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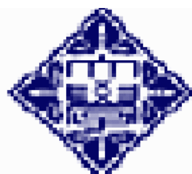
# Kanazawa Workshop on Atomic Force Microscopy

## 15-18 January, 2007

## Kanazawa New Grand Hotel, Kanazawa, Japan



## Program

Day 1 Monday 15 January, 2007

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|---|---|
| 16:00~17:00 | Registration |
| 17:00~19:00 | Mixer |

Day 2 Tuesday 16 January, 2007

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|---|---|
| 9:00~9:10 | Opening Remark |
| | SESSION I (Chaired by Christian Le Grimellec) |
| 9:10~9:50 | Kunio Takeyasu (Invited)<br>(Graduate School of Biostudies, Kyoto Univ., Japan)<br>"Biological application of nano-scale imaging and single-molecule manipulation techniques" |
| 9:50~10:30 | Suzanne Jarvis (Invited)<br>(Trinity College Dublin, Ireland)<br>"Using nano-mechanics to explore biological function" |
| 10:30~10:50 | Break |
| | SESSION II (Chaired by Simon Schuring) |
| 10:50~11:30 | Paolo Facci (Invited)<br>(S3-INFM-CNR, Physics Dep., Univ. of Modena, Italy)<br>"Retrieving functional and conformational information from single proteins: towards an AFM-based approach" |
| 11:30~12:10 | Peter Hinterdorfer (Invited)<br>(Institute of Biophysics, Johannes Kepler Univ. Linz, Austria)<br>"Molecular recognition force microscopy/spectroscopy " |
| 12:10~14:00 | Lunch at French Restaurant “Roi” (12F) |
| | SESSION III (Chaired by Sonia Antranz Contera) |
| 14:00~14:40 | Atsushi Ikai (Invited)<br>(Tokyo Institute of Technology, Japan)<br>"Wedding of biochemistry and mechanics by force" |
| 14:40~15:00 | Masaru Kawakami<br>(JAIST, Japan)<br>"Novel force ramp AFM technique adopting single molecule events for dynamic force spectroscopy " |
| 15:00~15:20 | Masami Kageshima<br>(Graduate School of Engineering, Osaka Univ., Japan)<br>"Equilibrium and non-equilibrium processes and internal friction in dynamics of single biopolymer" |
| 15:20~15:40 | Break |
| | SESSION IV (Chaired by Suzanne Jarvis) |
| 15:40~16:00 | Masaki Tanemura<br>(Nagoya Institute of Technology, Japan)<br>"Small-scale batch fabrication and characterization of carbon nanofiber probes" |
| 16:00~16:40 | Takeshi Fukuma (Invited)<br>(Trinity College Dublin, Ireland)<br>"Direct imaging of water/lipid interface by frequency modulation AFM at sub-angstrom resolution" |
| 16:40~17:00 | Jason Cleveland<br>(Asylum Research, USA)<br>"Dual frequency AFM" |
| 17:00~17:15 | Platinum Sponsor Lecture by Veeco Japan<br>Mayumi Misawa<br>"The new Nanoscope V SPM controller" |
| 17:15~17:30 | Platinum Sponsor Lecture by Olympus<br>Akitoshi Toda<br>"Small cantilevers to penetrate the market" |

Day 3 Wednesday 17 January, 2007

| | SESSION V (Chaired by Sonia Antoranz Contera) |
|---|---|
| 9:00~9:40 | Toshio Ando (Invited)<br>(Dep. of Physics, Kanazawa Univ., Japan)<br>"Instrumentation of high-speed AFM and observation of protein dynamics" |
| 9:40~10:20 | Chia-Hsiang Menq (Invited)<br>(Dep. of Mechanical Engineering, The Ohio State Univ., USA)<br>"Control of tip position using co-located magnetic actuation for high-speed AFM" |
| 10:20~10:40 | Break |
| | SESSION VI (Chaired by Chia-Hsiang Menq) |
| 10:40~11:20 | Levent Degertekin (Invited)<br>(Georgia Institute of Technology, USA)<br>"AFM probe structures with integrated interferometric sensing and electrostatic actuation" |
| 11:20~12:00 | Hirofumi Yamada (Invited)<br>(Graduate School of Engineering, Kyoto Univ., Japan)<br>"Subnanometer-resolution imaging in liquid by frequency modulation atomic force microscopy" |
| 12:00~14:00 | Lunch at French Restaurant “Roi” (12F) |
| | SESSION VII (Chaired by Kunio Takeyasu & Takayuki Uchihashi) |
| 14:00~14:20 | Noriyuki Kodera<br>(Dep. of Physics, Kanazawa Univ., Japan)<br>"Structural dynamics of acto-myosin V revealed by high-speed AFM" |
| 14:20-14:40 | Daisuke Yamamoto<br>(Dep. of Physics, Kanazawa Univ., Japan)<br>"Chaperonin GroELGroES action captured by high-speed AFM" |
| 14:40~15:20 | Christian Le Grimellec- (Invited)<br>(Centre de Biochimie Structurale, Univ. of Montpellier I, France)<br>"Alkaline phosphatase interactions with ordered membrane domains" |
| 15:20~16:00 | Simon Scheuring (Invited)<br>(Institut Curie - Research, UMR-CNRS 168, France)<br>"High-resolution AFM of the supramolecular assembly of membrane proteins in native membranes" |
| 16:00~16:20 | Break |
| 16:20~17:00 | Sonia Antranz Contera (Invited)<br>(Biotechnology IRC, Physics Dep., Univ. of Oxford, UK)<br>"Relating structure, biomechanics and function of single membrane proteins" |
| 17:00~17:40 | Takayuki Uchihashi (Invited)<br>(Dep. of Physics, Kanazawa University, Japan)<br>"Improvements in the high-speed AFM and observation of membrane protein dynamics" |
| 17:40~17:55 | Platinum Sponsor Lecture by RIBM, Co. Inc.)<br>Takashi Morii<br>"New lineup of RIBM products: SXM-advanced, SXM-basic and high-speed AFM NanoLiveVision" |
| 18:30~21:00 | Dinner Party at a Japanese Restaurant |

Day 4 Thursday 18 January, 2007

| | SESSION VIII (Chaired by Peter Hinterdorfer) |
|---|---|
| 9:00~9:40 | Andrew E. Pelling (Invited)<br>(Dep. of Medicine and the LCN, Univ. College London)<br>"Mechanobiology: Non-imaging applications of AFM in cell biology" |
| 9:40~10:20 | Chikashi Nakamura (Invited)<br>(AIST, Japan)<br>"Cell surgery: A novel living cell manipulation technology using nanoneedle and AFM" |
| 10:20~10:40 | Takaharu Okajima<br>(Nanotechnology Research Center, Hokkaido Univ., Japan)<br>"Viscoelastic properties of living cells investigated by time-domain AFM analysis" |
| 10:40~11:00 | Break |
| 11:00~12:00 | Business Meeting for Discussion of International Collaboration<br>(Kanazawa University and Foreign Scientists) |
| 12:00~17:00 | Lunch & Excursion |

Excursion Schedule

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| 13:00 | Bus leaves the Kanazawa New Grand Hotel |
| 13:30 | Ando's Lab (demonstration of high-speed AFM) |
| 14:30 | Bus leaves the Kanazawa University |
| 15:00 | Walking around the Kenroku Park and Kanazawa Castle |
| 16:00 | "Yuzen" Kimono painting at the Traditional Industry Hall |
| 17:30 | Bus arrives at the Kanazawa Railway Station |
| 18:00 | Arriving at the Kanazawa New Grand Hotel |

# Preface

# BIOLOGICAL APPLICATION OF NANO-SCALE IMAGING AND SINGLE-MOLECULE MANIPURATION TECHNIQUES

*Kunio Takeyasu, Masatoshi Yokokawa, Hirohide Takahashi, Yasuhiro Hirano, R.L. Ohniwa, Kohji Hizume and Shige H. Yoshimura*

Graduate School of Biostudies, Kyoto University, Kyoto, Japan
*Corresponding author: takeyasu@lif.kyoto-u.ac.jp (Tel: +81 75 753 7905)

We have been using atomic force microscopy (AFM) for studying the structural organization and dynamics of various biological macromolecules and assemblies [1-5]. Here we shall summarize our most recent results using AFM.

