Atomic Force Microscopy in the Pharmaceutical Sciences: Drug Interactions and Disease Mechanisms

By: John T. Thornton
Veeco Metrology Group

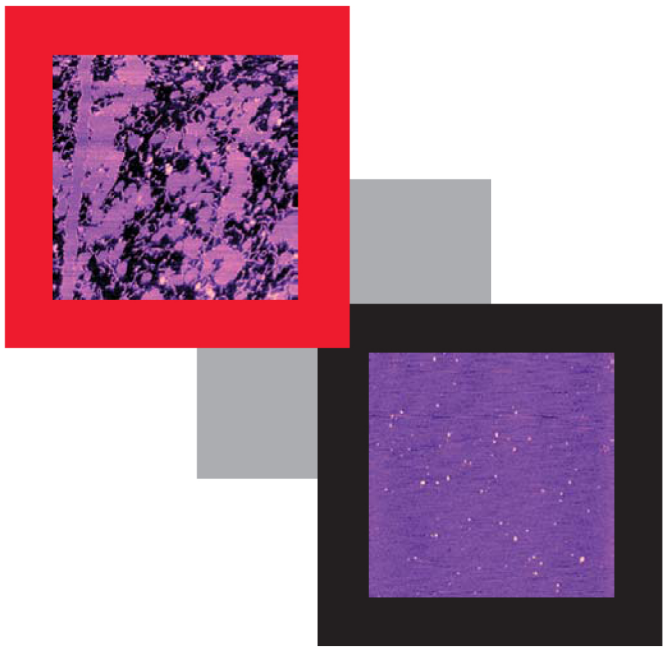
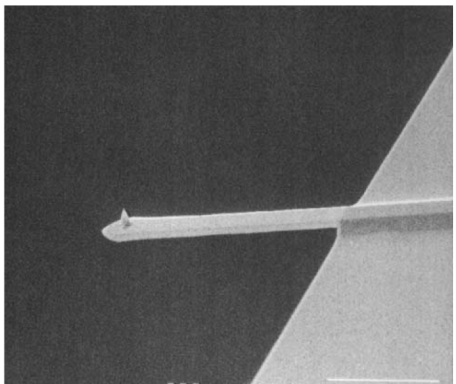


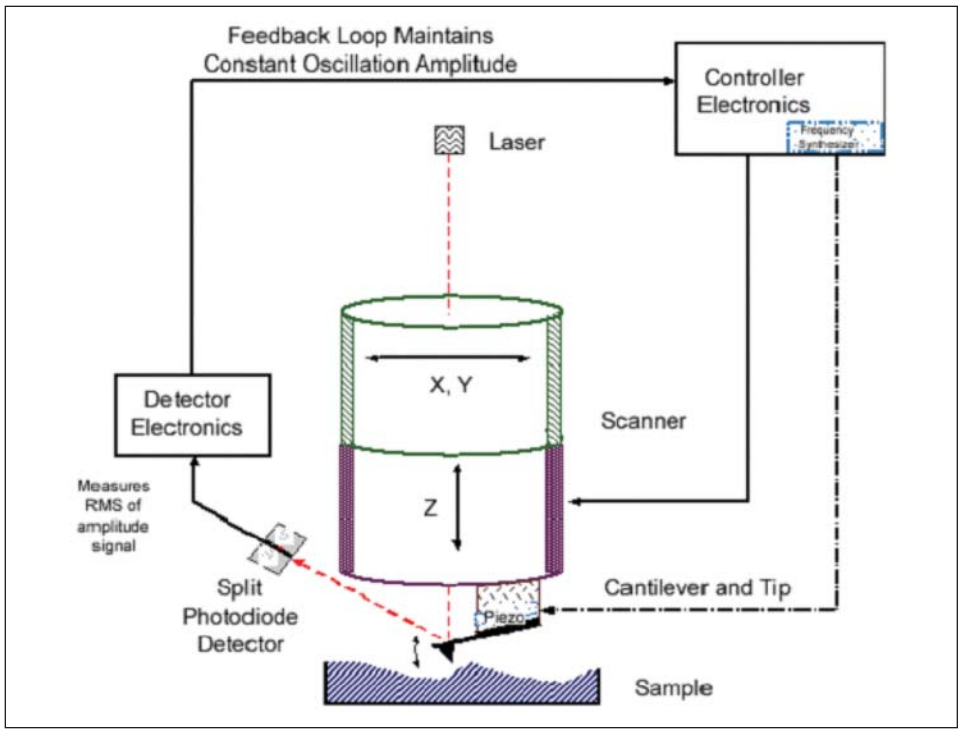
Figure 1
SEM image of an integrated single crystal silicon cantilever and tip with an end radius of 5 - 10nm. Magnification 300X.



