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Origins of phase contrast in the atomic force microscope in liquids

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We study the physical origins of phase contrast in dynamic atomic force microscopy (dAFM) in liquids where low-stiffness microcantilever probes are often used for nanoscale imaging of soft biological samples with gentle forces. Under these conditions, we show that the phase contrast derives primarily from a unique energy flow channel that opens up in liquids due to the momentary excitation of higher eigenmodes. Contrary to the common assumption, phase-contrast images in liquids using soft microcantilevers are often maps of short-range conservative interactions, such as local elastic response, rather than tip-sample dissipation. The theory is used to demonstrate variations in local elasticity of purple membrane and bacteriophage λ29 virions in buffer solutions using the phase-contrast images.

atomic force microscopy | liquid environments | energy dissipation | higher eigenmodes | momentary excitation

Dynamic atomic force microscopy (dAFM) is an essential experimental tool for the study of conservative and dissipative forces on surfaces at nanometer length scales, which has major implications for the physics of biomolecular interactions, chemical bond kinetics, adhesion, wetting and capillary action, friction and elasticity on material surfaces (1, 2). dAFM techniques have been developed to distinguish between dissipative (friction, viscoelastic, bond breaking, surface hysteresis, capillary condensation) and conservative (elastic, magnetic, electrostatic) forces between a sharp oscillating tip and the surface. In amplitude-modulated AFM (AM-AFM) phase-contrast imaging, the variation in the phase of the oscillating probe tip with respect to the drive signal is mapped over the sample. For the past decade phase contrast has been intimately connected with variation in tip-sample dissipation over the sample (2–6). Phase-contract imaging is widely recognized as perhaps the most important AM-AFM mode for the measurement of compositional contrast.

The connection between phase contrast and tip-sample dissipation rests on the assumption that the cantilever dynamics can be modeled by a single eigenmode (point-mass) oscillator. With this assumption, the tip-sample dissipation can be equated to the difference between work input to the oscillator and energy dissipated into a surrounding viscous medium (4, 5). This theory forms the bedrock upon which phase-contrast imaging is currently based, at least under ambient and vacuum conditions.

dAFM is now a well-known and broadly extended technique for nanoscale imaging and force spectroscopy in the biology community (7–10). Because the natural medium for the study of biological samples is liquid, it is of fundamental importance to develop a proper description of the different working modes of dAFM when the probe and sample are immersed in liquids. In particular, there is little work on understanding the origins of phase contrast in liquids (6) where soft cantilevers (stiffness ≤ 1 N/m) with low-quality factors (Q ≤ 5) are routinely used for the imaging of soft biological samples. It is important to note that all prior theoretical works on phase contrast, both in air and liquids, are based on the assumption that a single eigenmode is sufficient to describe the microcantilever dynamics (2).

In this article, we build on a recent result (11)—valid for soft microcantilevers tapping on a sample in liquids—that shows the second eigenmode is momentarily excited near times of tip-sample contact. This finding implies that the dynamics of soft microcantilevers in liquids is naturally multimodal. We show that this opens up a new energy flow channel for the soft microcantilevers in liquids and that phase contrast really measures the extent of energy transferred to the second eigenmode via tip-sample interaction rather than tip-sample dissipation. We demonstrate that in situations where electrostatic bilayer forces and tip-sample adhesion are negligible, phase contrast becomes a mapping of local elasticity variations of the sample. Experimental phase-contrast images on purple membrane and λ29 viral capsids in buffer solution are used to verify the theoretical findings by linking these images with the local variations of the sample stiffness.