The topics include: (1) Similarities and differences between the eukaryotic and prokaryotic genome organizations in cells. (2) Importance of the topology controls of DNA in architecturing the higher-order structures. (3) Application of fast-scanning AFM to the analyses of enzyme reaction. (4) Development of a novel method for a site-specific attachment of any glutathione S-transferase (GST)-fused proteins to the cantilever in a desired direction, which allows the applications to the measurement of interaction between chromatin and inner nuclear membrane proteins such as the lamin B receptor (LBR). (5) Successful application of the PicoTrec™ mode that can simultaneously obtain a topographic image together with a recognition signal by using protein- (antibody-) coupled cantilever (recognition imaging). Using the PicoTrec™ mode combined with our GSH- and antibody-cantilevers, we could detect specific interactions between LBR and chromatin, and between DNA and nuclear matrix proteins such as SP120.

**[References]**

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5. R.L. Ohniwa, K. Morikawa, J. Kim, T. Ohta, A. Ishihama, C. Wada & K. Takeyasu (2006) Dynamic state of DNA topology is essential for genome condensation in baceteria. ***EMBO J.,*** 25: 1-13.

# Using nano-mechanics to explore biological function

*Suzanne P. Jarvis*

Centre for Research on Adaptive Nanostructures and Nanodevices, Trinity College Dublin, Lincoln Place Gate, Dublin 2, Ireland.
E-mail: suzi.jarvis@tcd.ie
Website: http://www.nanofunction.org

Using a range of atomic force microscope techniques we have explored biological function across a broad range of systems and length scales. To this end we have utilized imaging, force-extension curves, indentation, and in some cases, AFM combined with fluorescence microscopy. Our measurements have included model systems investigated at the submolecular level, for example, to understand interactions between hydrophilic residues and their aqueous environment. We have also explored more complex systems *in vitro* and *in vivo* where mechanical responses have helped us to explain the beneficial mechanical properties of physiological amyloid fibrils. The most complex systems we have studied to date involve measurements at the single cell level, where we have used AFM both to measure and also to mechanically stimulate single cells. We have focused on cell types where mechanics is believed to be particularly important including mesenchymal stem cells where mechanical stimulus is thought to be important for differentiation, and cells of the lamina cribrosa, one of the regions in the intraocular portion of the optic nerve chronically exposed to a mechanically dynamic environment.

I will give a brief overview of the methods used and the systems studied so far, highlighting in particular our work on more complex biological systems.

This work was performed in collaboration with Prof. P. Prendergast and colleagues at the Centre for Bioengineering, Trinity College Dublin and the group of Prof. C. O'Brien, Mater Hospital, Dublin.

# Retrieving functional and conformational information from single proteins: towards an AFM-based approach

*Paolo Facci*

National Research Center "nanoStructures and bioSystems at Surfaces-S3" of CNR-INFM, Via G. Campi 213/A, I-41100 Modena, Italy.
E-mail: p.facci@unimo.it

Large classes of different proteins (e.g. redox metalloproteins, ion channels) whose functional tasks are fundamental in life-sustaining processes, function by eliciting a current flow (ions, electrons) through them. Due to the intimate connection between structure and function in proteins, understanding their correlation appears of paramount importance. Scanning probe techniques are believed to bear the potentialities for investigating the functional behavior of particular biomolecules [1,2] at the level of the single molecule while retrieving, simultaneously, conformational information. However, due to the particular imaging environment, the accomplishment of this task is not trivial and requires special solutions and experimental set-up.
In this talk I will present the results of our research efforts towards the aforementioned goal [3,4], outlining the chosen technical solutions and the open issues that still prevent us from implementing an AFM which can measure, at the same time, topography and current in water-based environments, and with molecular resolution.

References

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# Molecular Recognition Force Microscopy/Spectroscopy

*Peter Hinterdorfer*

Institute for Biophysics, Johannes Kepler University Linz, Altenbergerstr. 69, A-4040 Linz, Austria
E-mail: peter.hinterdorfer@jku.at

In molecular recognition force microscopy (MRFM), ligands are covalently attached to atomic force microscopy tips for the molecular recognition of their cognitive receptors on probe surfaces. Using an appropriate tip surface chemistry protocol with 6-nm long heterobifunctional crosslinkers as key elements, the ligand density on the AFM tip is sufficiently dilute for allowing single molecule studies. Our crosslinker library possesses many different chemical endgroups for various functional coupling strategies. Interaction forces between single receptor-ligand pairs are measured in force-distance cycles. A ligand-containing tip is approached towards the receptors on the probe surface, which possibly leads to formation of a receptor-ligand bond. The tip is subsequently retracted until the bond breaks at a certain force (unbinding force). In force spectroscopy (FS), the dynamics of the experiment is varied, which, in case of a single sharp activation barrier, reveals a logarithmic dependence of the unbinding force on the force velocity. From this curve the barrier height and width can be deduced, as shown on virus/cell receptor interactions. A more complex energy landscape dominates the interaction of the nuclear import receptor importin β with the small GTPase Ran. The complex switches between two distinct conformational states of different binding strength. Our results support a model whereby functional control of Ran-importin β is achieved by a population shift between pre-existing alternative conformation.

In another study it was shown that single molecule force measurements between single-stranded DNA containing multiple methylcytosines and an anti-methylcytosine antibody can survey the distances between methylcytosines with single nucleotide resolution. Two step unbinding events in force curves corresponded to sequential dissociation of two Fab-domains of one antibody from a single DNA molecule, with a distance in excellent agreement with the contour length of nucleotides in between two methylcytosines. Using different DNA sequences, the applicability for methylcytosine sequencing and the detection of single nucleotide polymorphism at the single molecule level was demonstrated.

Finally, a method for the localization specific binding sites and epitopes with nm positional accuracy by combining dynamic force microscopy with single molecule recognition force spectroscopy is presented. A magnetically driven AFM tip containing a ligand covalently bound via a tether molecule was oscillated at 5 nm amplitude while scanning along the surface. Since the tether had a length of 6 nm, the ligand on the tip was always kept in close proximity to the surface and showed a high probabilty of binding when a receptor site was passed. Topography and recognition images were obtained simultaneously using a specially designed electronic circuit. Maxima ($U_{up}$) and minima ($U_{down}$) of each sinusoidal cantilever deflection period were depicted, with $U_{down}$ driving the feedback loop to record a height (topography) image and $U_{up}$ providing the data for the recognition image. In this way, topography and recognition image were gained simultaneously and independently with nm lateral resolution. This method is capable of localizing distinct histones in chromation preparations and can visualize nm-sized receptor domains on cell surfaces.

# Wedding of Biochemistry and Mechanics by Force

*Atsushi Ikai*, *Rehana Afrin*, *Hironori Uehara*, *Taichi Tamanaka*
*Hiroshi Sekiguchi and Toshiya Osada*

Laboratory of Biodynamics
Graduate School of Bioscience and Biotechnology
Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku,
Yokohama 226-8501, Japan
TEL: +81 45-924-5828 FAX: +81 45-924-5806
ikai.a.aa@m.titech.ac.jp

Traditionally, chemistry and mechanics have been treated as two separate disciplines, one concentrating on the conversion of molecules from one form to another and the other focusing on the deformation of macroscopic materials under compressive or tensile stresses. However, the advent of nanotechnologies has meant that we are now able to compress or stretch individual molecules and measure their mechanical response; closing the traditional gap between the two disciplines. DNA has been shown by Bustamante and his colleagues to be stretched beyond the contour length of the standard B-form resulting in the new S-form that is stable only under a tensile stress of about 80 pN [1]. Protein molecules are also unfolded under an applied tensile force; so exposing the hitherto unknown mechanics of intramolecular segmental interactions [2,3,4,5]. By compressing single protein molecules, one can obtain Young's modulus of globular proteins under native and denaturing conditions [6]. A single synthetic polymer chain has been extended from its two ends allowing researchers to compare the experimental stretch curve with various theoretical models of polymer extension [7]. We have been applying the most sophisticated single molecule manipulation technology available to the development of surgical techniques in single living cells by inserting plasmid DNA into or extracting mRNA out of a live cell [8]. We are also pulling membrane proteins to probe their interaction with intracellular structures such as the cytoskeleton [9]. I will give an overview of the new technology being developed in our laboratory for the single molecule and single cell manipulation and its application to bio-medical fields.

