

Localization of Single Avidin–Biotin Interactions Using Simultaneous Topography and Molecular Recognition Imaging

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Simultaneous topography and recognition imaging (TREC),^[1,2] a recent development in dynamic force microscopy, has proven to be a powerful technique in biophysical research. In contrast to the common force mapping mode,^[3–7] the slow imaging speed and the low lateral resolution are overcome by oscillating a functionalized tip close to its resonance frequency during the scan across the surface.^[1,2,8] In these studies, the topographical imaging of receptor molecules is combined with molecular recognition by their cognate ligands bound to the atomic force microscope (AFM) tip via a distensible tether. The binding sites are evident from the reduction in the oscillation amplitude, as a result of specific recognition during the lateral scan. The receptors are recognized by the ligand on the scanning tip with a lateral resolution of a few nanometers, yielding a topographic image and a separate map of recognition sites from a single scan.^[1,2]

Because this new technique opens a broad range of biological applications, a stable and easy-to-use setup is of key interest for new users. Using well-characterized, high-affinity biological binding partners, such as avidin–biotin, for TREC imaging enables a better understanding of the key factors for optimizing the scanning parameters.

The robust and well-described avidin–biotin interaction offers an ideal pair of binding partners. Avidin–biotin is 1) robust and reliable, 2) well-known in terms of binding properties,^[9] 3) easy to prepare with commercially available components. Moreover, 4) avidin can simply be adsorbed to mica, and mica is an ideal support for AFM imaging. Finally, 5) the biotin–PEG (poly(ethylene glycol)) tether is commercially available and can be attached to amino-functionalized AFM tips in one step.

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Single avidin molecules can easily be immobilized onto mica via electrostatic adsorption by virtue of their positive net charge at neutral pH. Using different concentrations of avidin in the adsorption buffer, the surface coverage of avidin on mica can be adjusted easily.^[9] In addition, a biotin residue was coupled to the AFM tip via a distensible PEG chain. More specifically, biotin was covalently tethered to the amino-functionalized tip in a single coupling step, using “biotin-PEG-NHS” which consists of a PEG chain with a biotin on one end and an amino-reactive *N*-hydroxysuccinimide ester function (NHS group) on the other.^[9]

With this configuration, two independent maps were simultaneously acquired, that is, a topography image of the immobilized avidin molecules and a lateral map of the corresponding recognition sites, both recorded at experimental times comparable to normal AFM imaging. Before being applied to TREC, the tips were examined for a functional biotin residue by force spectroscopy experiments on a mica surface densely covered with avidin molecules.^[9]

Force spectroscopy with an oscillating tip offers an important tool for adjusting the scanning parameters in TREC microscopy. In simultaneously recorded amplitude–distance and force–distance cycles (Figure 1), the oscillation amplitude and the cantilever bending force, respectively, were investigated.

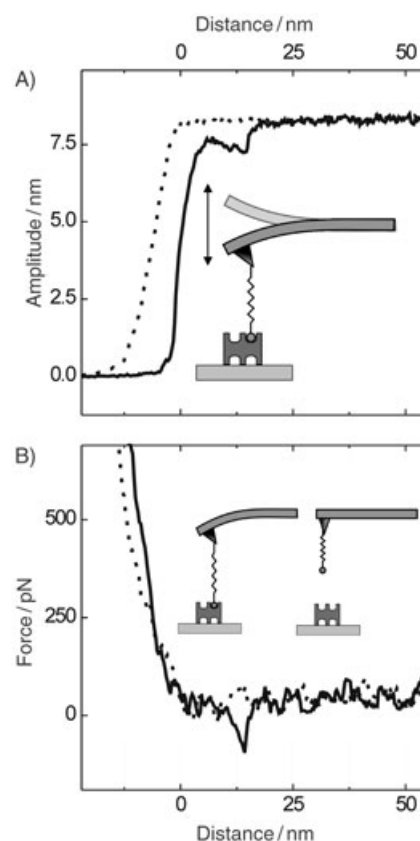


Figure 1. Raw data of an amplitude–distance and a force–distance cycle showing a single avidin–biotin unbinding event. A) In the amplitude–distance cycle, a single unbinding event is shown at 14 nm, resulting in an amplitude reduction of ≈ 1 nm at 8 nm free amplitude. B) The simultaneously acquired force–distance cycle exhibits the same unbinding event with an unbinding force of ≈ 80 pN.

