Probing protein-protein interactions by dynamic force correlated spectroscopy (FCS)

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We develop a formalism for single molecule dynamic force spectroscopy to map the energy landscape of protein-protein complex ($P_1P_2$). The joint distribution $P(\tau_1, \tau_2)$ of unbinding lifetimes $\tau_1$ and $\tau_2$ measurable in a compression-tension cycle, which accounts for the internal relaxation dynamics of the proteins under tension, shows that the histogram of $\tau_1$ is not Poissonian. The theory is applied to the forced unbinding of protein $P_1$, modeled as a wormlike chain, from $P_1P_2$. We propose a new class of experiments which can resolve the effect of internal protein dynamics on the unbinding lifetimes.

Many biological functions are mediated by interactions between biomolecules under mechanical stress. Protein-DNA interactions involve force-induced motion of proteins \cite{Ref1, Ref2}. Similarly, specific protein-protein interaction in cell-protein complexes are important in molecular recognition \cite{Ref3}. Dynamic force spectroscopic techniques probe these interactions by forced unbinding of protein-protein complexes using forces in the 1pN–100pN range and can be used to map the complex energy landscape underlying protein-protein association \cite{Ref1, Ref2, Ref4, Ref5, Ref6, Ref7, Ref8}. Atomic force microscopy (AFM) has been employed in the studies of protein-protein interactions involving immunoglobulins \cite{Ref9}, molecular motors \cite{Ref7, Ref10} and cell adhesion complexes \cite{Ref3, Ref6, Ref8}.

In constant force-induced unbinding of single protein-protein complexes, the histograms of unbinding lifetimes is fit using the Poisson distribution

$$P_u(\tau; f_{\text{ext}}) = k_1(f_{\text{ext}}) \exp[-k_1(f_{\text{ext}})\tau].$$

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The dependence of the unbinding rate constant \( k_1 = 1/\tau_u \) (\( \tau_u \) is the lifetime of the complex) on the external force \( f_{ext} \) is given by the Bell model \([11]\), \( k_1(f_{ext}) = k_{10} \exp[f_{ext} \sigma/k_B T] \). The parameter \( \sigma \) is the maximum protein-protein bond extension before rupture, and \( k_{10} \) is the force-free unbinding rate of the bound complex \( P_1 P_2 \). Because the Poisson approximation ignores the intrinsic dynamics of proteins (i.e. conformational motions and rearrangements), this analysis can only be used when \( \tau_u \) exceeds the timescale of internal protein motion, \( \tau_R \). However, NMR studies of relaxation dynamics of proteins show that \( \tau_R \) of single chain proteins ranges from nanoseconds to tens of milliseconds \([12]\). Lifetime measurements of a single P-selectin receptor with specific ligand PSGL-1 show that \( \tau_u \) varies between miliseconds and few seconds depending on the magnitude of \( f_{ext} \) \([3, 6]\). Because the lifetimes of the protein-protein complex under force become comparable to \( \tau_R \), the interpretation of the unbinding data is complicated by protein motion. Thus, Eq. \( (1) \) cannot be used to describe experimental histograms of the lifetimes. To account for the competing timescales \( (\tau_R \) and \( \tau_u \) \) a theoretical framework that probes correlations between intrinsic relaxation and unbinding dynamics is needed to analyze experimental data.

In typical AFM experiments, the cantilever tip coated with protein \( P_1 \) is brought into contact with the surface-attached protein \( P_2 \), and allowed to interact for a time \( \Delta t \) so that the complex \( P_1 P_2 \) can form (compression cycle). The tip is then retracted to a prescribed distance which results in the complex feeling a constant force \( f = f_{ext} x \) in the direction \( x \) perpendicular to the surface (tension cycle). The lifetime \( \tau \) at which \( P_1 P_2 \) bond breaks is recorded. However, if \( \tau_u \sim \tau_R \), there is a finite time \( (\sim \tau_R) \) for propagation of the constant tension from the pulled terminus of \( P_1 \) to the binding interface of the \( P_1 P_2 \) complex. Thus, the average time \( \tau_u \) to break the \( P_1 P_2 \) bond (assuming that cantilever spring constant is stiff compared with the non-covalent linkages that stabilize \( P_1 \) and \( P_2 \)), is enhanced by \( \tau_R \) resulting in the "apparent" lifetime \( \tau \approx \tau_R + \tau_u \) of the complex.

