Multiplexed fluctuation-dissipation-theorem calibration of optical tweezers inside living cells

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In order to apply optical tweezers-based force measurements within an uncharacterized viscoelastic medium such as the cytoplasm of a living cell, a quantitative calibration method that may be applied in this complex environment is needed. We describe an improved version of the fluctuation-dissipation-theorem calibration method, which has been developed to perform *in situ* calibration in viscoelastic media without prior knowledge of the trapped object. Using this calibration procedure, it is possible to extract values of the medium's viscoelastic moduli as well as the force constant describing the optical trap. To demonstrate our method, we calibrate an optical trap in water, in polyethylene oxide solutions of different concentrations, and inside living fission yeast (*S. pombe*). Published by AIP Publishing.
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I. INTRODUCTION

Optical manipulation of individual biomolecules, and associated single-molecule force measurements, has revolutionized our understanding of a number of key biological processes.^{1–19} Most studies of this sort have been carried out *in vitro* in dilute solutions, where well-established calibration protocols, such as the power spectrum method, can be applied.^{20–23} Generally, these calibration methods rely on prior knowledge of the viscosity of the solution that surrounds the molecule of interest.

By contrast, measurements carried out inside living cells represent a new, emerging frontier for optical tweezers-based force spectroscopy.^{24–29} Optical and magnetic tweezers are uniquely capable for such measurements because they can reach nondestructively within living cells. However, the environment of biomolecules inside living cells is dense, viscoelastic, heterogeneous, and unknown *a priori*. Moreover, the optical trap stiffness, which determines the forces that can be applied and measured via optical tweezers, is dependent on the local optical properties of the cellular milieu. Therefore, to enable *in vivo* intracellular force measurements, more sophisticated calibration methods are required.

Recently, a very promising approach for *in vivo* calibration has been introduced and developed, called the fluctuation-dissipation-theorem (FDT) method.^{30–35} The FDT procedure combines passive and active approaches. The passive measurement records the thermal motion of an optically trapped particle. In the active portion of the procedure, either the trap position or the sample stage is oscillated transverse to the trapping beam, and the particle's positional response is tracked simultaneously. This measurement is then repeated at multiple frequencies to obtain the frequency-dependent response function. By combining the results of the undriven and driven parts of the calibration procedure, while exploiting the connection between response and fluctuations demanded

by the fluctuation dissipation theorem,^{36–38} the unknown system parameters can be determined, including the trap stiffness, the voltage-to-displacement conversion constant, and the medium's viscoelastic properties. The FDT method has already been demonstrated to probe forces exerted on cargoes during intracellular transport using phagocytosed latex beads³¹ as well as to measure directional stall forces of kinesin and dynein in A549 human epithelial cells and *Dictyostelium discoideum*.³⁴

Here, we demonstrate a multiplexed version of a laser-driven FDT method, using back-focal-plane interferometry (BFPI), that simultaneously collects data from many frequencies.^{20,23} Our method maintains the general advantages of laser driving compared to stage driving³⁰ and the experimental simplicity of a single-laser setup. Most importantly, laser driving allows for measurements at higher frequencies because the optical element responsible for driving, namely, an acoustic-optic deflector (AOD), can achieve much higher frequencies than the mechanical element responsible for stage driving, namely, a piezo stage. To our knowledge, previously, only sinusoidal driving has been used for FDT calibration.^{30–33} However, with sinusoidal driving, a separate measurement is required for each data point in the frequency spectrum, rendering it tedious to acquire the frequency response over a wide range of frequencies. The key innovation of our work is that we take advantage of square-wave driving, with concomitant multiple higher harmonics that enable calibration over a wide range of frequencies simultaneously. From this procedure, similar to Ref. 31, both the spontaneous fluctuations and the forced response of the bead to the driven trap can be fit globally, based on an assumed viscoelastic model with fitting parameters that describe both the medium and the trap. Our method significantly decreases the time for FDT calibration, thus, in principle, would help facilitate the study of time-dependent phenomena. Another (initially unanticipated) feature of square-wave driving is that it is then

straightforward to verify that signal detection is occurring in the back focal plane, as required for correct interpretation of BFPI measurements.

The purpose of this paper is to present our version of FDT calibration. To this end, we first demonstrate the method for polystyrene beads in a simple, viscous medium (water). Then, we apply this approach to polystyrene beads suspended in viscoelastic solutions of long-chain polyethylene oxide (PEO). Finally, we apply our method to endogenous lipid droplets within the cytosol of fission yeast (*S. pombe*), thereby extracting both the cytosol's viscoelasticity and the trap properties.