Theoretical Considerations

Mathematical Modeling. We begin with a general mathematical model governing the dynamics of a soft cantilever tapping on a sample in liquid, for which at least a 2-eigenmode model is required (11):

$$\frac{\ddot{q}_1}{\omega_1^2} + \frac{\dot{q}_1}{\omega_1 \Omega_1} + q_1 = F_1 \cos(\omega t) + \frac{F_{a}(d, d, m)}{k_1} \tag{1a}$$

$$\frac{\ddot{q}_2}{\omega_2^2} + \frac{\dot{q}_2}{\omega_2 \Omega_2} + q_2 = F_2 \cos(\omega t) + \frac{F_{a}(d, d, m)}{k_2} \tag{1b}$$

where $q_i$ is the contribution to tip deflection of the $i$th eigenmode and dots represent temporal derivatives. $F_i$, $k_i$, $Q_i$, and $\omega_i(i = 1, 2)$ refer to the equivalent forcing amplitudes and stiffnesses (12), quality factors, and natural frequencies of the first 2 eigenmodes respectively. $\omega$ is the drive frequency and $T = 2\pi/\omega$ is the excitation time period. Moreover, $\omega \approx \omega_1$ for the conventional tapping-mode imaging where the drive frequency is tuned to the natural frequency of the first eigenmode. $F_{a}(d, d, m)$ is the nonlinear tip-sample interaction force, where $d = Z + q_1 + q_2$ is the instantaneous tip-sample gap for a base-sample separation $Z$ and $m$ is a Boolean variable indicating the state of the tip-sample contact in the case of hysteretic force models; i.e. $m = 1$ for contact and $m = 0$ otherwise (13, 14). For steady-state oscillations, let $A_i$ and $\phi_i$ refer to the amplitude and phase lag, respectively.


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of the first harmonic of the first eigenmode response given by \( A_1 \cos(\omega t - \phi_1) \). Far from the sample (\( F_{s} = 0 \)), \( A_1 \) tends to the unconstrained amplitude \( A_0 \). We calculate \( F_2/F_1 = -0.55 \) for a uniformly distributed forcing and \( k_2/k_1 = 39 \) from Bernoulli–Euler beam theory.

The total tip-sample interaction force \( F_{ts}(d, d, m) \) can be decomposed additively into conservative forces \( F_{tsc}(d) \) and nonconservative (dissipative) forces \( F_{tsnc}(d, d, m) \). Conservative forces obey \( F_{ts}(d) = -\partial V_s(d)/\partial d \) for a potential function \( V_s(d) \) and cannot change the total mechanical energy of the probe upon completion of a periodic orbit. In liquids, the principal conservative tip-sample forces arise from elastic contact forces and the so-called DLVO (Derjaguin–Landau–Verwey–Overbeek) forces (14), which include attractive van der Waals and repulsive electrostatic double-layer forces. Hydrophobic and hydrophilic interactions can also contribute to conservative forces; however, these forces are relevant only for special tip-sample combinations. Finally, hydration layers on ordered surfaces cause oscillatory tip-sample forces (14); however, these forces are typically too small to detect with AM-AFM. Nonconservative tip-sample interaction forces of the form \( F_{tsnc}(d, d, m) \) dissipate mechanical energy of the probe upon interaction. Nonconservative forces typically encountered in dAFM include viscoelastic forces of the form \( F_{tsnc}(d, d) \), hysteretic adhesion forces of the form \( F_{tsnc}(d, m) \), or combinations of the two (15). Common hysteretic forces in liquids include specific bond-forming/-breaking between a functionalized tip and complimentary molecule on the surface (16), or hysteretic adhesion between the tip and a highly adhesive soft surface predicted by the Johnson–Kendall–Roberts (JKR) theory (17).

Here we focus on the common scenario, where a nonfunctionalized tip interacts with a soft biological sample supported on a stiff substrate in a high-ionic-concentration buffer where the DLVO forces are highly screened (18, 19) and adhesion hysteresis is negligible. Accordingly, interactions between the tip and sample can be modeled by a rate-dependent (viscous) force \( F_{tsnc}(d, \dot{d}) \) combined with a conservative contact force \( F_{tsc}(d) \). An example of such a model is Hertz contact (20) combined with Kelvin–Voigt viscoelastic forces (15). However, the phenomenon described here also applies for other contact theories, such as Chadwick theory for thin membranes (21) or linear contact theory for viral capsids (10). The Hertz contact model (20) is given by

\[
F_{tsc}(d) = \begin{cases} 
0, & d > 0 \\
\frac{4}{3} E_s \sqrt{R(d)}^{3/2}, & d \leq 0,
\end{cases}
\]

