A main goal of single-molecule experiments is to evaluate equilibrium free energy differences by applying fluctuation relations to repeated work measurements along irreversible processes. We quantify the error that is made in a free energy estimate by means of the Jarzynski equality when the accumulated work expended on the whole system (including the instrument) is erroneously replaced by the work transferred to the subsystem consisting of the sole molecular construct. We find that the error may be as large as 100%, depending on the number of experiments and on the bandwidth of the data acquisition apparatus. Our theoretical estimate is validated by numerical simulations and pulling experiments on DNA hairpins using optical tweezers. © 2009 American Institute of Physics. [DOI: 10.1063/1.3155084]

I. INTRODUCTION

In a typical single-molecule pulling experiment, an individual molecular construct is stretched by means of a device [e.g., optical or magnetic tweezers, atomic force microscope (AFM), etc.] able to measure both the applied force, usually on the piconewton scale, and the end-to-end molecular extension, typically expressed in nanometers. Many interesting kinetic and thermodynamical properties of the stretching process can be inferred from the resulting force-extension curve; in particular, the free energy difference between the folded and the unfolded states can be evaluated by exploiting a well-known result of nonequilibrium thermodynamics, the Jarzynski equality

$$\beta W_{\text{rev}} = -\log(\exp(\beta W(\Gamma)))_{\Gamma},$$

where $W(\Gamma)$ is the amount of work performed on the system throughout the stretching process $\Gamma$, $\beta$ is as usual the inverse of the thermal energy $k_BT$, and $W_{\text{rev}}$ is the reversible work, i.e., the work needed to perform the pulling experiment in quasiequilibrium conditions. Since a single molecule is a small system, the work $W(\Gamma)$ is affected by thermal fluctuations; the angular brackets $(\cdots)_{\Gamma}$ thus stand for an average over all possible realizations of the same experimental protocol. In fact, a generalization of the Jarzynski equality due to Hummer and Szabo makes it possible to reconstruct the whole free energy landscape as a function of the molecular extension. This program has been successfully applied to the experimental study of multidomain proteins.

Many a research have been devoted to the practical difficulties that arise when Eq. (1) is applied to the free energy reconstruction problem, e.g., the bias induced by the finite number of experimental attempts, the role played by the resolution of the measuring apparatus, or the effect of instrument noise and experimental errors. The present article deals with yet another possible source of error, which, though already known, has generally been dismissed as negligible without a compelling argument. The point is that in most experimental settings the molecular extension is not the proper control parameter, so that it is not correct to interpret the area below the FEC as the work that appears in Eq. (1). If the control parameter is the total distance the area under the force-distance curve (FDC) should be used instead.

Here we thoroughly analyze under which conditions the use of the wrong definition for the work can appreciably affect the estimate of free energy differences by means of Eq. (1). The conclusion, in a nutshell, is that the error induced by the substitution may be as large as 100%, depending on the number of experiments and on the data acquisition frequency. Also important are the details of the data analysis procedure: How the integration extrema are chosen, what method is used to integrate the FEC, and how different FECs are aligned to correct for instrumental drift effects.

The paper is organized as follows: First, we get some theoretical insight by considering our problem in its simplest possible setting (Sec. II). Then, we validate our conclusions with an experimental test implemented with optical tweezers and DNA hairpins (Sec. III). A recapitulation of our results (Sec. IV) and an appendix with some technicalities round off this article.

II. A TOY MODEL

A detailed model for single-molecule experiments with optical tweezers has been discussed elsewhere. Here we consider a simplified version of it that conserves only the physical features directly relevant to our problem. Although the toy model in this section is phrased in the optical tweezers language, it takes no effort to translate it into an AFM nomenclature, the mathematics being just the same.

In our model, graphically depicted in Fig. 1, the optical trap is moved by the experimenter, hence the proper control
parameter is the trap-pipette distance $\lambda$, while the end-to-end molecular extension is a quantity subject to fluctuations denoted by $x$. The trap is a harmonic potential with stiffness $k_0$, while $k_m$ is the stiffness of the molecular construct comprising hairpin and handles. Given a fixed value of the control parameter $\lambda$, the state of the system is specified by the pair $(x, \varsigma)$, where $\varsigma$ is a label taking values 0 if the hairpin is closed (or folded) and 1 if it is open (unfolded). The hairpin itself is a pure two-state system whose state transitions of the hairpin are governed by a simplified Kramers–Bell kinetics, with rates for opening $k_+$ or closing $k_-$ given by

$$k_+ = k_0 \exp \left( \frac{w_0 f_0(x)}{k_B T} \right),$$

$$k_- = k_0 \exp \left( \frac{-w_1 f_1(x) + \Delta G_0}{k_B T} \right),$$

where $w_0$ and $w_1$ represent the distances from the barrier to the closed and the open states, respectively, $f_0$ and $f_1$ are two functions of $x$ with physical dimensions of a force, and $k_0$ is the attempt frequency. The rates just defined must respect the detailed balance condition

$$\frac{k_-}{k_+} = \exp \left[ -\frac{H^{(\lambda)}(x, 1) - H^{(\lambda)}(x, 0)}{k_B T} \right],$$

for each $\lambda$ and for each $x$. This requirement implies

$$w_0 f_0(x) + w_1 f_1(x) = \frac{k_0}{2} (\ell_1 - \ell_0)[2x - (\ell_1 + \ell_0)].$$

Our choice here is to take simply $f_0(x)$ = $f_1(x)$, so that $w_0 + w_1 = \ell_1 - \ell_0$.

