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High Spatial Resolution Imaging with Near-Field Scanning Optical Microscopy in Liquids

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The mechanism of tuning fork-based shear-force near-field scanning optical microscopy is investigated to determine optimal experimental conditions for imaging soft samples immersed in liquid. High feedback sensitivity and stability are obtained when only the fiber probe, i.e., excluding the tuning fork prongs, is immersed in solution, which also avoids electrical shorting in conductive (i.e., buffer) solutions. Images of MEH-PPV were obtained with comparable spatial resolution in both air and water. High optical resolution (∼160 nm fwhm) was observed.

Near-field scanning optical microscopy (NSOM) has emerged as a versatile analytical tool that combines the high resolution of scanning probe microscopy with the noninvasiveness of optical microscopy.[1] Despite rapid advances in NSOM for subwavelength imaging of a wide variety of samples, it has not yet been applied with routine success for imaging live biological samples. Imaging of hydrated cells avoids morphology perturbations due to the drying or freezing of the samples required by other imaging techniques, e.g., transmission electron microscopy (TEM), and also allows for the exciting possibility of measuring dynamic processes in real time. Unfortunately, imaging soft samples immersed in solution with noncontact NSOM is not trivial. Several problems, including compensating for evaporation to maintain a stable feedback and accommodating the decrease in sensitivity of the feedback mechanism due to the liquid viscosity and drag, must be addressed before NSOM imaging in solution can be performed with the same high spatial resolution that can be achieved in air or vacuum.[2,3]

In the past few years, several groups have explored the NSOM design using shear force feedback, wherein tip vibrations are monitored as a function of tip−sample separation, to image samples in a liquid environment.[4−9] To date, all experiments have produced low resolution, unstable feedback, and low feedback sensitivity, which is exhibited by the significant decrease (>40%) in the quality factors when the mechanical detector is immersed in liquid.[10] Large piezoelements are routinely used for mechanical detection but exhibit multiple resonance frequencies with relatively low *Q*-factor values (<200) in air. Even upon shallow immersion in liquid, this method suffers from a large decrease in the mechanical detector's sensitivity.[4−7] Karrai and Grober developed a shear-force feedback mechanism using a low resonance frequency quartz tuning fork (ω_0 ∼33 kHz) with an optical fiber probe mounted to one of the prongs.[8] This technique provides a single resonance frequency and produces high topographical resolution at low tip amplitudes (<1 nm) in air but also exhibits significantly decreased feedback sensitivity when immersed in liquid.

Recently, Rensen et al.[9] employed a high resonance frequency tuning fork (ω_0 ∼97 kHz) for liquid NSOM, which provides greater sensitivity and more stable feedback than a 33-kHz quartz tuning fork.[9,11] In their work, the *Q*-factors decreased as a function of tuning fork immersion depth, but after immersing the prongs more than halfway up the tuning fork tines, the *Q*-factor was observed to increase. Based on this observed *Q*-factor recovery, they suggest complete immersion of the prongs to achieve optimal conditions for scanning in water. Moreover, with the prongs immersed completely, the solution level does not need to be absolutely constant, and as a result, no special considerations were needed to accommodate evaporation.

Here we present an alternative method of performing NSOM in liquids, demonstrating that optimal scanning and higher spatial resolution in solution are achieved with only the fiber probe, which is mounted on a high-frequency tuning fork, immersed in liquid. A sample cell (Figure 1) was constructed to maintain a constant liquid level throughout the scanning period, and we modified a chemically etched, uncoated SiO_2 fiber-optic tip to overhang the fork prong edge ∼3× more (∼600 μm) than a commercial (Thermomicroscopes) tip (∼200 μm, Figure 2). These modifications allowed us to obtain resonance frequencies higher than 90 kHz and high *Q*-factors (∼400) when only the fiber-optic probe was immersed. Keeping the tuning fork dry also facilitated scanning in a greater variety of liquids, including buffer solutions and organics, which was not possible with the method of Rensen et al. due to electrical shorting problems.

