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Perspective

Two-Dimensional Fluctuation Correlation Spectroscopy (2D-FlucCS): A Method to Determine the Origin of Relaxation Rate Dispersion

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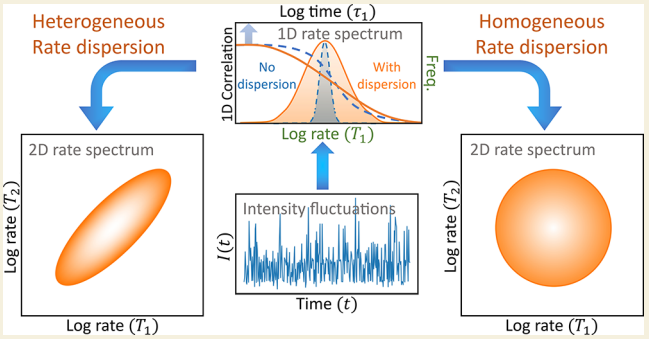
ABSTRACT: Relaxation rate dispersion, i.e., nonexponential or multicomponent kinetics, is observed in complex systems when measuring relaxation kinetics. Often, the origin of rate dispersion is associated with the heterogeneity in the system. However, both homogeneous (where all molecules experience the same rate but inherently nonexponential) and heterogeneous (where all molecules experience different rates) systems can exhibit rate dispersion. A multidimensional correlation analysis method has been demonstrated to detect and quantify rate dispersion observed in molecular rotation, diffusion, solvation, and reaction kinetics. One-dimensional (1D) autocorrelation function detects rate dispersion and measures its extent. Two-dimensional (2D) autocorrelation function measures the origin of rate dispersion and distinguishes homogeneous from heterogeneous. In a heterogeneous system, implicitly there exist subensembles of molecules experiencing different rates. A three-dimensional (3D) autocorrelation function measures subensemble exchange if present and reveals if the system possesses static or dynamic heterogeneity. This perspective discusses the principles, applications, and potential and also presents a future outlook of two-dimensional fluctuation correlation spectroscopy (2D-FlucCS). The method is applicable to any experiment or simulation where a time series of fluctuation in an observable (emission, scattering, current, etc.) around a mean value can be obtained in steady state (equilibrium or nonequilibrium), provided the system is ergodic.



KEYWORDS: *Relaxation Rate Dispersion, Rate Heterogeneity, Heterogeneous Kinetics, Nonexponential Kinetics, Fluctuation Correlation Spectroscopy*

INTRODUCTION

Relaxation in simple systems is characterized by exponential decay or single-rate kinetics, which results in a narrow distribution of rates on a rate spectrum.[1] Deviation from exponentiality, or multirate kinetics, i.e., nonexponential relaxation, are widely observed when measuring relaxation in complex systems such as polymers, unconventional liquids, nanostructures, and biological systems.[2−17] This leads to broad distribution on a rate spectrum.[18] For instance, the rotational rates of a small molecule in a polymer near its glass transition are distributed over a wide range of values.[2,15] The solvation rate around a small organic probe molecule in ionic liquids is dispersed over multiple decades.[3,4] Multiexponential behavior is reported in translational diffusion and solvation in deep eutectic solvents.[5] Dynamics of protein molecules are spread over several decades in time.[6,19] Broad distribution of diffusion time is observed in the cytoplasm of living cells.[7,20] Conformational changes in proteins are often associated with observed nonexponential relaxation kinetics.[13,21]

Nonexponential kinetics manifests relaxation rate heterogeneity, i.e., the existence of multiple rates, rate dispersion.[22] Often, observation of rate dispersion is believed to originate from heterogeneity in the system.[20,23,24] It is also discussed that nonexponential kinetics observed when studying relaxation in proteins may not originate from conformational substates; rather, they may be an inherent property of the protein.[14] A nonexponential decay can originate from both heterogeneous and homogeneous systems.

A system is said to be heterogeneous when different particles experience different relaxation rates; for instance, different rates due to particles experiencing different local environments display rate dispersion when measured in an ensemble. In

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contrast, a homogeneous system is when all the particles experience identical relaxation rates which can be inherently nonexponential, perhaps due to complex relaxation pathways or locally correlated dynamics exhibiting rate dispersion. In the presence of rate dispersion, characterizing an unknown system at equilibrium as homogeneous or heterogeneous is not straightforward, if possible.

Despite its widespread observation, a dedicated and robust method has been lacking to determine the origin of relaxation rate heterogeneity. Current methods, such as multidimensional ultrafast spectroscopies, are being used to characterize spectral line widths.[25−27] However, these techniques are perturbation−response based and fail to report on processes occurring at equilibrium. Traditional ensemble-averaged methods are fundamentally inadequate in quantifying relaxation rate heterogeneity. Unlike these conventional methods, single-molecule spectroscopy offers a unique advantage by circumventing molecular averaging, enabling a closer examination of the true nature of kinetics at the single-molecule level.[28]

Fluorescence correlation spectroscopy (FCS) a technique that measures the diffusion of fluorescent molecules in a sample, coupled with fluorescence lifetime data acquired by time correlated single photon counting (TCSPC), was employed to deconvolute the heterogeneity inherent in an ensemble-averaged fluorescence decay profile. This methodology was effectively applied to understand the conformational changes in a fluorescently labeled polypeptide.[29] Additionally, the application of 2D fluorescence lifetime correlation spectroscopy (2D-FLCS) to a dye mixture (Cy3 and TMR) revealed distinct species within an inhomogeneous sample based on their fluorescence lifetimes.[30]

Tracking the single-molecule Förster resonance energy transfer (sm-FRET) signal between the A-site tRNA and L27 protein in a single ribosome, seven ribosomal subpopulations, and a scheme of spontaneous exchange was identified among these subpopulations.[31] sm-FRET has long been demonstrated to be an effective tool to study the evolution of intramolecular distances as folding/unfolding progresses, and this was further extended to reveal the presence of protein subpopulations in unfolded states when the conditions favor the native structure.[32] sm-FRET has also been demonstrated to be capable of detecting the presence of heterogeneity in a variety of systems, such as conformational fluctuations in proteins,[21] immiscible binary mixtures, etc.[33]