Introduction

Since atomic force microscopy (AFM) provides the ability to investigate surface structure at nanometer-to-subangstrom resolution in ambient and liquid environments, it has been used routinely over the past two decades in many scientific fields. Indeed, AFM has contributed to ground-breaking research in the investigation of DNA, proteins, and cells in biological studies;[1] structure and component distribution in polymer science;[2] piconewton force interactions and surfactant behavior in colloid science;[3,4] and physical/mechanical properties and fabrication variables in the material sciences.[5,6] Pharmaceutical research often consists of a combination of these scientific branches, making it a particularly viable field for the application of AFM.[7] The ability of AFM to provide high-resolution, three-dimensional surface structure, regardless of sample conductivity, makes it a powerful complement to other common analytical techniques currently available.[8] This application note examines how AFM has been applied to pharmaceutical research studies of drug interactions and disease mechanisms.

Figure 2
Schematic of the major components of an atomic force microscope, showing the feedback loop for TappingMode operation.



AFM History and Methods

AFM is the most commonly used form of the scanning probe microscopy (SPM) family of techniques. The origin of SPM began with the development of the scanning tunneling microscope (STM) in 1982 by researchers at IBM, Zurich.[9] The ability of the STM to resolve atomic structure on a sample surface earned the inventors the Nobel Prize in 1986.

However, the STM can only be applied to conductive or semiconductive specimens. To broaden this type of microscopy to the study of insulators, the atomic force microscope was developed in collaboration between IBM and Stanford University in 1986.[10] AFM is performed by scanning a sharp tip on the end of a flexible cantilever across a sample surface, while maintaining a small, constant force (see fig. 1). The tips typically have an end radius of 5 nm to 10 nm, although this can vary depending on tip type. The scanning motion is conducted by a piezoelectric tube scanner that scans the tip in a raster pattern with respect to the sample (see fig. 2). The tip-sample interaction is monitored by reflecting a laser off the back of the cantilever onto a split-photodiode detector. The two most commonly used modes of operation are contact mode AFM and TappingMode™ AFM, which are conducted in air and liquid environments.

In contact mode AFM, a constant cantilever deflection is maintained by a feedback loop that moves the scanner vertically (z) at each lateral (x,y) data point to form the topographic image. By maintaining a constant deflection during scanning, a constant vertical force is maintained between the tip and sample. Applied forces during imaging typically range between 0.1 and 100 nN. Although contact mode has proven useful for a wide range of applications, it sometimes has difficulty on relatively soft samples.

TappingMode AFM consists of oscillating the cantilever at its resonance frequency (typically ~300 kHz) and scanning across the surface with a constant, damped amplitude. The feedback loop maintains a constant root-mean-square (RMS) amplitude by moving the scanner vertically during scanning, which correspondingly maintains a constant applied force to form a topographic image. The advantage of TappingMode is that it typically operates with a lower vertical force than that possible with contact mode, and it eliminates the lateral, shear forces that can damage some samples. Thus, TappingMode has become the preferred technique for imaging soft, fragile, adhesive, and particulate surfaces.

Although the initial use of SPM was to produce high-resolution topographic images, a number of SPM techniques have been developed to study physical and materials properties about sample surfaces. These techniques are commonly used to investigate differences in friction, adhesion, elasticity, hardness, magnetic and electrostatic fields, carrier concentration, conductivity, and temperature distribution. Fundamental force studies are also conducted to study adhesive, attractive, and repulsive interactions between specimens.

One of the important innovations that has been integrated into commercial atomic force microscopes is environmental control of the sample. Imaging at elevated temperatures

has made it possible to study thermal phase transitions in ambient or inert gas conditions. Imaging in fluid at elevated temperatures has provided the ability to image structures at a physiological temperature of 37°C. Atmospheric hoods are also commonly used in AFM to control humidity or to conduct experiments under specific atmospheric conditions.

