Noise in combined optical microscopy and dynamic force spectroscopy: Toward *in vivo* hydration measurements

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Noncontact atomic force microscopy (AFM) using frequency modulation (FM) detection allows atomic resolution to be obtained in vacuum on a variety of insulating surfaces and molecular deposits. This technique has recently been extended to liquid environments, and, in addition to atomic scale contrast, FM-AFM in liquid allows measurement of ordered liquid layers above surfaces. The role of water and ions in biological processes is of great interest and in order to localize fluorescently tagged structures, such as proteins, optical microscopy combined with AFM provides an invaluable tool. Thus, to take advantage of the wealth of optical identification techniques available in biology, the AFM must be coupled to an optical microscope. Such systems are commercially available, but mechanical noise due to vibrations is a major concern compared with the compact, specialized instruments used to measure hydration structure to date. In this article the authors demonstrate, through both modeling and measurement, that hydration structure can be measured on such a commercial "bio-AFM," despite the additional noise sources present in these instruments and that with the addition of a bandpass filter and amplifier it can be done "out-of-the-box" using only commercial electronics and tips. Thus, hydration structure measurements are accessible to virtually any laboratory with such a system. © *2010 American Vacuum Society.* [DOI: 10.1116/1.3368462]

I. INTRODUCTION

Noncontact atomic force microscopy (NC-AFM) using frequency modulation (FM) detection, exploits the change in resonance frequency of an oscillating cantilever to measure the interaction between a sharp tip and sample.[1] In vacuum environments, NC-AFM using FM detection is becoming a well developed technique giving atomic and molecular resolution in many systems.[2–4] More recently FM detection has been applied in liquid environments. The inherent difficulties of measuring the frequency shift in an environment where the Q factor of the cantilever is 1000–10 000 times lower than in vacuum have been overcome and atomic and molecular resolution obtained.[5–7] The potential of the FM-AFM technique to study, particularly by dynamic force spectroscopy, the molecular arrangement of water and ions around biomolecules and cells in physiological conditions makes this an extremely important development in AFM, and it has already been applied to model lipid systems.[8–10]

Forces arising from confined liquids have been studied for many years using surface force apparatus, which measures the force between, for example, two curved mica surfaces as they are squeezed together.[11,12] This instrument allows the hydration structure above surfaces to be measured, but the combination of AFM's ability to image and manipulate at the nanometer scale, as well as measure the oscillatory hydration force, makes it attractive particularly for investigations at the single molecule level allowing local measurement of the hydration structure.

The types of AFMs used in these investigations are usually specialized instruments built for the purpose such as those based on ultralow noise deflection sensors,[7] thermal-mechanical noise measurements,[13] using specialized carbon nanotube tips[14] or magnetically actuated tips.[15] However, in terms of enabling hydration structure measurements in biological systems, in particular live cells, the work is greatly facilitated if the AFM is mounted on an inverted optical microscope to take advantage of the myriad of fluorescent labels, including genetic tagging with fluorescent proteins, to target the measurement to a structure of interest. Such "bio-AFMs" are commercially available from a number of vendors and have reached a stage of sophisticated integration between the optical and scanning probe microscopes. Access to simultaneous optical information is particularly important if one wishes to investigate the hydration structure near a feature (e.g., an ion channel or other protein of interest) which can be genetically tagged with a fluorescent protein. This allows the experiment to be localized to the structure of interest while it is in a physiologically relevant state, i.e., part of the cell membrane rather than isolated and deposited on a mica surface. In this article we show that noise characterization of a commercially available bio-AFM, an Asylum MFP-3D-BIO, and modeling indicate that hydration structure can indeed be detected by a commercial AFM on an inverted optical microscope using only commercial components and tips. We have performed measurements in water and octamethylcyclotetrasiloxane (OMCTS) above mica to demon-

strate, via histograms of measured intermolecular spacings, that this in fact can be achieved. Furthermore, we show that hydration structure can also be detected in phase modulation (PM) as well as FM mode. All major commercial AFMs provide an amplitude modulation imaging mode (ac mode in the Asylum Research software, for example) and allow the phase between the cantilever oscillation and its drive signal to be collected as complementary data. If this mode is used for dynamic force spectroscopy, hydration structure is evident in the phase-distance curve, just as it is in the frequency shift distance curve in FM mode. This means that with the addition of a bandpass filter and amplifier a commercial bio-AFM is ready to measure hydration structure out-of-the-box.

