Supporting Information

Ribeuzzi-Crivellari and Ritort 10.1073/pnas.1320006111

S1 Text

In S1 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 S1 Text, section S1 we introduce the model in the symmetric case. In S1 Text, section S2 we prove that \( x_{\lambda} \) and \( x_{\gamma} \) are independent quantities in our model, which is a central result in our discussion. In S1 Text, section S3 we do the same for \( W_{+} \) and \( W_{-} \). These conclusions are supported by a statistical analysis of the experimental data. In S1 Text, sections S4 and S5 we comment on the definition and meaning of the hydrodynamic parameters of our model. One of these parameters is \( \gamma_{\lambda} \), 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 S1 Text, section S6 we discuss free-energy inference in asymmetric setups and in S1 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

\[
U_{\text{TOT}}(y_A, y_B) = \frac{k}{2} y_A^2 + \frac{k}{2} (y_A - \lambda)^2 + U_m(y_A - y_B). \tag{S1}
\]

A more general discussion, taking into account asymmetric set-

ups, 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 \( \lambda \) 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,

\[
\dot{y} = -\nu \nabla U_{\text{TOT}}(y_A, y_B, \lambda) + \eta, \tag{S2}
\]

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

\[
\langle \eta_i(s) \eta_j(t) \rangle = K_B T \mu_{ij} \delta(t - s), \quad \text{with} \quad \mu_{ij} = \langle \eta_i \eta_j \rangle. \tag{S3}
\]

i.e., that the covariance of the noise is proportional to the mobility \( \mu \). In the absolute 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:

\[
\mu = \begin{pmatrix}
\gamma_{\lambda}^+ & \Gamma_{\lambda}^+ \\
\Gamma_{\lambda}^- & \gamma_{\lambda}^-
\end{pmatrix},
\]

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 \( \gamma, \Gamma \) between the beads do not significantly change in the force range of a pulling experiment. In S1 Text, section S4 we show that this condition is met for tethered molecules ranging from 20 nm to 1 \( \mu \)m in contour length to a 10% accuracy.

This guarantees that the mobility matrix is symmetric and constant:

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

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

\[
U_{\text{TOT}}(x_-, x_+; \lambda) = U_-(x_-, \lambda) + U_+(x_+; \lambda).
\]

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

- It diagonalizes the mobility tensor \( \mu \) in the form

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

- It diagonalizes the noise covariances; i.e., it gives rise to two statistically independent noise contributions, \( \eta_+ \) and \( \eta_- \), which separately affect the new coordinates \( x_+ \) and \( x_- \):

\[
\langle \eta_+(s) \eta_+(t) \rangle = K_B T \delta(t - s), \quad \langle \eta_-(s) \eta_-(t) \rangle = 0. \tag{S4}
\]

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:

\[
\gamma_+ x^+_t = -\partial_{y_A} U^+ + \eta^+_t \tag{S5}
\]

\[
\gamma_- x^-_t = -\partial_{y_A} U^- + \eta^-_t. \tag{S6}
\]

S2. The Differential \( X_+ \) and Center-Of-Mass \( X_c \) 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: $U(x, y)$, which takes the form $U_1(x, y) + U_2(x, y)$ for a general transformation, $x_+ = x_1(x, y)$ and $x_- = x_2(x, y)$. In this situation, $x_+$ and $x_-$ are statistically independent,

$$P(x_+ | x_-) = P(x_+) \propto \exp(-\beta U_+(x_+)).$$  \[S7\]

and in particular have zero covariance:

$$\langle x_+ x_- \rangle = \langle x_+ \rangle \langle x_- \rangle.$$  \[S8\]

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:

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

Indeed,

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

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

$$f_+^e = \cos(\phi) f_A + \sin(\phi) f_B$$  \[S11\]

$$f_-^e = -\sin(\phi) f_A + \cos(\phi) f_B.$$  \[S12\]

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. S2B), whereas they have a different definition (i.e., they are not generated by a $\pi/4$ rotation) in the asymmetric case (Fig. S2D). 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 $W_-,$

$$W_\pm = \int dx_\pm (x_\pm, \lambda) d\lambda.$$  \[S13\]

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 $\chi^2$ 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 $\chi^2$ 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:

$$e_A^f = \sum_i^N \delta(A_i \in [a_i, a_{i+1})$$  \[S13\]

$$e_B^f = \sum_i^N \delta(B_i \in [b_i, b_{i+1}))$$  \[S14\]

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

$$\chi^2 = \sum_{i,j} \frac{(e_{ij} - e_A^f e_B^f)^2}{e_A^f e_B^f}$$  \[S15\]

with $e_A^f = \sum_i \delta(A_i \in [a_i, a_{i+1}))$ and $e_B^f = \sum_i \delta(B_i \in [b_i, b_{i+1}))$. The value obtained through Eq. S15 is then compared with the $\chi^2$ distribution. The two variables are considered independent if the $\chi^2$ 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 $W_+, W_-$ 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 $\chi^2$ test detects dependence between $W_+, W_-$ but not between $W_+, W_-.$ (in the $\chi^2$ 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:

