

**PROBING LIGAND-RECEPTOR INTERACTIONS WITH ATOMIC FORCE  
MICROSCOPY**

by

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## I INTRODUCTION

In recent years new technology has been developed to directly measure the forces involved in protein-protein interactions. Previously, studies of ligand-receptor interactions usually involved biochemical methods of binding affinities or rate constants. Although this kind of measurement remains an essential part of protein interaction studies, it lacks information about the influence of internal and external forces that receptors associated with plasma membrane are constantly exposed to. For example, during cell migration adhesion receptors undergo cycles of adhesion and deadhesion, and lymphocytes attached to blood vessel walls are constantly being perturbed by blood flow currents. Now, however, the field has broadened to include measurements revealing the mechanical properties of biomolecules under applied force. These direct measurements can be used to determine the dynamic strength and characterize the changes in free energy (i.e., energy landscape) during breakage of the complex. Techniques that have been employed include the use of microneedles (Kishino and Yanagida 1988), optical tweezers (Ashkin et al. 1990; Svoboda et al. 1993), magnetic beads (Smith et al. 1992), the biomembrane force probe (BFP) (Evans et al. 1995) and atomic force microscopy (AFM) (Burnham and Colton 1989). Initial pioneering work include force measurements of actin filament (Kishino and Yanagida 1988), DNA (Perkins et al. 1994) and ligand-receptor systems (Florin et al. 1994). Although each of the different methods have their own merits, in this chapter we focus on the use of the AFM in measuring ligand-receptor interactions. Also

included in this review are detailed methods and experimental protocols that can be applied toward studying most protein-protein interactions.

The AFM was originally designed to be an imaging tool (Binnig et al. 1986). Modified from the design of the scanning tunneling microscope (STM), the AFM acquires topographic images by methodically scanning the specimen with a flexible cantilever that bends according to the contours of the surface. Atomic level resolution is acquired by translating the deflection of the cantilever into an image map of surface height differences. Since mapping of the surface can be conducted in both air and aqueous environments, imaging studies of biomolecules are possible under near physiological conditions, thus, enabling researchers to examine the subtle details of biological structures such as biomembranes (Zasadzinski et al. 1988), bacteriorhodopsin (Butt et al. 1990) and DNA (Hansma et al. 1992). For excellent reviews on AFM imaging see Heinz et al. (1999) or Engel et al. (1999).

The AFM can also be operated in the force scan mode in which its ultra sensitivity can be used to measure interactions between two apposing surfaces down to the single molecule level. In studies of ligand-receptor forces, the ligand is immobilized on the surface of a flexible AFM cantilever while the receptor is attached to a suitable substrate. The interaction force is acquired from the deflection of the cantilever during the approach and withdrawal of the cantilever from the substrate. Using this method Lee et al (1994a) were able to directly measure the unbinding force of a single ligand-receptor interaction. This novel application led to the use of the AFM as an ultra-sensitive force transducer for probing biomolecular interactions. A partial list of the

unbinding forces of measured ligand-receptor pairs is given in Table 1. In recent years, this non-imaging AFM technique (often referred to as force spectroscopy) has also been employed to study the unfolding of individual proteins (Rief et al. 1997a; Marszalek et al. 1999; Oesterhelt et al. 2000).

The AFM force measurements of ligand-receptor interactions can be interpreted within the theoretical framework proposed by Bell (Bell, 1978). In this model the application of a mechanical force to a ligand-receptor bond is predicted to reduce the activation energy and hence accelerate the dissociation of the bond. Moreover, the unbinding force should increase with the logarithm of the rate at which an external mechanical force is applied toward unbinding of adhesion complexes (i.e. loading rate). Confirming the Bell model, recent studies using the BFP and the AFM have shown that increases in loading rate cause an increase in rupture force between individual complexes of streptavidin/biotin (Merkel et al. 1999; Yuan et al. 2000). The same relationship has been shown for fluorescein and its different antibodies (Schwesinger et al. 2000), concanavalin A (Con A)/D-mannose (Chen and Moy 2000), cadherins (Baumgartner et al. 2000), and complementary DNA strands (Lee et al. 1994b; Strunz et al. 1999).

