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I. Conformational Dynamics of Biological Macromolecules by Polarization-Modulated Fourier Imaging Correlation Spectroscopy

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Abstract

Experiments that optically probe the translational motions and internal conformational transitions of biological macromolecules have the potential to enable mechanistic studies of biochemical processes in living cells. This work presents a novel 'phase-selective' approach to fluorescence fluctuation spectroscopy that simultaneously monitors protein conformational transitions and nanometer center-of-mass displacements. Polarization- and intensity-modulated photo-excitation is combined with phase-sensitive signal detection to monitor the collective coordinate fluctuations from a large population of fluorescent molecules ($N \sim 10^6$). Test experiments are performed on DsRed, a tetrameric complex of fluorescent protein subunits. Thermally induced conformational transitions of the complex lead to fluctuations in the optical dipolar coupling between adjacent chromophore sites. Polarization-resolved equilibrium fluctuation trajectories provide the raw data necessary to determine time-correlation functions and probability distributions of coordinate displacements, which characterize conformational transitions of the DsRed complex.

Keywords

Anisotropy; Fluorescence fluctuations; multi-dimensional spectroscopy; Förster energy transfer; phase-selective measurement

1. Introduction

Experiments that probe fluorescence fluctuations of individual molecules ¹, or the collective fluctuations of a finite population ², are well-suited to observe the slow (microseconds - several seconds) structural changes of biological macromolecules. Direct measurements of molecular fluctuations can reveal details of bio-molecular processes that are otherwise impossible to observe by ensemble-averaged measurements ³. Unfortunately, many single-molecule experiments must deal with the difficulties of observing a low signal in the presence of heterogeneity. For example, ambiguities can arise when attempting to separate the fluctuating signal due to a protein's conformational transitions from those due to molecular diffusion, which can occur on overlapping time scales.

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This work presents a novel high signal-to-noise experimental scheme to probe conformational dynamics of biological macromolecules in the presence of diffusion. Polarization-modulated Fourier imaging correlation spectroscopy (PM-FICS) is based on the idea that changes in molecular structure can be reflected by small variations of a phased optical signal. The technique and mathematical analyses bare similarities to those employed in multi-dimensional NMR and optical experiments⁴. PM-FICS experiments use a spatially modulated optical grating to electronically excite a moderately large number of fluorescent molecules ($N \sim 10^6$), which freely diffuse in dilute solution. Fluctuations of the polarized, steady-state emission is continuously monitored at ~ 1 ms intervals, over a period of ~ 512 s. It is shown below how phase-synchronous detection of the polarized fluorescence signal, with respect to the phase of the exciting laser field, can be used to separately isolate molecular translational and optical anisotropy fluctuations. In the current work, the polarized fluorescence fluctuations result from changes in the incoherent dipolar coupling between rigidly oriented chromophore sites of a fluorescent protein complex, DsRed. The protein is suspended in viscous 95% glycerol / water solution, so that on the time scale of the fluorescence lifetime ($\sim$ nanoseconds), rotational diffusion is very slow ($\sim$ microseconds) and the rate of fluorescence depolarization is unaffected by molecular rotation. However, the polarized emission is strongly influenced by intra-molecular energy transfer, which is sensitive to the relative orientation, separation, and transition energies of adjacent chromophore sites.

In the following experiments, we examine the slow polarized fluorescence fluctuations that result from conformational transitions of DsRed, a multicolored fluorescent protein derived from the coral *Discosoma sp.* Fluorescent proteins (FPs) are widely used in biotechnological applications as reporters of gene expression and other singular events of cell activity⁵. It has been hypothesized that FPs from reef-building corals function as part of an adaptive mechanism to optically interact and to regulate the symbiotic relationship between corals and photosynthetic algae⁶. It is therefore interesting to examine the conformational transitions that affect the protein's ability to transfer optical excitation energy. DsRed is an efficient fluorophore, with an exceptionally high extinction coefficient ($\epsilon = 3 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$, $\lambda_{\text{ex}} \sim 532$ nm), orange-red emission that is easily separated from excitation light, and a relatively high fluorescence quantum yield ($q_f = 0.79$)⁷. Much is known about DsRed's structure⁸⁻¹⁰, its photo-physical properties¹¹⁻¹⁷, and its behavior as a molecular energy transfer complex^{11, 12, 14, 15}, making it a suitable system to demonstrate the utility of the PM-FICS approach.

In the remainder of this paper we outline the basic experimental implementation of PM-FICS, including a brief theoretical description of the observables. Using DsRed as a model test system, we demonstrate how PM-FICS can separately and simultaneously measure partially averaged molecular center-of-mass and conformational coordinate fluctuations over a wide range of time scales (1 ms - 500 s) much longer than the excited state lifetime.

2. Experimental Methods

2.1 Experimental Layout

In Fig. 1A is shown a schematic diagram of the PM-FICS apparatus. Additional details about instrumentation, sample preparation, and data collection are described by Fink *et al.*¹⁸. We cross left and right circularly polarized laser beams to simultaneously generate an intensity interference fringe pattern and a linear polarization grating at the focal plane of a fluorescence microscope. The wave vector of the optical pattern $\mathbf{k}_G$ is oriented along the $\hat{a}$ direction, as shown. The laser resonantly excites freely diffusing DsRed molecules in 95% glycerol / water solution ($\lambda_{\text{ex}} = 532$ nm, sample thickness ~ 10 μm , excitation intensity at sample $= 1.27 \times 10^{-4} \mu\text{W } \mu\text{m}^{-2}$). The concentration of DsRed is dilute enough (~ 10 nM) so that the mean separation between molecules nearly matches the experimentally adjustable fringe spacing ($d_G \sim 1$ μm). The phase Φ of the spatially modulated intensity pattern is swept at the frequency

~ 1 MHz, much faster than a molecule can undergo a measurable change in its conformation, or in its center-of-mass position, but much slower than the rate of fluorescence ($\tau_f \sim 3.2$ ns)¹⁴. The back-emitted fluorescence, which is detected through the focusing objective, is similarly modulated at the sweep frequency. A polarizing beam splitter separates the emission into orthogonal plane polarized channels, which are simultaneously and phase-synchronously detected.