Integrating the capabilities of 2D-FLCS with sm-FRET, a comprehensive insight into the conformational dynamics and ligand binding mechanisms of the *Bsu* preQ$_1$ aptamer and its energy landscape was unveiled on a microsecond time scale. This synergistic approach provided a detailed understanding of the gene regulation process occurring in bacteria.[34]

Linear dichroism experiments have provided valuable information about the local heterogeneity in polymers nearits glass transition by measuring molecular rotation at the single-molecule level.[35] The breakdown of rotational−translational decoupling in violation of Debye−Stokes−Einstein (DSE) prediction in polymers has long been thought to be the result of ensemble averaging. A recent study shows simultaneous measurements of rotation and translation in polystyrene near its glass transition temperature (T_g). Measurements revealed that rotational−translational decoupling in violation of DSE predictions persisted even at single-molecule levels.[36]

Despite the remarkable insights offered by single-molecule-based approaches in unraveling heterogeneity, a notable limitation arises from the necessity to measure each molecule for a sufficiently extended duration to acquire reliable data. This becomes particularly challenging when the probe molecule undergoes photophysical or photochemical changes induced by perturbations from high-power excitation sources, as commonly employed in single-molecule spectroscopy. For a measurement to provide statistically reliable information on the relaxation process involved, it needs to be more than $\sim 10^4$ times the relaxation time.[37] Insufficient length of the trajectory results in spurious behavior of the correlation curves and improper rate spectra.[28] This limitation underscores the significance of 2D-FlucCS, providing a pivotal solution to address these challenges and provide us with a robust platform to decipher the origin of rate dispersion. Recently, multidimensional correlation analysis methods have been applied to systems at equilibrium measured using single-molecule experiments and computer simulations to provide a clear definition and straightforward quantification of heterogeneity and subensemble exchange in glass-formers and ionic liquids, and conformational pathways in biological molecules.[36,38−40] The unique capabilities of 2D-FlucCS make it a crucial tool for probing rate heterogeneity in systems where conventional approaches face limitations, ensuring a more comprehensive and accurate exploration of intricate relaxation processes.

In this perspective, we provide a brief idea of the working principles of two-dimensional fluctuation correlation spectroscopy (2D-FlucCS) as a method to determine the origin of rate dispersion. Calculation of one-dimensional and two-dimensional autocorrelation functions and the information they carry is presented. Recent advances made by applying these methods on several systems using different techniques are discussed. An outlook and possible future directions are offered toward the application of these methods to interesting problems in different branches of science.

Rate Dispersion and Its Origin

Any relaxation process is characterized by the time it takes to complete and is usually measured from a decaying curve (Figure 1, top panel). The inverse of the characteristic time is defined as the rate of that process. Simple processes in simple environments are characterized by a single rate corresponding to a narrow distribution on a rate spectrum (Figure 1, top panel−blue curve) and are said to have no rate dispersion. A simple process in a complex environment or a complex process in a simple environment can lead to a combination of rates that appear as a broader distribution on the rate spectrum (Figure 1, top panel−orange curve) which is defined as rate dispersion. Rate dispersion can be defined as both heterogeneous and homogeneous rate dispersion originating from distinct underlying processes.

Heterogeneous Rate Dispersion. In a system, if the dynamics of the molecules (or particles) are different from each other, i.e., they have different relaxation rates, then the system is said to have a heterogeneous rate dispersion, meaning rate dispersion is due to heterogeneity in the system (Figure 1, middle-left panel). This could be due to molecules experiencing different local environments, such as in the case of heterogeneous systems like polymers near glass transitions, compartments of a biological cell, etc. Conversely, local environments are identical, but particles themselves are different in physical properties. For example, in an ensemble of nanostructures, subensembles of different sizes may exist. In a different scenario, in an isotropic media, protein molecules

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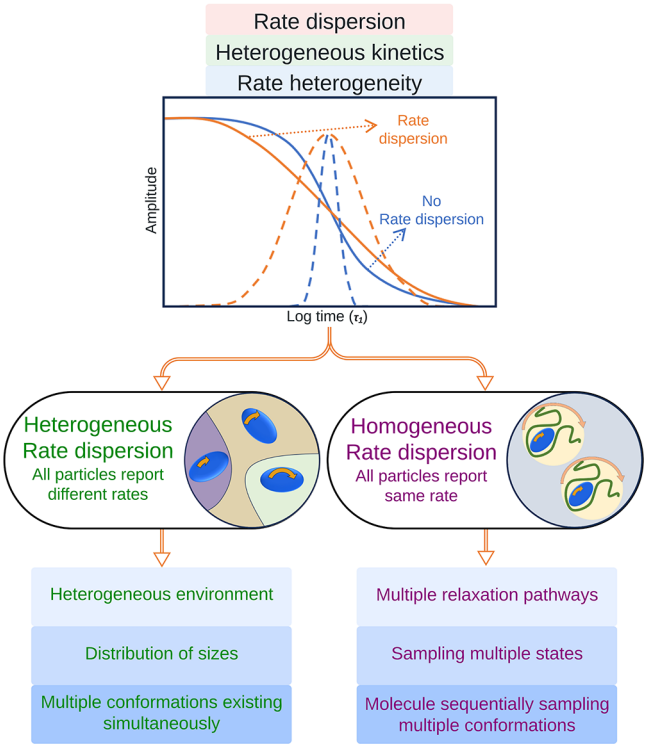

Figure 1. Flowchart distinguishing the heterogeneous and homogeneous mechanisms behind rate dispersion in a system. Top panel: 1D decay curves and their corresponding rate spectra for a system without rate dispersion (blue) and with rate dispersion (orange). Middle panel: two possible mechanisms behind rate dispersion in a system. Lower panel: their various origins.

may simultaneously exist in multiple conformations, resulting in different hydrodynamic radii, which make the system heterogeneous. The heterogeneous surroundings, or heterogeneity in the physical properties of the molecule or a combination of both, lead to heterogeneous rate dispersion (Figure 1, middle-left panel).

