

Antibody recognition imaging by force microscopy

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We have developed a method that combines dynamic force microscopy with the simultaneous molecular recognition of an antigen by an antibody, during imaging. A magnetically oscillated atomic force microscopy tip carrying a tethered antibody was scanned over a surface to which lysozyme was bound. By oscillating the probe at an amplitude of only a few nanometers, the antibody was kept in close proximity to the surface, allowing fast and efficient antigen recognition and gentle interaction between tip and sample. Antigenic sites were evident from reduction of the oscillation amplitude, as a result of antibody-antigen recognition during the lateral scan. Lysozyme molecules bound to the surface were recognized by the antibody on the scanning tip with a few nanometers lateral resolution. In principle, any ligand can be tethered to the tip; thus, this technique could potentially be used for nanometer-scale epitope mapping of biomolecules and localizing receptor sites during biological processes.

Keywords: Force microscopy, molecular recognition, antibody-antigen, receptor-ligand, lysozyme

The atomic force microscope (AFM)¹ has yielded nanometer-scale topographical images of biological molecules in aqueous buffers²⁻⁴. It has also been used to detect molecular recognition events between single molecules through either normal⁵⁻¹⁵ or lateral¹⁶ force interactions. In these experiments, ligands are bound to AFM tips for the molecular recognition of receptors on the surface. Normal forces are measured by monitoring the cantilever deflection during approach-retraction cycles at a constant lateral position. This allows for the detection of the unbinding force (the maximum force at the moment of receptor-ligand detachment), which is obtained upon retraction of the cantilever from the surface. Recently, unbinding forces were quantified for various ligand-receptor pairs, including biotin-avidin^{5,6,12}, DNA bases⁷, antibody-antigen^{8-11,13,14}, and cell recognition proteins¹⁵. It was also shown that the value of the unbinding force for a certain receptor-ligand pair is not unique but rather is dependent on the dynamics of the experiment¹⁷.

Simultaneous information for topography and forces was recently obtained by lateral force mapping (i.e., using a biotinylated AFM tip to perform an approach-retract cycle in every pixel of a micrometer-sized streptavidin pattern)¹⁸. In a first attempt at single-molecule force mapping, the binding probability between an antibody tethered to an AFM tip and antigens on the surface was measured as a function of the lateral position⁸, allowing determination of antigenic sites with nanometer positional accuracy⁸. Using a similar configuration, height and adhesion force images were simultaneously obtained with resolution approaching the single-molecule level¹⁴. These force mapping strategies, however, lack high lateral resolution¹⁸ and/or are much slower in data acquisition^{8,10,14,18} than topography imaging, since the frequency of the retract-approach cycles performed in every pixel is limited by hydrodynamic forces in the aqueous solution (to ~20 Hz at maximum). In addition, obtaining the force image requires that the ligand be disrupted from the receptor in each retract-approach cycle. For this, the z-amplitude of the retract-approach cycle must be at least 50 nm, and therefore the lig-

and on the tip lacks access to the receptor on the surface for most of the duration of the experiment.

We present a new imaging method, which uses dynamic force microscopy¹⁹⁻²¹ to map antigenic sites on a surface by molecular recognition of an antigen by an antibody tethered to an AFM tip^{8,13,14}. This technique, formerly used to record high-resolution topography images, provides very gentle interactions between the tip and surface. We extend this method by coupling an antibody to the AFM tip through a flexible tether, such that specific interaction between the antibody on the tip and the antigen on the surface can be used to localize antigenic sites. The magnetically coated tip was oscillated by an alternating magnetic field while being scanned along the surface. With this methodology, topography and recognition image can be obtained at the same time, and distinct receptor sites in the recognition image can be assigned to structures from the topography image.

Results and discussion

Half-antibodies were used as monovalent ligands on the tip. The concentration used was such that only one half-antibody had access to the lysozyme molecules on the surface. (This was tested on a densely packed lysozyme multilayer, with the tip used showing not more than one binding event per approach-retract cycle). The antigen, lysozyme, was tightly adsorbed to mica in conditions that yielded a high surface coverage. A topographic image of this preparation was first recorded in buffer using a bare AFM tip as a control (Fig. 1A). The surface appears very smooth, as expected from a densely packed protein multilayer (Fig. 1A).

