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Atomic force microscopy and manipulation of living glial cells

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The atomic force microscope (AFM) is capable of imaging surfaces at very high resolution. The AFM has been used to image living glial cells in culture. Typical images reveal the three-dimensional shape of the cell and often internal cellular structures are visible. In this report, it is shown that by increasing the imaging force, cells can be removed from the surface on which they are grown. Although the forces involved in this process are complex, it is possible to compare relative adhesion of different types of living cells to a particular substrate.

I. INTRODUCTION

The atomic force microscope (AFM)[1] is, in principle, a surface probe. Recently, several laboratories have shown that in addition to this capability, the AFM can resolve subsurface features in living and fixed cells.[2–6] The mechanism by which this is accomplished is not completely understood although an intuitively obvious model is that the cell membrane conforms to the contours of the underlying structures as the scanning tip traces over the membrane. However, it is also possible that the scanning tip penetrates the plasma membrane and makes contact with subsurface structures directly.[3] Despite our lack of knowledge about the imaging mechanism, the ability to image living cells, and their subsurface components, indicates that the AFM is evolving into a powerful high resolution imaging tool for molecular cell biology.

Glial cells play several important roles in the development, maintenance, and regeneration of the nervous system. Thus, the modes of interaction between glial cells and neurons is of great interest to neurobiologists. Glia have many of the physiological properties associated with neurons. In addition, neurons are often found in intimate association with glial cells, and it has been proposed that such interactions may be important during development of the nervous system.[7] For example, extracellular matrix molecules produced by glia as well as cell surface-bound molecules may provide substrates that can assist in the guidance of migrating postmitotic neurons and also direct the outgrowth of axons to their proper targets. Therefore, we are exploiting some of the unique properties of the AFM to study glia and their interactions with different surfaces.

Many studies have demonstrated the AFM's ability to manipulate objects, including living cells, at a very fine scale.[3,6,8–13] In this study, we have used the AFM to manipulate living glial cells and detach them from the substrate upon which they are grown. By increasing the applied vertical force during the imaging process, cells can be removed from the surface at defined force levels. Although it is difficult to relate the scanning probe forces to the absolute adhesive force between the cell and substrate, it should be possible by this method to measure relative adhesivity of cells to different substrates and from this information gain insight into how glial cells might direct neuron outgrowth *in vivo*.

II. MATERIALS AND METHODS

A. Cell preparation and culture

Glial cells were prepared from rat hippocampi as previously described.[6] Briefly, hippocampi were freshly dissected from one to four days old rat pups (Sprague–Dawely). Tissue was dissociated through enzymatic treatment (20 U/ml papain) and subsequent trituration. The cells were plated onto poly-*L*-lysine coated glass coverslips (Fisher Scientific No. 1, 12 mm diam) inlayed in a 35 mm culture dish. Hippocampal cells were grown in medium consisting of Eagle's minimum essential medium (Earle's salts; Gibco) supplemented 10% with heat-inactivated fetal bovine serum (Sigma) and containing 40 mM glucose, 2 mM *L*-glutamine, 1 mM pyruvate, 14 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin. All experiments were performed on cells that had been in culture for two to four days. In separate experiments, glial cells were identified in hippocampal cultures using antibodies directed against glial fibrillary acidic protein (GFAP; 1:1000, Sigma) and an Elite Kit (Vector).

The XR1 glial cell line is an established cell line derived from *Xenopus laevis* embryonic stages 24–26 retinal neuroepithelium.[14] XR1 cells were plated onto acid washed glass coverslips (12 mm diam) which had been coated with collagen (rat tail type IV, Sigma) or ECL (basement membrane preparation composed mainly of entactin, collagen, and laminin, Upstate Technology, Inc.) as previously described.[3] XR1 cells were plated at a density of 3000 or 4000 cells/ml and grown in L15 medium (60%) supplemented with fetal calf serum (10%), fungibact (1%), and embryo extract and maintained at 22 °C.

