Supporting Information

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SI Text

In *SI Text* we analyze in further detail several aspects of the paper regarding theory and experiments. Concerning the theory we study in detail a dynamical model describing the experiments reported in the main text. This model is then used to shed light on several aspects of the discussion in the main text. In *SI Text*, section S1 we introduce the model in the symmetric case. In *SI Text*, section S2 we prove that x_+ and x_- are independent quantities in our model, which is a central result in our discussion. In *SI Text*, section S3 we do the same for W_+ and W_-. These conclusions are supported by a statistical analysis of the experimental data. In *SI Text*, sections S4 and S5 we comment on the definition and meaning of the hydrodynamic parameters of our model. One of these parameters is γ_+, which was used in estimating the missing contribution to the total dissipation when using the wrong work definition W'. We also recall how to measure these parameters from equilibrium experiments and present measurements on different DNA tethers. In *SI Text*, section S6 we discuss free-energy inference in asymmetric setups and in *SI Text*, section S7 we present measurements at lower pulling speeds. These measurements show that correct work measurement is important even at low irreversibility conditions.

S1. A Model for the Experimental Setup

We consider a model for the experimental dual-trap setup shown in Fig. S1. In this setup two focused laser beams form two optical traps, which allow us to manipulate a dumbbell formed by two trapped beads coupled through a molecular tether. The model is one dimensional: The dynamics take place along the direction of the tether, the y axis in Fig. S1. Should the tether not be oriented in the plane perpendicular to the direction of the laser beams, the analysis is more involved as detailed in ref. 1. In this section we assume the two traps are identical and harmonic, so that the total potential energy reads

$$U_{\mathrm{TOT}}(y_A, y_B) = \frac{k}{2} y_B^2 + \frac{k}{2}(y_A - \lambda)^2 + U_m(y_A - y_B). \quad \textbf{[S1]}$$

A more general discussion, taking into account asymmetric setups, is deferred to section S6. Note that we choose the center of trap B (left trap) as the origin of our reference frame and that λ is the position of trap A relative to trap B. A dynamical model for our system can be obtained using Langevin equations subject to a total potential as defined in Eq. **S1**,

$$\dot{\mathbf{y}} = -\overline{\mu}\nabla U_{\mathrm{TOT}}(y_A, y_B, \lambda_t) + \boldsymbol{\eta}, \quad \textbf{[S2]}$$

where $\mathbf{y} = (y_A, y_B)$, $\nabla = (\partial_{y_A}, \partial_{y_B})$, $\eta = (\eta_A, \eta_B)$ is a vector formed by the noise terms affecting the two beads, and $\overline{\mu}$ is the mobility tensor. We assume the noise to be δ correlated in time and the fluctuation–dissipation theorem to be fulfilled,

$$\langle \boldsymbol{\eta}_i(s)\boldsymbol{\eta}_j(t)\rangle = K_{\mathrm{B}} T \mu_{ij}\delta(t-s), \quad \textbf{[S3]}$$

i.e., that the covariance of the noise is proportional to the mobility μ. In the above expression K_{B} is the Boltzmann constant and T the absolute temperature. In the vector Eq. **S2** the mobility is a tensor or matrix. Its diagonal terms describe the friction affecting the motion of the beads, whereas the off-diagonal terms describe hydrodynamic interactions between the beads:

$$\overline{\mu} = \begin{pmatrix} \gamma_A^{-1}(y_A, y_B) & \Gamma_{AB}^{-1}(y_A, y_B) \\ \Gamma_{BA}^{-1}(y_A, y_B) & \gamma_B^{-1}(y_A, y_B) \end{pmatrix}.$$

The validity of Eq. **S3** for hydrodynamic fluctuations in a pulling experiment is a standard assumption, and it is a necessary hypothesis in the derivation of fluctuation relations (FRs) such as Jarzynski equality or the Crooks fluctuation relation. The analysis of this equation is greatly simplified by the following two features, which can be easily matched in our experimental setup:

- The setup is fully symmetric, meaning not only $k_A = k_B$ but also $r_A = r_B$, where r_A, r_B are the bead radii.
- The hydrodynamic coefficients γ, Γ between the beads do not significantly change in the force range of a pulling experiment. In *SI Text*, section S4 we show that this condition is met for tethered molecules ranging from 20 nm to 1 μm in contour length to a 10% accuracy.

This guarantees that the mobility matrix is symmetric and constant:

$$\overline{\mu} = \begin{pmatrix} \gamma^{-1} & \Gamma^{-1} \\ \Gamma^{-1} & \gamma^{-1} \end{pmatrix}.$$

Under these conditions, the transformation $x_+ = (y_A + y_B)/2, x_- = y_A - y_B$, decouples the potential into two separate terms,

$$U_{\mathrm{TOT}}(x_-, x_+; \lambda) = U^-(x_-; \lambda) + U^+(x_+; \lambda).$$

Moreover, the symmetry requirements cited above have the following two important consequences:

- It diagonalizes the mobility tensor μ in the form

$$\overline{\mu} = \begin{pmatrix} \gamma_+^{-1} & 0 \\ 0 & \gamma_-^{-1} \end{pmatrix}.$$

- It diagonalizes the noise covariance; i.e., it gives rise to two statistically independent noise contributions, η_+ and η_-, which separately affect the new coordinates x_+ and x_-:

$$\langle \eta_\pm(s)\eta_\pm(t)\rangle = K_{\mathrm{B}} T \gamma_\pm \delta(t-s), \quad \langle \eta_+(s)\eta_-(t)\rangle = 0. \quad \textbf{[S4]}$$

In the main text we have shown how the partition functions for x_- and x_+ decouple. From previous equations one can easily see that the full right–left symmetry of the setup guarantees that also the equations of motion for x^+ and x^- decouple completely:

$$\gamma_+ \dot{x}_t^+ = -\partial_{x^+} U^+(x_t^+, \lambda_t) + \eta_t^+ \quad \textbf{[S5]}$$

$$\gamma_- \dot{x}_t^- = -\partial_{x^-} U^-(x_t^-, \lambda_t) + \eta_t^-. \quad \textbf{[S6]}$$

S2. The Differential X_- and Center-Of-Mass X_+ Coordinates Are Statistically Independent

The factorization of the partition function (Eq. **8** in the main text) plays an important role in the derivation of our results. For symmetric systems such factorization is directly related to the statistical independence of the center-of-mass and differential coordinates. Here we experimentally check this factorization by showing how we can generally describe the state of our system, using a decomposition of the measured forces into two statistically independent coordinates. For linear systems such decomposition is always possible whereas for general nonlinear systems such decomposition is possible only in the symmetric case. Imagine that the equilibrium distribution of our system is dictated by a Hamiltonian depending on two coordinates: $U(y_A, y_B)$, which takes the form $U_+(x_+) + U_-(x_-)$ for a general transformation, $x_- = x_-(y_A, y_B)$ and $x_+ = x_+(y_A, y_B)$. In this situation, x_+ and x_- are statistically independent,

$$P(x_+|x_-) = P(x_+) \propto \exp(-\beta U_+(x_+)), \qquad \textbf{[S7]}$$

and in particular have zero covariance:

$$\langle x_+ x_- \rangle = \langle x_- \rangle \langle x_- \rangle. \qquad \textbf{[S8]}$$

A further consequence of this decomposition of the Hamiltonian is the factorization of the partition function discussed in the main text. For simplicity we consider forces instead of distances, as our instrument directly measures forces. The independence of the coordinates x_+ and x_- directly translates into the independence of the corresponding forces:

$$f_+ = -\partial U_+(x_+), \quad f_- = -\partial U_-(x_-). \qquad \textbf{[S9]}$$

Indeed,

$$\langle f_+ f_- \rangle = \int dx_+ dx_- f_+ f_- e^{-\beta U} = \int dx_+ f_+ e^{-\beta U_+} \int dx_- f_- e^{-\beta U_-} = \langle f_+ \rangle \langle f_- \rangle. \qquad \textbf{[S10]}$$

We use the covariance as a measure of linear dependence and a Pearson's χ^2 test as a measure of dependence in general. The χ^2 test is described in *SI Text*, section S3. The transformations we use to uncouple the 2 df are rotations of the form

$$f_+^\phi = \cos(\phi) f_A + \sin(\phi) f_B \qquad \textbf{[S11]}$$

$$f_-^\phi = -\sin(\phi) f_A + \cos(\phi) f_B. \qquad \textbf{[S12]}$$

We present measurements of equilibrium force fluctuations obtained using different tethers. In Fig. S2 we describe measurements performed on 1-kb dsDNA tethers stretched at 2 pN. Experiments were performed using PBS buffer (pH 7.5), 1 M NaCl at 25 °C just as in the pulling experiments described in the main text. The force of 2 pN was chosen to fall in the range explored in the pulling experiments. The first set of measurements was performed using symmetric traps $k_A \simeq k_B \simeq 0.02$ pN/nm. The second set of experiments was performed with asymmetric traps, $k_A = 0.012$ pN/nm, $k_B = 0.003$ pN/nm. In both cases it is possible to find a coordinate system such that the two coordinates are independent and in both cases this coordinate system is obtained by a rotation of the vector (f_A, f_B) (Fig. S2 *A* and *C*). The difference is that in the symmetric case the new variables correspond to the center-of-mass and differential coordinates as discussed in the main text (Fig. S2*B*), whereas they have a different definition (i.e., they are not generated by a $\pi/4$ rotation) in the asymmetric case (Fig. S2*D*). In Fig. S3 we report similar measurements performed on a DNA hairpin (the sequence is the same as reported in the main text) in a symmetric setup. Equilibrium traces were acquired at different forces. From *Top* to *Bottom* in Fig. S3 we can see the hairpin is (*i*) completely folded, (*ii*) preferentially folded, (*iii*) preferentially unfolded, and (*iv*) completely unfolded. Force fluctuations are clearly non-Gaussian, a characteristic of nonlinear two-state systems. Again, for the symmetric setup, our data show that the center-of-mass (x_+) and differential (x_-) coordinates are independent. We note that in the more general nonlinear case and for asymmetric setups no independent coordinates can be defined.

S3. The Differential Work W_- and W_+ Are Statistically Independent

An immediate consequence of the decoupling of the equations of motion is the independence of W_+ and W_-:

$$W_\pm = \int \partial_\lambda U_\pm(x_\pm, \lambda) \dot{\lambda} dt.$$

Now, because W^+ depends only on x^+ and W^- depends only on x^-, we can conclude that the two quantities are independent. Of course this result holds under specific assumptions and is, in the end, just a property of the model. From the experimental point of view one can test the independence of W^+ and W^- by statistical analysis, studying their linear (Pearson) correlations or using a Pearson's χ^2 test for dependence. The first method is sensitive to linear correlation between two observables, whereas the second, more stringent test, is sensitive to generic correlations. The χ^2 test goes as follows: To test the independence of two random variables (A, B) from a finite number of measurements we first define the empirical marginals, i.e., separate histograms of the two variables:

$$e_j^a = \frac{\sum_i^N \delta\left(A_i \in [a_j, a_{j+1})\right)}{N} \qquad \textbf{[S13]}$$

$$e_j^b = \frac{\sum_i^N \delta\left(B_i \in [b_j, b_{j+1})\right)}{N}. \qquad \textbf{[S14]}$$

Here $\delta(x \in \mathcal{C})$ is 1 if x belongs to the set $\mathcal{C}$ and is 0 otherwise. We then compare the actual 2D histogram, e_{ij} to the product of the marginal,

$$\chi^2 = \sum_{k,l} \frac{\left(e_{kl} - e_k^a e_l^b\right)^2}{e_k^a e_l^b}, \qquad \textbf{[S15]}$$

with $e_{jk} = \sum_i \delta(A_i \in [a_j, a_{j+i}))\delta(B_i \in [b_k, b_{k+i}))/N$. The value obtained through Eq. **S15** is then compared with the χ^2 distribution. The two variables are considered independent if the χ^2 value satisfies the 5% significance test. The results of the two statistical tests are reported in Tables S1 and S2. Tables S1 and S2 compare the results of the tests performed on the pair W, W' or on the pair W_+, W_-, for the forward, the reverse, and the cyclic protocols. In each case three different pulling speeds are considered. Table S1 and Table S2 refer to two different dumbbells, respectively. In most cases the pair W, W' shows higher covariance than the pair W_+, W_-. Moreover, the χ^2 test detects dependence between W, W' but not between W_+, W_- (in the χ^2 column 1 denotes a positive test for dependence and 0 a negative test).