II. NOISE CHARACTERIZATION

The noise sources and dependence on parameters in the frequency shift signal in FM-AFM has been presented in the literature and is well described in a recent paper for low Q environments such as liquids.[16] However, all models to date neglect the influence of vibrations which are not compensated for by the vibration isolation of the microscope. In most dedicated FM-AFM systems, these are not large compared to other noise sources and can safely be neglected. However, for the case where we wish to combine AFM with optical microscopy in order to access additional biologically relevant information, the AFM is mounted on an inverted microscope which presents a greater challenge in vibration reduction. In our case this is a MFP-3D-BIO (Asylum Research, Santa Barbara CA) mounted on an inverted fluorescence microscope (Olympus IX71). The microscope is inside an acoustic isolation chamber (BCH-45 from TMC) and sits on an active vibration isolation table (TS300 from TableStable). AFM vendor specifications typically state 50 pm of mechanical noise at the sample stage (i.e., the residual vibrations originating in the environment which are attenuated by the vibration isolation system) in a 1.5 kHz bandwidth. The noise level for this specification is usually assessed on a rigid substrate such as mica mounted on a standard 1 in. × 3 in. × 1 mm microscope slide in ambient conditions (air at room temperature). This does not correspond to realistic conditions for bio-AFM experiments where the sample is typically supported on a thin (~170 μm) no. 1.5 coverslip in a fluid cell, possibly with an oil immersion objective in contact with the glass and a cooled charge-coupled device (CCD) camera running.

We have made measurements to quantify various experimental scenarios in our system. Results are shown in Fig. 1. Figure 1 shows the deflection noise density for a tip in contact with a mica surface glued to a 25 mm round no. 1.5 coverslip submerged in milli-Q filtered distilled water in the Asylum Research closed liquid cell. Following the procedure recommended by the vendor to establish that the instrument is meeting the noise specification, the tip (a silicon nitride cantilever which gives a sensitivity of ~33 mV/nm with the MFP-3D deflection sensor) is brought into contact with the mica to a set deflection of ~30 nm at which point the feedback is decreased to the lowest possible value. Without an
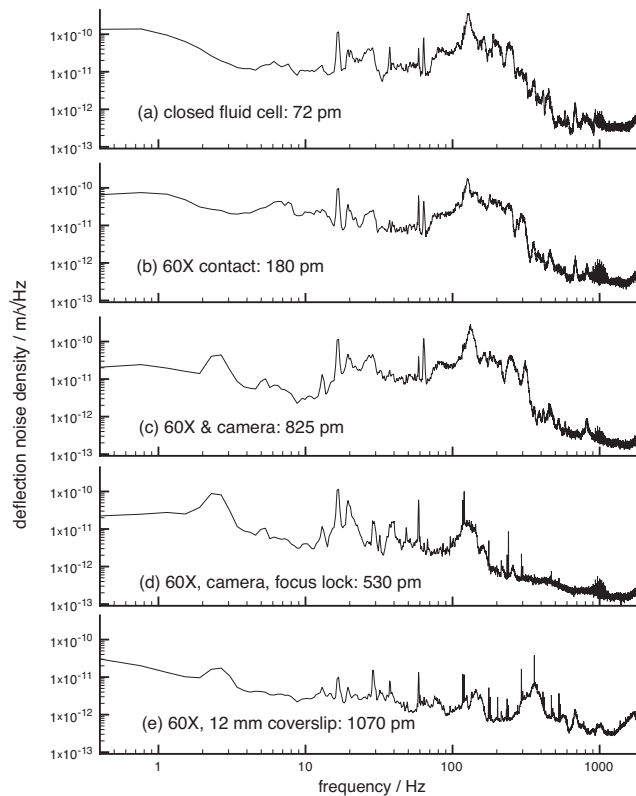