B. Force microscopy

All images were collected with a Nanoscope III using a fluid cell (Digital Instruments, Inc., Santa Barbara, CA). Glial cells plated on glass coverslips as described above were mounted on magnetic pucks with double stick tape or super glue and placed on the piezo tube taking care not to let them dry out. A small drop ($\sim 100\ \mu l$) of growth media

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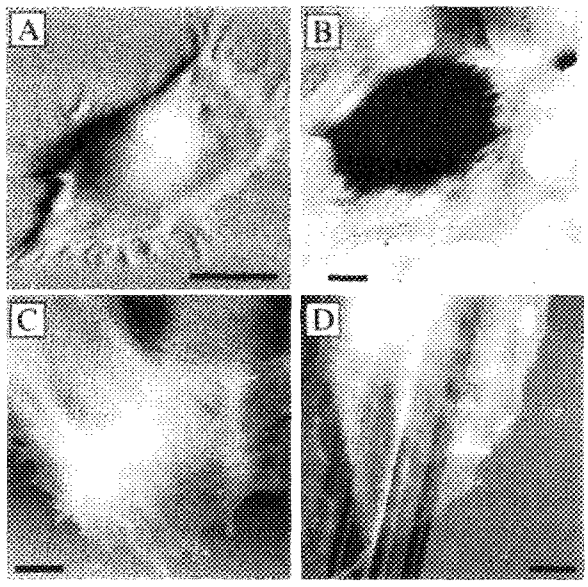

FIG. 1. AFM images of living cells. In (A), a rat hippocampal glial cell is shown in error signal mode. In this image only large features (e.g., nucleus) are apparent. In (B), a height mode image of an XR1 cell is shown using an inverted gray scale to emphasize the fibers within the cell. These are *f*-actin stress fibers. In (C), a second example of stress fibers in a living XR1 cell is shown (height mode). In (D), a height mode image of an XR1 cell growing over a scratch in the substrate is shown. The cell has a transparent quality which is unexpected for a surface probe such as the AFM and may be related to an imaging mechanism in which the tip penetrates the cell membrane during imaging (Ref. 3). Bar = 20 μm.

(XR1 cells) or imaging solution (hippocampal cells) containing 150 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.35), 330 mOSm, was placed directly on the cantilever in the fluid cell which was subsequently lowered onto the coverslip. The fluid formed a column maintained by surface tension and no o-ring was used to seal the chamber. This arrangement avoids loading of the piezo crystal by the o-ring and is stable for a least 1–2 h. Cells were scanned at 5 Hz or less (see Sec. IV) to minimize image distortion due to piezo induced cell vibration. The applied vertical forces in the repulsive mode are reported in the text and figures. For data presentation, Nanoscope image files were imported into "Image 1.44" (Macintosh image analysis freeware from NIH), converted to PICT format and configured in "Freehand" (Aldus) on a Macintosh IIfx.

III. RESULTS

Imaging living cells; the glial cells in the study were ioslated from rat hippocampus[6] or frog retina (XR1 cell line).[14] Figure 1(A) shows a typical error signal mode (ESM; an imaging mode that reveals detail in the surface under scrutiny but does not give accurate height information)[15] image of a rat hippocampal glial cell. In this case, large features like the nucleus are apparent but the subcellular structures are not obvious. This may be related to the structure of the scanning tip and/or the cell type (see Sec. IV). In the height mode (HM; an imaging mode that gives accurate height information) images, [Figs. 1(B), 1(C), and 1(D)] the cells have a transparent appearance. This is especially apparent in Fig. 1(D) which shows a height mode image of a portion of an XR1 glial cell growing over a scratch made in the glass substrate. The apparent transparency of these cells is probably due in part to the fact that they are very flat and follow the contours of the substrate upon which they are grown. However, it is also possible that the scanning tip penetrates the cells in some locations during imaging and this gives the appearance of seeing through the cell body.[3] For example, Figs. 1(B) (in which the gray scale is inverted) and 1(C) are height mode images of XR1 cells in which *F*-actin stress fibers running throughout the cell are obvious.[3]

The adhesive force between cells and their growth substrate can be qualitatively assessed by AFM. Figure 2 shows an example of a force manipulation experiment on a pair of rat hippocampal glial cells. As the cell pair was scanned the applied vertical force was increased from 24 to 72 nN. At 24 nN, the cells appear relatively unperturbed. However, as the force was increased the cells became more rounded and large holes in the expanded peripheral veil (the flattened cellular expansion at the cell periphery) appeared. Small structures such as filopodial spikes were removed, without apparent injury to the cells. Finally, at 72 nN applied vertical force, the cells were removed from the surface leaving behind a region of cellular material. A previous study[6] has shown that a clear difference exists in the minimum forces required to remove glia (60–84 nN) and neurons (5–36 nN) from the same growth substrate. Thus, even though the absolute adhesive force between the cell and the surface is not known, the relative adhesion of different cell types for a particular surface can be measured by this method.