Homogeneous Rate Dispersion. In contrast to heterogeneous rate dispersion, homogeneous rate dispersion arises from all the molecules (or particles) experiencing identical but complex dynamics, inherently nonexponential. This may arise due to the involvement of multiple relaxation pathways, or compounded motions: for instance, a trapped molecule rotating inside a cavity formed by a long polymer chain while the polymer chain also rotates at a different rate. Transitions to different states may also give rise to inherently nonexponential dynamics. A protein molecule switching between different conformations in equilibrium may lead to dispersed rates when measuring molecular motion. Molecules behaving identically but in an inherently complex fashion give rise to a rate dispersion defined as a homogeneous rate dispersion (Figure 1, middle-right panel).

METHODS

Autocorrelation Analysis

1D Autocorrelation—Measures Rate Dispersion. One-dimensional autocorrelation is defined as

$$G(\tau_1) = \langle I(\tau_1)I(0)\rangle \tag{1}$$

Here, the delay time τ_i is the delay between the absolute times t_i, such that $\tau_i = t_i - t_{i-1}$: i.e., $\tau_1 = t_1 - t_0$.

The mean-corrected intensity signal is given by $I(t) = F(t) - \langle F(t)\rangle$, where $F(t)$ is the intensity fluctuations obtained from experiments or simulations. Figure 2 (left panel) shows a typical 1D autocorrelation curve obtained from eq 1. If the 1D autocorrelation can be modeled with a model involving a single time constant, then the curve is said to be free from rate dispersion. If multiple time constants are needed to model the curve, then the curve has rate dispersion. The nature of the 1D autocorrelation curve establishes the presence of rate dispersion in the system. However, standalone 1D analysis is unable in determining the origin of rate dispersion; at least 2D analysis is necessary to understand the origin of rate dispersion.

2D Autocorrelation—Measures Origin of Rate Dispersion. The two-dimensional autocorrelation function is given by

$$G(\tau_2, \tau_1) = \langle I(\tau_2 + \tau_1)I(\tau_1)I(0)\rangle \tag{2}$$

Figure 2 (right panel) shows a 2D autocorrelation surface obtained from eq 2. For further analysis, time slices over any one of the time axes are plotted. A 2D autocorrelation analysis works on the principle of rate filtering (Figure 3) and can decipher whether the rate dispersion observed by 1D analysis is heterogeneous or homogeneous rate dispersion or a combination of both.

Rate Filtering. 2D analysis differentiates between the system with homogeneous dispersion from that with heterogeneous dispersion. This ability of differentiation arises from rate-filtering capability. While calculating 2D autocorrelation curves, we can select a given rate and remove all the faster rates from contributing to 2D autocorrelation curves. If a system has heterogeneous rate dispersion, i.e., there exists a distribution of rates experienced by different molecules, rate filtering slows down the 2D correlation curves upon increasing

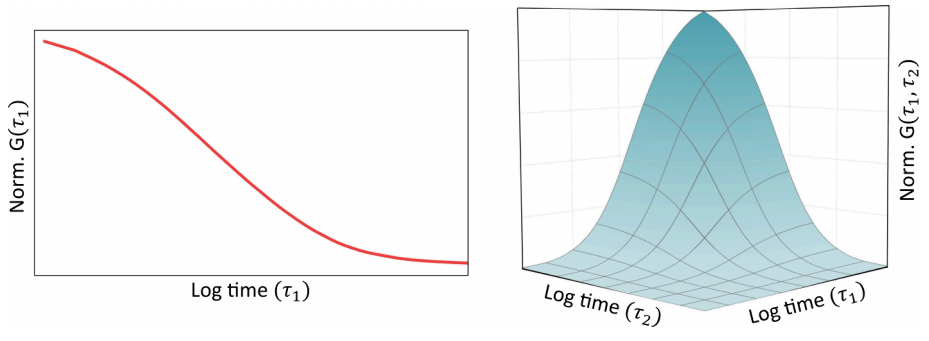

Figure 2. Left panel: 1D autocorrelation curve obtained using eq 1. Right panel: 2D autocorrelation surface obtained using eq 2.

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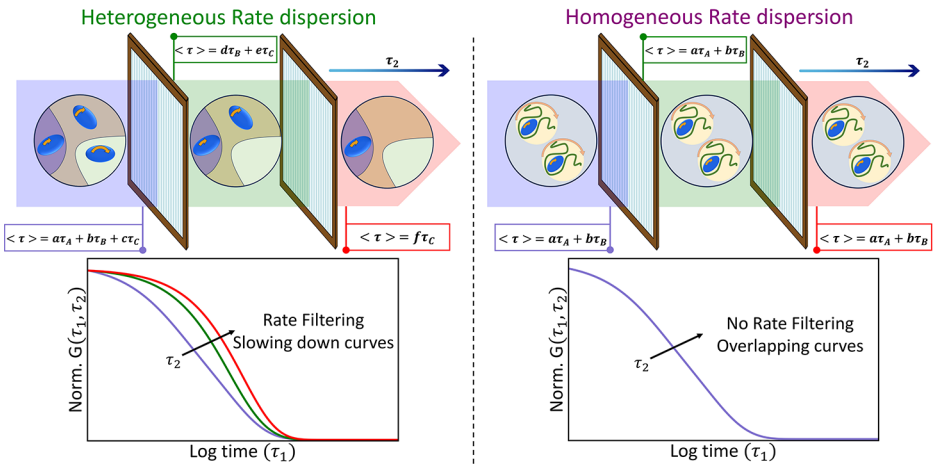

Figure 3. Illustrative depiction of the rate-filtering process. Top-left panel: in the context of heterogeneous rate dispersion, iterative filtering progressively eliminates faster-relaxing components. Bottom-left panel: transformation of the decay curve from multicomponent to that of a single component as a function of τ_2. Top-right panel: conversely, in instances of homogeneous rate dispersion, rate filtering is absent. Bottom-right panel: decay curve remains multicomponent upon variation of τ_2.

the second delay time, τ_2 (Figure 3, left panel). In contrast, in a system with homogeneous rate dispersion all the molecules experience same distribution of rates; in this case rate filtering has no effect and yields overlapping 2D slices (Figure 3, right panel) (Note S1, Supporting Information).

