Spatially resolved force spectroscopy of biological surfaces using the atomic force microscope

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The spatial distribution of intermolecular forces governs macromolecular interactions. The atomic force microscope, a relatively new tool for investigating interaction forces between nanometer-scale objects, can be used to produce spatially resolved maps of the surface or material properties of a sample; these include charge density, adhesion and stiffness, as well as the force required to break specific ligand–receptor bonds. Maps such as these will provide fundamental insights into biological structure and will become an important tool for characterizing technologically important biological systems.

Long- and short-range interactions between macromolecules and macromolecular assemblies are central to the dynamic behavior of biological systems. Many interactions have been examined in great detail using thermodynamic and kinetic approaches. However, there is little direct information about how the interaction energy between two structures is distributed in space because it is difficult to measure explicitly the interaction energy as a function of separation distance. The most direct way to obtain this kind of data is to measure the force (the derivative of energy with respect to distance) between two structures.

A wide range of instruments and approaches has been developed for measuring forces between small structures, including mechanical springs made from glass fibers, optical tweezers, vesicle-based force transducers and the surface-forces apparatus (SFA). Other approaches to investigating intermolecular forces include optical or diffraction methods to monitor the displacement of structures under a load resulting from hydrodynamic or osmotic forces. Using optical methods, it is also possible to monitor the thermally driven motion of particles in solution and from that motion to infer interactions that particles have with the media and nearby structures.

The atomic force microscope (AFM) is a relatively new tool for measuring intermolecular forces between nanometer-scale objects. The AFM has many similarities to several of the force-sensing instruments. In one view, the AFM is much like a miniature SFA in which a mechanical spring is used to measure force as a function of distance between two structures; however, because the AFM uses a probe with a radius of curvature typically on the order of 10–100 nm (compared with the ~1 cm effective radius of curvature of the SFA), the AFM can measure local interactions with a high spatial resolution. Thus, it can be applied to highly heterogeneous surfaces, albeit at the expense of sensitivity.

One reason the AFM is considered to be a separate technology is because it was first developed as an imaging tool, something neither the SFA nor glass fibers had been used for at the time. The relative ease of use and the commercial availability of the AFM make it one of the most widely used instruments for measuring intermolecular forces. In this article, we review the use of the AFM as a tool for force spectroscopy (measuring forces as a function of distance) in biomolecular systems. We will cover only DC-type force measurements; AC-based measurements, including thermal-noise-based approaches, are not included.

Basic elements of the AFM

A useful way to consider the AFM is the ‘small ball on a weak spring’ model (Fig. 1a). In this model, a weak mechanical spring is used to measure the forces between a probe (the ball) and a sample whose position may translate relative to the probe. In practice, the spring is composed of a cantilever and the ball is a tip at the free end of the cantilever. The tip and sample are translated relative to each other using piezoelectric ceramics, and movements of the spring are measured using optical methods. The detailed implementation of AFM has been reviewed and a common implementation is shown in Fig. 1b for reference.

Approximating the AFM tip as a sphere is reasonable for many interaction forces. For some forces, analytical expressions describing the tip as a cone or a pyramid exist. In other cases (noted below), the specific geometry and area of the tip are key determinants of the interaction force. The surface chemistry of the tip is also critical to the interaction with the sample and there is a great deal of ongoing research aimed at modifying and characterizing tip chemistry.
Nanotechnology

Curve, which is a plot of cantilever deflection, the purposes of presentation, we will divide the force varies as it is moved toward or away from the sample, for as the probe approaches the sample and is retracted to determining the point of contact very difficult. (long-range forces and sample elasticity) that can make contact with the sample. In practice, there are two factors ing is called a force–distance curve. Notice that determin-
curve as a straight line of unit slope. A corrected curve with the sample movement; this appears in the force curve in which the cantilever deflection is coupled 1:1 face, zero separation is defined as the region in the force diction distance (Eqn 1). It assumes a simple relationship (i.e. describe force as a function of the probe–sample sepa-
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The interpretation of AFM force curves relies almost entirely on established force laws, particularly those determined using the SFA14,15. These force laws describe force as a function of the probe–sample separation distance (D) rather than as a function of the z-piezo position. Thus, to be useful, the force curves must be transformed into descriptions of force as a function of distance, F(D).