The dynamics of our model is ruled by the overdamped Langevin equation

$$\gamma \frac{dx}{dt} = f_\lambda(x(t), \varsigma) + \sqrt{2k_B T} \xi(t),$$

where $\xi(t)$ is a Gaussian white noise

$$\langle \xi(t) \rangle = 0,$$

$$\langle \xi(t) \xi(t') \rangle = \delta(t - t').$$

The experimental protocol is defined by the choice of a function $\lambda(t)$. Here we consider a constant velocity pulling $\lambda(t) = \lambda_0 + vt$.

### A. Accumulated vs transferred work

For the toy model introduced in the previous section, $\lambda$ is the control parameter which can be directly manipulated, while the molecular extension $x$ is subject to Brownian fluctuations. Therefore, the work performed on the system throughout a pulling experiment $\Gamma$ that starts at time $t_i$ from $\lambda = \lambda_i$ and terminates in $\lambda = \lambda_f$ at time $t_f = t_i + (\lambda_f - \lambda_i)/v$ is properly defined as

$$W(\Gamma) = \int_{t_i}^{t_f} \frac{\partial H^{(\lambda)}(x, \varsigma)}{\partial \lambda} d\lambda = \int_{\lambda_i}^{\lambda_f} f_\lambda(\lambda, x) d\lambda,$$

where we used Eq. (5) and $f_\lambda(\lambda, x) = k_b(\lambda - x)$ is the force induced by the displacement of the bead in the trap. Such work is measured in practice as the area under the FDC [see Fig. 2(a)]. Note that for all single-molecule techniques that we are aware of, $f_\lambda$ is actually the only one force experimentally measurable. In the following we will, for simplicity, drop the subscript and write $f$ instead of $f_\lambda$.

The area under the FEC [see Fig. 2(b)], on the other hand, is what in Ref. 19 is called transferred work [as opposed to the accumulated work $W(\Gamma)$].
The thermodynamic force-extension curve (TFEC) is the quasi-equilibrium pulling experiment plotted as a function of the molecular extension \( x \). If we define \( W_{\text{rev}} \) as the area under the TFEC, then Eq. (14) yields

\[
W(\Gamma) = W'(\Gamma) + \frac{f_i(\Gamma)^2 - f_j(\Gamma)^2}{2k_b},
\]

where \( f_i \) and \( f_j \) are the (trajectory-dependent) initial and final values of the force, respectively. The difference between \( W \) and \( W' \) is therefore a pure boundary term.

### B. The reversible work

If we realize the pulling experiment in conditions of quasiequilibrium, that is at infinitesimally small velocity \( v \rightarrow 0 \), then we obtain the thermodynamic force-distance curve (FDC), whose analytical expression is given by Eq. (A5). The area under the FDC is the reversible work \( W_{\text{rev}} \), equal to the free energy difference between the final and initial states of the system. From an experimental perspective, however, the really interesting quantity is rather the free energy difference \( \Delta G_0 \) between the open and closed states of the hairpin at zero external force. According to Eq. (A13), this is given by

\[
\Delta G_0 = W_{\text{rev}} - \frac{\langle f_i \rangle^2 - \langle f_j \rangle^2}{2k_{\text{eff}}},
\]

where \( \langle f \rangle_{i(f)} \) is the equilibrium initial (final) value of the force, and \( k_{\text{eff}} \) is the effective stiffness

\[
\frac{1}{k_{\text{eff}}} = \frac{1}{k_b} + \frac{1}{k_m}.
\]

The analogous quantity obtained using the transferred work is

\[
\beta \tilde{W} = -\log \frac{1}{n} \exp(-\beta W_{\text{rev}}).
\]

So we see that either \( W_{\text{rev}} \) or \( W_{\text{rev}}' \) are equally useful to extract the free energy of formation \( \Delta G_0 \) of the hairpin. The problem is that it is often impractical (and sometimes impossible) to achieve quasiequilibrium conditions. Here comes into play the Jarzynski equality, as we see in the next section.

### C. Jarzynski estimator

The Jarzynski equality [Eq. (1)] gives us a recipe to compute the reversible work, given a suitably sized collection of irreversible processes. The work that appears in Eq. (1) is the accumulated work \( W(\Gamma) \) defined in Eq. (11); nonetheless, in some cases it happens that the most readily available data for the experimenter is the FEC, therefore the work that is measured is in fact the transferred work \( W'(\Gamma) \) of Eq. (12). In such occasions, the transferred work has been used in the Jarzynski equality, under the assumption that the resulting error is smaller compared to other sources of experimental uncertainty.

In this section, we answer the following question: How large of an error is made in the evaluation of \( \Delta G_0 \) if the transferred work \( W'(\Gamma) \) is used instead of the accumulated work \( W(\Gamma) \)?

Let us call \( \tilde{W} \) the Jarzynski estimate of the reversible work \( W_{\text{rev}} \), based on \( n \) experiments that produce the set of work measurements \( \{W_i\} \)

\[
\beta \tilde{W} = -\log \frac{1}{n} \exp(-\beta W_i).
\]

The analogous quantity obtained using the transferred work is

\[
\beta \tilde{W}' = -\log \frac{1}{n} \exp(-\beta W_i').
\]

The quantity \( \tilde{W} \) is guaranteed by Eq. (1) to be an estimator of the reversible accumulated work \( W_{\text{rev}} \), whereas \( \tilde{W}' \) is not the proper way to compute the reversible transferred work \( W_{\text{rev}}' \) (a bona fide way to estimate \( W_{\text{rev}}' \) is discussed in Ref. 19). We now set out to evaluate the difference \( \tilde{W} - \tilde{W}' \).