In this review, we discuss ligand-receptor interaction studies and begin by first introducing AFM instrumentation, including a list of potential commercial suppliers and a protocol for calibrating the AFM cantilever. Next, we provide experimental protocols for measuring ligand-receptor interactions by probing receptor protein immobilized on agarose beads or on the surface of living cells. Finally, we conclude by describing exciting advances that

have been made in AFM force spectroscopy techniques and discuss future directions for the field.

## **II. AFM INSTRUMENTATION**

Before discussing the basic mechanisms of the AFM let us first examine the steps of a simple force scan measurement. At the start of an experiment a flexible cantilever, coated with the ligand of interest, is brought in contact with receptor protein attached to an opposing substrate. The cantilever is then subsequently withdrawn until the final bond separates. Throughout the measurement changes in cantilever deflection are detected by a 2-segment photodiode that senses alterations in the reflective path of a laser spot focused on the back of the cantilever. The signals from the photodiode are then amplified and sent to a computer for analysis. Thus, the essential components for an AFM include a piezoelectric translator that displaces the cantilever, a laser and photodiode to detect changes in cantilever deflection and a computer to control movement of the cantilever as well as to process and record data.

To carry out the above procedures we use a home-made AFM (Fig. 1) in our laboratory, as modification of the standard AFM design used for imaging can improve the quality of the signal acquired in AFM force measurements. For instance, we have found that uncoupling the mechanism for lateral and vertical scans reduced mechanical and electrical noise and improved the sensitivity of the instrument. In our apparatus, the vertical movement of the cantilever is controlled by mounting it on a piezoelectric translator (Physik Instrumente, model P-821.10)

which expands or contracts depending on the applied voltage. By changing the voltage across the piezo, the protein-coated cantilever and ligand can be brought into binding contact. The deflection of the cantilever is monitored by focusing a laser beam from a 3mW diode laser (Oz Optics; em. 680 nm) on the upper surface of the cantilever. Changes in the reflection path of the laser are monitored by a 2-segment photodiode (UDT Sensors; model SPOT-2D). The differential signal from the photodiode segments is then digitized by a data acquisition system equipped with an 18-bit optically isolated analog-to-digital converter (Instrutech Corp., Port Washington, NY). Control of the piezoelectric translator and timing of the measurements is done through custom software. Further reduction of mechanical vibration and temperature fluctuation is achieved by suspending the entire apparatus by bungee cords inside of a large evacuated refrigerator. The detection limit of our AFM system is in the range of 20 piconewtons (pN). A piconewton sensitive commercial AFM with necessary software is readily available from Asylum Research (Molecular Force Probe; Santa Barbara, CA) and is specially designed to carry out force measurements similar to those carried out in our laboratory. A listing of other AFM manufacturers is given below for the interested reader.

**A. Manufacturers of AFM instrumentation.**

The following is a partial list of AFM manufacturers.

Burleigh Instruments Inc., New York, USA.

<http://www.burleigh.com/Pages/surface.htm>

Digital Instruments, California, USA.

<http://www.di.com/>

Molecular Imaging Corporation, Arizona, USA.

<http://www.molec.com/index.html>

OMICRON, GmbH, Germany.

<http://www.omicron-instruments.com/>

ThermoMicroscopes/Park Scientific Instruments, California, USA.

<http://www.thermomico.com/>

TopoMetrix, California, USA.

<http://www.topometrix.com/>

WITec GmbH, Germany.

<http://www.WITec.de/>

**B. Cantilever Calibration**

In order to translate the deflection of the cantilever,  $x$ , to units of force,  $F$ , it is necessary to determine the spring constant of the cantilever,  $k_c$  (i.e.,  $F = k_c x$ ). There are several techniques for calibrating tips and theoretical methods that provide an approximation of  $k_c$  (Sader 1995). Determination of  $k_c$  using empirical methods involves taking measurements of

cantilever deflection with application of a constant known force (Senden and Ducker 1994) or measurements of the cantilever's resonant frequency (Hutter and Bechhoefer 1993). The method we use for calibrating cantilevers is based on Hutter and Bechhoefer (1993) and is briefly outlined below.