It is shown in the Appendix that the detected signals, polarized along the $\hat{\alpha}$ and $\hat{\chi}$ directions, can be approximated by

$$Z_{k_G}^{\alpha} \simeq \frac{I_o}{V} \sum_{n=1}^N |\widehat{\mu}_n^a|^2 |\widehat{\mu}_n^e|^2 \left[\frac{1}{6} \exp i(\mathbf{k}_G \cdot \mathbf{r}_n) + \frac{1}{4} \exp i(\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{ae}) \right] \quad (1)$$

and

$$Z_{k_G}^{\chi} \simeq \frac{I_o}{V} \sum_{n=1}^N |\widehat{\mu}_n^a|^2 |\widehat{\mu}_n^e|^2 \left[\frac{1}{3} \exp i(\mathbf{k}_G \cdot \mathbf{r}_n) - \frac{1}{2} \exp i(\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{ae}) \right]. \quad (2)$$

In Eqs. (1) and (2), $\widehat{\mu}_n^a$ and $\widehat{\mu}_n^e$ are the absorption and emission transition dipole moments of optically coupled sites in the DsRed conformation, and θ_n^{ae} is the angle (assumed fixed on the time scale of fluorescence) between the two dipole moments. The two transition dipoles are assumed to couple via a resonant incoherent Förster mechanism. Because the fluorescence lifetime ($\tau_f \sim 3.2$ ns) is much shorter than the rotational diffusion time of DsRed in 95% glycerol / water (viscosity ~ 780 cP, $\tau_R \sim 100$ ns), the polarized fluorescence signals do not depend on the rotational diffusion of DsRed, but are sensitive to the conformation angle θ_n^{ae} .

Linear combinations of Eqs. (1) and (2) are used to isolate either the first or second of the two terms. The local number density observable $Z_{k_G}^{ND} \equiv Z_{k_G}^{\chi} + 2Z_{k_G}^{\alpha} \propto \langle \exp i(k_G x) \rangle$, and the local anisotropy density observable $Z_{k_G}^{AD} \equiv Z_{k_G}^{\chi} - 2Z_{k_G}^{\alpha} \propto \langle \exp i(k_G x - 2\theta^{ae}) \rangle$ are thus defined, where the angle brackets indicate the sum over N molecules. As discussed further below, the complex signals contain information about partially averaged molecular center-of-mass and anisotropy coordinates.

2.2 Cumulant approximation

We assign a microscopic interpretations to the number density and the anisotropy density using standard methods of statistical mechanics and probability theory^{19, 20}. It is convenient to begin with a discussion of the number density. In the FICS experiment, the parameter

$Z_{k_G}^{ND}(t)$ is sequentially sampled over time. Because the grating wave vector $\mathbf{k}_G$ points in the $\hat{\alpha}$ -direction, only the x -components of the N molecular center-of-mass positions contribute to the signal. The positions $\{x_1(t), x_2(t), \dots, x_N(t)\}$ behave as continuous random variables, and fluctuate about their mean according to $x_n(t) = \delta x_n(t) + \langle x \rangle_{eq}$. We define the equilibrium probability $P_{eq}(x)$ of observing a randomly selected molecule with coordinate x , with mean $\langle x \rangle_{eq} = \int_{-\infty}^{\infty} x P_{eq}(x) dx = 0$ and variance $\sigma_{eq}^2 = \int_{-\infty}^{\infty} x^2 P_{eq}(x) dx$. At a given instant, the FICS

observable samples a Fourier component of a subset $P_N[x; t] = \frac{1}{N} \sum_{n=1}^N A_n(t) \delta[x - x_n(t)]$ of the equilibrium distribution. According to the central limit theorem, the sampled distribution has

mean value $\bar{x}_N(t) = \int_{-\infty}^{\infty} x P_N[x; t] dx = \frac{1}{N} \sum_{n=1}^N x_n(t) A_n(t)$ and variance

$\sigma_N^2 = \left[\delta \bar{x}_N(t) \right]^2 = \sum_{n=1}^N x_n^2(t) A_n(t) \propto \sigma_{eq}^2 / N$, which is narrowed relative to the equilibrium distribution by the factor N^{-1} ¹⁹. The number density fluctuation $Z_{k_G}^{ND}(t)$ is the Fourier transform (also known as the characteristic function) of the sampled distribution $P_N[x;t]$. We approximate $Z_{k_G}^{ND}(t)$ using a cumulant expansion of the sampled distribution, truncated to second order¹⁹:

$$Z_{k_G}^{ND}(t) \simeq \exp \left\{ i k_G \bar{x}_N(t) - \frac{1}{2} k_G^2 \left[\delta \bar{x}_N(t) \right]^2 \right\} \quad (3)$$

