). In this analysis, a start time and an end time, t_{start} and t_{end}, were scanned to chose the calculation of cross-correlation function from a two-band signal intensity time trajectory, $\{I_1(t)\}$, $\{I_2(t)\}$. The two scanning parameters, t_{start} and t_{end}, define the start time (t_{start}) and the time lapse (from t_{start} to t_{end}) of a cross-correlation function calculation window along a two-band fluctuation signal trajectory. This 2D calculation gives a cross-correlation for defined segments from t_{start} to t_{end} as

$$\begin{aligned} C_{cross}(\tau, t_{start}{:}t_{end}) &= \int_{t_{start}}^{t_{end}} I_1(t)\, I_2(t - \tau)\, dt \\ &= \sum_{t_{start}}^{t_{end}} I_1(t)\, I_2(t - \tau) \end{aligned} \quad (4)$$

The window of t_{start} to t_{end} is scanned in a range through the intensity trajectories. A cross-correlation function is calculated from a two-band fluctuation trajectory for each scanned pair of t_{start} to t_{end}. The initial amplitude of $C(\tau, t_{start}{:}t_{end})$ was presented by the difference between the first n points and the next $n + m$ points from $\tau = 0$:

$$\zeta = \{\langle C(1{:}n)\rangle\} - \{\langle C(n + 1{:}n + m)\rangle\} \quad (5)$$

The index n and m defines the precision of calculated initial amplitude, ζ, of the correlation function. In our analysis, we chose $n = m = 3$, which is sufficient to obtain a reliable value of ζ from the calculated cross-correlation function. As a function of t_{start} and t_{end}, the value of ζ is plotted as a two-dimensional map of t_{start} to t_{end}. A hot color represents a positive amplitude of $C(\tau)$, and a cold color represents a negative amplitude of $C(\tau)$. Positive amplitude indicates correlation, and negative amplitude indicates anticorrelation.

The significant advantage of TCAD is that both the correlated and anticorrelated spectral intensity fluctuations can be identified pixel by pixel for each pair of t_{start} to t_{end} based on calculated cross-correlation function pixel by pixel (Figure 1). Specifically, for an analysis of the single-molecule FRET D−A two-band fluctuation trajectories, (1) the parameter of the Z-axis is the amplitude of the cross-correlation function from a FRET donor−acceptor two-band fluorescence intensity time trajectory. A positive amplitude means (rep-

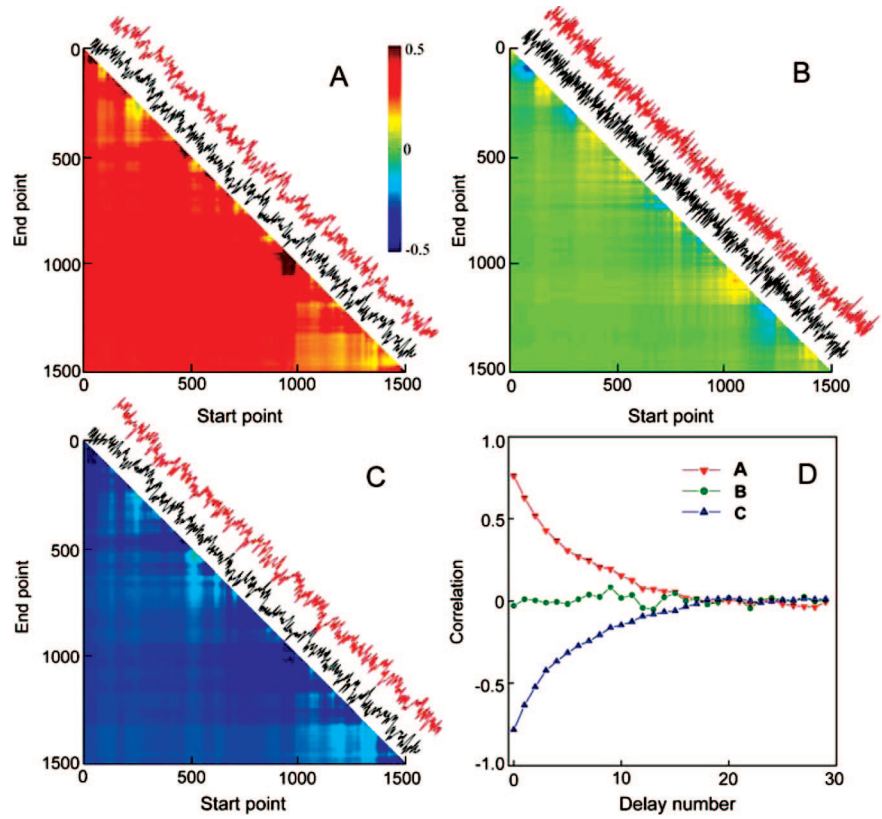

Figure 1. Simulation results of the two-dimensional cross-correlation function amplitude distribution based on three simulated two-band fluctuation trajectories, which are (A) correlated with positive correlation amplitude, (B) uncorrelated with zero correlation amplitude, and (C) anticorrelated with negative correlation amplitude, respectively. (D) Correlation function decay curves corresponding to the data of parts A−C.

