Simultaneous *T*opography and *REC*ognition Mapping with PicoTREC™: A Powerful New Technology That Can Be Used To Map Nanometer-Scale Molecular Binding Sites On A Variety Of Surfaces

W. T. Johnson[*], G. Kada[*], C. Stroh[**], H. Gruber[**], H. Wang[***], F. Kienberger[**], A. Ebner[**], S. Lindsay[***], and P. Hinterdorfer[**]

[*]Molecular Imaging, Tempe, AZ, USA, www.molec.com
[**]Johannes Kepler University of Linz, Linz, Austria, www.jku.at
[***]University of Arizona, Tempe, AZ, USA, www.asu.edu

ABSTRACT

PicoTREC™ is a powerful new chemical/biological detection technology that combines nanoscale *T*opographic imaging the AFM with single molecule *REC*ognition mapping. The technique has been used to map interactions between, for example charged species, antibodies/antigens, and ligands/receptors in order to analyze the chemical composition of a variety of samples. PicoTREC combines *in-situ* nanometer scale, resolution imaging with the selectivity and sensitivity of single molecule, piconewton sensitivity to resolve and detect recognition events. It is label-less, so it is not dependent on fluorescence, radioactivity, or enzyme-linked detection schemes. When a ligand for a particular receptor is attached to an AFM tip, the AFM tip becomes a chemically selective sensor so that the forces required to break chemical bonds with the specific binding epitopes on target molecules can be detected and resolved. Consequently, maps of binding sites across a variety surfaces can quickly and easily be obtained.

Keywords: AFM, biosensor, antibody-antigen, ligand-receptor, nanobiotechnology

1 INTRODUCTION

AFM offers a unique solution to study biological process at the nanometer scale. The development of the atomic force microscope (AFM) has allowed scientists and engineers to visualize, probe, and analyze the molecular structure of biological molecules and other substrates in their native environments with unprecedented resolution and without the need for rigorous sample preparation or labeling. AFM can be used to study the nanomechanical properties of a wide variety of biological, organic and inorganic samples, for example, adhesion, hardness, and elasticity of sample surfaces [1]. Nanoscale biological adhesion events affect a variety of important physiological phenomena, for example, DNA replication, RNA transcription, cellular growth and differentiation, tissue growth, the action of drugs, hormones, and toxic substances, to the performance of the immune system.

AFM is unique in its ability to detect piconewton single molecule interaction forces with nanoscale resolution [2]. This has opened the possibility of measuring inter- and intra-molecular forces of biomolecules on the single molecule level. TREC (*T*opography and *REC*ognition imaging) combines the high resolution imaging capability of AFM, with its ability to detect single molecule binding events on the piconewton scale [3,4]. Consequently, it is now possible to investigate interactions of single molecules with their specific receptors while simultaneously recording a high-resolution topography image. Here we will review the principles of TREC together with some applications to various biological systems.

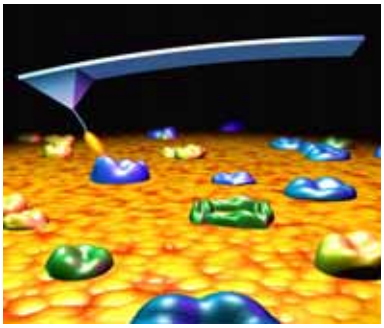

Figure 1: A specific ligand, attached to an AFM tip via a flexible PEG tether, binds to a target molecule.

2 WHAT IS PICOTREC?

PicoTREC is new technique in which sensor molecules are attached to the AFM tip on the end of an elastic polyethylene glycol (PEG) tether. This PEG tether gives the sensor molecule the freedom to reorient itself properly to bind to its target on the surface. When a recognition event occurs, the AFM detects a variety of signals. Included are the signals that correspond to the forces required to break hydrogen bonds and the weaker short range interactions that are involved in specific molecular binding events. PicoTREC resolves these signals and plots them separately. This technique is being detect the binding forces between antibodies and antigens, drug or hormones and their receptors, and protein-DNA complexes. When combined

with MAC Mode™ AFM (Molecular Imaging, Tempe, AZ), entire maps of specific binding sites can be obtained in real time. PicoTREC records two separate images by scanning the tethered sensor molecule across the surface to detect binding events. One image provides the topography of the target molecules on the surface. The second image displays a map of specifically recognized target molecules on the surface. Using MAC Mode, PicoTREC, and optimized cantilevers, a ligand can be kept in close proximity to the surface, allowing efficient recognition and gentle interaction between tip and sample during scanning. PicoTREC resolves molecular recognition during the lateral scan by processing the asymmetric reduction of the oscillation amplitude. In this way, the locations of the target molecules are easily determined from their coordinates on the recognition image.