For the current situation in which center-of-mass motion is expected to occur through a free diffusion mechanism, the above approximation is expected to be accurate, since the equilibrium distribution is well characterized as Gaussian²⁰. In such cases, higher-order moments of

$P_{eq}(x)$ are negligible. Equation (3) expresses the phase and amplitude of $Z_{k_G}^{ND}(t)$ in terms of the mean value and the variance of the sampled distribution $P_N[x;t]$, respectively. Thus, in the cumulant approximation the number density samples the distribution size N , and the deviation of the sampled mean position about the equilibrium distribution,

$$\delta \bar{x}_N(t) = \bar{x}_N(t) - \langle x \rangle_{eq}.$$

In analogy to the above treatment for the number density, an approximation for the anisotropy density is

$$Z_{k_G}^{AD}(t) \simeq \exp \left\{ i \left[k_G \bar{x}_N(t) + 2\theta_N^{-ae}(t) \right] - \frac{1}{2} \left[k_G \delta \bar{x}_N(t) + 2\delta \theta_N^{-ae}(t) \right]^2 \right\}. \quad (4)$$

Similar interpretations apply to the phase and amplitude of $Z_{k_G}^{AD}(t)$ as to those for the number density, but with the additional contributions from the mean depolarization angle of the

sampled distribution $\theta_N^{-ae}(t) = \sum_{n=1}^N \theta_n^{ae}(t) P_N[\theta_n^{ae}(t)]$ and the variance $\left[\delta \theta_N^{-ae}(t) \right]^2$. The argument

of Eq. (4) generally contains coupling terms proportional to $k_G^2 \left[\delta \bar{x}_N(t) \right]^2 \left[\delta \theta_N^{-ae}(t) \right]^2$. Such terms vanish if it is assumed that the molecular center-of-mass positions are statistically uncorrelated with the depolarization angles. In this case, an additional approximation for the anisotropy

density is given by $Z_{k_G}^{AD}(t) = Z_{k_G}^{ND}(t) Z^A(t)$, where the anisotropy is defined by

$Z^A(t) \equiv Z_{k_G}^{AD}(t) / Z_{k_G}^{ND}(t) \propto \langle \exp i [2\theta_n^{ae}(t)] \rangle$. This factorization is invoked for the remainder of the paper. While both the number density and the anisotropy density are k_G -dependent, the anisotropy depends only on internal molecular degrees of freedom, and is independent of the specifically probed length scale.

The accuracy of the approximations given by Eqs. (3) and (4) depends on the relative importance of higher moments of the equilibrium distributions, $P_{eq}(x)$ and $P_{eq}(\theta^{ae})$. Were the equilibrium distributions nearly Gaussian, their higher moments would be small, and in such cases the second-order cumulant is an adequate model. For distributions with greater complexity, the second order cumulant approximation does not necessarily account for signal contributions from non-zero higher moments. Nevertheless, such higher moments may be less important when considering the time-dependent fluctuations of the optical signals. This point can be investigated through computer simulation and experiment. In the current study, we

regard equations (3) and (4) as convenient starting points for the development of future models to interpret PM-FICS data.

2.3 Two-point time-correlation and distributions functions

Two-point time-correlation functions (TCFs) provide a useful characterization of the average time intervals over which statistically correlated events occur in equilibrium systems²⁰. The TCF of the complex-valued operator $Z(t)$ is defined as

$\langle Z^*(0) Z(t_{21}) \rangle = \lim_{\tau \rightarrow \infty} \frac{1}{\tau} \int_0^\tau Z^*(t') Z(t_{21} + t') dt'$, where the angle brackets indicate the integration over time, and $t_{21} = t_2 - t_1 \geq 0$ is the time interval separating two successive measurements. Using the cumulant approximations of Eqs. (3) and (4), the number density and the anisotropy density TCFs are written

$$C_{ND}^{(2)}(t_{21}) = \langle Z_{k_G}^{ND*}(0) Z_{k_G}^{ND}(t_{21}) \rangle = \left\langle \exp \left[-\frac{1}{2} k_G^2 \delta \Delta \bar{x}_N^2(t_{21}) \right] \right\rangle \left\langle \exp i \left[k_G \Delta \bar{x}_N(t_{21}) \right] \right\rangle \quad (5)$$

and

$$C_{AD}^{(2)}(t_{21}) = \langle Z_{k_G}^{AD*}(0) Z_{k_G}^{AD}(t_{21}) \rangle = C_{ND}^{(2)}(t_{21}) C_A^{(2)}(t_{21}). \quad (6)$$

The second term on the right hand side of Eq. (6) is the anisotropy TCF, given by

$$C_A^{(2)}(t_{21}) = \langle Z^{A*}(0) Z^A(t_{21}) \rangle = \left\langle \exp \left[-2 \delta \Delta \theta_N^{ae2}(t_{21}) \right] \right\rangle \left\langle \exp i \left[2 \Delta \theta_N^{ae}(t_{21}) \right] \right\rangle. \quad (7)$$

Equation (5) defines the mean center-of-mass displacement $\Delta \bar{x}_N(t_{21}) = \bar{x}_N(t_{21}) - \bar{x}_N(0)$ of the sampled distribution during the time interval t_{21} , and the variance $\delta \Delta \bar{x}_N^2(t_{21}) = \left[\Delta \bar{x}_N(t_{21}) \right]^2$.