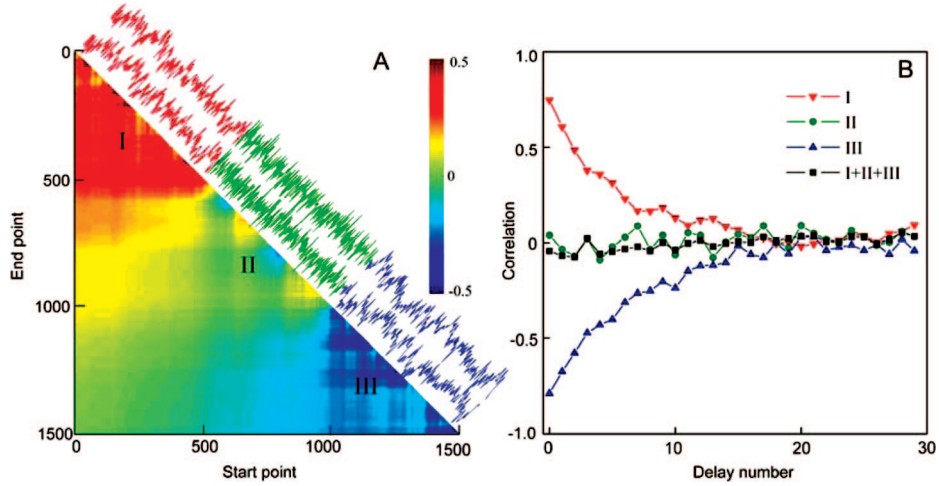

Figure 2. TCAD map and cross-correlation functions calculated from a simulated two-band fluctuation trajectory that consists of three sections: cross-correlated (I: 1−500 data points), noncorrelated (II: 501−1000 data points), and anticorrelated (III: 1001−1500 data points). (A) 2D regional correlation analysis by TCAD mapping. The hot color represents positive amplitude, and the cold color represents negative amplitude. (B) Correlation functions calculated from the three sections of correlated, noncorrelated, and anticorrelated fluctuation data corresponding to sections I, II, and III, and the whole data trajectory (I + II + III). It is evident that a conventional correlation calculation from the whole data trajectory gives no correlation amplitude, whereas the 2D regional correlation analysis gives definitive analysis of the correlation behavior for each specific fluctuation in the long trajectory.

resented by a hot color) that the two bands ($I_1(t)$ and $I_2(t)$) in an intensity trajectory fluctuate correlatively (both go up and down together) (Figure 1A and D), whereas a negative amplitude (represented by a cold color) means that the two bands ($I_1(t)$ and $I_2(t)$) in an intensity trajectory fluctuate anticorrelatively (one goes up while the other one goes down, and vise versa) (Figure 1C and D); a zero amplitude (represented by a green color) means that the two bands ($I_1(t)$ and $I_2(t)$) in an intensity trajectory fluctuate randomly without any correlation (Figure 1B and D). (2) The parameters of $X-Y$ axes represent the time window between t_{start} and t_{end} for calculating the cross-correlation functions from the FRET donor−acceptor two-band fluorescence intensity time trajectories. The t_{start} and t_{end} of the TCAD define the time window (position and width) for the calculated cross-correlation function, and the width and position of the window are scanned through the intensity trajectories by scanning all the possible values of t_{start} and t_{end}.

Simulation and Demonstration of the 2D Regional Correlation Analysis of Fluctuation Time Trajectories

(1) Simulated Fluctuation Signal Trajectories. We demonstrate the power of the 2D regional correlation analysis to identify a correlated or an anticorrelated two-band signal fluctuation from a simulated two-band intensity time trajectory. Here, for a numerical simulation, the time variable (t) is replaced by a data point index (n) that represents a time unit. Thus, the "start point" and "end point" in index numbers have the same meaning as the "start time" and "end time" in time units, respectively. We use eq 6 to construct simulated fluctuation data,[18,79] which possesses an autocorrelation function with an exponential decay pattern $e^{-1/\tau}$

$$f(n) = f(n-1) \times \exp(-1/\tau) + \theta \times N(n) \qquad (6)$$

$f(1) = 0$, $N(n)$ is the noise component of a normal distribution with mean at zero and variance 1, and the ratio of noise to signal θ (=0.5) is the coefficient defining the noise level. In this simulated data, we define $\tau = 5$, which gives the autocorrelation function of $f(n)$ in the form of $e^{-1/5}$.