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

When considering equilibrium fluctuations at constant trap-to-trap distance $\lambda,$ 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
\[
\frac{\partial}{\partial x} U_{\text{TOT}}|_{x_0^-} = 0.
\]

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 \( \lambda \). In this approximation the equations of motion in Eq. S5 become
\[
\gamma_+ \frac{d}{dt} x_+ = -2k \left( x_+ - \frac{\lambda}{2} \right) + \eta_+ , \quad [S18]
\]
\[
\gamma_- \frac{d}{dt} x_- = - \left( \frac{k}{2} + k_m \right) (x_- - x_0^-) + \eta_- . \quad [S19]
\]

In particular, \( \gamma_+ \) is the friction affecting the center of mass of the dumbbell, whereas \( \gamma_- \) affects the dynamics of the differential coordinate. After Batchelor (3), we set
\[
\gamma_+^{-1} = \frac{\gamma^{-1} + \Gamma^{-1}}{2}, \quad \gamma_-^{-1} = 2(\gamma^{-1} - \Gamma^{-1}). \quad [S20]
\]

In brief, \( \gamma \) is the hydrodynamic friction coefficient for a single bead, whereas \( \Gamma \) is the intensity of hydrodynamic interactions between the two beads. It is important to bear in mind that in principle \( \gamma \) and \( \Gamma \) 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,
\[
Q_{\text{eq}}(x_+) = \frac{1}{Z} exp \left( -\beta k \left( x_+ - \frac{\lambda}{2} \right)^2 \right) . \quad [S21]
\]
\[
P_{\text{eq}}(x_-) = \frac{1}{Z} exp \left( -\beta k \frac{k_m}{4} (x_- - x_0^-)^2 \right) , \quad [S22]
\]
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
\[
\sigma_+^2 = \frac{K_b T}{2k}, \quad \sigma_-^2 = \frac{2K_b T}{k + 2k_m} . \quad [S23]
\]

Information about the hydrodynamic interactions can be obtained from the time-dependent correlation functions of \( x_+ \) and \( x_- \),
\[
C_+(t) = \left< \left( x_+(0) - \frac{\lambda}{2} \right) \left( x_+(t) - \frac{\lambda}{2} \right) \right> \quad [S24]
\]
\[
C_-(t) = \left< \left( x_-(0) - x_0^- \right) \left( x_-(t) - x_0^- \right) \right> , \quad [S25]
\]
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
\[
\beta C_+(t) = \frac{e^{-kt_2^2t}}{2k} , \quad [S26]
\]
\[
\beta C_-(t) = \frac{2e^{-\left( k + 2k_m \right)t}t}{k + 2k_m} . \quad [S27]
\]

where \( \beta = (K_b T)^{-1} \). From Eqs. S24 and S25 the correlation function at time 0 equals the variances given in Eq. S23:
\[
C_+(0) = \sigma_+^2, \quad C_-(0) = \sigma_-^2 . \quad [S28]
\]

The hydrodynamic parameters can be retrieved from the correlation functions, as the stiffnesses, \( k, k_m \), are known from Eq. S23,
\[
\frac{1}{\gamma} + \frac{1}{\Gamma} = -\frac{1}{2k} \frac{d}{dt} \log(C_+) \Big|_{t=0} , \quad [S29]
\]
\[
\frac{1}{\gamma} + \frac{1}{\Gamma} = -\frac{2}{k + 2k_m} \frac{d}{dt} \log(C_-) \Big|_{t=0} , \quad [S30]
\]

where we used Eqs. S20, S26, and S27. As discussed in the main text, \( \gamma_+ \) 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: \( \gamma^{-1} \) and \( \Gamma^{-1} \). 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 \( \rho = \langle x_- \rangle / r_t \), with \( r_t \) the radius of the beads and \( \langle x_- \rangle \) the mean of the differential coordinate. (Note that \( \langle x_- \rangle > 2r_t \) 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:
\[
\gamma^{-1} \approx \frac{1}{6\pi \eta b} \left( 1 + \frac{15}{4\rho^3} + O(\rho^{-6}) \right) \quad [S31]
\]
\[
\Gamma^{-1} \approx \frac{1}{6\pi \eta b} \left( \frac{3}{2\rho} - \rho^3 + O(\rho^{-7}) \right) . \quad [S32]
\]