Similar definitions hold for the mean depolarization displacement $\Delta \theta_N^{ae}(t_{21})$ and its variance $\delta \Delta \theta_N^{ae2}(t_{21})$ given by Eq. (7). Equations (5) - (7) assume statistical independence between the means and variances of the sampled distributions. The TCFs are generally complex-valued, with phase factors that depend on the mean coordinate displacements, and amplitudes that depend on the variances. These functions decay, on average, on a time scale for which the magnitude of the phase displacement exceeds $\sim \pi/4$. The number density TCF described by Eq. (5) is known as the self-part of the intermediate scattering function of liquid state theory. In the absence of long range molecular interactions, this expression can be further simplified using the Gaussian model for single particle motion²⁰. In this approximation, Eq. (5) becomes $C_{ND}^{(2)}(t_{21}) \propto \exp \left[-k_G^2 D_s t_{21} \right]$, where D_s is the self-diffusion coefficient. Equation (6) suggests that the anisotropy TCF can be determined by the ratio $C_{AD}^{(2)}(t_{21}) / C_{ND}^{(2)}(t_{21})$.

While TCFs convey the average time scales of molecular coordinate displacements, more detailed information about the weights and magnitudes of the sampled displacements are contained in two-point distribution functions (DFs). We define $P_N^{(2)} \left[\Delta \bar{x}_N(t_{21}) \right] d\Delta \bar{x}_N$ as the probability of sampling N molecules whose mean center-of-mass coordinate has undergone a displacement in the range $\Delta \bar{x}_N$ and $\Delta \bar{x}_N + d\Delta \bar{x}_N$, during the time interval t_{21} . We construct this distribution by sampling pair-wise products of the form

$Z_{k_G}^{ND*}(0)Z_{k_G}^{ND}(t_{21}) = \exp\left[-\frac{1}{2}k_G^2\delta\Delta\bar{x}_N^{-2}(t_{21})\right] \exp i\left[k_G\Delta\bar{x}_N(t_{21})\right]$ From the two-point products we compile histograms of the displacements $\Delta\bar{x}_N(t_{21})$, weighted by the sampled inverse variance ($\propto N$). Referring to the central limit theorem, if $\Delta\bar{x}_N(t)$ behaves as a Gaussian random variable, $P_N^{(2)}\left[\Delta\bar{x}_N(t_{21})\right]$ is expected to be Gaussian with center $\langle\Delta\bar{x}_N\rangle=0$. Furthermore, the variance of this distribution should scale linearly with time according to $\delta\Delta\bar{x}_N^{-2}(t_{21})=2D_s t_{21}$, in analogy to the self-part of the van Hove function²⁰.

We similarly define the joint probability $P_N^{(2)}\left[\Delta\bar{x}_N(t_{21});\Delta\theta_N^{-ae}(t_{21})\right]$ of sampling N molecules with mean depolarization angle $\Delta\theta_N^{-ae}(t_{21})$ and mean displacement $\Delta\bar{x}_N(t_{21})$ during the time interval t_{21} . $P_N^{(2)}\left[\Delta\bar{x}_N(t_{21});\Delta\theta_N^{-ae}(t_{21})\right]$ is constructed from the anisotropy density according to $Z_{k_G}^{AD*}(0)Z_{k_G}^{AD}(t_{21})=Z_{k_G}^{ND*}(0)Z_{k_G}^{ND}(t_{21})Z^{A*}(0)Z^A(t_{21})$, where we assume no correlation between molecular positions and depolarization angles. An absence of coupling between the parameters $\Delta\bar{x}_N(t_{21})$ and $\Delta\theta_N^{-ae}(t_{21})$ implies that $P_N^{(2)}\left[\Delta\bar{x}_N(t_{21});\Delta\theta_N^{-ae}(t_{21})\right]=P_N^{(2)}\left[\Delta\bar{x}_N(t_{21})\right]P_N^{(2)}\left[\Delta\theta_N^{-ae}(t_{21})\right]$. The DF for the anisotropy fluctuations is thus determined by the ratio $P_N^{(2)}\left[\Delta\theta_N^{-ae}(t_{21})\right]=P_N^{(2)}\left[\Delta\bar{x}_N(t_{21});\Delta\theta_N^{-ae}(t_{21})\right]/P_N^{(2)}\left[\Delta\bar{x}_N(t_{21})\right]$.

3. Results and Discussion

In Fig. 2 are shown experimental trajectories of the continuously sampled observables $Z_{k_G}^{ND}(t)$ and $Z_{k_G}^{AD}(t)$ taken at acquisition frequency 1 kHz, and with fringe spacing $d_G = 1.06$ μm . Data runs were recorded over a period of 512 s. As in our previous work,¹⁸ signal count rates were typically in the range 250,000 - 400,000 cps using laser excitation intensity (measured at the sample) of 1.27×10^{-4} $\mu\text{W } \mu\text{m}^{-2}$. In the left column are shown the number density, and in the right column, the anisotropy density. In panels A and B, the observables are presented in terms of their corresponding phases, while in panels C - F, they are represented as real and imaginary parts. As shown in Fig. 2A, fluctuations of the mean center-of-mass coordinate on the order of ~ 10 nm are discernible. Similarly, Fig. 2B shows that fluctuations of the phase of the anisotropy density on the order of $\sim 2^\circ$ can be resolved. While both $Z_{k_G}^{ND}(t)$ and $Z_{k_G}^{AD}(t)$ exhibit rapid sub-second fluctuations, the anisotropy density contains at least one additional, slowly varying contribution on the time scale of several seconds. From Figs. 2E and 2F, it appears that photo-bleaching of the signal is not significant over the 512 s data acquisition period.