II. THEORY

In a simple viscous fluid of known viscosity, optical tweezers can be calibrated by analyzing only the thermal fluctuations of a trapped bead. This is the widely used “power spectrum” calibration method.^{21,22} In viscoelastic media, however, the elastic response and frictional behavior—its storage and loss moduli—are generally unknown. As a consequence, the Brownian motion of a trapped particle alone is inadequate to calibrate the optical trap. To elucidate what is necessary to calibrate an optical trap in an uncharacterized viscoelastic medium, we start with the generalized Langevin equation of motion for a particle, trapped in such a medium (assumed to be isotropic),

$$m\ddot{x}_{\text{bead}} = -\gamma\dot{x}_{\text{bead}} - k_m(t)x_{\text{bead}} - k(x_{\text{bead}} - x_{\text{trap}}) + F_{\text{random}}(t), \quad (1)$$

where x_{bead} is the position of the bead, γ is its drag coefficient, $k_m(t)$ describes the mechanical response of the medium, k is the stiffness of the optical trap, and $x_{\text{trap}} = \frac{A}{2} \cos \Omega t$ with Ω the drive frequency and A the peak-to-peak drive amplitude. At frequencies relevant to our experiments, the mass term in Eq. (1) is negligible and henceforth will be dropped, leading to

$$\gamma\dot{x}_{\text{bead}} + k_m(t)x_{\text{bead}} + kx_{\text{bead}} = kx_{\text{trap}} + F_{\text{random}}(t). \quad (2)$$

Since Eq. (2) is linear, its general solution can be written as a sum of two terms,

$$x_{\text{bead}}(t) = x_{\text{response}}(t) + x_{\text{thermal}}(t), \quad (3)$$

where

$$x_{\text{response}}(t) = \Re\left[\frac{kA}{2(k_m(\Omega) + k - i\Omega\gamma)}e^{-i\Omega t}\right], \quad (4)$$

(where $\Re[\dots]$ denotes the real part), while $x_{\text{thermal}}(t)$ is conveniently specified via its Fourier transform,

$$x_{\text{thermal}}(\omega) = \frac{F_{\text{random}}(\omega)}{k_m(\omega) + k + i\omega\gamma}. \quad (5)$$

In our experiments, detection of the bead's position is achieved by means of a quadrant photodiode (QPD) located in the back focal plane of the condenser, as necessary for BFPI. As a result, the QPD signal contains a sinusoidal voltage (V), proportional to $x_{\text{bead}} - x_{\text{trap}}$,

$$V(t) = \Re\left[-\beta^{-1}\frac{A}{2}\frac{k_m(\Omega) - i\Omega\gamma}{k + k_m(\Omega) - i\Omega\gamma}e^{-i\Omega t}\right], \quad (6)$$

where β specifies the conversion between position and voltage. To allow for a possible time delay between trap position readout and bead position readout, we incorporate a time lag (t_0) into Eq. (6), which then becomes

$$V(t) = \Re\left[-\beta^{-1}\frac{A}{2}\frac{k_m(\Omega) - i\Omega\gamma}{k + k_m(\Omega) - i\Omega\gamma}e^{-i\Omega(t-t_0)}\right]. \quad (7)$$

We expect (and find) that the time lag is fixed for given data acquisition hardware.

In the case of square-wave driving, Eq. (7) must be modified to account for the higher harmonics now present, with the result that this contribution to the QPD signal becomes

$$V(t) = \Re\left[\sum_{n=1,3,5,\dots}^{\infty} -\beta^{-1}\frac{A}{n\pi}\frac{k_m(n\Omega) - in\Omega\gamma}{k + k_m(n\Omega) - in\Omega\gamma}e^{-i(n\Omega(t-t_0))}\right]. \quad (8)$$

For a bead whose motions are described by Eq. (2), the fluctuation dissipation theorem (FDT) informs us that the power spectral density (PSD) of the bead's spontaneous thermal positional fluctuations (in $\text{V}^2 \text{Hz}^{-1}$) is^{36–38}

$$P_{\text{thermal}}(\omega) = -\beta^{-2}\frac{2k_B T}{\omega} \Im\left[\frac{1}{k_m(\omega) + k + i\omega\gamma}\right], \quad (9)$$

where $\Im[\dots]$ denotes the imaginary part.

Superimposed on this thermal spectrum is the contribution to the PSD corresponding to the bead's response to the laser driving. If the time series of the bead displacement is truncated to be an integer multiple of the driving period, there are single-data-point spikes at all odd harmonics of the driving frequency. The height of each of these spikes is proportional to the measurement time T_m .²³ Thus,

$$P_{\text{response}}(\omega) = \sum_{n=1,3,5,\dots}^{\infty} \beta^{-2}\frac{A^2 T_m}{n^2 \pi^2} \left| \frac{k_m(n\Omega) - in\Omega\gamma}{k + k_m(n\Omega) - in\Omega\gamma} \right|^2 \delta_{\omega, n\Omega}, \quad (10)$$

where $\delta_{\omega, n\Omega}$ is the Kronecker delta, equal to 1 for $\omega = n\Omega$ and zero otherwise.

Equations (4), (9), and (10) are also the basis for previous implementations of FDT calibration. However, in Refs. 32 and 33, β was determined by independent video microscopy measurements. Then, with the knowledge of β , the trap stiffness k was calibrated from the thermal spectrum and the active spectrum in a model-independent fashion.^{30,32,33} Alternatively, in Ref. 31, the thermal spectrum and the forced response of the bead to the perturbations are fit globally to a specific model that depends on k , β , and viscoelastic parameters of the medium.³¹ Both of these approaches drive the trapped bead sinusoidally, so that individual measurements must be made at many different frequencies in order to carry out the calibration. By contrast, we drive the trapped bead with a square wave, which contains multiple higher harmonics of the fundamental.

