Developing the atomic force microscope for studies of living cells

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Atomic force microscopy (AFM) is capable of imaging living cells in their native environment, at magnifications and resolutions exceeding those of both transmission and scanning electron microscopes. The AFM measures cell topography by moving a sharp probe mounted on the end of a flexible cantilever across the surface of the cell. However, unique physiological and physical properties of living cells make them very difficult to consistently image with the AFM. This article discusses the development of a system that achieves high-resolution imaging of living cells by AFM.

Maintenance of steady-state culture conditions

The authors found the most critical issue for consistent AFM imaging of living cells is the maintenance of steady-state culture conditions. Spurious cell movement is easily induced by changes in the cell's micro-environment and adversely affects AFM imaging. The technology used for studying living cells by AFM was developed using the thermostatted, controlled-environment culture system[1] shown in *Figure 1*. Coverglass cell cultures are mounted for inverted light microscopy (LM) in the bottom of Dvorak-Stotler culture chambers[2] (**Lucas-Highland Co.,** Chantilly, VA); the upper coverglass is omitted.

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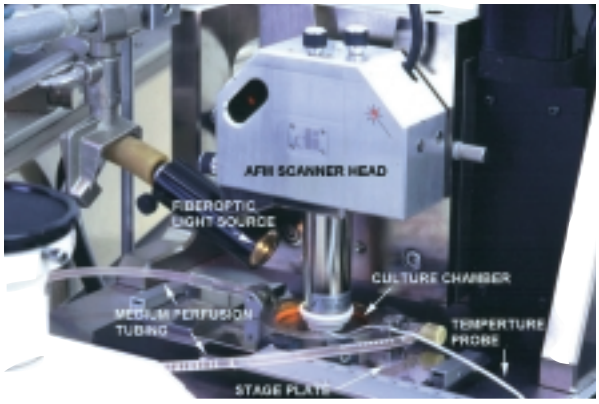

Figure 1 Close-up view of BioScope AFM scanner in place over the Dvorak-Stotler controlled-environment culture chamber.

The input and output ports of the chamber are fitted with 20-in.-long, sterile perfusion tubes. The distal ends of the perfusion tubes are attached to 10-mL syringes. The syringe at the input end of the chamber is filled with preequilibrated complete medium; the syringe at the output end of the chamber is filled with approx. 0.5 mL of complete medium. The chamber is mounted onto a custom-built stage plate on the AFM and the syringes are mounted on a modified infusion/withdrawal pump (model 940, **Harvard Apparatus,** South Natick, MA). The pump was modified such that infusion and withdrawal occurred simultaneously and in synchrony. The LM and AFM (BioScope, **Digital Instruments,** Santa Barbara, CA) are mounted on a vibration isolation table (model VW-306, **Newport Corp.,** Irvine, CA) and enclosed (except for the frontal area above the top of the air table) with a custom-built acoustic isolation chamber made of an aluminum shell covered on the inside wall with soundproofing insulation (no. AFBF-TP, **Acoustic Solutions Inc.,** Richmond, VA). The open area at the front of the isolation chamber is closed with two full-length,

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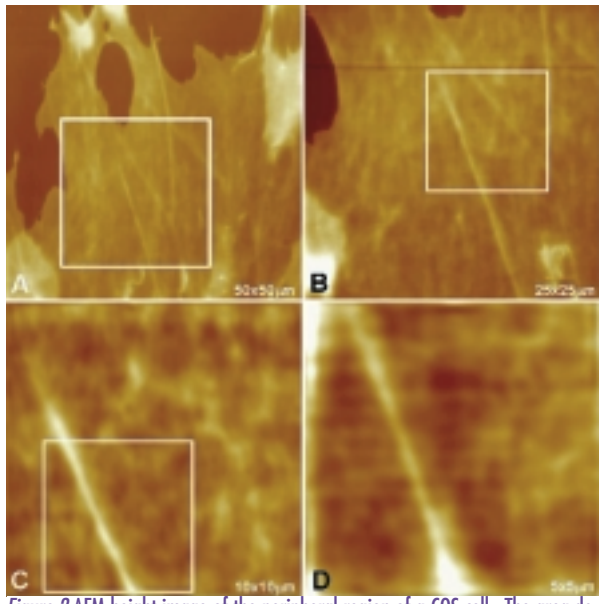

Figure 2 AFM height image of the peripheral region of a COS cell. The area demarcated by the square in A was magnified 2× in B, 5× in C, and 10× in D.