To carry out recognition imaging, the tip must be in the vicinity of the antigen long enough for a binding event to occur. This requires careful control of the tip amplitude and frequency of oscillation, both of which are provided by magnetic excitation¹⁹⁻²¹. The tip is oscillated at an amplitude less than the length of the polymer tether that holds the antibody, and the on time (τ_{on}) for the anti-

body–antigen interaction must be much faster than the dwell time (τ_D) of the tip over the antigen. $\tau_{on} = (ck_{on})^{-1}$ where c , the concentration of antibody over an antigen, is given by $(N_A\pi L^2h)^{-1}$ with N_A being the Avogadro constant; L , the length of the antibody tether; and h , the amplitude of the tip oscillation. Using $k_{on} = 1.65 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (S.J.S.-G., unpublished data), $L = 6 \text{ nm}$, and $h = 2.5 \text{ nm}$, we obtain $\tau_{on} 1 \text{ ms}$, which is sufficiently short compared with a dwell time of $\sim 12 \text{ ms}$ for typical tip scan speeds of $1 \mu\text{m/s}$.

Imaging with a half-antibody tethered to the tip in conditions identical to those used to obtain the topographical multilayer image (Fig. 1A) gave strikingly different images (Fig. 1B). At certain lateral positions of the image, profiles with apparent heights of 1–2 nm became visible. These recognition profiles arose from the binding of the half-antibody on the AFM tip to lysozyme molecules on the surface, which led to a reduction in the amplitude of the oscillating tip. Apparently, at positions where recognition profiles were detected, the antibody binding site of the lysozyme molecules faced toward the AFM tip, and in positions lacking recognition profiles, the antibody binding site of lysozyme was buried. The lysozyme appeared to be randomly oriented in the multilayer preparation used.

The specificity of the recognition profiles was supported by an antibody blocking experiment (Fig. 1C). Injection of free antibody led to the disappearance of recognition profiles (Fig. 1C). This can be explained by free antibody binding to the antibody binding sites of the lysozyme molecules, thereby blocking recognition by the antibody on the tip. The binding of antibodies to the lysozyme multilayer was imaged as new structures; therefore, the antibody block image (Fig. 1C) does not appear quite as smooth as the lysozyme multilayer topography image before addition of the blocking antibodies (Fig. 1A).

The resolution of the method was tested on singly distributed lysozyme molecules. A typical topographic image of a lysozyme preparation that yielded a low surface coverage²² was recorded in buffer using a bare AFM tip (Fig. 2A). Single lysozyme molecules

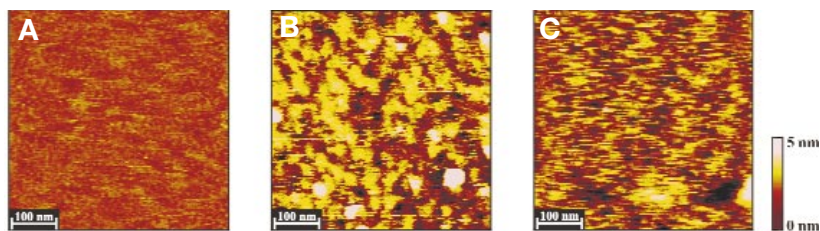


Figure 1. (A) Multilayer topography image. Image size was 500 nm. False color bar for heights from 0 (dark) to 5 (bright) nm. (B) Multilayer recognition image. Imaging was performed using an AFM tip carrying one half-antibody with access to the antigens on the surface. The bright dots representing recognition profiles indicate lateral positions where antibody–antigen recognition occurred. (C) Multilayer block image. The image was obtained with the same half-antibody tip and conditions as in (B) in the presence of free antibody in solution.

were clearly resolved (Fig. 2A). A cross-section analysis (Fig. 2D, profile in black) revealed that the molecules appear 8–12 nm in diameter and 2.0–2.5 nm in height. The larger sizes measured by AFM, as compared with a size of $4.5 \times 3.0 \times 3.0 \text{ nm}$ determined by X-ray crystallography²³, can be explained by tip broadening²² and compression of the molecule due to the applied force (Fig. 2A).

Imaging of singly distributed lysozyme molecules with a half-antibody tethered to the tip (see Fig. 2E as a typical example) yielded recognition profiles similar to those observed in Figure 1B. These recognition profiles differ significantly both in height and diameter compared with the profiles of the single-molecule topography image (Fig. 2C). Cross-section analysis (Fig. 2D, trace in red) reveals a height of 3.0–3.5 nm and a diameter of 20–25 nm. Profiles obtained from the single-molecule recognition image (Fig. 2E) appear at least 1 nm higher and 10 nm broader than profiles from the single-molecule topography image (Fig. 2C).