For a cross-correlation simulation, the following equations are used to construct correlated two-band fluctuating data trajectories, $\{I_1(n)\}$ and $\{I_2(n)\}$:

$$I_1(n) = f(n) + \theta \times N_1(n)$$

$$I_2(n) = f(n) + \theta \times N_2(n) \qquad (7)$$

$f(n)$ is the simulated data from eq 6, and $N(n)$ and θ are the same as those in eq 6. $N_1(n)$ and $N_2(n)$ are independent. In eq 7, when $\theta = 0$, I_1 is same as I_2, which means that I_1 and I_2 are totally correlated, namely, $C_{cross}(0)$ is equal to 1 in eq 1.

(2) Using the Two-Dimensional Correlation Amplitude Distribution (TCAD) to Identify Different Correlation Fluctuations at Different Time Segments in a Two-Band Intensity Fluctuation Trajectory. To demonstrate the capability of the 2D regional correlation analysis to identify the fluctuation dynamics, we calculated a TCAD map from a simulated two-band data trajectory that contains three segments of correlated (red), noncorrelated (green), and anticorrelated (blue) fluctuations. The TCAD map clearly shows the positive amplitude obtained for the correlated region (I), the zero amplitude for the noncorrelated region (II), and the negative amplitude for the anticorrelated region (III). With this TCAD map identification, we are able to analyze the cross-correlation function pixel by pixel, which reveals the dynamics of the different fluctuations (Figure 2A and B).

If two intensity trajectories are dominated by anticorrelated fluctuation, such as single-molecule FRET of D−A fluorescence intensities, the two-band intensities should have an anticorrelated fluctuation characterized by a negative amplitude of cross-correlation function (Figure 2B-III). On the other hand, if the measurement noise or fast fluctuations beyond the instrumental response time dominate the trajectory, then the two-band intensity fluctuation is noncorrelated with a zero amplitude of the cross-correlation function (Figure 2B-II). In real measurements under room temperature, single-molecule fluorescence fluctuations often involve a certain degree of thermally induced fluorescence intensity fluctuations. The thermally induced fluctuations typically give correlated fluctuations of two-band D−A FRET fluorescence intensities, giving a component of positive amplitude of cross-correlation functions (Figure 2B-I). The ability to identify a FRET related fluorescence intensity fluctuation is dependent on the relative amplitudes of FRET associated anticorrelated fluctuation, thermally induced correlated fluctuations, and the noncorrelated noise background. Conventional second-order cross-correlation analysis only calculates cross-correlation functions from fluctuation trajectories, and the overall amplitude of the cross-correlation function is low or vanished due to the cancelation between the cross-correlation amplitudes of correlated thermally induced and anticorrelated FRET fluctuations (Figure 2B-I+II+III). The real anticorrelated FRET intensity fluctuations are often buried under high thermally induced fluctuation and noise background. If a conventional approach of cross-correlation function calculation is used to include the whole trajectory of three segments, then the anticorrelated behavior is averaged out as in Figure 2B-I+II+III. This is a widely encountered problem for a single-molecule FRET fluorescence fluctuation analysis, especially for the intramolecular FRET in single-molecule proteins.

The 2D regional correlation analysis is highly capable of identifying the informative regions from the white noise regions in a fluctuation trajectory (Figure 2A). Then, a selective correlation analysis is able to reveal the meaningful fluctuation dynamics of the system measured by a cross-correlation function calculated for the identified segments in the long trajectory (Figure 2B). Otherwise, a conventional cross-correlation function calculation directly treating the fluctuation trajectory as a whole is not able to reveal a significant correlation fluctuation contained in the fluctuation trajectory (Figure 2B-I+II+III). Especially, when a conventional cross-correlation function analysis gives a zero correlation amplitude, the typical conclusion will be that the fluctuation is dominated by noise so that there is no correlation in the fluctuation. However, if a two-band fluctuation trajectory contains both correlated and anticorrelated fluctuations, the cross-correlation function can also only show a small correlation amplitude or even no correlation amplitude, which is like a correlation function calculated from a noncorrelated noise trajectory (Figure 2B-II and I+II+III).

(3) 2D Regional Correlation Analysis Is Capable of Characterizing Fluctuations at a High Noise Background. To evaluate the signal-to-noise limit of the 2D regional correlation analysis in identifying correlated and anticorrelated fluctuations from a noncorrelated noise background, we calculate the TCAD maps from a simulated two-band trajectory with different levels of noise background, noise-to-signal ratio θ = 0, 0.5, 1, and 2. The results (Figure 3) show that the identification of the positive and negative correlation amplitudes can be effective and beyond the standard deviation up to the noise-to-signal ratio of 2:1, which is adequate for analyzing most single-molecule FRET fluorescence intensity trajectories.