3D Autocorrelation—Measures Rate Exchange. A generalized 3D autocorrelation function is defined as follows:[39]

$$G(\tau_3, \tau_2, \tau_1) = \langle I(\tau_3 + \tau_2 + \tau_1)I(\tau_2 + \tau_1)I(\tau_1)I(0)\rangle \quad (3)$$

A 3D autocorrelation analysis can decipher whether there is any rate exchange between the different subensembles comprising the varying relaxation rates in a system. Taking slices (a set of 2D correlation curves) along τ_3, one can measure the exchange times or spectral diffusion time. If the rate exchange is slower than the slowest relaxation rate, then the system appears to have no rate exchange. On the other hand, if the rate exchange is faster than the fastest relaxation rate, the system appears to be homogeneous. In the limit of intermediate exchange, 3D analysis provides accurate information on rate exchange.

Rate Spectra

Rate spectra, often encountered in the context of time-resolved measurements, provide a visual representation of the distribution of relaxation rates within a dynamic system. They offer insights into how different processes contribute to the overall relaxation behavior of the system.

3D Rate Spectra. The 3D rate correlation spectra, $\hat{G}^{(3)}(\tau_3, T_2, T_1)$, is given by

$$G^{(3)}(\tau_3, \tau_2, \tau_1) = \int_0^\infty dT_2 \int_0^\infty \hat{G}^{(3)}(\tau_3, T_2, T_1) e^{-\tau_2/T_2} e^{-\tau_1/T_1} dT_1 \quad (4)$$

where T_1 and T_2 are the inverse rates. A complete 3D rate spectrum can be constructed by calculating 2D rate spectra piece by piece as a function of τ_3. Rate exchange in a system can be measured as a function of τ_3. These rate spectra can be obtained using Laplace transform.

2D Rate Spectra. The 2D rate correlation spectra, $\hat{G}^{(2)}(T_2, T_1)$, given by[39]

$$G^{(2)}(\tau_3 = 0, \tau_2, \tau_1) = \int_0^\infty dT_2 \int_0^\infty \hat{G}^{(2)}(\tau_3 = 0, T_2, T_1) e^{-\tau_2/T_2} e^{-\tau_1/T_1} dT_1 \quad (5)$$

is an analogue to the multidimensional frequency correlation spectra often used in NMR and various other spectroscopies. A pictorial representation is provided in Figure 4. 2D analysis is

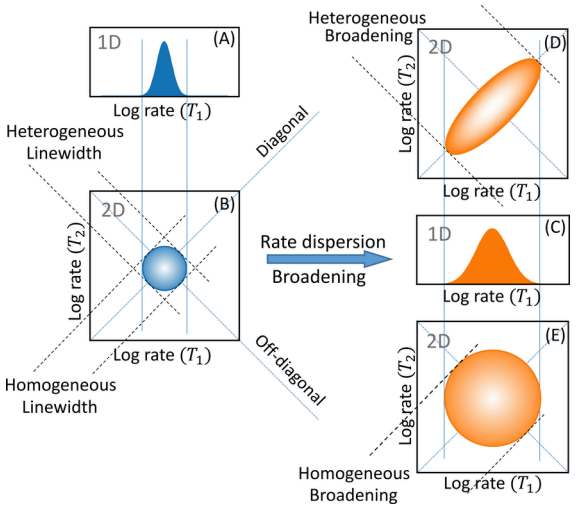

Figure 4. (A) 1D rate spectra of a system with no rate dispersion. (B) 2D rate spectra of the same system. (C) 1D rate spectra of a system with rate dispersion, which can have two possible origins. (D) Heterogeneous rate dispersion: asymmetric elongation along the diagonal and antidiagonal. (E) Homogeneous rate dispersion: symmetrical elongation along the diagonal and antidiagonal.

the limiting case of 3D analysis in the limit that $\tau_3 = 0$. Here, T_1 and T_2 are the inverse rates. Due to a logarithmic scale, there is an equivalence between rate and time constant, and they only differ in sign. A rate−rate correlation spectra refers to a statistical measure that quantifies the degree to which two variables change together. A uniform elongation of peaks along the diagonal and antidiagonal implies that each component/particle in the system experiences all the relaxation rates. Hence, each inverse rate correlates with itself; such a system is

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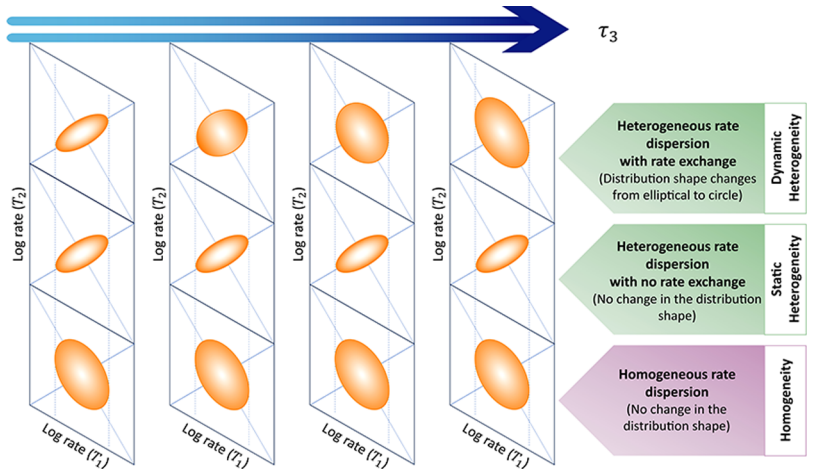

Figure 5. 2D rate spectrum as a function of time τ_3. Top panel: heterogeneous rate dispersion with rate exchange. middle panel: heterogeneous rate dispersion with no rate exchange. Lower panel: homogeneous rate dispersion where no rate exchange exists.