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# Novel force ramp AFM technique adopting single molecule events for dynamic force spectroscopy

[1,2]Masaru Kawakami and [2]Alastair Smith

[1]School of Materials Science, Japan Advanced Institute of Science and Technology
[2]School of Physics and Astronomy, University of Leeds
E-mail: kmasaru@jaist.ac.jp

Recently developed techniques such as atomic force microscopy (AFM), the biomembrane force probe (BFP) and optic/magnetic tweezers have allowed us to manipulate biomolecules at the single molecule level and have provided a wealth of information about their mechanical property. In addition, single molecule experiments with these techniques have enabled us to study the energy landscapes of conformational change or unbinding of biomolecules. It is called dynamic force spectroscopy, where the force loading rate is altered and the loading rate dependence of the force at which the reactions occur is investigated. In general, the force loading rate is altered by changing the constant retraction velocity of the scanner (piezo) or the optic/magnetic beads. However, biomolecules have a non-linear elasticity against force, giving non-linear changes of force as a function of time, while the force loading rate is usually determined by assuming that the biomolecule under study has a constant elasticity.

Recently, a real force ramp technique has been developed by the introduction of an analogue PID feedback circuit allowing the kinetic parameters $(\Delta x, k_0)$ of the unfolding reaction of ubiquitin and titin to be calculated [1,2]. In the force ramp experiment, homopolymers of proteins are chosen and stretched. However, with these molecules the multiple unfolding events occur sequentially during the force ramp, which is not taken into account and the force ramp is executed “abruptly”. Consequently the values of the kinetic parameters given with this technique were much smaller than those from the constant velocity experiments.

In this study, a novel force ramp technique capable of executing a true force ramp which takes multiple unfolding events into account has been developed. This is enabled by using a software controlled PID feedback that monitors protein unfolding events during the force ramp. In the talk details of this technique will be presented. Using this technique we obtained the parameters which are almost identical to those determined by the conventional loading velocity experiments, indicating the validity of this technique and importance of consideration of the multiple unfolding events during the force ramp.

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# Equilibrium and non-equilibrium processes and internal friction in dynamics of single biopolymer

Masami Kageshima[1,2], Yoshimasa Nishihara[1], Yoshiki Hirata[3], Takahito Inoue[3]
Sumiko Kimura[4], Yoshitaka Naitoh[1] and Yasuhiro Sugawara[1]

[1]Deptartment of Applied Physics, Osaka University, Suita, Osaka 565-0871, Japan
[2]JST-PRESTO, Kawaguchi, Saitama 332-0012, Japan
[3]National Inst. of Adv. Industrial Sci. and Technol., Tsukuba, Ibaraki 305-8568, Japan
[4]Department of Biology, Chiba University, Chiba 263-8522, Japan
E-mail: kage@ap.eng.osaka-u.ac.jp

Folding/unfolding dynamics of single biopolymer occupies essential part of biological functions. Even more macroscopic biological phenomenon like molecule-membrane interaction or cell adhesion also can be attributed to this kind of microscopic dynamics. It is well known that thermodynamical or equilibrium processes classified into enthalpic and entropic responses contribute macroscopic elasticity of the single molecule. On the other hand, mechanical response of the molecule also includes various types of non-equilibrium processes like relaxation or denaturing due to external forces. While the classical thermodynamics can only deal with a phenomenon simply as a difference between two equilibrium states, non-equilibrium approaches may provide detailed understanding of the transition itself. A key to the dynamic process is energy dissipation or internal friction. In the present study, force spectroscopy technique based on atomic force microscopy (AFM) is intensified by introducing the idea of viscoelasticity measurement. An AFM cantilever is magnetically excited at a particular frequency well below its resonance and the resultant responses in its amplitude and phase are analyzed to extract elastic and viscous properties of the molecule during the course of its forced unfolding. As a model system for the present study, a titin (or connectin) single molecule, which exists in each sarcomere in muscle was chosen. It has a characteristic modular structure of immunoglobulin (Ig) and fibronectin-3 (Fn3) domains. While the stiffness during unfolding of each domain proved to approximate a derivative of the DC force profile relatively well, it did not reflect the characteristic transition to an unfolding intermediate that was observed in the DC force. This means that the transition can be, at least in the time-scale of the present modulation frequency, can be regarded as a non-equilibrium process. In addition, a particular domain was observed to exhibit a characteristic slow unfolding process in contrast to the others that were mostly denatured in ca. 10 msec. or faster. The process had 2 stages of relaxation prior to ordinary random-coil-like extension profile, and in the latter of the two a peaking in the drag coefficient was observed as shown in Fig. 1. This characteristic viscosity is discussed from a viewpoint of internal friction in a polymer chain.

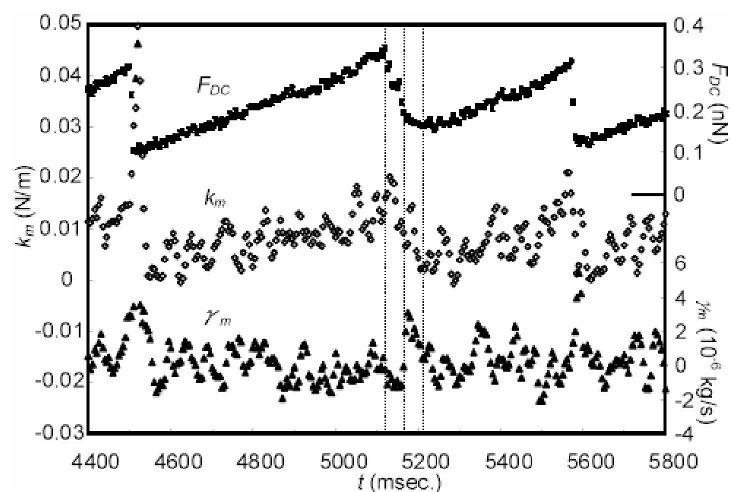


Fig. 1 Simultaneously measured force ($F_{DC}$), molecular stiffness ($k_m$) and drag coefficient ($\gamma_m$) of a titin single molecule showing characteristic slow decay process from t=5120 to 5210 msec.

# Small-Scale Batch Fabrication and Characterization of Carbon Nanofiber Probes

*M.Tanemura*[1], *M. Kitazawa*[1, 2], *J.Tanaka*[1], *Y. Sugita*[1] *and R.Ohta*[2]

[1] Department of Environmental Technology, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan
[2] Olympus Co. Ltd., 6666 Inatomi, Tatsuno, Kami-Ina-Gun, Nagano 399-0495, Japan
E-mail: tanemura.masaki@nitech.ac.jp

Due to their high aspect ratios, nanoscale tip radii, high chemical stability and high mechanical strength, carbon nanotubes (CNTs [1]) and carbon nanofibers (CNFs) are thought to be an ideal probe for scanning probe microscopes (SPMs). Thus, much effort has been devoted to fabricate CNT- or CNF-based SPM probes since the discovery of CNTs [2]. Nevertheless, the batch fabrication of CNT- or CNF-tipped probes is still quite challenging because of several unsolved difficulties in conventional fabrication methods, such as the manual attachment of single CNTs or chemical vapor deposition growth of CNTs onto SPM chips.

Here we challenged the batch-growth of linear-shaped single CNFs onto commercially available Si cantilevers (3 – 9 chips / batch) using a newly developed $Ar^+$-ion-irradiation method [3]. In the present work, the growth parameters were optimized and the electric properties of ion-induced CNF probes were revealed.

Single CNFs pointing in the $Ar^+$-ion-beam direction grew on the tips of arrayed chips (Fig. 1). CNFs increased in length with an increase in the growth time, and the discrepancy in length was estimated to be typically +/- 10 % in an array of 9 SPM chips grown under the optimized condition. CNFs grown at room temperature, for instance, reached about 1 um in length for the 10 min-growth. In the I-V measurements, commercial-type Si probes (without CNF) showed a typical semiconductive characteristic. By contrast, Si probes with ion-induced CNFs (CNF probes) displayed a metallic characteristic with a high signal-to-noise ratio in the I-V curves and possessed a high AFM resolution. Thus, it was believed that batch-fabricated ion-induced CNFs were quite promising as practical SPM probes.

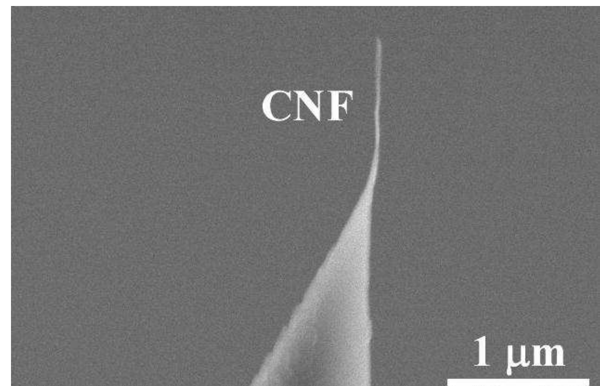

Fig. 1 SEM image of a typical CNF probe taken after repeated AFM measurements.