The antibody–antigen recognition process during imaging is depicted in Figure 2B. Approaching the antigen in a lateral scan from the left, the antibody on the tip binds to the antigen $\sim 10 \text{ nm}$ before the tip end is above the antigenic site (Fig. 2B, left tip) because of the flexible tethering provided by the crosslinker^{8,13}. In the bound state, the z-oscillation of the cantilever is further reduced by the attractive

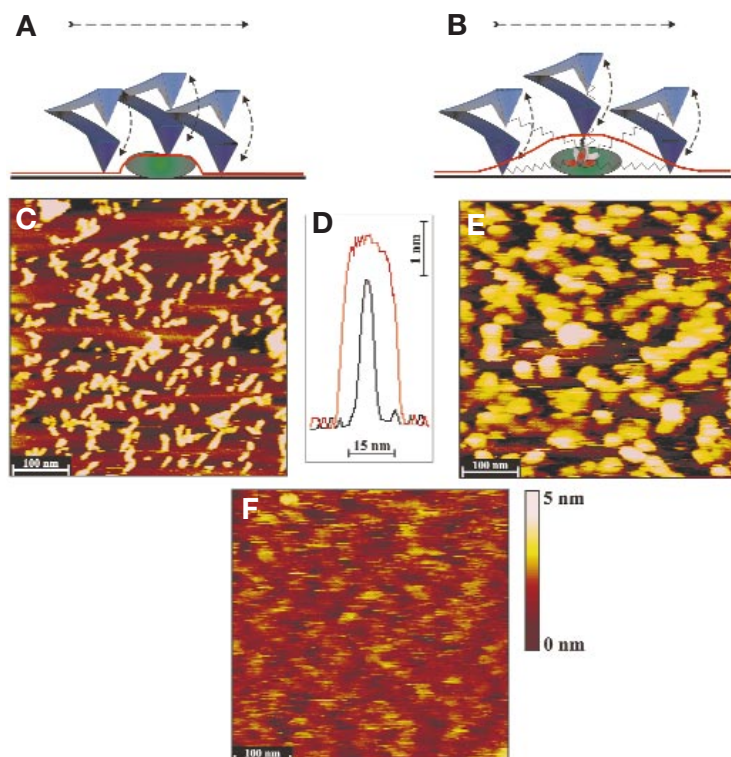


Figure 2. (A) AFM tip–lysozyme interaction during topography imaging. The red line indicates the height profile obtained from a single lysozyme molecule (shown in green) with the AFM using a bare tip. (B) AFM tip–lysozyme interaction during recognition imaging. Half antibodies (shown in red) are bound to the AFM tip by a flexible tether^{8,13,14} (jagged line) for the recognition of lysozyme (shown in green) on the surface. Imaging results in a height profile as indicated (red line). (C) Single-molecule topography image. Single lysozyme molecules can be clearly resolved. Sometimes small lysozyme aggregates are observed. Image size was 500 nm. False color bar for heights from 0 (dark) to 5 (bright) nm. (D) Height profiles. Cross-section profiles of single lysozyme molecules obtained from the topography (black line) and the recognition (red line) image. (E) Single-molecule recognition image. The bright dots represent recognition profiles of single lysozyme molecules. Imaging was performed using an AFM tip carrying one half-antibody with access to the antigens on the surface. Conditions were exactly the same as in (C). (F) Single-molecule block image. The image was obtained with the same half-antibody tip and conditions as in (E) in the presence of free antibody in solution. Recognition is blocked as apparent from the lack of recognition profiles.

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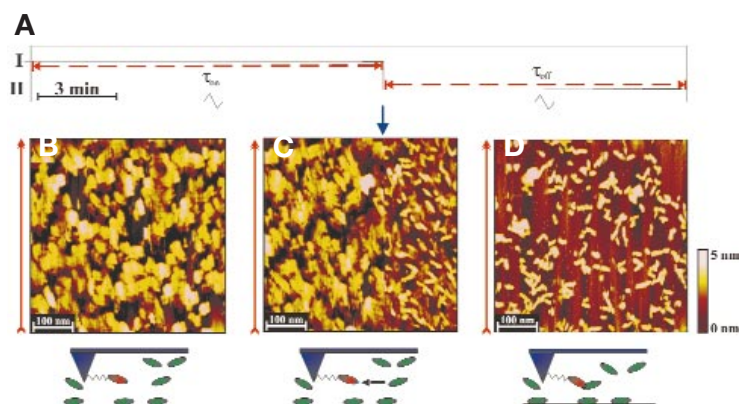


