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Probing Membrane Proteins Using Atomic Force Microscopy

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Abstract To gain insights into how biological molecules function, advanced technologies enabling imaging, sensing, and actuating single molecules are required. The atomic force microscope (AFM) would be one of novel potential tools for these tasks. In this study, techniques and efforts using AFM to probe biomolecules are introduced and reviewed. The state-of-art techniques for characterizing specific single receptor using the functionalized AFM tip are discussed. An example of studying the angiotensin II type 1 (AT1) receptors expressed in sensory neuronal cells by AFM with a functionalized tip is given. Perspectives for identifying and characterizing specific individual membrane proteins using AFM in living cells are provided. Given that many diseases have their roots at the molecular scale and are best understood as a malfunctioning biological nanomachines, the prospects of these unique techniques in basic biomedical research or in clinical practice are beyond our imagination. J. Cell. Biochem. 97: 1191–1197, 2006. © 2006 Wiley-Liss, Inc.

Key words: single molecule recognition; AFM; tip functionalization; membrane protein; AT1 receptor; sensory neuron

As we enter into the post-genomic era, increased attentions have been directed to characterization of structure and function of biomolecules. Understanding the location, structure, and molecular dynamics of these molecules is of fundamental importance to elucidate their function. To gain insights into how these biomolecules operate, advanced technologies are required for gaining information at the level of single molecules. The atomic force microscope (AFM) would be one of such novel tools for the task. AFM was initially developed as an instrument mainly used for surface science research [Binning et al., 1986]. Research efforts in the past few years indicate that AFM is a potentially powerful tool for biochemical and biological research [Henderson, 1994]. Recent progress in the spatial resolution of AFM technology has made topographical image of single protein a routine work [Baker et al., 2000; Fotiadis et al., 2002]. The unique capability of AFM to directly observe single molecules in their native environments has provided insights into the interaction of proteins that form functionality assemblies. While recognition of individual protein such as specific cell membrane receptor is still a challenge, the technique to functionalize an AFM probe with specific molecules such as antibodies establishes a promising way to identify proteins in a specific manner. It has been shown that individual receptors can be identified by an AFM tip functionalized with antibodies through a force mapping technique [Ludwig et al., 1997; Willemsen et al., 1998] or directly from a phase image in tapping mode [Raab et al., 1999; Stroh et al., 2004]. These results, however, were obtained by imaging processed samples putting on substrate surfaces. In practice, it is still very difficult to image single receptors in their original biological environments, such as cell membranes due to the topographical interference and

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softness of the membrane. Fortunately, this problem has been solved by the use of recently developed lift-up interleave scan tapping phase imaging mode [Li et al., 2005].

In this study, past efforts to probe individual biomolecules using AFM are reviewed, followed by the introduction of the state-of-art techniques enabling recognition of single molecules using the functionalized AFM tip. An example of studying angiotensin II type 1 (AT1) receptors expressed in sensory neuronal cell membrane by AFM with the functionalized tip is given. Finally, perspectives for detecting individual cell membrane proteins using AFM in living cells are provided.

TIP FUNCTIONALIZATION AND SURFACE MODIFICATION

By immobilizing samples on a very flat surface such as mica, individual bio-molecules can be observed by AFM. For example, the major intrinsic proteins have been immobilized on a freshly cleaved mica surface by incubating with carboxypeptidase at room temperature overnight [Fotiadis et al., 2000]. The mica surface was silanzied in a solution of 2% 3-aminopropytrithoxysilane in toluene for 2 h [Ros et al., 1999] or by exposing it to the vapor of 3-aminopropytrithoxysilane for several minutes [Baker et al., 2000].