Conventional correlation analysis treats a fluctuation trajectory as a whole, rather than analyzing the fluctuation region by region, to calculate a correlation function, which often presents a noneffective approach since a signal fluctuation may only dominate the noise background in intermittent time segments but not all over the whole time course of a trajectory. In our approach, we use the 2D regional correlation analysis to identify the time segments where the correlated or anticorrelated fluctuations are identifiable beyond the noncorrelated noise background; then, we specifically calculate the cross-correlation functions for each specific region to reveal the fluctuation dynamics that otherwise is averaged out in a conventional correlation analysis. To further demonstrate the significance of regional calculation of correlation function compared to a calculation over the whole trajectory, we simulate a signal trajectory that contains a half of signal fluctuation and a half of

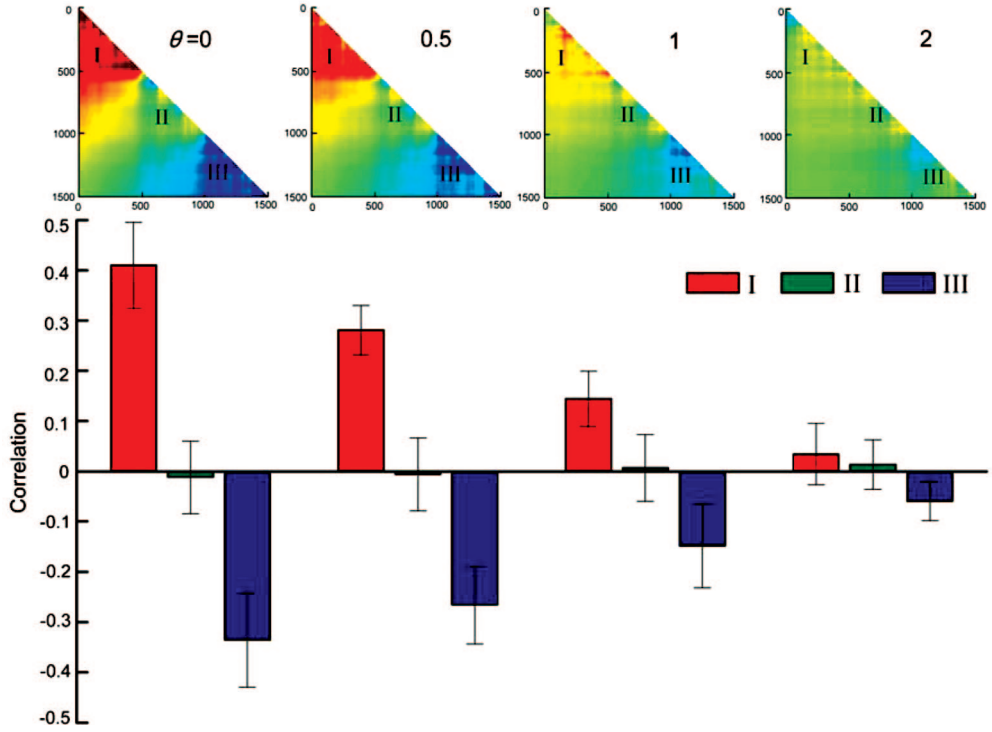

Figure 3. Sensitivity of the 2D regional correlation analysis on fluctuation under a noise background. Upper panel: comparison of the calculated TCAD maps under different noise levels with $\theta = 0$, 0.5, 1, and 2. Bottom panel: mean values and error bars of standard deviation of the regions calculated from each TCAD map.

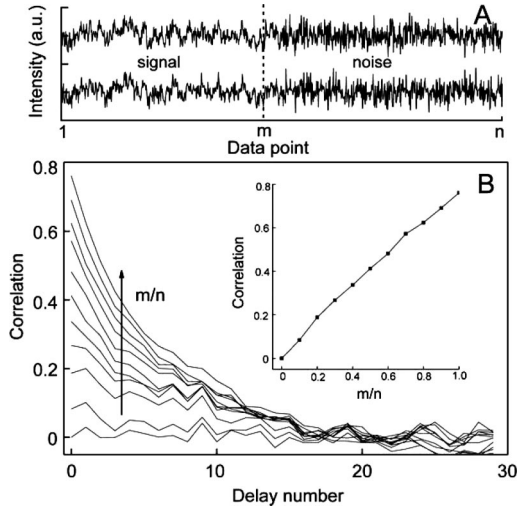

Figure 4. Influence of data point number ratio of signal to noise on the cross-correlation function. In a pair of trajectories with n data points, the first m data points are correlated with $\theta = 0.5$, and from m to n are noncorrelated noise. (A) A simulated two-band fluctuation trajectory containing signal segment (from 1 to m) and noise segment (from m to n). (B) The correlation function decay curves under different m/n values. The arrow indicates the increase of m/n, and the inset plot shows the value of correlation amplitude changes with the m/n ratio.