However, current AFMs do not have an independent measure of D. Instead, the transformation to D is achieved by subtracting the cantilever deflection from the z-piezo movement (Fig. 2b,16). For a very hard surface, zero separation is defined as the region in the force curve in which the cantilever deflection is coupled 1:1 with the sample movement; this appears in the force curve as a straight line of unit slope. A corrected curve is called a force–distance curve. Notice that determin-
ing D by this approach requires that the tip make contact with the sample. In practice, there are two factors (long-range forces and sample elasticity) that can make determining the point of contact very difficult.

A complete force curve includes the forces measured as the probe approaches the sample and is retracted to its starting position. Because the forces on the tip can vary as it is moved toward or away from the sample, for the purposes of presentation, we will divide the force curve into approach and retraction portions and consider them separately.

Approach For a simple approach between two hard surfaces in the absence of any long-range interactions, van der Waals forces will dominate. The attractive van der Waals force varies with separation distance as D−5 for a (large) sphere approaching a flat surface. As the tip dimensions decrease to those of a single atom, the D−3 dependence becomes a D−6 dependence17. This force appears in the approaching force curve as small downward deflection just prior to contact (Fig. 3a). The downward deflec-
tion is often very steep and is sometimes associated with a jump-to-contact event. The jump results when the force gradient between the tip and the sample exceeds the stiffness of the cantilever, causing instability in the position of the cantilever17. The force also depends on the tip, sample and medium composition.

Two long-range forces that give rise to force curves with a similar morphology (a smooth, exponentially increasing repulsive force) are electrostatic (for like charges) and polymer-brush forces. Quantitative electrostatic measurements with the AFM are based on forces produced from overlapping electrical double layers, and the associated ion gradients, as a charged probe is brought near a charged sample surface (Fig. 3b): SFA work has shown that the Gouy–Chapman theory [the electrostatic part of Derjaguin, Landau, Verwey and Overbeek (DLVO) theory] can relate force measurements to surface charge and poten-
tial14,15. Ducker and colleagues showed that this also holds for AFM measurements15. Subsequently, a num-
ber of groups have measured surface-charge density and Debye length as a function of pH, electrolyte type and concentration with the AFM, and found agreement

Figure 1
(a) Small ball on weak spring model of an atomic force microscope (AFM). This model highlights the central features of the AFM as a me-
chanical sensor for probing interaction forces. (b) Schematic diagram of a common implementation of an AFM. The sample is mounted on a stage that can move in the x, y and z directions, typically using a piezoelectric actuator. The deflection of the cantilever in response to interactions at the tip is detected by an optical lever, in which the movements of a laser reflected off the cantilever are detected by a split-segment photodiode. The deflection data are passed to a computer controller that provides appropriate feedback to the stage and collects data. The essential elements of an AFM are: a probe (tip) attached to a spring (cantilever); a means of measuring deflections of the spring (optical lever and split-segment photodiode); a sample; and a means for moving the sample and probe relative to each other (piezoelectric tubes). Deflections of the spring are then used to measure interactions of the probe tip with the sample.

Force curves
Force is measured in an AFM by collecting a force curve, which is a plot of cantilever deflection, d, as a function of sample position along the z-axis (i.e. toward or away from the probe tip; the z-piezo position) (Fig. 2a). It assumes a simple relationship (i.e. Hooke’s Law) between the force, F, and the cantilever deflection (Eqs 1):

\[ F = -k \cdot d \]  

(1)

where k is the spring constant of the cantilever.