Functionalization of AFM tips by chemically and biologically coating with molecules, for example biotin–avidin pairs [Florin et al., 1994; Lee et al., 1994] and antigen–antibody pairs [Hinterdofer et al., 1996; Ros et al., 1998], has opened a new research area for studying interactions of molecules at the molecular level. Chemical coating of probes is mainly done by silanization or by functionalized with thiols and is often the first step before biological functionalization. Many protocols have been used for attaching proteins to an AFM tip. There are two main ways to functionalize the AFM tip with antibodies. One is to direct coat the antibody on a silanized tip, and the other is to tether the antibody on a tip by a linker. The direct coating method is simple and results in high lateral resolution. The tethering method involves much more complicated steps, but it gives better antigen recognition given that the interaction between antibody and antigen is highly specific which involves a high degree of spatial and orientational specificity. The drawback of the tethering method is that the lateral resolution is low. The detailed steps of these two methods are discussed below.

Several direct coating methods are available, and most of them have been based on silanizing a solid surface. Here is an example that has been used by us for directly attaching antibodies to an AFM tip. The silicon nitride tips were treated with 10% nitric acid solution which was left in a silicone bath for 20 min at 80°C. This causes the formation of surface hydroxyl groups on the SiN tips. The tips were then thoroughly rinsed with distilled water, placed into 2% APrMDEOS solution in toluene, and kept in a desiccator purged with argon gas for 5 h. This treatment provides reactive primary amine groups on the nitride surface. The tips were washed thoroughly with PBS and placed into a solution of 2 μg/ml Anti-AT1 IgG for 10 min. The antibody-conjugated tips were then washed thoroughly with PBS and distilled water to remove loosely attached antibodies. These tips should be used immediately before being dried.

Functionalization of the AFM tip with antibody using the tethering method involves much more steps than the direct coating method. It usually needs a spacer to covalently bind the protein in order to orient the protein to expose specific site(s) of the protein. Polyethyleneglycol (PEG) is a common spacer to be used. A terminal thiol group can be first attached to PEG, which then will be attached to a gold-coated silicon nitride tip. An amine group at the other end of the PEG molecule attaches proteins (e.g., antibodies) via a covalent bond [Hinterdofer et al., 1996].

Tip functionalization with antibody can be completed using the following steps as illustrated in Figure 1. The first step is to modify the antibody with *N*-Succinimidyl 3-(Acetylthio) propionate (SATP). The second step is to modify AFM tips with aminopropyltriethoxysilane (APTES). The third step is to tether the cross-linkers (spacer) on tips. The final step is to link the SATP-Labeled antibody to tips. The detailed protocol can be found in Reference [Stroh et al., 2004]. After tip functionalization, the tips can be stored in PBS buffer at 4°C for 2 weeks.

PROBING RECEPTOR–LIGAND INTERACTIONS

AFM is capable of measuring forces in the piconewton scale by nature, a property that has been exploited to examine receptor–ligand