For ligand-receptor force measurements we use triangle-shaped unsharpened gold-coated silicon-nitride cantilever tips that have spring constants ranging from 10 mN/m to 50 mN/m. The cantilever tip can be treated as a simple harmonic oscillator whose power spectrum of thermal fluctuation can be used to derive the spring constant. In brief, the cantilever is raised several microns from the surface and its natural frequency of vibration (resonant frequency) is monitored for 2-3 seconds. Since each vibration mode of the cantilever receives the thermal energy commensurated to one degree of freedom,  $k_B T / 2$ , the measured variance of the deflection  $\langle x^2 \rangle$  can be used to calculate the spring constant (i.e.,  $\frac{1}{2} k_B T = \frac{1}{2} k_C \langle x^2 \rangle$ , where  $k_B$  and  $T$  are Boltzmann's constant and temperature, respectively). To separate deflections belonging to the basic (and predominant) mode of vibration from other deflections or noise in the recording system, the power spectral density of the temperature-induced deflection is determined, and only the spectral component corresponding to the basal mode of vibration is used to estimate the spring constant. Using this approach, the spring constants of cantilevers can be calibrated in either air or solution. The calculated spring constant  $k_C$  can then be used to calculate, rupture force,  $F$ , by  $F = k_C \Delta V$ , where  $\Delta V$  is the change in voltage detected by the photodiode just

prior to and immediately after the rupture event and  $C$  is a calibration constant that relates deflection and photodiode voltage.  $C$  is determined from the deflection of the cantilever pressed against a rigid surface, such as the bottom of a plastic petri dish.

### **III. AFM APPLICATIONS FOR LIGAND-RECEPTOR INTERACTION STUDIES**

The AFM can be a powerful tool to determine dynamic strength of ligand-receptor bonds. There are certain issues one must consider, however, when acquiring direct force measurements. First, appropriate measures must be taken to identify and/or eliminate any possible non-specific binding forces. Second, the strength of a ligand-receptor bond is affected by the applied loading rate of the measurement. In other words, the binding properties can be influenced by the speed at which ligand and receptor are separated and the elasticity of the surfaces to which the proteins are anchored. In the following paragraphs we go into greater detail about these two points and then follow with protocols for carrying out AFM force measurements on agarose beads and cells.

Since the non-specific forces involved in the tip-sample interaction are typically electrostatic in origin, simple measures can be taken to reduce non-specific binding. Minimization of these forces can be accomplished by carrying out experiments in ionic solutions, such as phosphate buffered saline (PBS), that shield charges on the surfaces being brought into contact. Adsorbing receptor proteins directly on surfaces that are intrinsically charged (e.g., glass has a layer of negative charges on its surface) should also be avoided. Instead uncharged substrates like agarose and dextran can be used to support the proteins. The addition of 0.1

mg/ml bovine serum albumin (BSA) to the sample reservoir may also help reduce non-specific interactions.

Another concern when acquiring AFM force measurements is the fact that the rate at which an external force is applied, i.e. the loading rate of the measurement, can influence the binding properties of the ligand-receptor interaction. At higher loading rates the rupture force is expected to be higher than at lower loading rates. Since loading rate is dependent on the speed at which the ligand and receptor are separated and the elasticity of the surfaces to which both ligand and receptor are attached, it is important to note the cantilever retract speed and support parameters (i.e surface elasticity) when comparing the rupture force of different ligand receptor pairs.

The following sections present experimental protocols for carrying out AFM force measurements of ligand-receptor interactions. In brief, the experiments use an AFM cantilever tip coated with the ligand or receptor of interest while its binding partner is immobilized on another surface. The interaction between the two functionalized surfaces is acquired from the deflection of the cantilever during the approach and separation of the surfaces. Ligand-receptor interaction is derived from the adhesive force between the surfaces.

#### **A. Protocol I: Cantilever Functionalization**

The ligand must be immobilized on the AFM tip in order to acquire direct force measurements of ligand-receptor pairs. The techniques commonly used involve either chemi-

adsorption (Moy et al. 1994) or covalent coupling of the ligand to the tip via an extended linker (Hinterdorfer et al. 1996). The linker between the tip and the ligand lends greater mobility and access to receptors on the surface being probed. The following outlines a method for functionalizing tips with streptavidin (Fig. 2). This method is advantageous since the streptavidin/biotin system has been well-characterized, is high-affinity, and the initial layer of biotin-BSA may help to mask any electrical charges on the cantilever tip that could lead to nonspecific binding.

Materials: AFM cantilevers (MLCT-AUHW; Thermomicroscopes, Sunnyvale, CA), biotin-BSA (A-6043; Sigma, St. Louis, MO), streptavidin (Sigma).

