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Detection and localization of single molecular recognition events using atomic force microscopy

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Because of its piconewton force sensitivity and nanometer positional accuracy, the atomic force microscope (AFM) has emerged as a powerful tool for exploring the forces and the dynamics of the interaction between individual ligands and receptors, either on isolated molecules or on cellular surfaces. These studies require attaching specific biomolecules or cells on AFM tips and on solid supports and measuring the unbinding forces between the modified surfaces using AFM force spectroscopy. In this review, we describe the current methodology for molecular recognition studies using the AFM, with an emphasis on strategies available for preparing AFM tips and samples, and on procedures for detecting and localizing single molecular recognition events.

Molecular recognition between receptors and their cognate ligands is important in life sciences. Such specific interactions include those between complementary strands of DNA, enzyme and substrate, antigen and antibody, lectin and carbohydrate, ligands and cell-surface receptors as well as between cell adhesion proteins. These interactions are involved in many important biological processes, including genome replication and transcription, enzymatic activity, immune response, initiation of infection, and many other cellular functions. Furthermore, their selectivity and specificity are widely exploited in nanobiotechnology for developing bioanalytical and biomedical devices such as biosensors[1]. Despite the vast body of available literature on the structure and function of receptor-ligand complexes, information about the molecular dynamics within the complexes during the association and dissociation process is usually lacking. Moreover, until recently, mapping the spatial distribution of individual binding sites on model or cellular surfaces was not accessible because of a lack of appropriate imaging techniques. Consequently, there is clearly a need to develop and exploit single molecule tools for sensing and mapping molecular recognition interactions on biosurfaces.

Owing to its capacity to allow observation and manipulation of biosurfaces under physiological conditions, the AFM[2] has revolutionized the way in which researchers now explore biological structures at the single-molecule level[3]. Although AFM imaging provides three-dimensional views of specimens with unprecedented resolution and with minimal sample preparation[4], AFM force spectroscopy allows measurement of piconetwon (10^{-12} N) forces associated with single molecules[5,6] thereby providing fundamental insights into the molecular basis of biological phenomena and properties as diverse as molecular recognition[7–9], protein folding and unfolding[10,11], DNA mechanics[12] and cell adhesion[13].

The main parts of the AFM are the cantilever, the tip, the sample stage and the optical deflection system consisting of a laser diode and a photodetector (**Fig. 1**). AFM images are created by scanning (in the *x* and *y* directions) a sharp tip, mounted to a soft cantilever spring, over the surface of a sample and by using the interaction force between the tip and the sample to probe the topography of the surface. Force spectroscopy relies on measuring this force with piconewton sensitivity as the tip is pushed toward the sample and retracts from it in the *z* direction. The sample is mounted on a piezoelectric

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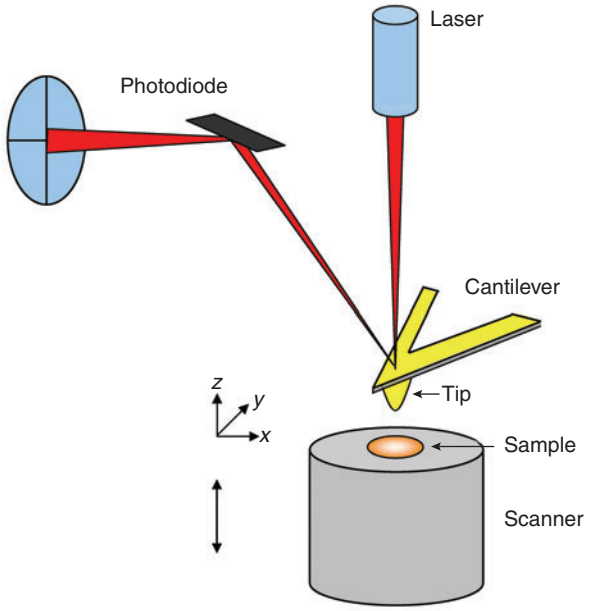

Figure 1 | General principle of atomic force microscopy. AFM imaging is performed by scanning a very sharp tip across the sample surface while the force of interaction between the tip and the sample is monitored with piconewton sensitivity. The sample is mounted on a piezoelectric scanner which ensures three-dimensional positioning with high resolution, and the force between tip and surface is monitored by measuring the cantilever deflection using an optical method (laser, photodiode). Beside imaging surfaces, AFM can be used in the force spectroscopy mode, in which the cantilever deflection is recorded as a function of the vertical displacement of the piezoelectric scanner, that is, as the sample is pushed towards the tip and retracted from it (bidirectional arrow).

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scanner which ensures three-dimensional positioning with sub-nanometer resolution. The force is monitored by measuring the deflection (vertical bending) of the cantilever. The cantilever deflection is usually detected by a laser beam focused on the free end of the cantilever and reflected into a photodiode; this deflection is directly proportional to the force. In so-called dynamic force spectroscopy, the rate of the increasing force (loading rate) is varied during the experiment, thereby providing insights into the molecular dynamics of recognition processes.

Here we describe current methodologies for sensing single molecular recognition events using AFM force spectroscopy. We highlight procedures and practical advice for preparing AFM tips and samples as well as for analyzing the forces, dynamics and localization of receptor-ligand interactions.