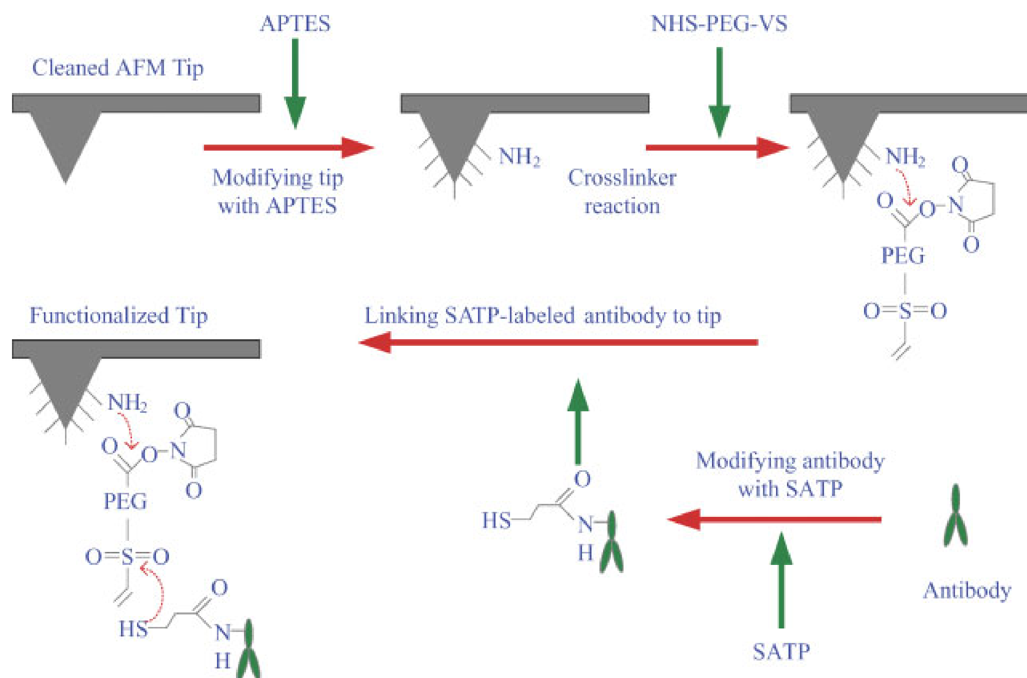

Fig. 1. The process of tip functionalization via a spacer. APTES, aminopropyltriethoxysilane; SATP, *N*-succinimidyl 3-(acetylthio)propionate; PEG, polyethylene glycol. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

interactions. An emerging body of literature employing AFM to measure and characterize these interactions is available. A wide range of receptor–ligand pairs has been studied by AFM with a functionalized tip. The first study focuses on very high affinity interactions, such as interactions between biotin and avidin [Florin et al., 1994; Lee et al., 1994; Moy et al., 1994]. In this study, a silicon nitride AFM tip was functionalized by avidin through the following steps: the tip was first cleaned and silanized, and then incubated in biotinylated bovine serum albumin for 24 h. After fixed in 1% glutaradehyde solution for 30 s, the avidin was added to bind to the biotin, and the AFM tip was functionalized with avidin. By immobilizing the biotin on an agarose bead, the rupture force between the biotin from the bead and the avidin from the tip was measured using the force modulation mode. When the tip was retracted, it detached from the surface in a series of discrete jumps with each corresponding to breakage of one or more biotin–avidin bindings. The total jump-off force was expected to consist of an integral multiple of single rupture force. Therefore, by constructing a histogram of rupture forces, the single pair unbinding force was measured. The rupture force has been calibrated from 160 pN for avidin–bioton pair to 260 pN for streptavidin–biotin pair [Moy et al., 1994]. As understanding and interpretation of these initial studies on the measurements of rupture forces by AFM have improved, more investigators reproduced and extended the avidin-biotin findings by determining the bond strength of other examples of receptor–ligand pairs. These studies of AFM binding have been given an extensive overview by Willemsen et al. [2000].

Antibody–antigen interaction is of importance in the immune system, which may vary considerably in affinity. Hinterdofer et al. [1996] were the first to determine the interaction between individual antibodies and antigens. In their work, they used flexible linkers to couple either the antigen or the antibody to the tip, which provided the antibody and antigen enough freedom to overcome problems of misorientation due to the high degree of spatial and orientational specificity between antibody and antigen. By coupling the antigens and antibodies to the tip and surface, respectively, via polyethylene glycol (PEG) spacer (8 nm in length), the binding probability has been significantly improved due to the large mobility of provided by the long spacer molecules.

When a tip is functionalized at very low antibody density such that only one single