In this Letter we propose a novel theoretical methodology for describing forced unbinding which allows for accurate estimation of protein-protein interaction parameters. The approach is based on analyzing not only the distribution of single lifetimes \( P(\tau) \) but also the joint distribution \( P(\tau_1, \tau_2; \Delta t) \) of lifetimes \( \tau_1 \) and \( \tau_2 \) separated by compression time \( \Delta t \). The distribution \( P(\tau_1, \tau_2; \Delta t) \) is measurable by constructing the joint histogram of lifetimes using current experimental methods. Because in current AFM assays \( \Delta t \) can be as short as microseconds \([13]\), \( \Delta t \)
can be varied by changing the frequency of the compression cycle; \( P(\tau_1, \tau_2; \Delta t) \) can be utilized to resolve \( \tau_R \) which in turn can be used to obtain \( \tau_u \), and free-energy landscape parameters \( \sigma \) and \( k_{10} \). The theory describes protein-protein complexes that obey \( P_1 + P_2 \Rightarrow P_1 P_2 \), and can be extended to more elaborate kinetic and protein models.

**Basic concepts:** Typically, for specific protein-protein complexes the binding rate for \( P_1 + P_2 \Rightarrow P_1 P_2 \) is fast, and \( \Delta t \) is controlled by the duration of the compression cycle. Because of the conformational fluctuations of \( P_1 \), the binding interface experiences a restoring force \( f(X, t) \) which tends to decrease the end-to-end distance \( X \). As \( t \) increases, the unbinding force along the coordinate \( X \) increases so that \( f(X, t) \rightarrow f_{\text{ext}} \) as \( t \rightarrow \infty \), and \( X(t) \) approaches the equilibrium force-dependent value \( \langle X(f_{\text{ext}}) \rangle \). Due to the conformational dynamics of the proteins, the unbinding rate, \( k_1(X, t) = k_{10} \exp[\sigma f(X, t)/k_B T] \), is a stochastic variable that depends on \( X \) through \( f(X) \). When application of \( f_{\text{ext}} \) does not result in complete stretching of \( P_1 \) (\( X = L \)), the instantaneous value of force along the \( P_1 P_2 \) bond is equal to the restoring force

\[
 f(X, \tau) = -k_B T \frac{1}{P(X, \tau)} \frac{\partial P(X, \tau)}{\partial X} \tag{2}
\]

where the probability that \( P_1 \) has end-to-end distance \( X \) at time \( t \) is given by

\[
P(X, t) = \frac{1}{N(t)} \int_0^L dX \psi_{\text{eq}}(X_0) G_0(X, t; X_0) \tag{1}
\]

and \( N(t) \) is a normalization constant. When \( f_{\text{ext}} \) is large to fully stretch \( P_1 \), the force felt by \( P_1 P_2 \) bond spikes up to \( f_{\text{ext}} \) at \( X = L \), i.e. \( f = f(X, \tau) h(L - X) + f_{\text{ext}} h(X - L) \), where \( h(X) \) is the Heavyside step function. In this Letter we consider \( f_{\text{ext}} \) that does not exceed the unfolding force threshold. The unbinding time distribution is given by the convolution of unbinding kinetics and dynamics of \( X \), i.e.,

\[
P(\tau, f_{\text{ext}}) = \frac{1}{N_1} \int_0^L dX_1 4\pi X_1^2 \int_0^L dX_0 4\pi X_0^2 P_u(X_1, \tau) G_{f_{\text{ext}}}(X_1, \tau; X_0) \psi_{\text{eq}}(X_0) \tag{3}
\]

where \( N_1 \) is a normalization constant. In Eqs. (2) and (3), \( G_0(X, t; X_0) \) and \( G_{f_{\text{ext}}}(X_1, t; X_0) \) are respectively the force-free and force-dependent conditional probability of \( X \) at time \( t \) and \( \psi_{\text{eq}}(X) \) is the equilibrium distribution of \( X \). The unbinding probability \( P_u(X, t) \) depends on \( X \) through \( k_1 \), i.e. \( P_u(X, t) = k_1(X, t) \exp[-k_1(X, t)t] \). The above equation is a generalization of Eq. (11) for force exerted on \( P_1 P_2 \) bond that continuously evolves from zero to \( f = f_{\text{ext}} \) over time \( \tau_R \).