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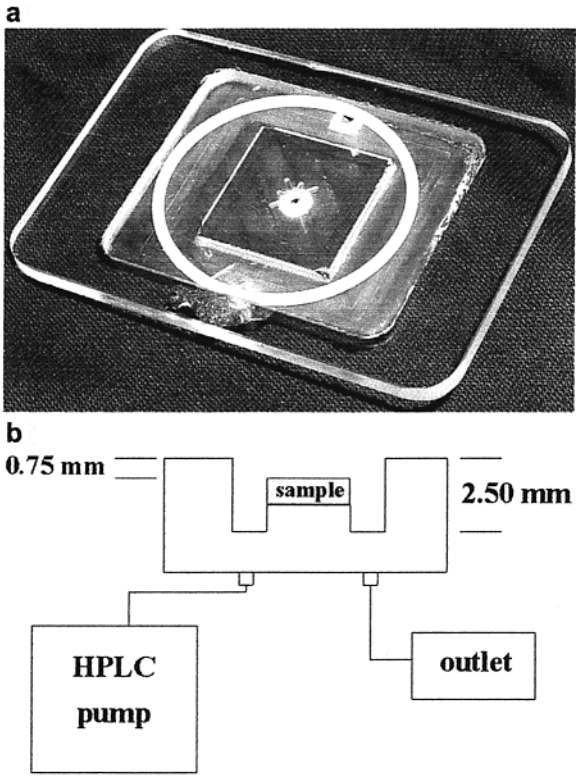

Figure 1. (a) Sample cell. (b) Side-on schematic diagram of the sample cell setup. The glass slide was milled to a depth of ~2.5 mm and the sample placed on an island that is located in the center of the well. An acrylic O-ring is used to create a negative meniscus for a thinner solution layer, and an inlet and outlet controls the flow of liquid into the cell. The liquid level is controlled by an HPLC pump which typically operated at a flow rate of ~50 μL/min to account for evaporation loss.

To analytically calculate the physical parameters of the tuning fork system upon immersion in water, salt, and phosphate buffer solutions, we use an externally driven, damped, simple harmonic oscillator model.[3,8] The equation of motion is

$$\ddot{x} + \beta\dot{x} + \omega_0^2 x = F_0 \cos \omega t \qquad (1)$$

where x is the displacement of the probe in the x-direction during oscillation, ω_0 is the resonance frequency in air, the cosine term describes the driving force, and β is the damping coefficient, defined as

$$\beta = b/2m \qquad (2)$$

Here, b is the damping constant and m is the mass of the tuning fork and probe. These measurements were performed far from the sample surface, and β represents only the liquid contribution to the damping of tip vibrations. Q is the quality factor, which is a measure of the damping effects on the probe and defines the sensitivity of the system, and is given by

$$Q = \omega_R/2\beta \approx \omega_R/\Delta\omega \qquad (3)$$

where ω_R is the resonance frequency in the liquid environment and $\Delta\omega$ is the fwhm of the resonance curve. The approximation

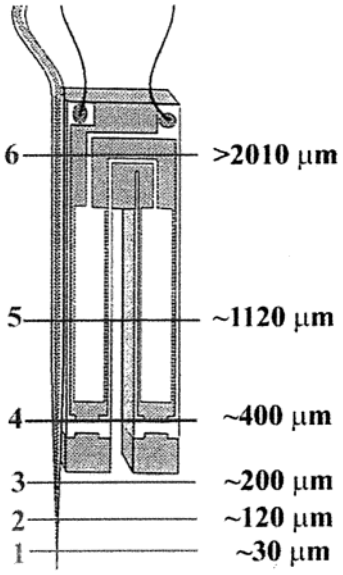

Figure 2. Depths of immersion. Lines 1−6 are at the following immersion depths: 1, ~30 μm; 2, ~120 μm; 3, ~200 μm; 4, ~400 μm; 5, ~1120 μm; 6, >2010 μm.[22]

in eq 3 is valid if the amplitudes are small;[12] in our system, although x_0 (the tip amplitude in air) is not directly measured, its amplitude is always less than 1 nm. In all calculations here, x_0 is taken to be 0.1 nm. The damping force is given by

$$F_D \cong k_0 x_0 / Q\sqrt{3} \qquad (4)$$

where k_0 is the spring constant of the tuning fork in air. The damping force includes the liquid contributions to the tip damping and the drag effects due to the scanning motion.