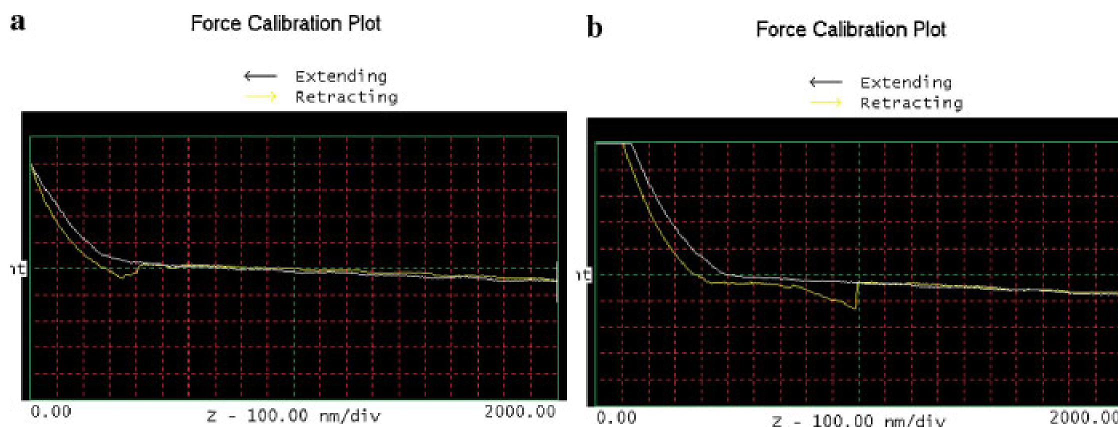

Fig. 2. Force-distance curve generated by force modulation mode AFM. The antibody is covalently bound to the tip. The antigen is fixed on cell membrane surface. **a**: There is no binding; (**b**) there is binding and it raptures during retracting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

antibody at the tip apex has chance to access an antigen on the surface, single molecular antibody–antibody complex could be examined. An example of rupture of such a complex is shown in Figure 2. The unbinding behavior can be monitored from the retrace curve at which the rupture occurs when the force suddenly changed as shown in Figure 2b. When there is no binding existing, the retracting is smooth as shown in Figure 2a.

SINGLE MOLECULE RECOGNITION

Although high-resolution images can provide some detailed conformal information of molecules, they may not provide information related to any specific protein. Because the interaction between ligands and receptors is highly specific and possesses a high degree of spatial and orientational specificity, the technique to functionalize an AFM tip with specific molecules make investigation of single-specific molecule possible. Rupture forces representing biomolecular specific interactions can also be exploited as a contrast parameter to create images in which the individual biomolecules can be recognized. It has been proven that single receptors can be recognized by an AFM tip functionalized with antibody through a force mapping technique [Ludwig et al., 1997; Willemsen et al., 1998]. In this study, the functionalized tip was raster-scanned over the surface while a force-distance curve was generated for every pixel. From the force distance curve, the surface parameters such as stiffness and adhesion force was extracted either by online in real time or by off-line analysis. The individual receptors were recognized through these so called adhesion mode AFM. However, the adhesion AFM image obtained by this method has low lateral resolution and the work is extremely time consuming.

Another way to recognize specific proteins like receptors is by the use of tapping-mode phase imaging. It can differentiate between areas with different properties regardless of their topographical nature [Cleveland et al., 1998; Tamayo and Garcia, 1998]. The phase angle is defined as the phase lag of the cantilever oscillation relative to the signal sent to the piezo driving the cantilever. Theoretical simulations and experiments of the cantilever dynamics in air have shown that phase contrast arises from differences in the energy dissipation between the tip and the sample. The phase shift is related analytically to the energy dissipated in the tip sample interaction by following equation [Cleveland et al., 1998, Tamayo and Garcia, 1998].

$$\sin\psi = \left(\frac{\omega}{\omega_0}\frac{A}{A_0}\right) + \frac{QE_D}{\pi kAA_0}$$

where ψ is the phase angle; ω/ω_0 is the working frequency/resonance frequency; A/A_0 is the set-point amplitude/free amplitude; Q is the quality factor; E_D is the energy dissipation; and k is the cantilever spring constant. The phase shift due to the tip–sample interaction, which involves energy dissipation, is the displacement of the non-contact solution to higher phase shifts and the intermittent contact solution to lower phase shift values. The more dissipative features will appear lighter in the non-contact regime, whereas they will appear darker in the intermittent-contact regime [James et al., 2001].

When scanning the proteins immobilized on mica surface using a tip functionalized with its antibody, the tip–sample interaction force will increase as the tip approaching to the AT1 receptor, thus a significant change of the phase shift will be generated. Since the topographical information is also convoluted to the phase contrast image but with low frequency, a band-pass filter can be used to remove the low frequency topographical information and the high frequency noise. After filtering the phase contrast image, only the receptors' image will be left on the surface. Individual surface receptor has been identified using these techniques [Raab et al., 1999; Stroh et al., 2004].

