Hybrid Scanning Ion Conductance and Scanning Near-Field Optical Microscopy for the Study of Living Cells

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ABSTRACT We have developed a hybrid scanning ion conductance and scanning near-field optical microscope for the study of living cells. The technique allows quantitative, high-resolution characterization of the cell surface and the simultaneous recording of topographic and optical images. A particular feature of the method is a reliable mechanism to control the distance between the probe and the sample in physiological buffer. We demonstrate this new method by recording near-field images of living cells (cardiac myocytes).

INTRODUCTION

Scanning probe microscopies have the potential to image living cells at high resolution, and hence follow cellular dynamics and map cell function. Contact atomic force microscopy (AFM) particularly the "tapping in liquid" mode of operation (Putman et al., 1994; Hansma et al., 1994; Ohnesorge et al., 1997) has been used to image living cells with a resolution that is one order of magnitude better than conventional optical microscopy. However, interpretation of the obtained results has been difficult because the nature of the interaction forces that come into play between the tip and the sample are not fully understood, as is the extent of deformation/perturbation of the "soft" cell membrane structure by the hard AFM tip. To date, scanning near-field microscopy (SNOM) imaging has been only performed on fixed cells (Muramatsu et al., 1995; Gheber et al., 1998). In this article we show that by combining scanning ion conductance microcopy with scanning near-field microcopy it is possible to record near-field images of living cells, and that this offers a reliable method for distance control.

Briefly, in SNOM, a near-field light source with an output aperture of sub-wavelength dimensions is scanned above the sample surface (for a review see Subramaniam et al., 1998). The interaction forces between the source and sample are used to maintain their separation at less than the sub-wavelength dimensions of the aperture, allowing simultaneous generation of optical and topographic images. As in far-field optical microcopy, all contrast mechanisms are available in SNOM, and in particular chemical imaging is possible by the use of fluorescent labels (Pohl and Courjon, 1993). For the imaging of biological samples in liquids it is still difficult to reliably control the sample- probe distance. To date the best reported resolution of SNOM operated in liquid is 60 nm (Keller et al., 1998) using non-contact AFM for distance control.

In SICM, an electrolyte-filled, glass micropipette is scanned over the surface of a sample bathed in an electrolytic solution (Hansma et al., 1989). The pipette-sample separation is maintained at a constant value by controlling the ion-current that flows via the pipette aperture. The optimum tip-sample separation that has allowed SICM to be established as a non-contact profiling method for elaborated surfaces is equal to one-half of the tip diameter (Korchev et al., 1997a). The tip's output is used to generate topographic features and/or images of the local ion-currents flowing through pores on the sample surface. The spatial resolution achievable using SICM is dependent on the size of the tip aperture: typically between 50 nm and 1.5 μm. However, it is possible to fabricate smaller apertures ($<$50 nm (Brown and Flaming, 1986)).

In this paper we report modification of an existing SICM setup such that simultaneous generation of SICM and SNOM images of living cardiac myocyte cells was possible. Cardiac myocyte cells were chosen because they are composed of light and dark bands of material/striations that periodically occur every 2.1 μm, which give them a distinct appearance and, therefore, make them a good model system for study. They are also important cells because they constitute heart muscle chambers that synchronously contract to produce the crucial pumping activity to circulate blood to the rest of the body. These cells have previously been studied using SICM (Korchev et al., 1997a).

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EXPERIMENTAL ARRANGEMENT

The SICM experiment consists of a scanning probe, piezo-actuator scanning elements, control electronics, and a computer. These components are built in and around an inverted microscope (Diaphot 200, Nikon Corporation, Tokyo, Japan) central to the experiment.

We fabricate our SICM probes by pulling borosilicate, glass microcapillaries with outer and inner diameters of 1.00 mm and 0.58 mm, respec-

tively, using a laser-based micropipette puller (Model P-2000, Sutter Instrument Co., San Rafael, CA). This reproducibly and easily produces probes with conical taper lengths and apex diameters of 200 nm, 400 nm, and 1.0 μm, respectively. The corresponding inner diameters are 100 nm, 200 nm, and 500 nm, respectively.