Preparing AFM tips and samples

Single molecule–recognition studies require functionalization of AFM tips (or cantilevers) and samples with relevant biomolecules (or cells). It must be emphasized that the quality and reproducibility of these preparation steps are the key factors that govern the success and the reliability of a single-molecule experiment (**Box 1**).

AFM tips. In the first pioneering studies of single receptor-ligand force measurements, strong physical adsorption of biotinylated bovine serum albumin (BBSA) was used to directly coat the tip[7] or a glass bead glued to it[14]. This physisorbed protein layer may be reacted with avidin or streptavidin (**Fig. 2a**), and then serve as a matrix for modification with biotinylated ligands[15]. In spite of the large number of probe molecules on the tip, the small fraction of properly oriented molecules and/or blockage of most reactive sites may be sufficient to allow the measurement of single receptor-ligand unbinding forces. But because parallel breakage of multiple bonds is often observed with this configuration, it is not ideally suited for single molecule–recognition studies. Another limitation of the biotin-avidin system is its fairly low binding strength compared to that of covalent bonds[16].

In fact, several important factors must be considered to achieve single molecular interaction detection. First, the binding of the molecules to the surfaces should be much stronger than the intermolecular force being studied. This is best achieved by using covalent bonds as they are at least ten times stronger (1–2 nN)[16] than typical receptor-ligand bonds. Second, the surface density of the molecules should be sufficiently low to ensure single-molecule interactions. Third, the molecules should retain sufficient mobility so that they can freely interact with complementary molecules, which is usually achieved by attaching the molecules on the surfaces via a flexible molecular spacer. Forth, unspecific adsorption on the modified surfaces should be inhibited to minimize the contribution of unspecific adhesion to the measured forces. Fifth, for oriented systems, site-directed coupling in which the molecule has a defined orientation may be desired. To fulfill these requirements, essentially two types of surface chemistries have been developed in different laboratories, which are based either on the strong chemisorption of thiols on gold surfaces or on the covalent attachment of silanes or alcohols on silicon oxide surfaces.

In the thiol approach (**Fig. 2b**), gold surfaces are first obtained by coating microfabricated cantilevers (or supports) by thermal evaporation with a thin adhesive layer of chromium (or titanium), followed by a 15–100-nm-thick gold layer. Then, proteins, oligonucleotides or carbohydrates that bear thiol groups can be attached directly on the gold surfaces. This type of surface chemistry has allowed the detection of intermolecular forces between complementary DNA oligonucleotides[8], between ferritin antibody and ferritin[17], between concanavalin and oligosaccharides[18], and between fibronectin and bacterial cells[19]. Alternatively, biomolecules can be attached onto gold via self-assembled monolayers (SAMs) of functionalized alkanethiols. SAMs are easily obtained by immersing gold surfaces in dilute (typically 1 mM) ethanol solutions of the selected alkanethiols. The latter can then be reacted with the biomolecule of interest in different ways, depending on their terminal functional groups. Alkanethiols that terminate in carboxyl functions can be reacted with amino groups of proteins using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) in aqueous solution[20]. Amino-functionalized surfaces prepared using either alkanethiols or silanes (see below) may also be used to attach proteins. In this context, carboxymethyl-amylose activated with NHS and EDC has been used to provide both firm attachment and molecular mobility[21,22]. This approach, however, is only of limited use as it usually gives broad unbinding force distributions owing to multiple attachment points.

For some studies, it may be desired to orient all the attached molecules in the same way, which can be achieved using the site-directed nickel nitrilotriacetate (Ni-NTA)-histidine system (**Fig. 2b**)[23–27]. In this system, recombinant histidine-tagged proteins are attached via their carboxy or amino termini onto an AFM tip coated with NTA-terminated alkanethiols. This coupling approach offers the

advantage of allowing an optimal exposure of the C-terminal or N-terminal domains. In addition, dilution of the NTA alkanethiols in a matrix of shorter tri(ethylene glycol) alkanethiols (**Fig. 2b**) confers high mobility to the attached proteins, minimizes nonspecific protein adsorption and allows low-density coupling to ensure single-molecule recognition[25,26]. A limitation of the Ni-NTA–His system is its low binding strength (150–200 pN)[23] compared to that of covalent bonds (1–2 nN)[16]. Therefore, it is important to emphasize that this approach may not be appropriate for studying strong receptor-ligand bonds, and that control experiments should be performed to confirm that the bond between His and Ni-NTA did not break during the experiment (for example, comparing the measured unbinding forces with those obtained for the bond between His and Ni-NTA using the same loading rate).

In contrast to the above procedures, silane or alcohol-based approaches can be applied directly to silicon tips (or supports), that is, without predepositing a gold layer (**Fig. 2c**). In particular, various amine-functionalization procedures using ethanolamine[9,28] or different silanization methods[29–32] are available to anchor biomolecules for single-molecule experiments. As the amine surface density determines, to a large extent, the number of ligands on the tip that can specifically bind to the receptors on the surface, the amine surface density has to be sufficiently low to guarantee single-molecular recognition events[9,28]. The amino-terminated surfaces readily react with a cross-linker, which provides the ligands with motional freedom and prevents their denaturation. Cross-linkers typically carry two different functional ends, for example, an amine-reactive NHS group on one end, and 2-pyridyldithiopropionyl (PDP)[33]

BOX 1 RECOMMENDATIONS FOR RELIABLE SINGLE-MOLECULE EXPERIMENTS

There are several important factors that determine the quality of a single molecular recognition experiment. Practical advice related to these factors is provided below.