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# Direct Imaging of Water/Lipid Interface by Frequency Modulation Atomic Force Microscopy at Sub-Angstrom Resolution

*Takeshi Fukuma*, *Michael J. Higgins, Suzanne P. Jarvis*

Centre for Research on Adaptive Nanostructures and Nanodevices, Trinity College Dublin, Lincoln Place Gate, Dublin 2, Ireland
E-mail: takeshi.fukuma@tcd.ie

Frequency modulation atomic force microscopy (FM-AFM) has been used in ultra high vacuum (UHV) environments for investigating subnanometer-scale structures and functions of various surfaces. Until recently, however, its operating environment had been limited only to UHV, which has prevented its practical applications in air and liquids. Recently, Fukuma *et al.* presented a way to overcome this limitation using an ultra low noise cantilever deflection detection system and thereby operating FM-AFM with extremely small cantilever oscillation amplitude [1]. This has made it possible to obtain true molecular [2] and atomic [3] resolution with FM-AFM in liquid. One of the most interesting applications of this new technique is high-resolution imaging of biological systems under physiological conditions. However, true subnanometer resolution with FM-AFM in liquid has not been demonstrated on biological systems.

Biological membranes are amongst the most fundamental elements in biological systems. They form the walls of cells and boundaries between the organelles therein with a selectively permeable structure. The structure and function of the membranes are determined by the chemical interactions between the constituent molecules mediated through water and ions in physiological solution. Thus, understanding of the interactions between lipid molecules (main constituents of biological membranes) and water or ions are of great importance. To date, various spectroscopic methods have been utilized for investigating water/lipid interface. However, these methods provide only global information averaged over micrometer-scale area and hence molecular-scale details of water-lipid and ion-lipid interactions have mostly remained unknown.

Here we investigate a dipalmitoylphosphatidylcholine (DPPC) lipid bilayer in phosphate buffer solution as a model biological membrane under physiological conditions by FM-AFM [4]. The force vs. distance curves measured between the bilayer and the AFM tip show oscillatory force profiles with a peak spacing of 0.28 nm, indicative of the existence of up to two hydration layers next to the membrane surface. FM-AFM imaging at the water/lipid interface visualizes individual hydration layers in three-dimensions with molecular-scale corrugations corresponding to the lipid headgroups. Furthermore, we visualize extensive lipid-ion interaction networks and their transient formation between headgroups in the bilayer. The spatial distribution of ion occupancy, visualized in real-space with the unprecedented lateral resolution of 90 pm, reveals the existence of two equivalent binding sites associated with the phosphate groups and the network formation between them.

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# Dual Frequency AFM

*Jason Cleveland*

Asylum Research, 341 Bolley Drive, Santa Barbara, CA 93117, USA

E-mail: jason@AsylumResearch.com

Scanning probe microscopy (SPM) uses a number of different scanning modes to characterize surface topography and other characteristics. We will present a new SPM imaging mode that goes beyond traditional phase image in measuring mechanical and chemical properties. In this new imaging mode, Dual AC™, a cantilever is driven at or near two of its flexural eigenmodes. For most cantilevers, these eigenmodes are non-harmonic. The 2nd eigenmode amplitude and phase show strikingly different contrast from the same fundamental eigenmode signals. As in traditional AC imaging, the cantilever and imaging parameters can be chosen such that the tip-sample interactions are either attractive or repulsive. In general, if the cantilever is maintained in the attractive state, the 2nd eigenmode is sensitive to long ranged forces and if the cantilevers is maintained in the repulsive state, the 2nd eigenmode is sensitive to mechanical properties of the sample. Data on Magnetic and Electric Force Microscopy (MFM and EFM) samples, collagen fibers, and λ-digest DNA be shown to support this.

# The New NanoScope V SPM Controller

*Mayumi Misawa*

Nihon Veeco K.K., 5-6-10 Tsukiji Chuou-ku Tokyo 104-0045
E-mail: mayumi-m@veeco.co.jp

Fast, Dependable Data Capture: The new NanoScope® V controller utilizes advanced electronics, including A/D and D/A converters operating at 50MHz, to deliver reliable, high-speed data capture. This state-of-the-art fifth-generation controller allows measurement of tip-sample/cantilever dynamics, enabling researchers to study the influence of mechanical properties on the physics of probe-sample interactions at timescales previously inaccessible to SPM users. It also allows calibration of the cantilever spring constant at resonant frequencies up to 2MHz. High-speed data capture is simultaneous with imaging or ramping and independent of microscope mode.

Flexible Controller Features: The NanoScope V enables up to eight images to be simultaneously displayed in real-time (and captured for analysis) with unprecedented signal-to-noise ratio. The controller incorporates three independent lock-in amplifiers and provides thermal tune measurements of cantilever resonance up to 2MHz. It also affords easy access to most input and output signals through front-panel BNCs. Input of data into the controller from an external source (e.g., photomultiplier tube) is supported, as is user access to lock-in amplifiers and to signals to/from a microscope (e.g., XYZ sensors, amplitude, phase).

Highest Pixel Density: The ability to acquire up to 5120 x 5120-pixel images eliminates the need to capture several images at lower pixel densities as well as the requirement for offset adjustments to correlate information from multiple images. The high pixel density saves time when searching for low-density features distributed over large areas and allows observation of large structures and small features in the same image.

Outstanding Software Functionality: Veeco's NanoScript™ open-architecture option provides a growing list of functions to control the SPM for custom experiments and nanoscale research (e.g., nanomanipulation in X, Y, Z; automated scanning; nanolithography with different tip-sample interactions). These functions can also be called from any programming language that can act as a client of Microsoft's Component Object Model (COM), including LabVIEW™, MATLAB®, Visual Basic, Ruby, Python, C++/MFC, Excel®, and Word®.

Easy-AFM, Remarkable Simplicity: For the ultimate in streamlined operational simplicity, Veeco's Easy-AFM™, offers an intuitive, easy-to-follow graphic user interface for new or infrequent SPM users. It reduces the time for initial setup by engaging the sample with the probe (in air), automatically adjusting the scanning parameters, and obtaining high-quality TappingMode™ images on most samples at a push of a button. Easy-AFM is ideal for multi-user environments.

# Small cantilevers to penetrate the market

*Akitoshi TODA*

OLYMPUS Corp. 2-3 Kuboyama-cho, Hachioji-shi, Tokyo 192-8512, Japan
E-mail: probe@olympus.co.jp

For a decade, a small cantilever has been believed as a foregone conclusion for scanning probe microscopy in biophysics field. However, it seems to be still in the process, at least in business. What has made us hesitate to getting on board a train?

A small cantilever sized in around 10 micron long and 2 micron wide has been used in the research of high speed AFM.(1),(2) But it is not compatible for commercially available AFMs. Therefore, many of researchers had less chance to use such an extremely small cantilevers. We assumed that a medium small size cantilever which is smaller than conventional cantilevers while being compatible with conventional AFM optical sensors would open the door of small world express. 'Bio-Lever mini', or BL-AC40TS-C2, has been introduced recently into the market, based on the assumption.(3) The cantilever sized in around 40 micron long and 15 micron wide shows a resonant frequency of 110 kHz in air and of 25 kHz in water while its spring constant of 0.1 N/m. It is higher than the resonant frequencies of the XY-Z scanner, the users can study at a maximum the effectiveness of the medium small cantilever with their AFMs. We believe that their experience must motivate to learn more about small cantilevers and that small, or perhaps smaller cantilevers penetrate the market in the near future.

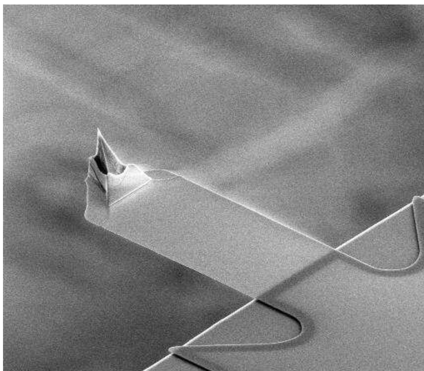

Fig.1 SEM micrograph of BioLever mini.
Lever sized in 37(L)x16(W) micrometers, has a tetrahedral silicon tipe near the free end.