Following the numerical procedure described by Fink et al.¹⁸, we calculate the number density and anisotropy density TCFs, defined by Eqs. (5) and (7). These calculations were repeated using independently acquired data sets, to insure their reproducibility. The TCFs are presented in Figs. 3A and 3B, displaced vertically for the five different fringe spacings $d_G = 1.6, 1.75, 2.12, 2.56$, and 2.92 μm . In Fig. 3A, the decays for $C_{ND}^{(2)}(t_{21})$ (black curves) are compared to model curves for single particle diffusion (white). The model curves are given by the function $\exp\left(-k_G^2 D_s t_{21}\right)$ where the value used for the self-diffusion coefficient ($D_s = 3.7 \times 10^{-10}$ $\text{cm}^2 \text{s}^{-1}$) is consistent with previous measurements of DsRed self-diffusion in 95% glycerol / water solution¹⁸. For each of the five fringe spacings, the time constants of the decays increase with increasing length scale. The excellent agreement between data and the Gaussian model strongly

suggests that molecular interactions are negligible, and that center-of-mass motions are uncorrelated. The assumption that the two coordinate displacements $\Delta \bar{x}_N(t_{21})$ and $\Delta \theta_N(t_{21})$ are statistically uncorrelated is tested by comparing results for $C_{AD}^{(2)}(t_{21})$ to model decays, which account for the factorization between the anisotropy and the number density [see Eq. (6)]. For this purpose, a single exponential decay, $\exp(-t_{21}/\tau_A)$, is used to represent the anisotropy TCF $C_A^{(2)}(t_{21})$. In Fig. 3B, results for $C_{AD}^{(2)}(t_{21})$ are compared to the model decays, $\exp\left[-\left(k_G^2 D_s + 1/\tau_A\right)t_{21}\right]$ with $\tau_A = 8$ s. Note that the TCFs $C_{AD}^{(2)}(t_{21})$ decay more rapidly than $C_{ND}^{(2)}(t_{21})$, and that the agreement between data and model curves is very good for all values of d_G . In Fig. 3C, the ratio of the decays $C_{AD}^{(2)}(t_{21})/C_{ND}^{(2)}(t_{21}) = C_A^{(2)}(t_{21})$, are compared to the model function $\exp(-t_{21}/\tau_A)$. The very favorable agreement between these results for $C_A^{(2)}(t_{21})$ and the single-exponential decay demonstrates a clear separation between the anisotropy and center-of-mass fluctuations. The value obtained for the relaxation time $\tau_A = 8$ s suggests this is the average time scale for internal conformational fluctuations of DsRed.

In Fig. 4 are presented DFs of the mean center-of-mass displacement $P_N^{(2)}\left[\Delta \bar{x}_N(t_{21})\right]$, and the depolarization angle $P_N^{(2)}\left[\Delta \theta_N(t_{21})\right]$. These DFs are constructed from the observables $Z_{k_G}^{ND}(t)$ and $Z_{k_G}^{AD}(t)$, measured with fringe spacing $d_G = 2.12$ μm . In both panels are shown the distributions vertically displaced for three different values of the time interval t_{21} . The sampled distributions are symmetric and centered about the origin, and their widths increase as a function of t_{21} . In Fig. 4A, the sampled distributions $P_N^{(2)}\left[\Delta \bar{x}_N(t_{21})\right]$ are compared to the self-part of the van Hove correlation function $G_s\left(\Delta \bar{x}_N, t_{21}\right) = (4\pi D t_{21})^{-3/2} \exp\left(-\Delta \bar{x}_N^2 / 4 D_s t_{21}\right)$ (dashed gray curves), where the value of D_s is the same as that used above for the model calculations of the number density TCF. The very good agreement between the van Hove function and the DF suggests that the Gaussian model provides an accurate picture of molecular self-diffusion in the DsRed system. It is important to emphasize that the comparison between theory and measurement at the level of the DFs is a more stringent test of the Gaussian model than the previous comparison at the level of the TCFs. The TCFs represent a statistical average over the distribution of sampled displacements, according to

$C_{ND}^{(2)}(t_{21}) = \int_{-\infty}^{\infty} \exp\left[-\frac{1}{2} k_G^2 \delta \Delta \bar{x}_N^2(t_{21})\right] \exp\left[i k_G \Delta \bar{x}_N(t_{21})\right] P_N^{(2)}\left[\Delta \bar{x}_N(t_{21})\right] d\Delta \bar{x}_N(t_{21})$. The DFs provide dynamical information that is often obscured in the TCFs by the statistical average.

In Fig. 4B is shown the DF of the mean depolarization angle $P_N^{(2)}\left[\Delta \theta_N(t_{21})\right]$. Although this distribution is approximately Gaussian for the time intervals shown, the width of the distributions ceases to broaden on time scales greater than ~ 70 ms.

4. Conclusions

In this work, a novel phase-selective fluorescence fluctuation method, polarization-modulated Fourier imaging correlation spectroscopy (PM-FICS), was applied to simultaneously monitor molecular center-of-mass and anisotropy fluctuations. This approach allows for the study of internal conformational fluctuations of the fluorescent protein complex DsRed in the presence of diffusion. The phase-selectivity of PM-FICS enables the determination of two-point distribution functions (DFs) and time-correlation functions (TCFs).