Preparing AFM tips. Functionalization of AFM tips using appropriate procedures is of key importance for the detection of single molecular recognition. As a general rule, the use of covalent binding to attach biomolecules on tips should be favored, which is generally obtained using either thiol or silane surface chemistries. Protocols should be carefully developed and tested to achieve strong attachment of the biomolecules at low surface density, to guarantee sufficient mobility and to inhibit unspecific adsorption. Orientation of the biomolecules can be achieved using the site-directed Ni-NTA–His system or sulfur chemistry after introduction of cysteine residues into the protein of interest; the first approach, however, is limited by its fairly weak binding strength. Protocols are also available that describe how to attach cells onto AFM cantilevers, among which there are elegant approaches relying on specific receptor-ligand interactions. For every new system, the quality of the surface modifications should be assessed using fluorescence or other surface analysis techniques.

Preparing supporting surfaces. Purified biomolecules can be attached to mica, glass and silicon supports using generally the same protocols as those available for tip functionalization. Procedures are also available to immobilize cells on supports. The use of chemical fixation and/or air-drying should generally be avoided as this may cause denaturation of the cell-surface constituents. As a general rule, it is recommended to keep functionalized tips and samples hydrated and in conditions where their functionality remains intact.

Recording force curves. Depending on the nature of both tip and sample, the lifetime of a functionalized tip may be very short because of tip contamination or damage. In particular, when the sample surface is fragile and coated with loosely bound material, as often the case for cell surfaces, the 'activity' of the biological tip may be lost after recording a single image or a few force curves. In these conditions, it may therefore be useful to visualize the morphology of the sample surface with an unmodified tip to identify a region that is sufficiently smooth, homogeneous and stable before engaging a functionalized tip on the same region. Then, it is recommended to record force curves immediately after the tip is engaged on a single spot and to assess how the force behavior evolves with time and when moving from one spot to another. Another important point to avoid tip and sample alteration, is to limit the maximum contact force to several hundred piconewtons. To measure discrete molecular recognition forces, it may sometimes be useful not only to dilute the surface density of biomolecules on the tip, but also to modulate the contact force and the contact time between tip and sample. Most importantly, quantitative force measurements require accurate determination of the cantilever spring constant, which can be achieved using different methods. How about statistics? To get reliable force data on a given molecular recognition system, users should record several hundred force-curves using many independent tips and samples.

Varying the loading rate. For force spectroscopy experiments on receptor-ligand complexes it is important to vary the loading rate over orders of magnitude. The loading rate is the force increase over time during pulling the receptor-ligand complex and can be approximated by the effective spring constant of the system (cantilever and bound molecules) multiplied by pulling velocity. It can be varied by changing the spring constant of the cantilever and/or by changing the retraction speed. The upper limits are given by the force sensitivity and the possibly occurring hysteresis between tip approach and tip retraction, respectively. The latter is caused by hydrodynamic forces acting on the cantilever during movement. Another limitation to the pulling speed is the resonance frequency of the cantilever[85]. As a rule of thumb, spring constants should not exceed 0.1 N/m, and pulling speeds should be lower than 5 μm/s.

Recording force maps. Microscopists may use either adhesion force mapping or dynamic recognition force mapping to localize specific binding sites on biosurfaces. The choice will depend on the type of microscope available, on the targeted application and on the desired information. Although the first method allows quantitative determination of forces, it is slow and offers fairly poor lateral resolution compared to the second method. In the future, we anticipate that an ideal combination will consist of using first dynamic recognition force mapping for identifying specific recognition sites and then exploiting force spectroscopy to provide quantitative force data.

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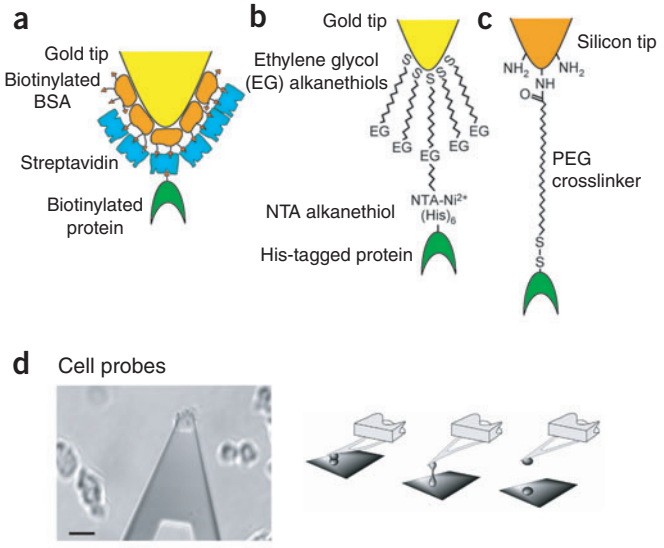

Figure 2 | Schematics of surface chemistries commonly used for modifying AFM tips for single-molecule recognition studies. (**a**) Physisorption of proteins such as BBSA[7,14]. The BBSA-coated tip may be reacted with streptavidin and then modified with biotinylated proteins. (**b**) Chemisorption of alkanethiols on gold[25,26]. Histidine-tagged proteins are attached onto a gold-coated tip modified with NTA-terminated alkanethiols. (**c**) Covalent coupling of silanes on silicon oxide[9,28]. Proteins are coupled to the tip via a heterobifunctional polyethylene glycol (PEG) crosslinker: the amine reactive NHS end of the crosslinker reacts with amines on the silicon tip, yielding a stable amide bond, and the reactive PDP group forms a bond with free thiols presented by cysteines in the protein, resulting in a stable disulfide bond. (**d**) Attachment of living cells by means of receptor-ligand interactions. Light-microscopy image of a *Dictyostelium discoideum* cell mounted on an AFM cantilever (left). By applying a repulsive contact force between the cantilever-mounted cell and a target cell at the bottom of a Petri dish, and then retracting the cantilever from the target cell (right schematics), specific cell-cell adhesion forces can be measured. Scale bar, 20 μm; **d** reproduced with permission from ref. 13.

