Near-field optical microscopy with a vibrating probe in aqueous solution

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We show that with an appropriately configured scanning quartz pipette coated with aluminum, a near-field scanning optical microscope (NSOM) can be constructed to operate in aqueous solution for applications in biology. Many of the technical limitations associated with a scanning pipette were circumvented by introducing a small modulation of the distance between the pipette and the sample. We show that this ac method allows the pipette to be positioned very close to the sample surface and is robust in obtaining reproducible NSOM images in solution. This approach is also compatible with fluorescence imaging and fluorescence resonance energy transfer, and should further facilitate the use of NSOM in various areas of cell biology where high resolution is considered to be critical. © *2001 American Institute of Physics.* [DOI: 10.1063/1.1361088]

The high resolution ability of near-field scanning optical microscopy (NSOM) has already been demonstrated in many laboratories with a spatial resolution approaching nanometer dimensions in some cases.[1–4] For many biological applications,[5–7] such high resolution is obviously advantageous and should be able to resolve some of the controversial issues, such as the existence of microdomains in cell membranes.[8,9] However, the shear force approach[2] that is highly successful in air has been shown to be ineffective in aqueous solution,[10] which is essential for biological NSOM.[11] This difficulty lies in the fact that failing to maintain the probe position at a fixed distance from the sample surface not only causes damage to the probe itself, but also leads to artifacts that are difficult to eliminate.[12–14] As a result, the application of NSOM in aqueous solution has been limited.[7,11,15–17]

A possible approach for control of the probe position in aqueous solution is to use a scanning pipette rendered optically opaque to form a near-field optical probe at the pulled end,[18] while its position is controlled like that in a scanning ion conductance microscope (SICM).[19–21] Since the ion current through the pipette is sensitive to the distance from the sample surface, a proper feedback system should be able to maintain a constant distance between the sample and the probe. However, a major problem with a conventional SICM is that the change in ion current is not sufficiently sensitive to tall features nearby. Therefore, if the probe is not scanned at a ''safe'' distance above the surface, the probe is often trapped in deep ''valleys,'' thus causing instabilities in the system. As a result, both the pipette and the sample are often damaged.[20] In addition, drifts due to contamination of the electrodes (Ag/AgCl) over time can also complicate attempts at getting stable images of a sample.[22] Because of these technical challenges, a recent exploration of this idea was only partially successful.[17] The probe had to be scanned at a distance in excess of 250 nm. The resolution achieved was modest, mostly because the illuminated area at this ''safe'' distance is much greater than the size of the aperture. Clearly, to fully utilize the advantage of a small aperture, one must be able to place the opening of the pipette as close as possible to the sample surface.

In this letter, we show that, by generating a small periodic distance modulation between the pipette and the sample at a fixed amplitude, the alternating ion current (ac) is more sensitive to the distance, and the pipette can be scanned only a few nm from the sample surface. This distance modulation can also eliminate the problem of trapping the probe in surface valleys, similar to that in tapping mode atomic force microscopy (AFM).[23,24] Such a system is ideally suited for imaging in solution, and is insensitive to long term drifts.

The basic layout of our SICM/NSOM is shown schematically in Fig. 1. A piezo tube scanner, capable of a 50 μm scan range laterally, is used to scan the pipette in three dimensions.[25] The ion current is converted to voltage, giving a sensitivity of 1 V/nA with the corner frequency at 9 kHz. A

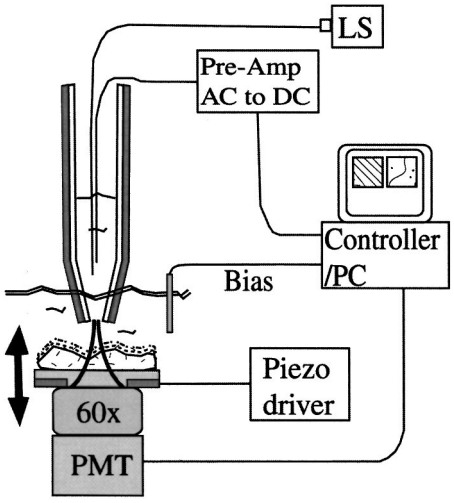

FIG. 1. Schematic illustration of the SICM/NSOM setup. LS: Light source; PMT: photomultiplier tube. The sample is oscillated by a thin piezo plate; the ac component of the ion current detected by the Ag/AgCl electrode is converted to rms, which is used as the feedback signal.