Direct observations of the number density and the anisotropy density fluctuations, $Z_{k_G}^{ND}(t)$ and $Z_{k_G}^{AD}(t)$, show that thermally driven relaxation processes in DsRed occur over a broad range of time scales. A fringe spacing dependent analysis of the TCFs, $C_{ND}^{(2)}(t_{21})$, $C_{AD}^{(2)}(t_{21})$ and $C_A^{(2)}(t_{21})$, demonstrates that it is possible to simultaneously determine the effects of center-of-mass and anisotropy fluctuations. The Gaussian form of the mean center-of-mass displacement DF suggests that DsRed translational motion occurs by Fickian diffusion. For such systems, the translational components of the DFs and TCFs can be clearly identified and removed to isolate the components that depend on internal conformation. While the DF of the anisotropy fluctuations do not broaden on time scales greater than ~ 70 ms, the anisotropy TCF $C_A^{(2)}(t_{21})$ decays on the average time scale of $\tau_A = 8$ s. The large disparity in relaxation time scales is consistent with a dynamically complex system composed of competing conformation processes that occur over a wide range of rates. A molecular level interpretation of these processes, and the role of heterogeneity in the DsRed system, is examined in more detail in our adjoining paper.

The information provided by the PM-FICS method should be useful to test fundamental models of protein dynamics. In the current work, the well-defined structure of the DsRed molecule and its favorable optical properties made this an appealing candidate to demonstrate the PM-FICS approach. Future extensions of the method should enable information-rich studies of the conformation dynamics of biological macromolecules of broad interest. The ability to perform such studies in solution and in cell compartments could facilitate future studies of *in vivo* enzymatic function.

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Appendix

Consider the fluorescence intensity from the n th DsRed molecule illuminated by the exciting laser beams (see Figs. 1B and 1C). If the molecule is initially excited at time $t = 0$, its fluorescence intensity at time t is proportional to ²¹

$$\left\langle \left| \widehat{\mathcal{E}}_{ex}(\phi) \cdot \widehat{\mu}_n^a(0) \right|^2 \left| \widehat{\mathcal{E}}_{det}^{a,x} \cdot \widehat{\mu}_n^e(t) \right|^2 \right\rangle \quad (\text{A1})$$

Here, $\widehat{\mu}_n^a$ and $\widehat{\mu}_n^e$ are the absorption and emission transition dipole moments of optically coupled sites in the DsRed conformation, respectively. The angle brackets $\langle \dots \rangle$ indicate an orientational average over the isotropic distribution of absorption and emission dipoles, assuming a fixed angle θ_n^{ae} between the two. Since the fluorescence lifetime $\tau_f \sim 3.2$ ns) is much shorter than the rotational diffusion time of DsRed in 95% glycerol / water $\tau_R \sim 100$ ns), the orientation of the emission dipole moment at the detection time is approximately the same as its orientation at the time of its excitation. Hence, it is assumed that $\widehat{\mu}_n^e(t) \simeq \widehat{\mu}_n^e(0)$. As shown in Fig. 1C, the unit vectors $\widehat{\alpha}$, $\widehat{\beta}$, and $\widehat{\chi}$ define the laboratory frame coordinate system. The rotating excitation electric field direction $\widehat{\mathcal{E}}_{ex}(\phi)$ lies in the $\widehat{\alpha} - \widehat{\chi}$ plane, and is oriented relative to the $\widehat{\chi}$ -direction by the polar angle ϕ . The detection electric field directions, $\widehat{\mathcal{E}}_{det}^a$ and $\widehat{\mathcal{E}}_{det}^x$, lie fixed and parallel to the $\widehat{\alpha}$ - and $\widehat{\chi}$ -directions, respectively.

A standard approach is applied to calculate Eq. (A1) for the experimental geometry²². First, it is necessary to calculate the probability $W(\theta, \varphi, \phi) d\theta d\varphi$ that the excited molecule has absorption dipole oriented with polar angle θ and azimuthal angle φ :

$$|\widehat{\mathcal{E}}_{ex}(\phi) \cdot \widehat{\mu}_n^a(\theta, \varphi)|^2 = W(\theta, \varphi, \phi) d\theta d\varphi = \frac{\cos^2(\theta + \phi) \sin\theta d\theta d\varphi}{\int_0^{2\pi} \int_0^\pi \cos^2(\theta + \phi) \sin\theta d\theta d\varphi}. \quad (\text{A2})$$

The denominator of the above expression,

$\int_0^{2\pi} \int_0^\pi \cos^2(\theta + \phi) \sin\theta d\theta d\varphi = (-2\pi/3) [-3 + \cos(2\phi)]$ counts over all possible orientations of $\widehat{\mu}_n^a$. Note that this normalization recovers the standard value of $4\pi/3$ for $\phi = 0$. In the remaining calculations, it is useful to approximate the factor $[-3 + \cos(2\phi)]^{-1}$ using the truncated expansion $-\frac{1}{3} - \frac{1}{9}\cos(2\phi)$.