where \( E_s = [(1 - 2 \nu_s^2)/E_s + (1 - 2 \nu_t^2)/E_t]^{-1} \) is the effective elastic modulus of the tip-sample combination, \( E_s, E_t, \nu_s, \nu_t \) are elastic moduli and Poisson’s ratios of the tip and sample, respectively, and \( R \) is the radius of the tip. Nonconservative, viscoelastic forces (15) are modeled by

\[
F_{tsnc}(d, \dot{d}) = \begin{cases} 
0, & d > 0 \\
-\eta_s \dot{d} \sqrt{R(d)}, & d \leq 0,
\end{cases}
\]

where \( \eta_s \) is the viscosity of the sample. In the numerical simulations presented here, all Poisson’s ratios are 0.3 and \( E_t = 130 \) GPa for a silicon tip with radius \( R = 20 \) nm.

**Numerical Simulations.** We begin with numerical simulations (22) of Eq. 1 for \( A_0 = 10 \) nm and a conventional excitation \( \omega_0 = \omega_1 \) for typical cantilevers in air and liquid environments. A dissipative sample modeled by Eq. 2 and Eq. 3 with \( E_s = 100 \) MPa (purple membrane), \( \eta_s = 10 \) Pa·s is chosen for the simulations. By comparison, membrane proteins have viscosities on the order of 1 Pa·s (23). The cantilevers are excited far from the sample, where they achieve steady periodic oscillations, and are then brought close to the sample by gradually reducing \( Z \). For the simulation in air, we consider a stiff rectangular cantilever \( C_1 \) (\( k_1 = 30 \) N/m, \( Q_1 = 400, Q_2 = 1200, \omega_1 = 2\pi \cdot 300 \) kHz, \( \omega_2 = 6.5\omega_1 \) typical for air operation. In liquids, we choose a soft rectangular cantilever \( C_2 \) (\( k_2 = 0.11 \) N/m, \( Q_1 = 1.85, Q_2 = 4.3, \omega_1 = 2\pi \cdot 3.9 \) kHz, \( \omega_2 = 7.7\omega_1 \) typical for liquid operation and the properties of which were measured experimentally (24). Note for both the soft and stiff microcantilevers considered here, the second eigenmode frequency is not near an integer multiple of the first, ensuring that resonant interactions between eigenmodes do not occur (25).

From these simulations, we find that the second eigenmode is significantly excited momentarily near times of contact with the sample in liquids, as compared with air, even though the drive frequency is tuned to the first eigenmode. The responses of the first and second eigenmodes are decomposed and plotted for 2 periods at an amplitude ratio of \( A_1/A_0 = 0.9 \) for the simulation in air (Fig 1A) and in liquid (Fig 1B). During interaction with the sample, some amount of energy \( E_{1-2} \) is transferred from the first to the second eigenmode. However, for the same conditions \( A_0 \) and \( A_1/A_0 \), the momentary excitation of the second eigenmode is at least 2 orders smaller in air than in liquid. Also, because \( Q_2 < \omega_2/\omega_1 \) in liquid, the momentary excitation of the second eigenmode decays during the oscillation time period \( T = 2\pi/\omega_1 \) (\( \omega_1 \approx \omega_0 \)) i.e., the energy transfer \( E_{1-2} \) eventually dissipates into the surrounding media.

To understand the origins of phase contrast in this situation, we perform a steady-state energy flow analysis for a single
excitation period \( T \) according to the diagram in Fig. 1C. The total work performed by the excitation source is divided into \( W_1 \) and \( W_2 \), supplied to the first and second eigenmodes, respectively. Energy can leave the first eigenmode either by dissipation into the surrounding media \( E_{med,1} \), dissipation into the sample \( E_s \) by nonconservative interactions or through a third energy flow channel. During interaction with the sample, the 2-eigenmode model allows energy \( E_{1-2} \) to flow from the first to the second eigenmode where it is subsequently dissipated into the surrounding media in the form of \( E_{med,2} \). The steady-state energy balance for the first eigenmode is

\[
W_1 = E_s + E_{1-2} + E_{med,1}.
\]

Considering an \( N \) harmonic approximation \( q(t) \approx \sum_{n=1}^{N} A_n \cos (\omega t - \phi_n) \) leads to \( W_1 = \int_0^T F_1 \cos (\omega t) q(t) dt = \pi F_1 A_1 \sin \phi_1 \).

