Analyzing Chromosomes, Ion Channels and Novel Nucleic Acid Structures by AFM

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Abstract. The atomic force microscope (AFM) is proving to be a powerful tool for analysis of biological samples. We provide three examples of the application of AFM to the study of biological questions. First, polytene chromosomes from *Drosophila* are imaged and manipulated by the AFM. Second, the localization of calcium channels on the release face of a nerve terminal is described. Finally, analyses of a new form of DNA, the G-wire, is presented. These examples illustrate the wide variety of biological questions to which AFM can contribute.

1. Introduction

The atomic force microscope (AFM) (1) has recently come into vogue as a tool for studying biological samples. Simple sample preparation requirements, generation of three-dimensional data sets, detection of numerous force interactions and the ability to manipulate as well as image samples have contributed to the popularity of the AFM. In this report we provide an overview of three studies using the AFM to address biological questions. The samples under investigation are polytene chromosomes, calcium channels and a novel DNA structure, the G-wire.

Polytene chromosomes from *Drosophila melanogaster* have several attributes that make them an excellent test system for analysis of chromosome and chromatin structure by AFM. They have been extremely well studied and mapped with light and electron microscopes. The locations of many genes are known with relatively high precision. The chromosomes are very large due to the many endoduplications of the DNA during larval development. Finally, polytene chromosomes contain interphase chromatin, rather than highly condensed metaphase chromatin, making them an ideal sample for study of the relationship between chromatin structure and gene expression.

The movement of calcium in neurons is critically linked to synaptic vesicle release and synaptic transmission. The flow of calcium into the nerve terminal triggers the fusion of secretory vesicles with the plasma membrane and release of their contents. Calcium enters the nerve terminal through a sub-group of membrane proteins, the calcium channels. The organization of these calcium channels in the nerve terminal is likely to be critical for fast (<l ms) synaptic transmission. However, previous studies have not identified these proteins, thus, their 3D organization is undefined.

G-rich DNA (and RNA) sequences can form novel structures stabilized by cation coordination and a tetrameric arrangement of guanines, each hydrogen bonded to two neighbors, called the G-quartet (2, 3). We have recently discovered conditions under which a simple synthetic oligonucleotide, d(GGGGTTGGGG), can spontaneously assemble into large superstructures stabilized by G-quartets. These structures are termed G-wires (4, 5). G-wires are extraordinarily stable and have unusual kinetic properties with regard to growth. These and other features make them an interesting subject for study by AFM.

In this paper we will provide an overview of these three projects, all of which have been presented in detail elsewhere (4, -8).

2. Experimental

2.1 Polytene Chromosome Preparation

Polytene chromosomes of salivary gland cells from *Drosophila melanogaster* were isolated and squashed as previously described (9). All of the imaging substrates were glass. Standard microscope slides (26 X 76 mm^2), 12 mm^2 round coverslips (Fisher), and #1 glass coverslips; either 22 X 22 mm^2 or 22 X 44 mm^2 (Corning) were used. Some of the coverslips were acid washed. There was no apparent difference between the acid washed and the untreated coverslips.

2.2 Chick Giant Synapse Preparation

Chick ciliary ganglion presynaptic nerve terminals (the calyx) were prepared and the release face identified by fluorescence microscopy using the vital dye 4-Di-2-Asp as previously described (10-12). The specimen was kept wet and fixed prior to imaging. The N+ type calcium channel toxin w-conotoxin GVIA (w-CTX), conjugated to biotin, was added to the preparation, allowed to bind to calcium channels, and the excess removed by rinsing. Streptavidin-gold (30 nm dia.) was added, allowed to bind to the biotinylated w-conotoxin, and the excess rinsed. Samples were then imaged in solution in contact mode as described below.

2.3 G-Wire Preparation

d(GGGGTTGGGG) was dissolved in the appropriate buffer (typically containing Na+ or K+ ions) as previously described (4, 5) at 1-10 mg/ml and incubated at 37°C for 12-24 h. After incubation the samples were diluted 1: 100 in 10 mM Tris-HCI, pH 7.5 and 1 mM $MgCl_2$.