3 SINGLE-MOLECULE RECOGNITION STUDIES WITH AFM

Force-spectroscopy is a type of molecular recognition experiment that is often used to study and quantify binding interactions between single molecule on an AFM tip and molecules immobilized on a surface [5]. In this technique, an AFM tip, to which ligands are immobilized, is brought into contact with a surface that contains complimentary binding sites or receptors to the ligands on the AFM tip. As the molecules on the AFM and those on the surface come into contact, a ligand-receptor bond is formed (figure 1). Upon retraction of the AFM tip away from the surface, the ligand-receptor binding complex deforms until the hydrogen bonds and other attractive interactions which hold the complex together reaches a critical force and the complex dissociates or unbinds. By performing numerous unbinding experiments with the AFM, rate constants and affinity of binding for the recognition and binding events can be calculated [6]. Structural data of the binding pocket can also be inferred from force-spectroscopy studies. AFM tip sensitivity, tip-sensor design, and attachment chemistry are all critical factors in recognition experiments.

4 ATTACHMENT CHEMISTRY FOR RECOGNITION STUDIES

Various strategies have been used, with varying success, to bind ligands such antibodies, drugs, vitamins, hormones, and nucleic acids to AFM tips for recognition studies [7]. In most of the early AFM-spectroscopy studies, ligands were directly bound to the AFM tip surface. However, direct attachment of a ligand to this solid surface does not always allow the ligand to orient itself freely away from the relatively massive AFM tip, so direct attachment can severely constrain the ligand and, more importantly interfere with its ability to bind correctly to the site of interest. Consequently, direct attachment of ligands to AFM tips may not provide the most favorable environment for specific recognition events to take place. Due to the limitations of direct attachment of ligands to AFM tips in recognition studies, flexible polymer linkers, which provide space between the ligand and the surface of the tip have been developed and utilized with great success. In the unbound state, flexible linkers allow an unbound ligand to freely orient and diffuse within a certain volume. Therefore, the ligand is allowed to diffuse about much like it would if it were in solution and less constrained binding of a ligand to its receptor can be achieved [8]. Furthermore, flexible PEG linkers can stretch, allowing movement of the AFM tip to some extent without severely disrupting the binding interaction (figure 2).

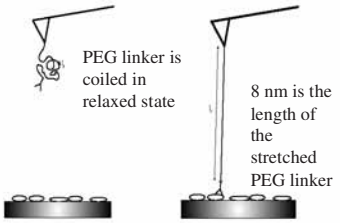

Figure 2: Flexible, heterobifunctional polyethylene glycol (PEG) linkers provide a means to attach ligands to AFM tips while permitting unconstrained binding to target molecules on the surface. The length of the linker is a critical parameter in TREC imaging as discussed below.

The PEG tether is a critical component for PicoTREC and recognition studies in general. In order to optimize the interactions between the ligand and it target, we use an elastic heterobifunctional polyethylene glycol (PEG) linker of ~8 nm length to attach the ligands to the AFM tip. Relatively short PEG linkers permit ligands on the AFM tip to diffuse within a defined volume. They impart additional degrees of freedom that are necessary so that the ligand can reorient itself to bind with an immobilized complimentary binding epitope in an optimal conformation.

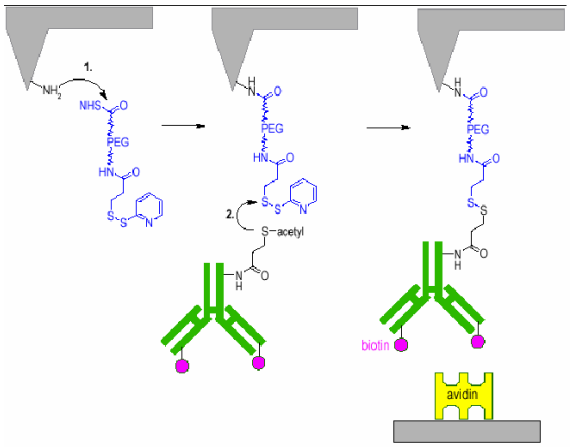

Figure 3: Immobilizing a specific ligand (biotinylated IgG) to an AFM tip and attaching a positively charged target (avidin) to negatively charged mica.

