Supplementary Material to "Force Spectroscopy with Dual-Trap Optical Tweezers: Molecular Stiffness Measurements and Coupled Fluctuations Analysis"

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S1 DERIVATION OF EQUATIONS (36-41) IN THE MAIN TEXT

If misalignment is negligible the experimental setup is described by two coordinates y_1, y_2, as discussed in the Main Text. The equilibrium probability distribution for y_1, y_2 is a Gaussian distribution:

$$P(y_1, y_2) = \frac{1}{Z} \exp\left(\frac{(y_1, y_2) \cdot \bar{\mathsf{K}}'(y_1, y_2)}{k_B T}\right), \tag{1}$$

so that the covariance matrix is easily obtained as:

$$\begin{aligned} \begin{pmatrix} \langle y_1^2 \rangle & \langle y_1 y_2 \rangle \\ \langle y_1 y_2 \rangle & \langle y_2^2 \rangle \end{pmatrix} &= k_B T \bar{\mathsf{K}}'^{-1} \\ &= \frac{k_B T}{k_1 k_2 + k_m k_1 + k_2 k_m} \begin{pmatrix} k_2 + k_m & k_m \\ k_m & k_1 + k_m \end{pmatrix}. \end{aligned} \tag{2}$$

If we set:

$$\kappa = k_1 k_2 + k_m k_1 + k_2 k_m \tag{3}$$

then, from (2) we get:

$$\frac{k_1}{\kappa} = \frac{\langle y_2^2 \rangle - \langle y_1 y_2 \rangle}{k_B T}, \tag{4}$$

$$\frac{k_2}{\kappa} = \frac{\langle y_1^2 \rangle - \langle y_1 y_2 \rangle}{k_B T}, \tag{5}$$

$$\frac{k_m}{\kappa} = \frac{\langle y_1 y_2 \rangle}{k_B T}. \tag{6}$$

Moreover, using the identity:

$$\frac{1}{\alpha} = \frac{\alpha}{\alpha^2} = \frac{k_1 k_2}{\alpha^2} + \frac{k_1 k_m}{\alpha^2} + \frac{k_m k_2}{\alpha^2} \tag{7}$$

we get:

$$\kappa^{-1} = \frac{\langle y_1^2\rangle\langle y_2^2\rangle - \langle y_1 y_2\rangle^2}{(k_B T)^2}. \tag{8}$$

In experimental set-ups where forces are measured directly, it is convenient to extract the trap stiffness on the basis of force fluctuation measurements. Force and bead positions have an affine relation:

$$f_i = k_i y_i + f_i^0, \tag{9}$$

where f_i^0 is the mean tension measured in trap i. This affine relation can be put in a matrix form as:

$$\mathbf{f} = \bar{\mathsf{k}}_D \mathbf{y} + \mathbf{f}_0 \tag{10}$$

with $\mathbf{f} = (f_1, f_2)$, $\mathbf{y} = (y_1, y_2)$ and

$$\bar{\mathsf{k}}_D = \begin{pmatrix} k_1 & 0 \\ 0 & k_2 \end{pmatrix}. \tag{11}$$

Given the affine relations, Eq. (9),(10), the force covariance matrix for the force is now given by:

$$\begin{aligned} \begin{pmatrix} \sigma_{11}^2 & \sigma_{12}^2 \\ \sigma_{12}^2 & \sigma_{22}^2 \end{pmatrix} &= k_B T \bar{\mathsf{k}}_D \left(\bar{\mathsf{K}}'\right)^{-1} \bar{\mathsf{k}}_D = \\ &= k_B T \begin{pmatrix} \frac{(k_2+k_m)k_1^2}{k_1k_2+k_1k_m+k_2k_m} & \frac{k_1k_2k_m}{k_1k_2+k_1k_m+k_2k_m} \\ \frac{k_1k_2k_m}{k_1k_2+k_1k_m+k_2k_m} & \frac{(k_1+k_m)k_2^2}{k_1k_2+k_1k_m+k_2k_m} \end{pmatrix}, \end{aligned} \tag{12}$$

with $\sigma_{ij}^2 = \langle f_i f_j \rangle - \langle f_i \rangle \langle f_j \rangle,\ i = 1, 2$. Using Eq. (12) it is easy to show that:

$$k_1 = \frac{\sigma_{11}^2 + \sigma_{12}^2}{k_B T} \tag{13}$$

$$k_2 = \frac{\sigma_{22}^2 + \sigma_{12}^2}{k_B T} \tag{14}$$

These formulae can be used to invert any element of the covariance matrix to get k_m:

$$k_m = \frac{1}{k_B T} \frac{\sigma_{12}^2 \left(\sigma_{11}^2 + \sigma_{12}^2\right) \left(\sigma_{22}^2 + \sigma_{12}^2\right)}{\sigma_{11}^2 \sigma_{22}^2 - \sigma_{12}^4} \tag{15}$$