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separate piezo plate is used to oscillate the specimen. The oscillation frequency can be adjusted between 3 and 20 kHz, and the amplitude of oscillation can be up to 20 nm. The NSOM is placed on an inverted optical microscope with a long working distance objective [60× at a numerical aperture (NA) of 0.7]. A photomultiplier tube (PMT) (R374, Hamamatsu, Japan), operated in the analog mode, is installed at the video port to collect the optical signal through the objective, which is directly sent to one of the auxiliary channels on a NanoScope IIIa controller (Digital Instruments, Santa Barbara, CA). The ion current is measured with Ag/AgCl electrodes, and the bias voltage is 30–500 mV, which is supplied by the NanoScope IIIa controller and can be set by the software. The electrolytic solution used is 350 mM KCl in both the pipette and the sample bath. The root-mean square (rms) of the ac signal is sent to the scanning tunneling microscopy (STM) current input on the NanoScope IIIa controller.

The pipettes are prepared by pulling thin wall quartz capillaries [1.00 mm in outer diameter (o.d.) and 0.70 mm in inner diameter (i.d.)] in a programmable CO_2 laser based microelectrode puller (Sutter Instruments, model P2000). Right after a pipette is pulled, a layer of aluminum, up to 100 nm, is deposited in a vacuum evaporator (at 10^{-6} Torr). The pipettes are first filled by boiling in water/ethanol for 10–15 min, followed by backfilling with 350 mM KCl. The standard test sample, an aluminum shadow mask of 2.2 μm polystyrene beads, is prepared by evaporating a thin film of aluminum, <30 nm, on a monolayer of beads adsorbed on a clean glass slide. The beads are then removed by sonication for 20 min in distilled water.

The relationship between the ion current and the distance above the surface has been described by Schwab[26] as

$$I = I_0 \frac{z}{z+k}, \tag{1}$$

where $k = 1.5 \ln(r_a/r_i)\ r_0 r_i / L_p$ and $I_0 = U/R_p$. U is the bias voltage, R_p the pipette resistance, r_a/r_i the ratio between the outer and inner radii at the pulled end, r_0 the inner radius close to the electrode, L_p the distance between the electrode and the pipette opening, and z the distance to the sample surface. If the distance, z, in Eq. (1) is substituted with the time dependent term, $z = z_0 + \delta \sin \omega_0 t$, and we expand Eq. (1) in terms of δ, the expression can be decomposed into a dc offset and a series of ac terms as follows:

$$I = I_0 \left\{ \frac{z_0}{z_0+k} + \delta \frac{k \sin(\omega_0 t)}{(z_0+k)^2} - \delta^2 \frac{k \sin^2(\omega_0 t)}{(z_0+k)^3} + \ldots \right\}. \tag{2}$$

The rms of the first term gives the following distance dependence:

$$\text{rms} = I_0 \frac{1}{\sqrt{2}} \frac{\delta k}{(z_0+k)^2}. \tag{3}$$

This rms average is even more sensitive to the distance than the dc term (at the same mean distance, z_0, from the surface), which demonstrates that the rms average can clearly be used for feedback control.

Light is directly delivered to the pipette with a 240 μm multimode optic fiber, which is coupled to a 150 W halogen quartz lamp without any wavelength selection. The optical power transmitted to the pipette in this arrangement is less than 60 μW. The light throughput declines sharply with the diameter of the pipette (Fig. 2), $\sim a^3$, where a is the diameter of the aperture. This is somewhat different from that prepared from optic fibers[12] and its theoretical basis has not yet been examined.

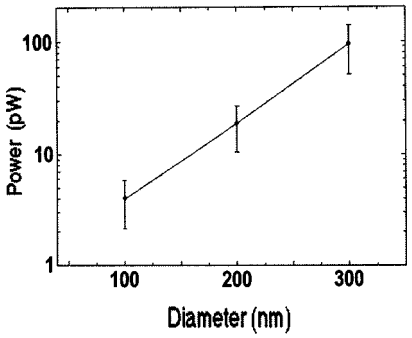

FIG. 2. Measured light emission from various diameter pipettes coated with aluminum. The pipettes are filled with water and the power input is held at ~ 60 μW. The light throughput is related to the third power of the diameter over the range measured.

Typical NSOM/SICM images are shown in Fig. 3. In this case, the sample is oscillated at 8.5 kHz with an amplitude of ~ 17 nm at pixel time of ~ 1 ms and the optical power delivered to the pipette is about 60 μW. On a large scale, the SICM image [Fig. 3(a)] shows excellent correspondence to the NSOM image [Fig. 3(b)]. The regular packing of the beads is clearly seen in these images. At higher resolution, the outer diameter of the probe is estimated at 400–500 nm based on SICM images [Fig. 3(c)], while in the NSOM image [Fig. 3(d)], the estimated resolution from the edges of the aluminum shadow is in the range of 200–300 nm. Since the aluminum coating on the pipette is around 100 nm, as shown by scanning electron microscopy (SEM) imaging, these measurements are consistent with an optical aperture of ~ 200 nm. Such images are very stable and can be obtained repeatedly without degradation of the pipette or the specimen. Based on calibrated current measurements, the closest distance between the pipette and the sample surface is less than 10 nm. Therefore, with distance modulation, the probe can be pushed to a position very close to the specimen, thus allowing the high resolution potential of NSOM to be realized. With this system, probe trapping or current drifting has not been a serious problem, and even thicker aluminum masks can be imaged as well. Without distance modulation, stable images at this close distance could not be obtained. The signal to noise ratio (defined as the mean signal/rms) in the optically transparent regions (aluminum free) is ~ 10. Since the dark current of the PMT (including contributions from the preamplifier) is equivalent to 5×10^4 photons in 1 ms and around 10%–20% of photons emitted from the pipette should be collected, the estimated signal to noise (S/N) ratio should be 15–20 based on 20 pW illumination (see Fig. 2). This slight discrepancy may be attributed to losses due to the various surfaces in the optical path that are not accounted for in this calculation.