2.4 AFM Methods

All AFM was performed using a Nanoscope III (Digital Instruments, Inc.) equipped with a J or D scanner and using either standard silicon nitride tips, oxide sharpened tips or silicon tapping tips. In some cases a BioScope^{Tm} (combined AFM/optical microscope) was used to facilitate localization of area of interest. Both contact and, when possible, TappingMode^{Tm} images were obtained. Images in air were typically collected at relative humidity ≤ 10%.

Polytene chromosomes were imaged either in air, under water or in phosphate buffered saline (PBS) as previously described (7, 8). Chromosome dissection experiments were carried out in phosphate buffered saline (PBS). A typical dissection was achieved by disabling the slow scan axis at the region to be cut and maximizing both the force and the scan rate (13, 14, 15). By making small adjustments in the scan angle we were able to increase the size of the cuts. This process can be accomplished in less than 2 minutes.

Chick ciliary ganglion presynaptic nerve terminal release faces were imaged in solution in contact mode. A statistical analysis of the occurrence of protuberances thought to correspond to gold particles in control and experimental samples was carried out (6). The relative spacing of the gold particles was measured using NIH Image (version 1.53).

For G-wires, 10µl of the G-wire preparation was deposited onto a freshly cleaved mica substrate. The G-wires were allowed to adsorb for 5 min., washed with 1 ml sterile water, and rapidly dried in a stream of N_2 gas. For contact mode imaging, the relative humidity was maintained at ≤ 10%.

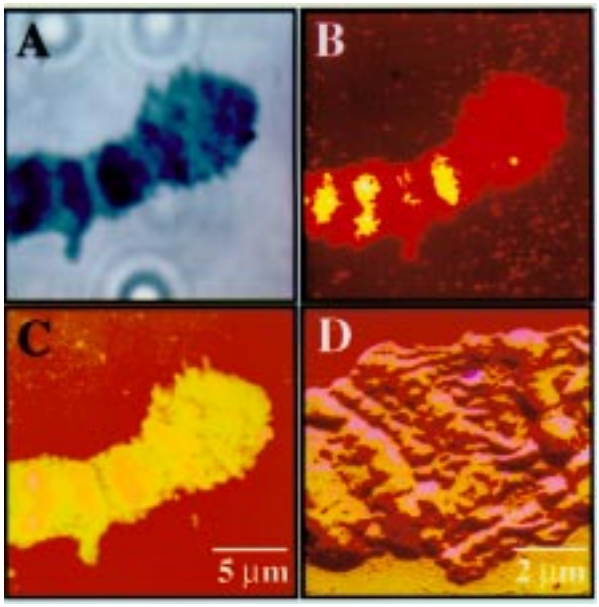

Figure 1. Images of a polytene chromosome. A, brightfield image of chromosome stained with commassie blue. B, The same portion of a chromosome stained with ethidium bromide homodimer I and imaged by scanning laser confocal fluorescence microscopy. C, AFM height image of the same chromosome taken in air. D, Higher resolution AFM deflection mode image of another portion of the same chromosome, imaged in PBS. Note the details in chromosome substructure.