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or vinyl sulfone[34] groups, which can be covalently bound to thiols, on the other (**Fig. 2c**). This sulfur chemistry is highly advantageous as it is very reactive and readily permits site-directed coupling via NTA groups[23]. In addition, the cross-linker may also be used as a spacer, which allows the molecule to freely orient and diffuse within a certain volume[9]. Free thiols on ligands can be generated by (i) binding *N*-succinnimidyl-3-(S-acethylthio)propionate to amines[33], (ii) cleaving antibodies according to a standard procedure[35], or (iii) attaching PDP hydrazide to oligosaccharide residues of antibodies[36,37]. Alternatively, proteins can be directly coupled via their lysines to aldehyde end groups of cross-linkers (L. Wildling, P.H. & H. Gruber; manuscript in preparation).

For any new system, it is strongly recommended to validate the quality of the tip surface modifications even when using well-established protocols from the literature. To this end, flat model supports such as silicon or mica are treated in parallel with the tips, and characterized by means of various analytical techniques, including fluorescence and AFM imaging. For fluorescence measurements[9,28], the attached molecules can either be directly fluorescence labeled or fluorescence-labeled secondary antibodies can be ligated to surface-bound primary antibodies. Alternatively, the density of specific sites can be determined with an enzyme immunological assay[29]. The latter two methods provide the advantage to test the functionality of the attached molecules on the surface, whereas the first determines only the total number density. Using standard amine-functionalization procedures as described above, values between 200 and 500 molecules/μm^2 were usually obtained with all three protocols. For a typical AFM tip radius of 20–50 nm, this value corresponds to about one molecule per effective tip area, which appears to be suited for single-molecule experiments. AFM topographic imaging in aqueous solution is also very useful to assess the quality of the functionalized surfaces as it allows determination of whether the modified surfaces are homogenous and devoid of aggregates[14,25]. Furthermore, scanning small areas at large forces results in the removal of the biomolecular layers and thereby allows direct determination of their thickness.

Notably, several techniques have also been developed to attach cells directly onto AFM cantilevers (**Fig. 2d**), allowing researchers to probe cell-cell or cell-support interactions. Strategies for creating cell-probes involve the use of specific receptor-ligand interactions[13,38], electrostatic interactions[39], glue[40] or chemical fixation[41]. Depending on the application, best results may be obtained using tip-less cantilevers, as cell contact is favored in this case. An important issue when applying these protocols is to make sure that the native surface of the attached cells is not altered or denatured. In this respect, an elegant approach is to attach individual cells to an AFM cantilever via lectins such as wheatgerm agglutinin. This technique allowed measurement of the specific adhesion force between two adjacent cells of *Dictyostelium discoideum* on a single-molecule level (**Fig. 2d**)[13]. Notably, this force could be ascribed to the discrete interaction between two cell adhesion glycoproteins engaged in cell aggregation.

Samples. Another crucial issue is that the receptors (or ligands) recognized by the functionalized tip need to be firmly attached to a solid support using appropriate, nondestructive methods. Mica, glass and silicon have proved to be excellent supports for immobilizing purified receptors. Muscovite mica, the most frequently used support for biological AFM, is a nonconducting layered mineral which can be easily cleaved with the help of an adhesive tape leading to clean, atomically flat surfaces. Because the mica surface is negatively charged at neutral pH, simple adsorption of positively charged proteins, such as lysozyme[35], may be sufficient to withstand the pulling force exerted by the tip during the force spectroscopy measurements. Physical adsorption is also a well-suited immobilization strategy for probing native membranes[4,42].

For most biomolecules, however, immobilization through covalent attachment is required. When using glass, silicon or mica, the immobilization schemes are very similar to those described above for tip functionalization, that is, supports can either be modified through thiol surface chemistry[43,44] after gold deposition, or directly subjected to silane[45] or ethanolamine[46] modification. It is worth noting that ultrasmooth gold-coated surfaces (roughness ~0.1 nm) may be obtained using template stripped gold methods[47]. The typical procedure involves depositing gold onto a smooth support such as mica, supporting the free gold surface by gluing it to a glass slide using epoxy glue and stripping the gold film from the support. Another important remark is that silanes, as opposed to alkanethiols, do not allow for high surface densities, that is, >1,000 molecules/μm^2. By comparison, the surface density of a monolayer of streptavidin is ~60,000 molecules/μm^2 and that of a phospholipid monolayer may exceed 10^6 molecules/μm^2.