Three-dimensional and high-precision movement of the probe relative to the sample is achieved by the piezo-translation stage (Tritor 100, Piezosystem Jena, Germany) on which the SICM probe is mounted. The stage has a range of 100 μm in the *x*, *y*, and *z* directions so that scanning over biological samples with features that scale up to 30–50 μm is possible.

The pipette-sample separation is maintained at a constant value by monitoring the ion-current that flows between Ag/AgCl electrodes in the micropipette and electrolyte solution in which the sample is immersed. For this work, phosphate-buffered saline (PBS) solution is used for both filling the micropipette and the electrophysiological medium of the cardiac myocytes so that concentration cell potentials and liquid junction potentials are not established. The ion-current is measured for DC voltages of 50 mV applied to the electrodes. It is amplified by means of a high-impedance operational amplifier (OPA129, Burr Brown International, U.S.A.) and converted to a voltage signal over a resistance of 10^8 Ω. This signal is then input into the control electronics where it is used for feedback control and data acquisition.

The micropipette is housed in a special, custom-made holder which is assembled together with the current amplifier and piezo-translation stage to comprise the SICM head. The SICM head is mounted onto a second *z*-translator on top of the inverted microscope that facilitates coarse vertical positioning of the micropipette relative to the sample immediately below it. The sample is contained in a petri dish placed on the microscope's stage. Movement of the sample relative to the micropipette is achieved by the *x*, *y* translation controls of the stage. The processes of monitoring the vertical position of the micropipette relative to the sample and selection of an area of interest on the sample can be viewed on a TV screen via a video camera (JVC TK-1280E, Victor Company, Japan).

Modifications were made to the experiment described above to permit simultaneous SICM and SNOM imaging. Continuous wave laser light (Laser 2000 Ltd, UK of wavelength, 532 nm, was coupled via a multimode fiber (FG-200-UCR; 3M Specialty Optical Fibers, West Haven, U.S.A.) into the micropipette. In order to confine light to the aperture, 100–150 nm of aluminum was evaporated onto the walls of the pipette. The scattered laser light was collected by a 60× long working distance objective and relayed by transfer optics onto a PMT (D-104–814, Photon Technology International, Surbiton, England) to record the optical signal. Simultaneous optical and topographic images of the sample were acquired using the control/data acquisition hardware and software produced by East Coast Scientific (Cambridge, UK). A diagram of the experimental apparatus is shown in Fig. 1.

Adult rabbit myocytes were isolated using a low-calcium solution (NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10 and nitrilotriacetic acid (NTA) 5 (mmol/l), preoxygenated with 100% O_2) and collagenase and protease enzymes as previously described (Jones et al., 1990). Cells were imaged on a glass coverslip at the bottom of the petri dish in a low-calcium medium at room temperature.

RESULTS

Figs. 2 and 3 show images of a living rabbit cardiac myocyte. The optical and SICM images were recorded simultaneously and it took ~20 min to record one set of images. The micropipette used is estimated, by the measured ion current, to have an internal diameter of ~500 nm and was held ~250 nm over the surface during imaging. The estimated external diameter is 1000 nm, and comprises the glass and metal coating. This means that these images were