To begin with, we sort the set \( \{W_i\} \) in ascending order
generally not the same that gives the minimum of $W/\text{H20850}$. The key observation is that the sum of exponentials in Eq. (15) and call $\Delta G'_0$ the result of setting $W'_{\text{rev}}=\tilde{W}'$ in Eq. (17), we get

$$\Delta G_0 - \Delta G'_0 = a - b \log n - \mu + \sqrt{2} \sigma z(n) - \frac{\langle f \rangle_z^2 - \langle f \rangle_z^2}{2k_b}. \quad (24)$$

A further simplification is possible: Taking the average of Eq. (14) and using Eq. (B12) we are left with the formula

$$\Delta G_0 - \Delta G'_0 = \frac{\sqrt{6}}{\pi} (\gamma - \log n) s + \sqrt{2} z(n) s', \quad (25)$$

where $s$ and $s'$ are the standard deviations of $\{W_i\}$ and $\{W'_i\}$, respectively, and $\gamma$ is the Euler–Mascheroni constant.

Equation (25) states that the error in the evaluation of the energy properties of the hairpin due to the substitution of $\{W_i\}$ with $\{W'_i\}$ in the Jarzynski equation depends on three factors: The standard deviations $s$ and $s'$ and the number of experiments $n$. There is a remarkable difference between the roles played by $s$ and $s'$: The standard deviation $s$ of the accumulated work generally depends only on the pulling rate $u$ and the chemical nature of the construct comprising molecule and handles; the standard deviation $s'$ of the transferred work, on the other hand, is also strongly dependent on the bandwidth of the data acquisition system.

The reason is easy to understand, while the area under the FDC [Fig. 2(a)] practically does not change if we smooth out the curve, the area under the FEC [Fig. 2(b)] is heavily dependent on the fluctuations of the extremal points $x_i$ and $x_f$ (see also Fig. 6). We will have more to say about this point in Sec. III.

In the derivation of Eq. (25) we have made use of three approximations:

1. we discarded all the contributions to the sum of exponentials in Eqs. (18) and (19) except the one coming from the minimum-work trajectory;
2. we assumed a normal distribution for $\{W'_i\}$;
3. we assumed a Gumbel distribution for $\{W_i\}$.

Although each one of them seems reasonable, it is not redundant, before discussing the experimental utility of Eq. (25), to check the final result against a numerical test.

D. A numerical test

In order to validate Eq. (25), we have performed a numerical simulation of Eq. (9), generating hundreds of thousands of curves like the two represented in Fig. 2. The effect of the instrumental bandwidth has been mimicked by applying different filters to the data, so that each point of the FDC or FEC represents actually an average over $m$ consecutive integration steps. In this way we have generated data in a fair range of values of $s'$. The results are illustrated in Fig. 4.

The first observation is that the error can be very large: As much as 50$k_BT$ in a system where the true $\Delta G_0$ is 57.7$k_BT$, that amounts to a relative error not far from 1000%. Then we observe that, in spite of the somewhat rough simplifications used in its derivation, the analytical prediction of

![Graph](image-url)

FIG. 3. (Color online) Dependence on the sample size $n$ of the mode $\omega'$ of $W_{(1)}$ (i.e., the maximum of the distribution for $W_{(1)}$, see Appendix B). The dimensionless variable $z$ is $(\mu - \omega')/(\sqrt{2}\sigma)$, where $\mu$ and $\sigma$ are the mean and standard deviations of the normally distributed transferred work $W'$. The represented curve is the numerical solution to Eq. (B9).

$$W_{(1)} \leq W_{(2)} \leq W_{(3)} \leq \cdots \leq W_{(n)}. \quad (20)$$

The key observation is that the sum of exponentials in Eq. (18) is dominated by the minimum-work trajectory of our sample

$$\beta \tilde{W} \approx \beta W_{(1)} + \log n. \quad (21)$$

Repeating the same argument for the set $\{W'_i\}$ that collects the measured values of the transferred work, we find

$$\tilde{W} - \tilde{W}' \approx W_{(1)} - W'_{(1)}. \quad (22)$$

Note that the trajectory that realizes the minimum of $\{W'_i\}$ is generally not the same that gives the minimum of $\{W'_i\}$.

In order to go further in our analytical approximation, we need to specify the distributions of $W$ and $W'$. Based on our experience with both experimental and simulated data, we assume that $W'$ is normally distributed (see Fig. 8) with mean $\mu$ and variance $\sigma^2$, while for $W$ we adopt a Gumbel distribution (see Fig. 9) with parameters $a$ and $b$ [which are related to the average and standard deviation of the accumulated work $W$ by means of Eq. (B12) in Appendix B]. This latter choice is the simplest distribution that exhibits the asymmetry we expect from a nonlinear system (in the case of linear systems the work distribution is Gaussian). Also, there are theoretical arguments suggesting that the Gumbel distribution may play a universal role for correlated random variables similar to the one played by the Gaussian distribution for uncorrelated ones.

We can now estimate the distribution of $W_{(1)}$ and $W'_{(1)}$. The details can be found in Appendix B, here we quote just the final result: The most likely value of $W_{(1)} - W'_{(1)}$ is approximately

$$a - b \log n - \mu + \sqrt{2} \sigma z(n), \quad (23)$$

where $z(n)$ is the function of the sample size represented in Fig. 3.
Eq. (25) fares reasonably well in the comparison with the simulated data, although there seems to be a small apparently systematic underestimation of \( \Delta G_0 - \Delta G'_0 \). Finally, a comment about the range of \( s' \): The standard deviation of \( \{ W'_i \} \) is a linear function of the amplitude of the fluctuations of \( x \), given by Eq. (A16); this fixes an upper limit to the range of \( s' \) that can be explored without changing the system.