When using single-molecule AFM force spectroscopy on cellular

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surfaces, a key issue is to immobilize the cells while preserving their viability and integrity. For animal cells, a simple preparation method is to exploit their ability to spread and adhere to solid surfaces[48–51]. Firm immobilization of weakly adhering cells can be achieved by various adhesive coatings such as Cell-Tak[52], gelatin, collagen or polylysine[21]. Alternatively, chemical fixation using cross-linking agents such as glutaraldehyde may also be applied. Although this approach may be relevant for topographic imaging[53], in most instances it will not be satisfactory for single-molecule recognition studies.

Unlike animal cells, microbial cells such as bacteria and yeast cannot spread on solid supports. Thus, immobilization by means of simple adsorption procedures is often inappropriate as this usually leads to cell detachment by the scanning tip. Stronger attachment may be achieved either by pretreating the support with polycations[54] or lectins[55], or by binding the cells covalently to the support[56]. An alternative approach is to immobilize the cells mechanically in a polymer membrane with a pore size comparable to the dimensions of the cell. This method allows one to perform repeated imaging and force spectroscopy on living cells without causing cell detachment or cell damage[57,58].

Measuring single-molecule recognition forces

Several powerful techniques are available for probing the interaction forces between biosurfaces; they include the use of shear flow detachment[59], surface force apparatus[60], biomembrane force probe[61], optical tweezers[62] and AFM. Remarkably, AFM is the force-measuring method with the smallest force sensor (AFM tip radii are in the 2–50 nm range) and therefore provides the highest lateral resolution. In fact, AFM is currently the only force technique that allows one to map and analyze single receptors with nanoscale lateral resolution.

Measuring molecular recognition forces by AFM requires recording so-called force curves between the modified tip and sample surface. AFM force curves are obtained by monitoring, at a given (x, y) location, the cantilever deflection (d) as a function of the vertical displacement of the piezoelectric scanner (z). This yields a raw 'voltage-displacement' curve, which can be converted into a 'force-displacement' curve using two conversions. Firstly, the sensitivity of the AFM detector, that is, the slope of the retraction curve in the region where tip and sample are in contact, is used to convert the voltage into a cantilever deflection. It is important to note that the estimated sensitivity is only valid when the sample behaves like a hard, nondeformable material, which is often true for purified molecules attached on hard supports. For soft cells, however, the value obtained for the sensitivity may be incorrect owing to sample deformation by the tip. In this case, it is mandatory to assess the sensitivity of the detector on a hard support, before or after the force measurements on cells. Secondly, the cantilever deflection is converted into a force (F) using Hooke's law: $F = k \times d$, where k is the cantilever spring constant. The force resolution of the AFM is in first approximation limited by the thermal noise of the cantilever that, in turn, is determined by its spring constant. In addition, the resonance frequency, the quality factor, and the measurement bandwidth can also substantially contribute[63]. Therefore, for single-molecule force measurements, best results are generally obtained with cantilevers exhibiting small spring constants (that is, in the range of 0.01 to 0.10 N/m) and short lengths (<50 μm), because they exhibit lower force noise. Notably, the actual spring constants may differ substantially from values quoted by the manufacturer, meaning researchers must determine spring constants experimentally to get accurate knowledge of the measured forces (see ref. 64 for a recent comparison of calibration methods).

Various features may be distinguished in a force curve. At large tip-sample separation distances, the force experienced by the tip is zero. As the tip approaches the surface, the cantilever may bend upwards owing to repulsive forces (electrostatic, hydration or most importantly steric forces) until the tip jumps into contact when the gradient of attractive forces exceeds the spring constant plus the gradient of repulsive forces. Upon retracting the tip from the surface, the curve often shows a hysteresis referred to as the adhesion 'pull-off' force (**Fig. 3a**). This value is most important in molecular recognition studies as it represents the unbinding force between complementary receptor and ligand molecules. In the presence of flexible molecules, such as long biomolecules or cross-linkers, an elongation force may develop before the unbinding event, reflecting an increase of the spring constant of the flexible molecule during extension.

A key feature in molecular recognition studies is to demonstrate the specificity of the measured unbinding forces, which can be achieved in different ways. Block experiments in which the receptor sites are masked by adding free ligands is the most simple and straightforward approach (**Fig. 3b**). Another, probably less convincing, approach is to use a tip that has been functionalized with biomolecules resembling the ligands but that do not have specificity for the receptors. For cell studies, mutants deficient in receptor production may provide elegant additional controls.

Concerning statistics, it is recommended to measure many unbinding events by recording several hundred force curves on different locations of the sample and to express the data as force