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 \( \rho \) 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 (\( \rho > 2 \)) to the lubrication limit (\( \rho \approx 2 \)). The experimental data for \( \gamma \) and \( \Gamma \) 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 \( \gamma^{-1} \) and \( \Gamma^{-1} \) with Eqs. S31 and S32 (solid lines). For every tether we assumed a single value of \( \rho \) and averaged values obtained at different forces. In the force range relevant for pulling experiments, changes in \( \gamma^{-1} \) and \( \Gamma^{-1} \) 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 \( \rho. \) 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 \( \gamma_s \) 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:

\[
P(W) = P(W - \Delta). \tag{S33}\]

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 \( \Delta \) 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,

\[
P(W)_{\Delta, \Sigma} = P^* \cdot N(\Delta, \Sigma), \tag{S34}\]

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

\[
P_{\Delta^*, \Sigma^*}(W) = P^* \cdot N(\Delta^*, \Sigma^*), \tag{S35}\]

satisfies the CFR. Then it is easy to check that \( P_{\Delta^*+\phi \sqrt{\Sigma^*+2\phi}/\beta} \) will satisfy the FR for any \( \phi (\beta = 1/k_B T \) 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

\[
AF(k_A, k_m, k_B) = \frac{\sigma_y^2 - \sigma_{y^*}^2}{\langle W \rangle - \langle W^* \rangle} = \frac{1}{\beta} \frac{k_A (k_B - k_m)}{k_B + 2k_m}, \tag{S36}\]

where \( \beta = 1/k_B T. \) 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 \( (\Delta, \Sigma) \) such that \( AF = \Sigma^2/\Delta \) and that \( P_{\Delta, \Sigma}(W) \) satisfies the fluctuation symmetry. These \( (\Delta, \Sigma) \) allow the reconstruction of \( P(W) \) and thus allow us to measure free-energy differences or even dynamical quantities like \( \gamma_s. \) 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. S6A.

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 \( U_{in}(x) = (1/2)k_m x^2 \) in Eq. S1 so that the total potential reads

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

To simplify the following discussion we now switch to a vector notation. From now on we denote \( \mathbf{X}(t) \) 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),

\[
\mathbf{X}(t) = (\lambda_t, 0), \tag{S38}\]

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

\[
\mathbf{U}(\mathbf{X}, \mathbf{X}(t)) = \frac{1}{2} (\mathbf{y} - \mathbf{y}^0) \cdot \mathbf{k} (\mathbf{y} - \mathbf{y}^0) + \frac{1}{2} k_{eff} \mathbf{A}^2, \tag{S39}\]

where \( \mathbf{k} \) is the stiffness tensor,

\[
\mathbf{k} = \begin{pmatrix} k_A + k_m & -k_m \\ -k_m & k_B + 2k_m \end{pmatrix}, \tag{S40}\]

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

\[
k_{eff} = \frac{k_B k_m}{\text{det}(\mathbf{k})}, \tag{S41}\]

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

\[
\mathbf{y}^0 = \begin{pmatrix} \lambda - \frac{k_B k_m}{\text{det}(\mathbf{k})} (\lambda) \\ \frac{k_B k_m}{\text{det}(\mathbf{k})} (\lambda) \end{pmatrix}. \tag{S42}\]

During a pulling experiment the trap-to-trap distance will be changed at a constant speed, \( v_t \):

\[
\lambda_t = \lambda_0 + vt. \tag{S43}\]
As a consequence the equilibrium positions of the beads will change in time:

$$y^0(t) = \left( \lambda_0 + vt - \frac{k_B k_m}{\det(k)} (\lambda_0 + vt), \frac{k_A k_m}{\det(k)} (\lambda_0 + vt) \right). \tag{S44}$$

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 $U$:

$$W = -v \int_0^t k_A (y_A(s) - X^T_A(s)) ds. \tag{S45}$$

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 ($B$),

$$W' = v \int_0^t k_B (y_B(s) - X^T_B(s)) ds, \tag{S46}$$

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 $(y, W)$ or equivalently from the corresponding Fokker–Planck equation. Here by we denote the usual vector containing the positions of the beads whereas $W = \langle y_A^n y_A^0(s) ds, y_B^n y_B^0(s) ds \rangle$. We use the vector $W$ from which the random variables $W$, $W'$ are obtained as

$$W = W_A, \quad W' = -W_B. \tag{S47}$$

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

$$\dot{y} = -\overline{\Pi} (y-y^0) + \eta, \tag{S48}$$

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

$$\overline{\Pi} = \left( \begin{array}{cc} \overline{\Pi}^{-1} & 0 \\ 0 & \overline{\Pi}^{-1} \end{array} \right). \tag{S49}$$

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

$Lf(y, W) = (\overline{\Pi} (y-y^0) \cdot \nabla_y + \beta^{-1} \overline{\Pi} \cdot \nabla_y \nabla_y - \overline{\Pi} \overline{k}_D (y - X^T) \cdot \nabla_y) f(y, W), \tag{S50}$

where

$$\overline{k}_D = \left( \begin{array}{cc} k_A & 0 \\ 0 & k_B \end{array} \right). \tag{S51}$$