S2 EXPERIMENTAL VARIANCE AS A FUNCTION OF MEASUREMENT LENGTH

The power spectrum of a fluctuating linear mode, x (Ornstein-Uhlembeck process) is:

$$S(\omega) = \sigma \frac{2\omega_c}{\pi(\omega^2 + \omega_c^2)}, \tag{16}$$

where σ is the variance $\langle \delta x^2 \rangle$ of the process and ω_c its corner frequency. Integrating the power spectrum in the range between the inverse of the acquisition time T and the acquisition bandwidth B yields the expected variance:

$$\langle \delta x^2 \rangle_{B,T} = \int_{2\pi/T}^{2\pi B} d\omega S(\omega) = \frac{2\sigma}{\pi} \left(\arctan\left(\frac{2\pi B}{\omega_c}\right) - \arctan\left(\frac{2\pi}{T\omega_c}\right)\right) \quad (17)$$

If the acquisition bandwidth is much larger than the corner frequency ($B \gg \omega_c$) this can be approximated as:

$$\langle \delta x^2 \rangle_T = \sigma \left(1 - \frac{2}{\pi} \arctan\left(\frac{2\pi}{T\omega_c}\right)\right). \quad (18)$$

If a signal y is the superposition of two linear modes with variances σ_1, σ_2 and corner frequencies ω_1, ω_2 the expected behavior for the variance $\langle \delta x^2 \rangle_T$ as a function of the acquired trace is:

$$\langle \delta y^2 \rangle_T = \sigma_1 \left(1 - \frac{2}{\pi} \arctan\left(\frac{2\pi}{T\omega_1}\right)\right) + \sigma_2 \left(1 - \frac{2}{\pi} \arctan\left(\frac{2\pi}{T\omega_2}\right)\right), \quad (19)$$

which is the form of the fit used in Figure 5B.

S3 DUMBBELL DYNAMICS

The discussion in the main text shows that, in absence of misalignment, using the differential and center of mass coordinates, the stiffness tensor is diagonalized and the four fluctuation modes are uncoupled. In non-ideal cases the decoupling is not complete, but the four dimensional problem is reduced into independent lower dimensional problems. Both in the case of trap and of tether misalignment the dynamics of the center of mass is decoupled from that of the differential coordinate: the off diagonal terms couple either y_- with z_- or y_+ with z_+ but never y_- with z_+ or y_+ with z_-. This fact does greatly simplify the description of the dynamics of the dumbbell in Fig. 3A of the main text: instead of considering a four dimensional problem we can consider two independent two dimensional problems:

$$\dot{\mathbf{R}} = \bar{\mu}_R \left(-\bar{\mathsf{K}}_+\mathbf{R} + \eta_R\right), \tag{20}$$

$$\dot{\mathbf{r}} = \bar{\mu}_r \left(-\bar{\mathsf{K}}_-\mathbf{r} + \eta_r\right). \tag{21}$$

Here we arranged the coordinates in two vectors: $\mathbf{R} = (y_+, z_+), \mathbf{r} = (y_-, z_-)$, $\bar{\mathsf{K}}_+$ is the subtensor of $\bar{\mathsf{K}}$ which affects y_+ and z_+, and $\bar{\mathsf{K}}_-$ is the subtensor which affects y_- and z_-. For example, in the case of tether misalignment:

$$\bar{\mathsf{K}}_- = \begin{array}{c} \\ y_- \\ z_- \end{array} \begin{array}{c} \begin{array}{cc} \quad y_- \quad & \quad z_- \quad \end{array} \\ \begin{pmatrix} k_y + 2u(\epsilon) & \epsilon w(\epsilon) \\ \epsilon w(\epsilon) & k_z + 2v(\epsilon) \end{pmatrix} \end{array}. \tag{22}$$

with,

$$u(\epsilon) = k_m(1-\epsilon^2) + \frac{f}{r_0}\epsilon^2 \tag{23}$$

$$v(\epsilon) = \frac{f}{r_0}(1-\epsilon^2) + k_m\epsilon^2 \tag{24}$$

$$w(\epsilon) = \left(k_m - \frac{f}{r_0}\right)\sqrt{1-\epsilon^2}, \tag{25}$$

and

$$\bar{\mathsf{K}}_{+} = \begin{array}{c} \\ y_+ \\ z_+ \end{array} \begin{array}{c} \begin{array}{cc} y_+ & z_+ \end{array} \\ \begin{pmatrix} k_y & 0 \\ 0 & k_z \end{pmatrix} \end{array}. \tag{26}$$

