Topography and Recognition Imaging with the Agilent 6000 ILM, an Integrated AFM/ILM

Application Note

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Figure 1. The Agilent 6000 ILM AFM on a Zeiss Axiovert X1 ILM.

Introduction

Atomic Force Microscopy (AFM) provides a means to study even very delicate biological entities far below the limits of optical resolution. Some interesting samples from a biological perspective that have been studied by AFM include DNA, RNA, phospholipids, polysaccharides, metalloproteins, titin, enzymes and other proteins, microtubules, antibodies, chromatin, purified receptors, as well as relatively larger structures such as viruses, sub-cellular components and organelles, bacteria, yeast, algae, live mammalian cells and even whole tissue samples. One of the most exciting, advances in nanobiotechnology has been the integration of an AFM with an inverted light microscope (ILM) [1]. These hybrid AFM/ILMs permit investigators to take advantage of the power and utility of both techniques simultaneously. An example of an AFM which was designed specifically for seamless integration with an optical microscope is the Agilent 6000 ILM (Figure 1).

A novel AFM technique called topography and recognition imaging (TREC) can be used relatively quickly to map the locations of picoNewton-scale ligand – receptor and antibody – antigen interactions with nanoscale lateral resolution [2–11]. PicoTREC was the result of a collaborative effort between scientists and engineers at The Institute for Biophysics at Johannes Keppler University in Linz, Austria, The Biodesign Center at Arizona State University and Agilent's Nanotechnology Measurements Division. It gives researchers the ability to detect and map molecular recognition events while concurrently generating high resolution AC mode topographic landscape and AFM phase images. With PicoTREC, researchers can quickly find molecules that are engaged in ligand – receptor or antigen – antibody interactions and it can often be used in place of slower and more tedious molecular recognition experiments while achieving similar results. PicoTREC utilizes MAC Mode (Magnetic AC Mode) to oscillate and

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scan a biochemically functionalized AFM probe in the most gentle manner possible while maintaining precise, close proximity between the probe tip and the sample. It utilizes hardware that is compatible with Agilent AFMs and biochemically modified AFM probes that each researcher functionalizes with specific biochemistries for their own particular applications.

Principle of PicoTREC

PicoTREC resolves biomolecular recognition events by processing the perturbation of an AC wave form that is generated by oscillating an AFM probe with a magnetic field. The perturbation is a consequence of biomolecular entities on the tip of the AFM probe binding with a specific molecular target on a sample. Topography and recognition images are generated simultaneously. The locations of antigens or receptors on the samples are easily determined by comparing the molecular binding sites that are displayed in the recognition image with the corresponding topography image.

More specifically, PicoTREC resolves single and multiple recognition events by processing the asymmetric reduction of the AC Mode signal that is caused by a biomolecular entity on the tip of the AFM probe binding with specific molecular targets that are immobilized on a sample surface under the probe [12]. For example, when a standard, commercially available MAC Lever is oscillated in MAC Mode at a distance far away from the sample surface, the time resolved signal is in the form a sinusoidal wave. In this case the drive signal and the detected signal are identical. When that same probe comes in closer proximity to a sample and interacts with the sample, the lower portion of the detected signal is damped due to probe – sample interactions. With PicoTREC, the MAC Lever is functionalized with a ligand or an antibody that is attached to the tip of the probe via a short and flexible tether, such as polyethylene glycol (PEG). In PicoTREC, the modified MAC Lever also oscillates as it is scanned over the sample surface using MAC Mode, so the bottom portion of the time resolved sinusoidal wave form is also altered when the tip of the probe is near the sample's surface. However, in this particular case, the sinusoidal wave form may also be altered because of ligand – receptor or antibody – antigen binding interactions that occur between the molecule or molecules on the tip of the probe and molecular binding sites on the sample surface. The probe will continue to oscillate and the upper portion of the sinusoidal wave will continue to be dampened until the tip moves away from the binding site laterally and the ligand – receptor or antibody – antigen interaction is broken. Then the wave returns to normal as with standard MAC Mode imaging. The damping effect on the upper portion of the sinusoidal wave form is detected, amplified and resolved by PicoTREC, which then generates a molecular recognition map that can be used, along with the topography image, to identify the precise locations of specific interactions between the molecules on the tip of the MAC Lever probe and binding sites on the sample surface.