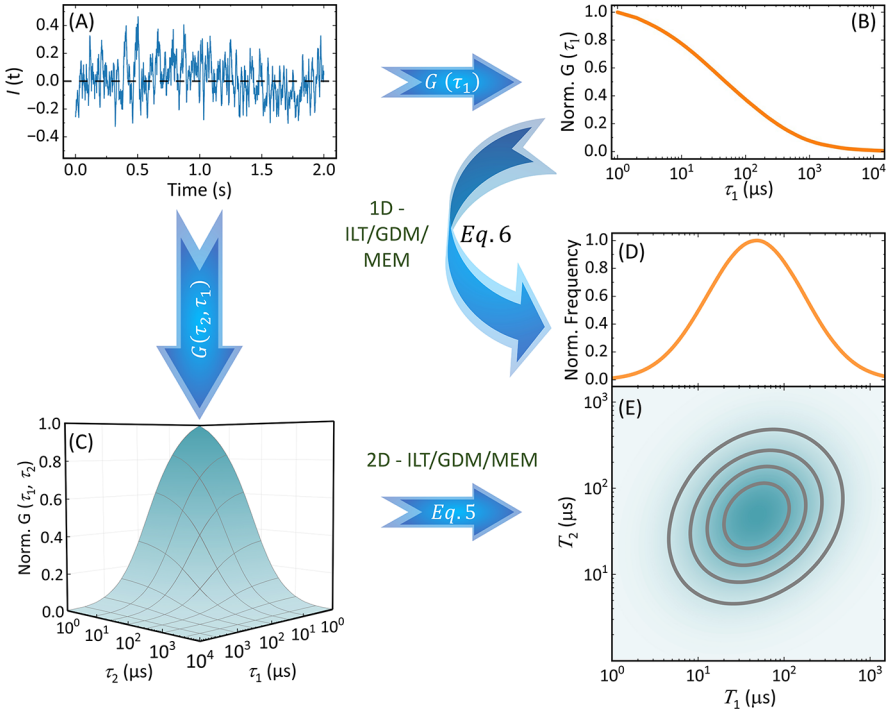

Figure 6. (A) Mean corrected intensity fluctuations $I(t)$. (B) 1D autocorrelation curve obtained from $I(t)$ using eq 1. (C) 2D autocorrelation surface obtained from $I(t)$ using eq 2. (D) Distribution of rates as a rate spectrum obtained by 1D ILT/GDM/MEM (eq 6). (E) Rate−rate spectra obtained by modeling the 2D correlation surface to 2D ILT/GDM/MEM.

said to be a homogeneous system. Whereas for a heterogeneous system, different particles experience different relaxation rates, and hence inverse rates only correlate with themselves and not with other rates; this leads to narrowing of the antidiagonal intensity.[18]

1D Rate Spectra. One-dimensional correlation curves can be transformed into a 1D rate spectrum

$$G^{(1)}(\tau_3 = \tau_2 = 0, \tau_1) = \int_0^\infty \hat{G}^{(1)}(\tau_3 = \tau_2 = 0, T_1) e^{-\tau_1/T_1}\, dT_1 \quad (6)$$

where T_1 is the inverse rate. A pictorial representation is shown in Figure 4. 1D analysis is a further limiting case of 3D in the limit that $\tau_3 = \tau_2 = 0$. These rate spectra give the distribution of rates along the temporal axis and their relative contribution to the resultant spectra via their respective amplitudes. A single-exponential decay results in the narrowest distribution of rates.

Rate Exchange. A rate dispersion can be due to either static or dynamic heterogeneity or a combination of both. Static heterogeneity is defined as the simultaneous existence of different relaxation rates experienced by different molecules, which leads to rate dispersion. Dynamic heterogeneity is defined as a sequential transformation of rates to different values on a given molecule, which also leads to rate dispersion.[2,41] In conventional one-dimensional experiments, without prior information about the system, it requires numerous control measurements to be able to characterize the system as having homogeneous or heterogeneous rate dispersion. Multidimensional correlation analysis methods do

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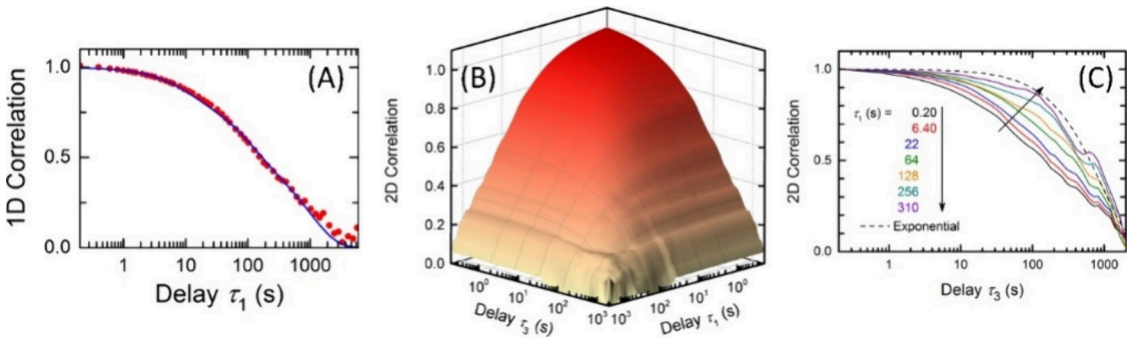

Figure 7. 2D-FlucCS of linear dichroism experiment. (A) The normalized 1D full-ensemble averaged autocorrelation curve. (B) The 2D correlation surface in τ_3−τ_1 coordinates. (C) A set of normalized slices of the surface taken at various τ_1, showing the correlation curve getting less dispersed as τ_1 increases. Reprinted from ref 38, with the permission of AIP Publishing.