The energy dissipated by the media is

\[
E_{med,1} = \frac{k_1}{\omega_1} \int_0^T q_1^2(t) dt = \frac{\pi}{k_1} \sum_{n=1}^{N} A_n^2 \omega_n^2 \text{which we define as } E_{med,1} \equiv \sum_{n=1}^{N} E_{med,1,n}, \text{ where } E_{med,1,n} \text{ is the energy dissipated into the surrounding media by the } n^{th} \text{ harmonic of the first eigenmode. Substituting the above relations into the energy balance (Eq. 4) yields}
\]

\[
\sin \phi_1 = \frac{g(\omega_1) A_1}{A_0} \left( 1 + \sum_{n=2}^{N} A_n^2 \omega_n^2 \right) \text{ where } \bar{w}_0 = \omega/\omega_0 \text{ and } g(\omega_1) \text{ is a gain factor predicted by the frequency response far from the sample that is used to eliminate the unknown } F_1. \text{ According to Eq. 5, when the amplitude setpoint } A_1/A_0 \text{ is held constant during a scan, the phase lag } \phi_1 \text{ is influenced by (i) the tip-sample dissipation } E_s (5), (\text{ii}) \text{ the higher harmonic content of the first eigenmode (which is relatively small)} \text{ (6) and (iii) the energy propagation between eigenmodes } E_{1-2}. \text{ Setting } \omega = \omega_1 \text{ and ignoring } E_{1-2} \text{ and the higher harmonic content } (A_n, n \geq 2), \text{ we recover the classical single harmonic approximation (5). Ignoring } E_{1-2} \text{ alone, we recover the higher harmonic correction (6). The critical question becomes How large is } E_{1-2} \text{ as compared with } E_s? \text{ The energy losses per drive cycle } E_s \text{ and } E_{1-2} \text{ can be expressed in terms of work integrals as}
\]

\[
E_s = - \int_0^T F_1 d\omega dt
\]

and

\[
E_{1-2} = \int_0^T F_1 d\omega dt,
\]

respectively, Eqs. 7 and 8 are numerically integrated in the simulations of the stiff cantilever C1 in air and the soft cantilever C2 in liquid, both tapping on the dissipative sample (\( E_s = 100 \text{ MPa, } \eta_s = 10 \text{ Pa s} \)) in Fig. 1D and E, respectively. In air, the \( E_{1-2} \) is \( \sim3 \) orders of magnitude smaller than \( E_s \), and the conventional theory (2, 4, 5) relating phase contrast to tip-sample dissipation holds. In the liquid simulation, however, we have the opposite scenario: \( E_{1-2} \) is nearly an order of magnitude greater than \( E_s \) and therefore becomes the primary source of phase contrast. To demonstrate the implications of the energy loss \( E_{1-2} \) on phase-contrast imaging in liquids, we compare simulations of phase lag for the stiff cantilever C1 in air and the soft cantilever C2 in liquid tapping on a heterogeneous sample consisting of 3 distinct regions (Fig. 2A): Region I (\( E_s = 100 \text{ MPa, } \eta_s = 10 \text{ Pa s} \)), Region II (\( E_s = 100 \text{ MPa, } \eta_s = 0 \)), and Region III (\( E_s = 1 \text{ GPa, } \eta_s = 0 \)). The simulations in air (Fig. 2B) agree with the conventional theory