IN SITU PROBING MEMBRANE PROTEINS

Although individual proteins can be recognized efficiently through the tapping-mode phase imaging by a functionalized tip, biomolecules have to be extracted, purified, and attached to a flat and rigid surface using the methods described above. Detecting directly membrane proteins in their native environments is daunting given the cell membrane surface topographical information will interfere with the antigen–antibody binding which "buries" the signal and make the recognition impossible. Fortunately, these problems can be solved by an interleaved lift-up scanning recently developed to remove the interference from topography. By scanning the same line twice, the topographical image can be obtained from the first scan, and the phase image can be obtained from the second lift-up scan. By choosing a proper lift up height of the AFM tip, the second scan will only contain the force information of the antigen and antibody interaction. After combining the phase image with the topographical image, the individual receptors can be located and labeled on the membrane surface. Using this technique, the AT1 receptor has been successfully recognized from a fixed neuronal cell membrane surface [Li et al., 2005]. In this study, an AFM tip was biologically functionalized with the AT1 antibody by a tethering method as discussed previously. A phase contrast image of the neuronal cell membrane is shown in Figure 3 that was obtained using the functionalized tip and then

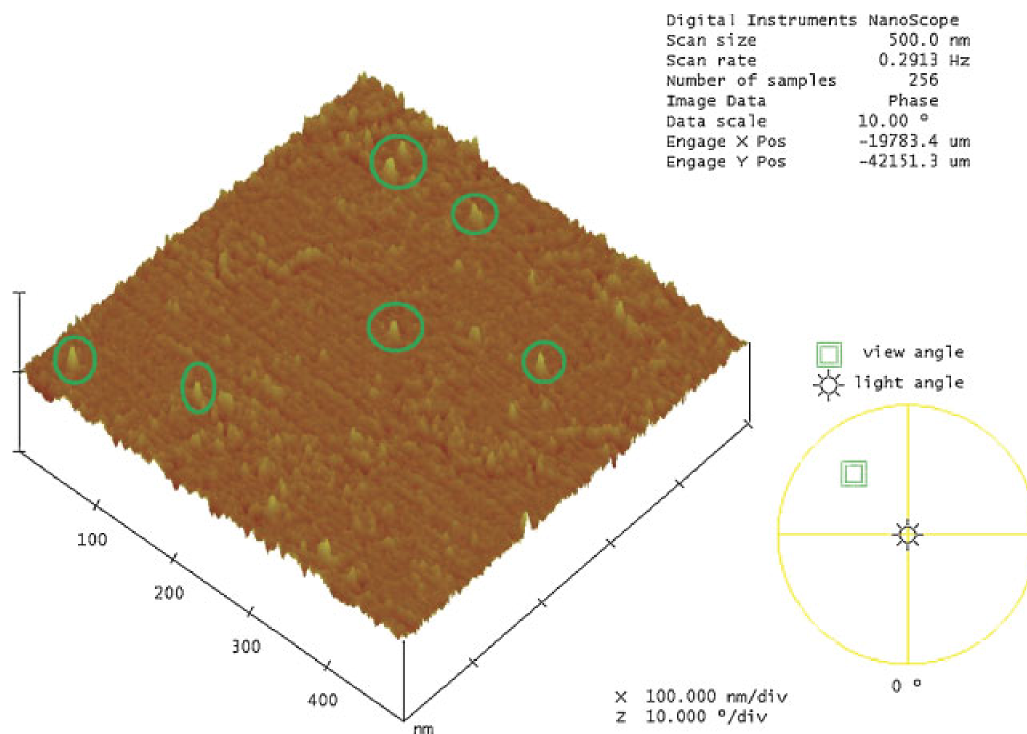

Fig. 3. 3-D view of a phase contrast imaging of cell membrane surface using tapping mode lift-up interleave AFM with scanning range of 500 nm. The image was obtained using a functionalized tip with AT1 antibody and then passed through a band-pass filter. Some AT1 receptors are clearly identified as shown in the image labeled by the circles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

processed with a band-pass filter. The experimental result shows that single biomolecule such as the AT1 receptor expressed in neuronal cell membrane can be recognized using the biologically functionalized tip through a lift-up interleaved tapping phase imaging mode AFM.