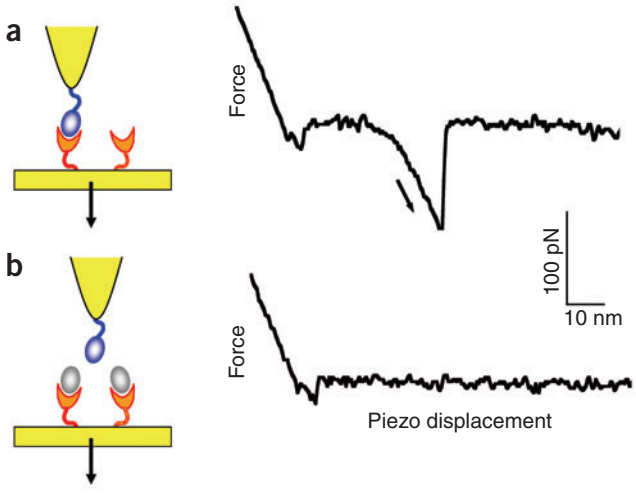

Figure 3 | Measurement of molecular recognition interaction forces. (**a**) Typical force-displacement curve obtained upon retracting (down arrow) an AFM tip functionalized with oligoglucose carbohydrates from a surface modified with the lectin concanavalin A. Force-displacement curves are obtained from raw 'voltage-displacement' curves using the sensitivity of the AFM detector and the cantilever spring constant. The curve shows an unbinding force of about 100 pN, attributed to the rupture of a single carbohydrate-lectin pair. The unbinding event is accompanied by a nonlinear elongation force (arrow on the right) reflecting essentially the stretching of the flexible spacer. (**b**) Blocking experiment demonstrating that the 100 pN unbinding force is not observed when the measurements are performed in the presence of blocking agents like glucose or mannose.

histograms showing the distribution of at least 100 unbinding force values. Also, the reliability and reproducibility of the measured unbinding forces should be demonstrated by comparing data obtained using many independent tips and samples. Finally, as we shall see below, unbinding force histograms should be generated while varying the loading rate over several orders of magnitude.

Exploring the dynamics of receptor-ligand interactions

Unbinding forces between receptors and ligands measured at constant pulling velocity represent only a single point in a continuous spectrum of bond strengths because theory predicts[65] and experiments confirm[15,38,61,66] that these depend on the loading rate, that is, the timely rate at which the load, that is, the force F, is applied to the bond. Therefore, users should always control, and when possible vary, the loading rate to compare unbinding forces from different experiments. As we shall see, this procedure is also very useful to assess kinetic parameters and shapes of energy landscapes of the unbinding process.

To understand the loading rate dependence, one must keep in mind that receptor-ligand bonds have limited lifetimes and that the lifetime is shortened when a force is applied to a bond owing to thermal activation. In fact, the thermal energy of the surrounding solution makes bonds also break in the absence of an external force, which is essential for rendering possible time-controlled regulation of ligand-stimulated processes. The characteristic time scale ($\tau(0)$) needed for this spontaneous dissociation (0 in τ indicates that no force is applied) is given by the inverse of the kinetic off-rate constant in solution (k_{off}), thus $\tau(0) = k_{off}^{-1}$. Pulled faster than $\tau(0)$, bonds will resist detachment and an unbinding force becomes measurable. The millisecond to second time scale accessible for AFM force experiments lies in the thermally activated regime, in which thermal impulses govern the dissociation process. Here, a Boltzmann ansatz can be used to describe how a force F acting on a complex lowers the dissociation barrier and shortens the bond lifetime: $\tau(F) = \tau(0)\exp(-x_\beta F/k_BT)$[65,67]; x_β marking the thermally averaged projection of the energy barrier along the direction of the force, $\tau(F)$ is the bond life time under force F, k_B is the Boltzmann constant, and T is the absolute temperature.

In a mode termed dynamic force spectroscopy, the unbinding force of a receptor-ligand complex is measured as a function of the actual loading rate ($r = dF/dt$), in which the latter can be derived from the product of the pulling velocity and the effective spring constant of the whole system (cantilever and involved molecules). This approach is actually very useful to assess kinetic parameters of the unbinding process, including length scales and relative heights of energy barriers. For most experimental configurations, the force applied to a receptor-ligand complex in force curves increases approximately linearly with time (**Fig. 3**). Bond rupture itself is a stochastic process, and the likelihood of bond survival can be expressed in a master equation as a time-dependent probability $N(t)$ to be in the bound state under a linearly increasing force (load), that is, $dN(t)/dt = -k_{off}(rt)N(t)$ (ref. 68). This, together with the equation for $\tau(F)$ given above, results in a distribution of unbinding forces $P(F)$ parameterized by the loading rate[65,68].

In experiments, careful analysis of the force distributions, from which each has to be constructed from the force values of many force curves, obtained at the different loading rates (**Fig. 4**)[66] is required to gain the variables mentioned above. The theory above[61,65,68] predicts that, if only one single, sharp energy barrier governs the dissociation process, the maxima of the distributions at different loading rates (F^*), are in logarithmic dependence on the loading rate, according to $F^* = F_\beta \ln(rk_{off}^{-1}/F_\beta)$, with F_β being the force scale set by the ratio of thermal energy (k_BT) to x_β[65,68]. Thus, a single energy barrier should lead to a simple, straight line in an F^* versus $\log(r)$ plot. Several examples of this behavior for a variety of receptor-ligand combinations are available in the literature[15,23,30]. But for cases in which