\[
(2-5, 7, 26) \text{ that the purely elastic regions (II, III) exhibit no phase contrast (26), and the dissipative region (I) has a greater phase lag, resulting in a positive phase contrast } \Delta \phi^{1-\text{II,III}} > 0 \text{ and } \Delta \phi^{1-\text{III}} < 0. \text{ Thus, when this sample is imaged in air with the stiff cantilever C1, Region I will appear brightest in the phase-lag image. However, Region III will appear brightest in the phase-lag image in liquid. The difference in phase contrast } \Delta \phi^{1-\text{III}} \text{ shown in Fig. 2} \text{ indicates that the primary sources of phase contrast are different in the simulations in air and liquid. In air, phase contrast derives from tip-sample dissipation } E_s, \text{ whereas in liquids, } E_{1-2} \text{ is the primary source of phase contrast. Although more viscous samples allow greater tip-sample dissipation, stiffer samples increase the momentary excitation of the second eigenmode (24), thus increasing the energy propagation } E_{1-2}. \text{ More precisely, it is the dominant short-range interaction between the tip and the sample (typically the elastic interactions) that governs the momentary excitation of the second eigenmode and hence the phase contrast. From Eq. 8, it is clear that the energy propagation } E_{1-2} \text{ can also be influenced by nonconservative tip-sample interaction forces. However, the viscoelastic contact forces described in Eq. 3, provide a poor mechanism for energy propagation } E_{1-2}. \text{ We demonstrate this concept in Fig. 3, where the } E_s-\eta_s \text{ parameter space for the viscoelastic contact modeled by Eqs. 2 and 3 with the soft cantilever C2 in liquid is shown. Simulations of the oscillating cantilever approaching the sample for both } A_0 = 10 \text{ nm and } A_0 = 1 \text{ nm are performed for a } 20 \times 20 \text{ grid of points equispaced in log scale for } E_s = [10 \text{ MPa, } 10 \text{ GPa}] \text{ and } \eta_s = [10 \text{ mPa s, } 100 \text{ Pa s}]. \text{ The phase-lag surface } \phi_1 (E_s, \eta_s) \text{ is constructed by collecting points on the phase-lag curves where } A_1/A_0 = 0.95 \text{ in each simulation. Fig. 3 demonstrates 3 important results. First, unless the sample is extremely soft and extremely viscous (in the neighborhood of } E_s = 10 \text{ MPa, } \eta_s = 100 \text{ Pa s}; \text{ see Fig. 3), phase lag is invariant to changes in viscosity. Second, for moderate- to low-viscosity samples, we find that phase lag } \phi_1 \text{ is monotonically increasing with respect to sample elasticity. Third, the trends in phase lag are quite robust—the phase lag simulated for an amplitude } A_0 = 10 \text{ nm (Fig. 3A) is almost identical to the simulations for } A_0 = 1 \text{ nm (Fig. 3B).}
\]
Fig. 3. Simulated phase lag $\phi_1$ vs. elastic modulus $E_s$ and viscosity $\eta_s$ for the soft cantilever C2 in liquid ($A_1/A_0 = 0.95$, $\alpha_1 = 0.9\alpha_0$) for (A) $A_0 = 10$ nm and (B) $A_0 = 1$ nm. In both cases, the phase lag $\phi_1$ is invariant with respect to viscosity except in the combination of extremely low elasticity and high viscosity. For moderate to low viscosities, $\phi_1$ is monotonically increasing with respect to $E_s$.

**Results and Discussion**

The present theory demonstrates that phase contrast for soft cantilevers in liquids can usually be attributed to energy propagation between eigenmodes, which is often mediated by conservative short-range interactions, rather than tip-sample dissipation. When elastic contact forces provide the dominant short-range interaction with the sample, phase contrast then maps the local elastic stiffness. Experimentally, we can determine the primary source of phase contrast based on the following argument. Because both energy losses, $E_s$ (dissipation) and $E_{1-2}$ (propagation), increase the phase lag, we can identify which is the primary energy loss given some prior knowledge of the sample’s dissipative properties. For example, when a soft, viscous (dissipative) biological sample is supported on a stiff, nondissipative substrate, then (i) if the soft biological materials have a greater phase lag and appear as a bright region in the phase-lag image in comparison with the stiff substrate (either mica or glass), then tip-sample dissipation is the primary source of phase contrast; or (ii) if the soft biological material should appear as a dark region in the phase-lag image in comparison with the stiff, nondissipative substrate, then $E_{1-2}$ must be the primary source of phase contrast. Finally, in the experiments there is typically a phase offset due to the electronics and/or excitation mechanism. However, this offset is not important to the interpretation of phase-contrast images. We need only to ensure that the image shows the variations in phase lag and not phase, since these are inverse images of each other.