Drug Interactions

Interactions between biological specimens, such as ligand-receptor[11] and protein-DNA[12] systems, have frequently been studied by AFM, and can also be applied to the study of drug interactions with a variety of biological specimens. For example, AFM-based immunological studies have investigated antibody-antigen binding interactions,[13–15] and drug-DNA complexes have been studied with AFM to determine DNA ligand mode-of-binding.[16–19] This is of considerable interest since nucleic acid ligands are commonly used as anticancer drugs and in the treatment of genetic diseases. However, determining whether they bind to DNA by intercalation within major and minor grooves, by "nonclassical" modes, or by a combination of these modes can often be difficult and labor intensive. AFM was used to study drug binding mode, affinity, and exclusion number by comparing the length of DNA fragments that have and have not been exposed to the drug.[17] It is known that if intercalative binding is occurring, the DNA strand increases in length. Furthermore, the degree of lengthening is informative in determining the binding affinity and the site-exclusion number. AFM was shown to be an effective means of seeing and measuring any changes in the DNA strand.

When exposed to ethidium, a well-characterized intercalator, the DNA strand was shown through AFM to have increased in length from 3300 nm to 5250 nm (see fig. 3). Similarly, AFM intercalative binding studies showed the increase in the DNA strand, from 3300 nm to 4670 nm, upon exposure to daunomycin, an anticancer drug used to treat leukemia. This technique has also successfully been applied to new drugs in which the mode of binding was unclear. Exposure of 2,5-bis(4-amidinophenyl) (APF), a new drug for the treatment of *Pneumocystis carinii* pneumonia, did not produce lengthening of the DNA strands, indicating that the drug binds by nonintercalative modes.[18]

Although many AFM studies have concentrated on interactions at the molecular level, responses of living cells have also been visualized. Since cells can be imaged in a physiological solution at 37°C, it is possible to monitor the interaction between a metabolically active cell and a chemical or biological additive to the solution environment. Work has been published on observing the interaction between living cells and drugs,[20,21] viruses,[22–24] and other chemicals.[25,26] Rotsch and Radmacher conducted "force mapping" studies on 3T3 and NRK fibroblast cells to visualize the effects of various drugs.[20] Force mapping, also referred to as "the force volume technique," conducts a lateral array of force curves across a sample surface that results in a force-interaction image.[27] Collecting force curves consists of recording the change in the cantilever deflection as it moves vertically toward the sample until it touches the surface, and then pulls away from the surface and retracts back to its starting position.[28] Force curves can detect repulsive, attractive, and adhesive interactions in the piconewton-to-nanonewton range.