S4. Direct Measurement of the Hydrodynamic Parameters

In the main text we have already shown how, in a symmetric setup, by switching to the coordinate system defined by the center-of-mass and differential coordinates x_+, x_-, the potential decouples into two terms:

$$U_{\mathrm{TOT}}(x_+, x_-) = k\left(x_+ - \frac{\lambda}{2}\right)^2 + \frac{k}{4}(x_- - \lambda)^2 + U_m(x_-). \qquad \textbf{[S16]}$$

When considering equilibrium fluctuations at constant trap-to-trap distance λ, a linear approximation of U_m in Eq. **S16** may be used. The energy has a minimum at $x_+ = \lambda/2$, $x_- = x_-^0$. The value of x_-^0 is defined by

$$\partial_{x_-} U_{\mathrm{TOT}}\Big|_{x_-^0} = 0. \qquad \textbf{[S17]}$$

Here x_-^0 is the (mechanical) equilibrium distance between the centers of the beads. The position of the minimum and the derivative of the potential at the minimum depend on the trap-to-trap distance, so that x_-^0 is a function of λ. In this approximation the equations of motion in Eq. **S5** become

$$\gamma_+ \dot{x}_+ = -2k\left(x_+ - \frac{\lambda}{2}\right) + \eta_+, \qquad \textbf{[S18]}$$

$$\gamma_- \dot{x}_- = -\left(\frac{k}{2} + k_m\right)\left(x_- - x_-^0\right) + \eta_-. \qquad \textbf{[S19]}$$

In particular, γ_+ is the friction affecting the center of mass of the dumbbell, whereas γ_- affects the dynamics of the differential coordinate. After Batchelor (3), we set

$$\gamma_+^{-1} = \frac{\gamma^{-1} + \Gamma^{-1}}{2}, \quad \gamma_-^{-1} = 2\left(\gamma^{-1} - \Gamma^{-1}\right). \qquad \textbf{[S20]}$$

In brief, γ is the hydrodynamic friction coefficient for a single bead, whereas Γ is the intensity of hydrodynamic interactions between the two beads. It is important to bear in mind that in principle γ and Γ depend on the differential coordinate x_-. This dependence is negligible in pulling experiments that induce a small change in x_-, but it becomes clear and measurable if x_- is changed to a larger extent, e.g., using molecules of different contour length. The equilibrium probabilities generated by **[S18]** and **[S19]** are given by Boltzmann's distribution,

$$Q_{\mathrm{eq}}(x_+) = \frac{1}{Z_+}\exp\left(-\beta k\left(x_+ - \frac{\lambda}{2}\right)^2\right), \qquad \textbf{[S21]}$$

$$P_{\mathrm{eq}}(x_-) = \frac{1}{Z_-}\exp\left(-\beta\frac{k+2k_m}{4}\left(x_- - x_-^0\right)^2\right), \qquad \textbf{[S22]}$$

with Z_+, Z_- normalization factors. The variance of equilibrium fluctuations in x_+ and x_- is connected to the elastic properties of traps and tether by

$$\sigma_+^2 = \frac{K_{\mathrm{B}}T}{2k}, \quad \sigma_-^2 = \frac{2K_{\mathrm{B}}T}{k+2k_m}. \qquad \textbf{[S23]}$$

Information about the hydrodynamic interactions can be obtained from the time-dependent correlation functions of x_+ and x_-,

$$C_+(t) = \left\langle \left(x_+(t) - \frac{\lambda}{2}\right)\left(x_+(0) - \frac{\lambda}{2}\right)\right\rangle \qquad \textbf{[S24]}$$

$$C_-(t) = \left\langle \left(x_-(t) - x_-^0\right)\left(x_-(0) - x_-^0\right)\right\rangle, \qquad \textbf{[S25]}$$

which characterizes the decay of fluctuations and allows us to distinguish the presence of different contributions to the total variance. The computation of the correlation functions yields

$$\beta C_+(t) = \frac{e^{-k\gamma_+^{-1}t}}{2k}, \qquad \textbf{[S26]}$$

$$\beta C_-(t) = \frac{2e^{-(k+2k_m)\gamma_-^{-1}t}}{k+2k_m}, \qquad \textbf{[S27]}$$

where $\beta = (K_{\mathrm{B}}T)^{-1}$. From Eqs. **S24** and **S25** the correlation function at time 0 equals the variances given in Eq. **S23**:

$$C_+(0) = \sigma_+^2, \quad C_-(0) = \sigma_+^2. \qquad \textbf{[S28]}$$

The hydrodynamic parameters can be retrieved from the correlation functions, as the stiffnesses, k, k_m, are known from Eq. **S23**,

$$\frac{1}{\gamma} + \frac{1}{\Gamma} = -\frac{1}{2k}\frac{d}{dt}\log(C_+)\Big|_{t=0} \qquad \textbf{[S29]}$$

$$\frac{1}{\gamma} - \frac{1}{\Gamma} = -\frac{2}{k+2k_m}\frac{d}{dt}\log(C_-)\Big|_{t=0}, \qquad \textbf{[S30]}$$

where we used Eqs. **S20**, **S26**, and **S27**. As discussed in the main text, γ_+ is needed to correct the error committed by using the force measured in the trap at rest in the Jarzynski equality (Eqs. **17** and **18** in the main text).

S5. A Closer Look at Hydrodynamic Interactions

In the previous section we have shown how the analysis of thermal fluctuations in a dual-trap setup allows the measurement of the two scalar parameters entering into the hydrodynamics: γ^{-1} and Γ^{-1}. The sum of the two parameters can be obtained through Eq. **S29** from the time correlation function for x_+, Eq. **S26**. The difference is instead obtained by a similar analysis on the decay rate of $C_-(t)$. Up to now we have neglected the dependence of the hydrodynamic parameters on the relative distance between the two beads. This is justified provided this relative distance does not change too much in the pulling experiment. Conversely, changing the contour length of the molecule by an order of magnitude leads to a larger and detectable change in the hydrodynamic parameters. This offers the possibility of testing our measurements against the theoretical prediction obtained using the Stokes equation and expressed as a power series in the reduced distance $\rho = \langle x_-\rangle / r_b$ with r_b the radius of the beads and $\langle x_-\rangle$ the mean of the differential coordinate. (Note that $\langle x_-\rangle > 2r_b$ and thus $\rho > 2$). The two quantities can be computed to arbitrary precision, taking the first two terms of the series expansion reported in ref. 4:

$$\gamma^{-1} \simeq \frac{1}{6\pi\eta r_b}\left(1 - \frac{15}{4\rho^4} + O\left(\rho^{-6}\right)\right) \qquad \textbf{[S31]}$$

$$\Gamma^{-1} \simeq \frac{1}{6\pi\eta r_b}\left(\frac{3}{2\rho} - \frac{1}{\rho^3} + O\left(\rho^{-7}\right)\right). \qquad \textbf{[S32]}$$

We measured fluctuations using dsDNA tethers of four different contour lengths: 8 μm (24 kbp), 1 μm (3 kbp), 300 nm (1.2 kbp), and 20 nm (58 bp). For each tether fluctuations were measured at different forces. The different lengths of the tethers used in the experiments are such that ρ assumes values from above 6 down to 2.03, near the minimum value 2 taken when the beads are in contact: Hydrodynamic interactions are monitored from the far-field regime ($\rho \gg 2$) to the lubrication limit ($\rho \simeq 2$). The experimental data for γ and Γ can be confronted with predictions, Eqs. **S31** and **S32**, without free parameters as the buffer viscosity and the bead radius are known.

This comparison is shown in Fig. S4, *Left*, where the plot compares the measured values for γ^{-1} and Γ^{-1} with Eqs. **S31** and **S32** (solid lines). For every tether we assumed a single value of ρ and averaged values obtained at different forces. In the force range relevant for pulling experiments, changes in γ^{-1} and Γ^{-1} were below 10%. Theory and data agree, within the error bars, for the longer tethers. The data for the shortest tether show a deviation from expression [S31] and probably more terms of the expansion would be needed. Nevertheless in this case the parameters are very close to the contact value, $\rho = 2$, $\gamma^{-1}(2)/\gamma^{-1}(\infty) = \Gamma^{-1}(2)/\gamma^{-1}(\infty) = 0.775$. The precision of these measurements is limited, in our case, by the error with which the radius of the bead is known. One can reduce the dependence of the measured value on the size of the bead by using the ratio between the two parameters, which does not depend on r_b if not through the definition of ρ. The results are shown in Fig. S4, *Right*, where the measured data are compared with those obtained from untethered beads. This allows us to conclude that, at least in our conditions and within our experimental resolution, the friction and/or hydrodynamic effect due to the presence of a polymer between the beads is not distinguishable. In particular, knowing the value of γ_+ allows an estimation of the systematic error on unidirectional free-energy estimates from single-molecule pulling experiments based on wrong work definitions, as detailed in the main text.

S6. Free-Energy Inference in Asymmetric Setups

In the case of asymmetric setups $P(W)$ and $P(W')$ do not have such a simple relationship as in the presence of symmetry. In this case a successful inference cannot be based on the FR alone: Some additional, system-specific, information must be provided. We focus on asymmetric systems in the Gaussian approximation and use pulling experiments on dsDNA in an asymmetric setup to test our predictions. Is it still possible to infer the full dissipation from partial work measurements? The answer is positive if we are given some equilibrium information on the system. In this case it is enough to know the trap and molecular stiffnesses k_B, k_A, k_m, i.e., equilibrium properties of the system. However, why does direct inference fail in the asymmetric case? When discussing symmetric systems we have shown that $P(W)$ and $P(W')$ are related by a simple shift:

$$P(W) = P'(W - \Delta). \qquad \textbf{[S33]}$$

Only the mean of the probability distribution had to be changed, as the variance of the two distributions is the same. In that case imposing the validity of the Crooks fluctuation relation (CFR) for $P(W)$ yields the unique value of Δ to be used in the reconstruction. This is not true anymore in asymmetric systems. Here both the mean and the variance of the work distribution must be changed, which can be achieved by convolution,

$$P(W)_{\Delta,\Sigma} = P' \star \mathcal{N}(\Delta, \Sigma), \qquad \textbf{[S34]}$$

where $\star$ denotes the convolution operator and $\mathcal{N}(\Delta, \Sigma)$ is a normal distribution with mean Δ and SD Σ. Starting from any distribution $P'(W')$ there are infinitely many choices of Δ and Σ that yield a $P(W)_{\Delta,\Sigma}$ satisfying the fluctuations theorem. Indeed, let us suppose the pair Δ^*, Σ^* is such that

$$P_{\Delta^*,\Sigma^*}(W) = P' \star \mathcal{N}(\Delta^*, \Sigma^*) \qquad \textbf{[S35]}$$

satisfies the CFR. Then it is easy to check that $P_{\Delta^*+\phi,\sqrt{(\Sigma^{*2}+2\phi/\beta)}}$ will satisfy the FR for any ϕ ($\beta = 1/K_BT$ as always). In Fig. S5 we show the effect of the convolution of $P'(W')$ with different Gaussian distributions. In the rightmost column of that plot we highlight three different pairs for which the convolved distribution satisfies the CFR. In this situation the inference cannot rest on the CFR alone, and additional information about the system is needed. In the general scheme of a Gaussian approximation, inference of $P(W)$ is still possible. In the next subsections we show that $P(W)$ and $P'(W')$ are related by an asymmetry factor (AF) given by

$$\mathrm{AF}(k_A, k_m, k_B) = \frac{\sigma_W^2 - \sigma_{W'}^2}{\langle W \rangle - \langle W' \rangle} = \frac{1}{\beta} \frac{4k_m(k_A - k_B)}{k_A(k_B + 2k_m)}, \qquad \textbf{[S36]}$$

where $\beta = 1/K_BT$. The AF is an equilibrium quantity: It depends only on the stiffnesses of traps and tether. Knowing the AF allows us to select the unique pair (Δ, Σ) such that $\mathrm{AF} = \Sigma^2/\Delta$ and that $P_{\Delta,\Sigma}(W)$ satisfies the fluctuation symmetry. These (Δ, Σ) allow the reconstruction of $P(W)$ and thus allow us to measure free-energy differences or even dynamical quantities like γ_+. The behavior of the AF factor as a function of $x = k_B/k_A$ and $y = k_m/k_A$ is shown in Fig. S6*A*.