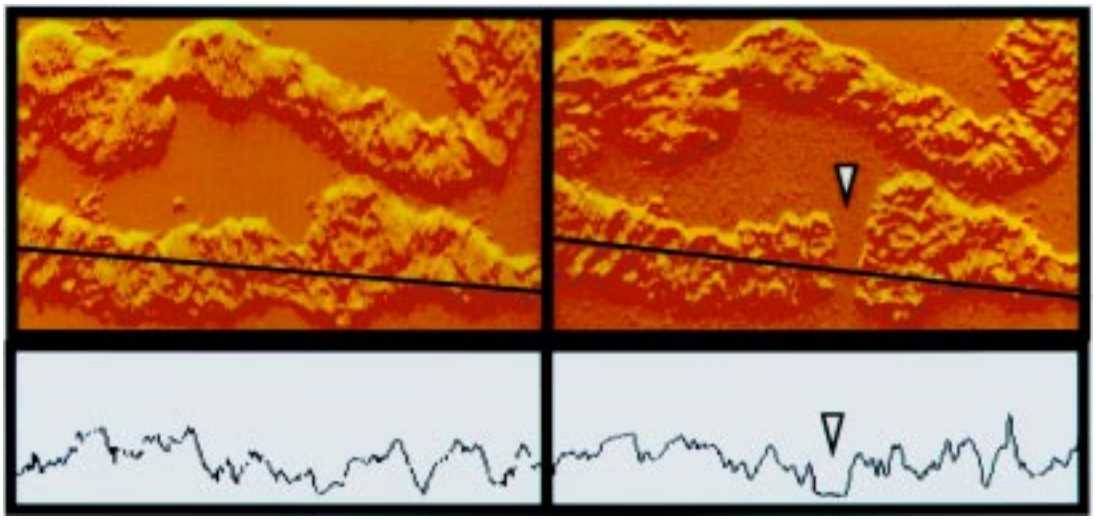

Figure 2. Chromosome dissection by AFM. The panels on the left show a deflection mode image of a polytene chromosome in PBS and a section through the chromosome (from a height mode image) prior to dissection. In the right hand panels, the same chromosomes are shown after dissection of a region in the lower chromosome (arrowhead).

3. Results and Discussion

3.1 Polytene Chromosomes

Polytene chromosomes were readily imaged in the AFM (Fig. 1). Their large size facilitated finding them on the substrate surface, even when present at low surface coverage. This feature, in conjunction with use of the combined optical/atomic force microscope (BioScope), greatly enhanced the rate of progress of this project.

Bands and interbands, similar to those observed by optical and electron microscopy, were observed. Bands often had bumpy surfaces corresponding to loops at the ends of chromatin strands attached at the base to the chromosome scaffold (Fig. 1D). Comparing DNA density (using the DNA binding dye ethidium homodimer I) with band height clearly showed that the DNA is packed in the Z dimension, rather than X or Y.

The structure of the strands in interband was complex. Numerous particles were apparently associated with the chromatin, most likely including transcription factors and polymerase complexes as well as structural proteins involved in chromosome architecture. A detailed discussion of interband structure will appear elsewhere.

By increasing the force applied by the AFM tip, and disabling scanning in the slow (Y) direction, it was possible to cut chromosomes (Fig. 2). After cutting, image quality is often compromised, suggesting that some of the excised chromatin had adhered to the scanning tip. Thus, the AFM may provide a rapid physical means for obtaining defined regions of chromosomes, which would facilitate gene mapping efforts. Details of these studies are presented elsewhere (7, 8).

3.2 Calcium Channels at Nerve Terminals

The general protocol for mapping calcium channels at the nerve terminals was to tag the channel with a toxin, w-conotoxin, conjugated to a gold ball through a streptavidin-biotin bridge (6). Control experiments in which various steps in the mapping procedure were blocked clearly showed that the increased incidence in the appearance of features on the surface of the release face corresponded to gold bound to calcium channels through the w-conotoxin-gold conjugate (Fig. 3). Statistical analysis of these data showed that the spacing of calcium channels is 40 nm or larger in increments of 20 nm. Since the gold used in this study was 30 nm in diameter, the 20 nm harmonic indicates that channels are in fact spaced at 20 nm intervals in the nerve terminal. This high density of calcium channels found in the nerve terminal ensures that synaptic vesicles (40-50 nm diameter) are likely to be immediately adjacent to calcium channels. A detailed description of this study is presented in (6).