PERSPECTIVES AND CONCLUSIONS

Although high-resolution images can provide some detailed conformal information of molecules, it is essential to recognize and characterize individual molecules such as receptors embedded in cell membranes in living cells. However, studies of living cells using AFM with high resolution have been hampered by cell deformation and tip contamination [Putman et al., 1994]. Different approaches have been used to obtain high-resolution images of soft biological materials. At low temperature, cells stiffen and high-resolution imaging become feasible [Prater et al., 1991]. Cells also become stiff after chemical fixation [Butt et al., 1990]. These circumstances, however, can hardly be called physiological. To reduce cell surface deformation and cantilever contamination in "authentic" physiological conditions, the applied cantilever loading forces needs to be much smaller than what is currently available commercially, which remains a technical challenge. Another solution is to use TMAFM (tapping mode AFM) in liquid, which gives a substantial improvement in imaging quality and stability over standard contact mode. Because of viscoelastic properties of the plasma membrane, the cell may harden when responding to externally applied high frequency vibration and hence is less susceptible to deformation. Moreover, with this imaging mode, the cantilever oscillates at its resonant frequency and is only in intermittent contact with the cell surface. As a result, the destructive shear force is minimized.

In order to reach the resolution of the molecule level in living cells, more challenges are ahead of us. We are in the process to develop techniques to tackle these challenges. For example, we are using a micro-grid to mechanically immobilize the surface of living cells to solve the problem of softness of cells. The micro-grid may help to constrain the membrane and to prevent it from deformation caused by the tapping force from the AFM tip. The AFM tip can touch the cell membrane through the small openings on the micro-grid, provided that the openings are big enough and that grid thickness is thin enough to allow the AFM tip contacting the cell surface through the hole. The opening size may vary from 2 to 10 μm depending on the cell size. The grid thickness varies from 10 to 500 nm depending on the fabrication techniques and its mechanical limitation. Figure 4 shows microgrid fabricated in our laboratory with thickness of 150 nm containing 3, 5, and 10 μm holes on it.

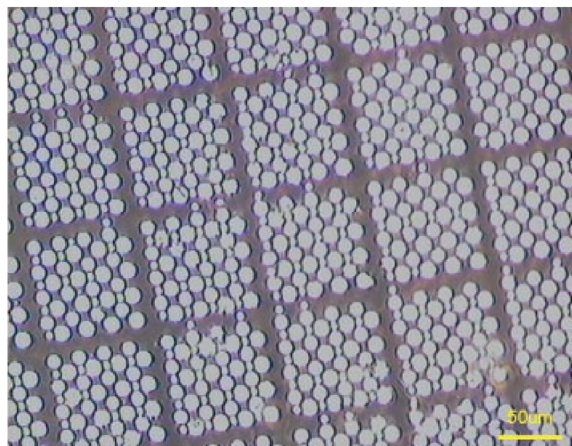

Fig. 4. Diamond microgrid with thickness of 150 nm containing 10, 5, and 3 μm holes on it. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In conclusion, the technique using a functionalized tip to measure the interaction force between ligands and receptors by AFM has been discussed for more than a decade, and single-molecule recognition using a functionalized tip from processed samples has been achieved. However, techniques for detecting and characterizing specific individual molecules from a living cell are still developing. Although barriers exist, they should be overcome in the very near future given the rapid advancement of nanotechnology. Once we have reached such breakthrough, further studies of membrane receptor functionalities including trafficking, signaling, and cross talk would be possible. Given that many diseases have their roots at the molecular scale and are best understood as a malfunctioning biological nanomachines, the prospects of these unique techniques in basic biomedical research or in clinical practice are only limited to our imagination.

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