In the first set of experiments, purple membrane (PM) is deposited on mica and gently imaged in buffer solution (see Materials and Methods for details). Fig. 4A was obtained with a soft, uniformly magnetically coated cantilever C1 that was excited magnetically. Dark regions in the phase-lag image correspond to the extracellular (EC) face of PM, confirming that energy propagation between eigenmodes is the primary source of the phase contrast.

Although the present theory considers magnetically excited cantilevers, the general form of the energy balance given in Eq. 4 applies to acoustically (base) excited cantilevers as well. Fig. 4B is obtained with a soft cantilever that is acoustically excited. The rough cytoplasmic (CP) face and smooth EC face can be identified in the topographic image. Again, mica appears bright in the phase-lag image, confirming that energy propagation between eigenmodes is the primary source of phase contrast. Furthermore, although it has been demonstrated in high salt-concentration buffers that the stiffnesses of the CP face and the EC face are indistinguishable by force-distance “jump-mode” imaging methods (18), the difference in stiffnesses between the CP and EC faces is clearly resolved in the phase-lag image. Moreover, the phase-lag image suggests that the CP face is slightly softer than the EC face as expected (18).

Fig. 4C contains high-resolution images of the EC face of PM, revealing the trimer assembly of bacteriorhodopsin (bR) which were obtained with acoustic excitation of the second eigenmode of a soft microcantilever (see Materials and Methods). The phase-lag image resolves the difference in stiffness between the bR assembly and surrounding lipids.

A second set of experiments was performed by using an acoustically excited Biolever, also in buffer solution. The topography and phase lag $\phi_1$ of a single $\phi 29$ virion with DNA packed inside and supported on a silanized glass substrate are shown in Fig. 5A and 5B, respectively. Fig. 5D shows the solid 3-dimensional electron microscopy (cryo-EM) reconstructed structure of a $\phi 29$ phage predicted by ref. 27 in blue. The cryo-EM reconstruction is imported into WSxM software (28) where a geometric tip dilation algorithm (29) is used to compute the expected dAFM topographic image accounting for the finite size of the tip. In this case, a tip radius $R = 8$ nm is assumed. Fig. 5 clearly resolves the capsid, collar, and tail knob of the virion.

The phase-lag image (Fig. 5B) clearly shows that the soft virus (stiffness $\approx 0.25$ N/m) appears dark compared with the glass ($E_s = 65$ GPa), again confirming that the source of phase contrast is energy propagation between eigenmodes. Interestingly, we find the stiffness of the virion changes along the longitudinal axis (Fig. 5C) in 3 distinct regions, showing how important features can be discerned from the phase-lag image. The phase-lag image clearly resolves the collar (green arrows), which appears as a bright region relative to the capsid and tail knob. We conclude that the collar is stiffer than the capsid. The tail knob is darkest in the phase-lag image, implying that its stiffness is least among...
gentle imaging conditions with high salt-concentration buffer solution where the electrostatic DLVO forces are screened (18, 19), and the short-range interactions with the sample are essentially local elastic contacts. Under these experimental conditions, phase-lag images can be interpreted as a mapping of local sample stiffness. It is only when the sample becomes extremely soft and viscous (see Fig. 3) that tip-sample dissipation plays a role in phase contrast.

Conventional theory that relates phase contrast, a key observable in dAFM, to tip-sample dissipation assumes that the cantilever motion can be described by using a single spatial eigenmode with, perhaps, higher harmonic temporal content. In ambient environments, high quality factors eliminate the energy propagation between eigenmodes, even in the case of bimodal (2-frequency) excitation (31, 32). We have shown that this assumption breaks down when soft cantilevers are used in liquids where the dynamics are naturally multimodal due to the momentary excitation of higher eigenmodes. For soft cantilevers in liquids, this leads to a new energy transfer channel where energy is transferred from the fundamental eigenmode to higher eigenmodes. In stark contrast to the situation in air or vacuum, this result implies that phase contrast of soft microcantilevers in liquids arises due to momentary vibrational energy transfer to the higher eigenmodes rather than tip-sample dissipation. Consequently, under controlled experimental conditions, phase-contrast images can be used to map intricate variations of local sample elasticity of soft biological samples in buffer solutions.