1. Soak cantilever for 5 min. in acetone and then UV irradiate for 15 min.
2. Incubate cantilever in a 50  $\mu$ l drop of biotin-BSA (0.5 mg/ml in 0.1 M sodium bicarbonate, pH 8.3) overnight at 37°C in a humidified incubator.
3. Wash cantilever 3x in PBS (pH 7.4) to remove unbound protein. (NOTE: At this point cantilevers can be stored in PBS at 4°C for up to a week)
4. Incubate cantilever in a 50  $\mu$ l drop of streptavidin (0.5 mg/ml in 0.01 M PBS, pH 7.4) for 10 min. at room temperature.
5. Wash cantilever 3x in PBS before use.

6. Biotinylated ligand may be coupled to the streptavidin-functionalized tip at this step (e.g., biotinylated concanavalin A; 0.5 mg/ml in PBS, 10 min incubation at room temperature).

Note:

1. It is important that biotin-BSA adsorption takes place at pH 8.3 or higher, as the basic conditions seem to facilitate BSA adsorption to the cantilever.
2. Kits for biotinylating the ligand of interest are readily available from Pierce Chemical Company.

### **B. Protocol III: AFM Measurement of ligand immobilized on agarose beads**

The following paragraphs discuss methods for acquiring force measurements of the streptavidin-biotin interaction. Numerous methods exist for immobilizing ligand to a variety of different substrates. Some commonly used ligands for affinity chromatography (e.g., biotin or D-mannose) can be found already attached to agarose beads from vendors such as Sigma (St. Louis, MO) and Pierce (Rockford, IL). As a substrate for the force measurements, the agarose beads provide several attractive features. The agarose matrix has low affinity for most proteins, keeping non-specific interaction at a minimum. Proteins can be readily coupled to activated agarose beads. Moreover, the elastic agarose substrate will conform to the shape of the

cantilever tip, thus increasing the contact surface area and creating a higher probability for receptor-ligand interactions (Moy et al. 1994). Ligand-receptor binding is also greatly enhanced by attaching receptors to molecular tethers (e.g., dextran and polyethyleneglycol) that allow for a wider range of lateral motion (Hinterdorfer et al. 1996; Rief et al. 1997b). In addition, tethers can provide latitude for proper reorientation of the molecule during stretching so that the external force being applied to the molecule is perpendicular to the surface.

The following is a protocol that we use for the preparation of biotinylated agarose beads for streptavidin-biotin rupture force measurements.

Materials: 35mm plastic petri dishes, streptavidin (Sigma), PBS (pH7.4), biotinylated agarose beads (Sigma).

1. Prepare streptavidin-coated dishes for immobilizing biotinylated agarose beads:
  - Place a 100  $\mu$ l drop of streptavidin (0.05 mg/ml in sodium bicarbonate, pH 9.6) on the bottom of a 35 mm plastic petri dish.
  - Incubate overnight at 37°C in a humidified incubator.
  - Rinse the dish 3x in PBS just before adding beads.
2. Add 100  $\mu$ l of biotinylated agarose beads to 1.5 ml PBS.
3. Centrifuge beads at 10,000 g for 10 seconds and remove supernatant.
4. Repeat wash 2x.
5. After removing supernatant from last wash, resuspend beads in 0.01% BSA in PBS.

6. Add 100  $\mu\text{l}$  of washed beads to the streptavidin-coated plate. Beads should adhere to the dish almost immediately.

Fig. 3 presents a representative AFM force measurement acquired using a streptavidin functionalized AFM cantilever and a biotinylated agarose bead. The measurement consisted of an approach trace and a retract trace. During the approach trace, expansion of the piezoelectric translator lowered the cantilever onto the agarose bead and pressed the AFM tip into the elastic bead. Surface contact is registered by an upward deflection of the cantilever and allowed for the formation of streptavidin-biotin complexes. The number of complexes formed depends on the area of surface contact, which can be estimated from the force exerted by the cantilever and the elasticity of the agarose bead. An applied force of one nanonewton frequently resulted in the formation of several complexes. The forced unbinding of these complexes is recorded in the retract trace. The sawtooth shape of the retract trace revealed that the unbinding the complexes does not necessarily occur simultaneously. The retract trace showed multiple transitions in force that are attributed to the breakage of one or more streptavidin-biotin bonds. The interaction between the AFM tip and the substrate can be reduced to a single streptavidin-biotin linkage by the addition of either soluble biotin or streptavidin. Under these conditions, the force transition at surface separation corresponds to the rupture force of the single streptavidin-biotin complex. Control experiments to verify the specificity of the streptavidin-biotin interaction were carried out with the addition of either free streptavidin or free biotin.