In the limit \( \tau_R \ll \tau_u \), \( P(\tau, f_{\text{ext}}) \) reduces to \( P_u(\tau, f_{\text{ext}}) \) given by Eq. (11).

**A model application:** To illustrate the consequences of the stochastic nature of \( k_1(X, t) \), which reflects the underlying dynamics of proteins, we assume that a thermally fluctuating worm-like
chain \((P_1)\) is in contact with the immobile \(P_2\). Upon application of force \(f_{ext}\), extension of \(P_1\) results in unbinding. In this example, the timescale for internal modes of \(P_1\) are comparable to the unbinding lifetime. The Hamiltonian for \(P_1\) is

\[
H = \frac{3k_BT}{2l_p} \int_{-L/2}^{L/2} ds \left( \frac{\partial \mathbf{r}(s, t)}{\partial s} \right)^2 + \frac{3l_p k_BT}{8} \int_{-L/2}^{L/2} ds \left( \frac{\partial^2 \mathbf{r}(s, t)}{\partial s^2} \right)^2 + f \int_{-L/2}^{L/2} ds \left( \frac{\partial \mathbf{r}(s, t)}{\partial s} \right)
\]

where \(l_p\) is the protein persistence length and \(\mathbf{r}(s, t)\) is the location of protein monomers \(-L/2 \leq s \leq L/2\) at time \(t\). The end-to-end vector is \(\mathbf{X}(t) = \mathbf{r}(L/2, t) - \mathbf{r}(-L/2, t)\), where \(L\) is the protein contour length. The statistics of \(X\) can be represented by a large number of independent modes when \(L/l_p \gg 1\). Thus, it is reasonable to assume that \(G_0(X(t); X_0)\) is a Gaussian. In the overdamped limit, when \(f_{ext}\) exceeds the unfolding threshold force, stretching of \(P_1\) is smooth and thus, preserves Gaussian statistics,

\[
G_0(X, t; X_0) = \left( \frac{3}{2\pi \langle X^2 \rangle} \right)^{3/2} \frac{1}{(1 - \phi^2(t))^{3/2}} \exp \left[ -\frac{3(X - \phi(t)X_0)^2}{2\langle X^2 \rangle(1 - \phi^2(t))} \right] \tag{5}
\]

specified by the mean value \(\langle X(t) \rangle = \phi(t)X_0\) and variance \(\sigma^2 = \langle X^2 \rangle - \langle X \rangle^2\), where the correlation function \(\phi(t) = \langle X(t)X(0)\rangle / \langle X^2 \rangle\). To construct \(G_0(X(t); X_0)\) we compute \(\langle X(t)X(0)\rangle\) and \(\langle X^2 \rangle = \lim_{t \to \infty} \langle X(t)X(0)\rangle\) with \(f_{ext} = 0\). By using Eq. \((4)\) and assuming that the dynamics of the worm-like chain in the overdamped random media is described by a stochastic force \(\gamma \xi(s, t)\) with white noise statistics, \(\langle \xi_\alpha(s, t) \rangle = 0\) and \(\langle \xi_\alpha(s, t) \xi_\beta(s', t') \rangle = 2\gamma k_BT \delta_{\alpha\beta} \delta(s - s') \delta(t - t')\), where \(\alpha = x, y, z\) and \(\gamma\) is the friction coefficient, we arrive at the Langevin equation:

\[
\gamma \frac{\partial}{\partial t} \mathbf{r}(s, t) + \epsilon \frac{\partial^4}{\partial s^4} \mathbf{r}(s, t) - 2\nu \frac{\partial^2}{\partial s^2} \mathbf{r}(s, t) = \xi(s, t) \tag{6}
\]

where \(\epsilon = 3l_p k_BT/4\) and \(\nu = 3k_BT/2l_p\). We solve Eq. \((6)\) for \(\mathbf{r}(s, t)\) with boundary conditions \([2\nu \frac{\partial^3}{\partial s^3} \mathbf{r}]_{\pm L/2} = 0\), \([2\nu_0 \frac{\partial^3}{\partial s^3} \mathbf{r}]_{\pm L/2} = 0\), where \(\nu_0 = 3k_BT/4\) to yield \([14]\):

\[
\langle X(t)X(0) \rangle_0 = 12k_BT \sum_{n=1}^{\infty} \frac{1}{\psi_n^2(L/2)} e^{-\gamma n^2/\gamma}, \quad n = 1, 3, \ldots, 2q + 1 \tag{7}
\]