EXPERIMENTAL SECTION

Sample Cell. To produce a stable liquid level over the sample, a glass sample cell was fabricated. A 5-mm-thick glass slide was milled to a depth of 2.5 mm to produce an island platform as shown in Figure 1. When placed on the platform, the sample is ~750 μm below the edge of the glass slide. An acrylic O-ring is placed around the sample island for additional control of the liquid level. To accommodate for evaporation, an inlet and outlet located at the well bottom were used. The inlet is connected to a LKB Bromma 2150 high-pressure liquid chromatography (HPLC) pump via a solvent splitter and needle valve.

Dipping Experiments. A commercial NSOM system (Thermomicroscopes, Lumina) equipped with the above nonoptical feedback system was employed for all measurements. Tip resonance frequency characterization experiments in air, water, sodium chloride, and phosphate buffer solutions were performed with an EG&G 7220 lock-in amplifier. Frequency scans were performed at different immersion depths of the tuning fork and probe as diagrammed in Figure 2. Lock-in frequencies were scanned between 80 and 100 kHz, and only one vibrational resonance was observed for each tuning fork. Tuning forks were allowed to dry for more than 10 min between different measure-

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Table 1. Effects of Water on ω_R and *Q*-Factor at Different Immersion Depths

depth	ω_R (Hz)	*Q*-factor
0	947 34	464
1	946 90	434
2	946 86	417
3	946 71	377
4	893 24	60
5	852 14	40
6	850 68	37

ments. For the salt and buffer solutions, salt residue was washed from the tuning fork with deionized water before drying.

Imaging. For topographical imaging, the polymer poly[2-methoxy-5-(2′-ethylhexyloxy)-1,4-phenylene vinylene] (MEH-PPV) was spin-cast onto glass slides.[13] The uncoated tip is dithered 5−10 nm[14] from the sample surface during the topography and optical image scans. Instead of raster scanning, forward and reverse motions of the sample were collected as separate images and used as a check of image reproducibility.

Linear reflectivity experiments[15] were performed on MEH-PPV films spin-cast from a 1% w/v solution in chlorobenzene with a Melles Griot HeNe laser (632.8 nm, ∼50 μW). Although there have been several studies utilizing NSOM to investigate the properties of this conjugated polymer,[13,16−18] none have studied MEH-PPV in a liquid environment. Because the polymer forms mesoscopic physical domains of a few hundred nanometers in diameter from the spin-cast process,[13] this sample was used to test the NSOM optical resolution in liquid.

RESULTS AND DISCUSSION

Resonance frequencies and quality factors were determined via lock-in frequency scans at a fixed set of immersion depths (Figure 2) in water, salt, and buffer solutions. Values of the resonance frequencies (ω_R) and *Q*-factors (*Q*) are summarized in Table 1. A *Q*-factor value of 464 was obtained for the tuning fork in air with a resonance frequency of 94.7 kHz. With only the fiber probe immersed in water, the *Q*-factors ranged from 434 (Figure 2, depth 1) to 377 (Figure 2, depth 3) while the resonance frequency shifted only slightly in all cases, i.e., less than 20 Hz (Table 1, Figure 3). In contrast, immersion of the fork prongs decreased the *Q*-factor by nearly an order of magnitude to values of 60, 41, and 36 as the water level increased to depths 4−6, respectively. Accordingly, it was also observed that the resonance frequency curve dramatically shifted to less than 90 kHz upon immersion of the tuning fork (Table 1, Figure 3).