FIG. 1. Deflection noise density characterization of the mechanical noise in our MFP-3D-BIO system. These curves show the power spectrum of the deflection signal of a cantilever in water in the Asylum Research closed liquid cell which is in contact with a mica surface on a 25 mm diameter no. 1.5 coverslip with the virtually zero feedback. The baseline noise is 72 pm rms. This increases to 180 pm when the 60× oil immersion objective is brought into contact with the coverslip. With the objective in contact and liquid cooled CCD running, the rms mechanical noise increases to 825 pm This can be mediated by increasing the focus tension of the optical microscope and locking the objective focus, reducing the value to 530 pm. Using smaller 12 mm diameter coverslips produces stronger mechanical coupling and 1070 pm rms.

objective in contact with the surface or the camera running the rms noise level is 72 pm. Once the objective, an oil immersion 60× high numerical aperture (NA) lens suitable for epifluoresence and total internal reflection fluorescence microscopy of single molecules (Olympus PLAPON60XOTIRFM), is brought into contact with the coverslip, the noise increases to 180 pm rms. With microscope's CCD camera (a liquid cooled elctron-multiplied Cascade 512B from Princeton Instruments) running the noise increases to 825 pm rms. Locking the objective and increasing the focus tension to maximum decreases the noise coupling giving a rms value of 530 pm while switching to a smaller 12 mm coverslip increases the noise coupling to 1070 pm rms.

While making hydration structure measurements in the presence of these noise levels seems daunting, since the intramolecular spacings in water are ~0.24 nm, fluorescence imaging using a nonimmersion high-NA lens (such as the Olympus UPLFLN 60X 0.9NA) and a triggering system to stop the camera cooling during acquisition of frequency shift distance curves may be a successful strategy to keep the

noise floor as close as possible to the base value. To investigate the feasibility of this experimental scenario, we model the frequency shift distance curves in the presence of our baseline noise.

III. NOISE MODELING

As mentioned above, a noise model for FM-AFM in liquid (low Q) environments, has been developed by Kobayashi *et al.*,[16] 2009. In this model the noise in the FM or frequency shift signal is given by the sum of a contribution from thermal noise, the deflection sensor noise, and the contribution of deflection sensor noise to the drive signal, so-called oscillator noise. Mechanical vibration noise is assumed small and neglected. Our noise characterization shows that, as expected for an AFM mounted on an inverted microscope system geared toward imaging a biological specimen optically as well as keeping it alive in a fluid cell, mechanical vibration noise is not negligible particularly for oil immersion objectives in contact with the coverslip and cooled, high sensitivity cameras running. A mechanical vibration noise term was added to the model using the expression

$$N_{\text{FM(vibs)}} = \frac{\partial \Delta f}{\partial z} \delta z \tag{1}$$

in analogy with scanning tunneling microscopy.[17] Additionally we have added a simple one pole low pass filter to simulate the low-pass filter (1 kHz, nonuser configurable) of our frequency detector (Nanosurf, EasyPLL). Calculated frequency shift noise densities are shown in Fig. 2. The dotted curves are calculated using the model in Kobayashi *et al.*,[16] 2009 and do not include vibrations. Curves are calculated for increasing values of the deflection sensor noise: 5, 10, 50, 100, 200, 500, and 1000 fm/$\sqrt{\text{Hz}}$. The solid group of curves includes the effects of vibrations at a level of 1.6 pm/$\sqrt{\text{Hz}}$ which corresponds to the baseline noise on an AFM mounted on an inverted optical microscope system. In this case the noise density is completely dominated by mechanical vibration noise for deflection sensors with noise $< \sim 200$ fm/$\sqrt{\text{Hz}}$. This is particularly interesting as much recent effort in liquid FM-AFM has gone into decreasing the deflection sensor noise density[5–7] but for combined optical and frequency shift measurement this may not be the limiting factor. The deflection sensor noise of our commercial system is 300 fm/$\sqrt{\text{Hz}}$ and, according to Fig. 2, the vibration noise term is still the most important contribution to the noise density even up to 500 fm/$\sqrt{\text{Hz}}$.