Moreover $\bar{\mu}_R, \bar{\mu}_r$ are tensors describing both viscous friction on each particle and hydrodynamic interactions while η_R, η_r are Gaussian noises with zero mean and correlations:

$$\langle \eta_R(t)\eta_R(s)\rangle = 2k_BT\bar{\mu}_R\delta(t-s)$$
$$\langle \eta_r(t)\eta_r(s)\rangle = 2k_BT\bar{\mu}_r\delta(t-s).$$

After Bachelor (1) we set:

$$\begin{aligned} \bar{\mu}_R = & \left(\gamma^{-1} + \Gamma^{-1}\right)\frac{\mathbf{r}_0 \otimes \mathbf{r}_0}{r_0^2} + \\ & + \left(\lambda^{-1} + \Lambda^{-1}\right)\left(I - \frac{\mathbf{r}_0 \otimes \mathbf{r}_0}{r_0^2}\right) \end{aligned} \tag{27}$$

and

$$\begin{aligned} \bar{\mu}_r = & \left(\gamma^{-1} - \Gamma^{-1}\right)\frac{\mathbf{r}_0 \otimes \mathbf{r}_0}{r_0^2} + \\ & + \left(\lambda^{-1} - \Lambda^{-1}\right)\left(I - \frac{\mathbf{r}_0 \otimes \mathbf{r}_0}{r_0^2}\right), \end{aligned} \tag{28}$$

where $\lambda, \gamma, \Lambda, \Gamma$ are scalar parameters depending on r_0. In brief, $\gamma\,(\lambda)$ is the hydrodynamic friction coefficient for motions along (perpendicular to) $\mathbf{r}_0$, while $\Gamma\,(\Lambda)$ is the intensity of hydrodynamic interactions along (perpendicular to) $\mathbf{r}_0$ (the vector connecting the centers of the beads in Fig.3A of the main text). It is important to bear in mind that $\bar{\mu}_R, \bar{\mu}_r, k_y, k_z, k_m, \mathbf{r_0}$ are functions of the trap–to–trap distance $\mathbf{R}^T$ or, equivalently, of the mean tension along the tether. The equilibrium probabilities generated by (20),(21) are given by the Boltzmann distribution i.e.:

$$Q_{eq}(\mathbf{R}) = \frac{1}{Z_R} \exp\left(-\frac{U(\mathbf{R})}{k_B T}\right), \quad (29)$$

$$P_{eq}(\mathbf{r}) = \frac{1}{Z_r} \exp\left(-\frac{V(\mathbf{r})}{k_B T}\right), \quad (30)$$

with

$$U(\mathbf{R}) = \frac{1}{2}\mathbf{R} \cdot \bar{\mathsf{K}}_+ \mathbf{R}, \quad (31)$$

$$V(\mathbf{r}) = \frac{1}{2}\mathbf{r} \cdot 2\bar{\mathsf{K}}_- \mathbf{r} \quad (32)$$

and Z_R, Z_r partition functions. The variance of equilibrium fluctuations in $\mathbf{R}$ and $\mathbf{r}$ is connected to the elastic properties of traps and tether by:

$$\langle \mathbf{R} \otimes \mathbf{R} \rangle = \bar{\mathsf{K}}_+^{-1} k_B T, \qquad \langle \mathbf{r} \otimes \mathbf{r} \rangle = \left(2\bar{\mathsf{K}}_-\right)^{-1} k_B T. \quad (33)$$

Information about hydrodynamic interactions can be obtained from the time-dependent correlation functions (tensors) of $\mathbf{R}$ and $\mathbf{r}$:

$$\bar{\mathsf{C}}_R(t) = \langle \mathbf{R}(t) \otimes \mathbf{R}(0) \rangle \quad (34)$$

$$\bar{\mathsf{C}}_r(t) = \langle \mathbf{r}(t) \otimes \mathbf{r}(0) \rangle, \quad (35)$$