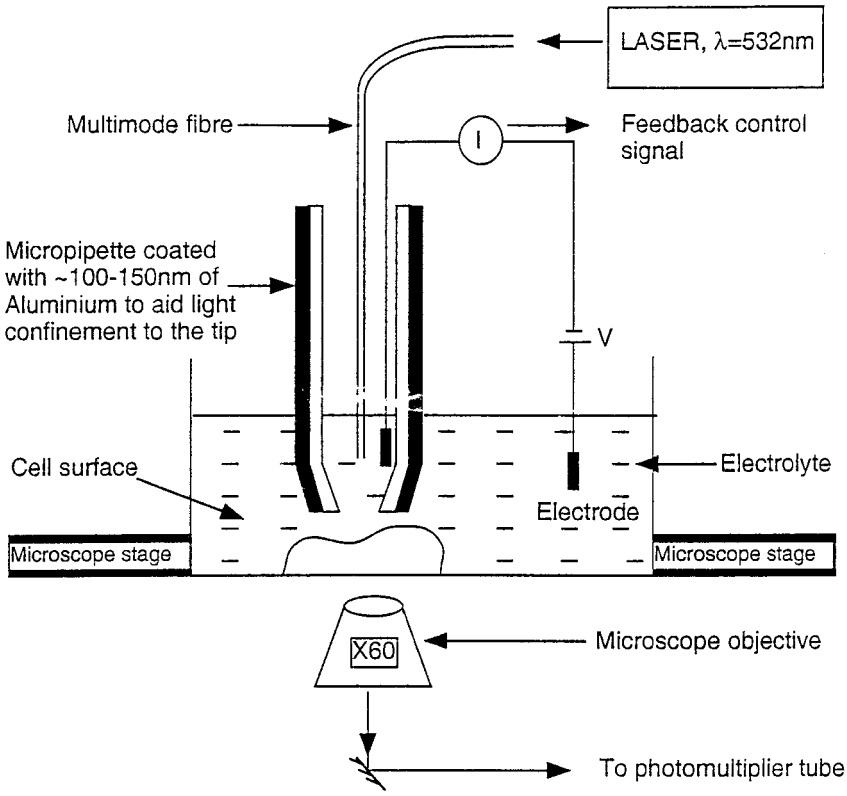

FIGURE 1 Schematic diagram of the hybrid SICM-SNOM apparatus.

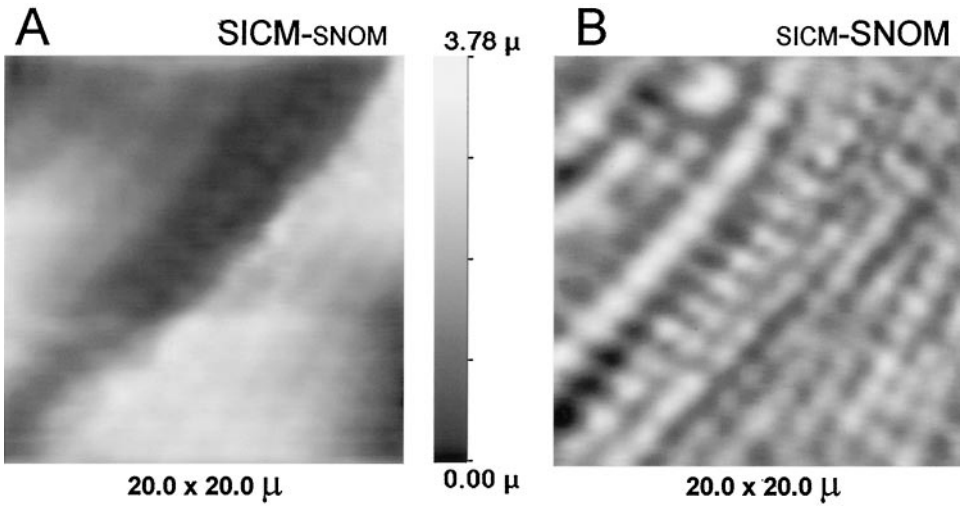

FIGURE 2 Topographical (SICM; A) and optical (SNOM; *B*) images of a rabbit cardiac myocyte obtained using the hybrid SICM-SNOM microscope. The characteristic striated pattern reflecting the sarcomeres is discernible in both the acquired images. The gray scale of the topographical image represents cell height. Cells were imaged on glass coverslips in a low-calcium solution to prevent spontaneous cell contraction (NaCl 120, KCl 5.4, $MgSO_4$ 2, $CaCl_2$ 0.5, glucose 10, HEPES 10 (mmol/l), pH 7.4).

recorded in the near field, less than a wavelength of light from the sample, with an aperture having a diameter comparable to the wavelength of light.