To determine the medium's viscoelasticity, our calibration procedure represents the viscoelastic response of the medium, $k_m(\omega)$, using an analytic form involving a small number of fitting parameters. For the systems studied in this paper, following Ref. 31, we have chosen

$$k_m(\omega) = k_{m0} + k_{m1} \frac{(i\omega)^\alpha}{\Gamma(\alpha)}, \quad (11)$$

where k_{m_0} , k_{m_1} , and α are fitting parameters. For spherical particles with radius R , the viscoelastic complex modulus of the medium, $G(\omega)$, can be linked to the parameters describing the medium

$$G(\omega) = G'(\omega) + iG''(\omega) = \frac{1}{6\pi R}(k_{m_0} + k_{m_1} \frac{(i\omega)^\alpha}{\Gamma(\alpha)} + i\omega\gamma). \quad (12)$$

The motivation for the particular form of Eq. (11) is that this function with $\alpha = \frac{1}{2}$ is theoretically predicted to describe the high-frequency response of solutions of flexible polymers, such as PEO.^{39,40} In addition, with $\alpha = \frac{3}{4}$, Eq. (11) has been predicted to describe the high frequency response of solutions of semi-flexible polymers,^{41,42} such as actin.^{40,43} Although Ref. 43 convincingly demonstrates that $\alpha = \frac{3}{4}$ describes the viscoelastic behavior of actin solutions at the highest frequencies, it also shows that an additional, empirical power-law term is necessary to describe the behavior over the full range of frequencies studied. Therefore, in the present paper, we have chosen to treat α as a fitting parameter to describe the viscoelastic properties of PEO solutions and the *S. pombe* cytosol. For different systems, alternative functional forms than Eq. (11) might be more appropriate. In such cases, we expect our method to be applicable *mutatis mutandis*.

By performing a global, simultaneous fit of the in-phase response versus frequency [Eq. (8)], the out-of-phase response versus frequency [Eq. (8)], and the thermal PSD versus frequency [Eq. (9)] to experimental measurements of these quantities, we obtain all of the unknown parameters, including k and β , thus calibrating the trap *in situ* within an arbitrary medium.

To assess the range of applicability of our approach, we performed simulations in which the trapped bead is coupled to Rouse-model-type polymer chains. The number of beads in the Rouse chain was varied to represent different types of viscoelastic media with a different number of modes. We simulated a trap with square-wave driving and performed our calibration procedure. In all cases tested with mode number varying from 2 to 100, our calibration well reproduces the conversion constant β and the trap stiffness k . Thus, we expect our calibration procedure with the chosen viscoelastic model works within a variety of viscoelastic media.

III. MATERIALS AND METHODS

A. Experimental setup

Our implementation of FDT calibration employs a 1064 nm-wavelength laser (Laser Quantum, Stockport, UK). Between the laser and the microscope objective (Nikon CFI $\times 100$, oil immersion, NA 1.25), the beam was incident on an acousto-optic deflector (AOD) (IntraAction DTD-274HA6). We programmed a field programmable gate array (FPGA) (Digilent BASYS) to control a direct digital synthesizer (DDS) (Analog Devices, ad9851), so that the DDS periodically outputs alternate frequencies to the AOD, which in turn drives the trap in a square-wave fashion. Position detection is by means of a QPD (Phresh Photonics SiQu50-M) situated at a plane conjugate to the back focal plane of the condenser lens. In such

a conjugate plane, high-resolution positional information is provided by the difference in intensity between quadrants. A CMOS camera (Thorlabs, DCC1645C) is used for bright-field imaging.

B. Sample preparation: Water and PEO solutions

To carry out the measurements in water and in polyethylene oxide (PEO) solutions, we prepared suspensions (1% w/v) of 1.09 μm -diameter polystyrene (PS) beads (SpheroTech, Lake Forest, IL) in flow cells consisting of a #1.5 microscope coverslip and a microscope slide, separated by a layer of 330 μm -thick, double-sided tape (3M, 9490LE) with a 5 mm $\times$ 45 mm rectangular cut-out. A stock solution (1.5 wt. %) of PEO was prepared by mixing PEO powder (5 MDa, Aldrich) in deionized water. After initial preparation, the stock solution was incubated overnight to ensure complete dissolution of the polymer. It was then diluted to yield ten solutions each containing a different PEO concentration, namely, 0.15 wt. %, 0.3 wt. %, 0.45 wt. %, 0.6 wt. %, 0.75 wt. %, 0.9 wt. %, 1.05 wt. %, 1.2 wt. %, 1.35 wt. %, and 1.5 wt. %. These solutions were then subject to subsequent study. For 5 MDa PEO solutions, the overlap concentration, which denotes the concentration at which the neighboring polymer coils start to overlap with each other, is approximately 0.04 wt. %. Therefore, the solutions under study are well within the semidilute regime. In addition, the mesh size for PEO at these concentrations is a few nanometers, which is much smaller than the bead size.^{44,45}

