

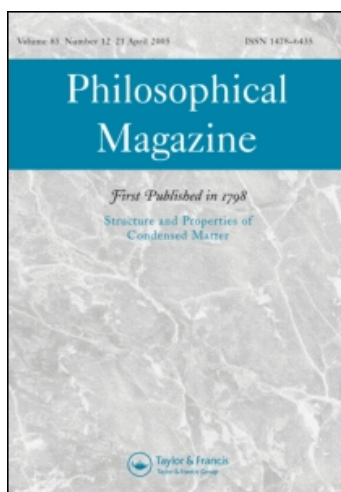
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Simultaneous measurement of fluorescence anisotropy and translational fluctuations by polarization-modulated MFICS

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A novel, high signal-to-noise strategy is presented for phase-selective, wavevector- and polarization-resolved fluorescence fluctuation spectroscopy. The approach is based on molecular Fourier imaging correlation spectroscopy (MFICS) and combines polarization- and intensity-modulated photoexcitation with phase-synchronous detection to simultaneously monitor centre-of-mass and slow fluorescence anisotropy fluctuations from a relatively large number ($\sim 10^6$) of fluorescent molecules. The method is demonstrated using DsRed, a tetrameric complex of fluorescent protein subunits, to unambiguously separate signal contributions due to slow optical conformational fluctuations from translational diffusion.

Keywords: anisotropy; fluorescence fluctuations; forster energy transfer; phase-selective measurement; fluorescent protein photodynamics

1. Introduction

Experiments that probe molecular translation and anisotropy, provide important information about the potential energy landscapes of complex systems, such as supercooled liquids and biological macromolecules. Although numerous experimental techniques probe either translation or anisotropy fluctuations separately, few current methods measure both types of motions simultaneously, and none do so unambiguously. Such experiments are needed to better understand how, decoupling occurs between slow molecular rotation and translation in supercooled fluids [1,2]. Similar experiments, performed on chromophore-labelled biological macromolecules, can help to elucidate conformational transition pathways.

In this paper, a unique experimental scheme is presented that is designed to achieve a clear separation between slow molecular centre-of-mass and optical polarization degrees of freedom of molecules diffusing in a viscous fluid. A phase-modulated optical grating was used to electronically excite a finite number of fluorescent ‘probe’ molecules ($N \sim 10^6$) that uniformly sample the ‘host’ fluid environment. The polarized, steady-state emissive fluctuations of the probes were continuously and phase-synchronously detected on millisecond time scales. By examining the scaling of the signals, with respect to the optical fringe spacing, d_G , the desired separation between fluorescence anisotropy and translational diffusion was obtained.

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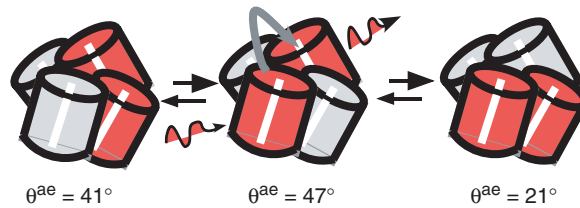


Figure 1. (Color online). Schematic representation of transitions between pair-wise coupled optical conformations in the DsRed complex, which were detected in our experiments.

1.1. Transitions between pair-wise coupled conformations in DsRed

In the following, we consider the slow (~ 8 s) anisotropy fluctuations resulting from changes in the incoherent dipolar coupling between adjacent chromophore sites in DsRed, a tetrameric complex of fluorescent protein subunits (see Figure 1). The cylinder-shaped subunits within the complex each contain an optical chromophore, with relative orientation of the transition dipole moments rigidly fixed [3]. The DsRed molecules are free to diffuse in 95% glycerol/water solution. On the time scale of the fluorescence lifetime ($\tau_f \approx 3.2$ ns) [4], rotational diffusion is very slow ($\tau_R \geq 100$ ns), so that the rate of fluorescence depolarization is unaffected by molecular rotation. However, fluorescence depolarization is strongly influenced by intramolecular energy transfer [4]. Because the relative distances and orientations between adjacent transition dipoles are small, an excited chromophore site can transfer its energy to one of its unexcited neighbours by a Förster dipole–dipole mechanism. When two sites in the DsRed complex are coupled, the excited state polarization rotates by the angle, θ^{ae} , which subtends the absorption dipole moment of the initially excited chromophore and the emission dipole moment of the emitting chromophore. Similarly to the behavior of the green fluorescent protein (GFP), the individual fluorescent protein subunits of DsRed can undergo so-called ‘flickering’ transitions between ‘bright’ and ‘dark’ states [5,6]. A locally resonant site (i.e. a ‘bright’ site) can reversibly interconvert to a non-resonant (‘dark’) site. Figure 1 schematically illustrates the inter-conversion between three possible pair-wise coupled optical conformations of the DsRed complex, with ‘bright’ subunits (shaded dark grey) and ‘dark’ subunits (shaded light grey). As local sites flicker between ‘bright’ and ‘dark’ states, the populations of distinct pair-wise coupled conformations are also expected to fluctuate, leading to fluctuations of the polarized fluorescence.