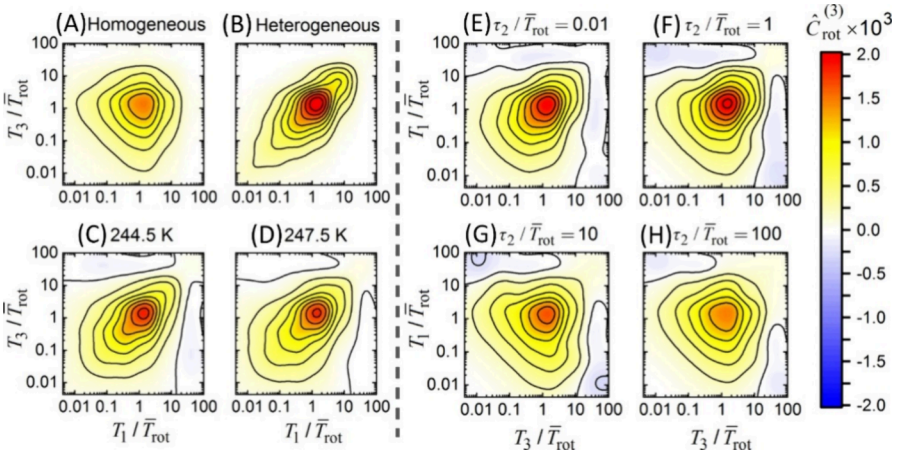

Figure 8. Rate−rate spectra obtained from 2D-FlucCS of linear dichroism experiment. Predicted spectra for (A) pure homogeneous dispersion and (B) pure heterogeneous dispersion. (C, D) spectra from the dichroism experiment at two different temperatures resembling heterogeneous model. (E−H) 2D spectra obtained as a function of time to show rate exchange. Reprinted figure with permission from ref 18. Copyright 2018 by the American Physical Society.

not require prior information about the system and define the system based on the information content of the observable time series. Analysis of 3D rate−rate spectra as a function of time provides information on rate exchange. If multiple rates exist simultaneously, i.e., the system exhibits heterogeneous rate dispersion, the shape of the 3D rate−rate spectrum will change over time to a symmetric distribution along both diagonals (Figure 5, top panel). This means the rate experienced by a given molecule samples a range of values over the timespan of the experiment and can be understood as dynamic heterogeneity. However, if all the molecules experience the same rates (inherently nonexponential), i.e., systems possess homogeneous rate dispersion, the 3D rate−rate spectrum will be invariable over time (Figure 5, lower panel). This reflects that the rate experienced by a given molecule is constant over time, although different molecules may have different rates, and it can be understood as static heterogeneity (Figure 5, middle panel).

Working Principle of 2D-FlucCS

2D-FlucCS is directly applicable to steady-state time series, at equilibrium or nonequilibrium, obtained from any experiment or simulation where 1D correlation functions are calculated and interpreted to reveal the underlying dynamics. For any experiment/simulation steady-state fluctuations in the observable once recorded can be used to calculate a multidimensional correlation function. As an example, let us consider the experimental setup of fluorescence correlation spectroscopy (FCS) and the subsequent application of 2D-FlucCS to the same. In FCS, the observable is recorded as fluctuations in the intensity $F(t)$. The length of trajectory is chosen such that it is 10^4 times the relaxation time to yield statistically reliable results. The first step includes mean correcting the signal such that $I(t) = F(t) - \langle F(t) \rangle$. The mean corrected intensity fluctuations are then used to calculate correlation curves according to eqs 4−6. To test for aging or any nonequilibrium fluctuations taking place in the system, symmetric and antisymmetric correlation functions can be obtained (Note S2, Supporting Information). Once the data have been validated to be free of any process other than the one, we are interested in the next step, which involves obtaining information about the distribution of rates present in the system under study.

The distribution of rates, i.e., a rate spectrum, can be obtained using a variety of methods such as inverse Laplace transform (ILT),[42] Gaussian distribution model (GDM),[43] maximum entropy method (MEM),[44] etc. The distribution of rates, along with the process for performing 2D-FlucCS, is depicted in Figure 6. An analysis of 2D rate spectra provides information about the origin of rate dispersion (Note S3, Supporting Information).

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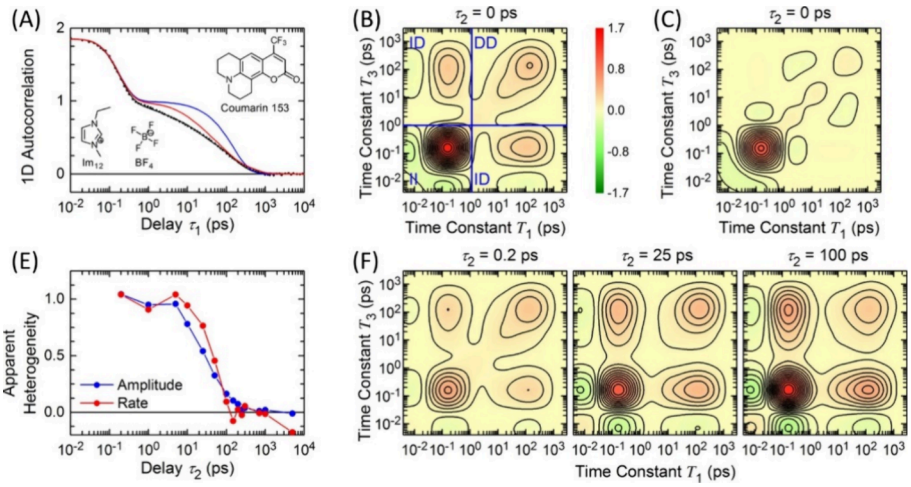

Figure 9. 2D-FlucCS of molecular dynamics in ionic liquids. (A) 1D autocorrelation. (B) 2D rate−rate spectra showing a mixture of homogeneous and heterogeneous dispersion. (C) Heterogeneous portion of the 3D spectrum at $\tau_2 = 0$. (D) Decay of apparent heterogeneity calculated from 3D autocorrelation. (E) 2D rate−rate spectra as a function of time showing loss of heterogeneity. Reprinted (adapted) with permission from ref 39. Copyright 2016 American Chemical Society.