C. Sample preparation: Living yeast cells

Live cell measurements were performed using wild-type fission yeast (*S. pombe*) cells. Cells were inoculated in YE5S media [0.5% yeast extract (BD Biosciences)], 3% glucose (Sigma), and supplements (adenine, histidine, leucine, lysine, and uracil at 2 mg/l; Sunrise Science Products) and grown overnight in glass culture tubes at 30 °C with orbital shaking at 220 rpm (New Brunswick Scientific Excella 24 shaking incubator). At the desired optical density ($\text{OD}_{600} \sim 0.5$; log-phase growth), cells were pelleted at 2000 rpm for 5 min (Clay Adams Dynac centrifuge 420101) in a 15 ml Falcon tube. To promote larger lipid droplet formation, cells were washed and resuspended in 4.5 ml pre-warmed 0.1% oleic acid (Ward's Science), 0.2% Tween 20 (Sigma), 0.5% peptone (BD Biosciences), 0.1% glucose, and 0.3% potassium phosphate (J.T. Baker) (YPO), and then incubated in YPO for an additional 3 h at 220 rpm at 30 °C to a final OD_{600} of 0.7-0.8. 1 ml of incubated cell solution was transferred to a 1.5 ml microcentrifuge tube, pelleted using a benchtop mini centrifuge (Benchmark Scientific MyFuge C1012), followed by aspiration of the supernatant. The pelleted cells were resuspended in twice the pellet volume of growth media. 1.4 μl of this concentrated cell solution was transferred to a $\sim 1 \text{ cm} \times 1 \text{ cm} \times 1 \text{ mm}$ agarose pad [1.4% agarose (Denville Scientific)] in Edinburgh minimal media plus five supplements (EMM5S; Sunrise Science Products) on a microscope slide. The cell pad was covered with a #1.5–22 $\times$ 22 mm coverslip, and edges were sealed with 1:1:1 petroleum jelly, lanolin and paraffin (VALAP) to limit evaporation during imaging.

D. Experimental procedure

First, a bead or a lipid droplet was trapped in a stationary trap. Then, a time series of this particle's Brownian motion was recorded, allowing computation of the particle's thermal PSD. Next, during the active part of the procedure, a 41.6 Hz square-wave signal was sent to the DDS from the FPGA, so that the AOD received one of two periodically alternating frequencies. Because the deflection angle of the AOD is proportional to input frequency, this signal causes the trap position to be driven back and forth in a square-wave fashion with an amplitude of 30 nm. This amplitude was chosen to be large enough to induce a good signal-to-noise ratio. The signal-to-noise ratio is about 30 for the PSD in Fig. 1(b). At the same time, however, it is small enough to ensure that the displacements of the trapped object remain within the linear region of QPD and are comparable to the displacements caused by thermal fluctuations, fulfilling Onsager's regression hypothesis. Because the harmonic content of a square wave falls with harmonic number n as $1/n^2$, whereas the response increases as n^2 (in viscous liquids) or less strongly than n^2 (in general viscoelastic media), then if the first harmonic is linear, so are all higher harmonics.^{46,47} Simultaneously, the particle displacement

signal, V , was recorded for a total duration of 1 s at a sampling rate of 20 kHz. The square-wave signal generated by the FPGA, which determines the position of the trap, was also recorded. The relationship between the FPGA signal and displacement of the trap position was directly determined by observing the laser focus via its back reflection from the coverslip-solution interface in bright-field microscopy images. For experiments in different media, we adjusted the laser power to ensure good trapping. As a result, the trap stiffnesses in water, in PEO solutions, and in yeast cells are not comparable.

To be able to apply the analysis of Sec. II, it is essential that the QPD is accurately located in the back focal plane of the trapped bead or droplet. Only then can the QPD correctly measure the difference in bead position and trap position, Δx . Ordinarily, in optical tweezers experiments that seek to measure a bead's passive thermal fluctuations, this alignment is neither especially critical nor is it possible to determine from the time series collected. Square-wave driving, however, provides us with a convenient way to find and verify the correct back focal plane: If the QPD is positioned away from the back focal plane, even when the bead is centered in the trap, the corresponding voltage signal will be different when the trap is located in each of the two different trap positions. Thus, the signature of correct alignment of the QPD is the absence of voltage differences for the two different trap positions during square-wave driving within a purely viscous medium [Fig. 1(a)].

IV. RESULTS AND DISCUSSION

A. Multiplexed FDT calibration in water

In order to test and validate our multiplexed version of the FDT method, we first conducted measurements on free polystyrene beads trapped in water, which is a simple viscous liquid with known viscosity. A typical time series of such a bead's response, during the active phase of the calibration in water, is shown as the thin, black trace in Fig. 1(a). Because we accurately position the QPD within the back focal plane, the response signal recorded by the QPD (V) is proportional to the difference in distance between the bead and the trap, Δx . As shown in the figure, this quantity displays two opposite-sense spikes in each period of the square wave. The corresponding theoretical response curve [Eq. (8)] is shown as the thick, red line in the figure. Clearly, the theoretical curve provides an excellent description of the experimental response.

The driven PSD, corresponding to the experimental trace of Fig. 1(a), is shown as the thin, black trace in Fig. 1(b). For comparison, superimposed onto the driven PSD is the undriven PSD [Eq. (9)], shown as the thick, red trace. Evidently, the thermal component of the driven PSD recapitulates the undriven PSD, realizing a constant value for frequencies from 10 Hz to about 2000 Hz, and then decreasing for frequencies increasing above about 2000 Hz. In addition, the driven PSD also includes a sequence of spikes, occurring at the harmonic frequencies of the square-wave driving [Eq. (10)], as expected.