S6.1. Pulling Experiments in Linear Systems. Free-energy inference in asymmetric setups is not as straightforward as it is in symmetric setups. To recover the full dissipation from partial work measurements we must complement these nonequilibrium measurements with some equilibrium information about the system. In this section we consider a Gaussian approximation for asymmetric setups that, as shown in Fig. 6 in the main text, agrees quite well with the experimental results. The Gaussian case is modeled by linear asymmetric systems, a class of statistical models for which inference is possible without symmetry restrictions. This amounts to choosing $U_m(x) = (1/2)k_m x^2$ in Eq. **S1** so that the total potential reads

$$U(y_A, y_B, \lambda) = \frac{k_m}{2}(y_B - y_A)^2 + \frac{k_B}{2}y_B^2 + \frac{k_A}{2}(\lambda - y_A)^2. \qquad \textbf{[S37]}$$

To simplify the following discussion we now switch to a vector notation. From now on we denote $\mathbf{X}^T(t)$ as the vector containing the positions of the traps (remember that B is the reference trap at rest with respect to water whereas trap A moves),

$$\mathbf{X}^T(t) = (\lambda_t, 0), \qquad \textbf{[S38]}$$

and $\mathbf{y}$ as the vector of bead positions: $\mathbf{y} = (y_A, y_B)$. In this vector notation the potential can be written in its normal form as

$$U\left(\mathbf{y}, \mathbf{X}^T(t)\right) = \frac{1}{2}\left(\mathbf{y} - \mathbf{y}^0\right) \cdot \bar{\mathsf{k}}\left(\mathbf{y} - \mathbf{y}^0\right) + \frac{1}{2}k_{\mathrm{eff}}\lambda^2, \qquad \textbf{[S39]}$$

where $\bar{\mathsf{k}}$ is the stiffness tensor,

$$\bar{\mathsf{k}} = \begin{pmatrix} k_A + k_m & -k_m \\ -k_m & k_B + k_m \end{pmatrix}; \qquad \textbf{[S40]}$$

k_{eff} is the effective stiffness of the dumbbell as a whole,

$$k_{\mathrm{eff}} = \frac{k_A k_B k_m}{\det(\bar{\mathsf{k}})}; \qquad \textbf{[S41]}$$

and $\mathbf{y}^0$ is the vector whose components are the equilibrium positions of the beads,

$$\mathbf{y}^0 = \left(\lambda - \frac{k_B k_m}{\det(\bar{\mathsf{k}})}(\lambda), \frac{k_A k_m}{\det(\bar{\mathsf{k}})}(\lambda)\right). \qquad \textbf{[S42]}$$

During a pulling experiment the trap-to-trap distance will be changed at a constant speed, v:

$$\lambda_t = \lambda_0 + vt. \qquad \textbf{[S43]}$$

As a consequence the equilibrium positions of the beads will change in time:

$$\mathbf{y}^0(t)=\left(\lambda_0+vt-\frac{k_Bk_m}{\det(\overline{\mathsf{k}})}(\lambda_0+vt),\ \ \frac{k_Ak_m}{\det(\overline{\mathsf{k}})}(\lambda_0+vt)\right). \qquad \textbf{[S44]}$$

During a pulling experiment the free energy of the system changes in time. FRs can be used to determine the free-energy change in the system from the distribution of the work performed on the system. As discussed in the main text, the correct definition of the work in such an experiment depends on the way in which the traps are moved. Work is identified as the time derivative of the total potential U:

$$W=-v\int_0^t k_A\left(y_A(s)-X_A^T(s)\right)ds. \qquad \textbf{[S45]}$$

Any different definition of work does not yield reliable free-energy differences. An alternative work definition can be based on the force measured in the trap at rest (B),

$$W'=v\int_0^t k_B\left(y_B(s)-X_B^T(s)\right)ds, \qquad \textbf{[S46]}$$

a choice made by many experimentalists. Is it possible to infer the distribution of W from the distribution of W' and thus to reliably measure free-energy differences? The answer to this is worked out in detail below.

S6.2. *W* and *W′* As Gaussian Random Variables. To answer the questions posed in the previous section we need to study the distributions of W and W'. In this linear model y_A and y_B are Gaussian random variables, so that W and W', being linear in y_A and y_B, are themselves Gaussian random variables too. The distribution of a Gaussian random variable is determined by its mean and variance. The computation of the mean and variance of the work requires the solution of a system of linear differential equations that can be obtained from the Markov generator for the joint process $(\mathbf{y},\mathbf{W})$ or equivalently from the corresponding Fokker–Planck equation. Here by $\mathbf{y}$ we denote the usual vector containing the positions of the beads whereas $\mathbf{W}=(v\int_0^t f_A(s)ds, v\int_0^t f_B(s)ds)$. We use the vector $\mathbf{W}$ from which the random variables W, W' are obtained as

$$W=\mathbf{W}_A,\quad W'=-\mathbf{W}_B. \qquad \textbf{[S47]}$$

Just as we did when discussing the measurement of hydrodynamic parameters, we use Langevin equations to describe the dynamics of our system,

$$\dot{\mathbf{y}}=-\overline{\mu}\overline{\mathsf{k}}\left(\mathbf{y}-\mathbf{y}^0\right)+\eta, \qquad \textbf{[S48]}$$

where $\overline{\mu}$ is the mobility tensor, describing both friction and hydrodynamic interactions,

$$\overline{\mu}=\begin{pmatrix}\gamma^{-1} & \Gamma^{-1}\\ \Gamma^{-1} & \gamma^{-1}\end{pmatrix}, \qquad \textbf{[S49]}$$

and η is a white noise compatible with the fluctuation–dissipation theorem; i.e., $\langle\eta_t\eta_s\rangle=2\beta^{-1}\overline{\mu}\delta(t-s)$. In this setting the Markov generator L for the process $(\mathbf{y},\mathbf{W})$ is given by

$$Lf(\mathbf{y},\mathbf{W})=\left(-\overline{\mu}\overline{\mathsf{k}}\left(\mathbf{y}-\mathbf{y}^0\right)\cdot\nabla_y+\beta^{-1}\overline{\mu}\cdot\nabla_y\nabla_y - v\overline{\mathsf{k}}_D\left(\mathbf{y}-\mathbf{X}^T\right)\cdot\nabla_w\right)f(\mathbf{y},\mathbf{W}), \qquad \textbf{[S50]}$$

where

$$\overline{\mathsf{k}}_D=\begin{pmatrix}k_A & 0\\ 0 & k_B\end{pmatrix}. \qquad \textbf{[S51]}$$

We recall that the Markov generator is the infinitesimal evolution operator in the sense that

$$\frac{\partial}{\partial t}\left\langle f\left(\mathbf{y}(t),\mathbf{W}(t)\right)\right\rangle_y=\left\langle Lf\left(\mathbf{y}(t),\mathbf{W}(t)\right)\right\rangle_y, \qquad \textbf{[S52]}$$

where $\langle\rangle_y$ denotes the average conditioned to initial condition $(y, 0)$. Applying the Markov generator to $\mathbf{y}$, $(\mathbf{y}-\langle\mathbf{y}\rangle_y)\otimes(\mathbf{y}-\langle\mathbf{y}\rangle_y)$, $\mathbf{W}$, $(\mathbf{W}-\langle\mathbf{W}\rangle_y)\otimes(\mathbf{W}-\langle\mathbf{W}\rangle_y)$ and then taking the average [$\mathbf{i}\otimes\mathbf{j}$ denotes the tensor $\overline{\mathsf{J}}$ such that $\overline{\mathsf{J}}\mathbf{k}=(\mathbf{i}\cdot\mathbf{k})\mathbf{j}$], we obtain the equations of motion for $\langle\mathbf{y}\rangle_y$, $\overline{\sigma}_y^2=\left\langle(\mathbf{y}-\langle\mathbf{y}\rangle_y)\otimes(\mathbf{y}-\langle\mathbf{y}\rangle_y)\right\rangle_y$, $\langle\mathbf{W}\rangle_y$, $\overline{\mathsf{C}}=\left\langle(\mathbf{W}-\langle\mathbf{W}\rangle_y)\otimes(\mathbf{y}-\langle\mathbf{y}\rangle_y)\right\rangle_y$, and $\overline{\sigma}_W^2=\left\langle(\mathbf{W}-\langle\mathbf{W}\rangle_y)\otimes(\mathbf{W}-\langle\mathbf{W}\rangle_y)\right\rangle_y$,

$$\frac{d}{dt}\langle\mathbf{y}\rangle_y=-\overline{\mu}\overline{\mathsf{k}}\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0(t)\right) \qquad \textbf{[S53]}$$

$$\frac{d}{dt}\overline{\sigma}_y^2=-2\,\mathrm{Sym}\left(\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_y^2+\frac{\overline{\mu}}{\beta}\right) \qquad \textbf{[S54]}$$

$$\frac{d}{dt}\langle\mathbf{W}\rangle_y=-\overline{\mathsf{k}}_D\left(\langle\mathbf{y}\rangle_y-\mathbf{X}^T(t)\right) \qquad \textbf{[S55]}$$

$$\frac{d}{dt}\overline{\mathsf{C}}=-\overline{\mu}\overline{\mathsf{k}}\overline{\mathsf{C}}-\overline{\sigma}_y^2\overline{\mathsf{k}}_D \qquad \textbf{[S56]}$$

$$\frac{d}{dt}\overline{\sigma}_W^2=-2\overline{\mathsf{k}}_D\overline{\mathsf{C}}, \qquad \textbf{[S57]}$$

where Sym(·) in **[S54]** is the operator that gives the symmetric part of its argument. These equations are explicitly derived in *SI Text*, section S6.5 and solved in *SI Text*, section S6.6, and we now comment on the results.