where the odd eigenfunctions are \([14]\):

\[
\psi_n(s) = \sqrt{c_n/L} \left( \frac{\alpha_n}{\cos \alpha_n L} \sin \alpha_n s + \frac{\beta_n}{\cosh \beta_n L} \sinh \beta_n s \right) \tag{8}
\]
with normalization constant $c_n$. The eigenvalues $z_n = \alpha_n^4 + 2\nu \alpha^2$ and the constants $\alpha_n, \beta_n$ are obtained by solving $\alpha_n \sin \left[ \frac{\alpha_n L}{2} \right] \cosh \left[ \frac{\beta_n L}{2} \right] - \beta_n^3 \cos \left[ \frac{\alpha_n L}{2} \right] \sinh \left[ \frac{\beta_n L}{2} \right] - \frac{1}{I_p} (\alpha_n^2 + \beta_n^2) \cos \left[ \frac{\alpha_n L}{2} \right] \cosh \left[ \frac{\beta_n L}{2} \right] = 0$ and $\beta_n^2 - \alpha_n^2 = \frac{1}{I_p}$. In the limit, $L/I_p \to \infty$, we arrive at the Rouse chain model describing the stretching modes $\psi_n^R = \sqrt{2/L} \sin \left( n \pi s/L \right)$ with eigenvalues $z_n^R = 3n^2 \pi^2 k_B T/2l_p L^2$. To construct force-dependent propagator $G_{fext}(X, t; X_0)$, we integrate Eq. (4) with $f_{ext} x$ added to $\xi(s, t)$ to obtain $\langle X^2 \rangle_{fext} = \langle X^2 \rangle_0 + f_{ext}^2 \sum_{n=1}^{\infty} \psi_n^2(L/2)/z_n^2$.

We computed $P(\tau, f_{ext})$ by integrating Eq. (3) at room temperature. The parameters $L$, $l_p$, and $\gamma = k_B T/DL$ determine the timescale of protein motion $\tau_R = \max \{ \gamma/z_n \}$. We set $k_{10} = 0.1 \mu s^{-1}$, $\sigma = 1.0 \text{nm}$, $L = 80 \text{nm}$, $l_p = 0.4 \text{nm}$ and $D = 10^{-8} \text{cm}^2/\text{s}$. The largest eigenvalue $z_1/\gamma = 0.2 \mu s^{-1}$ determines the longest relaxation timescale $\tau_R \approx 5 \mu s$. In left panels of Figure 1 we compare $P(\tau, f_{ext})$ for WLC and Rouse model (Eq. (3)) with the Poisson approximation $P_u(\tau, f_{ext})$ (Eq. (1)) for $f_{ext} = 1pN, 3pN$ and $10pN$. At $f_{ext} = 3pN$ and $10pN$, $P(\tau, f_{ext})$ for WLC model is in good agreement with $P(\tau, f_{ext})$ computed for the Rouse model. A slight overestimate in $P(\tau)$ at short $\tau$’s and lower $f_{ext} = 1pN$ is due to faster relaxation of the Rouse modes. For $k_1 \sim z_1/\gamma$, Poisson approximation $P_u(\tau)$ deviates noticeably from $P(\tau)$. Deviations grow as $f_{ext}$ is increased from $1pN$ to $10pN$; $P_u(\tau)$ overestimates $P(\tau)$ at shorter $\tau$ and underestimates $P(\tau)$ at longer $\tau$, predicting shorter lifetimes. Therefore, in cases where protein conformational relaxation and unbinding dynamics occur on similar timescales the use of Poisson approximation leads to inaccurate estimates of $k_{10}$ and $\sigma$. In the right panels of Figure 1 we compare $P(\tau, f_{ext})$ for the WLC and Rouse models with Poisson approximation $P_u(\tau, f_{ext})$ for $z_1/\gamma = 2 \mu s^{-1} \gg k_{10}$. A tenfold increase in $z_1/\gamma$ corresponds to less overdamped environment with larger $D = 10^{-7} \text{cm}^2/\text{s}$ (the other parameters are same as in left panels). Because, it now takes an order of magnitude shorter time to propagate $f_{ext}$ from the pulled end of $P_1$ to the $P_1P_2$ interface, Poisson distribution $P_u$ follows closely $P(\tau, f_{ext})$ at lower $f_{ext} = 1pN$ and $3pN$. However, $P_u$ deviates from $P(\tau, f_{ext})$ at higher $f_{ext} = 10pN$ due to rapid force-induced increase in the unbinding rate $k_1$. Thus, even when propagation of tension is rapid there are substantial deviations from Poisson distribution of bond lifetimes at higher $f_{ext}$.