III. AN EXPERIMENTAL TEST

This section reports the results of an experimental test of Eq. (25), whose theoretical derivation has been presented in Sec. II. The instrument we employed is a dual-beam miniaturized optical tweezers with fiber-coupled diode lasers (845 nm wavelength) that produce a piezoecontrolled movable optical trap and measure force using conservation of light momentum.\(^{30,31}\) The molecule is a DNA hairpin of sequence 5'-GCGAGGCTTACTCTCATCTGGAAACAGATGAAATGGCTGCG-3' hybridized to two double-stranded DNA (dsDNA) handles (29 base-pairs long). Pulling experiments were performed at 25 °C in a buffer containing tris-HCl pH 7.5, 1M ethylene diamine tetra-acetic acid (EDTA), and 1M NaCl. The data that we show (see Table I) have been measured from seven specimens in hundreds of stretching-releasing cycles performed at pulling speed of 200 nm/s (equivalent to a loading rate of 13.8 pN/s). The use of DNA hairpins presents several advantages\(^{32-35}\) over the RNA hairpins that were used in pioneering experiments of this kind.\(^{23,24}\)

In order to measure the dependence of \( \Delta G_0 - \Delta G'_0 \) on the bandwidth, we employed a fast analog-to-digital converter that makes possible to increase the data acquisition frequency from the standard value of 1 kHz to as much as 100 kHz (20 kHz, however, is larger than the corner frequency of the bead, around 10 kHz, and proved to be enough for this test). The availability of high-frequency data is a good start, but is not enough without a data analysis procedure that carefully preserves the statistical properties of the boundary term [see Eq. (14)]. Here are the main steps of the data analysis that we performed:

1. The stream of data is split into single unfolding or refolding events.
2. Taking advantage of the fact that the elastic response of the short dsDNA handles is with a good approximation Hookean, we fit the FDC folded and unfolded branches with straight lines.
3. The unavoidable small instrumental drift (which is manifested in the unphysical increasing or decreasing of the measured value of the trap position \( \lambda \)) is corrected by shifting the FDC in such a way that the

![Graph showing discrepancy between simulations and analytical approximation](image)

**FIG. 4.** (Color online) Numerical test of Eq. (25). The theoretical prediction is compared to the results of numerical simulations of Eq. (9). In abscissa, \( s' \) is the standard deviation of the transferred work values \( \{ W'_i \} \); different values of \( s' \) are obtained by varying the filter applied to the data. In ordinate, we represent the error (due to the erroneous use of \( s' \)) in the Jarzynski estimator \( \Delta G_0 - \Delta G'_0 \) (in k_BT units) on the determination of the free energy of formation of the hairpin. Each point represents the result of the analysis of \( n=9000 \) trajectories.

**TABLE I.** Experimental results: Comparison between the experimental (also shown in Fig. 5) and the theoretical [based on Eq. (25)] values of \( \langle \Delta G_0 - \Delta G'_0 \rangle / (k_BT) \). The data sets labeled “1 kHz” and “20 kHz” refer to the same experiment, with the standard (low-frequency) and the new (high-frequency) data acquisition system. The stiffness of the trap \( k_s \) is measured in pN/\( \mu \)m, while \( n \) is the number of trajectories.

| \( k_s \) (kHz) | From unfolding | From refolding |
|-----------------|---------------|---------------|
|                 | exp. | n  | th. | n  | exp. | th. |
| mol1 1 kHz      | 79     | 249 | 16.5 | 11.6 | 249 | 8.7   | 12.2 |
| mol1 20 kHz     | 79     | 200 | 31.4 | 20.6 | 202 | 15.1 | 20.2 |
| mol2 1 kHz      | 63     | 473 | 25.6 | 23.3 | 473 | 20.6 | 22.8 |
| mol2 20 kHz     | 63     | 364 | 24.7 | 29.5 | 362 | 33.8 | 31.2 |
| mol3 1 kHz      | 79     | 219 | 15.9 | 17.5 | 218 | 12.8 | 14.7 |
| mol3 20 kHz     | 79     | 169 | 31.2 | 20.1 | 166 | 19.8 | 19.0 |
| mol4 1 kHz      | 64     | 174 | 31.3 | 13.8 | 174 | 3.7   | 9.6  |
| mol4 20 kHz     | 64     | 143 | 18.2 | 17.4 | 138 | 14.4 | 15.8 |
| mol5 1 kHz      | 79     | 635 | 26.2 | 9.1  | 633 | 13.5 | 8.1  |
| mol5 20 kHz     | 79     | 501 | 19.2 | 15.2 | 499 | 17.0 | 14.6 |
| mol6 1 kHz      | 83     | 490 | 14.7 | 15.5 | 492 | 14.1 | 12.6 |
| mol6 20 kHz     | 83     | 386 | 27.0 | 18.6 | 384 | 15.4 | 16.6 |
| mol7 1 kHz      | 77     | 272 | 14.0 | 11.9 | 277 | 9.9   | 11.6 |
| mol7 20 kHz     | 77     | 215 | 27.8 | 20.1 | 219 | 25.9 | 19.1 |
straight line fitting the folded branch crosses $\lambda=0$ at the same value of the force in any event.