The calculations reported in the next sections show that, after neglecting transients,

$$\left\langle\mathbf{W}(t)\right\rangle=-v\int_0^t\overline{\mathsf{k}}_D\left(\mathbf{y}^0(s)-\mathbf{X}^T(s)\right)ds+\overline{\mathsf{k}}_D\overline{\mathsf{k}}^{-1}\overline{\mu}^{-1}\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D\dot{\mathbf{X}}^Tvt \qquad \textbf{[S58]}$$

and

$$\overline{\sigma}_W^2=2\frac{\overline{\mathsf{k}}_D\overline{\mathsf{k}}^{-1}\overline{\mu}^{-1}\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}v^2t. \qquad \textbf{[S59]}$$

The first term on the right-hand side of **[S58]** is the integral:

$$v\overline{\mathsf{k}}_D\left(\int_0^t-\left(\mathbf{y}^0(s)-\mathbf{X}^T(s)\right)ds\right). \qquad \textbf{[S60]}$$

The difference $\overline{\mathsf{k}}_D(\mathbf{y}^0(s)-\mathbf{X}^T(s))$ gives the equilibrium force in each trap at time s; this is just the force that would be obtained at the corresponding trap-to-trap distance λ if the pulling were carried out reversibly. The two components of the vector $\overline{\mathsf{k}}_D\left(\int_0^t(\mathbf{y}^0(s)-\mathbf{X}^T(s))ds\right)$ are of course equal in size but different in sign:

$$v\overline{\mathsf{k}}_D\left(\int_0^t -\left(\mathbf{y}^0(s)-\mathbf{X}^T(s)\right)ds\right)=\left(v\int_0^t f_{\mathrm{rev}}(s)ds,\ -v\int_0^t f_{\mathrm{rev}}(s)ds\right). \quad \text{[S61]}$$

The above expression is then just the reversible work,

$$W_{\mathrm{rev}}=k_{\mathrm{eff}}\left(\lambda_0 vt+\frac{1}{2}v^2t^2\right), \quad \text{[S62]}$$

where we used Eqs. **S39** and **S43**. The second term in [**S58**] is the dominant nonequilibrium contribution (by the way, this is the only term that is linear in time), which arises from the finite pulling speed of the protocol. A key element in this dissipation term is

$$\overline{\Omega}=\overline{\mathsf{k}}_D\overline{\mathsf{k}}^{-1}\overline{\mu}^{-1}\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D=\overline{\mathsf{p}}^T\overline{\mu}^{-1}\overline{\mathsf{p}}. \quad \text{[S63]}$$

To give some insight into the physical meaning of this quantity we note that $\overline{\mathsf{p}}$ transforms the trap position vector $\mathbf{X}^T$ into the equilibrium position vector $\mathbf{y}^0$:

$$\mathbf{y}^0=\overline{\mathsf{p}}\mathbf{X}^T. \quad \text{[S64]}$$

This dissipation term stems from friction ($\overline{\mu}^{-1}$), the relevant velocity being that of the equilibrium positions of the beads. The same element $\overline{\Omega}$ appears in the variance. Now, coming to W and W' we have

$$\langle W\rangle=W_{\mathrm{rev}}+\overline{\Omega}_{AA}v^2t \quad \text{[S65]}$$

$$\sigma_W^2=\frac{2}{\beta}\overline{\Omega}_{AA}v^2t \quad \text{[S66]}$$

$$\langle W'\rangle=W_{\mathrm{rev}}-\overline{\Omega}_{AB}v^2t \quad \text{[S67]}$$

$$\sigma_{W'}^2=\frac{2}{\beta}\overline{\Omega}_{BB}v^2t. \quad \text{[S68]}$$

Based on these expressions we introduce the asymmetry factor:

$$\mathrm{AF}=\frac{\sigma_W^2-\sigma_{W'}^2}{\langle W\rangle-\langle W'\rangle}. \quad \text{[S69]}$$

This quantity relates the distribution of W to W' based on equilibrium information. The AF does not depend either on the pulling speed, which is evident from Eqs. **S65–S68**, or on the hydrodynamic parameters. An explicit computation gives

$$\mathrm{AF}=\frac{1}{\beta}\frac{4k_m(k_A-k_B)}{k_A(k_B+2k_m)}. \quad \text{[S70]}$$

S6.3. The AF Can Be Measured from Equilibrium Force Traces. The AF can be directly measured from the equilibrium force traces. The equilibrium distribution for bead positions follows the Boltzmann distribution with respect to the potential in Eq. **S39**; i.e.,

$$P(\mathbf{y})=Z^{-1}\exp\left(-\frac{\beta}{2}\left(\mathbf{y}-\mathbf{y}^0\right)\cdot\overline{\mathsf{k}}\left(\mathbf{y}-\mathbf{y}^0\right)\right). \quad \text{[S71]}$$

According to such distribution the variance of $\mathbf{y}$ is

$$\mathrm{var}(\mathbf{y})=\beta^{-1}\overline{\mathsf{k}}^{-1}. \quad \text{[S72]}$$

Moreover, because forces and bead positions are linearly related, $\mathbf{f}=\overline{\mathsf{k}}_D(\mathbf{y}-\mathbf{X}^T)$, we have

$$\begin{aligned}\beta\mathrm{var}(\mathbf{f})&=\overline{\mathsf{k}}_D\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D\\&=\frac{1}{k_Ak_B+k_mk_A+k_mk_B}\begin{pmatrix}k_A^2(k_B+k_m) & k_Ak_Bk_m\\ k_Ak_Bk_m & k_B^2(k_A+k_m)\end{pmatrix},\end{aligned} \quad \text{[S73]}$$

where $\beta=(K_BT)^{-1}$. Using this formula we get

$$k_A=\beta\,\mathrm{var}(\mathbf{f})_{AA}+\beta\,\mathrm{var}(\mathbf{f})_{AB} \quad \text{[S74]}$$

$$k_B=\beta\,\mathrm{var}(\mathbf{f})_{BB}+\beta\,\mathrm{var}(\mathbf{f})_{AB} \quad \text{[S75]}$$

$$k_m=k_Ak_B\frac{\mathrm{var}(\mathbf{f})_{AB}}{\beta\det(\mathrm{var}(\mathbf{f}))}. \quad \text{[S76]}$$

These formulas assume that the dynamics of the dumbbell are strictly one dimensional and take place in the optical plane, i.e., that plane perpendicular to the optical axis. If this is not the case, this simplified treatment is not valid anymore and out-of-plane fluctuations must be taken into account. A discussion of these effects can be found in ref. 1. Once k_A, k_B, and k_m are known, the AF can be easily computed by Eq. **S70**. The present method for measuring rigidities from equilibrium force traces was applied and discussed in detail, in a totally different context, in ref. 2. We used it in this study to extract the values of k_A, k_B, and k_m for the asymmetric setup.

S6.4. Derivation of the Expression for the AF. The AF, as given in Eq. **S70**, can be obtained once the elements of $\overline{\Omega}$ are known. We start from the definition of $\overline{\Omega}$,

$$\overline{\Omega}=\overline{\mathsf{p}}^T\overline{\mu}^{-1}\overline{\mathsf{p}}, \quad \text{[S77]}$$

where

$$\overline{\mathsf{p}}=\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D=\frac{1}{\mathcal{E}}\begin{pmatrix}k_A(k_B+k_m) & k_Bk_m\\ k_Ak_m & k_B(k_A+k_m)\end{pmatrix}, \quad \text{[S78]}$$

with $\mathcal{E}=k_Ak_B+k_Ak_m+k_Bk_m$. Here it is useful to switch to a representation in which $\overline{\mu}^{-1}$ is diagonal. This can be achieved with a $\pi/4$ rotation:

$$\overline{\mathsf{R}}=\frac{1}{\sqrt{2}}\begin{pmatrix}1 & -1\\ 1 & 1\end{pmatrix}. \quad \text{[S79]}$$

Using $\overline{\mathsf{R}}$ we can write

$$\overline{\Omega}=\overline{\mathsf{p}}^T\overline{\mathsf{R}}\,\overline{\mathsf{R}}^T\overline{\mu}^{-1}\overline{\mathsf{R}}\,\overline{\mathsf{R}}^T\overline{\mathsf{p}}, \quad \text{[S80]}$$

where, as in *SI Text*, section S1, $\overline{\mathsf{R}}^T\overline{\mu}^{-1}\overline{\mathsf{R}}$ is diagonal. The nontrivial contribution that must still be computed is

$$\begin{aligned}\overline{\mathsf{p}}'=\overline{\mathsf{R}}^T\overline{\mathsf{p}}&=\frac{1}{\sqrt{2}\mathcal{E}}\begin{pmatrix}k_A(k_B+2k_m) & k_B(k_A+2k_m)\\ -k_Ak_B & k_Bk_A\end{pmatrix}\\&=\frac{1}{\sqrt{2}\mathcal{E}}\begin{pmatrix}A & B\\ -C & C\end{pmatrix}.\end{aligned} \quad \text{[S81]}$$

We can thus conclude that

$$\begin{aligned}\overline{\Omega}&=\frac{1}{2\mathcal{E}^2}\begin{pmatrix}A & -C\\ B & C\end{pmatrix}\begin{pmatrix}\gamma_+A & \gamma_+B\\ -\gamma_-C & \gamma_-C\end{pmatrix}\\&=\frac{1}{2\mathcal{E}^2}\begin{pmatrix}\gamma_+A^2+\gamma_-C^2 & \gamma_+AB-\gamma_-C^2\\ \gamma_+AB-\gamma_-C^2 & \gamma_+B^2+\gamma_-C^2\end{pmatrix}.\end{aligned} \quad \text{[S82]}$$

By definition we have

$$\mathrm{AF}=\frac{2}{\beta}\frac{\overline{\Omega}_{AA}-\overline{\Omega}_{BB}}{\overline{\Omega}_{AA}+\overline{\Omega}_{AB}}=\frac{2}{\beta}\frac{A-B}{A}=\frac{1}{\beta}\frac{4k_m(k_A-k_B)}{k_A(k_B+2k_m)}. \quad \textbf{[S83]}$$

S6.5. Derivation of the Equations. To derive the equations of motion **[S53]**–**[S57]** we must apply the Markov generator to the proper quantities, recalling that

$$\frac{d}{dt}\left\langle f(\mathbf{y}(t),\mathbf{W}(t))\right\rangle_y=\left\langle Lf(\mathbf{y}(t),\mathbf{W}(t))\right\rangle_y. \quad \textbf{[S84]}$$

We start by deriving Eq. **S53**. In this case we have that only the first term in the generator Eq. **S50** contributes as the second and third terms involve higher derivatives or derivatives with respect to **W**. We conclude that

$$\frac{d}{dt}\langle\mathbf{y}(t)\rangle_y=\langle L\mathbf{y}(t)\rangle_y=-\overline{\mu}\overline{\mathsf{k}}\left(\langle\mathbf{y}(t)\rangle_y-\mathbf{y}^0(t)\right). \quad \textbf{[S85]}$$

In the same way we can derive the equation for $\overline{\sigma}_y^2$, it is useful to recall that

$$\left\langle\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\otimes\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\right\rangle_y=\langle\mathbf{y}\otimes\mathbf{y}\rangle_y-\langle\mathbf{y}\rangle_y\otimes\langle\mathbf{y}\rangle_y, \quad \textbf{[S86]}$$

so that

$$\frac{d}{dt}\overline{\sigma}_y^2=\langle L(\mathbf{y}\otimes\mathbf{y})\rangle_y-\frac{d}{dt}\langle\mathbf{y}\rangle_y\otimes\langle\mathbf{y}\rangle_y. \quad \textbf{[S87]}$$