A practical methodology that can be used in conjunction with experimental data to accurately estimate of $k_{10}$ and $\sigma$ is required. Dynamical signatures of protein motion can be assessed by computing the joint distribution $P(\tau_1, \tau_2; \Delta t)$ of consecutive unbinding times, $\tau_1$ and $\tau_2$. 


separated by compression time $\Delta t$,

$$P(\tau_1, \tau_2; \Delta t, f_{ext}) = \frac{1}{N_2} \int_0^L dX_34\pi X_3^2 \int_0^L dX_24\pi X_2^2 \int_0^L dX_14\pi X_1^2 \int_0^L dX_04\pi X_0^2$$

$$\times P_u(X_3, \tau_2)G_{f_{ext}}(X_3, \tau_2; X_2)P_b(X_2, \Delta t)G_0(X_2, \Delta t; X_1)$$

$$\times P_u(X_1, \tau_1)G_{f_{ext}}(X_1, \tau_1; X_0)\psi_{eq}(X_0)$$

(9)

where $P_b(t)$ is the binding probability for $P_1 + P_2 \rightarrow P_1P_2$ and $N_2$ is a normalization constant. In Eq. (9), $G_0(X_2, t; X_1)$ is the force free propagator representing correlations of two interaction events decaying over $\tau_R$. When $\tau_R > \Delta t$, $P(\tau_1, \tau_2; \Delta t)$ is a sensitive measure of protein motion and thus, can be employed to estimate $\tau_R$. When $\tau_R \ll \Delta t$, unbinding events are independent, $\lim_{\Delta t \to \infty} G_0(X_2, \Delta t; X_1) \to \psi_{eq}(X_2)$, and hence, $P(\tau_1, \tau_2) \to P(\tau_1)P(\tau_2)$.

We computed $P(\tau_1, \tau_2; \Delta t)$ for $\Delta t=1\mu s \ll \gamma/z_1$, $\Delta t=10\mu s \sim \gamma/z_1$ and $\Delta t=500\mu s \gg \gamma/z_1$ for $f_{ext}=3.0pN$ and $k_{10}=0.1\mu s^{-1}$, $\sigma=1.0nm$, $L=80nm$, $l_p=0.4nm$ and $z_1/\gamma=0.01\mu s^{-1}$ (Fig. 2). We assumed that protein binding ($P_1 + P_2 \rightarrow P_1P_2$) is independent of the dynamics of $X$, i.e. once $P_1$ reached the vicinity of binding interface of $P_2$ it binds, and set $P_b(X, \Delta t)=P_b=1$ in Eq. (9). A short $\Delta t=1\mu s$ and $10\mu s$ peak in $P(\tau_1, \tau_2)$ (top and middle panels) is washed out at longer $\Delta t=500\mu s$ (bottom). Striking asymmetry of the contour plots at short $\Delta t$ is due to the dependence of shorter $\tau_2$-events on longer $\tau_1$-events. During the first interaction the constant force felt by $P_1P_2$ bond is ramped up from $f=0$ to $f=f_{ext}$ following the restoring force $f(X, t)$ thus, prolonging $\tau_1$. When $\Delta t \ll \tau_R \sim \gamma/z_1$, the next binding event takes place (at $t=\Delta t$ after the first unbinding) when $P_1$ is partially or fully stretched. As a result, the binding interface experiences non-vanishing restoring force from the beginning of the second interaction event and $\tau_2<\tau_1$. Contour plots of $P(\tau_1, \tau_2)$ become more symmetric as $\Delta t$ is increased to $10\mu s$ which implies growing statistical independence of unbinding events. At $\Delta t=500\mu s \gg \tau_R$, $P(\tau_1, \tau_2)$ is symmetric density, which results in factorization $P(\tau_1, \tau_2)=P(\tau_1)P(\tau_2)$. Thus, to obtain statistically meaningful distributions of uncorrelated unbinding times, unbinding events must be separated by much longer $\Delta t$ compared to $\tau_R$ whose a priori determination is difficult.