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

$$\frac{\partial}{\partial t} \langle f(y(t), W(t)) \rangle_y = \langle Lf(y(t), W(t)) \rangle_y, \tag{S52}$$

where $\langle \cdot \rangle_y$ denotes the average conditioned to initial condition $(y, 0)$. Applying the Markov generator to $y$, $(y - (y)_y) \otimes (y - (y)_y)$, $W$, $(W - (W)_y) \otimes (W - (W)_y)$ and then taking the average $[1 \otimes j]$ denotes the tensor $J$ such that $\mathbf{1}_k = (1 \cdot k)_J$, we obtain the equations of motion for $y$, $\overline{\sigma}_y^2 = \langle (y - (y)_y) \otimes (y - (y)_y) \rangle_y$, $\langle W \rangle_y, \overline{\zeta} = \langle (W - (W)_y) \otimes (y - (y)_y) \rangle_y$, and $\overline{\sigma}_W^2 = \langle (W - (W)_y \otimes (W - (W)_y) \rangle_y$,

$$\frac{d}{dt} \langle y \rangle_y = -\overline{\Pi} \langle y - y^0 \rangle \tag{S53}$$

$$\frac{d}{dt} \overline{\sigma}_y^2 = -2 \text{Sym} (\overline{\Pi} \overline{\sigma}_y^2 + \overline{\Pi}) \tag{S54}$$

$$\frac{d}{dt} \langle W \rangle_y = -\overline{k}_D \langle y - X^T \rangle \tag{S55}$$

$$\frac{d}{dt} \overline{\zeta} = -\overline{\Pi} \overline{\zeta} - \overline{\sigma}_D \tag{S56}$$

$$\frac{d}{dt} \overline{\sigma}_W^2 = -2 \overline{k}_D \overline{\zeta}, \tag{S57}$$

where $\text{Sym}(\cdot)$ 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,

$$\langle W(t) \rangle = -v \int_0^t \overline{k}_D \langle y^0(s) - X^T(s) \rangle ds + \overline{k}_D \overline{\sigma}_D \overline{\sigma}_y^2 \overline{\Pi} X^T \tag{S58}$$

and

$$\overline{\sigma}_W^2 = \frac{2 \overline{k}_D \overline{\sigma}_y^2 \overline{\sigma}_D \overline{\sigma}_y^2 \overline{\Pi}}{\beta} \tag{S59}$$

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

$$\overline{k}_D \left( \int_0^t (y^0(s) - X^T(s)) ds \right). \tag{S60}$$

The difference $\overline{k}_D \langle y^0(s) - X^T(s) \rangle$ 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 $x$ if the pulling were carried out reversibly. The two components of the vector $\overline{k}_D \langle y^0(s) - X^T(s)ds \rangle$ are of course equal in size but different in sign:

Ribezi-Crivellari and Ritort www.pnas.org/cgi/content/short/1320006111
\[ \nu \kappa_D \left( v \int_0^1 (y^0(s) - X^T(s)) \, ds \right) \left( v \int f_{\text{rev}}(s) \, ds, -v \int f_{\text{rev}}(s) \, ds \right). \]

The above expression is then just the reversible work,

\[ W_{\text{rev}} = k_{\text{eff}} \left( \lambda_0 \nu t + \frac{1}{2} \gamma \nu^2 t \right), \]

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

\[ \Omega = \kappa_D \nu^{-1} \nu^{-1} \kappa_D = \nu^3 \nu^{-1} \nu. \]

To give some insight into the physical meaning of this quantity we note that \( \nu \) transforms the trap position vector \( X^T \) into the equilibrium position vector \( y^0 \):

\[ y^0 = \nu X^T. \]

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

\[ \langle W \rangle = W_{\text{rev}} + \Omega_{\text{AA}} \nu^2 t \]

\[ \sigma^2_W = \frac{2}{\beta} \Omega_{\text{AA}} \nu^2 t, \]

\[ \langle W' \rangle = W_{\text{rev}} - \Omega_{\text{AB}} \nu^2 t \]

\[ \sigma^2_{W'} = \frac{2}{\beta} \Omega_{\text{AB}} \nu^2 t. \]

Based on these expressions we introduce the asymmetry factor:

\[ \text{AF} = \frac{\sigma_{W'} - \sigma_W}{\langle W' \rangle - \langle W \rangle}. \]

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

\[ \text{AF} = \frac{2 k_m (k_A - k_B)}{\beta k_A (k_B + 2 k_m)}. \]

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.,

\[ P(y) = Z^{-1} \exp \left( -\frac{\beta}{2} (y - y^0) \cdot \nu (y - y^0) \right). \]