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

$$\frac{\bar{\mathsf{C}}_R(t)}{k_B T} = e^{-\bar{\mu}_R \bar{\mathsf{K}}_+ t} \bar{\mathsf{K}}_+^{-1} \tag{36}$$

$$\frac{\bar{\mathsf{C}}_r(t)}{k_B T} = e^{-\bar{\mu}_r (2\bar{\mathsf{K}}_-) t} \left(2\bar{\mathsf{K}}_-\right)^{-1} . \tag{37}$$

S4 ANALYSIS OF FLUCTUATIONS: THE UNCOUPLED CASE $\epsilon = 0$

The simplest and most desirable experimental condition is that in which the tether is perfectly aligned to the y axis ($\epsilon = 0$, Fig.3B). In this case fluctuations along the two axis are uncoupled. In the model this corresponds to the vanishing of all off-diagonal elements in the hydrodynamic and elastic tensors. Indeed, when $\epsilon = 0$,

$$\bar{\mu}_R = \begin{pmatrix} \gamma^{-1} + \Gamma^{-1} & 0 \\ 0 & \lambda^{-1} + \Lambda^{-1} \end{pmatrix}, \tag{38}$$

$$\bar{\mu}_r = \begin{pmatrix} \gamma^{-1} - \Gamma^{-1} & 0 \\ 0 & \lambda^{-1} - \Lambda^{-1} \end{pmatrix}, \tag{39}$$

$$\bar{\mathsf{K}}_+ = \begin{pmatrix} k_y & 0 \\ 0 & k_z \end{pmatrix} \tag{40}$$

$$\bar{\mathsf{K}}_- = \begin{pmatrix} k_y + 2k_m & 0 \\ 0 & k_z + 2\frac{f}{r_0} \end{pmatrix}. \tag{41}$$

The correlation functions are also diagonal in this case:

$$\frac{\bar{\mathsf{C}}_R(t)}{k_BT} = \begin{pmatrix} \frac{e^{-\nu_+ t}}{k_y} & 0 \\ 0 & \frac{e^{-\nu_- t}}{k_z} \end{pmatrix} \tag{42}$$

$$\frac{\bar{\mathsf{C}}_r(t)}{k_BT} = \begin{pmatrix} \frac{e^{-\omega_+ t}}{k_y+2k_m} & 0 \\ 0 & \frac{e^{-\omega_- t}}{k_z+2f/r_0} \end{pmatrix}. \tag{43}$$

The above expressions shows the presence of 4 different frequencies in the fluctuation spectrum:

$$\nu_+ = \left(\frac{1}{\gamma} + \frac{1}{\Gamma}\right) k_y \tag{44}$$

$$\nu_- = \left(\frac{1}{\lambda} + \frac{1}{\Lambda}\right) k_z \tag{45}$$

$$\omega_+ = \left(\frac{1}{\gamma} - \frac{1}{\Gamma}\right) (k_y + 2k_m) \tag{46}$$

$$\omega_- = \left(\frac{1}{\lambda} - \frac{1}{\Lambda}\right) \left(k_z + 2\frac{f}{r_0}\right). \tag{47}$$

From the measurement of $\bar{\mathsf{C}}_R(t)$ and $\bar{\mathsf{C}}_r(t)$ it is possible to obtain the stiffness of both traps and molecule:

$$k_y = \frac{k_BT}{\left(\bar{\mathsf{C}}_R(0)\right)_{yy}} = \frac{k_BT}{\left(\bar{\sigma}_R^2\right)_{yy}} \tag{48}$$

$$k_m = \frac{1}{2}\left(\frac{k_BT}{\left(\bar{\mathsf{C}}_r(0)\right)_{yy}}\right). \tag{49}$$

The time correlation function for fluctuations of $\mathbf{R}$ and $\mathbf{r}$, Eq. (42),(43) carries further information regarding hydrodynamic interactions, which can be retrieved once the stiffnesses k_y, k_m are known:

$$\frac{1}{\gamma} + \frac{1}{\Gamma} = -\frac{1}{k_y}\frac{d}{dt}\log\left(\left(\bar{\mathsf{C}}_R\right)_{yy}\right)\Big|_{t=0} \tag{50}$$

$$\frac{1}{\gamma} - \frac{1}{\Gamma} = -\frac{1}{2k_m}\frac{d}{dt}\log\left(\left(\bar{\mathsf{C}}_r\right)_{yy}\right)\Big|_{t=0}. \tag{51}$$