Fig. 2 shows a 20 $\times$ 20 μm scan of the cardiac myocyte surface. The sarcomeric structure running from bottom left to top right is clearly visible in both the SICM and SNOM image. It is notable that there is a large hollow in the SICM image and that the optical image provides more detailed information than that obtained by SICM. The sarcomeres, which are spaced $\sim$2.1 μm apart, are clearly visible in the optical image as alternating dark and bright bands. Note also the optical image appears to be generated only at the surface of the cell, as expected using a scanning probe technique. The sarcomeres are visible in both images, although clearer in the optical image. Fig. 3 shows a large scan range. Note the excellent correspondence between the optical and SICM images. The Z lines and Z grooves of the cardiac myocyte run from top right to bottom left in the image. These appear as the dark regions in the optical image and the grooves in the topographical image. From the smaller scan range images shown in Fig. 3 we estimate our resolution to be $\sim$500 nm.

DISCUSSION

The results presented show that scanning ion conductance microscopy provides a reliable control mechanism for SNOM imaging of live cells. The images of cardiomyocytes we record have the widely accepted structure and dimensions. The observation of the dark bands in our image corresponding to the Z-lines suggests the contrast in the image is due to the local interaction of the near-field probe with the cell surface, resulting in reduction of the transmitted light.

In SICM imaging reliable control is possible as the pipette diameter is reduced because the ion current is still above the noise level and 50 nm resolution is possible (Korchev et al., 1997a, b). The distance between sample and micropipette is 250 nm, which is significantly larger than in shear force detection SNOM, where the distance is 1–10 nm. However, having demonstrated that hybrid SICM-SNOM is feasible it should be straightforward to reduce this distance if we used a 100 nm diameter micropipette. This would result in a probe-sample distance of 50 nm, comparable to SNOM using non-contact AFM in liquids (Keller et al., 1998). Because a living cell is dynamic and motile, however, ordinary SNOM may be less applicable, as high-resolution imaging still requires a fast scan time; otherwise, the cell structure will have changed during the scan. Furthermore, the probe is close to the cell surface and this distance cannot be easily increased for a larger probe. For contact mode AFM or shear force control there is also the possibility of the probe altering or damaging the soft cell sample. Our system is more flexible because in SICM the optimum micropipette-sample distance is the radius of the pipette tip. Thus we can either image at a greater distance from the sample with a large pipette tip. This will be an intense near field source allowing faster imaging at low resolution. Alternatively, we can image closer to the sample with a smaller pipette tip at higher resolution. In our experimental set-up the use of a higher numerical aperture objective and photon counting should largely compensate for the decrease in light intensity using a small pipette or allow more rapid imaging with a larger pipette. This flexibility should be very useful for imaging of a dynamical system such as the cell surface, depending on the time and spatial resolution required.

SUMMARY

We have shown that by using the ion current to control the distance between a coated micropipette and the sample it is possible to obtain simultaneous optical and SICM images of