The amplitude–distance cycle yields the value of the stretched crosslinker length, which is important for adjusting the actual oscillation amplitude. As a working principle of TREC, the oscillation amplitude must be smaller than the stretched crosslinker length. In addition, the amplitude reduction arising from the avidin–biotin interaction can be detected (Figure 1). The hysteresis in Figure 1 is caused by a small tip-to-surface adhesion. Because this hysteresis is only observable in the amplitude–distance cycle, and not in the force–distance cycle, the enormous sensitivity of amplitude–distance cycles can be seen.

The amplitude of the cantilever oscillation during approach (Figure 1, trace, dashed line) remains constant far away from the surface. As the tip is pushed into the surface, the amplitude decreases until it is fully reduced to zero at full contact. Upon retraction of the cantilever from the surface (retrace, solid line), the amplitude starts to increase and reaches the free amplitude again as the tip loses contact. During further retraction, a second amplitude reduction signal occurs, arising from the stretching of the tether, which has bound to an avidin molecule on the mica via its biotin end.

Finally, the steadily increasing tip–surface distance leads to dissociation of the avidin–biotin bond. The latter event is accompanied by a sharp increase in the cantilever oscillation amplitude of about 1 nm (corresponding to $\approx 10\%$ of the free amplitude) at 14 nm tip–surface distance (Figure 1 A, solid line). This amplitude reduction is governed by the ratio of the cantilever spring constant and the tether spring constant at the moment of unbinding.^[10,11] Using a tip oscillation frequency of 8 kHz at 300 nm ramp size and 1 Hz ramp frequency performed during this amplitude–distance cycle resulted in ≈ 200 tip oscillations without loss of binding during a cantilever travel distance of 14 nm at an effective loading rate of $\approx 250 \text{ nNs}^{-1}$, which reflects the high stability of the biotin–avidin bond.

The shape of the unbinding event in the simultaneously monitored force–distance cycle (Figure 1 B, solid line) is dominated by the nonlinear stretching behavior of the tether upon tip retraction from the surface.^[11] In contrast to the amplitude–distance cycle, the force–distance cycle monitors the cantilever deflection, which directly translates into a force according to Hook's law. Avidin–biotin unbinding results in a sudden jump to zero deflection (retrace in Figure 1 B). Both the amplitude– and force–distance cycles show clear unbinding events that are easily discernable from the cantilever noise, most likely because of the high affinity of the avidin–biotin bond.

The specificity of binding was proven by adding free streptavidin to the solution. Streptavidin has a negative net charge, so it does not stick to the negatively charged mica (in contrast to the positively charged surface-bound avidin) and is therefore optimal for blocking without covering the surface. This resulted in an effective block of the avidin–biotin interaction, as deduced from the absence of any unbinding event in the force– and amplitude–distance cycles.

Cantilevers that showed unbinding events in the force– and amplitude–distance cycles were used for TREC imaging. They had a low Q -factor (≈ 1 in liquid) and were driven at frequencies below resonance, for separating the topography signal

from the recognition signal.^[1,2,12] The principle of TREC (Figure 2 A) is explained in the following. The time-resolved deflection signal of the oscillating cantilever is low-pass filtered to remove the thermal noise, DC-offset leveled, and amplified,

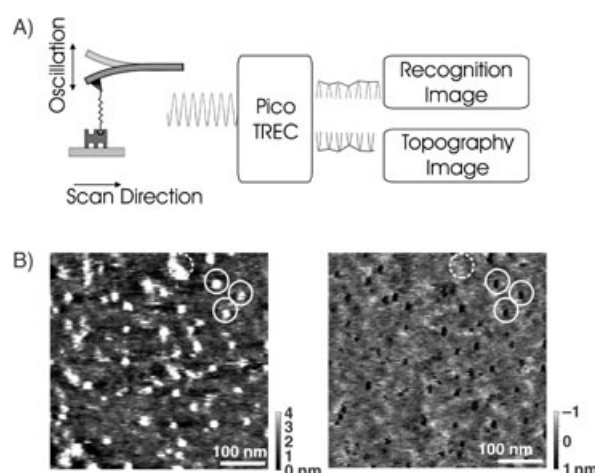


Figure 2. TREC imaging. A) Principle: The cantilever oscillation is split into lower and upper parts, resulting in simultaneously acquired topography and recognition images. B) Avidin was electrostatically adsorbed to mica and imaged with a biotin-tethered tip. A good correlation between topography (left image, bright spots) and recognition (right image, dark spots) was found (solid circles). Topographical spots without recognition denote structures lacking specific interaction (dashed circle). The scan size was 500 nm.

before it is split into the lower and upper parts of the oscillations. The signal passes a trigger threshold on each path and the lower and upper peaks of each oscillation period are determined by means of sample and hold analysis. Successive peaks result in a staircase function, which is subsequently filtered and fed into the microscope controller.