In contrast to the work of Rensen et al.,[9] we did not observe any recovery of the *Q*-factor when the prongs were completely immersed. Although complete immersion of the prongs would

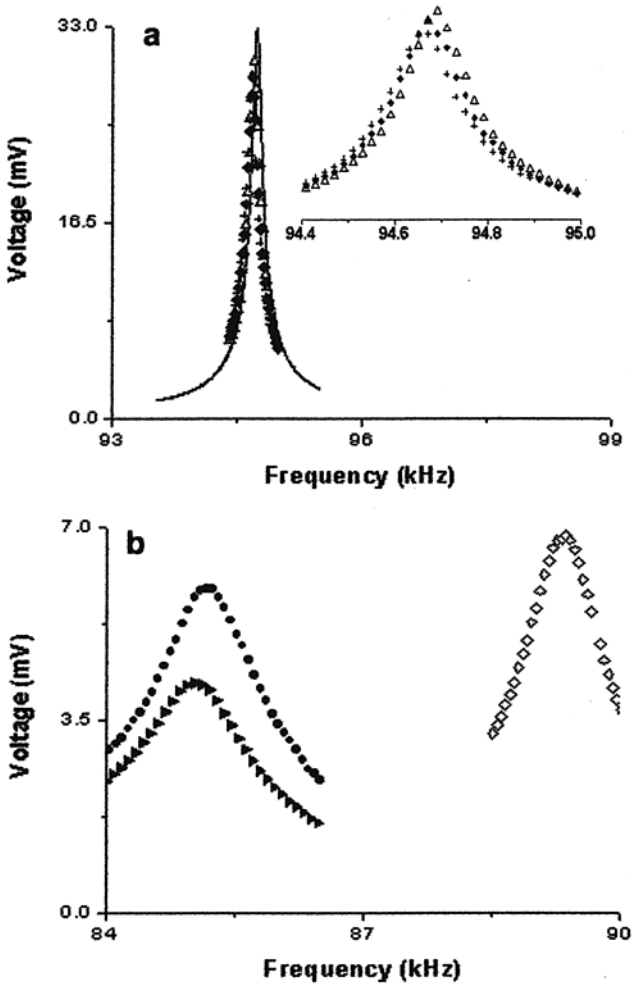

Figure 3. (a) Resonance curves in air (solid line) and at immersion depths 1−3 (△, ◆, and +, respectively). (b) Resonance curves at depths 4−6 (◇, ●, and ▲, respectively). Data are summarized in Table 1.

indeed facilitate a simplified experimental setup in that the exact liquid level is less crucial, we believe that the large *Q*-factor decrease, and hence the greatly decreased sensitivity of the feedback mechanism, reduces the image quality too much for this approach to be useful. By stabilizing the liquid level with our experimental design, the high *Q*-factor values that are obtained when only the probe is immersed indicates that optimal scanning conditions are achieved by this method.

Good feedback in ionic solution is also essential because in vivo imaging of biological samples requires immersion in a buffer solution due to osmotic pressure otherwise experienced by the cells. When the prongs are immersed in 0.2 M NaCl, 1.0 M NaCl, and phosphate buffer saline (PBS) solutions, the feedback current is destabilized and feedback cannot be obtained due to electrical shorting. Rensen et al. did not observe the shorting problem because they were working exclusively in (nonconductive) water. In all subsequent measurements in salt and buffer solutions, only the probe was immersed (Table 2). Table 2 shows that the damping force increases as the probe was immersed deeper into the liquid, and the greatest increase of damping force is ∼43% when the probe is completely immersed in PBS buffer. Although the damping force increases in ionic solutions, we were still able to maintain relatively high *Q*-factor values (ΔQ <30% in PBS