In Rotsch and Radmacher's study, force volume was used to map cell

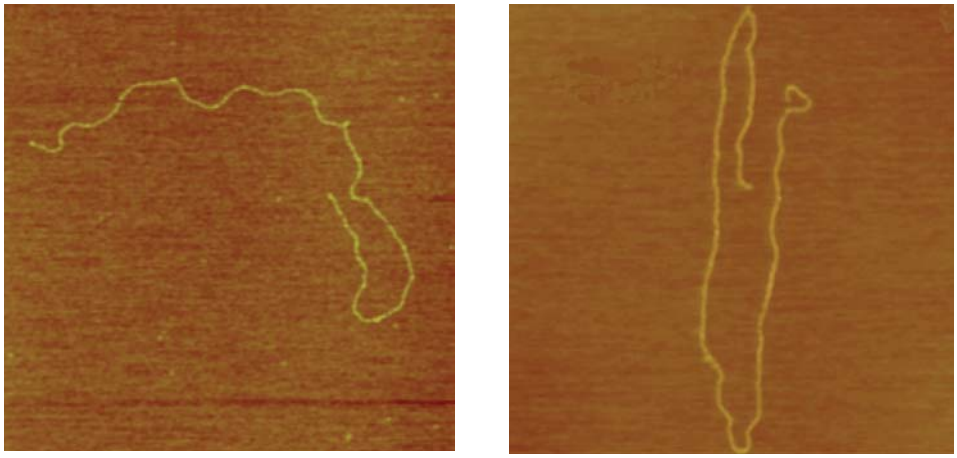
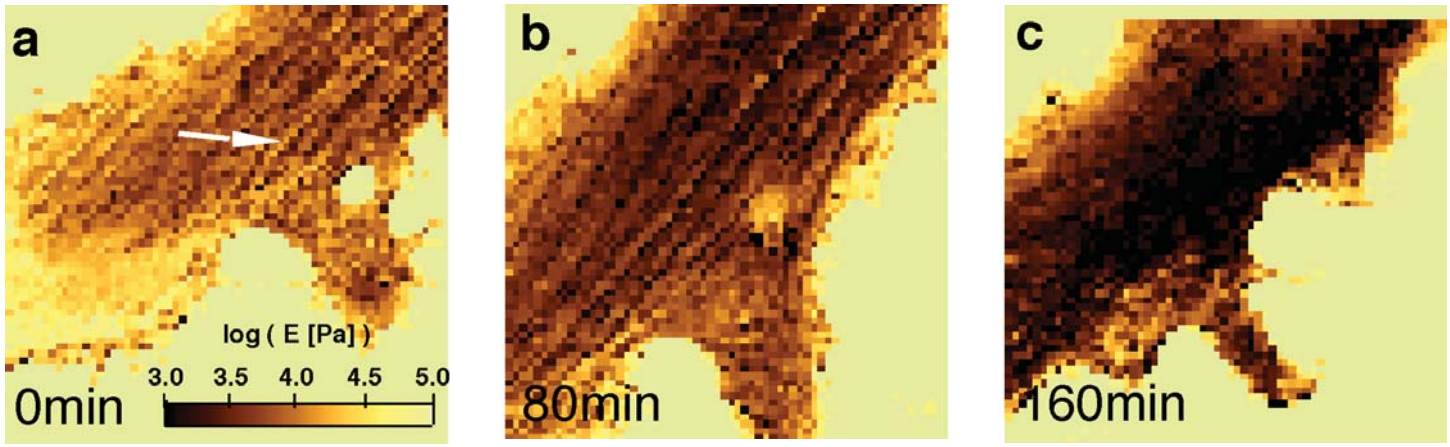


Figure 3
2.5 µm scans showing *(left)* a ligand-free DNA strand measuring ~3300 nm; *(right)* an ethidium-DNA complex measuring ~5250 nm. Images courtesy of L.A. Bottomley, Georgia Institute of Technology. [Reference 17]

Figure 4
Elasticity maps showing the dissaggregation of actin filaments in 3T3 fibroblasts due to interaction with Cytochalasin B. Images courtesy of Rotsch and Radmacher, Universität München, Germany. 230µm scan size. [Reference 20]



elasticity changes over time due to the interaction with f-actin disabling drugs. Actin filaments form a network that provides mechanical stability to the cell. By adding f-actin disabling drugs (Cytochalasin B and D, Latrunculin A, and jasplakinolide) to the solution environment of the living cells, the disaggregation of the actin filaments could be studied directly by observing the change in the elasticity of the cells. In AFM, a reduced amount of cantilever deflection when the tip is in contact with the cell indicates a decrease in elasticity. In **figure 4**, the effect of Cytochalasin B on the elastic modulus of the 3T3 fibroblast can be seen over time. Before addition of the drug, the actin filaments can be clearly seen in the force volume image (arrow in **fig. 4a**) due to their high stiffness with respect to the rest of the cell. After addition of Cytochalasin B to the fluid chamber with the cells, the disaggregation of the actin network produces a decrease in elasticity, which is indicated by the darker areas in the images. Over the 160-minute time frame, the elasticity (Young's modulus) was seen to decrease by a factor of 3.1. With each of the drugs applied, the effects of drug concentration on the rate of interaction and the decrease in elasticity were successfully studied.

Disease Mechanisms

AFM is also commonly used to gain a better understanding of the physiological mechanisms associated with disease. Some examples of this include AFM studies associated with Alzheimer's disease,[29–32] Parkinson's disease,[33,34] diabetes,[35] pancreatitis,[36] and cancer.[37] One of the advantages in using an atomic force microscope for this type of work is its ability to perform these investigations in situ.[38]