FIG. 1. The trapped bead response versus time and frequency. (a) Averaged QPD signal versus time over one period of the fundamental. Experimental data, averaged (for clarity) over ten periods and fifty independent data sets, consist of a thermal background due to Brownian motion plus two spikes caused by the square-wave laser driving. The theoretical curve (thick, red curve) calculated on the basis of Eq. (1) provides an excellent description of the data (thin, black curve). (b) Power spectral density versus frequency. The driven PSD (thin, black curve) is a sum of the thermal PSD (thick, red curve) and multiple spikes at odd harmonic frequencies. At low frequencies, (<5 Hz), the upturn in the experimental power spectrum is the result of low-frequency noise external to the system of interest.²¹

FIG. 2. Comparison between experimental measurements in water and best-fit model predictions for (a) the thermal PSD, (b) the in-phase, and (c) the out-of-phase Fourier amplitudes. The data points are indicated by the solid, black disks. The error bars were calculated from data within blocks.^{21,23} The block intervals were chosen to be of equal size on the logarithmic axis. Consequently, the number of points in a block grows exponentially with the frequency and the error bars decrease exponentially with increasing frequency. The best-fit model curves are the thick, red lines.

The thermal PSD is reproduced in Fig. 2(a), as the thin, black curve. The black disks in Figs. 2(b) and 2(c) show the measured in-phase (V') and out-of-phase (V'') Fourier amplitudes of the response at the harmonic frequencies of the square-wave drive. Since water is a simple viscous medium, here we used a model in which all parameters related to elasticity are set to zero,

$$P_{\text{thermal}}(\omega) = \beta^{-2} \frac{2k_B T \gamma}{k^2 + \gamma^2 \omega^2}, \quad (13)$$

$$V'(n\Omega) = -\beta^{-1} \frac{A}{\sqrt{2\pi}} \frac{n\gamma^2 \Omega^2 \cos n\Omega t_0 + k\gamma \Omega \sin n\Omega t_0}{k^2 + n^2 \gamma^2 \Omega^2}, \quad (14)$$

and

$$V''(n\Omega) = -\beta^{-1} \frac{A}{\sqrt{2\pi}} \frac{k\gamma \Omega \cos n\Omega t_0 - n\gamma^2 \Omega^2 \sin n\Omega t_0}{k^2 + n^2 \gamma^2 \Omega^2}. \quad (15)$$

To determine the parameters of this model, namely, β , k , γ , and t_0 , we simultaneously fit Eqs. (13)–(15) to measurements of the corresponding quantities (Fig. 2). In these fits, the parameters were constrained to be positive so that they have physically sensible meanings. The initial guesses for parameters were chosen by hand to ensure good fits; although, of course, the exact values chosen did not affect the final best-fit parameter values. Figure 3 shows that the values of k and β are independent of the amplitude of the square wave drive amplitude. To avoid the effect of low-frequency filtering from our silicon-based QPD, we restricted the fit to frequencies less than 2000 Hz.^{22,49} The resultant best-fit parameter values are $\beta = (1.11 \pm 0.35) \mu\text{m V}^{-1}$, $k = (0.179 \pm 0.011) \text{ pN nm}^{-1}$, $\gamma = (1.72 \pm 0.14) \times 10^{-8} \text{ kg s}^{-1}$, and $t_0 = (1.59 \pm 0.02) \text{ ms}$. Because the viscosity of water (η) is related to γ by $\eta = \gamma/6\pi R$, this value of γ represents a measurement of water's viscosity of $\eta = (8.37 \pm 0.68) \times 10^{-4} \text{ Pa s}$. In comparison, the literature viscosity of water at 25 °C, $\eta = 8.90 \times 10^{-4} \text{ Pa s}$, is in good agreement.⁵⁰ Equations (13)–(15), evaluated using the best-fit parameters, are shown as the thick, red lines in Figs. 2(a)–2(c). In every case, the model provides a good

FIG. 3. Best-fit values of (a) the trap stiffness k and (b) the volt-to-micron conversion constant β with different amplitudes of square-wave excitation. The values of k and β are independent of the amplitude of the square wave. When the amplitude is small, lower signal-to-noise ratios result in larger errorbars.

description of the experimental data, confirming that our procedure can successfully calibrate the optical trap.

B. Multiplexed FDT calibration in PEO solutions

To further test our method, we next examined solutions of 5 MDa PEO of varying concentration. Figure 4 shows both (a) the time-domain and (b) the frequency-domain responses to square-wave driving of a PS bead in several PEO solutions. For low PEO concentrations, the bead returns to the trap center in a relatively short time, similar to the response in water. By contrast, at higher PEO concentrations, the trapped bead recovers its equilibrium position much more slowly or not at all, within the drive period, indicating a viscoelastic response.