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Table 2. Effects of Liquid Environment on the Physical Parameters of the Tuning Fork System at Different Depths[a]

	depth	ω_R (Hz)	ΔQ (%)[c]	$\Delta\beta$ (%)[c]	ΔF_D (%)[c]
water	0	940 90	0.0	0.0	0.0
	1	939 30	2.8	2.7	3.0
	2	939 28	9.9	10.9	10.9
	3	939 13	11.3	12.2	12.9
0.2 M NaCl[b]	0	941 07	0.0	0.0	0.0
	1	939 30	7.4	7.8	8.1
	2	939 02	10.6	11.5	12.1
	3	938 97	15.5	15.1	15.8
1.0 M NaCl[b]	0	941 07	0.0	0.0	0.0
	1	939 54	10.6	11.5	12.1
	2	939 15	10.6	11.5	12.1
	3	938 91	13.9	15.6	15.4
PBS buffer[b]	0	941 07	0.0	0.0	0.0
	1	939 55	25.0	33.0	33.6
	2	939 13	26.9	36.2	36.9
	3	938 82	29.6	41.7	42.3

[a] Only the probe was immersed. All measurements were performed on the same tip. [b]To compensate for salt residue, measurements in air are the average obtained from the water experiments in air. [c]For the *Q*-factor, the percent change is decreasing, while for the damping coefficient and the damping force, the percent change is increasing.

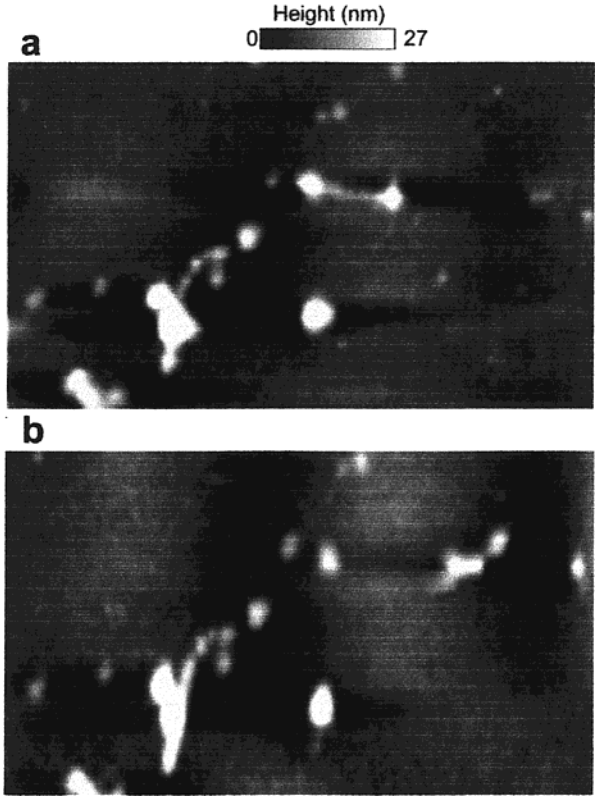

Figure 4. Topographical images of MEH-PPV in air (a) and water (b) in a 5 μm $\times$ 10 μm area.

buffer), which are greater than previous reports performed in water.[4−10] Significantly, this is a smaller decrease than those observed with the large piezoelements used for mechanical detection, which exhibit *Q*-factor degradation by >40% with shallow immersion in water.[4−7]

Figure 4 shows the topography scans of the same sample area in air and water at a depth of <200 μm. To obtain greater feedback