According to such distribution the variance of \( y \) is

\[ \text{var}(y) = \beta^{-1} \nu^{-1}. \]

Moreover, because forces and bead positions are linearly related, \( f = k_D (y - X^T) \), we have

\[ \beta \text{var}(f) = \kappa_D \nu^{-1} \kappa_D \]

\[ = k_A k_B + k_m k_A + k_m k_B \left( \begin{array}{cc} k_A k_B k_m & k_A k_B k_m \\ k_A k_B k_m & k_B (k_A + k_m) \end{array} \right). \]

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

\[ k_A = \beta \text{var}(f)_{AA} + \beta \text{var}(f)_{AB} \]

\[ k_B = \beta \text{var}(f)_{BB} + \beta \text{var}(f)_{AB} \]

\[ k_m = k_A k_B + \frac{\text{var}(f)_{BB}}{\beta \det(\text{var}(f))}. \]

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 symmetric setup.

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

\[ \Omega = \nu^2 \nu^{-1} \nu, \]

where

\[ \nu^2 = \nu^{-1} \nu = \frac{1}{\varepsilon} \left( \begin{array}{cc} k_A (k_B + k_m) & k_B k_m \\ k_A k_B k_m & k_B (k_A + k_m) \end{array} \right). \]

with \( \varepsilon = k_A k_B + k_A k_m + k_B k_m \). Here it is useful to switch to a representation in which \( \nu^{-1} \) is diagonal. This can be achieved with a \( \pi/4 \) rotation:

\[ \Omega = \frac{1}{\sqrt{2}} \left( \begin{array}{cc} 1 & -1 \\ 1 & 1 \end{array} \right). \]

Using \( \Omega \) we can write

\[ \Omega = \nu^2 \nu^{-1} \nu \]

where, as in SI Text, section S1, \( \nu^2 \nu^{-1} \nu \) is diagonal. The nontrivial contribution that must still be computed is

\[ \nu^2 = \frac{1}{\sqrt{2 \varepsilon}} \left( \begin{array}{cc} k_A (k_B + 2 k_m) & k_B (k_A + 2 k_m) \\ -k_A k_B & k_B k_A \end{array} \right) \]

\[ = \frac{1}{\sqrt{2 \varepsilon}} \left( \begin{array}{cc} A & B \\ -C & C \end{array} \right). \]

We can thus conclude that

\[ \Omega = \frac{1}{2 \varepsilon} \left( \begin{array}{cc} A & -C \\ B & C \end{array} \right) \left( \begin{array}{cc} \gamma_A A & \gamma_A B \\ -\gamma_A C & \gamma_A C \end{array} \right) \]

\[ = \frac{1}{2 \varepsilon} \left( \begin{array}{cc} \gamma_A A + \gamma_A C^2 & \gamma_A AB - \gamma_A C^2 \\ \gamma_A AB - \gamma_A C^2 & \gamma_A B + \gamma_A C^2 \end{array} \right). \]
By definition we have
\[
\frac{\partial F}{\partial \beta} = \frac{2}{\beta} \frac{\partial \Omega_{AA} - \Omega_{AB}}{\partial \beta} = \frac{2}{\beta} A - B - \frac{1}{\beta} 4k_m(k_A - k_B)
\]
[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
\[
\frac{d}{dt} \left( f(y(t), W(t)) \right) = \left\{ Lf(y(t), W(t)) \right\}_y
\]
[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
\[
\frac{d}{dt} \left( y(t) \right) = \left\{ L(y(t)) \right\}_y = -\mu \kappa \left( \left\langle y(t) \right\rangle - y_0 \right).
\]
[S85]

In the same way we can derive the equation for \( \pi_y^2 \), it is useful to recall that
\[
\left\langle \left( y - y_h \right) \otimes \left( y - y_h \right) \right\rangle = \left\langle y \otimes y \right\rangle - \left\langle y \right\rangle \otimes \left\langle y \right\rangle,
\]
[S86]

so that
\[
\frac{d}{dt} \left( \pi_y^2 \right) = \left\langle L( y \otimes y ) \right\rangle_y - \frac{d}{dt} \left( y \otimes y \right)_y.
\]
[S87]

We work out the two terms of the right-hand side of [S87] separately, starting from the first:
\[
\left\langle L( y \otimes y ) \right\rangle_y = \left\langle \left( \mu \kappa (y - y_0) \cdot \nabla_y + \beta^{-1} \mu \cdot \nabla_y \right) y \otimes y \right\rangle_y.
\]
[S88]

The third term in the generator was neglected because it involves a derivative with respect to W. Acting with the derivatives on \( y \otimes y \), gives
\[
\left\langle L( y \otimes y ) \right\rangle_y = \left\langle -\mu \kappa \left( (y - y_0) \otimes y \right) - \left( y \otimes (y - y_0) \right) \right\rangle + 2\beta^{-1} \mu
\]
\[= -2\text{Sym} \left( \mu \kappa \left( (y - y_0) \otimes y \right) \right) + 2\beta^{-1} \mu.
\]
[S89]