Materials and Methods

Numerical Simulations. All numerical simulations were performed in the open-access, web-based simulation suite VEDA v2.0 (22). Non-smooth/discontinuous models for tip-sample interaction forces often encountered in dAFM require special treatment in numerical studies (33–35). Accurate and efficient numerical integration of Eq. 1 for nonsmooth/discontinuous interaction models is achieved with the DDASKR routine with a root-finding algorithm based on the DASPK differential-algebraic equations software package (36, 37). The key advantage to the DDASKR routine over conventional routines is the ability to solve for the precise location of the sample boundary in state space and proceed to take the appropriate adaptive time steps while the tip is indenting the sample.

Experimental Setups. In the first set of experiments, the extracellular face of wild-type PM was deposited on mica in salt buffer. Wild-type bacteriorhodopsin isolated from Halobacterium salinarum strain S9 as PM in the form of lyophilized powder was obtained from Sigma-Aldrich. The PM was deposited on freshly cleaved mica in salt buffer [300 mM KCl, 20 mM Tris-HCl (18)] and imaged by using an Agilent 5300 AFM system. Results are shown in Fig. 4A was obtained with a soft, magnetically coated cantilever C1 that was excited magnetically (38) at $\omega_0 = 15$ nm/s ($A_0 = 0.8$). Fig. 4B was obtained with an Olympus cantilever (OMCL-RC800PB: $k_1 = 0.58$ N/m, $Q_1 = 3.3$, $\omega_0 = 2\pi \times 9.3$ kHz, $\omega_0/\pi = 7.1$) nominal stiffness $k_1 = 0.73$ N/m by using an acoustic excitation at $A_0 = 1.4$ nm ($A_1/A_0 = 0.65$). Fig. 4C was obtained with an Olympus cantilever (OMCL-RC800PB: $k_1 = 0.99$ N/m, $Q_1 = 1.0$, $Q_2 = 3.8$, $\omega_0 = 2\pi \times 4.4$ kHz, $\omega_0/\pi = 7.3$) nominal stiffness $k_1 = 0.10$ N/m, with an acoustic excitation of the second eigenmode at $A_0 \approx 0.3$ nm ($A_1/A_0 = 0.9$). Images were rendered in WSxM software (28). No filters were applied to the images except for those in Fig. 4C, which were filtered using the Mexican hat wavelet filter (39) at a scale of 1.2 nm.

In a second set experiments, a Staphylococcus virus capsid on a glass substrate was imaged in buffer solution by using an Olympus Biolever (BL-RC150VB; $k_1 = 0.036$ N/m, $Q_1 = 1.2$, $Q_2 = 2\pi \times 9.3$ kHz, $\omega_0/\pi = 7.6$) nominal stiffness $k_1 = 0.03$ N/m, that was acoustically excited at a $A_0 = 9$ nm ($A_1/A_0 = 0.8$).

High-resolution micrographs of mature virions were performed by using an atomic force microscope in a Nanosurf Electro
c

Lab Scan probe microscopes. A single drop of 20 ml of stock solution virions was deposited on silicon wafer and imaged by using a Nanosurf Electro
c

Lab Scans, which were filtered using the Mexican hat wavelet filter (39) at a scale of 1.2 nm.


dagnosis of local elastic stiffness and geometric tip dilation (29). The structural features of $\phi 29$, such as the collars (green arrows) and the hollow tail knobs are clearly resolved in the phase-lag image demonstrating the power phase-lag imaging as a mapping of local elastic stiffness/structural integrity of the virion.