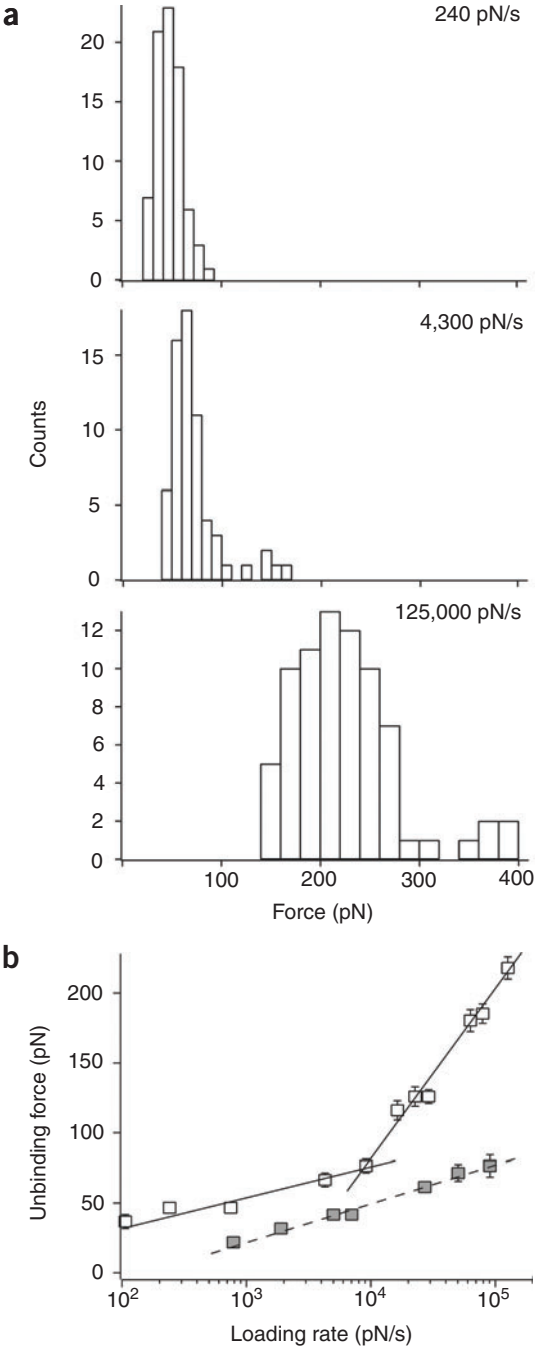

Figure 4 | Dynamic force spectroscopy of a single receptor-ligand bond. (**a**) Unbinding force histograms of the sialyl Lewis X (sLeX)–P-selectin interaction recorded at the indicated loading rates. (**b**) Dynamic force spectra of the sLeX–P-selectin interaction in the absence (open symbols) and presence (closed symbols) of EDTA. Data represent the mean ± s.e.m. Reproduced with permission from ref. 66.

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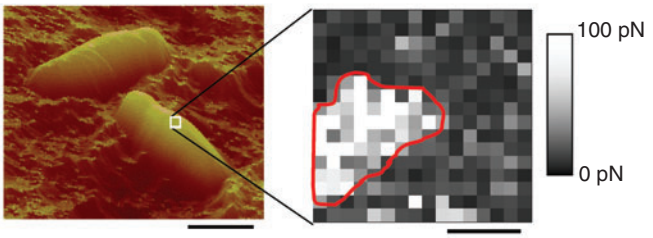

Figure 5 | Mapping molecular recognition sites on living cells. Topographic image (left) showing two living mycobacteria on a polymer support and adhesion force map (right) recorded on a single cell with a heparin-modified tip. In localized regions, the map reveals adhesion events (clear pixels) owing to the presence of adhesion proteins referred to as heparin-binding haemagglutinin adhesin (HBHA). Notably, the adhesin distribution is not homogeneous, but apparently concentrated into nanodomains that may have an important role in mediating the attachment of mycobacteria to epithelial cells. Scale bars, 2 μm (left) and 100 nm (right). Adapted from ref. 25.

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different barriers become dominant at varying loading rates, the curve will follow a sequence of linear regimes, each marking a particular barrier[61,65]. Transition from one regime to another is associated with an abrupt change of slope determined by the inverse of the characteristic barrier length scale x_β (**Fig. 4b**)[66]. Notably, length scales and relative heights of energy barriers can be obtained by measuring the bond strength over a broad range of loading rates[38]. One may also attempt to extract dissociation rate constants by extrapolation to zero force[15] and compare the values with those obtained with ensemble-average experiments like surface plasmon resonance[26]. Nevo and coworkers[69] recently found that the force histograms of the interaction between nuclear pore proteins show two maxima, strongly suggesting the coexistence of two complex populations, one with high affinity and the other with lower affinity.

Mapping molecular recognition events

There is increasing evidence indicating that eukaryotic cell membranes show lateral heterogeneities that are enriched in (glycol)sphingolipids, cholesterol and specific membrane proteins[70]. These nano- and microdomains are thought to have important roles in a variety of cellular functions including signaling, cell adhesion and membrane trafficking. It is also increasingly recognized that bacterial cell walls are dynamic structures that can show selective accumulations of constituents at particular locations[71]. This emphasizes the need to develop high-resolution imaging tools for localizing specific sites on eukaryotic and prokaryotic cell surfaces. In this context, AFM offers exciting opportunities for mapping individual binding sites with nanoscale resolution, thereby providing information that is complementary to that obtained by fluorescence and electron microscopy methods.

Adhesion force mapping. A first approach to map molecular recognition sites is to record arrays of force curves in the x, y plane using functionalized tips. Typically, 16×16 or 32×32 force curves are recorded on areas of a given size, the unbinding force value is estimated for each curve and then displayed as grey pixels, the brightness of which reflects the magnitude of the unbinding force (**Fig. 5**). The first proof of concept of this 'affinity imaging' mode has been provided by imaging microscale streptavidin patterns using a biotinylated tip[72]. The feasibility of using a lectin-modified tip for mapping wall polysaccharides on living yeast cells has also been demonstrated[55]. Since then, this method has been exploited for mapping binding sites on various types of living cells, including red blood cells[21], osteoclasts[73], endothelial cells[50] and mycobacteria[25] (**Fig. 5**). Although adhesion force mapping provides a quantitative analysis of unbinding forces, it is limited by its time resolution. The time currently required to record a map is on the order of 2–15 min, depending on the acquisition parameters, which is much greater than the time scale at which dynamic processes usually occur in biology.