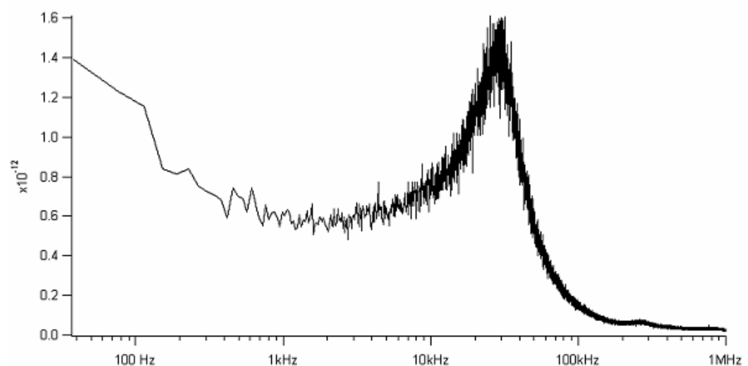


Fig.2 Thermal vibration spectrum in water of BioLever mini. The resonant peak is at 25 kHz.

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# Instrumentation of the High-speed AFM and Observation of Protein Dynamics

Toshio Ando

Dep. of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
CREST/JST, Kawaguchi, Saitama 332-0012, Japan

E-mail:tando@kenroku.kanazawa-u.ac.jp
WWW: http://www.s.kanazawa-u.ac.jp/phys/biophys/index.htm

X-ray crystallography and NMR have been successful in determining the protein 3D structure at atomic level. However, the obtainable structure is a static one averaged over many molecules and hence cannot reveal how protein molecules behave dynamically when they are functioning in solution. Currently-prospering single molecule analysis by fluorescence microscopy can detect dynamic behavior of protein at work but the spatial resolution is not high enough to visualize protein structure. Atomic force microscopy (AFM) does not possess spatial resolution as high as x-ray crystallography and NMR but very unique in its ability to visualize individual protein molecules in solution at (sub) nanometer resolution. However, its imaging rate is too low to capture dynamically moving molecules because of the slow scan speed due to the slow mechanical response of the cantilever and scanner. In addition, the tip-sample interaction force is large, which disturbs weak protein-protein interaction and sometimes leads to destruction of protein. In order to afford AFM an ability to trace moving protein molecules without disturbing their physiological function, we have been developing various devices over the past decade; for example, small cantilevers[1] with a high resonance frequency (0.6-1.2 MHz in water) and a small spring constant (~0.2 N/m), an optical deflection detection system[1,2] compatible with small cantilevers, a high-speed scanner[3] that does not vibrate when operated in z-direction at 150 kHz, a dynamic PID controller[4] that does not lower the feedback bandwidth even with the amplitude set point very close to the cantilever's free oscillation amplitude, a high-speed phase detector[5] that allows simultaneous capturing of topography and phase-contrast images. By these devises, it has recently become possible to image at (near) video rate, without disturbing protein's physiological functions[6,7]. For example, hand-over-hand movement of myosin V along actin filaments is clearly imaged. The negatively cooperative binding events between Gro-ES and the two rings of GroEL are successfully captured. These demonstrate that high-speed AFM is truly useful for studying protein's dynamic action and will surely open a new way of elucidating the mechanisms of protein functions.

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# Control of tip position using co-located magnetic actuation for high-speed AFM

*Chia-Hsiang Menq, Younkoo Jeong and G. R. Jayanth*

Department of Mechanical Engineering. The Ohio State University
201 West 19th Avenue, Columbus, OH, 43210
E-mail: menq.1@osu.edu

AFM imaging utilizes a sensitive feedback mechanism to achieve a specific control objective by adjusting the tip-to-sample distance through z-motion control. For example, the control objective of contact mode AFM is to maintain a constant deflection of the cantilever while the oscillation amplitude or frequency is regulated in usual dynamic mode AFM. Recently, direct control of tip-sample interaction force was also proposed. In AFM imaging, as a sample is scanned at higher speed, topographic details present themselves to the z-control loop as disturbances at higher frequencies. Therefore, the bandwidth of the z-control loop is one of the key factors that limit imaging rate. The z-control loop in dynamic mode AFM involves various dynamic processes, including z-scanner dynamics, cantilever dynamics, and tapping dynamics. Over the past 15 years, smaller AFM cantilevers as well as smaller piezoelectric actuators, in conjunction with active Q-control, have been proposed, and significant improvements for high-speed AFM have been made.

In this presentation, a co-located scheme for z-motion control is proposed, in which a magnetic actuator is introduced to work together with the regular z-scanner in a dual-control loop scheme aiming to directly control the tip position and hence the tip-to-sample distance. The magnetic actuator overcomes the bandwidth limitations of the z-scanner and does not introduce undesirable under-damped dynamics. Moreover, since the magnetic force is applied directly at the location where the motion being controlled, it is much easier and reliable to use a model cancellation method to compensate the dynamics of the cantilever and to elevate the speed of the tip-position control. This additional magnetic actuator serves to make the entire cantilever bandwidth available for tracking topographic variations at specified tip-sample interaction force. In high speed imaging, it will pick up high spatial-frequency surface topography and regulate the tip-sample interaction force while the regular z-scanner provides the necessary motion range.

A fast programmable electronics board (Field Programmable Gate Array) was employed to implement the proposed dual-control-loop scheme, in which model cancellation algorithms were realized to enhance the bandwidth of the magnetic coil and to replace the lightly damped dynamics of the cantilever with an over-damped system. It allows the cantilever to position the tip very rapidly without introducing unwanted transient dynamics. Experimental results will be presented to illustrate the effectiveness of the propose method. For tip-position control, it is shown that while an ordinary cantilever is excited by the magnetic actuator to oscillate around its resonance frequency (34.8 kHz), the same actuator is actively controlled to move up the mean position of the tip by 20nm within one cycle. Other preliminary results and potential issues in relation to high-speed AFM imaging and direct tip-sample interaction force control will also be discussed.

# AFM probe structures with integrated interferometric sensing and electrostatic actuation

*F. Levent Degertekin, A. Guclu Onaran, Hamdi Torun, Mujdat Balantekin, Krishna Sarangapani, and Cheng Zhu*

Georgia Institute of Technology
G.W. Woodruff School of Mechanical Engineering
801 Ferst Dr. NW, Atlanta, GA 30332, U.S.A.
E-mail: levent@gatech.edu

In this talk, we summarize our efforts in development of novel AFM probes and actuators based on extended use of micromachining techniques with a focus on applications in liquid media. The first type of device uses a surface micromachined membrane structure as force sensor which is directly actuated using the built-in electrostatic actuator [1]. This enables fast actuation of the probe tip limited only by the membrane dynamics. The motion of the tip is measured with high sensitivity using an integrated optical interferometer. Membrane structures suitable for in-liquid operation are fabricated on transparent substrates made of quartz or glass. A reflective metal grating is formed on the surface which also serves as one of the actuator electrodes. The membrane is made of dielectric layers, a silicon nitride - silicon oxide stack, or a polymer such as parylene over a sealed gap. The top metal actuator electrode – optical reflector layer is buried in this dielectric layer for electrical isolation in conductive liquids such as buffer solutions. To illustrate application of these probes in single molecule mechanics experiments, they were used to measure unbinding forces between L-selectin reconstituted in a polymer-cushioned lipid bilayer on the membrane and an antibody adsorbed on an AFM cantilever. Piconewton range forces between single pairs of interacting molecules were measured from the cantilever bending while using the membrane as an actuator. The integrated diffraction-based optical interferometer of the probe was demonstrated to have <10 fm/√Hz noise floor for frequencies as low as 3 Hz with a differential readout scheme. With softer membranes, this low noise level would be suitable for direct force measurements without the need for a cantilever. Furthermore, the probe membranes were shown to have 0.5 μm actuation range, with a flat response up to 100 kHz enabling measurements at high speeds [2]. We also describe a second type of device, the acoustic radiation force (ARP) actuator, for fast imaging in liquids. The ARP actuator uses focused acoustic waves at RF frequency range (100-300MHz) to induce localized forces on AFM cantilevers in liquids. The actuator has an actuation bandwidth in excess of 1MHz and it can be used with any type of AFM cantilever without a need for any magnetic or piezoelectric film. ARP actuator has been integrated to a commercial AFM system and fast tapping mode imaging without a Z-piezo has been demonstrated. Furthermore, single molecule force spectroscopy experiments were conducted using the same system [3].