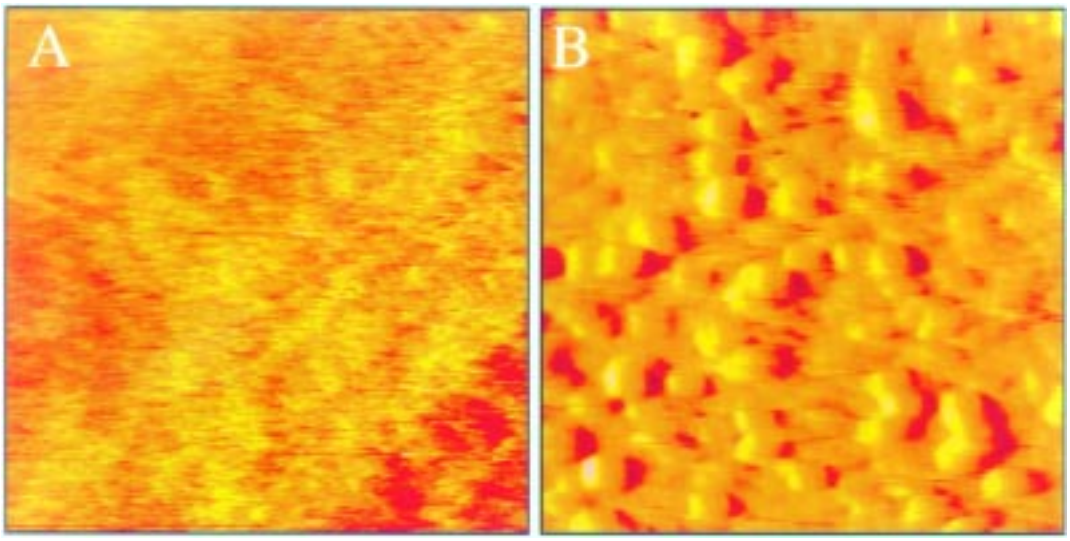

Figure 3. Tagging calcium channels at the nerve terminal with an w-conotoxin-biotin-streptavidin-colloidal gold conjugate. Panel A shows a calyx surface from a control experiment in which the sample was pre-blocked with non-biotinylated w-conotoxin. Panel B shows the experimental result. Both images were acquired in deflection mode. The field size is 1 μm.

3.3 G-Wires

Biochemical data had strongly suggested that the oligonucleotide d(GGGGTTGGGG) assembled into higher order structures under the appropriate conditions (5), but the morphological nature of these structures was unknown. AFM analyses of G-wires immediately gave significant insight into their structural nature (4) (Fig. 4). They are long, linear polymers. Their measured height is approximately 25-30 Å, which agrees well with the diameter of a G-quartet determined by NMR and x-ray crystallography (16-18). This is an interesting finding because the height of B-DNA in the AFM is typically 5-7Å, which is less than half its width determined by X-ray crystallography. Thus, it appears that G-wires are more robust than B-DNA in the sense that they are not compressed by the scanning tip. An additional possibility is that B-DNA undergoes a severe conformation change when absorbed to a surface, but G-wires do not. However, previous work determining the helical period of B-DNA on mica surfaces suggests that it is not significantly altered upon binding to the mica (19).

The studies briefly described here illustrate the breadth of application of AFM to biological questions. As the interest of biological investigators in AFM methods co-evolves with improved and specialized AFM instrumentation, it is likely that this technology will make an increasingly significant contribution to life sciences research.