We work out the two terms of the right-hand side of **[S87]** separately, starting from the first:

$$\langle L(\mathbf{y}\otimes\mathbf{y})\rangle_y=\left\langle(-\overline{\mu}\overline{\mathsf{k}}(\mathbf{y}-\mathbf{y}^0)\cdot\nabla_y+\beta^{-1}\overline{\mu}\cdot\nabla_y\nabla_y)\mathbf{y}\otimes\mathbf{y}\right\rangle_y. \quad \textbf{[S88]}$$

The third term in the generator was neglected because it involves a derivative with respect to **W**. Acting with the derivatives on $\mathbf{y}\otimes\mathbf{y}\rangle_y$ gives

$$\begin{aligned}\langle L(\mathbf{y}\otimes\mathbf{y})\rangle_y&=-\left\langle\overline{\mu}\overline{\mathsf{k}}\left((\mathbf{y}-\mathbf{y}^0)\otimes\mathbf{y}\right)-\left(\mathbf{y}\otimes(\mathbf{y}-\mathbf{y}_0)\right)(\overline{\mu}\overline{\mathsf{k}})^T\right\rangle_y+2\beta^{-1}\overline{\mu}\\&=-2\mathrm{Sym}\left(\left\langle\overline{\mu}\overline{\mathsf{k}}((\mathbf{y}-\mathbf{y}^0)\otimes\mathbf{y})\right\rangle_y\right)+2\beta^{-1}\overline{\mu}.\end{aligned} \quad \textbf{[S89]}$$

The second term in **[S87]** is just a time derivative, which can be worked out because of **[S85]**:

$$\begin{aligned}\frac{d}{dt}\langle\mathbf{y}\rangle_y\otimes\langle\mathbf{y}\rangle_y&=-\overline{\mu}\overline{\mathsf{k}}\left((\langle\mathbf{y}\rangle_y-\mathbf{y}^0)\otimes\langle\mathbf{y}\rangle_y\right)-\left(\langle\mathbf{y}\rangle_y\otimes(\langle\mathbf{y}\rangle_y-\mathbf{y}^0)\right)(\overline{\mu}\overline{\mathsf{k}})^T\\&=-2\mathrm{Sym}\left(\overline{\mu}\overline{\mathsf{k}}\left(\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0\right)\otimes\mathbf{y}\right)\right).\end{aligned} \quad \textbf{[S90]}$$

An explicit result for the time derivative of $\overline{\sigma}_y^2$ is obtained by taking the difference of **[S89]** and **[S90]**:

$$\begin{aligned}\frac{d}{dt}\overline{\sigma}_y^2&=-2\mathrm{Sym}\left(\left\langle\overline{\mu}\overline{\mathsf{k}}((\mathbf{y}-\mathbf{y}^0)\otimes\mathbf{y})\right\rangle_y\right.\\&\qquad\left.-\overline{\mu}\overline{\mathsf{k}}\left(\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0\right)\otimes\langle\mathbf{y}\rangle_y\right)\right)+2\beta^{-1}\overline{\mu}\\&=-2\mathrm{Sym}\left(\langle\overline{\mu}\overline{\mathsf{k}}(\mathbf{y}\otimes\mathbf{y})\rangle_y-\overline{\mu}\overline{\mathsf{k}}\left(\langle\mathbf{y}\rangle_y\otimes\langle\mathbf{y}\rangle_y\right)\right)+2\beta^{-1}\overline{\mu}\\&=-2\mathrm{Sym}\left(\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_y^2\right)+2\beta^{-1}\overline{\mu}.\end{aligned} \quad \textbf{[S91]}$$

In the second step above we used the fact that

$$\left\langle\left((\mathbf{y}-\mathbf{y}^0)\otimes\mathbf{y}\right)\right\rangle_y=\langle(\mathbf{y}\otimes\mathbf{y})\rangle_y-\left(\mathbf{y}^0\otimes\langle\mathbf{y}\rangle_y\right)$$

whereas

$$\left(\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0\right)\otimes\langle\mathbf{y}\rangle_y\right)=\left(\langle\mathbf{y}\rangle_y\otimes\langle\mathbf{y}\rangle_y\right)-\left(\mathbf{y}^0\otimes\langle\mathbf{y}\rangle_y\right).$$

Following the order of Eqs. **S53–S57** we should now compute the average of **W**. This is again a simple matter, as the computation proceeds almost exactly as in the case of $\langle\mathbf{y}\rangle_y$, with the only difference that now only the last term in Eq. **S50** for the generator matters. The result is

$$\frac{d}{dt}\langle\mathbf{W}\rangle_y=\nu\overline{\mathsf{k}}_D\left(\langle\mathbf{y}\rangle_y-\mathbf{X}^T(t)\right). \quad \textbf{[S92]}$$

In the case of $\overline{\mathsf{C}}$, the cross-correlation of **y** and **W** involves some additional computations. The function $\overline{\mathsf{C}}$ is linear in both **W** and **y** so that only the first and third terms in the generator contribute. The time derivative of $\overline{\mathsf{C}}=(\mathbf{W}-\langle\mathbf{W}\rangle_y)\otimes(\mathbf{y}-\langle\mathbf{y}\rangle_y)$ again yields two pieces, as in the case of **[S87]**, and we proceed in a similar way:

$$\frac{d}{dt}\overline{\mathsf{C}}=\langle L(\mathbf{W}\otimes\mathbf{y})\rangle_y-\frac{d}{dt}\langle W\rangle_y\otimes\langle\mathbf{y}\rangle_y. \quad \textbf{[S93]}$$

Again we compute the two parts separately:

$$\begin{aligned}\langle L(\mathbf{W}\otimes\mathbf{y})\rangle_y&=\left\langle-(\mathbf{W}\otimes(\overline{\mu}\overline{\mathsf{k}}(\mathbf{y}-\mathbf{y}^0)))+\left((\nu\overline{\mathsf{k}}_D(\mathbf{y}-\mathbf{X}^T))\otimes\mathbf{y}\right)\right\rangle_y\\&=\left\langle-\overline{\mu}\overline{\mathsf{k}}(\mathbf{W}\otimes(\mathbf{y}-\mathbf{y}^0))+\nu\left((\mathbf{y}-\mathbf{X}^T)\otimes\mathbf{y}\right)\overline{\mathsf{k}}_D\right\rangle_y\end{aligned} \quad \textbf{[S94]}$$

and

$$\begin{aligned}\frac{d}{dt}\langle\mathbf{W}\rangle_y\otimes\langle\mathbf{y}\rangle_y&=-\overline{\mu}\overline{\mathsf{k}}\left(\langle\mathbf{W}\rangle_y\otimes\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0\right)\right)\\&\quad+\nu\left(\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0\right)\otimes\langle\mathbf{y}\rangle_y\right)\overline{\mathsf{k}}_D.\end{aligned} \quad \textbf{[S95]}$$

Taking the difference of these two terms and using

$$\langle(\mathbf{W}\otimes(\mathbf{y}-\mathbf{y}^0))\rangle_y=\langle(\mathbf{W}\otimes\mathbf{y})\rangle_y-\left(\langle\mathbf{W}\rangle_y\otimes\mathbf{y}^0\right)$$

and

$$\left(\langle\mathbf{W}\rangle_y\otimes\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0\right)\right)=\left(\langle\mathbf{W}\rangle_y\otimes\langle\mathbf{y}\rangle_y\right)-\left(\langle\mathbf{W}\rangle_y\otimes\mathbf{y}^0\right),$$

it is possible to conclude that

$$\begin{aligned}\frac{d}{dt}\overline{\mathsf{C}}&=-\overline{\mu}\overline{\mathsf{k}}\left\langle\left(\mathbf{W}-\langle\mathbf{W}\rangle_y\right)\otimes\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\right\rangle_y\\&\quad+\left\langle\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\otimes\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\right\rangle_y\overline{\mathsf{k}}_D\\&=-\overline{\mu}\overline{\mathsf{k}}\overline{\mathsf{C}}+\nu\overline{\sigma}_y^2\overline{\mathsf{k}}_D.\end{aligned} \quad \textbf{[S96]}$$

The only equation left to derive is that for $\overline{\sigma}_W^2=\langle(\mathbf{W}-\langle\mathbf{W}\rangle_y)\otimes(\mathbf{W}-\langle\mathbf{W}\rangle_y)\rangle_y$. This computation involves only the last term in the generator:

$$\begin{aligned}\frac{d}{dt}\left\langle\left(\mathbf{W}-\langle\mathbf{W}\rangle_y\right)\otimes\left(\mathbf{W}-\langle\mathbf{W}\rangle_y\right)\right\rangle_y&=\langle L(\mathbf{W}\otimes\mathbf{W})\rangle_y\\&\quad-\frac{d}{dt}\left(\langle\mathbf{W}\rangle_y\otimes\langle\mathbf{W}\rangle_y\right).\end{aligned} \quad \textbf{[S97]}$$

As usual we evaluate the two terms separately and put them together as a second step:

$$\langle L(\mathbf{W}\otimes\mathbf{W})\rangle_y = \nu\langle \bar{\mathsf{k}}_D(\mathbf{W}\otimes(\mathbf{y}-\mathbf{X}^T)) + ((\mathbf{y}-\mathbf{X}^T)\otimes\mathbf{W})\bar{\mathsf{k}}_D\rangle_y, \quad \textbf{[S98]}$$

$$\frac{d}{dt}\left(\langle\mathbf{W}\rangle_y\otimes\langle\mathbf{W}\rangle_y\right) = \nu\bar{\mathsf{k}}_D\left(\mathbf{W}\otimes\left(\langle\mathbf{y}\rangle_y-\mathbf{X}^T\right)\right) + \left(\left(\langle\mathbf{y}\rangle_y-\mathbf{X}^T\right)\otimes\mathbf{W}\right)\bar{\mathsf{k}}_D. \quad \textbf{[S99]}$$

The last equation of motion is thus

$$\begin{aligned}\frac{d}{dt}\bar{\sigma}^2_W &= +\nu\bar{\mathsf{k}}_D\left\langle\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\otimes\left(\mathbf{W}-\langle\mathbf{W}\rangle_y\right)\right\rangle_y \\ &\quad +\nu\left\langle\left(\mathbf{W}-\langle\mathbf{W}\rangle_y\right)\otimes\left(\mathbf{y}-\langle\mathbf{y}\rangle_y\right)\right\rangle_y\bar{\mathsf{k}}_D \\ &= +2\mathrm{Sym}\left(\nu\bar{\mathsf{k}}_D\bar{\mathsf{C}}\right).\end{aligned} \quad \textbf{[S100]}$$

S6.6. Solution of the Equations. In the previous section we introduced an average operation $\langle\cdot\rangle_y$ without specifying with respect to which measure the average is taken. In this section we suppose that the initial probability distribution for the process is $p(\mathbf{y},0)=\delta(\mathbf{y}-y)$, a deterministic initial condition. The work is by definition equal to 0 at $t=0$, again a deterministic initial condition, and the same is true for the different variances and covariances because of the initial conditions. The initial conditions for the equations of motion will then be

$$\begin{cases}\langle\mathbf{y}(0)\rangle_y = y \\ \langle\mathbf{W}(0)\rangle_y = \bar{\sigma}^2_{\mathbf{y}}(0) = \bar{\mathsf{C}}(0) = \bar{\sigma}^2_{\mathbf{W}}(0) = 0.\end{cases} \quad \textbf{[S101]}$$

All of the equations we have to solve are linear equations that can be solved either by variation of constants or by direct integration. The first equation, that for $\langle\mathbf{y}\rangle_y$, is a case for variation of constants:

$$\begin{cases}\langle\mathbf{y}(0)\rangle_y = y \\ \dfrac{d}{dt}\langle\mathbf{y}\rangle_y = -\bar{\mu}\bar{\mathsf{k}}\left(\langle\mathbf{y}\rangle_y-\mathbf{y}^0(t)\right).\end{cases} \quad \textbf{[S102]}$$