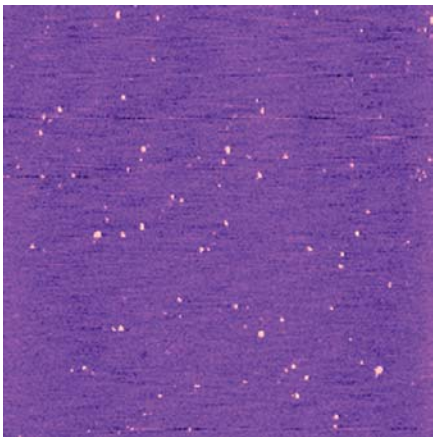
Yip and McLaurin used AFM to study the mechanisms of amyloid-ß (Aß) fibrillogenesis, which plays a role in Alzheimer's disease.[29] In situ TappingMode imaging was performed on total brain lipid bilayers to study the role of membrane composition and peptide structure. Brain lipid bilayers were deposited onto mica and imaged in phosphate buffer solution (PBS). After introducing the monomeric Aß peptides into the buffer solution, Aß1-40 molecules were found to be partially inserted into the bilayer surface (see **fig. 5a**). After 15 hours, fibril growth was initiated from these sites, resulting in membrane disruption (see **fig. 5b**). To study the specificity of lipid bilayer composition and Aß sequence, the same experiment was conducted with DMPC bilayers and Aß1-28 peptides. From these studies it was determined that the fibril formation occurs in the presence of acidic lipids, and that the peptide requires the hydrophobic C-terminal domain, which is critical for anchoring to the lipid to induce fibrillogenesis. Without these critical requirements, membrane disruption is produced by the formation of Aß aggregates without evidence of fibril formation. Based on these AFM observations, a better understanding of the mechanisms that result in the Aß fibrillogenesis was formed.

Summary

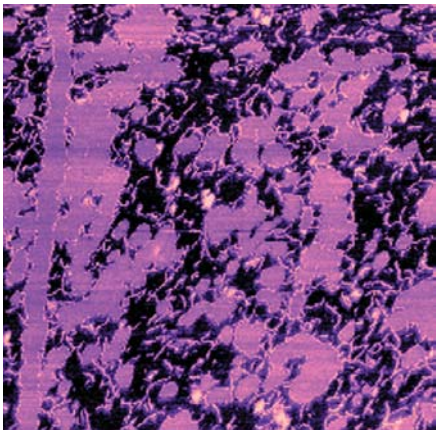
Atomic Force Microscopy (AFM) has many uses in the field of pharmaceutical research, including the investigation of in situ processes, interaction mechanisms, behavioral properties, and structure-function relationships. Although these examples are just a sampling of the work that has been conducted in drug interaction and disease mechanism studies, they indicate how important the atomic force microscope can be in furthering pharmaceutical sciences. The complementary nature of AFM with other analytical techniques will certainly result in many more applications as it is further adopted by the pharmaceutical community and as new AFM/SPM techniques are developed.

Figure 5

Images of brain lipid extract bilayers imaged by TappingMode in PBS. After introducing the amyloid-ß peptides (Aß1-40) into the buffer solution, they were found to be partially inserted into the bilayer surface (a). Fibril growth was initiated from these sites, resulting in membrane disruption. The image in (b) was acquired 15 hours after (a). Images courtesy of C. Yip, University of Toronto. 10µm scan size. [Reference 29]



a.)



b.)

