

## Basic Principles of Molecular Recognition Force Microscopy

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In molecular recognition force microscopy (MRFM), ligands are covalently attached to atomic force microscopy tips for the molecular recognition of their cognitive receptors on probe surfaces. Interaction forces between single receptor-ligand pairs are measured in force-distance cycles. Force spectroscopy (FS) varies the kinetics of these experiment is to yield further structural and dynamic information of the recognition process. By combining dynamic force microscopy (DFM) with recognition force spectroscopy the lateral position of receptors on surfaces is detected with nm resolution. A ligand-containing tip is oscillated along a surface with a few nm amplitude and antigenic positions are detected from the reduction of the oscillation amplitude upon ligand binding.

### 1. Introduction

This method is specifically designed for the need in bioscience to identify imaged structures with particular biomolecules, and to study biomolecular recognition at the surface of bioprobes, for instance of a ligand by a membrane receptor. For this, ligands are bound to AFM tips which are specifically recognized by the receptor molecule to be mapped or studied. So far applications were to isolated biomolecules on substrates, including measurements of recognition forces between biotin-avidin (Lee et al., 1994; Florin et al., 1994; Wong et al., 1998), complementary nucleotides (Boland et al., 1995), cell adhesion proteins (Fritz et al., 1998), and antibody-antigen recognition (Hinterdorfer et al., 1996; Dammer et al., 1996; Allen et al., 1997; Hinterdorfer et al., 1998; Willemsen et al., 1998; Ros et al., 1998; Raab et al., 1999). First examples of molecular recognition force microscopy on living cells was recently published, where the interaction forces between ligands on the AFM tip and integrin receptors in osteoclasts were measured (Lehenkari et al., 1999) and binding of antibodies and ligands to membrane-transporters were studied (Wielert-Badt et al., 2002).

Force spectroscopy experiments probe the molecular dynamics of ligand-receptor binding by varying the kinetics. This renders estimation of affinity, rate constants, and energy barriers as well as the bond width of the binding pocket possible (Hinterdorfer et al., 1996, Evans and Ritchie, 1997, Merkel et al., 1999, Schwesinger et al., 2000, Strunz et al., 2000). It also allows detection of association and different functional and conformational states of proteins. Besides studying ligand-receptor recognition processes itself, the localization of receptor binding sites by molecular recognition of a ligand is of particular interest. Simultaneous information for topography and ligand-receptor interaction is obtained by recognition imaging (Raab et al., 1999), where receptor sites are localized with nanometer positional accuracy. This opens new perspectives for nanometer-scale epitope mapping of bio-molecules and localizing receptor sites during biological processes.

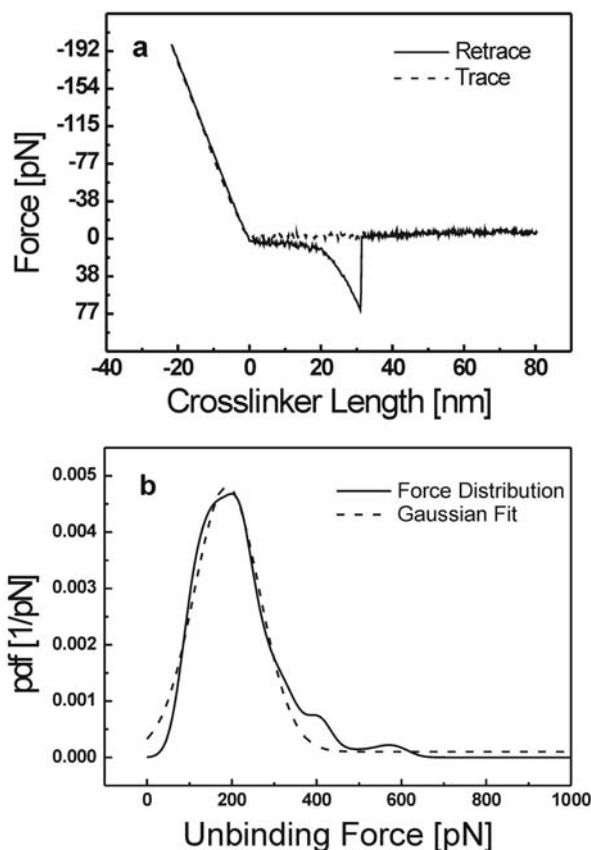
### 2. Basic Principles

For AFM molecular recognition experiments the bio-molecules are to be tightly attached to the AFM tip. We use an anchoring strategy by coupling ligands covalently with free thiols to tips (Hinterdorfer et al., 1996, 1998) via a flexible and distensible crosslinker (Haselgrübler et al., 1995). In this way, the site-directed coupled ligand can freely diffuse about the tip for unconstrained receptor-recognition. The crosslinker is a heterobifunctional PEG derivative of 18 units corresponding to 8 nm extended length. The surface density of ligands on tips is adjusted to a sufficiently dilute value ( $500/\mu\text{m}^2$ ) so that only one ligand on the AFM tip has access to receptors on the surface, which is suited for the detection of single molecule recognition events. The PEG crosslinker used for spacing the ligand several nanometers away