2. Experimental methods

Figure 2a shows a diagram of the optical layout of our experimental apparatus. Additional instrumentation and sample preparation details are described by Fink et al. [7]. We cross two orthogonal, elliptically 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 laser resonantly excites the sample with $\lambda_{ex} = 532$ nm, and sample thickness ~ 10 μ m. The concentration of DsRed is dilute enough (~ 10 nM) so that the mean separation between molecules nearly matches the experimentally adjustable fringe spacing ($d_G \sim 1$ μ m). We sweep the phase Φ of the spatially modulated intensity pattern at the frequency ~ 10 MHz, much faster than a molecule can undergo

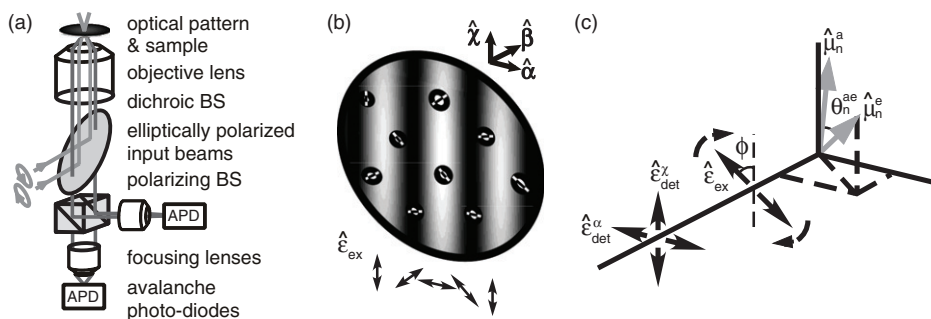


Figure 2. (a) Experimental apparatus. The sample is excited in a microscope using two orthogonal elliptically polarized laser beams. Plane-polarized fluorescence signals are phase-synchronously detected. (b) The two beams create a spatially modulated intensity and polarization pattern. The mean distance between probe molecules roughly matches the optical fringe spacing. At any instant, the sample is optically anisotropic. (c) Each optical chromophore is characterized by its absorption and emission dipole moments ($\hat{\mu}_n^a$ and $\hat{\mu}_n^e$, respectively), and its depolarization angle θ_n^{ae} . The polarized fluorescence is projected onto orthogonal lab-frame directions. The rotating exciting field imparts a modulation to the polarized fluorescence.

a conformational transition or a measurable change in its centre-of-mass position, but much slower than the fluorescence lifetime. The front-face detected fluorescence is similarly modulated at the sweep frequency. A polarizing beam splitter separates orthogonal plane polarized fluorescence signals, which are phase-synchronously detected at an acquisition frequency of ~ 1 kHz.

2.1. Polarization-dependent signal

We consider the fluorescence intensity from the n th molecule, illuminated by the exciting laser beams (see Figures 2b and 2c), proportional to $\langle |\hat{\epsilon}_{ex}(\phi) \cdot \hat{\mu}_n^a|^2 |\hat{\epsilon}_{det}^{\alpha,\chi} \cdot \hat{\mu}_n^e|^2 \rangle$ [8], where $\hat{\mu}_n^a$ and $\hat{\mu}_n^e$ are the absorption and emission transition dipole moments, and the angle brackets $\langle \dots \rangle$ indicate an orientation average over the isotropic distribution of absorption dipoles. We have defined the fixed laboratory frame detection electric field directions $\hat{\epsilon}_{det}^{\alpha}$ and $\hat{\epsilon}_{det}^{\chi}$, and the rotating excitation field direction $\hat{\epsilon}_{ex} = \sin \phi \hat{\alpha} + \cos \phi \hat{\chi}$. After performing the orientation average for our experimental geometry, we obtain first-order approximations to the plane-polarized, steady-state intensities:

$$A_n^{\chi}(\theta_n^{ae}, \Phi) \simeq |\hat{\mu}_n^a|^2 |\hat{\mu}_n^e|^2 \left[\frac{11}{24} - \frac{7}{360} \cos(2\theta_n^{ae}) + \frac{1}{9} \cos(2\theta_n^{ae} - 4\Phi) - \frac{1}{45} \cos(2\theta_n^{ae} + 4\Phi) \right] \tag{1}$$

and

$$A_n^{\alpha}(\theta_n^{ae}, \Phi) \simeq |\hat{\mu}_n^a|^2 |\hat{\mu}_n^e|^2 \left[\frac{11}{48} + \frac{7}{720} \cos(2\theta_n^{ae}) - \frac{1}{18} \cos(2\theta_n^{ae} - 4\Phi) + \frac{1}{90} \cos(2\theta_n^{ae} + 4\Phi) \right]. \tag{2}$$

In Equations (1) and (2), we have expressed the excitation polarization angle ϕ in terms of the phase of the intensity pattern: $\Phi = \phi/2$.

The total fluorescence collected from the N molecules is given by [7]

$$I_f^{\alpha,\chi}(\mathbf{k}_G, \Phi) = \frac{I_0}{V} \sum_{n=1}^N A_n^{\alpha,\chi}(\theta_n^{\text{ae}}, \Phi) [1 + \cos(\mathbf{k}_G \cdot \mathbf{r}_n + \Phi)], \quad (3)$$

where $\mathbf{k}_G = (2\pi/d_G)\hat{\alpha}$ is the wavevector of the optical grating. We apply phase-sensitive detection and low-pass filtering techniques to determine the in-phase [$X_{\mathbf{k}_G}^{\alpha,\chi} = I_f^{\alpha,\chi}(\mathbf{k}_G, \Phi = 0)$] and in-quadrature [$Y_{\mathbf{k}_G}^{\alpha,\chi} = I_f^{\alpha,\chi}(\mathbf{k}_G, \Phi = \pi/2)$] projections of the polarized fluorescence. We thus determine the complex signals [$Z_{\mathbf{k}_G}^{\alpha,\chi} = X_{\mathbf{k}_G}^{\alpha,\chi} + iY_{\mathbf{k}_G}^{\alpha,\chi}$]:

$$Z_{\mathbf{k}_G}^{\chi} \simeq \frac{I_0}{V} \sum_{n=1}^N |\hat{\mu}_n^{\text{a}}|^2 |\hat{\mu}_n^{\text{e}}|^2 \left[\frac{11}{24} \exp i(\mathbf{k}_G \cdot \mathbf{r}_n) + \frac{1}{18} \exp i(-\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{\text{ae}}) \right], \quad (4)$$

and

$$Z_{\mathbf{k}_G}^{\alpha} \simeq \frac{I_0}{V} \sum_{n=1}^N |\hat{\mu}_n^{\text{a}}|^2 |\hat{\mu}_n^{\text{e}}|^2 \left[\frac{11}{48} \exp i(\mathbf{k}_G \cdot \mathbf{r}_n) - \frac{1}{36} \exp i(-\mathbf{k}_G \cdot \mathbf{r}_n - 2\theta_n^{\text{ae}}) \right]. \quad (5)$$

Using Equations (4) and (5), we construct linear combinations to isolate the local number density fluctuation $Z_{\mathbf{k}_G}^{ND} \equiv Z_{\mathbf{k}_G}^{\chi} + 2Z_{\mathbf{k}_G}^{\alpha} \propto \langle \exp i(\mathbf{k}_G \cdot \mathbf{r}_n) \rangle$, and the local anisotropy density fluctuation $Z_{\mathbf{k}_G}^{AD} \equiv Z_{\mathbf{k}_G}^{\chi} - 2Z_{\mathbf{k}_G}^{\alpha} \propto \langle \exp i(\mathbf{k}_G \cdot \mathbf{r}_n + 2\theta_n^{\text{ae}}) \rangle$.

