Non-contact surface force microscopy for molecular interaction study

Takaaki Aoki†
Creation of Innovative Technology by Integration of Nanotechnology with Information, Biological and Environmental Technologies, JST
c/o Laboratory for Nanobiology, Graduate School of Frontier Bioscience,
Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871 Japan

Yoshiyuki Sowa
Graduate School of Engineering, Nagoya University, Furou-cho, Nagoya 464-8603, Japan

Toru Ide
Creation of Innovative Technology by Integration of Nanotechnology with Information, Biological and Environmental Technologies, JST
c/o Laboratory for Nanobiology, Graduate School of Frontier Bioscience,
Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871 Japan,

Toshio Yanagida
Graduate School of Frontier Bioscience, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871 Japan
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In order to detect and visualize the electrostatic features of biological macromolecules in a non-contact mode, we have refined the technique of scanning probe microscopy. The forces in the sub-piconewton range between the probe stylus and the sample surfaces have been measured with a gap distance controlled with nanometer accuracy. Images of the electrostatic surface forces of myosin filaments were detected in pure water using positively charged whiskers as cantilever probe tips. The images were consistent with the structure of myosin filaments that have a bipolar spindle shape; they were charged with a great number of negative charges in the central bare zone compared with the rest of the filament. Thus, in this non-contact mode, the electrostatic features of the protein surface rather than the surface topography were measured. This method has been further extended to measure forces exerted between protein molecules. Long-range interaction between kinesin and microtubules has been examined. It is likely that long-range attractive forces, in the order of several nanometers, exist between kinesin and microtubules.

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I. INTRODUCTION

Atomic force microscopy (AFM) has been proven to be a powerful tool to visualize fine surface structures particularly in the field of material science [1]. Recently AFM has also been used to study the mechanical properties of biological macromolecules, such as the unbinding strength between biomolecules [2] and the unfolding processes of single protein molecules as a result of external mechanical forces [3].

In contrast to these forces that are in the order of several tens of piconewton, the interaction forces between protein molecules for functioning are thought to be in the piconewton range. To measure such interaction forces between the protein molecules and the surface forces generated on protein molecules, scanning probe microscopy techniques have been refined in this study. The sensitivity of the force measurements of conventional AFM has been improved by approximately 100-fold [4, 5]. Very flexible cantilevers with a spring constant of approximately 0.1 pN·nm$^{-1}$ were made and a feedback system with a laser radiation pressure was introduced to control the fluctuations of the cantilever. Using this novel probe microscopy called Intermolecular Force Microscopy (IFM), the surface forces from single protein filaments [6] and long-range protein-protein interaction in non-contact region[7] have been measured. These results showed that the newly developed techniques could be a powerful method to resolve the biological functions of interacting macromolecules.

II. EXPERIMENTAL

A. Intermolecular Force Microscopy (IFM)

Details of the set-up originally developed for intermolecular force microscopy (IFM) has been previously described [5]. Cantilever probes with a sensitivity of 100 times higher than commercially available ones were made by heating and pulling glass thin coverslips by a Pt heater. ZnO whiskers were attached to the cantilevers as scanning stylus under a binocular microscope, and their rear surfaces were coated with gold thin film to reflect the laser lights (Fig. 1(a)). In order to reduce the thermal fluctuations of the flexible cantilevers, a position clamping technique using laser radiation pressure feedback system was also introduced (Fig. 1(b)). These developments allowed the force-distance relationship between the probe stylus and the sample surface to be measured with nanometer accuracy.
FIG. 1: The non-contact Intermolecular Force Microscope (nc-IFM). (a) Top view of a cantilever. The dotted line represents the area where gold thin film was coated to reflect the lasers and the asterisk represents the ZnO whisker crystal. (b) Intensity changes of the position sensing laser diode ($\lambda = 780$ nm) was detected by a photodetector (PD) after being reflected onto the lever and the signal was then amplified. The force data were obtained from feedback signals leading to a position clamping laser diode ($\lambda = 680$ nm). Rhodamine-labeled proteins were illuminated by an Nd-YAG laser ($\lambda = 532$ nm) and the fluorescence images were observed using an intensified SIT camera. Electrostatic repulsive forces between the positively charged ZnO whisker tip and the glass surface obtained in solutions with different salt concentrations (c-e). The white lines show the two-component exponential least-square curves [9] fit to the data.

B. Force measurements

Force-distance relationships arisen from the interactions of like-charges between the positively charged glass surfaces and a ZnO stylus were obtained in solution when the specimen was moved towards a ZnO tip in the z direction under the feedback control of thermal fluctuations of the probes [5].

Electrostatic surface force maps of single myosin filaments were obtained by scanning a positively charged stylus over the sample surface in an $x-y$ directions. During scanning the gap distance between the probe tip and proteins was kept constant at several tens of nanometer. The distribution of the electrostatic force profile was reconstructed as a 2D map [6].