The solution is

$$\langle\mathbf{y}(t)\rangle_y = e^{-\bar{\mu}\bar{\mathsf{k}}t}\left(y+\int_0^t\bar{\mu}\bar{\mathsf{k}}e^{\bar{\mu}\bar{\mathsf{k}}s}\mathbf{y}^0(s)ds\right), \quad \textbf{[S103]}$$

as it can be directly checked. We recall the definition of $\mathbf{y}^0(t)$,

$$\mathbf{y}^0(t) = \left(\lambda+\nu t-\frac{k_Ak_m}{\det(\mathsf{k})}(\lambda_0+\nu t), \frac{k_Bk_m}{\det(\mathsf{k})}(\lambda_0+\nu t)\right). \quad \textbf{[S104]}$$

In the following it is useful to write $\mathbf{y}^0(t)=\psi+\xi t$, where

$$\psi = \left(\lambda_0-\frac{k_Ak_m}{\det(\mathsf{k})}\lambda_0, \frac{k_Bk_m}{\det(\mathsf{k})}\lambda_0\right) \quad \textbf{[S105]}$$

$$\xi = \left(\nu-\frac{k_Ak_m}{\det(\mathsf{k})}\nu, \frac{k_Bk_m}{\det(\mathsf{k})}\nu\right). \quad \textbf{[S106]}$$

In terms of these quantities **[S103]** reads

$$\langle\mathbf{y}(t)\rangle_y = e^{-\bar{\mu}\bar{\mathsf{k}}t}\left(y+\int_0^t\bar{\mu}\bar{\mathsf{k}}e^{\bar{\mu}\bar{\mathsf{k}}s}(\psi+\xi t)ds\right). \quad \textbf{[S107]}$$

Integrating by parts we get

$$\begin{aligned}\langle\mathbf{y}(t)\rangle_y &= e^{-\bar{\mu}\bar{\mathsf{k}}t}\left(y+\int_0^t\bar{\mu}\bar{\mathsf{k}}e^{\bar{\mu}\bar{\mathsf{k}}s}(\psi+\xi t)ds\right) \\ &= e^{-\bar{\mu}\bar{\mathsf{k}}t}\left(y+e^{\bar{\mu}\bar{\mathsf{k}}s}(\psi+\xi s)\Big|_0^t-\int_0^t e^{\bar{\mu}\bar{\mathsf{k}}s}\xi ds\right) \\ &= e^{-\bar{\mu}\bar{\mathsf{k}}t}\left(y+e^{\bar{\mu}\bar{\mathsf{k}}t}(\psi+\xi s)-\psi-(\bar{\mu}\bar{\mathsf{k}})^{-1}e^{\bar{\mu}\bar{\mathsf{k}}s}\xi\Big|_0^t\right) \\ &= e^{-\bar{\mu}\bar{\mathsf{k}}t}\left(y+e^{\bar{\mu}\bar{\mathsf{k}}t}(\psi+\xi t)-(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(e^{\bar{\mu}\bar{\mathsf{k}}t}-1\right)\xi\right) \\ &= \left(e^{-\bar{\mu}\bar{\mathsf{k}}t}(y-\psi)+(\psi+\xi t)-(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(1-e^{-\bar{\mu}\bar{\mathsf{k}}t}\right)\xi\right) \\ &= e^{-\bar{\mu}\bar{\mathsf{k}}t}(y-\psi)+\mathbf{y}^0(t)-(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(1-e^{-\bar{\mu}\bar{\mathsf{k}}t}\right)\xi.\end{aligned} \quad \textbf{[S108]}$$

The average $\langle\mathbf{W}\rangle_y$ is given by

$$\begin{aligned}\langle\mathbf{W}\rangle_y &= \bar{\mathsf{k}}_D\int_0^t\langle\mathbf{y}(s)\rangle_y-\mathbf{X}^T(s)ds = \\ &= \bar{\mathsf{k}}_D\int_0^t e^{-\bar{\mu}\bar{\mathsf{k}}s}(y-\psi)+\mathbf{y}^0(s)-(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(1-e^{-\bar{\mu}\bar{\mathsf{k}}s}\right)\xi-\mathbf{X}^T(s)ds \\ &= \bar{\mathsf{k}}_D\left(\int_0^t\left(\mathbf{y}^0(s)-\mathbf{X}^T(s)ds\right)-(\bar{\mu}\bar{\mathsf{k}})^{-1}e^{-\bar{\mu}\bar{\mathsf{k}}s}(y-\psi)\Big|_0^t\right. \\ &\qquad\left.-(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(s+(\bar{\mu}\bar{\mathsf{k}})^{-1}e^{-\bar{\mu}\bar{\mathsf{k}}s}\right)\Big|_0^t\xi\right) \\ &= \bar{\mathsf{k}}_D\left(\int_0^t\left(\mathbf{y}^0(s)-\mathbf{X}^T(s)ds\right)-(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(e^{-\bar{\mu}\bar{\mathsf{k}}s}-1\right)(y-\psi)\right. \\ &\qquad\left.+(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(t+(\bar{\mu}\bar{\mathsf{k}})^{-1}\left(e^{-\bar{\mu}\bar{\mathsf{k}}t}-1\right)\right)\xi\right).\end{aligned} \quad \textbf{[S109]}$$

The equation for the variance $\bar{\sigma}^2_y$ requires some more attention, and we first prove a useful identity: Let A and B be symmetric matrices. The product AB is not in general symmetric:

$$(AB)^T = BA.$$

As a consequence the exponential of AB, e^{AB}, is not symmetric. We want now to prove that

$$\left(A^{-1}e^{AB}\right) = \left(A^{-1}e^{AB}\right)^T = e^{BA}A^{-1}. \quad \textbf{[S110]}$$

Using the definition of the matrix exponential we have

$$\begin{aligned}A^{-1}e^{AB} &= A^{-1}\sum_{k=0}^{\infty}\frac{(AB)^k}{k!} = A^{-1}+B+BAB+\ldots \\ &= (1+BA+BABA+\ldots)A^{-1} = \sum_{i=0}^{\infty}\frac{(BA)^i}{i!}A^{-1} = e^{BA}A^{-1}.\end{aligned} \quad \textbf{[S111]}$$

Similarly one can prove

$$\left(Ae^{AB}\right)=\left(Ae^{AB}\right)^{T}=e^{BA}A. \qquad \textbf{[S112]}$$

These results will prove useful in solving the equation for $\overline{\sigma}_y^2$. We first solve

$$\begin{cases}\overline{\sigma}_{\mathbf{y}}^2(0)=0\\ \dfrac{d}{dt}\overline{\sigma}_y^2=-2\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_y^2+2\dfrac{\overline{\mu}}{\beta}\end{cases} \qquad \textbf{[S113]}$$

and then prove that the solution of [**S113**] also solves [**S54**]. Eq. **S113** can again be solved by variation of the constants:

$$\overline{\sigma}_{\mathbf{y}}^2(t)=e^{-2\overline{\mu}\overline{\mathsf{k}}t}\int_0^t e^{2\overline{\mu}\overline{\mathsf{k}}s}2\frac{\overline{\mu}}{\beta}ds=e^{-2\overline{\mu}\overline{\mathsf{k}}t}\left(e^{2\overline{\mu}\overline{\mathsf{k}}s}\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\frac{\overline{\mu}}{\beta}\right)\Bigg|_0^t$$
$$=\left(1-e^{-2\overline{\mu}\overline{\mathsf{k}}t}\right)\frac{\overline{\mathsf{k}}^{-1}}{\beta}. \qquad \textbf{[S114]}$$

The original equation for $\overline{\sigma}_y^2$ contains $\mathrm{Sym}(\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_y^2)$. Using [**S110**] and [**S112**] we can prove that

$$\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_{\mathbf{y}}^2(t)=\overline{\mu}\overline{\mathsf{k}}\left(1-e^{-2\overline{\mu}\overline{\mathsf{k}}t}\right)\frac{\overline{\mathsf{k}}^{-1}}{\beta}=\frac{\overline{\mathsf{k}}^{-1}}{\beta}\left(1-e^{-2\overline{\mu}\overline{\mathsf{k}}t}\right)^{T}k\mu=\left(\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_{\mathbf{y}}^2(t)\right)^{T}. \qquad \textbf{[S115]}$$

In other words,

$$\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_{\mathbf{y}}^2(t)=\mathrm{Sym}\left(\overline{\mu}\overline{\mathsf{k}}\overline{\sigma}_{\mathbf{y}}^2(t)\right),$$

so that if $\overline{\sigma}_{\mathbf{y}}^2(t)$ solves [**S113**], it also solves [**S54**]. The equation for $\overline{\mathsf{C}}$ is again solved by variation of constants:

$$\begin{cases}\overline{\mathsf{C}}(0)=0\\ \dfrac{d}{dt}\overline{\mathsf{C}}=-\overline{\mu}\overline{\mathsf{k}}\,\overline{\mathsf{C}}+v\overline{\sigma}_y^2\overline{\mathsf{k}}_D.\end{cases} \qquad \textbf{[S116]}$$

The solution is

$$\overline{\mathsf{C}}(t)=ve^{-\overline{\mu}\overline{\mathsf{k}}t}\int_0^t e^{\overline{\mu}\overline{\mathsf{k}}s}\overline{\sigma}_{\mathbf{y}}^2(s)\overline{\mathsf{k}}_Dds$$
$$=ve^{-\overline{\mu}\overline{\mathsf{k}}t}\int_0^t e^{\overline{\mu}\overline{\mathsf{k}}s}\left(1-e^{-2\overline{\mu}\overline{\mathsf{k}}s}\right)\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=ve^{-\overline{\mu}\overline{\mathsf{k}}t}\int_0^t\left(e^{\overline{\mu}\overline{\mathsf{k}}s}-e^{-\overline{\mu}\overline{\mathsf{k}}s}\right)\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=ve^{-\overline{\mu}\overline{\mathsf{k}}t}\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(e^{\overline{\mu}\overline{\mathsf{k}}s}+e^{-\overline{\mu}\overline{\mathsf{k}}s}\right)\Big|_0^t\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=ve^{-\overline{\mu}\overline{\mathsf{k}}t}\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(e^{\overline{\mu}\overline{\mathsf{k}}t}+e^{-\overline{\mu}\overline{\mathsf{k}}t}-2\right)\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta} \qquad \textbf{[S117]}$$
$$=v\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(1+e^{-2\overline{\mu}\overline{\mathsf{k}}t}-2e^{-\overline{\mu}\overline{\mathsf{k}}t}\right)\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=v\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(1-e^{-\overline{\mu}\overline{\mathsf{k}}t}\right)^2\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}.$$

As in the case of $\langle W\rangle_y$ the equation for $\overline{\sigma}_W^2$ is solved by direct integration:

$$\overline{\sigma}_W^2=\int_0^t 2v^2\overline{\mathsf{k}}_D\overline{\mathsf{C}}(s)ds=\int_0^t v\overline{\mathsf{k}}_D\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(1-e^{-\overline{\mu}\overline{\mathsf{k}}s}\right)^2\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=2v^2\int_0^t\overline{\mathsf{k}}_D\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(1-2e^{-\overline{\mu}\overline{\mathsf{k}}s}+e^{-2\overline{\mu}\overline{\mathsf{k}}s}\right)\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=2v^2\overline{\mathsf{k}}_D\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(s+2\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}e^{-\overline{\mu}\overline{\mathsf{k}}s}-\frac{\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}}{2}e^{-2\overline{\mu}\overline{\mathsf{k}}s}\right)\Bigg|_0^t\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}$$
$$=2v^2\overline{\mathsf{k}}_D\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(t+2\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}\left(e^{-\overline{\mu}\overline{\mathsf{k}}t}-1\right)-\frac{\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-1}}{2}\left(e^{-2\overline{\mu}\overline{\mathsf{k}}s}-1\right)\right)$$
$$\times\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}=v^2\overline{\mathsf{k}}_D\left(\overline{\mu}\overline{\mathsf{k}}\right)^{-2}\left(2\overline{\mu}\overline{\mathsf{k}}t+2\left(e^{-\overline{\mu}\overline{\mathsf{k}}t}-1\right)-\left(e^{-\overline{\mu}\overline{\mathsf{k}}s}-1\right)^2\right)\frac{\overline{\mathsf{k}}^{-1}\overline{\mathsf{k}}_D}{\beta}. \qquad \textbf{[S118]}$$

Keeping terms of order $\mathcal{O}(t)$ in Eqs. **S108** and **S118**, we get the expressions anticipated in Eqs. **S58** and **S59**.