The changing character of the response with increasing concentration is shown in the frequency domain in Fig. 5, which shows PSDs obtained for different PEO concentrations, displayed on a log-log scale. The PSDs obtained at the lowest PEO concentrations are similar to those of beads in water, which are flat in the low frequency region and only decrease for frequencies increasing above the corner frequency. By contrast, the PSDs obtained at higher PEO concentrations appear progressively steeper in the low frequency region, suggesting that a power-law response increasingly dominates at the frequencies in question.

FIG. 4. (a) Time series of bead traces. From the innermost to the outermost curve, the PEO concentrations are 0.15 wt. %, 0.6 wt. %, 1.05 wt. %, 1.5 wt. %, respectively. Thick, red lines represent the theoretical curves. Voltage zero indicates no relative displacement between the trapped bead and the center of the trap. (b) Power spectral density versus frequency. The driven PSD (thin, black curve) is a sum of the thermal PSD (thick, red curve) and multiple spikes at odd harmonic frequencies.

FIG. 5. PSDs at different PEO concentrations. For comparison, the bottom curve is the PSD of water. From the second from bottom to the top curve, the PEO concentrations are 0.15 wt. %, 0.3 wt. %, 0.45 wt. %, 0.6 wt. %, 0.75 wt. %, 0.9 wt. %, 1.05 wt. %, 1.2 wt. %, 1.35 wt. %, and 1.5 wt. %, respectively. For clarity, successive PSDs are displaced one from another by a factor of 10. The experimental data are the solid, black disks, and the best-fit models are the thick, red lines.

Following our FDT calibration procedure, we performed simultaneous, global fits to V' , V'' , and the PSD to independently calibrate the trap at each of the ten different PEO concentrations studied. We varied k , β , k_{m1} , and α in these fits. We fixed $k_{m0} = 0$ and γ to the value corresponding to water because doing so yielded excellent agreement between the data and the model at all concentrations. α is constrained to lie between 0 and 1. A value of 0 indicates a purely elastic medium, while a value of 1 indicates a purely viscous medium. The other parameters including the trap stiffness and the conversion constant were constrained to be positive so that they have physically sensible meanings. The initial guesses were chosen by hand to ensure good fits for each sample but did not affect the final best-fit parameters. Figure 6 shows representative experimental measurements [thin, black lines in (a), and solid disks in (b) and (c)], together with the corresponding best-fit models (thick, red lines) of the PSDs and of the in-phase and out-of-phase responses for a 0.9 wt. % PEO solution. Evidently, the model well describes all of these quantities. The thick, red lines in Fig. 5, corresponding to the best-fit model PSDs at each PEO concentration, illustrate that the best-fit parameters provide an accurate description of the data across the range of concentrations studied.

The best-fit values of k and $k_{m1}\omega_0^\alpha$ at $\omega_0 = (2\pi \times 10)$ rad s $^{-1}$ are plotted versus PEO concentration in Fig. 7(a), while the best-fit values of β and α are plotted versus PEO concentration in Figs. 7(b) and 7(c), respectively. Inspection of these figures reveals that although k_{m1} and α vary significantly and systematically with concentration, the best-fit values of k and β show relatively small, random variations about their mean values across the range of concentrations studied. This observation, that our procedure yields similar values of k and β for optically similar but mechanically different samples, is just as expected, given that the same-sized beads (nominally) and the

FIG. 6. Comparison between experimental measurements in 0.9 wt. % PEO solution and best-fit model predictions for (a) the thermal PSD, (b) the in-phase, and (c) the out-of-phase Fourier amplitudes. The data points are indicated by the solid, black disks. The best-fit model curves are the thick, red lines.

same laser power were used throughout. The observed small variations in k and β likely reflect variations in bead size and the depth of the trap beyond the coverslip and point to the desirability of performing a trap calibration in each individual experiment, which can be quickly and easily accomplished using the procedure described here. The exponent, α , varies monotonically from a value near 0.7 at the lowest concentrations to a value near 0.4 at the highest concentrations. Such an evolution is often taken to be an indicator of an increasingly elastic medium.⁵¹

The frequency-dependent storage and loss moduli of PEO solutions, determined from Eq. (12), evaluated with the best-fit parameters, are shown in Fig. 8. The interpretation that PEO solutions are increasingly elastic for increasing PEO concentration is bolstered by the observation that at low PEO concentration, G'' is larger than G' in the relevant frequency

FIG. 7. Best-fit values of (a) the trap stiffness k (black disks) and $k_{m1}\omega_0^\alpha$ (blue diamonds) at $\omega_0 = (2\pi \times 10)$ rad s^{-1} , (b) the volt-to-micron conversion constant β , and (c) the stretching exponent, α , versus PEO concentration. The points at 0 wt. % correspond to water. The error bars, calculated from Hessian matrices returned by the MATLAB `fmincon` algorithm, represent the standard error of each parameter.

range, while for solutions with the highest PEO concentrations, the opposite is the case. Our results for G' and G'' are of similar magnitude to the moduli found by diffusing wave spectroscopy (DWS) for 4 wt. % solutions of 900 kDa PEO, reproduced as dotted and dashed-dotted red lines in Fig. 8.⁴⁸

C. Multiplexed FDT calibration in living yeast cells

Finally, we also performed our calibration method inside living fission yeast (*S. pombe*).³³ An essential assumption of all versions of the FDT calibration method is that the quantitative