Figure 3. (A) Time course of antibody state. Upon recording images (B–D), the antibody on the tip switched in between two different states I and II with characteristic times τ_{on} and τ_{off} . State I reflects the unbound state and state II the bound state. (B–D) Consecutive images with an antibody-carrying tip and free antigen in solution. Recognition imaging (Fig. 2B and first part of Fig. 2C) changes at blue arrow to topography imaging (second part of Fig. 2C and D). The red arrow indicates fast scan direction. Image size was 500 nm. False color bar for heights are 0–5 nm. Sketches below images show the binding of free lysozyme (green obovates) to the half-antibody (red obovate) on the tip during recording images (B–D). Recognition and topography images are shifted against each other by the distance the antibody–crosslinker complex is bound to the tip apart from the imaging atom (a few nanometers at most). Switching effects, as presented in this figure, were only observed with both free antigen present in solution and a half-antibody tip used for scanning.

force of the crosslinker antibody–antigen connection, which acts as a nonlinear spring^{8,13}. As the AFM detects the z-projection of the force, the amount of the attractive force measured increases when the tip moves farther to the right and reaches its maximum just above the position of the antigenic site (Fig. 2B, tip in middle). This amplitude reduction leads to an increasing tip–surface separation induced by the feedback loop of the AFM. Upon further tip movement to the right, the z-component of the attractive force decreases again, resulting in a decreasing tip–surface separation. At lateral distances comparable to the length of the antibody–crosslinker connection, the antibody on the tip dissociates from the antibody on the surface, and the attractive force goes to zero.

The diameter of cross-section profiles obtained from the single-molecule recognition image (Fig. 2D, red trace) corresponds to about twice the length of the crosslinker (6 nm) plus antibody (6 nm). Increased heights detected in comparison to profiles of the topography image (Fig. 2D, black trace) reflect the amplitude reduction owing to antibody–antigen recognition. Cross-section profiles of the recognition image (Fig. 2D, red trace) were fitted with a truncated power law function. Maxima of the profiles indicate the position of the antigenic site. The accuracy of maximum, which in turn reflects the positional accuracy of antigenic site determination, was 3.1 nm (ref. 1).

The specificity of the antibody–antigen interaction was tested by injecting free antibody into the liquid cell to block antigenic sites on the surface. The resulting images showed a reduction of apparent height (Fig. 2F). Recognition profiles as observed in Figure 1E were absent (Fig. 2F), as the free antibodies bound to the lysozyme molecules on the surface blocked recognition by the antibody on the tip. The surface in Figure 2F appears even smoother than in the topography image of Figure 2C, which is attributed to the unspecific adsorption of free antibodies leading to coverage of the mica surface. The blocking effect was even more dramatic when the same tip was used to image the same molecules with free antigen in the solution. Recognition imaging occurred when the antibody on the tip was unbound by free antigen, and topographical imaging occurred when the antibody was bound by free antigen.

Recognition and topography imaging can be clearly distinguished

in a row of consecutive single-molecule images recorded in the presence of free antigen using a half-antibody tip (Fig. 3B–D). Antigen was injected into the fluid cell at a concentration (10 nmol/L) such that τ_{on} was comparable to the image acquisition time. The bright enlarged dots in the first image (Fig. 3B) indicate that the antibody on the tip recognized the antigens on the surface because the antibody on the tip is not bound by free antigen. After 35 min (Fig. 3C, blue arrow) the dots became abruptly smaller both in size and height. The recognition image turned into a topography image. Free antigen in solution bound to the antibody on the tip (sketch below Fig. 3C), thereby switching the antibody from the unblocked to the blocked state (I to II, see blue arrow in Fig. 3A) and preventing molecular recognition of the antigens on the surface. As long as the antigen remained bound, the tip continued to record topography (Fig. 3D and sketch below). Recognition imaging was again recovered when the tip was lifted for about 10 min because the antigen dissociated, thereby switching the antibody back into the unblocked state. In this way, recognition and topography images of the same region can be observed in cycles. Two full block–recover cycles were recorded, from which parts are presented in Figure 3B–D.