overlapping curtains of plastic bubble wrap consisting of individual bubbles approx. 25 mm in diameter. Temperature within the acoustic isolation chamber is controlled within 0.2 °C with an air stream incubator (model ASI, **Nevtek,** Burnsville, VA) and monitored by a scanning recording telethermometer (model 47, **YSI,** Yellow Springs, OH) using a thermistor probe attached to the AFM stage. The light sources for the microscopes are located outside of the environmental enclosure to prevent the heat generated by the light sources from influencing the temperature within the enclosure and, consequently,

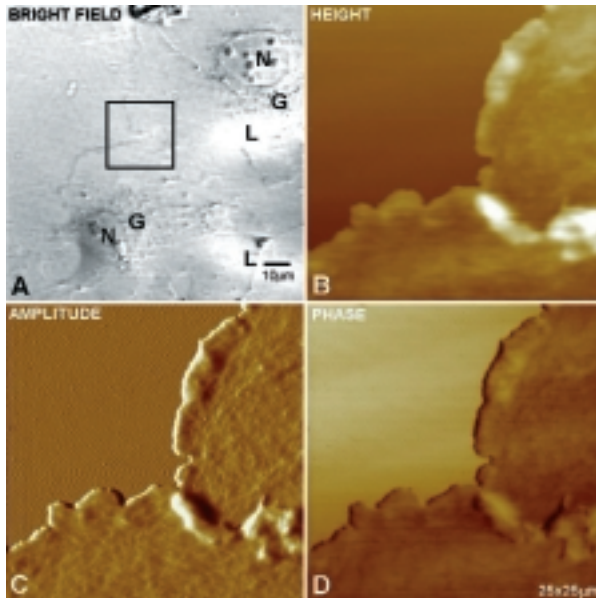

Figure 3 Brightfield LM and AFM images of a peripheral cytoplasmic region in COS-1 cells. A) Brightfield image, B) height, C) amplitude, and D) phase images at a scan size of 25 × 25 µm. The area demarcated by the squares was imaged in the AFM. (N, nucleus; G, golgi; L, AFM laser at edges of probe.)

the stability of the AFM. This system provides the living cells with a high degree of thermal stability as well as continual perfusion with preequilibrated medium. It not only maintains optimum physiologic conditions for the cells, but also minimizes cantilever drift by controlling temperature-induced variations.[3]

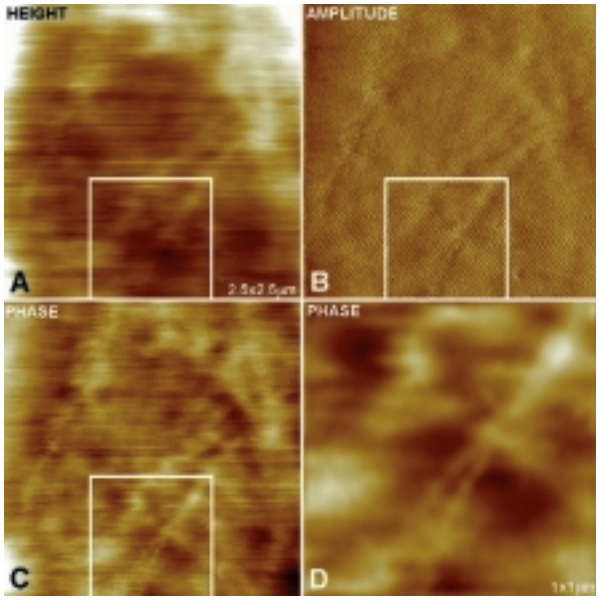

Figure 4 AFM images of the peripheral region of a COS cell. A) height, B) amplitude, and C) phase images at a scan size of 2.5 × 2.5 µm, D) enlarged phase images of the area demarcated by the square in C.

Tapping mode AFM imaging

In conventional contact mode, the AFM probe is in continuous contact with the surface of the cell being scanned. However, compression and shear forces generated by the probe tip as it traverses the cell surface damage the cell,[4,5] resulting in rapid image degradation as well as cell death. These problems can be markedly reduced by using tapping mode AFM. In tapping mode AFM, the cantilever is oscillated at a high frequency. These cantilever oscillations are reduced due to energy loss when the probe contacts the cell surface. This oscillation reduction can be used to identify and measure surface features of the cell. Tapping mode AFM greatly reduces the lateral forces present in contact mode AFM imaging.[6] However, reports of tapping mode AFM imaging of living cells are sparse.[6–8] The flat

Phase mode imaging is a variant of tapping mode AFM imaging and a new technology that permits visualization of localized variations in stiffness, adhesion, and viscoelasticity.

cytoplasmic region of a COS cell, which is too fragile to be imaged by contact mode AFM, could be successfully imaged with this system in tapping mode AFM at various magnifications (*Figure 2*). The authors found markedly fewer subsurface fibers in COS cells. This lower fiber density probably explains the fragility of COS cells when imaged by contact mode AFM. The area demarcated by white lines shows the actual scan area for each subsequent image. The cell moved slightly and changed shape between successive scans. However, the authors observed a quasi-regular pattern at the cell surface, which became apparent in a 10 × 10 µm scan (Figure 2*C*) and was essentially unchanged when the magnification was doubled (Figure 2*D*). Continuous imaging of the relatively flat cytoplasmic regions of COS cells was possible at a lateral resolution of approx. 70 nm and a vertical resolution of approx. 3 nm. The authors believe that tapping mode AFM imaging may well become a critical technique for studies of living homoiothermic vertebrate cells.