The present theory is also inadvertently supported by prior experimental results using soft cantilevers in liquids. For example, in experiments (30) performed on a patterned surface of hydroxyl- and carboxyl-terminated self-assembled monolayers in 10-mM phosphate buffer, the soft carboxyl region appears dark in the phase-lag image. In experiments (7) performed on PM deposited on a mica substrate in buffer solution (300 mM KCl, 10 mM Tris-HCl), PM appears dark in the phase-lag image. Conventional theory (2, 4, 7, 30) relating phase-lag to tip-sample dissipation predicts the soft, viscous (dissipative) regions will appear brighter in the phase-lag image (greater phase lag) compared with the theoretical situation in air or vacuum, this result implies that phase contrast of soft microcantilevers in liquids arises due to momentary vibrational energy transfer to the higher eigenmodes rather than tip-sample dissipation. Consequently, under controlled experimental conditions, phase-contrast images can be used to map intricate variations of local sample elasticity of soft biological samples in buffer solutions.

Materials and Methods

Numerical Simulations. All numerical simulations were performed in the open-access, web-based simulation suite VEDA v2.0 (22). Non-smooth/discontinuous models for tip-sample interaction forces often encountered in dAFM require special treatment in numerical studies (33–35). Accurate and efficient numerical integration of Eq. 1 for nonsmooth/discontinuous interaction models is achieved with the DDASKR routine with a root-finding algorithm based on the DASPK differential-algebraic equations software package (36, 37). The key advantage to the DDASKR routine over conventional routines is the ability to solve for the precise location of the sample boundary in state space and proceed to take the appropriate adaptive time steps while the tip is indenting the sample.

Experimental Setups. In the first set of experiments, the extracellular face of wild-type PM was deposited on mica in salt buffer. Wild-type bacteriorhodopsin isolated from Halobacterium salinarum strain S9 as PM in the form of lyophilized powder was obtained from Sigma-Aldrich. The PM was deposited on freshly cleaved mica in salt buffer [300 mM KCl, 20 mM Tris-HCl (18)] and imaged by using an Agilent 5300 AFM system. Results are shown in Fig. 4A was obtained with a soft, magnetically coated cantilever C1 that was excited magnetically (38) at $A_0 = 15$ nm ($A_1/A_0 = 0.8$). Fig. 4B was obtained with an Olympus cantilever (OMCL-RC800PB: $k_1 = 0.58$ N/m, $Q_1 = 3.3$, $\omega_0 = 2\pi \times 9.3$ kHz, $\omega_0/\pi = 7.1$) nominal stiffness $k_1 = 0.73$ N/m by using an acoustic excitation at $A_0 = 1.4$ nm ($A_1/A_0 = 0.65$). Fig. 4C was obtained with an Olympus cantilever (OMCL-RC800PB: $k_1 = 0.99$ N/m, $Q_1 = 1.0$, $Q_2 = 3.8$, $\omega_0 = 2\pi \times 4.4$ kHz, $\omega_0/\pi = 7.3$) nominal stiffness $k_1 = 0.10$ N/m, with an acoustic excitation of the second eigenmode at $A_0 \approx 0.3$ nm ($A_1/A_0 = 0.9$). Images were rendered in WSxM software (28). No filters were applied to the images except for those in Fig. 4C, which were filtered using the Mexican hat wavelet filter (39) at a scale of 1.2 nm.

In a second set experiments, a Staphylococcus virus capsid on a glass substrate was imaged in buffer solution by using an Olympus Biolever (BL-RC150VB; $k_1 = 0.036$ N/m, $Q_1 = 1.2$, $Q_2 = 2\pi \times 9.3$ kHz, $\omega_0/\pi = 7.6$) nominal stiffness $k_1 = 0.03$ N/m, that was acoustically excited at a $A_0 = 9$ nm ($A_1/A_0 = 0.8$).

High-resolution micrographs of mature virions were performed by using an atomic force microscope in a Nanosurf Electro
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Lab Scan probe microscopes. A single drop of 20 ml of stock solution virions was deposited on silicon wafer and imaged by using a Nanosurf Electro
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Lab Scans, which were filtered using the Mexican hat wavelet filter (39) at a scale of 1.2 nm.