**C. Protocol IV: AFM measurements on living cells**

The surface of a living cell is far more complicated than that of a bead or mica coated with protein. Furthermore, the low density of receptors on the cell surface and non-specific interactions introduce additional challenges. Lehenkari and Horton (1999) were able to measure RGD-integrin binding on the surface of osteoblasts and osteoclasts, and recently, we measured the binding force between Concanavalin A and its receptor on the surface of NIH-3T3 fibroblasts (Chen and Moy 2000). A brief generalizable protocol used to perform this work is provided following.

The AFM tip was functionalized with biotinylated concanavalin A (Con A) (C2272, Sigma) prior to the measurement (see IIIA). Measurements were carried out at room temperature in glucose-free RPMI media supplemented with 0.01% BSA and 0.01 mM  $MnCl_2$ . Glucose was eliminated from the culture medium to prevent potential competitive binding with the Con A-functionalized tip and Con A receptors on the cell. BSA was added to reduce non-specific binding as well as to promote adhesion of cultured NIH-3T3 fibroblast to the bottom of an uncoated plastic tissue culture dish. Measurements were carried out on both unfixed cells and cells lightly fixed with glutaraldehyde to determine if cross-linking of Con A receptors would have an effect on receptor unbinding strength. Compared to measurements on agarose beads and fixed cells, unfixed cells had much longer regions of stretch before final separation between the

tip and membrane (compare Fig. 4A with Fig. 3 and 4B). Typical distances for unfixed cells spanned 500 nm. Thus, Con A receptors seemed to be anchored to cell membrane tethers that stretched as the receptor was pulled. Rupture force measurements revealed a stronger rupture force for chemically-fixed cells ( $173 \pm 6.1$  pN) compared to unfixed cells ( $86 \pm 2.6$  pN) (Fig. 4C & 4D). Moreover, differences in cell elasticity were readily apparent from the slope of the retract trace as the tip pulled on the surface of the cell. Force histograms revealed multiple quantal peaks that were absent in the unfixed cell histograms (Fig. 4D) suggesting that much of the increase in rupture force was due to a shift toward cooperative binding of cells.

Using this method it is possible to acquire direct measurements of adhesion down to the level of single molecule pairs, unlike earlier cell adhesion assay studies and biochemical methods that relied on indirect measurements and could only access receptor group dynamics. Moreover, since the AFM is capable of both applying and detecting minute force changes on a cell or protein, simultaneous measurements of adhesion force and cell elasticity are possible at the cell surface.

#### **IV. FUTURE DIRECTIONS**

Recent experiments in affinity imaging and cell-cell adhesion point to promising future directions in the studies of ligand-receptor interaction by AFM. Affinity imaging combines the force measurements of ligand-receptor interaction with the imaging function of AFM. When the cantilever tip is coated with a specific receptor or ligand, AFM can provide an adhesion map

detailing the density of the binding partner on a surface (Ludwig et al. 1997). Affinity images of antigen immobilized on a substrate were obtained using antibody-functionalized cantilevers (Willemsen et al. 1998; Raab et al. 1999). This technique has been extended to soft cellular surfaces. Based on the specific interaction between *Helix pomatia* lectin and Group A red blood cell plasma membrane proteins, Grandbois et al. (2000) were able to discriminate Group A red blood cells from Group O red blood cells.

AFM has also been adapted for studies in cell-cell adhesion (Razatos et al. 1998; Benoit et al. 2000). These experiments differed from those discussed earlier in that a cell is attached to the end of the cantilever and used as a probe in cell adhesion studies. This approach allowed for both the ligand and receptor to be studied under conditions close to the native environment. Potential applications of this approach include the study of modulated adhesion following cell activation.

In the previous sections, we outlined several experimental approaches of AFM. Although AFM is still being improved to enhance sensitivity (Viani et al. 2000), the major challenge for AFM research may stem from its restricted accessibility to a limited number of laboratories. With the near completion of the genome project, many protein sequences have been revealed, however, their biophysical properties and functions still need to be elucidated. The AFM will be an important tool in this research.