APPLICATIONS

Simple chemical[45] and biological[46] systems at a phase transition[38] and even fundamental relaxation processes such as molecular rotation,[38] solvation,[39] energy transfer,[47] and diffusion[48] often exhibit rate dispersion, i.e., nonexponential kinetics. Understanding the underlying mechanisms driving nonexponential kinetics is of paramount importance in unraveling the dynamics and interactions that govern system behavior, with significant implications in diverse scientific fields.

Multidimensional correlation analysis when applied to single-molecule linear dichroism experiments, measuring molecular rotation in polymers near the glass transition (1D correlation curve and 2D correlation surface shown in Figure 7), revealed that the rate dispersion observed in molecular rotation is due to the heterogeneity in local viscosity and the heterogeneous surroundings experienced by different molecules leading to observed rate dispersion.[38] The rate−rate spectra for a similar experiment are obtained (as shown in Figure 8), showing the similarity in the experimentally obtained spectra with that of the heterogeneous model. Rate−rate spectra as a function of time provides information about the subensemble exchange.[18]

Molecular dynamics simulation trajectories obtained for the solvation energy of a probe molecule in an ionic liquid were analyzed using these methods (time slices along the τ_2 axis are shown in Figure 9) and demonstrated that the rate dispersion observed in solvation dynamics in a prototypical ionic liquid is due to variations in the local environment formed as a result of segregation of ionic and nonionic groups.[39] Rate−rate spectra show a mixture of heterogeneous and homogeneous dispersion. Rate−rate spectra as a function of time display loss of apparent heterogeneity as a consequence of rate exchange. These methods, when applied to single-molecule Förster resonance energy transfer experiments (shown in Figure 10), revealed a possible critical role of translocation of intermediate bound states in the assembly mechanism at deoxyribonucleic acid (DNA) junctions.[40] Very recently, interconversion on the millisecond time scale among three microstates having distinct end-to-end distances has been identified in DNA constructs using these methods.[49] Analysis

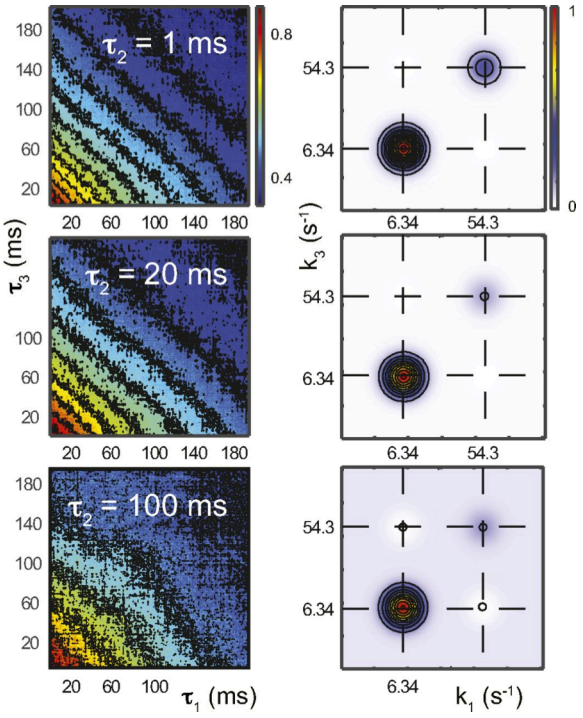

Figure 10. 2D-FlucCS of sm-FRET experiment to understand the dynamics of 3′-Cy3/Cy5-p$(dT)_{15}$-p/t DNA in the presence of 0.1 μM gp32. (Left panel) 2D autocorrelation maps at various τ_2. (Right panel) associated decay rate−rate spectra showing loss of heterogeneity with increasing τ_2. Reproduced or adapted with permission from ref 40. Copyright 2017 PNAS USA.

methods based on multidimensional correlation functions are able to detect the origin of rate dispersion and provide quantitative information.

Recently, we have successfully addressed a specific question; how to differentiate between two samples having similar rate dispersion in translational diffusion originating from different underlying mechanisms.[50] Translational diffusion, in an environment akin to FCS experiments, is simulated by a continuous time random walk. We have simulated a pure system (no dispersion, single component diffusion), a system

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with homogeneous dispersion (all particles exhibiting identical dynamics, inherently multicomponent diffusion), and a system with heterogeneous dispersion (a mixture of particles having different single component diffusion). Intensity fluctuation trajectories obtained from simulations were analyzed using 1D and, for the first time, 2D autocorrelation functions (rate spectra for the same are shown in Figure 11). 1D correlation