Biotin – Avidin Interactions

Avidin is a tetrameric glycoprotein with a molecular weight of 68 kDa that is produced in the egg whites of many land vertebrates, including, birds, reptiles and amphibians [13–15]. Avidin has an extremely high affinity and specificity for a small water soluble vitamin, vitamin H or vitamin B7, which is more commonly referred to as biotin. The biotin – avidin interaction is unique by the fact that it is one of the strongest known noncovalent interactions, having a Kd of 10^{-15} M. Commercially available avidin is derived from chicken egg whites, and it accounts for nearly 2 mg per egg. Avidin has a bacterial counterpart, streptavidin, which is a 53 kDa protein that is purified from a bacterium, *Streptomyces avidinii,* and also binds tightly to biotin. Since eukaryotes and prokaryotes are believed to have diverged very early in evolution, approximately 1.8 billion years ago, it is not surprising that streptavidin has only 30% sequence identity with avidin [16]. What is surprising is that avidin and streptavidin both have such strong, specific affinities for biotin. The Kd for the biotin-streptavidin bond is on the order of approximately 10^{-14} M, so it has only slightly less affinity for biotin than avidin. Furthermore, they have nearly identical secondary, tertiary and quaternary structures. Like avidin, streptavidin is also a tetramer, with each monomer having identical binding sites for biotin.

Biotin – Avidin Applications

The strength and the affinity of the biotin-avidin interaction has been utilized as a powerful tool in the biological sciences [17–23]. Biotin can be conjugated to a variety of biological molecules, including antibodies and antibody fragments, enzymes, other proteins, DNA, RNA, molecular dyes, and many other molecules. This makes the complex useful in a variety of assays, including Western blots, ELISAs and pull-down assays. Biotin – avidin permits the use of greatly diluted primary antibodies in immunoassays, enables shorter incubation times, and since each protein can bind four biotins, it can be used to amplify otherwise weak signals. Biotin – avidin and biotin – streptavidin interactions can also be used to purify proteins. For example, biotin is often used to tag molecules of interest and avidin can then used to extract the biotin-tagged protein [24, 25]. Biotin – avidin affinity labeling systems can even be found in clinical diagnostic assays, medical devices and in specific pharmaceutical applications [26, 27].

Avidin has at least one advantage over streptavidin when it comes to immobilization on solid surfaces. For example, streptavidin has a slightly acidic isoelectric point (pI), approximately 5, so it has relatively few positively charged groups at neutral pH. In contrast, avidin has a pI of approximately 10, so it possesses many more positively charged lysine residues at neutral pH. It is this fact that permits avidin to be immobilized much more easily to negatively charged substrates such as mica, without the need for elaborate covalent surface chemistries.

Experimental

Probe and Sample Preparation

The scheme used to prepare biotinylated AFM probes and avidin substrates for PicoTREC imaging is summarized in Figure 2.

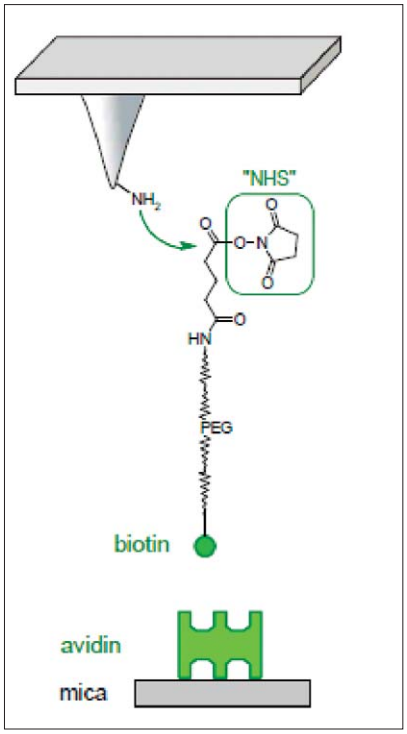


Figure 2. PicoTREC imaging requires that ligands and their receptors, or antigens and antibodies, be immobilized on the tip of an AFM probe and a suitable AFM substrate. In this case, biotin was attached to the tip of an AFM probe using a heterobifunctional PEG linker. The linker was covalently attached to the probe by amine – NHS ester chemistry. Avidin molecules were immobilized on freshly cleaved mica by electrostatic interactions in low salt buffer.

Probe Preparation

Amine-functionalized silicon nitride MAC Levers (TypeVI MAC Levers, Agilent Technologies) were prepared in close analogy to the protocol established by Riener et al 2003 [8, 11, 28–30]. In a chemical fume hood, five MAC Levers were placed in a crystallization dish that was filled with 10 mL of dichloromethane and the dish was covered. After 5 minutes, the MAC Levers were placed directly into a custom-built UV-ozone cleaner with the tips of the probes facing upwards. The ozone cleaner was turned on and the MAC Levers were allowed to stay in the apparatus for 2 hours. In the meantime, the lids of two microcentrifuge vials were placed inside a desiccator bell jar (5 L). The jar was purged with argon for 5 minutes to remove air and moisture. 30 µL of APTES (3-aminopropyl-triethoxysilane packaged under argon; SureSeal from Aldrich) and 10 µL of diisopropylethylamine (Aldrich) were pipetted into the lids of the microcentrifuge vials. The cleaned MAC Levers were then placed directly from the ozone cleaner onto a high density polypropylene surface in the bell jar. The bell jar was purged with argon for an additional 2 minutes and sealed. After 120 minutes the bell jar was purged with argon and the MAC Levers were removed. They were used immediately in the next step or stored under argon for no more than two days.