**Table 1: Summary of reported unbinding force of ligand-receptor bonds**

Ligand-receptor pair	unbinding force (pN)	Loading rate (pN/s)	references
Streptavidin – biotin	120-300	100-5000	Yuan et al. 2000
	5-170	0.05-60,000	Merkel et al. 1999
	200	N/A	Wong et al. 1998
Avidin-biotin	115-170	100-5000	Yuan et al. 2000
	5-170	0.05-60,000	Merkel et al. 1999
W120F <sup>1</sup> -biotin	90-170	100-5000	Yuan et al. 2000
P-selectin-Glycoprotein ligand-1	115-165	*	Fritz et al. 1998
VE-cadherin-FC pair	35-55	*	Baumgartner et al. 2000
DNP-hapten <sup>2</sup> -antibody (ANO2)	60±30	*	Heymann et al. 1999
Cell adhesion proteoglycans	40±15	N/A	Dammer et al. 1995
Meromyosin-actin	15-25	N/A	Nakajima et al. 1997
Ferritin-antibody	49	N/A	Allen et al. 1997
Human serum albumin-antibody	244	N/A	Hinterdorfer et al. 1996

<sup>1</sup>W120F: a streptavidin mutant in which tryptophan 120 was replaced by a phenylalanine.

<sup>2</sup>DNP-hapten: spin-labeled dinitrophenyl hapten.

\*: Authors studied unbinding force vs. different pulling velocities, we were unable to convert the velocity to loading rates.

**FIGURE LEGENDS**

**Fig. 1** Schematic of AFM.

**Fig. 2** Schematics for the functionalization of AFM tips with streptavidin.

**Fig. 3** Force vs. displacement curves of the interaction between a streptavidin-functionalized tip and a biotinylated agarose bead. The measurement recorded the force on the AFM cantilever on approach and retraction of the cantilever from the agarose bead. The inserts illustrate the deflection of the cantilever at different points during the measurement.  $f$  is the rupture force.

**Fig. 4** Force versus extension curves acquired from Con A-functionalized AFM tips interacting with Con A receptors on the surface of NIH-3T3 cells that were (A) not fixed and (B) fixed with glutaraldehyde. Histograms of rupture force between Con A-functionalized AFM tips and Con A receptors on (C) untreated cells and (D) glutaraldehyde-fixed cells. Arrows in D indicate quantized peaks at 80 pN, 160 pN, and 240 pN following fixation of cells in glutaraldehyde.

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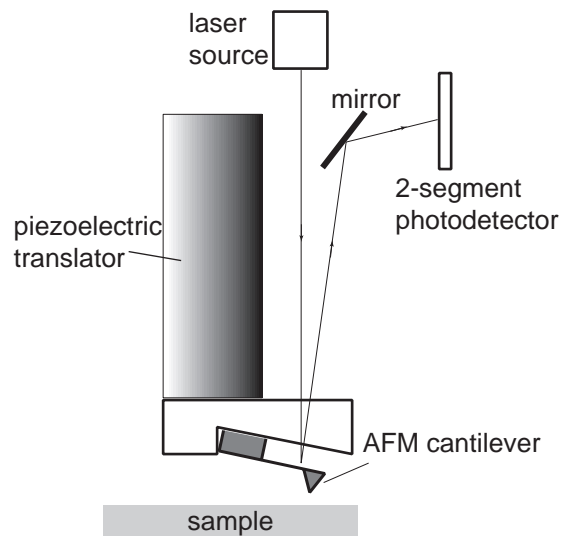