S7. Experiments at Lower Pulling Speed

In the main text we present experiments performed at high pulling speed, to enhance the dissipation associated with the movement of the center of mass. This was done to highlight the fact that W' does not fulfill the CFR. Nevertheless such violation is still evident at lower pulling speeds. Just for completeness in Fig. S7 we show the distributions of W_-, W, W', and W_+ in a pulling experiment performed on a dsDNA tether (experimental conditions are identical to those reported in the main text) at a pulling speed of 500 nm/s with $\Delta\lambda$ = 400 nm.

1. Ribezzi-Crivellari M, Ritort F (2012) Force spectroscopy with dual-trap optical tweezers: Molecular stiffness measurements and coupled fluctuations analysis. *Biophys J* 103(9): 1919–1928.
2. Ribezzi-Crivellari M, Huguet JM, Ritort F (2013) Counter-propagating dual-trap optical tweezers based on linear momentum conservation. *Rev Sci Instrum* 84(4):043104.
3. Batchelor GK (1976) Brownian diffusion of particles with hydrodynamic interaction. *J Fluid Mech* 74(1):1–29.
4. Happel J, Brenner H (1983) *Low Reynolds Number Hydrodynamics* (Springer, New York).

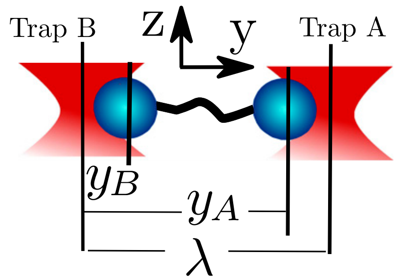

Fig. S1. Experimental setup. Two laser beams, oriented along the *z* direction, are used to create two optical traps. A dumbbell is formed by two optically trapped beads and a molecular tether. The tether is oriented along the *y* direction, perpendicular to the optical axis *z*. We choose the center of one trap (trap *B*) as the origin of our coordinate system. λ denotes the trap-to-trap distance and y_A and y_B denote the positions of the centers of the beads with respect to the reference trap *B*.

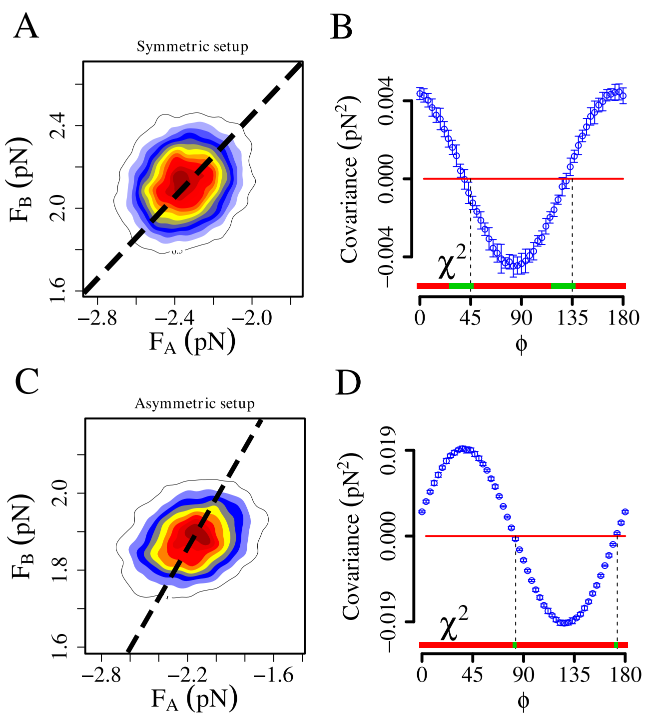

Fig. S2. Independent coordinates in linear systems. (*A*) Two-dimensional histograms of the forces measured in a symmetric dual-trap setup. The dashed line forms a $\pi/4$ angle with the coordinate axes and corresponds to the definition of f_+. (*B*) Covariance $\langle f_+^\phi f_-^\phi \rangle$ as a function of ϕ in a symmetric setup. The red line denotes 0 covariance (i.e., linear independence). At the bottom of the graph we report the result of a 1% significance χ^2 test, with red meaning dependent and green meaning independent. (*C*) Two-dimensional histograms of the force measured in an asymmetric dual-trap setup. The dashed line forms a $\pi/4$ angle with the coordinate axes and corresponds to the definition of f_+. In this asymmetric case f_+, f_- do not correspond to the principal axes of the histogram. (*D*) Covariance $\langle f_+^\phi f_-^\phi \rangle$ as a function of ϕ in an asymmetric setup. The red line denotes 0 covariance (i.e., linear independence). It is still possible to define a coordinate system where the 2 df are uncoupled, but now the definition is different from that given in the main text. At the bottom of the graph we report the result of a 1% significance χ^2 test, with red meaning dependent and green meaning independent. Tests were performed on 3-s data traces with 1-kHz acquisition rate.

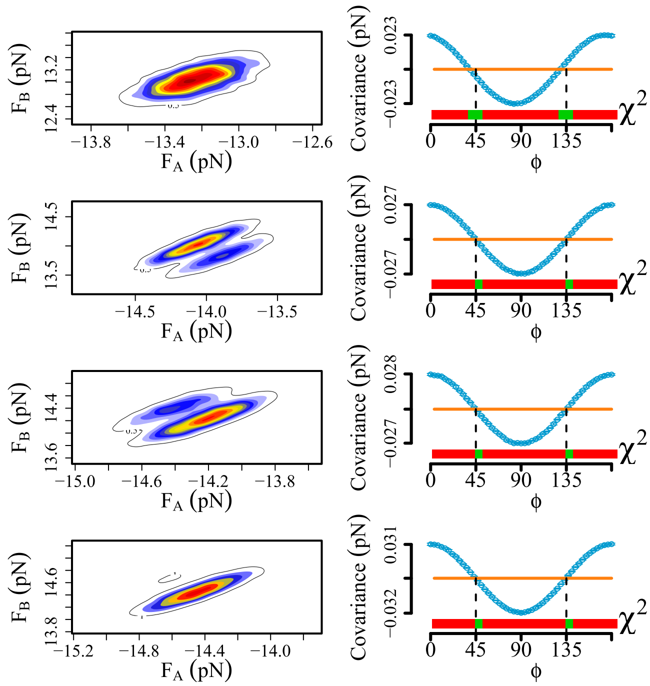

Fig. S3. Independent coordinates in nonlinear systems. (*Left*) Force fluctuations of a two-state DNA hairpin. Force increases from *Top* to *Bottom* and the hairpin is (*i*) completely folded, (*ii*) preferentially folded, (*iii*) preferentially unfolded, and (*iv*) completely unfolded. (*Right*) Covariance $\langle f_+^{\phi} f_-^{\phi} \rangle$ as a function of ϕ. The red line denotes 0 covariance (i.e., linear independence). At the bottoms of the graphs we report the result of a 1% significance χ^2 test, with red meaning dependent and green meaning independent. Data show that the center of mass (x_+) and the differential coordinate (x_-) are independent. Tests were performed on 3-s data traces with a 1-kHz acquisition rate.

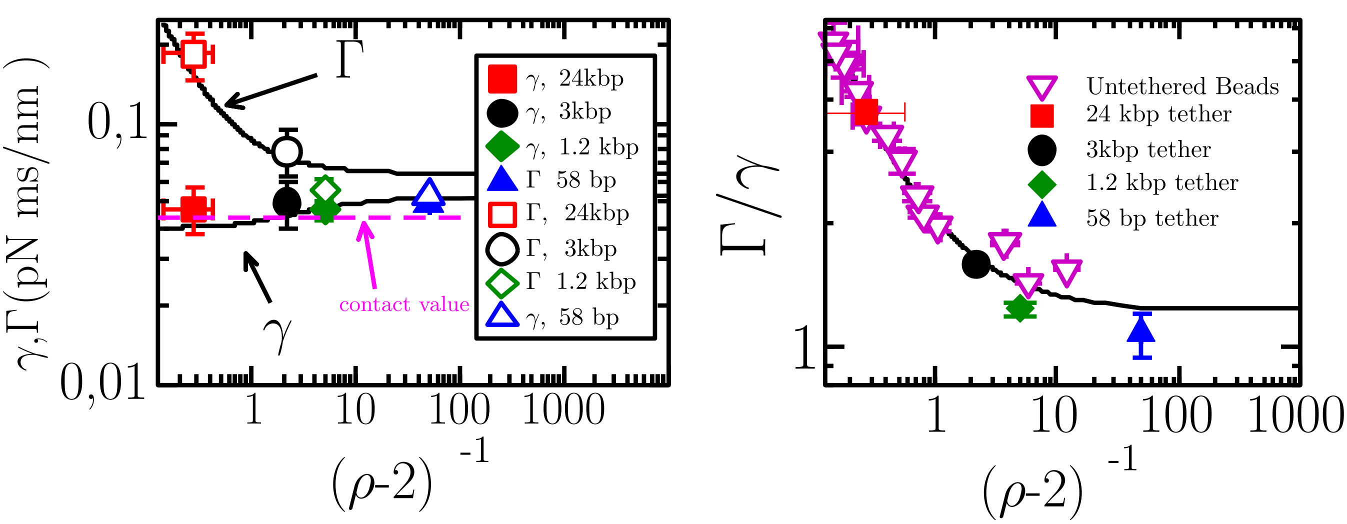

Fig. S4. Direct measurements γ and Γ. (*Left*) Hydrodynamic friction (γ, solid symbols) and interaction coefficient (Γ, open symbols) measured from the decay rate of thermal fluctuations. Each symbol is the average of measurements over five different molecules. Solid lines are the theoretical predictions (Eqs. **S31** and **S32**). The horizontal dashed line marks the exact theoretical value at contact ($\rho = 2$). (*Right*) Ratio between the hydrodynamic coefficients (Γ/γ) as a function of $(\rho - 2)^{-1}$. This quantity does not depend directly on r_b. Open symbols represent measurements obtained with untethered beads at different separations, and solid symbols show measurements obtained with tethered beads. The solid line gives the theoretical prediction according to the first two terms in the expansion as in Eqs. **S31** and **S32**.