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# Subnanometer-resolution Imaging in Liquid by Frequency Modulation Atomic Force Microscopy

*Hirofumi Yamada, Kei Kobayashi*

Kyoto University
Katsura, Nishikyo, Kyoto 615-8510, Japan
E-mail: h-yamada@kuee.kyoto-u.ac.jp

High-resolution imaging in liquid by FM-AFM is severely hindered by the extreme reduction of the Q-factor due to the hydrodynamic interaction between the cantilever and the liquid. We recently found that the use of the small amplitude mode and the large noise reduction in the cantilever deflection sensor brought a great progress in FM-AFM imaging in liquid. The force sensitivity is increased by FM detection with small amplitude oscillation because of the increase in the duration of the proximity interactions. Note that the small amplitude mode can be used only when the noise in the deflection sensor is sufficiently reduced down to a level of the thermal fluctuation of the cantilever. We found that the noise was effectively suppressed by decreasing the laser light coherence, which was experimentally performed by modulating the laser power with a high frequency signal (300-500 MHz).

In this presentation we describe subnanometer-resolution imaging of organic molecules including biomolecules in liquid using the improved FM-AFM. Figure 1 shows an FM-AFM image of a muscovite mica surface taken in pure water. The honeycomb structure of SiO4 tetrahedrons with a period of 0.52 nm is clearly seen. We also succeeded in obtaining high-resolution FM-AFM images of *bacteriorhodopsin* protein molecules hexagonally packed in a purple membrane as well as GroEL molecules, the chaperonin of *E. coli*, with a sevenfold-symmetric structure. The images were taken in buffer solution. In addition, hydration structures on a mica surface in water were investigated by FM-AFM with small amplitude oscillation. Figure 2 shows a frequency shift (corresponding to conservative force) vs. distance curve ($\Delta f$-$d$), where clear oscillation with a spacing of about 0.2nm due to the hydration structure was observed. The value is close to the size of water molecule (0.26nm). The success in high-resolution FM-AFM imaging in liquid has opened the new way to direct visualization of *in vivo* molecular-scale biological process.

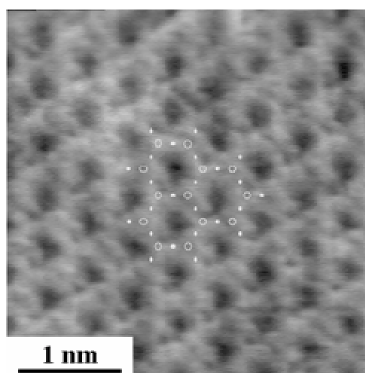



Fig. 1. FM-AFM image of a mica surface taken in pure water. ($\Delta f$ = +20 Hz, $A$ = 0.24 nm)

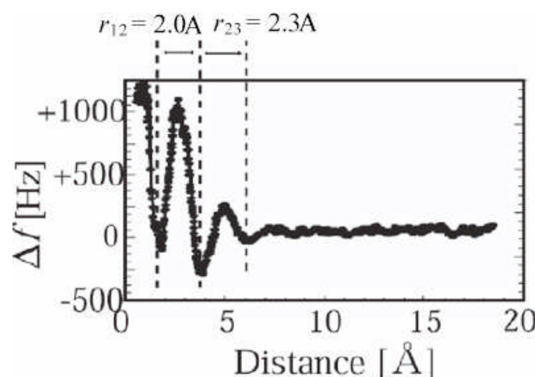



Fig. 2. Frequency shift vs. distance curve measured on a mica surface in water.

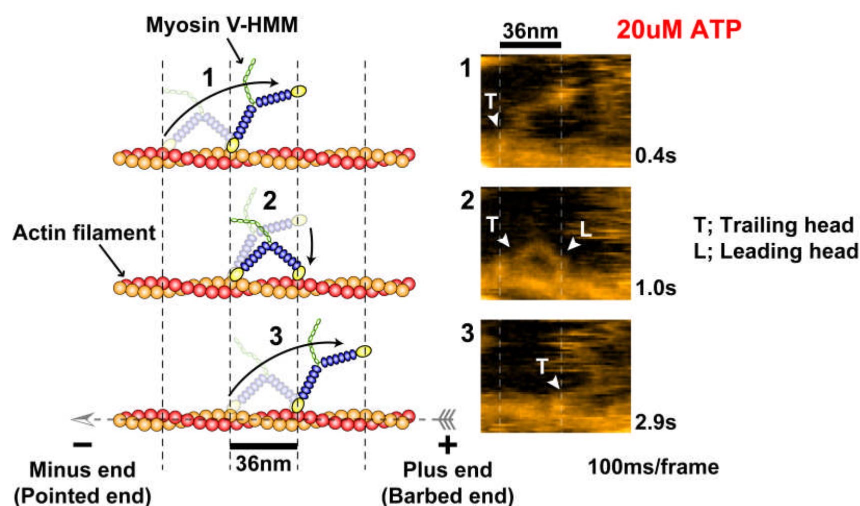
# Structural dynamics of acto-myosin V revealed by high-speed AFM

*Noriyuki Kodera*[1], *Daisuke Yamamoto*[1,2] & *Toshio Ando*[1,2]

[1]Dep. of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
[2]JST/CREST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
E-mail: ikouyo@kenroku.kanazawa-u.ac.jp

Myosin V is a two-headed molecular motor that delivers intracellular cargos over a long distance by moving processively along actin filaments. Its chemical kinetics and mechanical properties have been elucidated in a series of biochemical and biophysical studies. The hand-over-hand model that explains the myosin V processivity has reached a consensus[1]. However, its structural dynamics details have not yet elucidated by any techniques. Thus, we tried to visualize them using an advanced high-speed atomic force microscope (AFM)[2] equipped with various superior controllers that could enhance the scan speed and reduce the tip-sample interaction force as much as possible.

Both in nucleotide-free and ADP-containing solutions, myosin V attached to actin filament rigidly with only one head. The bound head formed an arrow-head-like structure, from which the polarity of the actin filament was identified. The bound head was the trailing head. By the addition of ATP, first both the heads of myosin V bound to the same actin filament at sites spaced about 36-nm apart. Then, the trailing head was detached from the actin filament, which was accompanied by bending of the leading head's neck region, so that the trailing head was quickly moved forward and was wavering like searching a next binding site on the actin filament. After wavering for a while, it landed on an actin site about 72-nm apart from the previous bound-site and thus became a new leading head. These AFM movies directly showed a series of structural changes in myosin V during the hand-over-hand movement.

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# Chaperonin GroEL-GroES action captured by high-speed AFM

Daisuke Yamamoto[1,2], Masaaki Taniguchi[1] and Toshio Ando[1,2]

[1]Department of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192 Japan,
[2]JST/CREST, 4-1-8, Honcho, Kawaguchi-shi, Saitama 332-0012 Japan

E-mail: yamadai@kenroku.kanazawa-u.ac.jp

The correct folding of many proteins in prokaryotes and eukaryotes requires the action of large protein structures known as chaperonins. GroEL, the chaperonin of *Escherichia coli*, is composed of 14 identical 57kDa subunits forming two heptameric rings, stacked back to back, each with a large central cavity. The binding of ATP and the co-chaperonin GroES, composed of seven 10kDa identical subunits, to GroEL double ring is required for productive folding of misfolded proteins. The asymmetry in the binding-release reaction between GroEL and GroES has been resolved through biochemical studies, including single ATP turnover experiments and the analysis of single ring mutants. Based of these observations, a model for a GroEL-GroES reaction cycle has been proposed. The GroEL has a negative cooperativity between the two rings and the GroES is assumed to stably bind only to either GroEL ring during ATP hydrolysis. However, this alternate GroES binding has not directly been evidenced by single molecule experiments.

Here, we describe the direct elucidation of this negatively cooperative binding action of the chaperonin system using a high-speed atomic force microscope (high-speed AFM). We first prepared a sample system in which GroEL lay down on a substratum. Because GroEL has a nature to attach onto bare mica surface in an end-up orientation, which keeps GroES from accessing to the both ends of GroEL, an appropriate substrate was needed. For this purpose, we performed two-dimensional crystallization of streptavidin on a supported phospholipid bilayer. Over a wide area the obtained streptavidin crystal was flat enough for AFM observation. To anchor the GroEL molecules on a substratum at their sidewalls, a GroEL mutant and its modification with biotin is needed; Asp at the equatorial domain was replaced with Cys and biotin molecule was attached to this residue. With these preparations it became possible to observe GroEL from its side by high-speed AFM. GroEL alone looked like a barrel, while GroEL associated with one GroES looked like a bullet. In the presence of GroES and ATP, dynamic changes in the GroEL appearance were successfully captured on video. The kinetics of dissociation of GroES from GroEL depicted from high-speed AFM observations showed two rate constants, contrary to the conventional model that assumes only one rate constant. Furthermore, a football shaped complex, in which the GroEL is bound by two GroES at the both ends, was confirmed to be formed during the chaperonin reaction cycles. We will report the details of these studies.