Hydration structure has been measured and the corresponding force calculated, such as Higgins *et al.*,[8] 2006. Using this force law as a template, we have found an empirical fitting function for the force law which gives us an analytic function for the force law and allows us to directly explore the effects of various parameters such as deflection sensor and vibration noise on an instrument's capacity to detect the peaks due to hydration structure. This allows the simple formula[18] to compute frequency shift from force to be used to generate model frequency shift distance curves. At this point, noise from the model above, can be added to the fre-
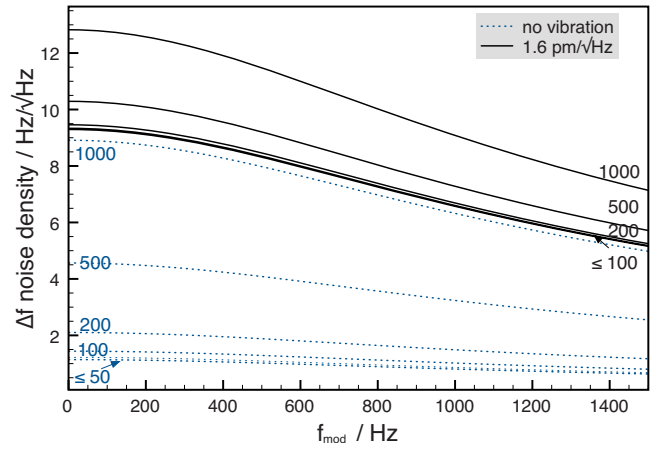

FIG. 2. (Color online) This plot shows the frequency shift noise density calculated using the model in Kobayashi *et al.* (Ref. 16) 2009 modified to include the noise due to vibration as well as the roll-off in frequency shift with increasing f_{mod}. The force law used in this calculation was estimated from frequency shift distance data measured in our AFM (Ref. 22). The noise density has been calculated for tip-sample separation of 1 nm and several deflection sensor noise levels (5, 10, 50, 100, 200, 500, and 1000 fm/$\sqrt{\text{Hz}}$). Other model parameters are as follows: an oscillation amplitude of 1 nm, a cantilever spring constant of 40 N/m, an oscillation frequency of 150 kHz, and Q factor of 6 in liquid. Solid black curves include vibrations, modeled with a flat frequency response, at a rms level corresponding to the baseline noise on an AFM mounted on an inverted optical microscope. Vibrations are excluded from the calculation in the dotted curves. For lower values of the deflection sensor noise (<100 fm/$\sqrt{\text{Hz}}$) the vibrations dominate the noise density and are a significant contribution at higher deflection sensor noise levels (Asylum AFM is ~300 fm/$\sqrt{\text{Hz}}$).