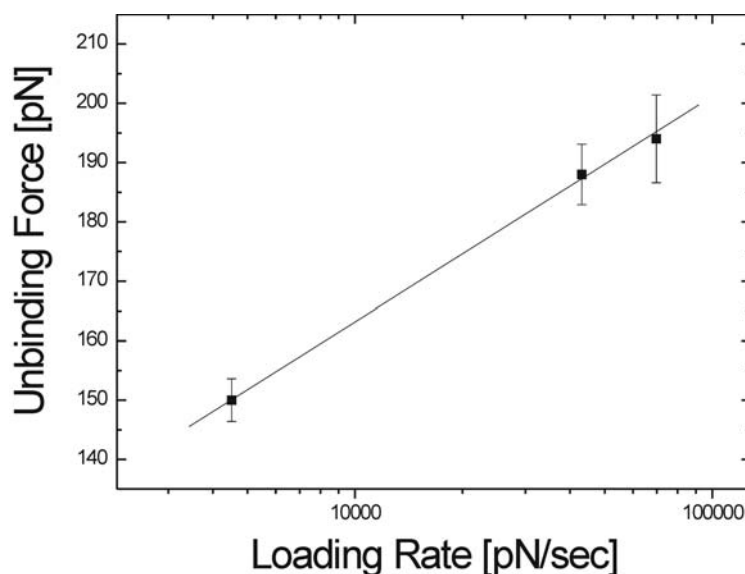
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from the AFM tip is a watersoluble, nontoxic, and nonadhesive polymer. Therefore, nonspecific tip-probe interactions are practically absent.

Single molecule ligand-receptor recognition events are measured in force-distance cycles as shown (Fig. 1a). At a fixed lateral position, a cantilever carrying a ligand is approached towards a probe surface to which receptors are attached, and subsequently retracted. The cantilever deflection, which directly relates to the tip-probe force according to Hook's law, is measured in dependence on the tip-surface separation  $\Delta z$ . During tip-surface approach (trace, dotted line) the cantilever deflection first remains zero because there is no detectable tip-surface interaction. Upon tip-surface contact ( $\Delta z = 0$  nm) a repulsive force develops that increases the more the tip is pushed into the surface. When the tip is subsequently retracted (retrace, solid line) the repulsive force decreases again. Since the ligand on the tip has bound to a receptor on the probe surface an attractive force develops (unbinding event) in the retrace ( $\Delta z = 0 - 21$  nm), which increases with increasing tip-surface separation. Its shape is determined by the elastic properties of the flexible PEG crosslinker (Kienberger et al., 2000a) and shows a nonlinear, parabolic-like characteristics. The physical connection between tip and surface connection sustains the increasing force until the ligand-receptor complex dissociates at a certain critical force (unbinding force), and the cantilever jumps finally back to the resting position (at  $\Delta z = 21$  nm).



**Fig. 1. (a) Single molecule recognition event.** Raw data from a force-distance cycle with 100 nm z-amplitude at 0.9 Hz measured in PBS. The attractive force signal developing in the retrace from 0 to 21 nm reflects single molecule recognition of a receptor on a surface by a ligand on the tip. **(b) Distribution of unbinding forces.** An empirical probability density function (pdf, solid line) was constructed from about 150 values of unbinding forces obtained in force-distance cycles. Data were fitted with a Gaussian function (dotted line). The maximum is at 150 pN.



**Fig. 2. Unbinding force  $f_u$  in dependence of the loading rate.** The maxima of the probability density functions (pdf) shown in Figure 4 are plotted on a half logarithmic scale at their different loading rates  $df/dt$ . The error bars are calculated from  $\sigma_U/n^{1/2}$ , with  $\sigma_U$  being the width of the force distribution and  $n$  the number of unbinding events per pdf.

### 3. Unbinding Force Distribution

For the quantification of the unbinding force, some ten force-distance cycles are usually recorded. Distributions of unbinding forces (Fig. 1b) are obtained by constructing empirical probability density functions from unbinding force measures (Hintendorfer et al., 1996, Baumgartner et al., 2000b). Single Gaussian functions of unitary area are calculated from the mean and variances of every value of the unbinding force. The Gaussians are added up and finally normalized, yielding the empirical probability density function. The advantage of this representation over simple histograms is that the data are weighted by their accuracy and, thus, yield a better resolution. Values of unbinding forces give a Gaussian-like distribution (Fig. 3b). For a typical example with one peak shown here (Kienberger et al., 2000b), the maximum is  $f \pm \sigma_u = 150 \pm 38$  pN (mean  $\pm$  SD). The uncertainty in determining  $f_u$  values, given by the thermal noise of the cantilever, was  $\sigma_0 \sim 10$  pN for the cantilever used. Therefore, unbinding forces were detectable at a signal to noise ratio of  $f/\sigma_0 = 15$ .