To obtain the probability of detecting the n th excited molecule's emission, polarized along the $\widehat{\alpha}$ - and $\widehat{\chi}$ -directions, one multiplies Eq. (A2) by the factor $|\widehat{\mathcal{E}}_{det}^{\alpha, \chi} \cdot \widehat{\mu}_n^e|^2$, and then integrates over all possible orientations. For the $\widehat{\alpha}$ -direction, this factor is equal to $[\sin(\theta + \theta_n^{ae}) \cos\varphi]^2$; for the $\widehat{\chi}$ -direction, it is equal to $\cos^2(\theta + \theta_n^{ae})$. The integrated intensities are

$$A_n^\alpha(\theta_n^{ae}, \phi) = \int_0^{2\pi} \int_0^\pi W(\theta, \varphi, \phi) \sin^2(\theta + \theta_n^{ae}) \cos^2\varphi d\theta d\varphi \quad (\text{A3})$$

and

$$A_n^\chi(\theta_n^{ae}, \phi) = \int_0^{2\pi} \int_0^\pi W(\theta, \varphi, \phi) \cos^2(\theta + \theta_n^{ae}) d\theta d\varphi. \quad (\text{A4})$$

Equations (A3) and (A4) are tested for the case of vertical polarization ($\phi = 0$). Substitution of Eq. (A2) into Eqs. (A3) and (A4), and using the normalization approximation stated above, leads to

$$A_n^\alpha(\theta_n^{ae}, \phi=0) \simeq \frac{2}{9} - \frac{2}{45}\cos(2\theta_n^{ae}) \quad (\text{A5})$$

and

$$A_n^\chi(\theta_n^{ae}, \phi=0) \simeq \frac{4}{9} + \frac{4}{45}\cos(2\theta_n^{ae}). \quad (\text{A6})$$

Equations (A5) and (A6) are first-order approximations to the exact solutions that would have been obtained [i.e., $A_n^\alpha(\theta_n^{ae}, \phi=0) = \frac{1}{4} - \frac{1}{20}\cos(2\theta_n^{ae})$ and $A_n^\chi(\theta_n^{ae}, \phi=0) = \frac{1}{2} + \frac{1}{10}\cos(2\theta_n^{ae})$] had the approximation to the normalization factor not been used. Equations (A3) and (A4) are next evaluated for the general case $\phi \neq 0$.

$$A_n^\alpha(\theta_n^{ae}, \Phi) \simeq |\widehat{\mu}_n^a|^2 |\widehat{\mu}_n^e|^2 \left[\frac{17}{72} + \frac{23}{360} \cos(2\theta_n^{ae}) - \frac{1}{9} \cos(2\theta_n^{ae} - \Phi) + \frac{1}{45} \cos(2\theta_n^{ae} + \Phi) - \frac{1}{72} \cos(2\Phi) - \frac{1}{48} \cos(2\theta_n^{ae} - 2\Phi) + \frac{1}{720} \cos(2\theta_n^{ae} + 2\Phi) \right], \quad (\text{A7})$$

and

$$A_n^x(\theta_n^{ae}, \Phi) \simeq |\widehat{\mu}_n^a|^2 |\widehat{\mu}_n^e|^2 \left[\frac{17}{36} - \frac{23}{180} \cos(2\theta_n^{ae}) + \frac{2}{9} \cos(2\theta_n^{ae} - \Phi) - \frac{2}{45} \cos(2\theta_n^{ae} + \Phi) - \frac{1}{36} \cos(2\Phi) + \frac{1}{24} \cos(2\theta_n^{ae} - 2\Phi) - \frac{1}{360} \cos(2\theta_n^{ae} + 2\Phi) \right]. \quad (\text{A8})$$

In Eqs. (A7) and (A8), the excitation polarization angle ϕ is expressed in terms of the phase of the intensity grating, $\Phi = 2\phi$. In the current experiments, a linear sweep is applied to the phase Φ at the frequency $\Omega/2\pi \sim 1$ MHz. Equations (A7) and (A8) show that the polarized intensities contain oscillating terms at the modulation frequency and at twice the modulation frequency.

The total fluorescence collected from the N molecules is given by¹⁸

$I_f^{\alpha,\chi} = \int_{-\infty}^{\infty} I_L(\mathbf{r}, \Phi) n^{\alpha,\chi}(\mathbf{r}, \theta^{ae}) d\mathbf{r}$, where $I_L(\mathbf{r}, \Phi) = I_0 [1 + \cos(\mathbf{k}_G \cdot \mathbf{r} + \Phi)]$ is the spatially modulated excitation intensity, $\mathbf{k}_G = (2\pi/d_G) \hat{\alpha}$ is the wave vector of the optical grating, and $n^{\alpha,\chi}(\mathbf{r}, \theta^{ae}) = \frac{1}{V} \sum_{n=1}^N A_n^{\alpha,\chi}(\theta_n^{ae}) \delta(\theta^{ae} - \theta_n^{ae}) \delta(\mathbf{r} - \mathbf{r}_n)$ is the polarization-dependent optical density of the sample. The focused laser beam waist (~ 50 μm) is much larger than the fringe spacing ($d_G \sim 1$ μm), so that $I_L(\mathbf{r}, \Phi)$ is well approximated as an infinite plane wave. Carrying out the spatial integration leads to

$$I_f^{\alpha,\chi}(\mathbf{k}_G, \Phi) = \frac{I_0}{V} \sum_{n=1}^N A_n^{\alpha,\chi}(\theta_n^{ae}, \Phi) [1 + \cos(\mathbf{k}_G \cdot \mathbf{r}_n + \Phi)]. \quad (\text{A9})$$

The phase $\Phi(t') = \Omega t' + \Phi_0$ is swept at the carrier frequency $\Omega/2\pi = 1$ MHz. The period of the modulation is much longer than the fluorescence lifetime and the rotational reorientation time, but short in comparison to the center-of-mass and conformational fluctuations of interest.