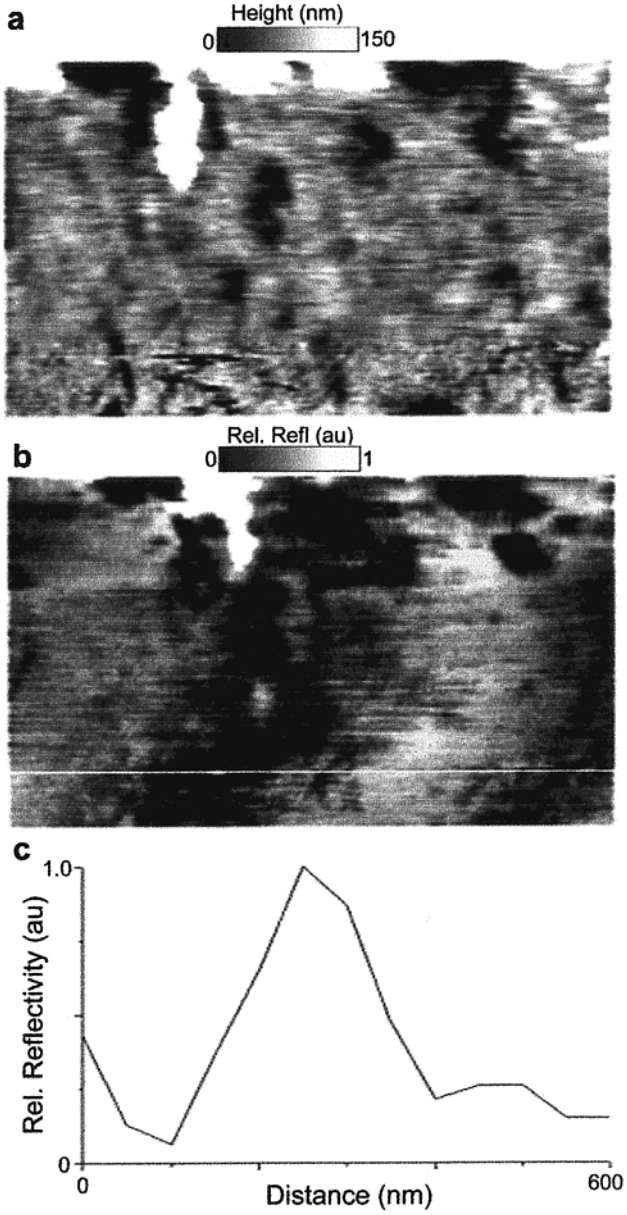

Figure 5. Linear reflectivity NSOM images and corresponding topography of MEH-PPV taken in water in a 5 μm $\times$ 10 μm area. (a) Topography of MEH-PPV in water. Maximum topographical height is 150 nm. (b) Linear reflectivity image of the same area produced at ω = 633 nm in water. (c) Line trace of optical image. The trace is taken along the white line shown in (b), and the resolution is determined by the full width half-maximum of the sharpest peak. Here, optical resolution is determined to be ~160 nm fwhm.

stability, we used a modified tip where the chemically etched fiber probe[19] is mounted on a bare tuning fork with an overhanging length of ~600 μm, ~3× longer than those commercially available. This helped in maintaining good feedback and achieving better image resolution. Good reproducibility is obtained in both forward and reverse scans, and the topographical spatial resolution in water is comparable to that obtained in air (~120 nm**).** Optical reflectivity images in water shown in Figure 5 also exhibit good reproducibility with a resolution of ~160 nm fwhm (~λ/4). Scans imaged in liquid in both Figures 4 and 5 show some "underwater"

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distortion, which can be avoided by decreasing the scan speed. We note that maintenance of a stable solution level is an absolute necessity for performing experiments that transmit optical fields through the solution. If the liquid level is unstable during scanning, the parallax of the excitation beam will also vary as the liquid level decreases. This will result in misalignment of the beam to the tip of the probe as parallax is dependent on the length of the beam path traversed in air before reaching the air−liquid interface. Thus, an unstable liquid level setup is limited to only transmission−collection, transmission−illumination, and collection−illumination NSOM modes.[20]

In summary, we have described an approach to implement NSOM in a liquid environment without great sacrifice of feedback sensitivity and stability. These results demonstrate that NSOM scanning in solution does not necessarily imply resolution degradation and indicates that NSOM imaging of biological samples in vivo is possible. Moreover, we have studied the solvatochromic shifts of MEH-PPV photoluminescence spectra in a variety of solvents[21] and are presently exploring the use of this new design for nonlinear near-field optical imaging of various samples in solution.

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