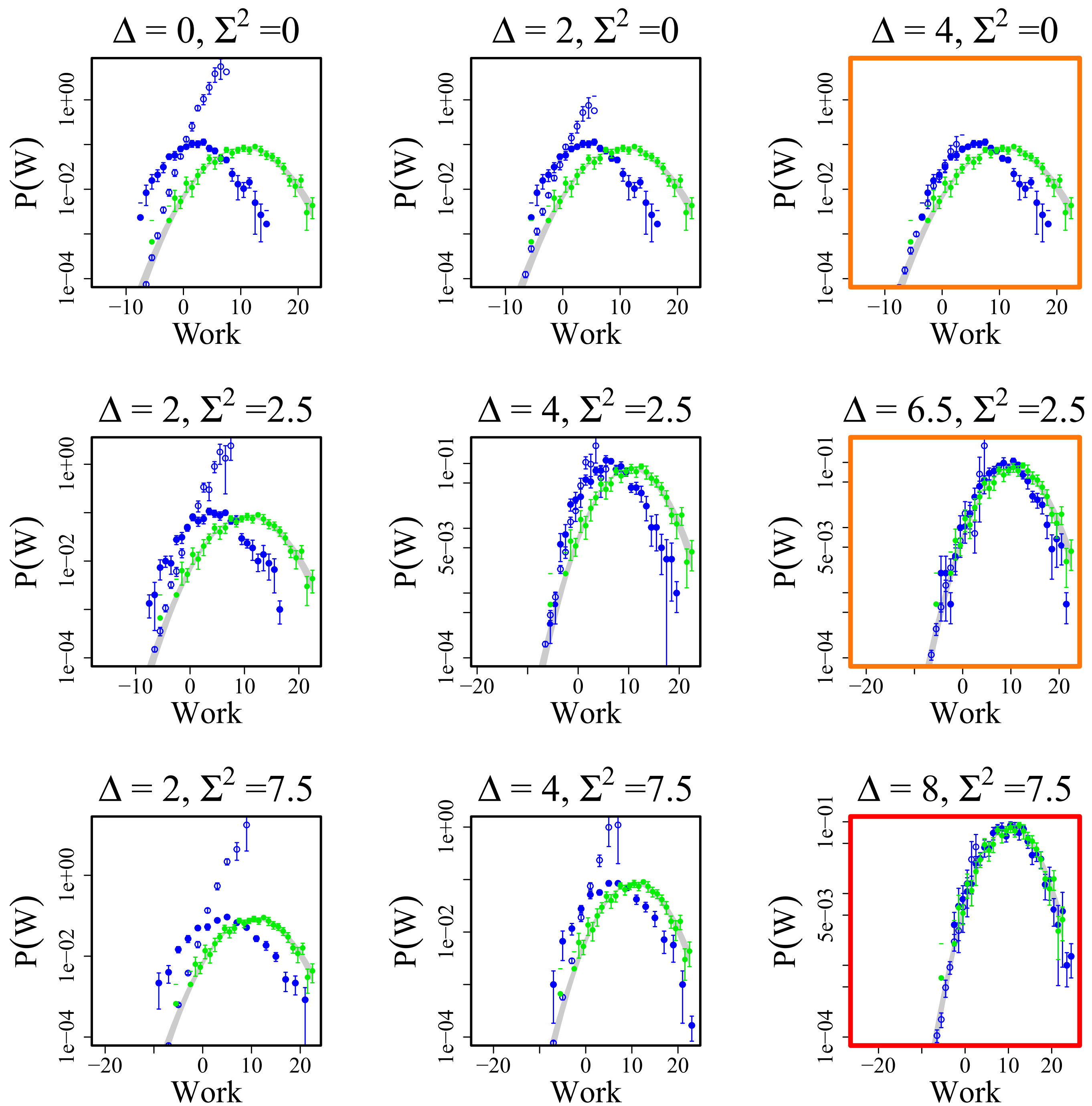

Fig. S5. Convolution and reconstruction of $P(W)$ from the CFR for the asymmetric case. Each row shows the result of a convolution by fixing the value of Σ and changing Δ. *Top*, *Middle*, and *Bottom* rows correspond to $\Sigma^2 = 0$, $\Sigma^2 = 2.5$, and $\Sigma^2 = 7.5$. In each panel we show $P_{\Delta,\Sigma}(W)$ (blue solid circles), together with $P_{\Delta,\Sigma}(-W)\exp(W)$ (blue open circles) and the experimentally measured $P(W)$ (green circles). Three different convolutions are found to fulfill the fluctuation symmetry $P_{\Delta,\Sigma}(W) = P_{\Delta,\Sigma}(-W)\exp(W)$, (*Right* column), but only one of them (*Bottom Right*) is compatible with the AF and matches the true $P(W)$.

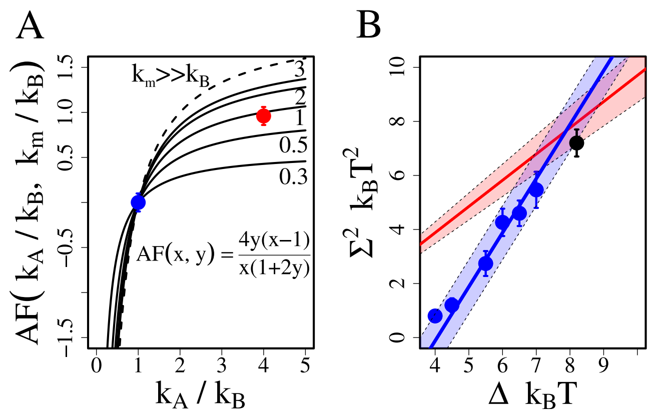

Fig. S6. Asymmetry factor and inference in asymmetric setups. (*A*) The asymmetry factor as a function of k_A/k_B and k_m/k_B. Different curves correspond to different values of k_m/k_B (0.3, 0.5, 1, 2, 3 from bottom to top). The dashed curve corresponds to the limit $k_m = \infty$. In the symmetric case ($k_A = k_B = 1$) the different curves coincide ($\Sigma^2 = 0$). The red circle denotes the asymmetric conditions in which experiments were performed. (*B*) Inference in the asymmetric case. Different pairs (Δ, Σ^2) yield probability distributions satisfying the CFR (blue circles and blue line). The asymmetry factor (red line) selects a narrow range of possible values, which is compatible with the experimentally measured values $\Delta = 8.2K_BT$ and $\Sigma^2 = 7.2(K_BT)^2$.

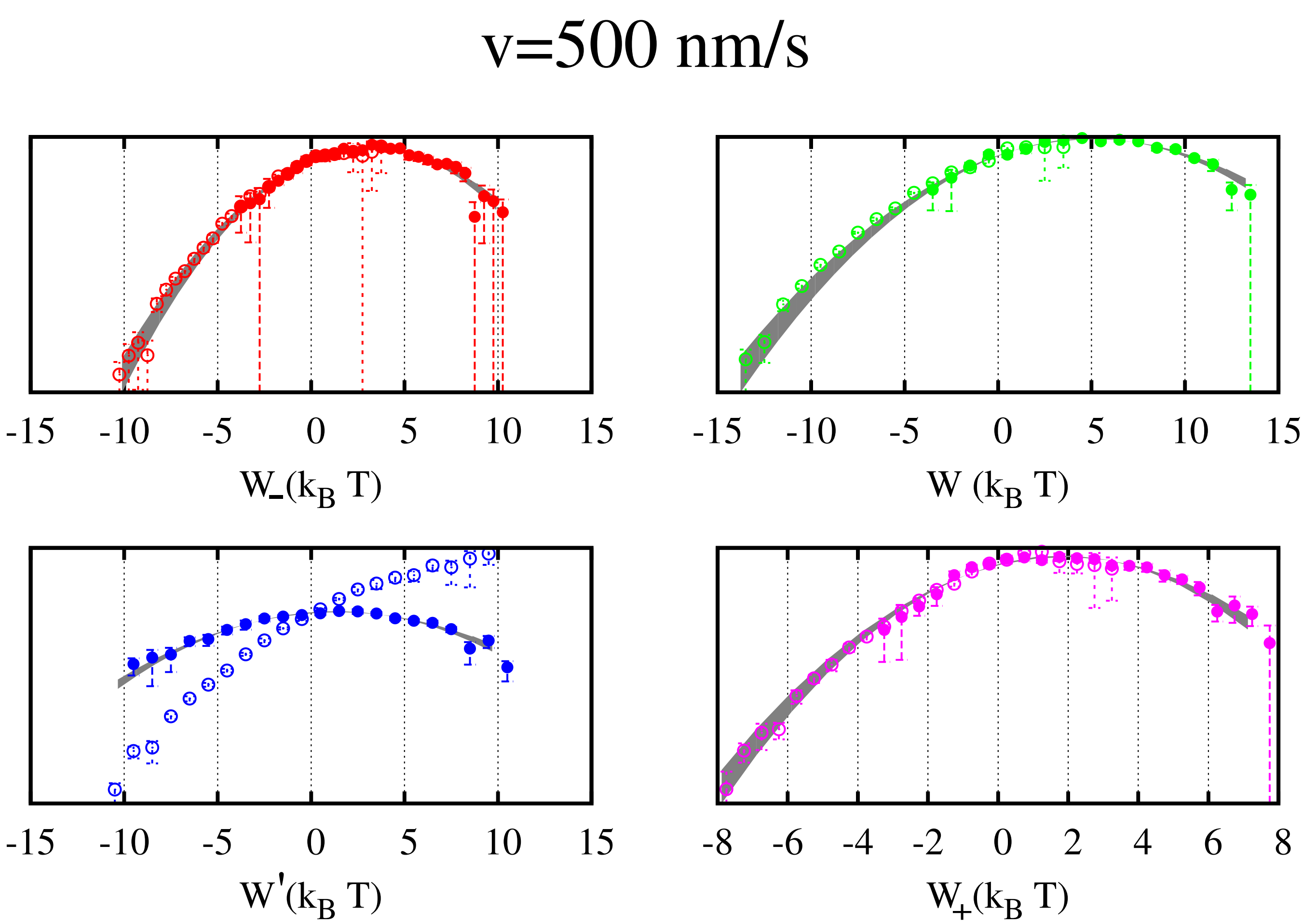

Fig. S7. Work measurements at lower pulling speed. The statistics of W, W', W_+, and W_- are shown. The pulling speed v = 500 nm/s is less than half of the lowest pulling speed presented in the main text, but the effect on the validity of the CFR for the different work quantities (W, W', W^+, W^-) is still visible. Five hundred nanometers per second lies in the typical range of pulling speed used in single-molecule pulling experiments.

Table S1. Molecule 1

Pulling speed	cov(W,W')	χ^2 test*	cov(W^+,W^-)	χ^2 test*
Forward protocol				
7.2 μm/s	0.41	1	0.08	0
4.3 μm/s	0.35	1	0.09	0
1.35 μm/s	0.38	1	0.05	0
Reverse protocol				
7.2 μm/s	0.17	0	0.018	0
4.3 μm/s	−0.05	0	−0.05	0
1.35 μm/s	0.36	1	0.20	0
Cyclic protocol				
7.2 μm/s	0.12	1	0.00	0
4.3 μm/s	0.12	1	0.01	0
1.35 μm/s	0.34	1	0.09	0

*1, dependent; 0, independent.

Table S2. Molecule 2

Pulling speed	cov(W,W')	χ^2 test*	cov(W^+,W^-)	χ^2 test*
Forward protocol				
7.2 μm/s	0.469377	1	−0.12	1
4.3 μm/s	0.6170601	1	−0.14	0
1.35 μm/s	0.5346567	1	0.09	0
Reverse protocol				
7.2 μm/s	−0.31	1	0.25	0
4.3 μm/s	−0.37	1	0.20	0
1.35 μm/s	−0.73	1	0.19	0
Cyclic protocol				
7.2 μm/s	0.26	1	−0.19	1
4.3 μm/s	0.34	1	−0.18	0
1.35 μm/s	0.42	1	−0.10	0

*1, dependent; 0, independent.