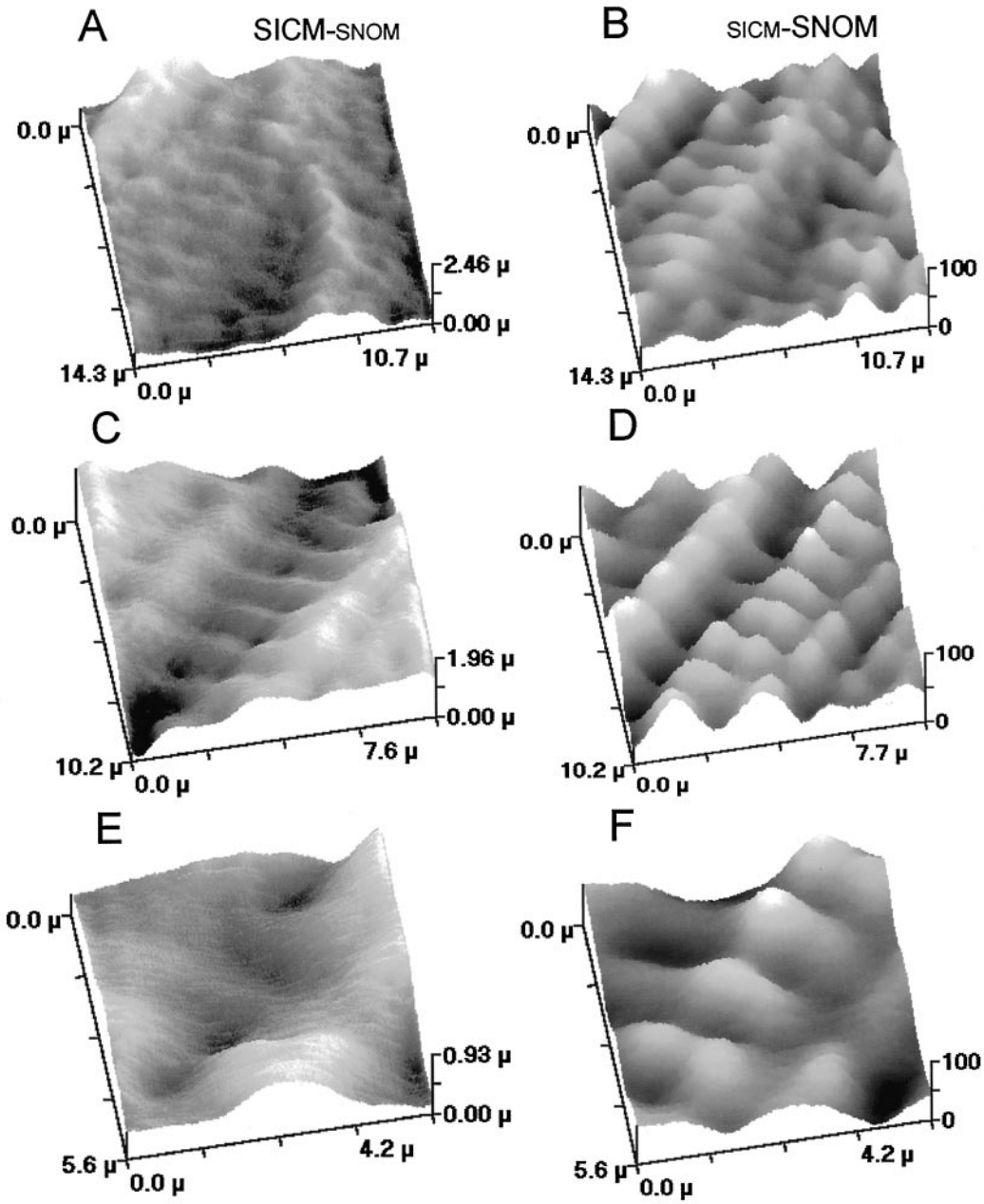

FIGURE 3 Topographical and optical images of a rabbit cardiac myocyte obtained using the hybrid SICM-SNOM microscope under identical conditions to Fig. 2. Comparison of SCIM-acquired images (*left-hand set*) and SNOM-acquired images (*right-hand set*). The sarcomeric striations are clear in the low power images (*A* and *B*). Higher magnifications also show the striations (*C* and *D*), but now the SICM image (*C*) reveals indentations that seem to correspond with discrete dark regions in (*D*), within the groves running from top left to bottom right. The striation distances, both SICM and SNOM, are commensurate with those found with the electron microscope (~2.1 μm). Still higher magnifications with the SICM (*E*) show more detail of the indentations, which could be the openings of the T tubules in the Z-grooves (Korchev et al., 1997a). These again appear to correspond to the black regions (this time larger than in (*D*)). The vertical axes in the optical images correspond to the relative intensity of light transmitted through the sample surface.

live cells in the near field. This appears to be a reliable way to perform SNOM imaging of live cells.

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