Force-distance relationships between kinesin and microtubules were measured by scanning the microtubules fixed on sample stage in the $x-z$ directions. While the cantilever coated with kinesin molecules was held at the same vertical position using feedback controls, the stage was manipulated so that the tip crossed the long axis of microtubules in the $x-z$ plane and approached toward the tip with a step of 8 nm in the $z$ direction [7].

III. RESULTS AND DISCUSSION

A. Cantilevers and position clamping system

Spring constants of the cantilevers were calculated from the thermal fluctuations of the displacements in solution by applying the principle of equipartition, and the val-
ues were approximately 0.1 pN·nm$^{-1}$. The spring constants were also directly determined by cross-calibration methods using glass microneedles [8]. In the IFM system, thermal fluctuations of the cantilever were reduced by laser radiation pressure feedback incorporated in the microscope. The rms displacement of the thermal fluctuations of the cantilever decreased from approx. 7 nm to 0.8 nm as a result of optical feedback clamping. Using this system, it was possible to maintain the gap between the probe stylus tip and the glass surface with nanometer accuracy. The sensitivity of force detection was determined by applying external oscillations to a cantilever holder and was confirmed to be 0.5 pN.

B. Electrostatic repulsion between positively charged surfaces

Electrostatic repulsive forces between a positively charged stylus and a glass surface in solution were measured (Figs. 1(c)-(e)). The surfaces of both the glass and the whiskers were modified with amino groups and the forces were measured as a function of gap distance. The force curves were well fitted with a theoretical electrostatic force-distance relationship between the charged conical stylus and the infinite surface [9, 10]. In the presence of 1 mM KCl, forces in the range of 1 pN or smaller at a gap distance of 5 nm could be clearly detected.

C. Surface force distribution of protein filaments

Surface electrostatic force images of myosin filaments were obtained in non-contact mode (Fig. 2(a)) using a stylus tip modified with positive charges. Therefore, the obtained images represent the electrostatic surface properties of the myosin filaments. In the non-contact surface force images, myosin filaments were observed as dark spindle-shaped images on a bright background. Repulsive forces resulting from the interaction of like-charges between the stylus tip and the glass surface contributed the bright background. Negatively charged myosin filaments neutralized the positive charge on the glass surface resulting in the dark images on a bright background. The force of the like-charge interactions between the stylus tips and the glass (~10 pN) suggests that the gaps in this experimental condition were ≥50 nm. The effects of the surface topology appear to be very small and thus, the images most likely only reflect the electrostatic features of the protein filaments.

The lateral section of an IFM image is showed in Fig. 2(b). In a single filament, there was a darker region in the center, indicating that the myosin filaments were more negatively charged at the center than the rest of the filament. Therefore, the electrostatic difference in the charge density between center and both ends of filaments is attributable to the electrostatic status of the myosin head because the heads exist on the filament except the central bare zone. This hypothesis has been tested by measuring the force-distance relationship between the positively charged tip and the self-assembled monolayer (SAM) of myosin heads (S1) on a gold thin layer [11], and the results indicate that myosin heads are positively charged [6]. From the experimental results of surface forces using the myosin filaments and the heads only, the number of charges on each head and rod per heavy chain were calculated statistically to be $+1.8e$ for the head part of the molecule and $-3.2e$ for the rod portion on average [6]. These values are in agreement with the values calculated from the amino acid sequence of myosin [12]. Thus, the non-contact images provide the electrostatic features of the protein surface, rather than the surface topography.
D. Long-range interactions between motor protein systems

Using non-contact IFM, it is possible to measure the forces exerted between the protein molecules in non-contact mode by attaching proteins to both the whiskers and the sample surface. This measurement will provide information on how protein molecules recognize their partner molecules for functioning. Kinesin is a motor protein that moves processively along microtubules, carrying organelles in neural axon. Observations using in vitro motility assay have suggested that kinesin binds to microtubules in a cooperative manner [13]. Therefore, the possible long-range interaction between kinesin and microtubules in the non-contact region has been investigated.

Kinesin was attached to a ZnO tip and the sample stage was scanned laterally and was approached vertically toward ZnO tip (Fig. 3(a)). Interaction forces were measured while the position of the cantilever was clamped by feedback and the gap distance was kept constant within the single x-scanning line (Fig. 3(b)). On the force signal channel, the cantilever was attracted to the stage at a certain Z position force of approx. 5 pN (line No. 3) while there are no responses on the cantilever deflection signal channel [7]. This suggests that long-range attractive force was exerted between kinesin and microtubules. The range over which the attractive force exist is considered to be between 1 to 15 nm because the rms displacement of the cantilever position with feedback clamping was 0.8 nm and the line interval of scanning in the z direction was 8 nm in the experimental condition. This extremely long-range attraction cannot be explained by simple electrostatic interactions because the salt concentration is high enough to shield the electrostatic effect over the nanometer range. To interpret this long-range interaction further, it will be necessary to make more detailed measurements.
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