Phase mode AFM imaging

In addition to its ability to image living cells, AFM can produce both qualitative and quantitative data relevant to physical properties of the cell.[9] Phase mode imaging is a variant of tapping mode AFM imaging and a new technology that permits visualization of localized variations in stiffness, adhesion, and viscoelasticity. In AFM phase imaging, the phase lag of the cantilever oscillation relative to the signal sent to the cantilever's piezoelectric driver is used as a basis for image generation. However, the collection and interpretation of phase images of living cells has not been elucidated. Recently, the authors succeeded in AFM phase imaging of living cells. AFM-derived phase control images of mica, glass, and collagen in culture medium were systematically analyzed using a variety of force parameters; the resulting data provided critical information for the interpretation of phase images of living cells. For example, phase images of a mica surface demonstrated that in fluid, height information is a complicating factor in phase images. The height information must be removed in order to obtain pure phase data. Phase images of collagen displayed marked force-dependent phase changes; these phase transitions are due to changes in both the viscoelasticity and stiffness of collagen fibers. The peripheral regions of COS cells were scanned at various tapping forces.

Figure 3 shows height, amplitude, and phase images of the area demarcated by the square in the brightfield LM image. Phase images at various tapping forces consistently show a more negative phase shift than the glass substrate. This implies that the tapping frequency used is high enough to ensure that phase shifts are governed primarily by cell stiffness. The peripheral regions of COS cells were also imaged at a scan size of 2.5 × 2.5 µm, as shown in *Figure 4*. The height image loses definition, and the amplitude image is nearly featureless at 2.5 × 2.5 µm. In contrast, both surface and subsurface features are clearly resolvable as positive (bright) phase shifts in the AFM phase image. The authors concluded that these positive phase shifts are undoubtedly due to localized fiber stiffness. The area of the cell membrane demarcated by the square in the 2.5 × 2.5 µm phase image (Figure 4*C*) is shown enlarged to 1 x 1 µm in Figure 4*D*. A bifurcating fiber with a lateral resolution of 30 nm is discernible only in the phase image. This resolution exceeds all previous reports of fiber measurements in living cells. AFM phase imaging was found to be a powerful tool, particularly at high magnification. The AFM phase technique is capable of very high-resolution imaging of living cells. The authors predict that AFM-based tapping mode imaging, especially phase imaging under steady-state culture conditions, will become an important and useful tool for medical and cell biological reseach.

References

1. Nagao E, Dvorak JA. An integrated approach to the study of living cells by atomic force microscopy. J Microsc 1998; 191:8–19.
2. Dvorak JA, Stotler WF. A controlled-environment culture system for high resolution light microscopy. Exp Cell Res 1971; 68:144–8.
3. Radmacher M, Cleveland JP, Hansma PK. Improvement of thermally induced bending of cantilevers used for atomic force microscopy. Scanning 1995; 17:117–21.
4. Fritz M, Radmacher M, Gaub HE. *In vitro* activation of human platelets triggered and probed by atomic force microscopy. Exp Cell Res 1993; 205:187–90.
5. Schoenenberger CA, Hoh JH. Slow cellular dynamics in MDCK and R5 cells monitored by time-lapse atomic force microscopy. Biophys J 1994; 67:929–36.
6. Putman CAJ, Van der Werf KO, De Grooth BG, Van Hulst NF, Greve J. Viscoelasticity of living cells allows high resolution imaging by tapping mode atomic force microscopy. Biophys J 1994; 67:1749–53.
7. Vesenka J, Moscher C, Schaus S, Ambrosio L, Henderson E. Combining optical and atomic force microscopy for life sciences research. Biotechniques 1995; 19:240–9.
8. Schaus SS, Henderson ER. Cell viability and probe-cell membrane interactions of XR1 glial cells imaged by atomic force microscopy. Biophys J 1997; 73:1205–14.
9. Dvorak JA, Nagao E. Kinetic analysis of the mitotic cycle of living vertebrate cells by atomic force microscopy. Exp Cell Res 1998; 69–74.