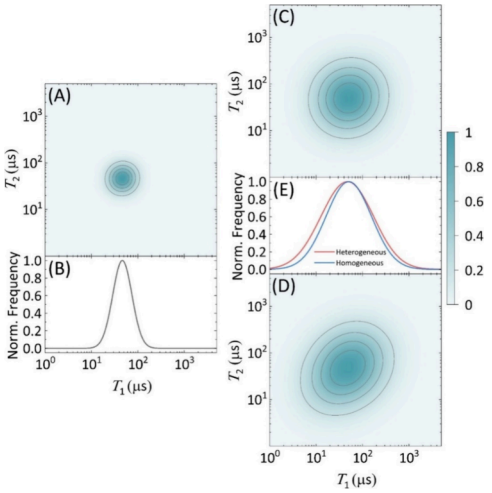

Figure 11. 2D-FlucCS analysis of time series data in FCS simulations. (A) 2D rate−rate correlation spectra of a dispersion free system. (B) 1D Projection of the 2D rate−rate correlation spectra for a dispersion free system. (C) 2D rate−rate correlation spectra of a system with homogeneous rate dispersion. (D) 2D rate−rate correlation spectra of a system with heterogeneous rate dispersion. (E) 1D projection of the 2D rate−rate correlation spectra for heterogeneous (red) and homogeneous (blue) systems. Reprinted from ref 50. Copyright 2023 with permission from Elsevier.

curves for both homogeneous and heterogeneous systems consist of similar rate dispersion. Rate filtering of 2D correlation curves shows apparent differences between both systems. A heterogeneous system shows the slowing of the correlation curve upon rate filtering, whereas a homogeneous system exhibits a relatively insignificant change. It is clearly demonstrated how 2D analysis can differentiate between homogeneous and heterogeneous origins of rate dispersion.

CONCLUSIONS AND FUTURE OUTLOOK

Application of multidimensional analysis methods to novel experiments to understand the kinetics behind nonexponential relaxation dynamics taking place in various systems is an emerging area spanning multiple scientific disciplines. In the case of biomolecules, conformational heterogeneity, i.e., the presence of multiple distinct conformers, such as in proteins, significantly influences dynamics and function.[19] Understanding the conformational landscape of a molecule is essential to understanding its function, which can be understood by observing its dynamics. However, studying the existence of conformational subensembles using ensemble-averaged spectroscopic techniques is exceptionally challenging if possible.[6] A promising solution is single-molecule spectroscopy, analyzing one molecule at a time.[51] This direct approach reveals underlying kinetics, providing valuable insights into molecular heterogeneity.

Generally, single-molecule fluorescence-based spectroscopy methods suffer from photobleaching, which limits the dynamic range of the experiments and contributes to overwhelming correlated statistical noise.[28] Ensemble-averaged multidimensional correlation analysis in single-molecule experiments provides quantitative insights into nonexponential kinetics in glass-formers. The vast potential of this method justifies its application in single-molecule studies across chemical, physical, and biological systems. Steady-state fluctuations in observables can be measured using a variety of techniques. Every measurement, whether an experiment or a simulation, is subjected to its subtleties and complexities. However, if measured fluctuations are analyzed and interpreted by means of 1D correlation functions, 2D-FlucCS can be applied directly to the same fluctuations and successfully yield the origin of observed rate dispersion.

Fluorescence-based single-molecule techniques such as single-molecule fluorescence (or Förster) resonance energy transfer (sm-FRET) and fluorescence correlation spectroscopy (FCS) are well-established methods to look at reaction kinetics at the single-molecule level and yield great details about molecular interactions and dynamics.[40,49] 2D-FlucCS can readily be applied on time series obtained by these experiments, and the origin of rate heterogeneity can be revealed. In the case of insufficiently long time series, multiple measurements can be taken, and ensemble-averaged 2D-FlucCS can be applied without compromising the reliable output.

A major limitation of fluorescent probes is their loss of information in the observables due to any photophysical and/or photochemical processes induced by high-intensity laser beams. An alternative to this is the use of a universal phenomenon of scattering exhibited by particles. Applying multidimensional methods to scattering fluctuations could reveal the kinetic schemes in systems that are difficult to probe using fluorescent molecules and markers. Dynamic light scattering (DLS) is widely used to measure the size and size distributions of objects.[52,53] As stated earlier, a broad distribution can originate from homogeneous and heterogeneous systems. DLS may yield a broad distribution for asymmetric particles, as the scattering cross-section will differ for differently oriented particles. In such a case, associating the broad distribution with size distribution would be incorrect. 2D-FlucCS has the potential to detect if the broad size distribution encountered in DLS measurements is from the distribution of spherical particles, i.e., heterogeneous systems, is due to orientation-dependent asymmetric scattering cross-section of the particles, i.e. homogeneous systems, or is a combination of both.

X-ray photocorrelation spectroscopy (XPCS) has been used to study structure and dynamics in polymers, gels, and nanocomposites.[54−56] In XPCS, an intensity−intensity autocorrelation function is measured in gels and nanocomposites that is related to the intermediate scattering function.[54] Two-time correlation functions are employed to observe time-dependent slowing down of the dynamics. The observations were associated with the aging process. It will be plausible to apply 2D-FlucCS and obtain a 2D rate spectrum as a function of time to obtain intricate details about dynamically heterogeneous systems.

Steady-state current fluctuations have recently been measured and analyzed by combining electrophysiology and autocorrelation functions to examine the mechanism of channel activation and the concurrent rearrangement of the gate in the narrow part of the pore.[57] It was inferred that conformational changes of a protein induced by ion binding

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propagate from the binding site in a sequential manner. The autocorrelations were found to have a distribution of relaxation rates and were assigned to different numbers of transitions between states in the underlying mechanism. Exposing the measured time series in these kinds of experiments to 2D-FlucCS shall render the true origin of various relaxation rates, sequential conformational change or molecular level inhomogeneity.

To determine whether an unknown system is homogeneous or heterogeneous, these methods require neither any prior information nor numerous control measurements. The time series contains all the information if obtained appropriately. These methods extract the information concealed in the time-dependent fluctuations of the observable due to fundamental dynamic processes. The potential of these methods is such that they are applicable to any steady-state time series, whether obtained at equilibrium or nonequilibrium, experimentally measured or simulated in a computer as long as it represents an ergodic system.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmeasuresciau.3c00048.

Notes corresponding to rate filtering, symmetric autocorrelation, rate−rate spectra and a figure corresponding to symmetric and antisymmetric 2D autocorrelation functions (PDF)

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Notes

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