(4) The FDCs are integrated between two fixed values $\lambda_i$ and $\lambda_f$. These integrations produce two sets of accumulated work values $\{W_i\}$: one for the unfolding and one for the refolding process.

(5) Each FEC is integrated between $x_i(\Gamma)=\lambda_i-f_i(\Gamma)/k_B$ and $x_f(\Gamma)=\lambda_f-f_f(\Gamma)/k_B$; note that, while $\lambda_i$ and $\lambda_f$ are the same for all trajectories, $f_i$ and $f_f$ depend on the trajectory $\Gamma$, and so do $x_i$ and $x_f$. In this way we obtain two sets of transferred work values $\{W'_i\}$: again, one for unfolding and one for refolding trajectories.

(6) The Jarzynski estimators $\tilde{W}$ and $\tilde{W}'$ are computed by means of Eqs. (18) and (19), and then Eqs. (15) and (17) give $\Delta G_0$ and $\Delta G'_0$.

Table I shows that Eq. (25) is generally quite close to the experimental results, most of the time predicting a discrepancy between $\Delta G_0$ and $\Delta G'_0$ within few $k_B T$ of the observed value. The occasional large deviations between theory and experiment should not be too surprising in view of the statistical nature of the quantity we are measuring and the approximate derivation of Eq. (25).

The data reported in Table I can be graphically represented in analogy with Fig. 4. In principle, we expect each data set to be represented by a slightly different straight line, as the number of trajectories $n$ varies from a minimum 143 to a maximum 635 (see Table I). However, in practice the differences are small enough that all the theoretically expected values are very close to the line that in Fig. 5 is denoted as “analytical approximation.”

Figure 6 shows a typical trajectory plotted as FDC and FEC, using 20 kHz and 1 kHz data. It can be immediately appreciated that, while the area under the FDC is insensitive to the sampling frequency, the area under the FEC may display important differences due to the fluctuations of the integration extrema.

A. Bidirectional methods

If the experimental situation makes it possible to implement not only the protocol $\lambda(t)$, but also the time-reversed protocol $\hat{\lambda}(t)=\lambda(\Delta t-t)$, where $\Delta t=t_f-t_i$ is the duration of the experiment, then a more efficient way of estimating free energy differences is to apply a bidirectional method, which takes advantage of the knowledge of both a “forward” and a “reverse” work distributions. Bidirectional methods are based on another fluctuation relation, the Crooks theorem:

$$\frac{\phi_{W_{\text{FOR}}}(w)}{\phi_{W_{\text{REV}}}(w)} = \exp\left(\frac{w-\Delta G}{k_B T}\right),$$

where $\phi_{W_{\text{FOR}}}(w)$ ($\phi_{W_{\text{REV}}}(w)$) is the probability density function (PDF) of the work along the forward (reverse) process. Also the Crooks theorem, like the Jarzynski equality, is written for the accumulated work $W$. Writing an analytical approximation of the error introduced by the erroneous use of

FIG. 5. (Color online) Experimental test of Eq. (25). In abscissa, $s'$ is the standard deviation of the transferred work values $\{W'_i\}$; different values of $s'$ are obtained by varying the stiffness of the trap and the bandwidth. In ordinate, we represent the error (due to the erroneous use of $W'$ in the Jarzynski estimator) $\Delta G_0-\Delta G'_0$ on the determination of the hairpin energy levels. See Table I for further details about the data.

FIG. 6. (Color online) (a) An experimental FDC observed with a high-frequency (20 kHz) and a low-frequency (1 kHz) data acquisition system. The area under the curve, which is a measure of the accumulated work $W$, practically does not change. (b) The FEC associated to the pulling experiment represented in (a). The area under the curve, which represents the transferred work $W'$, depends on the frequency of the data acquisition system because of the large fluctuations of the integration extrema. Insets: magnified views of the region around the maximum of the force.
the transferred work $W'$, in the style of what we did in Sec. II, looks quite more complicated, but a direct evidence of the role of the bandwidth is given in Fig. 7, where $\log(\phi_{W,FDR}(w)/\phi_{W,REV}(-w))$ is plotted as a function of $(w-\Delta G)/(k_BT)$ for two values of the bandwidth.

The experimental results are summarized in Table II. Even if the Crooks theorem is not satisfied, the estimate of the transferred work $W'$ for the accumulated work $W$ is not as bad as the one obtained by using the Jarzynski equality.

B. Role of the data analysis technique

The data analysis protocol detailed in Sec. III may be the best suited to the task of verifying Eq. (25), but is not feasible if one’s experimental setting only provides access to the transferred work $W'$ (and makes it difficult to accurately estimate the stiffness $k_s$ of the trap). If this is the case, then one either employs a version of the fluctuation theorem written for $W'$ (as in the already cited Ref. 19), or uses $W'$ in Eq. (1), but takes care of minimizing the error on the determination of $\Delta G_0$, approximately given by Eq. (25). For example, the folded and unfolded branches of the FEC can be smoothed (by application of a filter, by spline-fitting, etc.) until the variance of $\{W'_d\}$ is entirely due to the distribution of the breaking point, in which case the difference between $\Delta G_0$ and $\Delta G'_0$ becomes negligible compared to other sources of experimental error. This is the reason why both Refs. 23 and 24 obtained an acceptable experimental test of the Jarzynski equality and the Crooks theorem, respectively, even if erroneously using the transferred work.