white noise (Figure 4A). Figure 4B shows that the amplitude of the cross-correlation function decreases as more noncorrelated noise time segments are included in the calculation. It is obvious that when the correlation function is calculated from the whole time trajectory, there is no resolved correlation amplitude. The correlation amplitude as well as the signal-to-noise ratio of the cross-correlation function increases as the calculation is more and more specific to the region of the correlated fluctuation trajectory segment. It is relevant and effective to identify the regions dominated by correlated fluctuation or noncorrelated noise before a cross-correlation function analysis, which is particularly important for the fluctuations involved in multiple origins, for example, for the FRET fluorescence fluctuation of single-molecule protein conformational dynamics.

Application of 2D Regional Correlation Analysis on the Experimental Single-Molecule FRET Fluctuation Analysis

We demonstrate the power of 2D regional correlation analysis using TCAD mapping to identify the inhomogeneous fluctuation dynamics by revealing the correlation function segment by segment for a real single-molecule FRET fluctuation trajectory recorded from a single-molecule Cy3−Cy5 labeled kinase enzyme protein.[80] The single-molecule two-band (Cy3−Cy5) fluorescence intensity fluctuation reflects the conformational motions of specific domains of the protein involved in an enzymatic reaction.[80] The calculated TCAD map (Figure 5A) from the 18 s long trajectory shows that the region of 0−7 s gives a higher negative cross-correlation amplitude, and the region of 7−18 s gives a weaker negative cross-correlation amplitude. The regional cross-correlation functions in Figure 5B show a striking difference of the functions in the regions of 0−7 and 7−18 s, 17.0 s^{-1} and 120.5 s^{-1}, respectively. This clearly indicates that the FRET fluctuation rate constant is time dependent from segment to segment along the same time trajectory. The fluctuation rate changes from time to time for the same single-molecule protein molecule, which reflects the dynamic disorder of the protein conformational dynamics.[2,14] Remarkably, the cross-correlation function calculated from the whole trajectory of 0−18 s gives only an averaged fluctuation rate (22.2 s^{-1}) compared to the fluctuation rates from the specific segments. It is evident that the conventional approach of cross-correlation analysis is not necessarily capable of characterizing the complex fluctuation dynamics from complex single-molecule spectroscopy fluctuation data, and our new approach is able to

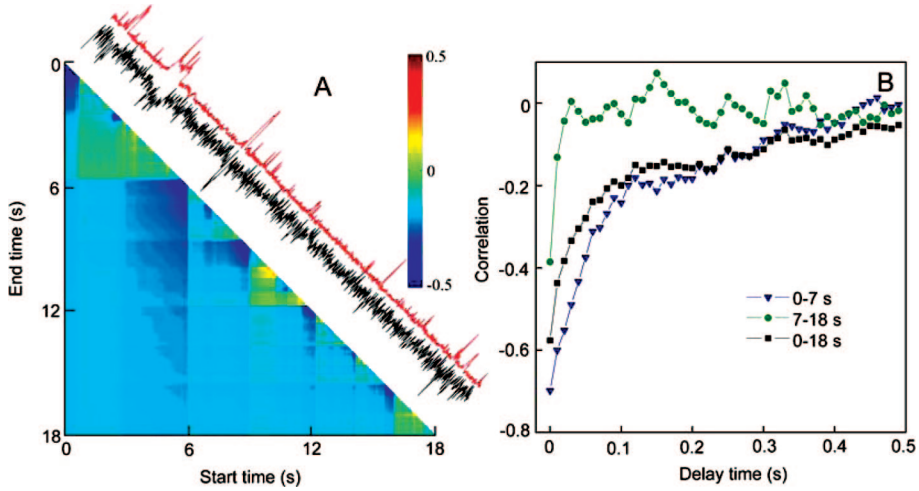

Figure 5. Application of 2D regional correlation analysis on single-molecule FRET fluctuation data analysis. The experimental FRET two-band (D−A) fluorescence intensity fluctuation trajectory is measured from a D−A labeled kinase enzyme protein molecule involving a conformational change fluctuation in a buffer solution. (A) A TCAD map calculated from a two-band (D−A) FRET fluorescence fluctuation trajectory. (B) Cross-correlation functions calculated from different sections of the trajectory.

improve the accuracy and specificity of the correlation analysis significantly.

Limitations and Possible Improvement of Our Approach

The 2D regional correlation analysis involves (1) calculation of TCAD maps of cross-correlation function amplitude from two-band fluctuation trajectories; (2) identification of correlated, anticorrelated, and noncorrelated region by the calculation of the TCAD maps; and (3) calculation of cross-correlation function and analysis of the fluctuation dynamics for the identified segments of the fluctuation trajectories. These analysis procedures require a higher computation resource than what is needed for a conventional cross-correlation analysis. With an ever-improving availability of the computational power in typical experimental laboratories, the 2D regional correlation analysis should be generally feasible.