# Alkaline Phosphatase Interactions with ordered membrane domains

*Christian Le Grimellec*[1], *Marie-Cécile Giocondi* [1],*Françoise Besson*[2], *Patrice Dosset*[1], *and Pierre-Emmanuel Milhiet*[1].

[1] INSERM U554, Nanostructures et Complexes Membranaires,Centre de Biochimie Structurale, F34090 Montpellier, France ; CNRS UMR5048, F34090 Montpellier, France ; Universités Montpellier 1 et 2, F34090 Montpellier, France.

[2]Laboratoire Organisation et Dynamique des Membranes Biologiques, CNRS UMR 5013,
Université Claude Bernard Lyon I, 43 boulevard du 11 novembre 1918,
F-69622 Villeurbanne Cedex, France

E-mail: clg@cbs.cnrs.fr

GPI-anchored proteins preferentially localize in the most ordered regions of the cell plasma membrane. Acyl and alkyl chain composition of GPI-anchors determine the association with the ordered domains. This suggests that changes in the fluid and in the ordered domains lipid composition affect the interaction of GPI-anchored proteins with membrane microdomains. Atomic force microscopy (AFM) shows that the spontaneous insertion of the GPI-anchored intestinal alkaline phophatase (BIAP) into the gel phase domains of dioleoylphosphatidyl-choline/dipalmitoyl-phosphatidyl-choline (DOPC/DPPC) and DOPC/sphingomyelin (DOPC/SM) also occurred in palmitoyloleoylphosphatidylcholine/SM (POPC/SM) gel-fluid phase separated membranes. However changes in the lipid composition of membranes had a marked effect on the bilayer topography: BIAP insertion was associated with a net transfer of phospholipids from the fluid to the gel (DOPC/DPPC) or from the gel to the fluid (POPC/SM) phases. For DOPC/SM bilayers, transfer of lipids was dependent on the homogeneity of the gel SM phase. In POPC/SM binary mixtures with the coexistence of fluid, gel and liquid ordered phases induced by cholesterol (POPC:SM:Chl, 1:1:0,35), BIAP preferentially localized in the more ordered phase, at room temperature. However, this distribution of BIAP between fluid and ordered phases was a function of temperature. How the AFM imaging of BIAP in model systems could contribute to the understanding of the behaviour of GPI-anchored proteins in biological membranes and what are the limitations of AFM in such studies will be discussed.

# Structure and assembly of membrane proteins in native membranes by atomic force microscopy (AFM)

*Simon Scheuring, Nikolay Buzhynskyy, Rui Pedro Goncalves, Szymon Jaroslawski*

Institut Curie, UMR-CNRS 168, 26 rue d'Ulm, 75248 Paris, France.
Email: simon.scheuring@curie.fr
www: http://perso.curie.fr/Simon.Scheuring/

The atomic force microscope (AFM) has become a powerful tool in structural biology allowing the investigation of biological samples under native-like conditions: experiments are performed in physiological buffer at room temperature and under normal pressure. Topographies of membrane proteins can be acquired at a lateral resolution of ~10Å and a vertical resolution of ~1Å. Importantly, the AFM features an extraordinary signal-to-noise ratio allowing imaging of individual membrane proteins in prokaryotic [1] and eukaryotic [2] native membranes that participate in supramolecular assemblies. These images can be docked with high precision by high-resolution structures resulting in atomic models of multiple proteins working together. The development of a novel 2-chamber AFM setup, in which membranes are deposited on nano-patterned surfaces, allows probing non-supported functional membrane proteins [3].

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Nature Methods 2006, 3 (12): 1007-1012
Rui Pedro Gonçalves, Guillaume Agnus, Pierre Sens, Christine Houssin, Bernard Bartenlian & Simon Scheuring*

# Relating structure, biomechanics and function of single membrane proteins

*Sonia Antoranz Contera*[1], *Kislon Voïtchovsky*[1], *Hayato Yamashita*[2], *Takayuki Uchihashi*[2], *Toshio Ando*[2], *J.F. Ryan*[1]

[1] Bionanotechnology IRC, Clarendon Laboratory, Physics Department, University of Oxford, Oxford OX4 3AP, UK
[2] Graduate School of Natural Science & Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan

E-mail: s.antoranzcontera1@physics.ox.ac.uk

One of the greatest challenges of biophysics is to understand how membrane protein sequences relate to the local forces that dynamically control their structure and drive their function. AFM is one of the most powerful techniques to address this problem, however, most of the single membrane protein unfolding AFM studies available fail to relate the potential barriers observed to specific molecular interactions relevant for the protein function.
In some of our recent papers, we have combined membrane indentation with AFM[1], AC-force spectroscopy[2], AFM unfolding and extraction of model peptides inserted planar lipid bilayers at different speeds[3], unfolding and extraction of bacteriorhodopsin (bR) from purple membranes[4] and comparison of pulling experiments with molecular dynamics simulations[3] to relate structure, nano-biomechanics, local hydration, mechanical properties and function of membrane proteins.
In particular, retinal proteins convert the energy of a single photon into large structural changes subsequently used to carry out various tasks. This is achieved by a complex combination of local dynamical interactions controlling the protein biomechanics, allowing efficient amplification of the retinal isomerization. In the case of the retinal containing proton-pump bR we have shown that steric, specific interactions create a rigid scaffold in the protein extracellular region[4]. This scaffold, which encloses the retinal, controls bR local biomechanical properties and anchors the protein into the membrane. In contrast, the cytoplasmic side of bR is mainly governed by relatively weak non-specific electrostatic interactions which provide the flexibility necessary for large cytoplasmic structural rearrangements during the photocycle. Finally we show that bR mechanical properties are part of the strategy adopted by bR to efficiently function in extreme halophilic environments.

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# Improvements in high-speed AFM and observation of membrane protein dynamics

*Takayuki Uchihashi, Hayato Yamashita, and Toshio Ando*

Dep. of Physics, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan
CREST-JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

E-mail: tuchi@kenroku.kanazawau-ac.jp

High-speed atomic force microscopy (AFM) is a unique tool to investigate the dynamic behaviors of proteins at works. Owing to the efforts for improving the scanning speed and feedback performance, an imaging rate of 50ms/frame has been achieved and then it is now possible to routinely observe dynamic actions of proteins such motor proteins. However, the imaging rate would be still insufficient to capture faster movement. Also the information we can take from the images was limited to only structure although tapping-mode AFM has a capability to sense chemical and mechanical properties of surface through detecting the phase difference of the cantilever oscillation. Here, we present some improvements to overcome these current limitations regarding the imaging rate and the phase imaging. Also, we show the recent results which show one application of high-speed AFM to observe dynamic behavior of membrane proteins.

### *I. Direct control of tip-surface distance using photo-thermal actuation of a cantilever.*

An intensity-modulated infrared laser beam was used to photo-thermally deflect small cantilevers. The slow response of the photo-thermal expansion effect was eliminated by inverse transfer function compensation. By regulating the laser power and hence regulating the cantilever deflection, the tip-sample distance was controlled, which was made much faster than conventional piezoactuator-based z-scanners because of the very high resonant frequency of the cantilevers. Using this control, video-rate imaging of protein molecules in liquids was achieved for the scan range of 250nm.

### *II. Fast phase imaging on polymer surface*

We developed a fast phase detector which can detect the phase difference between the cantilever oscillation and the excitation signal at each oscillation cycles. The phase-shift images clearly revealed the compositional heterogeneities in styrene-butadiene-styrene block copolymer films even at an imaging rate of more than 100 ms/frame.

### *III. Dynamic observation of purple membrane*

We applied high-speed AFM to image purple membrane (PM) in a buffer solution. We observed fluctuations of the crystal structures at the edge of PM due to desorption of bacteriorhodopsin (bR) trimers, diffusion of bR trimers in lipids, and decrystallization process of PM induced by photo bleaching. We also succeeded in imaging bR trimers faster than video rate. These results will open up new possibilities for studying membrane assembling processes and protein-protein interactions in membrane.

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## New lineup of RIBM products: SXM-advanced, SXM-basic, and high-speed AFM NanoLiveVision

*Takashi Morii*

Research Institute of Biomolecule Metrology, Co, Inc.