The second term in [S87] is just a time derivative, which can be worked out because of [S85]:
\[
\frac{d}{dt} \left( y \otimes y \right) = -\mu \kappa \left( \left( (y - y_0) \otimes (y - y_0) \right) - \left( y \otimes (y - y_0) \right) \right) T_y
\]
\[= -2\text{Sym} \left( \mu \kappa \left( (y - y_0) \otimes y \right) \right).
\]
[S90]

An explicit result for the time derivative of \( \pi_y^2 \) is obtained by taking the difference of [S89] and [S90]:
\[
\frac{d}{dt} \left( \pi_y^2 \right) = -2\text{Sym} \left( \mu \kappa \left( (y - y_0) \otimes y \right) \right) - \mu \kappa \left( \left( (y - y_0) \otimes (y - y_0) \right) - \left( y \otimes (y - y_0) \right) \right) T_y
\]
\[+ 2\beta^{-1} \mu
\]
[S91]

In the second step above we used the fact that
\[
\left\langle \left( y - y_0 \right) \otimes \left( y - y_0 \right) \right\rangle = \left\langle (y \otimes y) \right\rangle - \left\langle y_0 \otimes y \right\rangle
\]
whereas
\[
\left\langle \left( y \right) \otimes \left( y \right) \right\rangle = \left\langle y \otimes y \right\rangle - \left\langle y_0 \otimes y \right\rangle
\]

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 \( y \), with the only difference that now only the last term in Eq. S50 for the generator matters. The result is
\[
\frac{d}{dt} \left( W \right) = \nu \kappa_D \left( y - X^T \right).
\]
[S92]

In the case of \( \zeta \), the cross-correlation of \( y \) and \( W \) involves some additional computations. The function \( \zeta \) is linear in both \( W \) and \( y \) so that only the first and third terms in the generator contribute. The time derivative of \( \zeta = (W - W_h) \otimes (y - y_h) \) again yields two pieces, as in the case of [S87], and we proceed in a similar way:
\[
\frac{d}{dt} \zeta = \left( L( W \otimes y ) \right)_y - \frac{d}{dt} \left( W \otimes y \right)_y.
\]
[S93]

Again we compute the two parts separately:
\[
\left( L( W \otimes y ) \right)_y = \left( -\mu \kappa \left( (W \otimes (y - y_0)) \right) + \nu \kappa_D \left( (y - X^T) \otimes y \right) \right)_y
\]
[S94]

\[
\left( W \otimes (y - y_0) \right)_y = \nu \kappa_D \left( (y - y_0) \otimes y \right)_y
\]
[S95]

Taking the difference of these two terms and using
\[
\left( (W \otimes (y - y_0)) \right)_y = \left( (W \otimes y)_y - (W) \otimes y_0 \right)
\]
and
\[
\left( (W) \otimes (y - y_0) \right)_y = \left( (W \otimes y)_y - (W \otimes y_0) \right),
\]
[S96]

it is possible to conclude that
\[
\frac{d}{dt} \zeta = -\mu \kappa \left( (W - W_h) \otimes (y - y_h) \right)_y
\]
\[+ \left( (y - y_h) \otimes (y - y_h) \right)_y \kappa_D
\]
[S96]

The only equation left to derive is that for \( \pi_w^2 \) is \( \left( (W - W) \otimes (W - W) \right)_y \). This computation involves only the last term in the generator:
\[
\frac{d}{dt} \left( (W - W) \otimes (W - W) \right)_y = \left( L( W \otimes W ) \right)_y
\]
[S97]
As usual we evaluate the two terms separately and put them together as a second step:
\[
\langle L(W \otimes W) \rangle = v \langle \bar{k}_D (W \otimes (y - X^T)) + ((y - X^T) \otimes W) \bar{k}_D \rangle. \tag{S98}
\]

\[
\frac{d}{dt} \left( \langle W \otimes (y) \rangle = v \langle \bar{k}_D (W \otimes (y - X^T)) + ((y - X^T) \otimes W) \bar{k}_D \rangle \right)
= + v \langle \bar{k}_D (W - (W) \otimes (y - (y))) \rangle.
\tag{S99}
\]

The last equation of motion is thus
\[
\frac{d}{dt} \bar{\sigma}_W = + v \bar{k}_D \langle (y - (y)) \otimes (W - (W)) \rangle
= + 2 v \text{Sym}(v \bar{k}_D \bar{C}).
\tag{S100}
\]