For extensive application of the 2D regional correlation analysis, the standards of the 2D regional correlation analysis will need to be justified for different field specialties. Since the 2D regional correlation analysis involves scanning the two-band fluctuation data trajectories and identifying the regions in a TCAD map, to be able to identify the length of the segments from the calculated TCAD map will be critical for the reliability and accuracy of the analysis. The identifiable length of a segment depends on the specific fluctuation variables and parameters of specific measurements. For example, in an analysis of single-molecule FRET D−A intensity fluctuation, we only consider that a segment region contains a data trajectory length to be a factor of 3 longer than the correlation decay time as a minimum length of the segment in a fluctuation trajectory; we treat any shorter segments as noise.

Concluding Remarks

In this Article, we introduce the 2D regional correlation analysis of complex correlated and anticorrelated fluctuation dynamics under a noncorrelated noise background. For typical single-molecule FRET fluctuations, if thermally induced correlated fluctuation and FRET induced anticorrelated fluctuation are at similar time scales, the cross-correlation amplitudes cancel each other and only the larger amplitude component is revealed, which results in a loss of information and sometimes even leads to a misleading conclusion. A thermally induced fluctuation involves multiple complex processes, and their fluctuation dynamics often covers a broad temporal range. Therefore, only when the FRET fluctuation amplitude is larger than that of the thermal processes, anticorrelated behavior can be revealed from a cross-correlation function. However, a single-molecule FRET fluctuation does not always have a larger amplitude if a cross-correlation function is calculated from a whole single-molecule FRET fluctuation trajectory. The usefulness of the 2D regional correlation analysis is (1) the ability to search for the time segments when the anticorrelated FRET fluctuation is relatively larger and then (2) specifically analyze the FRET fluctuation dynamics for that identified segment. We emphasize that our 2D regional correlation analysis has a more general implication beyond analyzing single-molecule FRET anticorrelated donor−acceptor intensity fluctuation under a high background. Our analysis approach can be applied to other types of correlated or random fluctuations, including thermally induced intensity fluctuations, fluctuation due to photobleaching of donor or acceptor probe molecules, and intensity fluctuations due to the cross-talking in detecting the donor−acceptor two-band intensity trajectories. Although we only demonstrated an application for analyzing intermittent single-molecule FRET fluctuation dynamics, our approach is much more general and potentially applicable to analyze other types of fluctuation dynamics beyond the chemistry and biology fields.

Acknowledgment. This work is supported by the Office of Basic Energy Sciences within the Office of Science of the U.S. Department of Energy (DOE).

References and Notes

(1) Lu, H. P.; Iakoucheva, L. M.; Ackerman, E. J. *J. Am. Chem. Soc.* **2001**, *123*, 9184.
(2) Lu, H. P.; Xun, L. Y.; Xie, X. S. *Science* **1998**, *282*, 1877.
(3) Yanagida, T.; Ueda, M.; Murata, T.; Esaki, S.; Ishii, Y. *Biosystems* **2007**, *88*, 228.
(4) Barkai, E.; Jung, Y.; Silbey, R. *Phys. Rev. Lett.* **2001**, *87*, 207403.
(5) Agmon, N. *J. Phys. Chem. B* **2000**, *104*, 7830.
(6) Dickson, R. M.; Cubitt, A. B.; Tsien, R. Y.; Moerner, W. E. *Nature* **1997**, *388*, 355.
(7) Edman, L.; Rigler, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8266.
(8) Edman, L.; Foldes-Papp, Z.; Wennmalm, S.; Rigler, R. *Chem. Phys.* **1999**, *247*, 11.
(9) Edman, L.; Mets, U.; Rigler, R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6710.