E-mail: morii@ribm.co.jp

# Mechanobiology: Non-imaging Applications of AFM in Cell Biology

*Andrew E. Pelling*[1], *Farlan S. Veraitch*[2], *Carol Chu*[2], *Chris Mason*[2], *Brian M. Nicholls*[1], *Martin Koltzenburg*[3], *Yaron R. Silberberg*[1] *and Michael A. Horton*[1]

[1]The London Centre for Nanotechnology and Department of Medicine
[2]Advanced Centre for Biochemical Engineering
[3]Institute for Child Health
University College London, United Kingdom.

Correspondence Email: a.pelling@ucl.ac.uk

Measurement of the dynamic mechanical characteristics of living cells can often reveal surprising insights into cell biology in addition to quantifying material properties. The atomic force microscope (AFM) is a powerful nanoscale imaging device but its split-personality also allows for non-imaging mechanical approaches which have powerful applications when studying the biology of living cells. The AFM is well suited to measuring dynamic changes in the mechanical properties of cell membranes as well as applying controlled forces to living cells and tissues. This creates both passive and non-passive approaches to cell mechanics through measurement of material properties in addition to controlling and/or directing biological responses through applied force. In this talk I will present recent work from the London Centre for Nanotechnology in which the dynamic mechanical properties of living cells are measured and altered with combined fluorescence/confocal-AFM approaches. The results reveal surprising insights into biochemical signalling pathways as well mechanical dynamics during biological processes. Examples will be provided detailing how AFM can be used to detect and/or alter biological processes in single cells and tissues during apoptosis, cardiac contractions, primary neuron mechanotransduction, and organelle rearrangement in response to applied loads.

# Cell Surgery: A Novel Living Cell Manipulation Technology Using Nanoneedle and AFM

*Chikashi Nakamura*

Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), 2-41-6, Aomi, Koto-ku, Tokyo 135-0064, Japan
E-mail: chikashi-nakamura@aist.go.jp

Recently, applications of atomic force microscopy (AFM) have been extended to the field of cell biology. Direct imaging of living cell is powerful tools to analyze the architecture of living cell surface without staining or chemical pretreatements. We are utilizing an AFM system to manipulate a living cell. We expect that substances on a surface of the AFM tip can be forcibly and precisely transferred into a living cell, like a kind of surgical operations. Here we show a new low invasive single cell manipulation and gene delivery technology using an ultra thin needle (nanoneedle) and AFM. An AFM tip is etched and sharpened using focused ion beam to form a needle shape of 200 nm in diameter and 10 μm in length. The insertion process of nanoneedle into a cell can be monitored with a force exerted to the AFM cantilever. A needle penetration event into a cell is represented as a sudden repulsive force relaxation appearing in the force curve (Fig. 1). The invasiveness of the nanoneedle of 200 nm is so low that even two hours continuous insertion of the nanoneedle does not cause any cell death. Therefore we can manipulate a single cell sequentially.

The nanoneedle insertion could be applied for high-efficiency DNA transfer to living cell. The needle surface was modified with a positively charged peptide, poly-lysine. The DNAs adsorbed on the surface of nanoneedle electro-statically at pH 7.4 same as a culture medium. When the DNA immobilized needle was inserted to a living cell and was kept for several minutes, DNA molecules were efficiently released from the needle surface in cytosol autonomously by decrease of pH. We achieved high-efficiency gene transfer into the human primary cultured mesenchymal stem cell, with over 70% efficiency.

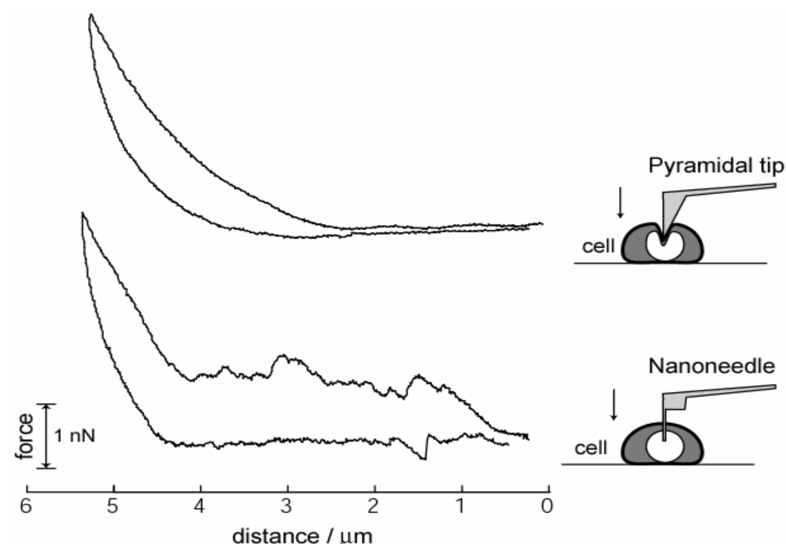


**Fig. 1** Force-distance curves when a normal AFM tip (upper) and nanoneedle (lower) were approached to a living cell.

# Viscoelastic Properties of Living Cells Investigated by Time-domain AFM Analysis

*T. Okajima, M. Tanaka, S. Tsukiyama, T. Kadowaki, S. Yamamoto, M. Shimomura, H. Tokumoto*

Nanotechnology Research Center, Research Institute for Electronic Science, Hokkaid o University, Creative Research Initiative "Sousei", Hokkaido University, N21 W 10 Kitaku, Sapporo, 001 -0021, Japan

E-mail: okajima@es.hokudai.ac.jp

Since living cells have high anisotropy as a consequence of their complex internal architecture, it is crucial to explore the relationship between structure and function at the micro-and nanoscale under physiological conditions. In this study, we measured mechanical relaxation of living cells by a time -domain AFM analysis, in which an indentation force was applied to the cell by the AFM tip, and then a time series of the cantilever deflection signal was measured at the fixed position of cantilever displacement (Fig.1 (left)). We used a commercial AFM apparatus, MFP-3D AFM (Asylum Research, Santa Barbara, CA), which was mounted on an inverted optical microscope (IX71, Olympus Co.) and a silicon cantilever "BioLever Mini" (BL -AC40TS, Olympus Co) whose spring constant and resonance frequency were l~0.1 N/m and ~30 kHz in liquids. Mechanical relaxation, i.e., a decay of the applied force, was clearly observed on human hepatoma cell line, HepG2 cells [1] and mouse fibroblast cell line, NIH3T3 cells [2]. The relaxation was well fitted to a stretched exponential function known as the Kohlrausch-Williams-Watts (KWW) function, which is empirically employed to represent dispersion processes of the system. The stretching exponent was estimated to be around 0.5, implying that the relaxation observed in HepG2 cells consisted of multiple relaxation process. The relaxation of cells was also measured by colloidal probe AFM, in which a silica bead with the diameter less than 2um was attached on the tip apex. The relaxations observed by the colloidal probe AFM were consistent with those by the sharp tip. This work was partly supported by Industrial Technology Research Grant Program in 2006 from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

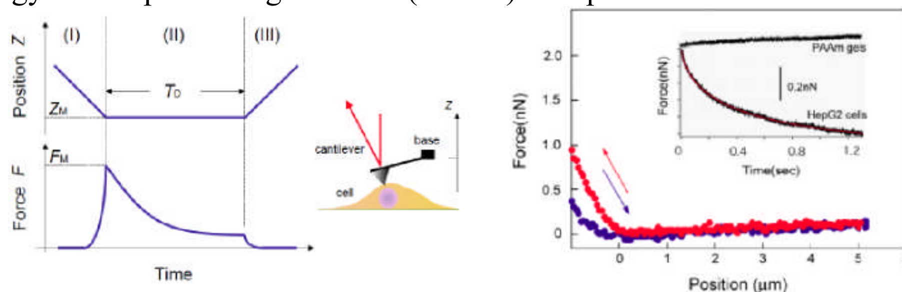


Fig. 1 (left) Schematics of stress (mechanical) relaxation measurement with AFM. The tip contacted the cell surface (region I), the position of the cantilever base Z was kept at a constant value (region II), and the tip was retracted (region III). (right) Typical approach and retraction force–distance curves measured in HepG2 cells. Inset shows the time series of the deflection signals observed on the HepG2 and a polyacrylamide (PAAm) gel.

[1] T. Okajima, M. Tanaka, S. Tsukiyama, T. Kadowaki, S. Yamamoto, M. Shimomura and H. Tokumoto, Nanotechnology in press.
[2] T. Okajima et al. JJAP in preparation.