S5 ANALYSIS OF FLUCTUATIONS WITH TETHER MISALIGNMENT $\epsilon \neq 0$

In presence of tether misalignment ($\epsilon \neq 0$) we have:

$$\bar{\mathsf{K}}_+ = \begin{pmatrix} k_y & 0 \\ 0 & k_z \end{pmatrix} \tag{52}$$

$$\bar{\mathsf{K}}_- = \begin{array}{c} \\ y_- \\ z_- \end{array} \begin{array}{c} \begin{array}{cc} y_- & z_- \end{array} \\ \begin{pmatrix} k_y + 2u(\epsilon) & \epsilon w(\epsilon) \\ \epsilon w(\epsilon) & k_z + 2v(\epsilon) \end{pmatrix} \end{array}. \tag{53}$$

$$\langle y_+^2 \rangle = k_B T \left(\bar{\mathsf{K}}_+^{-1}\right)_{y_+ y_+} = \frac{k_B T}{k_y} \tag{54}$$

$$\begin{aligned} \langle y_-^2 \rangle &= k_B T \left(2\bar{\mathsf{K}}_m^{-1}\right)_{yy} = \\ &= \frac{k_B T}{2k_m}\left(1 + \left(\frac{r_0 k_m}{f} - 1\right)\epsilon^2\right) + \mathcal{O}(\epsilon^3). \end{aligned} \tag{55}$$

Since we will be interested in tether misalignment for short tethers (≤ 3 kbp), in the last expression we have neglected the trap stiffness with respect to the tether stiffness: ($k_m \gg k_y, k_z; f/r_0 \gg k_z$). Note that whereas the variance of $\mathbf{R}$ is not affected by the coupling ϵ, the variance of $\mathbf{r}$ does. The increased $\langle y_-^2 \rangle$ is due to the superposition of two contributions, one due to fluctuations in the optical plane and the other due to fluctuations along the optical axis. In order to separate these two types of fluctuations we need to characterize their correlation function. The $\bar{\mu}_r$ appearing in the correlation function Eq.(37) is left invariant under a rotation of r_0. This is also approximately true for $\bar{\mathsf{K}}_-$ if $k_m \gg k_y, k_z; f/r_0 \gg k_z$. As a consequence the correlation function in presence of coupling can be computed as a rotation of $\bar{\mathsf{C}}_r(t)$ obtained in the previous section (Eq. (43)). If we denote by $\bar{\mathsf{C}}_r(t, \epsilon)$ the correlation function in presence of a coupling of strength ϵ and by $\bar{\mathsf{R}}(\epsilon)$ a rotation of an angle θ ($\epsilon = \sin\theta$) we get:

$$\bar{\mathsf{R}}(\epsilon) = \begin{pmatrix} \sqrt{1-\epsilon^2} & \epsilon \\ -\epsilon & \sqrt{1-\epsilon^2} \end{pmatrix} \tag{56}$$

and

$$\bar{\mathsf{C}}_r(t, \epsilon) = \bar{\mathsf{R}}(\epsilon)^T \bar{\mathsf{C}}_r(t) \bar{\mathsf{R}}(\epsilon), \tag{57}$$

$$\frac{\left(\bar{\mathsf{C}}_r(t, \epsilon)\right)_{yy}}{k_B T} = \left(1 - \epsilon^2\right) \frac{e^{-2\omega_+ t}}{2k_m} + \epsilon^2 \frac{r_0 e^{-2\omega_- t}}{2f}. \tag{58}$$

Similar although more cumbersome formulas can be obtained in more general cases, i.e. when the trap stiffness k_y, k_z are comparable or larger than $k_m, f/r_0$ respectively. Summarizing, in presence of misalignment along the z axis we expect the correlation function of the relative distance to be a double exponential exhibiting two widely separated timescales: a fast timescale (ω_+^{-1}) due to fluctuations in the optical plane and a slow timescale

(ω_-^{-1}) due to fluctuations along the optical axis. Once the two components have been separated through fitting, as shown in the Main Text, the same analysis as in the uncoupled case can be performed on the fast component of the correlation function, to measure the molecular stiffness and the hydrodynamic parameters. From the slow contribution of the correlation function (second term in the r.h.s. of Eq. (58)) it is also possible to extract the coupling parameter, since the ratio f/r_0 is independently known. In all the experiments presented in this paper, the coupling parameter was not higher than 0.25, which corresponds to an angle $\theta \simeq 15^o$. In our setup, especially for short tethers, we can have $\epsilon^2 \simeq \alpha$, making the slow contribution to the variance (55) comparable or even bigger than the one due to fast fluctuations.

References

1. Batchelor, G. K., 1976. Brownian diffusion of particles with hydrodynamic interaction. *Journal of Fluid Mechanics* 74:1–29.