(10) Jia, Y. W.; Talaga, D. S.; Lau, W. L.; Lu, H. S. M.; DeGrado, W. F.; Hochstrasser, R. M. *Chem. Phys.* **1999**, *247*, 69.
(11) Lu, H. P.; Xie, X. S. *Nature* **1997**, *385*, 143.
(12) Maiti, S.; Haupts, U.; Webb, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11753.
(13) Schenter, G. K.; Lu, H. P.; Xie, X. S. *J. Phys. Chem. A* **1999**, *103*, 10477.
(14) Kamper, N. G. V.; Oppenheim, I. *Stochastic processes in physics and chemistry*; MIT Press: Cambridge, MA, 1992; p 335.
(15) Wang, J.; Wolynes, P. *Phys. Rev. Lett.* **1995**, *74*, 4317.
(16) Wang, J.; Wolynes, P. *J. Chem. Phys.* **1999**, *110*, 4812.
(17) Xie, X. S.; Lu, H. P. *J. Biol. Chem.* **1999**, *274*, 15967.
(18) Zwanzig, R. *Acc. Chem. Res.* **1990**, *23*, 148.
(19) Lu, H. P. *Acc. Chem. Res.* **2005**, *38*, 557.
(20) Chen, Y.; Hu, D. H.; Vorpagel, E. R.; Lu, H. P. *J. Phys. Chem. B* **2003**, *107*, 7947.
(21) Lu, H. P.; Iakoucheva, L. M.; Ackerman, E. J. *Biophys. J.* **2000**, *78*, 262a.
(22) Yang, S. L.; Cao, J. S. *J. Phys. Chem. B* **2001**, *105*, 6536.
(23) Xie, X. S. *J. Chem. Phys.* **2002**, *117*, 11024.
(24) Lu, Q.; Wang, J. *J. Am. Chem. Soc.* **2008**, *130*, 4772.
(25) Lu, Q.; Lu, H. P.; Wang, J. *Phys. Rev. Lett.* **2007**, *98*, 128105.
(26) Wang, J.; Lu, Q.; Lu, H. P. *PLoS Comput. Biol.* **2006**, *2*, 842.
(27) Wang, J. *Chem. Phys. Lett.* **2006**, *418*, 544.
(28) Lee, C.; Lin, C.; Stell, G.; Wang, J. *Phys. Rev. E* **2003**, *67*, 41905.
(29) Barnes, M. D.; Ng, K. C.; Whitten, W. B.; Ramsey, J. M. *Anal. Chem.* **1993**, *65*, 2360.
(30) Schmidt, T.; Schutz, G. J.; Baumgartner, W.; Gruber, H. J.; Schindler, H. *J. Phys. Chem.* **1995**, *99*, 17662.
(31) Gohde, W.; Fischer, U. C.; Fuchs, H.; Tittel, J.; Basche, T.; Brauchle, C.; Herrmann, A.; Mullen, K. *J. Phys. Chem. A* **1998**, *102*, 9109.
(32) Xie, X. S.; Trautman, J. K. *Annu. Rev. Phys. Chem.* **1998**, *49*, 441.
(33) Moerner, W. E.; Orrit, M. *Science* **1999**, *283*, 1670.
(34) Rigler, R.; orrit, M.; Basche, T. Nobe Conference Lectures; Springer-erland: Berlin, 2003.
(35) Ishii, Y.; Yanagida, T. *Single Molecules* **2000**, *1*, 5.
(36) Barkai, E.; Jung, Y. J.; Silbey, R. *Annu. Rev. Phys. Chem.* **2004**, *55*, 457.
(37) Tan, X.; Hu, D. H.; Squier, T. C.; Lu, H. P. *Appl. Phys. Lett.* **2004**, *85*, 2420.
(38) Tan, X.; Nalbant, P.; Toutchkine, A.; Hu, D. H.; Vorpagel, E. R.; Hahn, K. M.; Lu, H. P. *J. Phys. Chem. B* **2004**, *108*, 737.
(39) Harms, G.; Orr, G.; Lu, H. P. *Appl. Phys. Lett.* **2004**, *84*, 1792.
(40) Harms, G. S.; Orr, G.; Montal, M.; Thrall, B. D.; Colson, S. D.; Lu, H. P. *Biophys. J.* **2003**, *85*, 1826.
(41) Liu, R. C.; Hu, D. H.; Tan, X.; Lu, H. P. *J. Am. Chem. Soc.* **2006**, *128*, 10034.
(42) Pappas, D.; Burrows, S. M.; Reif, R. D. *TrAC, Trends Anal. Chem.* **2007**, *26*, 884.
(43) Weiss, S. *Science* **1999**, *283*, 1676.
(44) Greulich, K. O. *ChemPhysChem* **2005**, *6*, 2458.
(45) Eggeling, C.; Fries, J. R.; Brand, L.; Gunther, R.; Seidel, C. A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1556.
(46) Ha, T. *Curr. Opin. Struct. Biol.* **2001**, *11*, 287.
(47) Ha, T.; Rasnik, I.; Cheng, W.; Babcock, H. P.; Gauss, G. H.; Lohman, T. M.; Chu, S. *Nature* **2002**, *419*, 638.
(48) Knemeyer, J. P.; Marme, N.; Sauer, M. *Anal. Chem.* **2000**, *72*, 3717.