FIG. 8. Comparison between storage modulus G' (solid, black lines) and loss modulus G'' (dashed, black lines) at different PEO concentrations. From the bottom pair to the top pair, the PEO concentrations are 0.15 wt. %, 0.6 wt. %, 1.05 wt. %, and 1.5 wt. %, respectively. For comparison, the red dotted line and the red dashed-dotted line are G' and G'' data points, respectively, for 4 wt. % solutions of 900 kDa PEO via diffusing wave spectroscopy.⁴⁸ We observe increases in both G' and G'' with concentration. G'' is larger than G' in the relevant frequency range at low PEO concentrations, while G' exceeds G'' in value for solutions with higher concentrations, showing increasing elasticity.

connection between spontaneous fluctuations and the response to an applied force is given by temperature alone. Inside living cells, there certainly exist non-thermal driving forces that could in principle limit the applicability of FDT calibration in some cases.⁵² Nevertheless, we proceed here similarly to Refs. 31 and 33 by assuming that the effect of active processes and non-equilibrium effects do not significantly affect our measurements and that the fluctuation dissipation theorem continues to hold within living cells at the frequencies studied (>1 Hz). Instead of a polystyrene bead, in this case, we trap an endogenous lipid droplet. Lipid droplets are readily visible via bright-field microscopy in *S. pombe* cells,⁵³ as shown in Fig. 9(a), which shows a bright-field-microscopy image, obtained on our optical trapping microscope, that visualizes several lipid droplets within each yeast cell. Lipid droplets satisfy two key requirements for strong trapping, namely, that there is a significant refractive index (n) difference between the cytosol ($n = 1.35$) and the lipid droplet ($n = 1.46$) and that their sizes are comparable to the wavelength of the trapping laser light.

Figure 9(a) shows wild-type *S. pombe* cells before trapping. The arrow indicates the lipid droplet that is about to be trapped. After initially trapping the droplet and then turning off the trap, the lipid droplet in question appears brighter and slightly larger in the bright-field image [arrow in Fig. 9(b)]. Trapping the droplet for a second time causes no further changes in its appearance. In addition, the power spectrum and response functions measured during the first period of trapping are indistinguishable from those obtained during the second period of trapping. We ascribe the change in droplet appearance upon initial trapping to a slight irreversible change in the position of the trapped droplet relative to the coverslip, as a result of trapping. Image analysis of the trapped lipid droplet in Fig. 9(b) reveals its diameter to be $(1.02 \pm 0.11) \mu\text{m}$.

Figure 10 shows both (a) the time-domain and (b) the frequency-domain responses of a representative lipid droplet

FIG. 9. Bright-field images of a lipid droplet in a *S. pombe* cell, (a) before and (b) after initial trapping. The circle in the inset of (b) indicates the diameter of the lipid droplet.

to square-wave driving. These data more closely resemble the response curves of trapped beads in high concentration PEO solutions, indicating that the *S. pombe* cytosol shows significant elasticity.^{33,54}

Following our FDT calibration procedure, we performed simultaneous, global fits to V' , V'' , and the PSD within each of 18 different *S. pombe* cells, effectively carrying out an independent calibration for each cell. We varied k , β , k_{m0} , k_{m1} , and α in these fits. The parameters were constrained in the same way as for the PEO solutions. γ was fixed to its value for water. Figure 12 shows representative experimental measurements (thin, black lines), together with the corresponding best-fit models (thick, red lines) of the PSD and of the in-phase and out-of-phase responses for a representative yeast cell. In Figs. 11 and 12(a), there are spikes at high frequencies in the PSDs. These spikes, which are related to the initial fast-decay behavior in Fig. 10(a), are not captured by our model-fitting curves. Nevertheless, the model well describes the data up to frequencies of about 3 kHz. Additional work is needed to understand the origin of these high frequency spikes.

Figure 11 shows the experimental PSDs (thin, black lines) of five different yeast cells, together with the corresponding best-fit model PSDs (thick, red lines). Although in every case the model well-describes the data, the PSDs show significant biological variability, either from variation between lipid droplets or individual cells. This biological variability is displayed in Fig. 13, which plots the best-fit parameters from all cells studied versus an integer label that specifies the cell.

FIG. 10. (a) Time-domain response to square-wave driving of a lipid droplet inside a *S. pombe* cell. The experimental trace (thin, black line) shown is an average over 500 periods. The best-fit model response is shown with the thick, red line. (b) Power spectral density versus frequency. The driven PSD (thin, black curve) is a sum of the thermal PSD (thick, red curve) and multiple spikes at odd harmonic frequencies. The spikes at even harmonic frequencies are more than two magnitudes smaller than the neighboring spikes at odd harmonic frequencies. The spikes at even harmonics are electronic artifacts within the QPD circuit.

The best-fit values of k , k_{m_0} , and $k_{m_1}\omega_0^\alpha$, where $\omega_0 = (2\pi \times 1) \text{ rad s}^{-1}$, are plotted versus cell label in Fig. 13(a), while the best-fit values of β and α are plotted versus the cell

FIG. 11. PSDs of five different yeast cells. The experimental data are the solid, black disks and the best-fit models are the thick, red lines. The PSDs show significant biological variability.