3. Results and discussion

From our measurements of the time-dependent fluctuations, we calculate the two-point time correlation functions (TCFs) for the number density, $C_{ND}^{(2)}(t_{21}) = \langle Z_{\mathbf{k}_G}^{ND*}(0)Z_{\mathbf{k}_G}^{ND}(t_{21}) \rangle$, and for the anisotropy density, $C_{AD}^{(2)}(t_{21}) = \langle Z_{\mathbf{k}_G}^{AD*}(0)Z_{\mathbf{k}_G}^{AD}(t_{21}) \rangle$. We plot these in Figures 3a

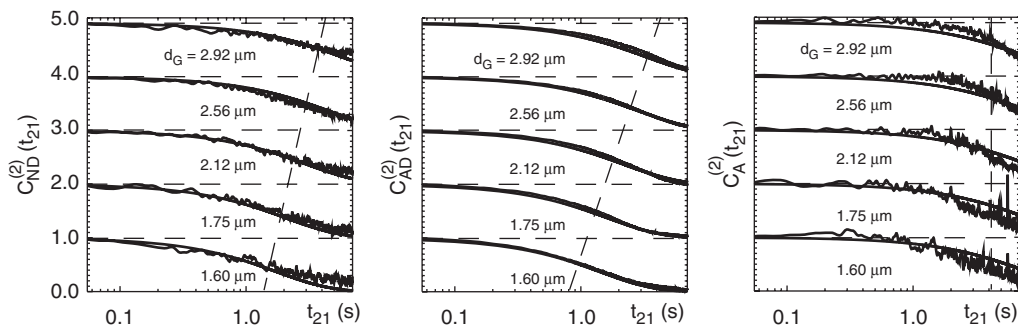


Figure 3. Two-point TCFs. (a) Number density $C_{ND}^{(2)}(t_{21})$ and (b) anisotropy density $C_{AD}^{(2)}(t_{21})$ TCFs, vertically displaced for five different fringe spacings. The experimental results (black) are compared to model decays (white, see text for details). (c) Anisotropy TCFs constructed from the ratio $C_A^{(2)}(t_{21}) = C_{AD}^{(2)}(t_{21})/C_{ND}^{(2)}(t_{21})$ of the decays shown in panels (a) and (b). Diagonal and vertical dashed lines roughly indicate the decay time scale.

and 3b, displaced vertically for five different fringe spacings. In Figure 3a, we compare our results for $C_{ND}^{(2)}(t_{21})$ (shown in black) to the Gaussian model for single particle diffusion (described by $\exp(-k_G^2 D_S t_{21})$, shown in white). For our calculations, we have used the value of the self-diffusion coefficient $D_S = 3.7 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$, which is consistent with our previous measurements of DsRed self-diffusion in 95% glycerol/water solution [7]. For each of the five fringe spacings, the agreement between our data and the Gaussian model is excellent. In situations where centre-of-mass and anisotropy fluctuations are statistically independent, we expect the anisotropy density TCF to be a product of terms $C_{AD}^{(2)}(t_{21}) = C_{ND}^{(2)}(t_{21})C_A^{(2)}(t_{21})$. For the current system, $C_A^{(2)}(t_{21})$ describes the decay of polarization memory due to internal fluctuations of the DsRed conformational states. In Figure 3b, we compare our measurements of $C_{AD}^{(2)}(t_{21})$ to the model decay $\exp[-(k_G^2 D_S + 1/\tau_A)t_{21}]$, where we have set $\tau_A = 8 \text{ s}$. Once again, agreement between our data and the theoretical model curves is very good. Figure 3c shows a comparison of the ratio of the decays shown in Figures 3a and 3b, $C_{AD}^{(2)}(t_{21})/C_{ND}^{(2)}(t_{21})$, to the model function $\exp(-t_{21}/\tau_A)$. The very favorable agreement we observe between our results for $C_A^{(2)}(t_{21})$ and the single-exponential decay for all length scales, suggests that $\tau_A \sim 8 \text{ s}$ is the slowest time scale that internal conformational fluctuations occur in DsRed.

4. Conclusions

We have demonstrated a novel approach, polarization-modulated MFICS, to simultaneously measure fluorescence anisotropy and translational motions in viscous fluids. We emphasize that, since our results for the anisotropy TCFs decay independently of the fringe spacing, we have demonstrated a clear and unambiguous separation between anisotropy and centre-of-mass fluctuations. Although not explicitly used here, the phase-selectivity of our measurements opens the possibility to construct distributions of molecular centre-of-mass and polarization displacements. We anticipate that future studies utilizing our approach will reveal detailed information about rotational-translational decoupling in super-cooled fluids, and conformational transition pathways in biological macromolecules.

Acknowledgements

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