Figure 1  
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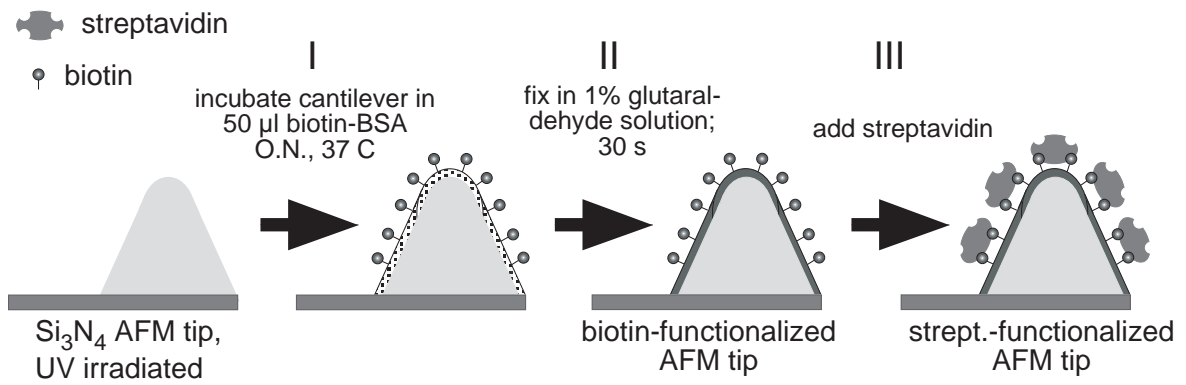


Figure 2  
Zhang et al. 2000

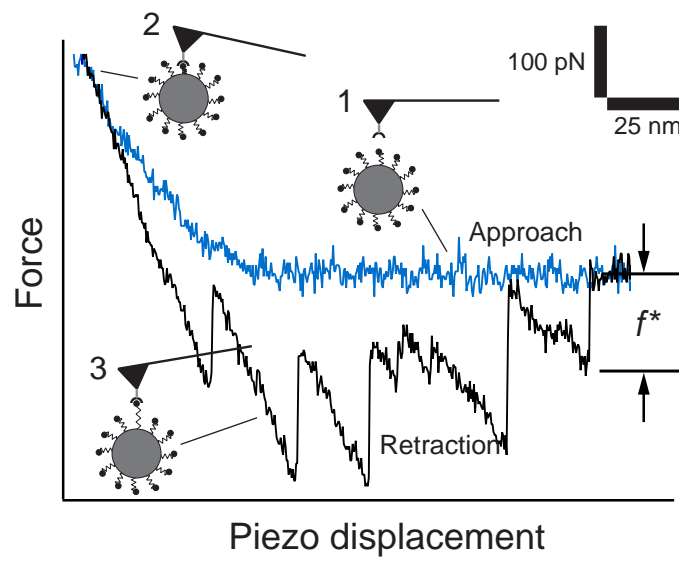


Figure 3  
Zhang et al., 2000

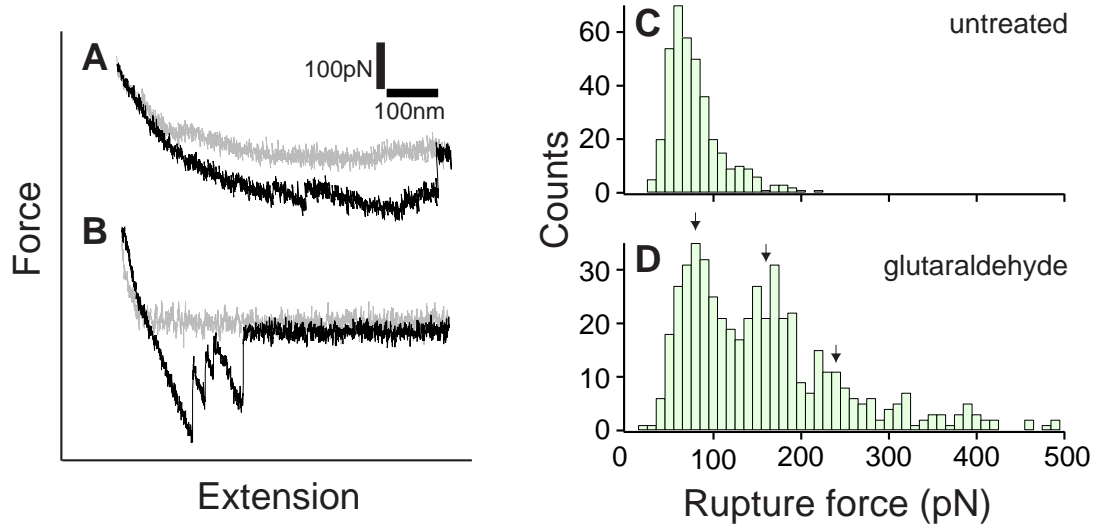


Figure 4  
Zhang et al., 2000