The lower parts (minima of oscillations) are used for driving the feedback loop to record the topographical image. A decrease in the lower parts is solely induced by topographical features and compensated by the feedback driven z piezo movement of the scanner. The upper parts (maxima of oscillations) provide the data for the image recognition and do not influence the piezo movement. Accordingly, the topographical image represents the true height of the sample, whereas the image recognition depicts the amplitude reduction arising from specific binding of the biotin on the tip to the avidin molecules on the surface. The tip oscillation amplitude ($\approx 8 \text{ nm}$) was adjusted to be slightly smaller than the extended crosslinker length ($\approx 14 \text{ nm}$) using the amplitude–distance cycle (Figure 1 A) to ensure that the biotin remains bound while the cantilever passes the avidin molecules, and to arrive at a significantly large amplitude reduction for the detection of recognition.^[2,8]

Because the oscillation amplitude was set to 8 nm and the maximum extension of PEG was 14 nm (Figure 1 A), the PEG spacer was stretched to a maximum of 60% (reached in each oscillation period at the highest tip position) during TREC imaging. This resulted in a spacer spring constant of about 2 pNm^{-1} and a maximum pulling force of 20 pN.^[11] The life-

time τ of a molecular bond under a force load is related to the thermal off-rate k_{off} in solution by $\tau^{-1} = k_{\text{off}} \cdot \exp(fx_{\beta}/k_{\text{B}}T)$,^[13] where f is the pulling force, x_{β} is the distance of the activation barrier for dissociation to the energy minimum of the bound state, and $k_{\text{B}}T$ is the thermal energy. τ can be experimentally determined from the loading-rate dependence of the unbinding force (for avidin–biotin see Merkel et al.^[13]). From these data,^[13] we obtained a lifetime of $\tau = 250$ ms at a constant applied pulling force of 20 pN. The dwell time of the biotin molecule on the tip over the avidin molecule on the surface during TREC imaging was significantly shorter (10 ms at a scan speed of 1 nm ms^{-1}). In addition, the force of 20 pN was only intermittently applied to the bond. Therefore, the biotin on the tip remained safely bound to the avidin molecule on the surface while scanning over it. In experiments using larger oscillation amplitudes (20 nm and more), the signals in the recognition image disappeared, because the threshold for unbinding is clearly exceeded (see Figure 1).

Figure 2B shows simultaneously acquired topography (left panel) and recognition images (right panel). Almost all avidin molecules visible in the topographical image (61 white spots in the left panel of Figure 2B) were recognized by the biotin attached to the AFM tip (55 dark spots in the right panel of Figure 2B), yielding an overall success rate of $p_{\text{B}} \approx 90\%$. The excellent correlation between topography and recognition images is also demonstrated on some single molecules (marked with solid white circles). Not all avidin molecules were recognized by the biotinylated tip (see dashed white circle), which could be caused by a partial loss in the functionality of avidin.

The specificity of the recognition signals was proven by acquiring topography and recognition images, and subsequently imaging the same place after blocking the tip with free streptavidin in solution (Figure 3). First, the topography (Figure 3A) and the recognition (Figure 3B) images are shown prior to blocking, again revealing a high yield of binding and a good correlation between the two images. Injecting free streptavidin in solution resulted in the disappearance of the dark spots in the recognition image (Figure 3D), while the corresponding topography image (Figure 3C) remained unchanged. Before blocking, all 94 avidin molecules (Figure 3A) were recognized (Figure 3B), but after the block only five spots with a smaller amplitude reduction remained in the recognition image (Fig-