IV. CONCLUSION

The output of a single-molecule pulling experiment can be graphically represented in the form of a FEC, where the externally applied force is compared to the molecular construct end-to-end distance, or a FDC, where the same force is represented against the physical control parameter, the length that can be directly manipulated by the experimenter. The area under the former curve is the work $W'$ transferred to the molecule subsystem, while the latter curve allows the measurement of the accumulated work $W$, the total amount of work expended on the whole system (experimental apparatus included).

The fluctuation theorems commonly used to compute free energy differences from these out-of-equilibrium processes apply to the work $W$, but not to the work $W'$. In this paper we quantified how large an error is likely to affect the estimate of the free energy at zero force $\Delta G_0$ of the molecule if $W$ is erroneously replaced with $W'$. We found an analytical approximated expression [Eq. (25)] that emphasizes the role of the data analysis procedure and of the bandwidth of the data acquisition system. We confirmed the validity of this approach by both numerical simulation of a toy model and experiments on a DNA hairpin. This work should resolve some issues about the proper way to measure work in single-molecule experiments that have generated discussion and controversy over the past years.

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| From unfolding | From refolding | Bidirectional |
|----------------|---------------|---------------|
| $\Delta G_0$   | $\Delta G'_0$ | $\Delta G_0$ | $\Delta G'_0$ | $\Delta G_0$ | $\Delta G'_0$ |
| 20 kHz         |               |               |               |               |               |
| ave 2          | 61.9          | 37.2          | 61.1          | 94.9          | 61.8          | 54.9          |
| ave 3          | 62.0          | 40.2          | 61.2          | 92.6          | 61.6          | 55.3          |
| ave 4          | 62.0          | 39.1          | 61.2          | 86.3          | 61.7          | 55.7          |
| ave 5          | 61.7          | 41.2          | 60.9          | 86.3          | 61.3          | 55.5          |
| ave 6          | 61.9          | 39.2          | 61.1          | 80.2          | 61.5          | 55.6          |
| ave 10         | 61.8          | 42.4          | 61.1          | 76.2          | 61.4          | 56.1          |
| ave 15         | 61.5          | 42.0          | 60.9          | 77.4          | 61.2          | 56.0          |
| ave 20         | 61.6          | 41.5          | 60.9          | 77.2          | 61.2          | 56.2          |
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**APPENDIX A: THERMODYNAMICS OF THE TOY MODEL**

The model defined in Sec. II is simple enough to allow the analytical solution of its equilibrium thermodynamics. The partition function of the system is

$$Z(\lambda) = \sum_{s \in \{0,1\}} \int_{-\infty}^{+\infty} dx \exp[-\beta H^s(x,s)],$$  

(A1)

where the Hamiltonian is given by Eq. (5). The integration is trivial, so we can immediately write the solution

$$Z(\lambda) = Z_0(\lambda) + Z_1(\lambda),$$  

(A2)

where

$$Z_s(\lambda) = \sqrt{\frac{2\pi}{\beta k_t}} \exp \left[ -\frac{\beta}{2} k_{\text{eff}} (\lambda - \ell_s)^2 - s \beta G_0 \right].$$  

(A3)

Given the partition function, we have access to all the thermodynamic properties of the model; the Gibbs free energy, in particular, is defined as

$$G(\lambda) = -k_t T \ln Z(\lambda),$$  

(A4)

and the TFDC is given by

$$\langle f(\lambda) \rangle = \frac{\partial G(\lambda)}{\partial \lambda} = k_{\text{eff}}[\lambda - P_1(\lambda) \ell_1 - P_0(\lambda) \ell_0],$$  

(A5)

where

$$P_s(\lambda) = \frac{Z_s(\lambda)}{Z(\lambda)},$$  

(A6)

is the probability of the state $s$ for a fixed value of $\lambda$. The coexistence value $\lambda_c$ is characterized by the fact that $P_0(\lambda_c) = P_1(\lambda_c)$, hence

$$\lambda_c = \frac{\Delta G_0}{k_{\text{eff}}(\ell_1 - \ell_0)} + \frac{\ell_1 + \ell_0}{2}.$$  

(A7)

The corresponding coexistence force is

$$f_c = \langle f(\lambda_c) \rangle = \frac{\Delta G_0}{\ell_1 - \ell_0}.$$  

(A8)

Notice that in the asymptotic region $\lambda \ll \lambda_c$, the probability of the open state is negligible, so the force goes as $k_{\text{eff}}(\lambda - \ell_0)$, while in the region $\lambda \gg \lambda_c$ it is the probability of the closed state that goes to zero, leaving a force dependence of the form $k_{\text{eff}}(\lambda - \ell_1)$.

From Eq. (A5) we can easily write down the reversible work

$$W_{\text{rev}} = \int_{\lambda_1}^{\lambda_2} \langle f(\lambda) \rangle d\lambda.$$  

(A9)

The integration can be done analytically using the fact that

$$\int \frac{dx}{a + e^{bx}} = x - \frac{1}{b} \ln(a + e^{bx}).$$  

(A10)

Some tedious algebraic manipulation is required before one can write for the reversible work the following exact formula:

$$W_{\text{rev}} = \Delta G_0 + \frac{k_{\text{eff}}}{2} \left[(\lambda_f - \ell_1)^2 - (\lambda_f - \ell_0)^2\right] - C,$$  

(A11)

where $C$ is a correction very small if $\lambda_f \ll \lambda_c \ll \lambda_f$ (that is the most common experimental condition) whose explicit form is

$$C = \frac{1}{\beta} \ln \left[ 1 + \exp(-\beta k_{\text{eff}}(\ell_1 - \ell_0)(\lambda_f - \lambda_c)) \right].$$  

(A12)

In practice, $\lambda_f \ll \lambda_c \ll \lambda_f$ so one can usually forget about $C$ and use Eq. (A5) to rewrite Eq. (A11) as

$$W_{\text{rev}} = \Delta G_0 + \frac{\langle f(\lambda) \rangle^2 - \langle f(\lambda) \rangle^2}{2 k_{\text{eff}}}.$$  

(A13)

where $\langle f(\lambda) \rangle = \langle f(\lambda_1) \rangle$ and $\langle f(\lambda) \rangle = \langle f(\lambda_f) \rangle$.