quency shift distance curve according to the calculated noise density. By inverting the problem, i.e., recalculating the force law from the noisy frequency shift distance curve, we can establish the parameter space for which hydration structure is observable by attempting to successfully fit the force law to recover the peak spacing. This calculation is shown in Fig. 3. Figure 3(a) shows the frequency shift computed from the empirical model based on Higgins *et al.*,[8] 2006. The points represent the same curve with the addition of noise characteristic of the deflection sensor and vibration of our MFP-3D-BIO AFM. Figure 3(b) shows the result of calculating the force from the noisy frequency shift distance curve. The hydration peaks are visible and the fitting function returns parameters close to those of the original input. The input spacing was 0.305 nm and the value estimated by nonlinear curve fitting of 30 simulated curves was 0.301 nm with a standard deviation of 0.07 nm. Among the parameters of the empirical force law, the peak spacing is particularly robust to noise. Although systematically offset from the input value, from simulation to simulation, the estimated peak spacing and its standard deviation deviated from the above by $<1\%$ while differences for the other parameters range typically range from 2%–24%. The noise due to vibrations has a less dramatic influence on hydration structure measurements than might be expected at first glance. This is due to the shape of the curve: at each hydration peak (and trough) $\partial \Delta f / \partial z$ will be small because the gradient of the frequency shift is ~ 0
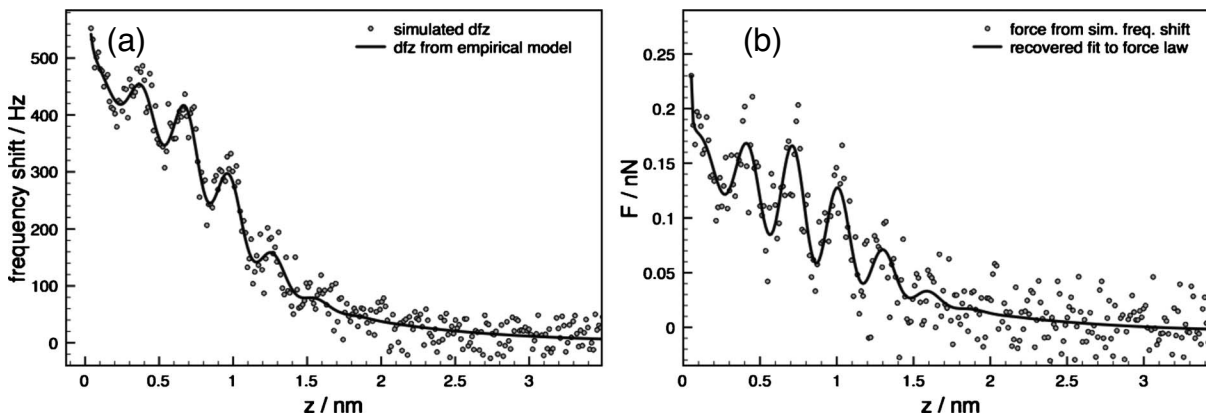
FIG. 3. Solid curve in (a) is the frequency shift distance curve calculated using the empirical results of Higgins *et al.*, 2006 (Ref. 8). The points represent the addition of noise appropriate to our AFM including vibrations (deflection sensor noise: 300 fm/$\sqrt{Hz}$ and vibration noise: 1.6 pm/$\sqrt{Hz}$). The problem is inverted in (b) where the data points are the force calculated from the frequency shift in (a) and the solid line is the empirical force law fit to the data. The parameters recovered from the fit are close to those of the original input.

near the minima and maxima, and Eq. (1) shows that this will limit the noise contribution from this term. Thus, the form of the hydration structure actually suppresses the effect of the noise.

IV. HYDRATION MEASUREMENTS

Our modeling predicts that the signature of hydration structure should be observable on a commercial AFM on an inverted optical microscope. To demonstrate this, we performed frequency shift distance measurements in water and OMCTS above mica mounted in the Asylum closed fluid cell. Figures 4(a) and 4(b) show typical frequency shift distance curves in water and OMCTS. These curves were collected using PPP-NCLR cantilevers (Nanosensors) with spring constants of ~40 N/m at sampling rates of 1000–1500 Hz. The resonance frequency in liquid for these cantilevers was typically 65–75 kHz and was located by looking at the nondriven, thermal spectrum. They were driven by self-oscillation with oscillator control electronics which consists of a phase shifter and an amplitude controller. (A modified NanoSurf EasyPLL was used for FM detection and excitation in a self-oscillation mode. The amplitude controller was modified to include both proportional and integral gain with automatic gain control which was not originally available on this model.) The deflection signal was bandpass filtered with a bandwidth of 7–10 kHz. The oscillation amplitude was between 0.7 and 1 nm.