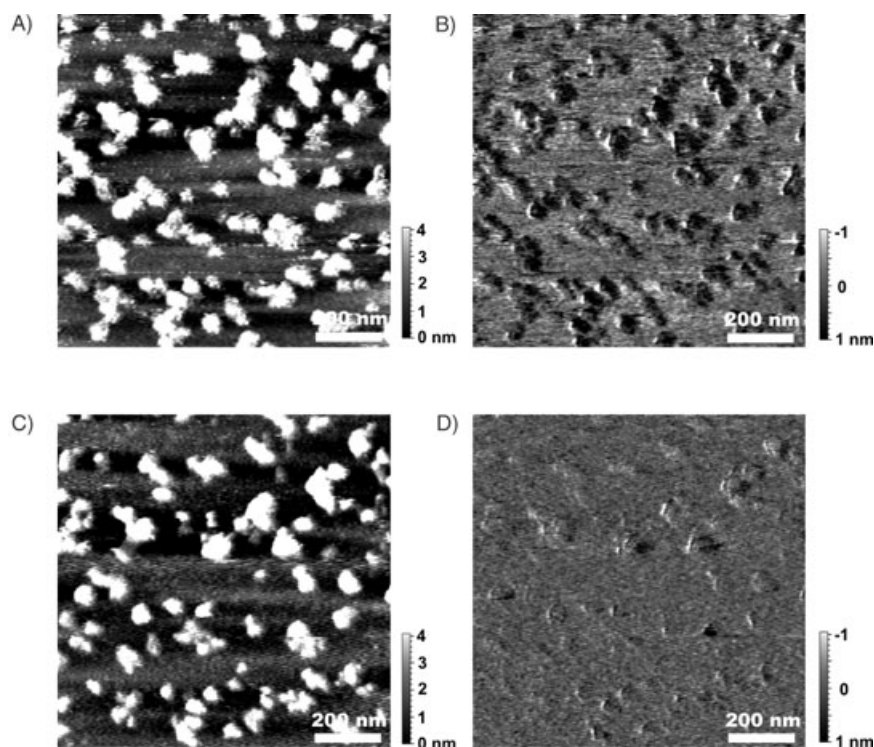


Figure 3. Specificity of TREC imaging. First, topography (A) and recognition (B) images were acquired. Then, the biotin on the tip was blocked by adding free streptavidin to the system while scanning the same position. No change in the topography image was observed (C). In contrast, almost all binding events disappeared in the recognition image (D). The scan size was 1000 nm.

ure 3D), corresponding to adhesive locations on the surface. This result clearly shows that the recognition events arise from the interaction of biotin on the tip with avidin on the surface, proving the overall specificity of the detected molecular recognition signals.

In conclusion, TREC is a new powerful AFM imaging tool in which topography and recognition of specific target sites are simultaneously mapped. The images are obtained in a dynamic force microscopy mode, and the target sites that are immobilized on the sample surface are recognized by sensor molecules covalently bound to the AFM tip. Avidin and biotin offer a well-characterized receptor–ligand pair that represents a prime example of TREC. Amplitude– and force–distance cycles thereby offer an important tool for TREC. Topography and recognition images were simultaneously acquired with unprecedented clarity. The images show that biotin covalently coupled to an AFM tip recognizes the specific binding sites (avidin) with a close-to-100% probability at nanometer lateral resolution and high specificity. Because of its broad applicability, this technique has potential for use in a wide range of biological systems.

Experimental Section

Tip and Surface Chemistry: Magnetically coated AFM tips (Molecular Imaging, Tempe, AZ, USA) were functionalized with ethanolamine and further derivatized with Biotin-PEG-NHS.^[9] Mica was incubated with an avidin solution ($0.5 \mu\text{g mL}^{-1}$ in 1 mM NaCl) for

10 min and subsequently rinsed with phosphate buffered saline (PBS) buffer solution.

TREC Imaging: Topography and recognition images were recorded in the magnetic AC (MAC) mode using a PicoSPM or a Pico Plus AFM (Molecular Imaging) with MAC levers (0.1 Nm^{-1} nominal spring constant). The excitation frequency of the cantilever was set about 10% below resonance (8 kHz).^[14] The free amplitude was between five and ten nanometers. The proportional and integral gains were set as high as possible, usually to 0.3–1. The topography and recognition data were recorded using either a home-built box or a commercially available PicoTREC (Molecular Imaging). In both cases, the oscillation amplitude was split into lower and upper parts. Adjusting the feedback to the lower amplitude was necessary to obtain topographical information free from recognition and adhesion information. The changes in the lower part of the amplitude were used to adjust the feedback of the servo. The scan speed was 1 Hz at 256 data points per line. To block the biotin on the tip, streptavidin was added into the fluid cell of the AFM at a final concentration of $50 \mu\text{g mL}^{-1}$ in PBS buffer solution.

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