FIG. 12. Comparison between experimental measurements of *S. pombe* cell and best-fit model predictions for (a) the thermal PSD, (b) the in-phase and (c) the out-of-phase Fourier amplitudes. The data points are indicated by the solid, black disks. The best-fit model curves are the thick, red lines.

number in Figs. 13(b) and 13(c), respectively. Figure 13(d) shows the measured size of the trapped droplets, determined from bright-field images. The best-fit values of k , k_{m_0} , $k_{m_1}\omega_0^\alpha$, and β all show significant variability. The scaling exponent, α , and the droplet diameter show smaller variations, with α taking an average value of $\alpha = 0.67 \pm 0.08$ across all cells studied. We ascribe the observed variations in k_{m_0} and $k_{m_1}\omega_0^\alpha$ to each lipid droplet experiencing a somewhat different local mechanical environment, depending on the particular cell and on the droplet's particular position within that cell. Similarly, we ascribe the observed variations in k and β to variations in the local optical environment about each droplet and in the depth of the trapped droplet beyond the coverslip. These observations again point to the importance of performing a trap calibration in each individual experiment, which can be easily accomplished using the procedure described here.

FIG. 13. Best-fit values of (a) the trap stiffness k (black disks), k_{m0} (red squares), and $k_{m1}\omega_0^\alpha$ (blue diamonds) at $\omega_0 = (2\pi \times 1) \text{ rad s}^{-1}$, (b) the volt-to-micron conversion constant β , (c) the stretching exponent α , and (d) the diameter of lipid droplets in 18 different *S. pombe* cells. In (a), k_{m0} is small compared to k , except for a few points. However, the frequency dependent elastic term related to k_{m1} is comparable to k at 1 Hz. The error bars in (a)–(c), are calculated from Hessian matrices returned by the MATLAB `fmincon` algorithm. The error bars in (d) are based on the pixel size of our bright-field microscope.

The mean storage modulus, G' , calculated from Eq. (11) using the best-fit parameters and averaged over all cells, is plotted in Fig. 14(a) as the solid red line. The extent of biological

variation in G' is represented in this figure by the shaded area, which is bounded by the lowest and highest storage moduli from the population of 18 *S. pombe* cells studied. Similarly, the mean loss modulus, G'' , is shown as the dashed red line in Fig. 14(b), where the shaded area is bounded by the lowest and highest loss moduli in the population.

These collected data reproduce a number of features seen in the FDT calibration procedure carried out in Ref. 33, also inside *S. pombe*. In Ref. 33, the trap stiffness shows comparable variability as is apparent in our work in Fig. 13(a). Reference 33 also reports that the measured frequency dependence of the storage and loss moduli of the *S. pombe* cytosol are well-described by $\alpha = 0.75$, similar to our value of $\alpha = 0.67 \pm 0.08$. The mean storage and loss moduli within *S. pombe* cells, determined in Ref. 33 are about three-fold smaller than our values for these quantities, as shown in Figs. 14(a) and 14(b), where the results from Ref. 33 are shown as the solid, black dots. However, the viscoelastic properties of the yeast cytosol have been shown to depend strongly on environmental conditions.⁵⁵ Therefore, we hypothesize that the factor of three difference in average moduli originates in differences in growing conditions and environments.

V. CONCLUSIONS

We have presented an improved FDT method for calibrating optical tweezers *in situ* in viscoelastic media, including living cells. The strengths of FDT calibration are as follows. First, it is independent of the viscoelastic character of the particular medium under study and therefore can be used inside living cells. The advantages of being able to apply known forces inside cells are myriad. In the context of our own research, we are seeking to investigate the mechanical properties of cell nuclei, thus *in situ* calibrated optical tweezers will make it straightforward to apply forces of known magnitude and direction while also monitoring the nuclear response. This provides several advantages over our previous force measurement experiments on isolated nuclei.⁵⁶ First, this simpler

FIG. 14. (a) Storage modulus G' (solid red) and (b) loss modulus G'' (dashed red) of *S. pombe* cell, calculated from the viscoelastic model and the best-fit fitting parameters. The thick, red lines are viscoelastic moduli averaged over all cells. The shaded areas are bounded by the highest and the lowest viscoelastic moduli from the population of 18 *S. pombe* cells studied. For comparison, G' (thin, solid black), G'' (thin, dashed black) of 1.5 wt. % PEO, G' of water (dot dashed blue), and viscoelastic moduli data of *S. pombe* cell by Mas *et al.*³³ (black dots) are also plotted.

experimental protocol removes the need to prepare and manipulate isolated nuclei, thereby avoiding the caveats associated with *ex vivo* approaches. Moreover, with this new *in vivo* tool, we will now be able to quantitatively assess the mechanics of very soft nuclei that could not be successfully purified previously due to mechanical instability. In principle, this approach can be applied to measure the force response of a variety of organelles in nearly any cell type; given the various physical interactions between organelles, the *in situ* nature of this method will likely prove ideal to address such areas of investigation. More generally, suitable calibration data to support the multiplexed FDT calibration method introduced in this paper can be collected in less than 3 s, thus permitting a significantly greater experimental throughput than previously developed approaches.

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