(49) Margittai, M.; Wodemgrem, J.; Scjweomberger, E.; Scjpder, G.; Felekyan, S.; Haistein, E.; Konig, M.; Fasshauer, D.; Grubmuller, H.; Jahn, R.; Seidel, C. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15516.
(50) Yang, H.; Luo, G. B.; Karnchanaphanurach, P.; Louie, T. M.; Rech, I.; Cova, S.; Xun, L. Y.; Xie, X. S. *Science* **2003**, *302*, 262.
(51) Ying, L. M.; Wallace, M. I.; Klenerman, D. *Chem. Phys. Lett.* **2001**, *334*, 145.
(52) Bagshaw, C. R.; Cherny, D. *Biochem. Soc. Trans.* **2006**, *34*, 979.
(53) Byassee, T. A.; Chan, W. C. W.; Nie, S. M. *Anal. Chem.* **2000**, *72*, 5606.
(54) Murakoshi, H.; Iino, R.; Kobayashi, T.; Fujiwara, T.; Ohshima, C.; Yoshimura, A.; Kusumi, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 7317.
(55) Sako, Y.; Minoghchi, S.; Yanagida, T. *Nat. Cell Biol.* **2000**, *2*, 168.
(56) Seisenberger, G.; Ried, M. U.; Endress, T.; Buning, H.; Hallek, M.; Brauchle, C. *Science* **2001**, *294*, 1929.
(57) Hu, D. H.; Lu, H. P. *Biophys. J.* **2004**, *87*, 656.
(58) Sakmann, B.; Neher, E. *Single Channel Recordings*, 2nd ed.; Kluwer: New York, 2001.
(59) Harms, G. S.; Cognet, L.; Lommerse, P. H. M.; Blab, G. A.; Kahr, H.; Gamsjager, R.; Spaink, H. P.; Soldatov, N. M.; Romanin, C.; Schmidt, T. *Biophys. J.* **2001**, *81*, 2639.
(60) Demuro, A.; Parker, I. *Biophys. J.* **2004**, *86*, 3250.
(61) Biju, V.; Micic, M.; Hu, D. H.; Lu, H. P. *J. Am. Chem. Soc.* **2004**, *126*, 9374.
(62) VandenBout, D. A.; Yip, W. T.; Hu, D. H.; Fu, D. K.; Swager, T. M.; Barbara, P. F. *Science* **1997**, *277*, 1074.
(63) Lu, H. P.; Xie, X. S. *J. Phys. Chem. B* **1997**, *101*, 2753.
(64) Pflug, L. A.; Ioup, G. E.; Ioup, J. W. *J. Acoust. Soc. Am.* **1998**, *103*, 2469.
(65) Oppenheim, I.; Shuler, K. E.; Weiss, G. H. *Stochastic processes in physics and chemistry*; MIT press: Cambridge, MA, 1977.
(66) Lippitz, M.; Kulzer, F.; Orrit, M. *ChemPhysChem* **2005**, *6*, 770.
(67) Mcquarrie, D. A. *Statistical Mechaniscs*; Harper & Row; New York, 1973.
(68) Edman, L. *J. Phys. Chem. A* **2000**, *104*, 6165.
(69) Rigler, R.; Vogel, H. *Single Molecules and Manotechnology*; Springer-Verlag: Berlin, Heidelberg, 2008.
(70) Erdmann, R.; Enderlein, J.; Seidel, C. *Cytometry* **1999**, *36*, 162.
(71) Bussian, D. A.; Summers, M. A.; Liu, B.; Bazan, G. C.; Buratto, S. K. *Chem. Phys. Lett.* **2004**, *388*, 181.
(72) Buranachai, C.; McKinney, S. A.; Ha, T. *Nano. Lett.* **2006**, *6*, 496.
(73) Shi, J.; Gafni, A.; Steel, D. *Eur. Biophys. J. Biophys. Lett.* **2006**, *35*, 633.
(74) Pan, D.; Hu, D. H.; Liu, R. C.; Zeng, X. H.; Kaplan, S.; Lu, H. P. *J. Phys. Chem. C* **2007**, *111*, 8948.
(75) Deniz, A. A.; Laurence, T. A.; Beligere, G. S.; Dahan, M.; Martin, A. B.; Chemla, D. S.; Dawson, P. E.; Schultz, P. G.; Weiss, S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5179.
(76) Ha, T. J.; Ting, A. Y.; Liang, J.; Deniz, A. A.; Chemla, D. S.; Schultz, P. G.; Weiss, S. *Chem. Phys.* **1999**, *247*, 107.
(77) Yasuda, R.; Masaike, T.; Adachi, K.; Noji, H.; Itoh, H.; Kinosita, K. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9314.
(78) Moerner, W. E.; Fromm, D. P. *Rev. Sci. Instrum.* **2003**, *74*, 3597.
(79) Hirano, T. *Electronics and Communications in Japan Part Iii-Fundamental Electronic Science* **1999**, *82*, 58.
(80) He, Y.; Mukherjee, S.; Yang, H.; Lu, H. P. *Biophys. J.* **2008**, *94*, 209a.

JP804453J