Application to Experiments: Using $D(\tau_1, \tau_2; \Delta t)=P(\tau_1, \tau_2; \Delta t)−P(\tau_1)P(\tau_2)$, correlations between $\tau_1$’s and $\tau_2$’s can be probed in AFM experiments. If $D\neq 0$, the unbinding events are influenced by conformational fluctuations of the protein. For the model parameters in Fig. 2 we show in Fig. 3 $D(\tau_1 = \tau_2; \Delta t)$ for $\Delta t=1\mu s$, $10\mu s$ and $500\mu s$. The peak of $D(\tau, \Delta t)/D(\tau, 0)$, which signifies the amplitude of correlations between the unbinding events, decays to zero as
\( \Delta t \) is increased from \( 1 \mu s \ll \gamma/z_1 \) to \( 500 \mu s \gg \gamma/z_1 \). An accurate statistical analysis of unbinding lifetimes can be made using the following steps. From the unbinding time histogram \( P(\tau,f_{ext}) \) and the “apparent” mean lifetime \( \tau_{app} \) the joint histogram \( P(\tau_1,\tau_2;\Delta t) \) for \( \Delta t \ll \tau_{app} \) is computed. The difference \( D(\tau_1,\tau_2;\Delta t) \) is evaluated using \( P(\tau,f_{ext}) \) and the experimentally determined \( P(\tau_1,\tau_2;\Delta t,f_{ext}) \). If \( D \approx 0 \), the unbinding events are uncorrelated, and \( k_1 \) can be estimated by fitting Eq. (1) to \( P(\tau,f_{ext}) \). If \( D > 0 \), the unbinding and protein motions are correlated. In this case the lifetime measurements must be repeated for longer \( \Delta t \). Using the new data, new distributions \( P(\tau_1), P(\tau_1,\tau_2;\Delta t) \) and \( D(\tau_1,\tau_2;\Delta t) \) can be calculated. The process is iterated until the requirement \( D \approx 0 \) is satisfied for the compression cycle of duration, say, \( \Delta t^* \). The protein relaxation time \( \tau_R \) is the minimum value of \( \Delta t = \Delta t^* \) at which \( D \approx 0 \). Uncorrelated lifetimes collected for \( \Delta t \gg \tau_R \approx \Delta t^* \) can then be binned to obtain the final histogram \( P(\tau) \). If \( \tau_R \ll \tau_{app} = \tau_R + \tau_u \) then \( \tau_{app} \approx \tau_u \), and \( k_{10} \) and \( \sigma \) can be estimated by fitting Eq. (1) to \( P(\tau,f_{ext}) \). However, if \( \tau_R \approx \tau_{app} \), \( P(\tau,f_{ext}) \) must be analyzed using Eq. (3) for given \( f_{ext}, L, \gamma = k_BT/DL \), and estimated \( \tau_R \). Thus, the theory presented here suggests a novel dynamic correlated force spectroscopy in which measurements of \( P(\tau_1,\tau_2;\Delta t) \) for varying \( \Delta t \) can be used to account for the influence of internal protein dynamics on forced unbinding of protein-protein complexes.

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FIGURE CAPTIONS

**Figure 1** The distribution of unbinding times $P(\tau, f_{ext})$ for WLC (solid) and Rouse model (dash-dotted lines) of protein and Poisson approximation $P_u(\tau, f_{ext})$ (dashed lines) for $f_{ext} = 1pN, 3pN$ and $10pN$ computed for $k_1 \sim z_1/\gamma$ (left) and $k_1 \ll z_1/\gamma$ (right panels).

**Figure 2** The joint distribution $P(\tau_1, \tau_2; \Delta t, f_{ext})$ of lifetimes $\tau_1$ and $\tau_2$ separated by $\Delta t=1\mu s$ (top), $10\mu s$ (middle) and $500\mu s$ (bottom panels) for $f_{ext}=3pN$. The contour plots of $P(\tau_1, \tau_2; \Delta t, f_{ext})$ are shown on the right.

**Figure 3** Normalized correlation amplitude $D(\tau, \Delta t)/D(\tau, 0)$ of equal lifetimes $\tau=\tau_1=\tau_2$ separated by $\Delta t=1\mu s$ (solid), $10\mu s$ (dash-dotted) and $500\mu s$ (dashed lines) for $f_{ext}=3pN$. 
Fig. 1 (V. Barsegov and D. Thirumalai)
Fig. 2 (V. Barsegov and D. Thirumalai)
Fig. 3 (V. Barsegov and D. Thirumalai)