For AFM imaging, the target molecules also must be bound to a proper substrate. The substrate must be as flat and as smooth as possible so that the AFM tip that can resolve sample features over any features that are present on the substrate. The chemistry of the substrate must permit firm attachment of the target in order to prevent the target molecules from being pushed away by the AFM tip as it moves across the surface, but the attachment must not perturb the integrity and morphology of target. Also, in recognition studies, the target molecules must be adhered to the substrate more firmly than the specific bonds between the ligand and the target. If the target molecules are too loosely bound, they will simply be pulled off of the surface as the AFM tip is retracted. There are as many attachment chemistries are there are classes of samples. Mica is a negatively charged substrate, so if the target has sufficient positive charge (e.g., avidin, lysozyme, ferritin), then the target may be sufficiently immobilized by electrostatic binding using appropriate the buffers. Some molecules of interest in imaging and recognition studies are negatively charged (e.g., DNA, RNA, and certain proteins) and these molecules can often be immobilized to mica and imaged by adding, for example, positively charged Magnesium, Nickel, or ammonium ions to the attachment and imaging buffers [9]. Other biological molecules lack the ionic properties that are necessary for electrostatic immobilization and, therefore, they need to be covalently immobilized to the substrate. If the target is a protein with lysine residues on the surface, such as is the case for the histone proteins found in chromatin, the sample can be bound to mica that has been pretreated with APTES (aminopropyltriethoxysilane) and glutaraldehyde [10].

5 TOPOGRAPHY AND RECOGNITION IMAGING WITH PICOTREC

As noted previously, force-spectroscopy experiments can unveil important information about specific interactions between molecules, such as rate constants and affinity of binding, and it these experiments are particularly useful on pure samples of known identity. Unfortunately, most biological samples are composed of more than one component. Important data such as the sample's size, shape, and nanoscale morphology is also lacking in force-spectroscopy recognition experiments. Furthermore, force-spectroscopy experiments tend to be lengthy (generally taking hours to complete), which is a negative factor when dealing with biological samples, which quickly and easily denature and degrade. Simultaneous size and morphology information is also needed to localize the ligand-receptor interactions to particular areas. It is critical to image and characterize the components of biological mixtures before the samples have a chance to denature or degrade. Therefore, it is apparent that high speed topographical and recognition results are especially useful or even necessary in AFM studies on biological samples that are composed of mixtures of discrete components [12]. For mixed samples, protein-protein or DNA-protein complexes, and biological surfaces such as cells or membrane fragments, high-resolution topography imaging should be combined with high speed chemical recognition mapping, so that binding sites can properly be assigned to topographical features.

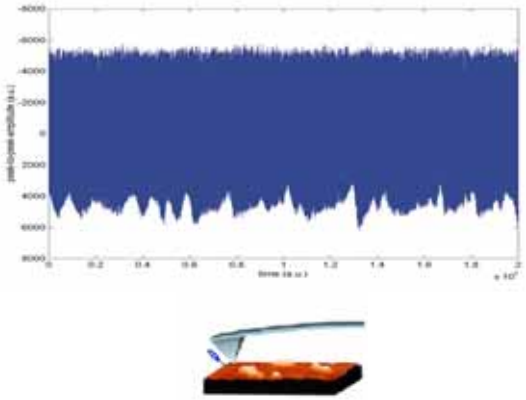

Figure 4A: MAC Mode signal in the absence of a recognition event

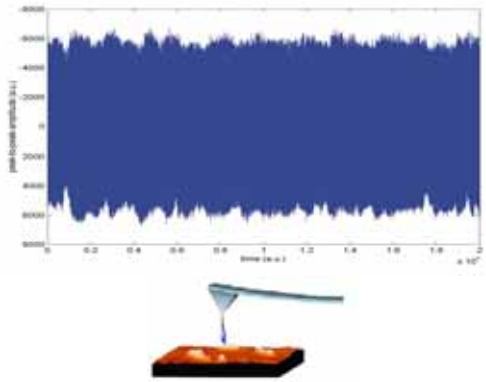

Figure 4B: MAC Mode signal in the presence of a recognition event

PicoTREC combines high resolution, fast, topographical information with specific recognition information. In PicoTREC, a magnetically coated AFM tip (MAC Mode Lever) is oscillated by an alternating magnetic field while being rastered over the sample. The magnetic field is generated by a magnetic coil that is placed either above or below the sample. The magnetically coated AFM tip comes in gentle contact with the sample, but only at the end of its downward movement, which reduces the contact time between the tip and the sample, and minimizes friction forces. As shown in figure 4A, this tip-surface interaction causes the amplitude of the oscillation to be reduced. The signal that arises from the oscillation is then processed into an image of the sample. Since the tip contacts the surface only intermittently, MAC Mode is an extremely gentle imaging tool that is useful for obtaining the highest