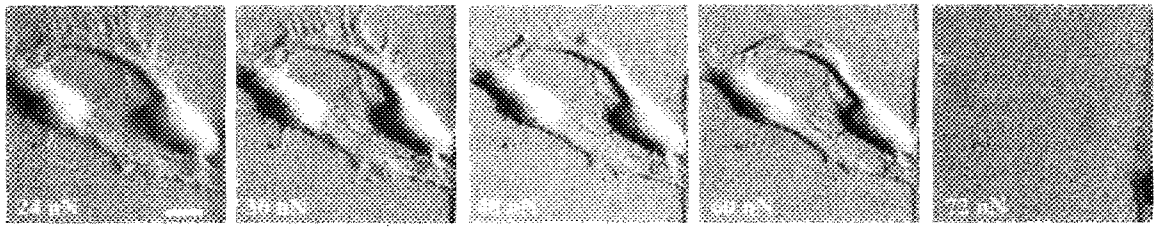

FIG. 2. The effect of increasing imaging force on cell adhesion to the growth substrate. Two rat hippocampal glial cells are shown in error signal mode images. As the applied vertical imaging force was increased (left-to-right, force indicated in lower left-hand corner of each frame) changes in cell morphology were apparent. Microspikes extending from the cell veil were removed and holes appeared in regions of the veil. At 72 nN applied vertical force, the cells were scraped away from the substrate. Bar = 20 μm.

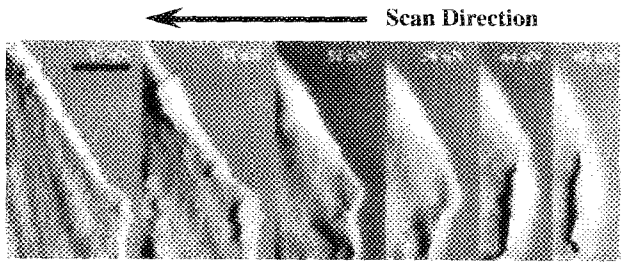

FIG. 3. A nanopeeling experiment on a living rat hippocampal glial cell. As the applied vertical imaging force was increased in the indicated direction the cell edge formed peaks and was then lifted from the surface and rolled back onto itself. At an applied vertical force of 73 nN, the cell was peeled away from the surface completely. Bar = 10 μm.

In other experiments in which only the edge of a rat hippocampal glial cell was imaged, we observed that as the force was increased portions of the veil folded back over the cell much like rolling a crepe until the cell was peeled away from the surface. Figure 3 shows an example of this type of "nanopeeling" experiment. As the scanning force was increased from 17 to 34 nN portions of the veil formed peaks which subsequently became absorbed back into more internal regions of the veil. Further increases in the scanning force from 52 to 69 nN resulted in rolling of the edge of the veil and blurring of the image. At 73 nN applied vertical force, the cell was peeled away from the surface completely.

IV. DISCUSSION

In this report we demonstrate the utility of the AFM for imaging and manipulating living glial cells. By systematically increasing the imaging force on living glial cells, we were able to determine at which force the cells became detacted from the surface. Comparison of the forces required to peel cells of various types from surfaces gives an important qualitative measure of relative attachment forces for different types of cells and surfaces.[3] In view of the critical role cell–cell and cell–matrix adhesions play in development, the ability of the AFM to generate information regarding relative adhesive forces could provide important insights to many developmental questions.

For successful imaging it was necessary to maintain the scanning rate below about 5 Hz. Presumably, this is because at higher scan frequencies, the inertia of the cell causes it to lag behind the piezo and results in blurring of the image. This is more of a problem for large or tall cells such as neurons. However, we have been able to obtain satisfactory images of living neurons using relatively small neurons isolated from rat hippocampus.[6]

Based on a limited number of experiments it is apparent that cells of the same general type (e.g., glia, but isolated from different organisms have very different imaging properties in the AFM. In general, flatter cells with large areas of substrate contact are easiest to image. A critical parameter for imaging soft samples like living cells is the transient force increase during initial deflection of the cantilever (to which the scanning tip is attached). This deflection generates the signal that results in compensatory movement of the piezo crystal supporting the sample in an attempt to maintain a "constant" imaging force. In the error signal imaging mode,[15] in which the initial deflection prior to piezo compensation is displayed, one can measure this transient force increase. With relatively stiff (e.g., $k = 0.5$ nN) cantilevers this force can be very high, easily doubling the equilibrium imaging forces in the 1–10 nN range. Therefore, it is best to use the most compliant cantilever (e.g., $k = 0.06$ nN) that produces satisfactory images of living cells.

Several laboratories have demonstrated that it is possible to image living cells with the AFM.[2,3,5,6] Although the application of AFM to living cells is a relatively recent effort, some key observations have been made regarding imaging parameters, cell type, and substrate preparation for successful imaging. If the resolution possible on other samples can be realized with living cells, the AFM will become an extremely powerful tool in molecular cell biology and related disciplines.

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