Dynamic recognition force mapping. In dynamic recognition imaging[74], molecular recognition signals are detected[9] during dynamic force microscopy imaging[75,76]. In more detail, AFM tips carrying ligands are oscillated at very small (5–10 nm) amplitudes while being scanned along the surface to which the cognate receptors are bound. Topography and recognition images are simultaneously obtained (by simultaneous topography and recognition (TREC) imaging) using an electronic circuit (PicoTrec; Molecular Imaging)[77,78]. Maxima (U_{up}) and minima (U_{down}) of each sinusoidal cantilever deflection period are depicted and fed into the AFM controller, with U_{down} driving the feedback loop to record the height (that is, the topography) image and U_{up} providing the data for construction of the recognition image (**Fig. 6a**). It is important to note that only for cantilevers with a low quality factor (~ 1 in liquid) driven at frequencies below resonance both types of information are independent. Using this approach, singly distributed avidin molecules were scanned with a biotinylated AFM tip[79], yielding topography and recognition images at the same time (**Fig. 6b**). The lateral positions of the avidin molecules obtained in the topography image were spatially well correlated with the recognition signals of the recognition image (**Fig. 6b**). Dynamic recognition imaging offers the advantage that topography and recognition images can be recorded

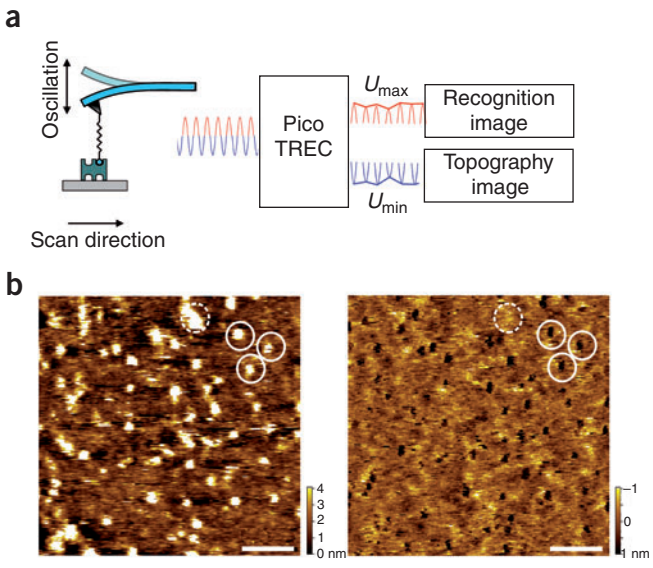

Figure 6 | Simultaneous topography and recognition imaging (TREC). (**a**) The cantilever oscillation signal is split into minima U_{min} and maxima U_{max}. (**b**) Singly distributed avidin molecules imaged with a biotin-tethered tip. The bright dots 2 to 3 nm in height and 15 to 20 nm in diameter visible in the topography image (left, solid circles) are single avidin molecules, and the black dots of the recognition image (right) arise from a decrease of the oscillation maxima that result from the physical avidin-biotin connection during recognition. Some topographical features lack specific interaction (dashed circle). Scan size was 500 nm. Scale bars, 100 nm. Reproduced with permission from ref. 79.

at the same speed as that used for conventional topographic imaging, typically 1–5 minutes per image. Yet, this is still slower than the rate of most dynamic processes, meaning that developing AFM instruments with increased imaging rates is an important challenge.

Conclusions

The methodology for exploring the forces and the dynamics of receptor-ligand interactions using AFM force spectroscopy is well established and should be increasingly used by biophysicists, chemical biologists, cell biologists and microbiologists. Remarkably, AFM is the only force-measuring technique that can map the nanoscale lateral distribution of single molecular recognition sites on biosurfaces. Yet, it is clear that the full potential of AFM will be best exploited when combined with other advanced microscopy and spectroscopy techniques.

Reliable protocols are available for attaching biomolecules or cells on the AFM tips and on supporting surfaces. Also, procedures to probe the forces, the dynamics and the localization of molecular recognition interactions are now well established. Nevertheless, it is fair to say that accurate data collection and interpretation remain often delicate and require strong expertise, especially when dealing with complex specimens like living cells. The main tasks are those associated with the quality of tip and support surface chemistries and with their possible alteration during data acquisition. Thus, a detailed understanding of the principle of the different modalities of AFM and of their limitations is essential before users start their first experiment.

In the future, we anticipate that the use of small cantilevers will improve the force resolution, thereby allowing measurement of smaller unbinding forces[63]. Nanotube tips functionalized with single biomolecules will permit the mapping of binding sites with a resolution that would be difficult to achieve with conventional tips[80]. In nanobiotechnology, functionalized cantilevers[81,82], combined with automatic analyses, will provide a basis for a new generation of highly sensitive biosensors, which may find important applications for the detection of toxins and for diagnosis and monitoring of diseases. Another big challenge is the development of fast scanning AFMs because the time resolution of this method is still a very limiting factor[83,84].

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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