### S6.6. Solution of the Equations.

In the previous section we introduced an average operation \( \langle \cdot \rangle \) 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 \( p(y, 0) = \delta(y - y), \) 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
\[
\begin{align*}
\langle y(0) \rangle &= y, \\
\langle W(0) \rangle &= \bar{\sigma}_W(0) = \bar{C}(0) = \bar{\sigma}_W(0) = 0.
\end{align*}
\tag{S101}
\]

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 \( \langle y \rangle, \) is a case for variation of constants:
\[
\begin{align*}
\frac{d}{dt} \langle y \rangle &= -v \bar{k}_D \left( \langle y \rangle - y^0(t) \right),
\end{align*}
\tag{S102}
\]

The solution is
\[
\langle y(t) \rangle = e^{-v \bar{k}_D} \left( y + \int_0^t p \bar{k}_D e^{-v \bar{k}_D} y^0(s) ds \right),
\tag{S103}
\]

as it can be directly checked. We recall the definition of \( y^0(t), \)
\[
y^0(t) = \left( \lambda + vt - \frac{k_A k_m}{\det(k)} \lambda_0 + vt, \frac{k_B k_m}{\det(k)} \lambda_0 \right).
\tag{S104}
\]

In the following it is useful to write \( y^0(t) = \psi + \xi t, \) where
\[
\begin{align*}
\psi &= \left( \lambda_0 - \frac{k_A k_m}{\det(k)} \lambda_0, \frac{k_B k_m}{\det(k)} \lambda_0 \right),
\xi &= \left( v - \frac{k_A k_m}{\det(k)} v, \frac{k_B k_m}{\det(k)} v \right).
\end{align*}
\tag{S105, S106}
\]

In terms of these quantities \( \langle y(t) \rangle \) reads
\[
\langle y(t) \rangle = e^{-v \bar{k}_D} \left( y + \int_0^t p \bar{k}_D e^{-v \bar{k}_D} (\psi + \xi t) ds \right).
\tag{S107}
\]

Integrating by parts we get
\[
\langle y(t) \rangle = e^{-v \bar{k}_D} \left( y + \int_0^t p \bar{k}_D e^{-v \bar{k}_D} (\psi + \xi t) ds \right)
= e^{-v \bar{k}_D} \left( y + e^{-v \bar{k}_D} (\psi + \xi s) \right)^t_0 - \int_0^t e^{-v \bar{k}_D} \bar{z} ds
= e^{-v \bar{k}_D} \left( y + e^{-v \bar{k}_D} (\psi + \xi s) - \bar{z} - \bar{z} \right)^t_0 e^{-v \bar{k}_D} \bar{z} ds
= e^{-v \bar{k}_D} \left( y + e^{-v \bar{k}_D} (\psi + \xi s) - \bar{z} - \bar{z} \right)^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \left( e^{-v \bar{k}_D} (y - \psi) + (\psi + \xi s) - \bar{z} - \bar{z} \right)^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \left( e^{-v \bar{k}_D} (y - \psi) + (\psi + \xi s) - (\bar{z} - \bar{z}) \right)^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \left( e^{-v \bar{k}_D} (y - \psi) + (\psi + \xi s) - (\bar{z} - \bar{z}) \right)^t_0 e^{-v \bar{k}_D} \bar{z} ds.
\tag{S108}
\]

The average \( \langle W \rangle \) is given by
\[
\langle W \rangle = \bar{k}_D \left[ \langle y(s) \rangle - X^T(s) ds \right] =
= \bar{k}_D \left[ \int_0^t \langle y(s) \rangle - X^T(s) ds \right] =
= \bar{k}_D \left[ \int_0^t \langle y^0(s) - X^T(s) ds \rangle - \bar{z} - \bar{z} \right]^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \bar{k}_D \left[ \int_0^t \langle y^0(s) - X^T(s) ds \rangle - \bar{z} - \bar{z} \right]^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \bar{k}_D \left[ \int_0^t \langle y^0(s) - X^T(s) ds \rangle - \bar{z} - \bar{z} \right]^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \bar{k}_D \left[ \int_0^t \langle y^0(s) - X^T(s) ds \rangle - \bar{z} - \bar{z} \right]^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \bar{k}_D \left[ \int_0^t \langle y^0(s) - X^T(s) ds \rangle - \bar{z} - \bar{z} \right]^t_0 e^{-v \bar{k}_D} \bar{z} ds
= \bar{k}_D \left[ \int_0^t \langle y^0(s) - X^T(s) ds \rangle - \bar{z} - \bar{z} \right]^t_0 e^{-v \bar{k}_D} \bar{z} ds.
\tag{S109}
\]

The equation for the variance \( \bar{\sigma}_W \) 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
\[
(ABA)^T = e^{BA} A^{-1}.
\tag{S110}
\]