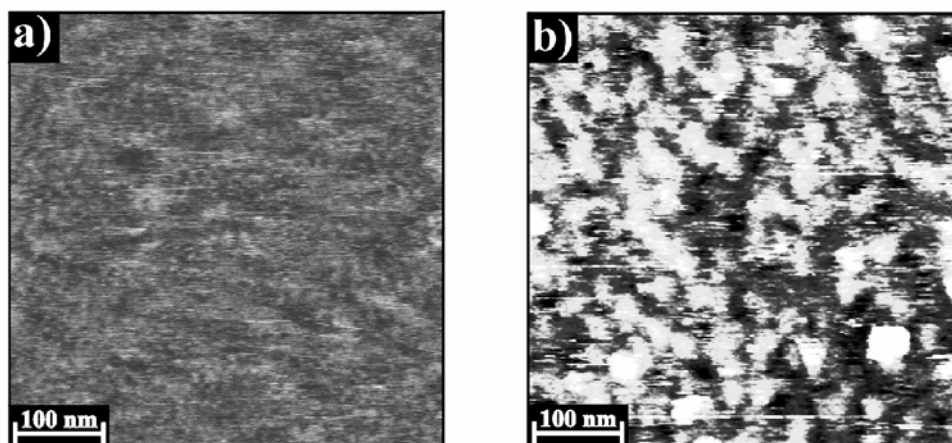
### 4. Force Spectroscopy

Unbinding forces of ligand-receptor bonds were found to assume not a unitary value but are dependent on the kinetics of the experiment (Evans and Ritchie, 1997, Fritz et al., 1998, Strunz et al., 1999, Baumgartner et al. 2000a, Kienberger et al., 2000b, Schwesinger et al., 2000). A force applied to a binding complex reduces its lifetime. During a force-distance cycle the force increases until the complex dissociates at force  $f$ . Therefore, the  $f$  values are dependent on the rate of force increase,  $df/dt =$  vertical scan velocity times spring constant. The unbinding force of ligand-receptor interaction was measured for different loading rates (Fig. 2) (Kienberger et al., 2000b) and rises linear on the half-logarithmic scale, which is characteristic for a single energy barrier in the thermally activated regime (Merkel et al., 1999, Strunz et al, 2000).

The input of the mechanical energy during pulling enhances the probability of ligand-receptor bond dissociation. An effective lifetime  $\tau(f)$  of the bond under an applied force  $f$  can be estimated by the time the cantilever spends in the force window spanned by the standard deviation  $\sigma_U$  of the  $f$  distribution. The time the force increases from  $f - \sigma_U$  to  $f + \sigma_U$  is then given by  $\tau(f) \approx 2\sigma_U/df/dt$  (Hinterdorfer et al., 1996). Bond lifetimes at zero force  $\tau(f)$  can be calculated using a Boltzmann ansatz (Bell, 1978), which yields the exponential lifetime-force relation  $\tau(f) = \tau(0) \cdot \exp(-l_r \cdot f/k_B \cdot T)$  for the reduction of the lifetime  $\tau(f)$  by the applied force  $f$  (Hinterdorfer et al., 1996). Fitting the data of Fig. 2 with this ansatz, results in a lifetime at zero force of  $\tau(0) = 15$  s and in an “effective bond length  $l_r$ ” of  $l_r = 0.2$  nm, respectively. From the kinetic binding rates determined by force spectroscopy, i.e.  $k_{\text{off}} = \tau_0^{-1}$  and  $k_{\text{on}}$  calculated from the time required for half-maximal binding and the effective ligand concentration on the tip, respectively, the equilibrium dissociation constant  $K_D$  may be estimated (Hinterdorfer et al., 1996, Baumgartner et al., 2000a).

## 5. Recognition Imaging

A new imaging method for the mapping of receptor sites on the surface due to molecular recognition by a ligand tethered to an AFM tip was recently developed (Raab et al., 1999) by combining dynamic force microscopy (DFM) (Han et al., 1996) with force spectroscopy. Hereby, the specific interaction of a ligand on the tip with receptors on the surface can be used to localize specific binding sites, which opens the possibility of recording recognition images. A magnetically coated tip was oscillated by an alternating magnetic field at 5 nm amplitude during lateral scans along the surface. The topography image (Fig. 3a) of a densely packed enzyme layer, recorded with a bare tip, appears very smooth. For obtaining the recognition image of the same layer (Fig. 3b), a ligand was coupled to the AFM tip via the crosslinker. Since the tether has a length of 8 nm the ligand on the tip has always a chance to bind when a receptor site is passed. Receptor-ligand recognition is monitored by reduction of the oscillation amplitude, as a result from antibody-antigen binding. These binding signals are apparent as additional bright dots in the recognition image and reflect the position of ligand binding-sites with nm lateral accuracy. With this methodology topography and recognition image can be obtained at the same time and distinct receptor sites in the recognition image can be assigned to structures from the topography image.



**Fig. 3. (a) Topography Image.** A densely packed enzyme layer was imaged with a bare tip in dynamic force microscopy. The free oscillation amplitude of the scanning tip was 5 nm and the feedback was installed to 1 nm amplitude reduction. **(b) Recognition Image.** An AFM tip, containing an antibody directed against the enzyme was scanned over the same enzyme layer. Imaging conditions were exactly the same as in (a). The bright dots correspond to recognition sites.

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