In order to use smaller pipettes[27] to achieve higher spatial resolution, both higher optical power and more sensitive detection methods will be required. Unlike in air where the maximum power is limited to <10 mW,[28] an additional advantage of this approach is that more optical power can be delivered to the pipette without damaging the aluminum

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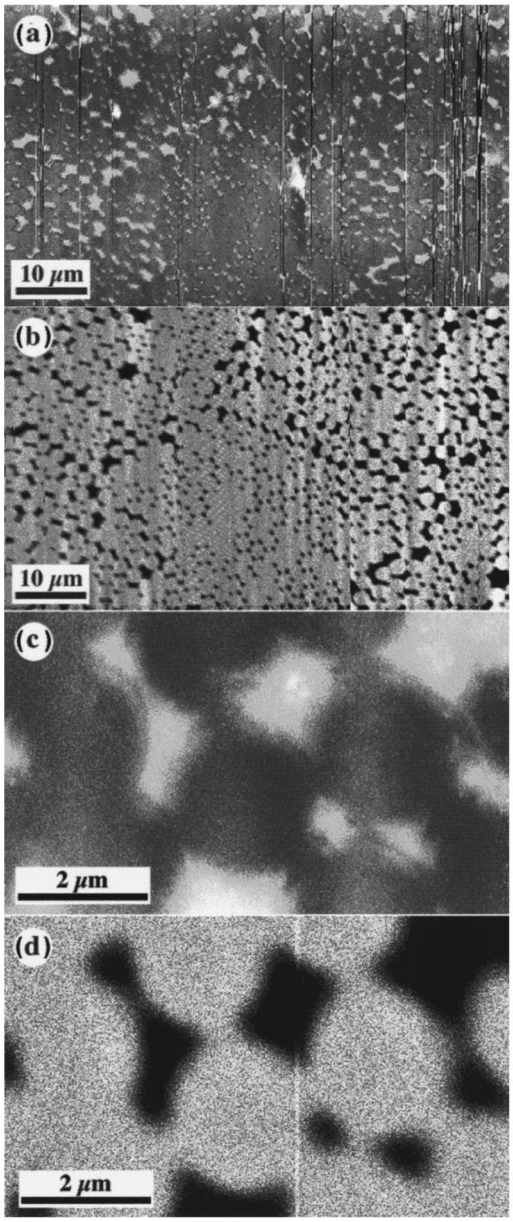

FIG. 3. Large scale images of the aluminum shadow prepared with adsorbed beads on glass are shown in (a) and (b). The topographic SICM image (a) corresponds well with the NSOM image (b). The close packing of the beads on the glass surface is clearly seen. In the SICM image (a), the deposited aluminum appears as isolated islands, while in the NSOM image, the same region is optically opaque. At higher resolution (smaller scan size), the estimated probe size of 400–500 nm based on SICM (c) is consistent with the resolution of ~200 nm, estimated from aluminum edges in the NSOM image (d), taking into account ~100 nm of deposited aluminum. The sharper features visible in the SICM image (c) are due to imperfections at the apex of the pipette which can also affect the ion current at close range. This is not seen in the NSOM image (d) because the optical signal is not sensitive to small distance changes. The pixel time is ~1 ms.

coating due to the efficient cooling in solution. Nearly a nanowatt of emitted light by a 50 nm pipette is obtainable at 50 mW. Using a photon counting PMT,[6,29] the calculated limit is about 20 fluorophores at a pixel time of 1–2 ms at a S/N of ~10 under this condition. Therefore, straightforward improvement of this approach should allow a direct examination of protein localization in the cell membrane at a resolution of around 50 nm, which should be sufficient to detect the anticipated small domains that are essential for organizing many of the signal transduction processes.[8,9] In addition to straightforward fluorescence imaging, fluorescence resonance energy transfer (FRET) is another simple and powerful capability that can be easily incorporated into this system.[30]

In summary, we have demonstrated the feasibility of using distance modulations in a NSOM/SICM for imaging in aqueous solution. Since the fabrication of small pipettes down to 20 nm is already well established,[27] this approach should open many novel applications of NSOM in biology under physiological conditions at much higher resolution than that achievable with far field imaging.

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