Using the definition of the matrix exponential we have
\[
A^{-1} e^{AB} = A^{-1} \sum_{k=0}^{\infty} \frac{(AB)^k}{k!} = A^{-1} + B + BAB + \ldots
= (1 + BA + BAB + \ldots) A^{-1} = \sum_{k=0}^{\infty} \frac{(BA)^k}{k!} A^{-1} = e^{BA} A^{-1}.
\tag{S111}
\]
Similarly one can prove
\[
(4e^{4B}) = (4e^{4B})^T = e^{4t}A. \quad \text{[S112]}
\]
These results will prove useful in solving the equation for \( \pi_i^2 \). We first solve
\[
\begin{aligned}
\pi_i^2(0) &= 0 \\
\frac{d}{dt} \pi_i^2 &= -2\pi_i^2 + 2\frac{\mu}{\beta}
\end{aligned}
\quad \text{[S113]}
\]
and then prove that the solution of [S113] also solves [S54]. Eq. S113 can again be solved by variation of the constants:
\[
\pi_i^2(t) = e^{-2\pi_i^2} \int_0^t e^{2\pi_i^2} 2\frac{\mu}{\beta} \, ds = e^{-2\pi_i^2} \left( e^{2\pi_i^2} (\frac{kD}{\mu}) \right) \frac{k}{\beta}
\]
\[
= \left( 1 - e^{-2\pi_i^2} \right) \frac{k}{\beta}
\quad \text{[S114]}
\]
The original equation for \( \pi_i^2 \) contains \( (e\mu)^2 \). Using [S110] and [S112] we can prove that
\[
\begin{aligned}
\pi_k \pi_i^2(t) &= \frac{1}{k} \left( 1 - e^{-2\pi_i^2} \right) \frac{k}{\beta}
\end{aligned}
\quad \text{[S115]}
\]
In other words,
\[
\pi_k \pi_i^2(t) = \text{Sym} (\pi_k \pi_i^2(t)),
\]
so that if \( \pi_i^2(t) \) solves [S113], it also solves [S54]. The equation for \( \pi_k \) is again solved by variation of constants:
\[
\begin{aligned}
\pi_k(0) &= 0 \\
\frac{d}{dt} \pi_k &= -\pi_k \pi_i + \pi_i^2 kD
\end{aligned}
\quad \text{[S116]}
\]
The solution is
\[
\begin{aligned}
\pi_k(t) &= \frac{1}{\pi_i^2} \int_0^t e^{\pi_i^2} \pi_k^2(s) kDds \\
&= \frac{1}{\pi_i^2} \int_0^t e^{\pi_i^2} \left( 1 - e^{-2\pi_i^2} \right) \frac{k}{\beta}
\end{aligned}
\quad \text{[S117]}
\]
As in the case of \( W \), the equation for \( \pi_i^2 \) is solved by direct integration:
\[
\begin{aligned}
\pi_i^2(t) &= \frac{1}{2\pi_i^2} \int_0^t \pi_k D \pi_i^2(s) kD \, ds = \frac{1}{2\pi_i^2} \int_0^t \pi_k D (\frac{kD}{\mu}) \frac{k}{\beta}
\end{aligned}
\quad \text{[S118]}
\]
Keeping terms of order \( O(t) \) 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 \( \Delta t = 400 \text{ nm} \).

---


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. $\lambda$ 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 $f_\perp$. (B) Covariance $\langle f_\perp f_\parallel \rangle$ as a function of $\phi$ 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 $\chi^2$ 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 $f_\perp$. In this asymmetric case $f_\perp$, $f_\parallel$ do not correspond to the principal axes of the histogram. (D) Covariance $\langle f_\perp f_\parallel \rangle$ as a function of $\phi$ 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 $\chi^2$ 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 \( f_{\phi} \) as a function of \( \phi \). The red line denotes 0 covariance (i.e., linear independence). At the bottoms of the graphs we report the result of a 1% significance \( \chi^2 \) test, with red meaning dependent and green meaning independent. Data show that the center of mass \( (x_c) \) 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 \( \gamma \) and \( \Gamma \). (Left) Hydrodynamic friction \( (\gamma, \text{solid symbols}) \) and interaction coefficient \( (\Gamma, \text{open symbols}) \) 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 \( (\Gamma/\gamma) \) 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 Σ = 0, Σ = 2.5, and Σ = 7.5. In each panel we show P_{Δ,S}(W) (blue solid circles), together with P_{Δ,S}(-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_{Δ,S}(W) = P_{Δ,S}(-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 ($\sum^2 = 0$). The red circle denotes the asymmetric conditions in which experiments were performed. (B) Inference in the asymmetric case. Different pairs ($\Delta, \sum^2$) 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 $\Delta = 8.2k_B T$ and $\sum^2 = 7.2(k_B T)^2$.

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

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<th>cov(W,W')</th>
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<th>cov(W',W)</th>
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*1, dependent; 0, independent.

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*1, dependent; 0, independent.