The APTES-treated (aminated) MAC Levers were pegylated using biotin-PEG-NHS [4, 28, 31, 32]. The aminated probes were placed in a glass crystallization dish that was filled with 10 mL of dichloromethane and the dish was covered. A 15 mg vial of biotin-PEG_{12}-NHS (Quanta Biodesign) was removed from a -80° C freezer and the vial was placed in a high density polypropylene container that had been filled with desiccant material. The vial was allowed to come to room temperature for 30 minutes in the presence of desiccant in order to prevent condensation. Upon removal from the desiccant jar, 0.5 mL of dichloromethane (Aldrich) and 7 µL of triethylamine (Aldrich) were added to the vial. The vial was shaken vigorously for several minutes until all solid substances were dissolved. Meanwhile, the amine-functionalized MAC Levers were removed from the crystallization dish and placed into a glass vial that had been dried in a 120° C oven and allowed to cool to room temperature in a desiccant jar. The dissolved biotin-PEG_{12}-NHS linker solution was carefully transferred to the glass vial, which was promptly sealed and allowed to stand at room temperature. After 2 hours, the biotin-PEG treated MAC Levers were removed from the vial, successively placed into two crystallization dishes filled with dichloromethane and allowed to stand in each dish for 5 minutes. Each MAC Lever was carefully dried under a stream of dry nitrogen and then placed in a gel pack AFM probe storage container. The biotin-PEG MAC Levers were used immediately or stored under argon at room temperature for up to 6 weeks.

Sample Preparation

An avidin coated mica substrate was prepared in such a manner that

individual avidin molecules and small clusters of avidin molecules were firmly affixed to mica by electrostatic interactions so that they could easily be distinguished from bare mica by MAC Mode AFM [4]. The surface coverage of avidin on the mica substrate was approximately 25–40%. A 1.25 µg/mL suspension of avidin (Aldrich) in "1/10X PBS" buffer (14.7 mM NaCl, 0.27 mM KCl, 0.81 mM Na_2HPO_4, 0.176 mM KH_2PO_4, pH 7.4) was mechanically vortexed for 3 minutes to break up any large clumps of avidin and 150 µL of the suspension was dispensed onto an extra large mica substrate (Agilent) which had been freshly cleaved using transparent adhesive tape. The avidin suspension was allowed to stand on the mica for 10 minutes after which time the mica was rinsed 10–20 times with 500 µL of "1/10x PBS" buffer. The mica was not permitted to dry but was immediately loaded onto an Agilent 6000 ILM cover slip liquid cell which was then immediately filled with 300 µL of "1/10X PBS" buffer. Higher concentrations of avidin and longer treatment times increased the amount of avidin bound to the mica.

Instrument Set Up and Imaging

The AFM was configured for MAC Mode imaging. PicoTREC was installed. PicoTREC performance is precisely correlated with AC oscillation amplitude, so the MAC mode amplitude must be identical to the effective length of the PEG-ligand complex that is attached to the tip of the MAC Lever. The oscillation amplitude was determined and confirmed by two separate methods, 1) MAC mode amplitude – distance cycles and 2) contact mode force – distance spectroscopy.

1. In order to adjust the AC mode oscillation amplitude by an amplitude – distance cycle, a piece of clean muscovite mica was added to a liquid cell which was filled was "1/10X PBS" buffer. The probe was tuned to its resonance frequency. The drive percent was set to achieve a free amplitude oscillation value (A_o) of approximately 4 nm. The tip – sample distance was slowly decreased until a "stop value" of 90% was achieved. Amplitude – distance sweeps were acquired while the drive percent value and the amplitude set point (A_{sp}) were adjusted until the peak-to-peak amplitude was 5.6 nm (the length of the PEG linker that was used in the experiment, NHS-PEG_{12}-biotin, was approximately 5.6 nm).

2. In order to adjust the AC mode oscillation amplitude (nm) via force – distance spectroscopy, the instrument was switched to contact mode and a clean mica sample was loaded on the sample plate. The deflection value was adjusted to -1.0 V. The tip – sample distance was decreased until the deflection value reached 0.0 V, at which point a force – distance curve was obtained. The sensitivity (nm/V) of the MAC Lever was calculated from the inverse of the slope of the contact region of the force – distance curve. The probe was withdrawn from the surface and the AFM was switched to MAC Mode. The free amplitude (A_o) was adjusted to 5.6 nm according to the following formula:

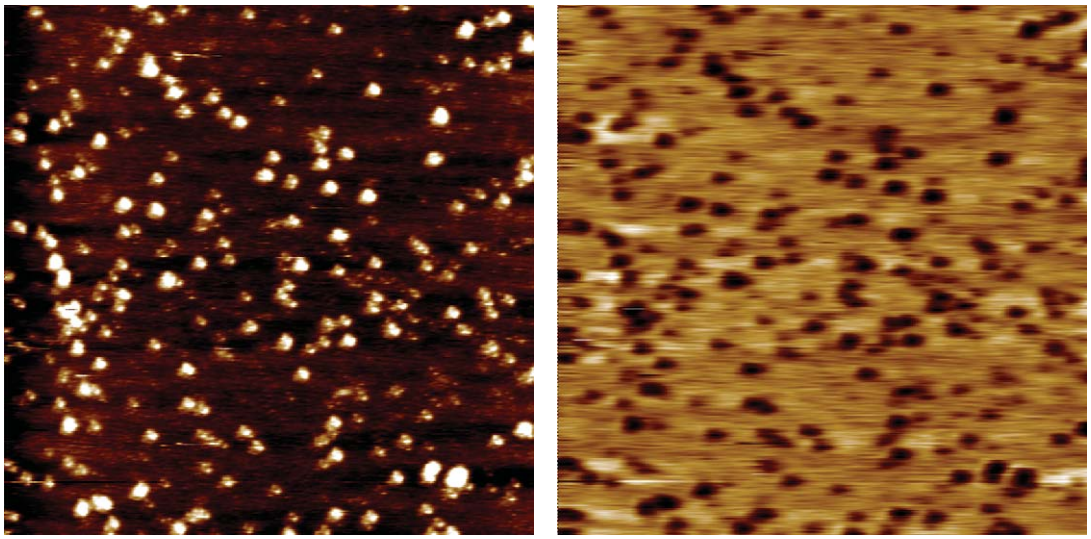
$$\text{Amplitude (nm)} = \frac{\text{Amplitude (V)}}{8 \cdot InputGain} \cdot Sensitivity\left(\frac{nm}{V}\right)$$

MAC Mode and PicoTREC Imaging

AFM images were acquired in MAC Mode using the Agilent 6000 ILM with PicoTREC. The 6000 ILM cover slip sample plate, which contained the sample in "1/10X PBS" buffer, was loaded onto the AFM and the probe was tuned to resonance. The A_o was set to 5.6 nm as described above. The distance between the tip of the AFM probe and the sample was decreased until a stop value of 85% was obtained, at which point scanning was initiated.

Results and Discussion

PicoTREC can detect specific single molecule interactions between a tethered ligand on the tip of a MAC Lever and immobilized receptors on a sample surface. Biotin – avidin interactions, which are of great importance in many research and diagnostic applications, and also a prototypical example of ligand – receptor interactions, were identified using PicoTREC and a biotinylated MAC Lever. MAC Mode was used to oscillate the biotinylated MAC Lever at its resonance frequency as the probe was gently scanned over the surface of the avidin molecules. Biotin – avidin interactions were detected by PicoTREC. The locations of the target molecules were identified by



A Avidin Topography Image **B** Avidin – Biotin Recognition Image

Figure 3. PicoTREC image of biotin – avidin interactions: During PicoTREC imaging, the AC sinusoidal wave was perturbed by avidin topographical features (topography image) and also by binding interactions between biotin molecules on the tip of the AFM probe and the immobilized avidin molecules. A) MAC Mode topography image of the avidin molecules. B) Corresponding biotin – avidin recognition image. Dark areas indicate regions of avidin that bound to pegylated biotin molecules on the tip of the AFM probe. The topography image and the recognition image were acquired simultaneously. The scan size was 3 µm and the scan rate was 1 Hz.

comparing the avidin topography images with the biotin – avidin PicoTREC recognition images (see Figure 3). Image 3A shows a MAC Mode topography image of avidin molecules on mica. Image 3B shows a biotin – avidin recognition image which was obtained simultaneously along with the topography image shown in 3A. Dark areas in the recognition image indicate locations where avidin molecules bound to pegylated biotin molecules on the tip of the AFM probe. Total image acquisition time was approximately 5 minutes.

Conclusion

Using PicoTREC, molecular interactions are quickly displayed and precisely identified. It records molecular recognition events by resolving the asymmetric perturbation of an AC oscillation signal, which occurs when molecules on the tip of a MAC Lever interact with binding sites on a sample. Using MAC Mode, molecules on the tip of the probe are kept in close proximity to the sample surface, allowing gentle interactions and efficient recognition events to occur during scanning. PicoTREC combines AC Mode AFM imaging with single molecule recognition imaging, giving rise to two separate images simultaneously. One of the images represents the topographical landscape of the sample. The other image represents a map of molecular interactions between the molecules on the tip of the AFM probe and the sample. The images are interrelated by their x-y coordinates.

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