The expectation value of the molecular extension is

$$\langle x(\lambda) \rangle = \frac{k_{\text{eff}}}{k_t} \lambda + \frac{k_m}{k_t} \left[ P_1(\lambda) \ell_1 + P_0(\lambda) \ell_0 \right].$$  

(A14)

This equation can be rephrased into an expression for the TFEC.

Another interesting quantity is the expectation value of $x^2$,

$$\langle x^2(\lambda) \rangle = \frac{1}{\beta k_t} + P_0 x^2(0) + P_1 x^2(1),$$  

(A15)

from which we easily obtain the variance for the equilibrium fluctuations of $x$

$$(\delta x)^2(\lambda) = \frac{k_{\text{eff}}}{k_t} + P_0(\lambda) P_1(\lambda) \frac{k_m^2}{k_t^2} (\ell_1 - \ell_0)^2.$$  

(A16)

The variance for the equilibrium fluctuations of the force are simply related to those of $x$

$$(\delta f)^2(\lambda) = k_{\text{eff}}^2 (\delta x)^2(\lambda).$$  

(A17)

**APPENDIX B: AN EXERCISE IN ORDER STATISTICS**

Let $\{Y_i\}$ be $n$ independent, identically distributed real-valued random variables with cumulative density function (CDF) $F(y) = \text{Pr}(Y_i \leq y)$. The PDF is defined as the derivative of the CDF $f(y) = F'(y)$. The PDF has the property $f(y) dy = \text{Pr}(y < Y_i \leq y + dy)$.

The minimum $Y_{(1)}$ of the set $\{Y_i\}$ is itself a random variable whose distribution can be deduced from the knowledge of $f(y)$ and $F(y)$. Indeed, the probability $F_{Y_{(1)}}(y)$ that the minimum is no more than $y$ is equal to the probability of having at least one $Y_i \leq y$. This is given by the binomial distribution as
Described by a normal distribution. 

In the rest of this section, we specialize these general formulas to the two distributions we used to describe the statistical behavior of the accumulated and transferred work.

1. Normal distribution

A normally distributed variable of mean $\mu$ and variance $\sigma^2$ is described by the CDF

$$\Phi^{(N)}(y) = \frac{1}{2} \left[ 1 + \text{erf} \left( \frac{y - \mu}{\sigma \sqrt{2}} \right) \right],$$

from which derives the PDF

$$\phi^{(N)}(y) = \frac{1}{\sigma \sqrt{2 \pi}} \exp \left[ - \frac{(y - \mu)^2}{2 \sigma^2} \right].$$

The distribution of the transferred work $W'$ is often well described by a normal distribution (see Fig. 8). It is convenient to define the reduced variable

$$\Phi_{Y_{(i)}}(y) = 1 - \left[ 1 - \Phi(Y) \right]^n,$$  \hspace{1cm} (B1)

Differentiating with respect to $y$ we find the corresponding PDF

$$\phi_{Y_{(i)}}(y) = n \left[ 1 - \Phi(Y) \right]^{n-1} \phi(Y).$$

The simplest way to characterize the most likely value of $Y_{(i)}$ is to consider the mode, that is, the point where the PDF has a maximum. This is given by solving with respect to $y$ the following equation:

$$[1 - \Phi(Y)] \phi'(y) = (n - 1) \phi^2(y).$$

In terms of which we can write the CDF of the minimum $Y_{(i)}$ of a sample of size $n$

$$\Phi_{Y_{(i)}}(z) = 1 - \left[ \frac{1}{2} + \frac{1}{2} \text{erf}(z) \right]^n,$$  \hspace{1cm} (B7)

and its PDF

$$\phi_{Y_{(i)}}(z) = \frac{n}{\sigma \sqrt{2 \pi}} \exp(-z^2) \left[ \frac{1}{2} + \frac{1}{2} \text{erf}(z) \right]^{n-1}.$$

The mode of the distribution $\Phi_{Y_{(i)}}(z)$ is the solution to the following transcendental equation:

$$\sqrt{n} \left[ 1 + \text{erf}(z) \right] = (n - 1) \exp(-z^2).$$

The numerical solution for $n \leq 10000$ is plotted in Fig. 3.

2. Gumbel distribution

In both our simulations and experiments, we find that the accumulated work is often adequately represented (see Fig. 9) by a random variable obeying the Gumbel distribution

$$\Phi_{G}(y) = 1 - \exp \left[ - \exp \left( \frac{y - a}{b} \right) \right],$$  \hspace{1cm} (B10)

$$\phi_{G}(y) = \frac{1}{b} \exp \left( \frac{y - a}{b} \right) \exp \left[ - \exp \left( \frac{y - a}{b} \right) \right].$$

The parameters $a$ and $b$ can be quickly estimated from the average $\bar{y}$ and the standard deviation $s$ of the sample $\{Y_i\}$ by means of the formulas

$$\bar{y} - \frac{s}{\sqrt{2 \pi}} = \frac{a}{b},$$

$$\frac{s}{\sqrt{2 \pi}} = \frac{b}{a}.$$
\[ b = s \frac{\sqrt{b}}{\pi} \quad a = \bar{y} + yb, \]  

where \( y \) is the Euler–Mascheroni constant 0.5772... The minimum value \( Y_1 \) over the sample is in this case distributed with PDF

\[ \phi_Y^{(G)}(y) = \frac{n}{b} \exp\left(\frac{y-a}{b}\right) \exp\left[ -n \exp\left(\frac{y-a}{b}\right) \right]. \]  

The mode of the minimum is therefore given simply by \( a - b \log n \).