At least 90% of the antigens were capable of binding antibodies, as estimated by comparing the recognition and topography image of Figure 3B and D. This suggests that, in contrast to the lysozyme multilayer preparation, singly distributed lysozyme molecules adsorb to mica in an orientation with the antibody binding site facing to the AFM tip. The latter result is not unexpected as the antibody binding site is close to the negatively charged enzymatic cleft of lysozyme (S.J.S.-G., unpublished data), which renders adsorption of this part of the protein to negatively charged mica surfaces very unlikely.

The times τ_{on} and τ_{off} (Fig. 3A) for switching the antibody on the tip from the unblocked to the blocked state and back again reflect the association rate constant k_{on} and dissociation rate constant k_{off} of a single antibody–antigen complex, respectively. Values were calculated according to $k_{on} = (\tau_{on}c)^{-1}$ and $k_{off} = \tau_{off}^{-1}$, where c is the antigen bulk concentration, τ_{on} is the association time, and τ_{off} is the dissociation time. For $c = 10$ nmol/L, $\tau_{on} = 35$ min, and $\tau_{off} = 10$ min, we obtain $k_{on} = 4.8 \times 10^4$ M⁻¹ s⁻¹ and $k_{off} = 1.7 \times 10^{-3}$ s⁻¹. These estimates are in reasonable agreement with $k_{on} 1.65 \times 10^5$ M⁻¹ s⁻¹ and $k_{off} = 2.9 \times 10^{-3}$ s⁻¹ measured by a macroscopic binding technique (S.J.S.-G., unpublished data).

Lateral scanning of antigens on a surface with an AFM tip, containing a tethered antibody and oscillating at 5 nm amplitude, enables fast and reliable detection and localization of antigenic sites with 3 nm resolution. Detecting switching of the antibody on the tip in between two different states allows measurement of the kinetics of antibody–antigen association and dissociation on the single-molecule level. As recognition and topography images can be obtained at the same time during this switching process and the methodology is applicable with any ligand, it should prove possible to recognize many types of proteins or protein layers and carry out epitope mapping on a nanometer scale. The gentle interaction inherent in magnetically excited dynamic force microscopy should permit extension of the technique to soft cellular surfaces where dynamic processes may be mapped over time scales from seconds to hours.

Experimental protocol

Coupling of half antibody to tips. Binding of ethanolamine HCl (Sigma, Vienna, Austria) to MacLevers (Molecular Imaging, Phoenix, AZ) that were coated with silicon on the tip side, and subsequent coupling of a heterobifunctional tether (polyethylene glycol derivative), synthesized in our lab²⁴ to amines on the tip surface was done as described^{8,13}. Half-antibodies (type Hy-Hel 26, provided by S.J.S.-G.) were produced by cleaving the disulfides in the

central region of the heavy chain with 2-mercaptoethylamine HCl (Sigma) according to a standard procedure (Pierce, Rockford, IL). They were then bound to the sulfur-reactive end of the tether in buffer containing 20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.4 (incubation time, 1.5 h). Tips were subsequently washed in the same buffer and stored in the cold room.

Binding of lysozyme to surface. Densely packed lysozyme multilayers were prepared by adsorbing lysozyme to mica at 12 µg/ml for 10 s in buffer containing 0.1 M sodium acetate, 0.5 M NaCl, pH 4.6, before they were extensively washed. For single-molecule preparations, lysozyme (Sigma) was adsorbed²² to mica at 1 mg/ml in a 1 mM NaCl buffer for 10 min, resulting in a surface concentration of about 300/µm². The mica sheets were then extensively washed in the same buffer and subsequently in phosphate-buffered saline (PBS).

AFM experiments. We used a Macmode PicoSPM magnetically driven dynamic force microscope (Molecular Imaging) with a Molecular Imaging Macmode interface driving a Nanoscope IIIa controller (Digital Instruments, Santa Barbara, CA). Images were recorded with either a bare MacLever's or one containing one half-antibody for recognition. Cantilevers had a nominal spring constant of 0.1 N/m. Measurements were performed in PBS with 5 nm free tip oscillation amplitude at 5 kHz driving frequency and 20% amplitude reduction. The lateral frequency of 1 Hz resulted in a total recording time of 9 min. Image size was 500 nm. For block-unblock cycles (Fig. 3), 10 nmol/L of lysozyme were injected during scanning in a buffer (PBS) where adsorption to the mica surface was unlikely to occur. The flowthrough cell was thoroughly flushed to guarantee complete intermixing.

Acknowledgments

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