Hundreds of frequency shift distance curves from different experiments and tips in water and OMCTS were analyzed using a supervised peak finding algorithm which applies a Savitzky–Golay filter and automatically locates peaks in the frequency shift distance curves. Molecular spacings were calculated while allowing user intervention to avoid obvious false positives. The raw (points) and filtered (lines) frequency shift distance curves are shown in Figs. 4(a) and 4(b). In water, the average peak spacing was found to be 0.24 nm and in OMCTS the average peak spacing was found to be

0.75 nm. Histograms of the peak spacings are shown in the insets in Figs. 4(a) and 4(b). These values are in good agreement with those reported using custom AFM's with carbon nanotube tips.[19] It should be noted that the intramolecular spacing of water continues to be a topic of intense research. Results from x-ray measurements[20] and Monte Carlo simulations[21] give a spacing of 0.13 nm. Reported values at NC-AFM 2009 range from 0.13 to 0.38 nm with some results indicating a distance dependent spacing. Accordingly, the asymmetry we observe in histogram of peak spacings for water is likely significant and further investigation is needed understand the origin of the distribution of molecular spacings.

In addition to PPP-NCLR tips, PPP-FMAuD (spring constant ~2 N/m, resonance frequency in water 25 kHz) were found to be an excellent commercial option for hydration measurements. given the stable nature of the gold coating in aqueous solution and the low spring constant. Using PPP-FMAuD tips, hydration structure measurements were taken in the usual way by measuring frequency shift distance curves and, in addition, by exciting the cantilever at a fixed frequency and monitoring the phase between the oscillation and the drive signal (PM mode). Figure 4(c) shows a frequency shift distance curve in OMCTS over mica acquired using a PPP-FMAuD and Fig. 4(d) shows a phase-distance curve. The initial oscillation amplitude (far from the sample surface) for these measurements was 0.84 nm. The oscillatory signature of the hydration structure is present in the phase-distance curve and a high signal-to-noise ratio of the PM measurement is evident. This enhanced signal may be due to the fact that in PM mode the amplitude also changes: Decreasing amplitude may give higher force sensitivity. The enhanced signal-to-noise may also be the result of the removal of the oscillator noise associated with the self-oscillation excitation used in FM mode. The practical advantage of using PM mode to measure hydration structure is that not only does it provide high signal-to-noise, allowing the molecular spacing to be determined, but also makes the mea-
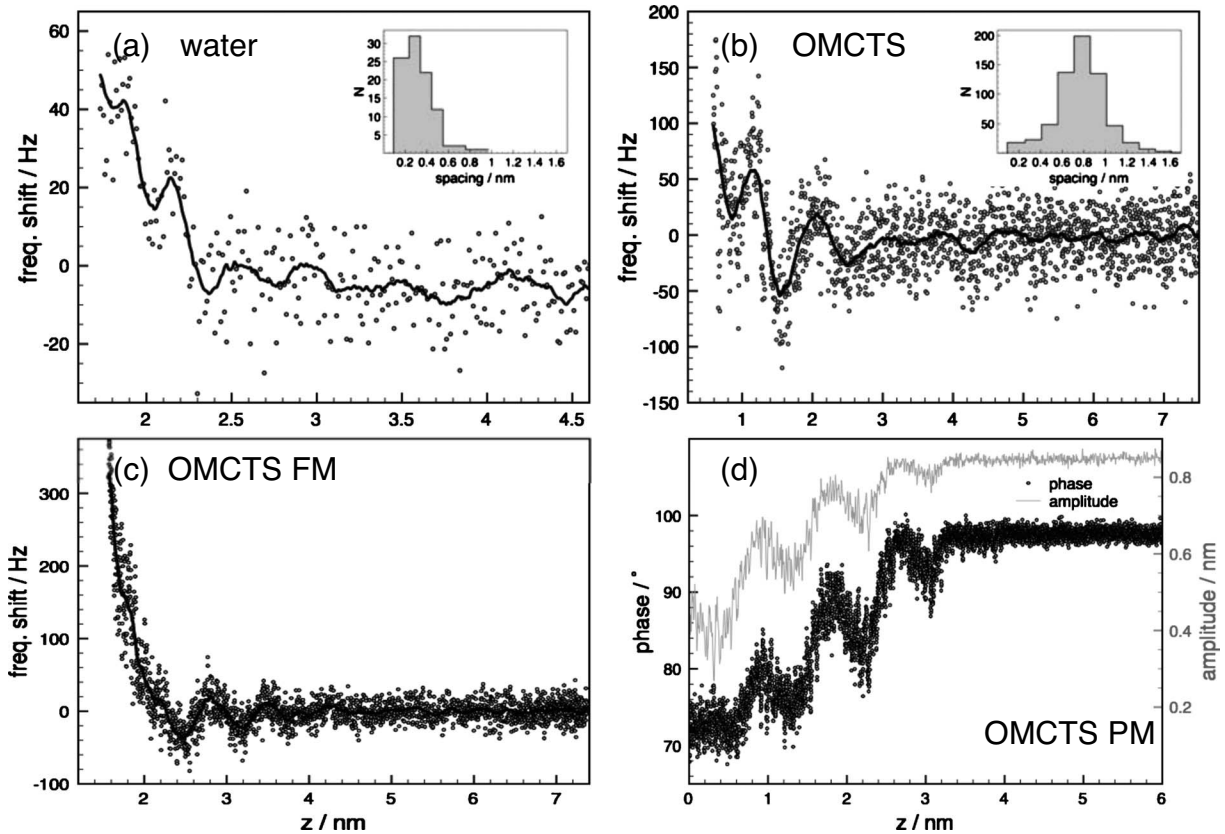
FIG. 4. Shown in (a) and (b) are sample frequency shift distance curves collected above mica in water (a) and mica in OMCTS (b). Raw data are shown as points while the lines have been filtered using a Savitzky–Golay filter for analysis using our supervised peak finding code. Hundreds of frequency shift distance curves from different experiments and tips in water and OMCTS were analyzed and molecular spacings calculated to generate the histograms shown in the insets in (a) and (b). In water, the average peak spacing was found to be 0.24 nm and in OMCTS the average peak spacing was found to be 0.75 nm in good agreement with reported values (Ref. 19). It should be noted that although the measured quantity is a frequency shift, under mechanical excitation in liquid, the measured frequency shift can be affected by parasitic resonances (Refs. 23 and 24). but peak spacings are unaffected. We also compared FM (c) and PM (d) measurements using Nanosensor PPP-FMAuD tips and found the hydration peaks to be readily observable in AM mode with very high signal-to-noise.