1. F. Ritort, J. Phys.: Condens. Matter 18, R531 (2006).
2. C. Hyeon and D. Thirumalai, J. Phys.: Condens. Matter 19, 113101 (2007).
3. M. Manosas and F. Ritort, Biophys. J. 88, 3224 (2005).
4. M. Manosas, D. Collin, and F. Ritort, Phys. Rev. Lett. 96, 218301 (2006).
5. C. Jarzynski, Phys. Rev. Lett. 78, 2690 (1997).
6. F. Ritort, Adv. Chem. Phys. 137, 31 (2008).
7. U. Marini Bettolo Marconi, A. Puglisi, L. Rondoni, and A. Vulpiani, Phys. Rep. 461, 111 (2008).
8. F. Ritort, C. R. Phys. 8, 528 (2007).
9. G. Hummer and A. Szabo, Proc. Natl. Acad. Sci. U.S.A. 98, 3658 (2001).
10. G. Hummer and A. Szabo, Acc. Chem. Res. 38, 504 (2005).
11. O. Braun, A. Hanke, and U. Seifert, Phys. Rev. Lett. 93, 158105 (2004).
12. A. Imparato and L. Pelti, J. Stat. Mech.: Theory Exp. 06, P03005 (2006).
13. C. Hyeon, G. Morrison, and D. Thirumalai, Proc. Natl. Acad. Sci. U.S.A. 105, 9604 (2008).
14. N. C. Harris, Y. Song, and C.-H. Kiang, Phys. Rev. Lett. 99, 068101 (2007).
15. A. Imparato, F. Sbrana, and M. Vassalli, Europhys. Lett. 82, 58006 (2008).
16. J. Gore, F. Ritort, and C. Bustamante, Proc. Natl. Acad. Sci. U.S.A. 100, 12564 (2003).
17. S. Rahav and C. Jarzynski, J. Stat. Mech.: Theory Exp. 07, P09012 (2007).
18. P. Maragakis, F. Ritort, C. Bustamante, M. Karplus, and G. E. Crooks, J. Chem. Phys. 129, 024102 (2008).
19. J. M. Schurr and B. S. Fujimoto, J. Phys. Chem. B 107, 14007 (2003).
20. F. Ritort, C. Bustamante, and I. Tinoco, Jr., Proc. Natl. Acad. Sci. U.S.A. 99, 13544 (2002).
21. R. D. Astumian, J. Chem. Phys. 126, 111102 (2007).
22. I. Tinoco, Jr., Annu. Rev. Biophys. Biomol. Struct. 33, 363 (2004).
23. J. Liphardt, S. Dumont, S. B. Smith, I. Tinoco, Jr., and C. Bustamante, Science 296, 1832 (2002).
24. D. Collin, F. Ritort, C. Jarzynski, S. B. Smith, I. Tinoco, Jr., and C. Bustamante, Nature (London) 437, 231 (2005).
25. A. Saha, and J. K. Bhattacharjee, J. Phys. A: Math. Theor. 40, 13269 (2007).
26. F. Douarche, S. Ciliberto, A. Petrosyan, and I. Rabbiosi, Europhys. Lett. 70, 593 (2005).
27. F. Douarche, S. Ciliberto, and A. Petrosyan, J. Stat. Mech.: Theory Exp. 05, P09011 (2005).
28. E. Bertin, Phys. Rev. Lett. 95, 170601 (2005).
29. E. Bertin and M. Clusel, J. Phys. A 39, 7607 (2006).
30. C. Bustamante and S. B. Smith, U.S. Patent No. 7 133 132, B2 (November 7, 2006).
31. S. B. Smith, Y. Cui, and C. Bustamante, in Biophotonics, Part B, Vol. 361, Methods in enzymology, edited by G. Marriott and I. Parker (Academic, New York, 2003), pp. 134–162.
32. M. T. Woodside, W. M. Behnke-Parks, K. Larizadeh, K. Travers, D. Herschlag, and S. M. Block, Proc. Natl. Acad. Sci. U.S.A. 103, 6190 (2006).
33. M. T. Woodside, P. C. Anthony, W. M. Behnke-Parks, K. Larizadeh, D. Herschlag, and S. M. Block, Science 314, 1001 (2006).
34. A. Mossa, M. Manosas, N. Forns, J. M. Huguet, and F. Ritort, J. Stat. Mech.: Theory Exp. 09, P02060 (2009).
35. M. Manosas, A. Mossa, N. Forns, J. M. Huguet, and F. Ritort, J. Stat. Mech.: Theory Exp. 09, P02061 (2009).
36. M. R. Shirts, E. Bair, G. Hooker, and V. S. Pande, Phys. Rev. Lett. 91, 140601 (2003).
37. D. D. L. Minh and A. B. Adib, Phys. Rev. Lett. 100, 180602 (2008).
38. G. E. Crooks, Phys. Rev. E 60, 2721 (1999).