References

1. Revenko, I., "Probing the Life Sciences with Atomic Force Microscopy," in Micro- and Nanostructures of Biological Systems, Proceedings of 1st Symposium held at the Martin Luther University Halle-Wittenberg at Halle, October 4–5, 2000, pp. 1–51.
2. Magonov, S.N., "Visualization of Polymers at Surfaces and Interfaces with Atomic Force Microscopy," Chp.10, Handbook of Surfaces and Interfaces of Materials, Vol. 2, ed. H.R. Nalwa, Academic Press, 2001, pp. 393–429.
3. Ducker, W. A., T.J. Senden, and R.M. Pashley, "Direct Measurement of Colloidal Forces Using an Atomic Force Microscope," Nature 353 (1991) 239.
4. Manne, S., J.P. Cleveland, H.E. Gaub, G.D. Stucky, and P.K. Hansma, "Direct Visualization of Surfactant Hemimicelles by Force Microscopy of the Electric Double Layer," Langmuir 10 (1994) 4409.
5. VanLandingham, M.R., S.H. McKnight, G.R. Palmese, J.R. Elings, X. Huang, T.A. Bogetti, R.F. Eduljee, and J.W. Gillespie, "Nanoscale Indentation of Polymer Systems Using the Atomic Force Microscopy," J. Adhesion 64 (1997) 31.
6. Strausser, Y.E., M. Schroth, and J.J. Sweeney, "Characterization of the Low-Pressure Chemical Vapor Deposition Grown Rugged Polysilicon Surface Using Atomic Force Microscopy," J. Vac. Sci. Technol. A 15 (1997) 1007.
7. Shakesheff, K.M., M.C. Davies, C.J. Roberts, S.J.B. Tendler, and P.M. Williams, "The Role of Scanning Probe Microscopy in Drug Delivery Research," Crit. Rev. Therap. Drug Carr. Syst.13 (1996) 225.
8. Russell, P., D. Batchelor, and J. Thornton,"SEM and AFM: Complementary Techniques for Surface Investigations," Microscopy and Analysis 49 (2000) 5.
9. Binnig, G., H. Rohrer, Ch. Gerber, and E. Weibel, "Surface Studies by Scanning Tunneling Microscopy," Phys. Rev. Lett. 49 (1982) 57.
10. Binnig, G., C.F. Quate, and Ch. Gerber, "Atomic Force Microscope," Phys. Rev. Lett. 56 (1986) 930.
11. Florin, E., V.T. Moy, and H.E. Gaub, "Adhesion Forces Between Individual Ligand-Receptor Pairs," Science 264 (1994) 415.
12. Wyman, C., E. Grotkopp, C. Bustamante, and H.C.M. Nelson,"Determination of Heat-Shock Transcription Factor 2 Stoichiometry at Looped DNA Complexes using Scanning Force Microscopy," The EMBO Journal 14 (1995) 117.
13. Allen, S., J. Davies, M.C. Davies, A.C. Dawkes, C.J. Roberts, S.J.B. Tendler, and P.M. Williams,"The Influence of Epitope Availability on Atomic-Force Microscope Studies of Antigen-Antibody Interactions," Biochem. J. 341 (1999) 173.
14. Quist, A.P., A.A. Bergman, C.T. Reimann, S.O. Oscarsson, and B.U.R. Sundqvist, "Imaging of Single Antigens, Antibodies, and Specific Immunocomplex Formation by Scanning Force Microscopy," Scanning Microscopy 9 (1995) 395.
15. Jones, V.W., J.R. Kenseth, M.D. Porter, C.L. Mosher, and E. Henderson, "Microminiaturized Immunoassays Using Atomic Force Microscopy and Compositionally Patterned Antigen Arrays," Anal. Chem. 70 (1998) 1233.
16. Coury, J.E., L. McFail-Isom, S. Presnell, L.D. Williams, and L.A. Bottomley, "Scanning Probe Visualization of Electrostatically Immobilized Intercalating Drug-Nucleic Acid Complexes," J. Vac. Sci. Technol. A 13 (1995) 1746.
17. Coury, J.E., L. McFail-Isom, L.D. Williams, and L.A. Bottomley, "A Novel Assay for Drug-DNA Binding Mode, Affinity, and Exclusion Number: Scanning Force Microscopy," Proc. Nat. Acad. Sci. USA 93 1996) 12283.
18. Coury, J. E., J. R. Anderson, L. McFail-Isom, L.D. Williams, and L.A. Bottomley, "Scanning Force Microscopy of Small Ligand-Nucleic Acid Complexes: Tris(ophenanthroline) rutheni um(II) as a Test for a New Assay." J. Am. Chem. Soc. 119 (1997) 3792.
19. Pope, L.H., M.C. Davies, C.A. Laughton, C.J. Roberts, S.J.B. Tendler, and P.M. Williams, "Atomic Force Microscopy Studies of Intercalation-Induced Changes in Plasmid DNA Teriatry Structure," J. Microscopy 199 (2000) 68.