surement available to a wide range of experimentalists with standard commercial AFM equipment. Custom tips, electronics, and AFM hardware are not needed: The addition of a bandpass filter and amplifier is sufficient.

V. CONCLUSIONS

Combined optical and atomic force microscopy is needed to take advantage of genetic labeling of proteins with fluorescent reporters so that they can be targeted for hydration structure measurements *in vivo*. We have shown that for an AFM mounted on an inverted optical microscope, with the sample in a fluid cell, the contribution to the noise density due to vibrations is significant and can dominate over noise from the deflection sensor for deflection sensor noise densities <200 fm/$\sqrt{Hz}$. Our modeling shows that despite the presence of the noise from all sources, including vibrations, the spacing of the hydration peaks can be recovered reliably by fitting an empirical force law. In fact, this parameter is particularly robust to the presence of noise which is likely due to the nature of the hydration force law: around the minima and maxima the gradient of the frequency shift is ~ 0 which means the contribution from vibrations is reduced. We

have demonstrated the measurement of hydration structure on our MFP-3D-BIO in water and OMCTS above mica. Average peak spacings of 0.24 nm for water and 0.75 nm for OMCTS were recovered. Additionally we compared FM and PM hydration measurements using Nanosensor PPP-FMAuD tips. We found the hydration peaks to be readily observable using the PM method. Further study is needed to determine if there is an intrinsic advantage to the PM measurement in liquid as practically it simplifies the experimental setup: with the addition of a bandpass filter and an amplifier these types of measurements can be made on a commercial bio-AFM out-of-the-box.

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