resolution images possible of soft biological samples in their native environments (e.g., physiological pH) including individual protein molecules and membranes [11]. When specific ligands or antibodies, that are specific for surface-immobilized receptors, are attached to the AFM tip and the tip is oscillated and rastered across the sample surface, the amplitude of the oscillation is further modified. PicoTREC resolves the topography signal from the recognition signal to provide simultaneous Topography and RECognition (TREC) imaging. Figure 4B shows what happens when a MAC Mode AFM tip that has been derivatized with a ligand binds to a receptor immobilized on the surface. As the specific ligand molecules on the AFM tip bind to and then unbind from their targets on the surface, the unbinding force is detected in the top portion of the oscillation.

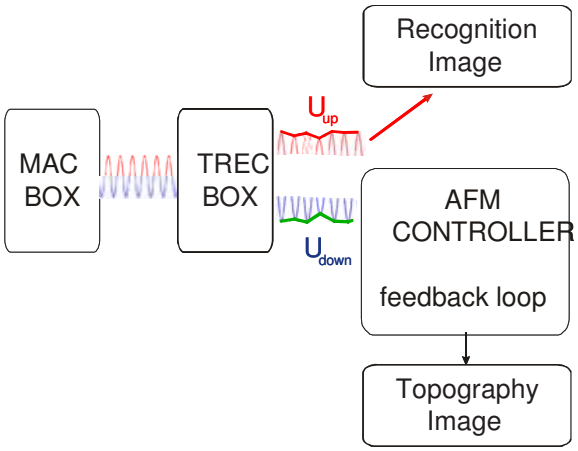

Figure 5: PicoTREC simultaneously records topography and recognition images using the maxima and minima of each sinusoidal cantilever oscillation

As shown in figure 5, the information from the unbinding event is resolved from the topographic information by PicoTREC. In this manner, molecules that bind to ligands on the AFM tip can be differentiated from molecules that do not bind.

The length of the PEG linker and the amplitude of MAC Mode oscillation (~5 nm) used with PicoTREC have been optimized and are extremely valuable components of the system. The amplitude is slightly smaller than the extended PEG tether length of ~8 nm (see figure 2) so that the ligand molecules on the tip can remain bound as the tip oscillates over the receptor-binding site. This results in high binding efficiencies and greater pixel densities in the recognition image. Figure 6 shows a TREC image of chromatin which was imaged with a MAC Mode AFM tip that was modified with a PEG linker and antihistone H3.

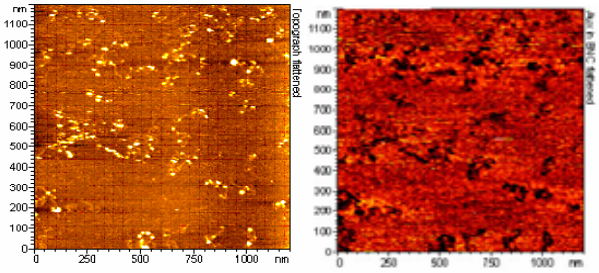

Figure 6: Simultaneous Topography (left) and RECognition (right) images of chromatin (histone-DNA complexes) using antihistone H3 modified AFM tips. Recognition sites are indicated by dark areas in the RECognition image.

6 SUMMARY

Simultaneous Topography and RECognition imaging has been demonstrated and proven to be a useful tool to identify molecules based on nanoscale topography and chemical composition. The technique has expanded AFM beyond basic imaging studies and quantifying molecular recognition events on mono-component samples to include pharmacology, toxicology, immunology, and the study of biological structure-function relationships on complex samples and mixtures of samples in real time. PicoTREC gives the atomic force microscope the ability to chemically distinguish between discrete molecular entities at the single-molecule level along with nanometer scale topographic imaging. The technique can be especially useful to analyze the components of heterogeneous samples and to resolve chemical information from the samples that can not be resolved from topographic images alone. PicoTREC has been demonstrated to be applicable to a variety of biological systems in their native environments (including antilysozyme-lysozyme, a variety of antihistone-chromatin interactions, and biotin-avidin).

7 REFERENCES

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