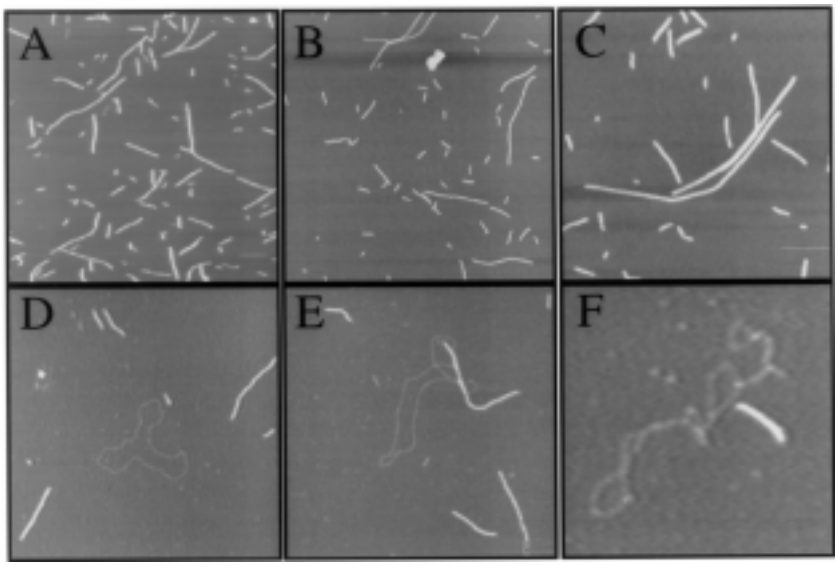

Figure 4. Height mode images of G-wires. All panels show G-wires grown in the presence of sodium ion and spermidine. Panels D-F show co-deposited G-wires and B-form plasmid DNA. Note the height difference (white to gray = tall to flat) between the plasmid DNA and the G-wires. Field sizes are: panels A, B, D and E, 1μm, panels C and F, 500 nm.

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5. References

1. Binnig, G., Quate, C.F. and Gerber, C. (1986) *Phys. Rev. Lett.* 56, 930-933.

2. Guschlbauer, W., Chantot, J.-F. and Thiele, D. (1990) *J. Biomol. Struct. Dyn.* 8, 491-511.

3. Williamson, J.R., Raghuraman, M.K. and Cech, T.R. (1989) *Cell* 59, 871-880.

4. Marsh, T.C., Vesenka, J. and Henderson, E. (1994) *submitted*

5. Marsh, T.C. and Henderson, E. (1994) *Biochemistry* 33, 10718-10724.

6. Haydon, P.G., Henderson, E. and Stanley, E.F. (1994) *Neuron* in press,

7. Jondle, D.M., Ambrosio, L., Vesenka, J. and Henderson, E. (1995) *Chrom. Res.* in press,

8. Mosher, C, Jondle, D., Ambrosio, L., Vesenka, J. and Henderson, E. (1995) *Scan. Micros.* in press.

9. Pardue, M.L. (1986) In Roberts, D.B. (ed.), *Drosophila* a practical approach. IRL Press, Oxford, Washington DC, pp. 111- 137.

10. Stanley, E.F. and Atrakchi, A.H. (1990) *Proc. Natl. Acad. Sci. USA* 87, 9683-9687.

11. Stanley, E.F. (1991) *Neuron* 7, 585-591.

12. Stanley, E.F. and Goping, G.J. (1991) *J. Neurosci.* 11, 985-993.

13. Henderson, E. (1992) *Nuc. Acids Res.* 20, 445-447.

14. Vesenka, J., Hansma, H., Siegerist, C, Siligardi, G., Schabtach, E. and Bustamante, C. (1992) *SPIE* 1639, 127-137.

15. Hansma, H., Vesenka, J., Siegerist, C, Kelderman, G., Morret, H., Sinsheimer, R.L., Elings, V., Bustamante, C. and Hansma, P.K. (1992) *Science* 256, 1180-1184.

16. Kang, C.H., Zhang, X., Ratliff, R., Moyzis, R. and Rich, A. (1992) *Nature* 356, 126-131.

17. Laughlan, G., Murchie, A.I.H., Norman, D.G., Moore, M.H., Moody, P.C.E., Lilley, D.M. and Luisi, B. (1994) *Science* 265, 520-524.

18. Smith, F.W. and Feigon, J. (1992) *Nature* 356, 164-168.

19. Rhodes, D. and Klug, A. (1980) *Nature* 286, 573-579.