20. Rotsch, C., and M. Radmacher, "Drug-Induced Changes in Cytoskeletal Structure and Mechanics in Fibroblasts: An Atomic Force Microscopy Study," Biophys. J. 78 (2000) 520.
21. Wu, H.W., T. Kuhn, and V.T. Moy, "Mechanical Properties of L929 Cells Measured by Atomic Force Microscopy: Effects of Anticytoskeletal Drugs and Membrane Crosslinking," Scanning 20 (1998) 389.
22. Haeberle, W., J.K.H. Hoerber, F. Ohnesorge, D.P.E. Smith, and G. Binnig, "In Situ Investigations of Single Living Cells Infected by Viruses."Ultramicroscopy 42-44 B (1992) 1161.
23. Ohnesorge, F. M., J.K.H. Horber, W. Haeberle, C.P. Czernyk, D.P.E. Smith, and G. Binnig, "AFM Review Study of Pox Viruses and Living Cells." Biophys. J. 73 (1997) 2183.
24. Cricenti, A., M.A. Scarselli, R. Generosi, P. Perfetti, S. Bach, and V. Colizzi, "Analysis of Infected Human Mononuclear Cells by Atomic Force Microscopy," Il Nuovo Cimento 20 (1998) 1153.
25. Nakamura, R., and M. Nakanishi, "Atomic Force Microscopy to Study the Degranulation of Rat Peritoneal Mast Cells After Activation," Immunology Letters 69 (1999) 307.
26. Rotsch, C., K. Jacobson, J. Condeelis, and M. Radmacher, "EGF-stimulated Lamellipod Extension in Adenocarcinoma Cells," Ultramicroscopy 86 (2001) 97.
27. Heinz, W.F., E. A-Hassen, and J.H. Hoh, "Applications of Force Volume Imaging with the Atomic Force Microscoy," Application Note, Digital Instruments/Veeco Metrology Group, 1998.
28. Prater, C.B., J.H. Maivald, K.J. Kjoller, and M.G. Heaton, "Probing Nanoscale Forces with the Atomic Force Microscopy," Application Note, Digital Instruments/Veeco Metrology Group, 1995.
29. Yip, C.M. and J. McLaurin, "Amyloid-beta Peptide Assembly: A Critical Step in Fibrillogenesis and Membrane Disruption," Biophys. J. 80 (2001) 1359.
30. Harper, J.D., S.S. Wong, C.M. Lieber, and P.TLansbury,"Assembly of Aß Amyloid Protofibrils: An in Vitro Model for a Possible Early Event in Alzheimer's Disease," Biochemistry 38 (1999) 8972.
31. Lambert, M.P., A.K. Barlow, B.A. Chromy, C. Edwards, C. Freed, M. Liosatos, T.E. Morgan, I. Rozovsky, B. Trommer, K.L. Viola, P. Wals, C. Zhang, C.E. Finch, G.A. Krafft, and W.L. Klein, "Diffusible, Nonfibrillar Ligands Derived from Aß1-42 are Potent Central Nervous System Neurotoxins," Proc. Nat. Acad. Sci. 95 (1998) 6448.
32. Blackley, H.K.L., N. Patel, M.C. Davies, C.J. Roberts, S.J.B. Tendler, M.J. Wilkinson, and P.M. Williams, "Morphological Development of ß(1-40) Amyloid Fibrils," Experimental Neurology 158 (1999) 437.
33. Conway, K.A., J.D. Harper, and P.T. Lansbury, "Accelerated in vitro Fibril Formation by a Mutant α-Synuclein Linked to Early-onset Parkinson Disease," Nature Medicine 4 (1998) 1318.
34. Conway, K.A., S-J. Lee, J-C. Rochet, T.T. Ding, R.E. Williamson, and P.T. Lansbury, "Acceleration of Oligomerization, Not Fibrillization, is a Shared Property of both α-synuclein Mutations Linked to Early-onset Parkinson's Disease: Implications for Pathogenesis and Therapy," Proc. Nat. Acad. Sci. 97 (2000) 571.
35. Goldsbury, C., J. Kistler, U. Aebi, T. Arvinte, and G.J.S. Cooper, "Watching Amyloid Fibrils Grow by Time-lapse Atomic Force Microscopy," J. Mol. Biol. 285 (1999) 33.
36. Slezac, L.A., A.S. Quinn, K.C. Sritharan, J.P. Wang, G. Aspelung, D.J. Taatjes, and D.K. Andersen, "Binding Forces of Hepatic Microsomal and Plamsa Membrane Proteins in Normal and Pancreatitic Rats: an AFM Force Spectroscopic Study," Microscopy Res. and Technique 44 (1999) 363.
37. Pietrasanta, L.I., A. Schaper, and T.M. Jovin, "Imaging Subcellular Structures of Rat Mammary Carcinoma Cells by Scanning Force Microscopy," J. Cell Sci. 107 (1994) 2427.
38. Stolz, M., D. Stoffer, U. Aebi, and C. Goldbury, "Monitoring Biomolecular Interactions by Time-Lapse Atomic Force Microscopy," J. Struct. Biol. 131 (2000) 171.