Substitution of Eqs. (A7) and (A8) into Eq. (A9), and retaining only the terms that are third-order in Φ leads to

$$I_f^\alpha(k_G, \Phi) \propto \frac{1}{6} \cos(k_G x_n + 3\Phi) + \frac{1}{4} \cos(k_G x_n - 2\theta_n^{ae} + 3\Phi) - \frac{1}{60} \cos(k_G x_n + 2\theta_n^{ae} + 3\Phi) \quad (\text{A9})$$

and

$$I_f^x(k_G, \Phi) \propto \frac{1}{3} \cos(k_G x_n + 3\Phi) - \frac{1}{2} \cos(k_G x_n - 2\theta_n^{ae} + 3\Phi) + \frac{1}{30} \cos(k_G x_n + 2\theta_n^{ae} + 3\Phi) \quad (\text{A10})$$

Using phase sensitive detection techniques described in reference ¹⁸, the above signals are demodulated at three times the carrier frequency. One thus determines the in-phase

$\left[X_{k_G}^{\alpha,\chi} = I_f^{\alpha,\chi}(k_G, 3\Phi=0) \right]$ and in-quadrature $\left[Y_{k_G}^{\alpha,\chi} = I_f^{\alpha,\chi}(k_G, 3\Phi=\pi/2) \right]$ projections of the polarized fluorescence. These components are combined according to $Z_{k_G}^{\alpha,\chi} = X_{k_G}^{\alpha,\chi} + iY_{k_G}^{\alpha,\chi}$ to obtain the complex-valued signals

$$Z_{k_g}^{\alpha} \propto \frac{1}{6} \exp(i(\mathbf{k}_G \cdot \mathbf{r}_n)) + \frac{1}{4} \exp(i(\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{ae})) - \frac{1}{60} \exp(i(\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{ae})) \quad (\text{A11})$$

and

$$Z_{k_g}^{\chi} \propto \frac{1}{3} \exp(i(\mathbf{k}_G \cdot \mathbf{r}_n)) - \frac{1}{2} \exp(i(\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{ae})) + \frac{1}{30} \exp(i(\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{ae})) \quad (\text{A12})$$

Because the final three terms of Eqs. (A11) and (A12) are small in comparison to the leading terms, the complex signals are well approximated by Eqs. (4) and (5).

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Figure 1.

(A) Schematic of the experimental apparatus. Two orthogonal, elliptically polarized laser beams are crossed at the sample plane of a fluorescence microscope. The spatially and temporally integrated fluorescence is split using a polarizing beam-splitter (BS), and detected in parallel using avalanche photo-diodes. (B) At the sample, the superposition of the two laser beams creates (simultaneously) a spatially modulated intensity interference pattern and a plane polarization grating. A 180° rotation of the polarization vector coincides to a full cycle modulation of the intensity pattern. Molecular chromophores are depicted as white circles bisected by line segments, indicating the orientations of transition dipoles. (C) Each optical chromophore is characterized by its absorption and emission dipole moments ($\vec{\mu}_n^a$ and $\vec{\mu}_n^e$, respectively), and its depolarization angle θ_n^{ae} . The polarized emission is projected onto orthogonal laboratory frame axes. The rotating polarization of the exciting field imparts a modulation to the detected depolarization angle.

Figure 2.

Experimental trajectories of the number density fluctuation $Z_{k_G}^{ND}(t)$ and the anisotropy density fluctuation $Z_{k_G}^{AD}(t)$, constructed from the demodulated polarized fluorescence signals, with $d_G = 2.12 \mu\text{m}$. These measurements are recorded from a 10 nM solution of DsRed in 95% glycerol / water, at a frequency of 1 kHz, and over an acquisition period of 512 s. In panels (E) and (F), the final 100 seconds of the data run are shown. In panels (A) - (D), the time axis is expanded to show the variation over a 1 second time window. In panels (A) and (B), the signals are represented in terms of the amplitudes and phases. In panels (C) - (F), the two signal quadratures are shown.

Figure 3.

Fringe spacing dependent two-point time-correlation functions (2P-TCFs) of the number density and the anisotropy density fluctuations, $C_{ND}^{(2)}(t_{21})$ and $C_{AD}^{(2)}(t_{21})$, respectively. Decays corresponding to different values of the fringe spacing d_G are vertically displaced. Diagonal and vertical dashed lines are guides to the eye to roughly indicate the decay time scale. In panel (A), we compare $C_{ND}^{(2)}(t_{21})$ to the Gaussian model for Fickian diffusion $\exp(-k_G^2 D_s t_{21})$, where $D_s \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$. In panel (B), we compare $C_{AD}^{(2)}(t_{21})$ to the function $\exp[-(k_G^2 D_s + 1/\tau_A)t_{21}]$ where $\tau_A = 8 \text{ s}$ is the average time scale of the anisotropy decay, due

to protein conformation fluctuations that affect energy transfer efficiency. In panel (C), we compare the ratio of the data shown in panels (A) and (B), $C_A^{(2)}(t_{21}) = C_{AD}^{(2)}(t_{21}) / C_{ND}^{(2)}(t_{21})$ to the model decay $\exp(-t_{21}/\tau_A)$.

Figure 4.

Two-point distribution functions of (A) the mean sampled center-of-mass displacement and (B) the mean sampled displacement of the depolarization angle. In each panel are shown distributions vertically displaced for three different time intervals. In panel (A) are comparisons between the measured distribution (black) and the self-part of the van Hove correlation function $G_s(\Delta\bar{x}_N, t_{21}) \propto \exp\left(-\Delta\bar{x}_N^2/4D_s t_{21}\right)$ with $D_s = 3.7 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$, (gray dashed curves). In panel (B), the distributions are compared to Gaussians with standard deviations 15.7°, 23°, and 23°.