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ber of groups have measured surface-charge density and Debye length as a function of pH, electrolyte type and concentration with the AFM, and found agreement
Polymers grafted onto a surface can, by virtue of their thermally driven (Brownian) motion, exert a repulsive force on a particle moving through their space. For a densely grafted polymer in an appropriate solvent, the force on a spherical probe can be described by the Alexander de Gennes’ theory (Fig. 3c).

Thus, a grafted polymer acts as an entropic spring, which can be examined with an AFM24,25. For a densely grafted polymer in an appropriate solvent, the force on a spherical probe can be described by the Alexander de Gennes’ theory (Fig. 3c).

The material properties of samples can be investigated with AFM force curves by relating the applied force to the depth of indentation as the tip is pushed against the sample. Although this is not an interaction force per se, we include it because it is a widely used application of AFM force spectroscopy and because surface elasticity is often convolved with real interaction forces. AFM force curves have been used to examine the viscoelastic properties of a wide range of biological structures (for a recent review, see Ref. 26), including cartilage27, lysozyme molecules 28, gelatin gels29, glial cells30, epithelial cells31,32 and mast cells33. Temporal changes in cellular mechanics have also been monitored34,35, which involves the technically challenging problem of maintaining the tip at a single point for long periods of time. One of the simplest and most widely used models for mechanical deformation that has been applied to AFM-force-curve data is the Hertz model, which gives the force on a spherical probe as a function of the elastic properties of the material, the radius of the probe and the indentation depth36 (Fig. 3d). However, when investigating viscoelastic samples, careful consideration must be given to the time-dependent viscous contributions, which strongly affect the rate of approach.

Adhesion

The retracting portion of the force curve sometimes follows the approach curve; however, there is often hysteresis. The most common type of hysteresis is due to some sort of adhesion, which appears in the force curve as a deflection below the zero-deflection line. The source of the adhesion can vary depending on the sample. In the ideal case of a sphere interacting with a flat surface, the adhesion force can be related to the radius of the sphere and the surface energies (Fig. 3e) of the two surfaces34. Under ambient conditions, the main source of adhesion is the formation of a capillary bridge between the tip and the sample (Fig. 3f). In air, most samples have several nanometers of water adsorbed to the surface; this water layer wicks up the tip and forms a ‘bridge’ between the tip and the sample. Pulling the tip out of that bridge requires a large force to overcome the surface tension37. In fluid, the adhesive force depends on the interfacial energies between the tip and sample surfaces, and the solution38, varying the solution can thus change the force of adhesion.

A different form of ‘adhesion’ occurs when a polymer is captured between the AFM tip and the substrate. In this case, there is a very distinctive ‘adhesive’ force as the tip is pulled away. Typically, these curves initially retrace the approach curve near the surface but, away from the surface, exhibit a smooth negative deflection as the polymer is stretched until it breaks or detaches from the tip or the substrate, and the cantilever returns to the zero-deflection line (Fig. 3g). If multiple polymer molecules attach to the tip and substrate, a
Examples of atomic force microscope (AFM) force curves and the force laws used to interpret them. Notice that the curves are shown as force versus sample-position curves, which is the most common way they are displayed in the literature; however, the force laws are all functions of the separation distance, $D$. (a–d) Approach curves. (e–h) Retraction curves. (a) An ideal, attractive, van der Waals force in the absence of other forces. (b) Repulsive electrostatic double-layer force in solution. (c) Polymer-brushing forces that result from the thermally driven motion of polymers grafted onto a solid surface in solution. (d) Indentation curve on an elastic sample; notice that, in this case, the true contact is near the point at which the cantilever begins to deflect. (e) Adhesion between a sphere and a plane in the absence of contaminating adsorbates (typically in a vacuum). (f) Capillary adhesion (very common under ambient conditions, under which many surfaces have thin layers of water) results from the formation of a water bridge between the tip and sample. (g) Polymer-extension force curves show a characteristic negative deflection far from the surface and a jump back to zero deflection as the polymer breaks or detaches from one of the surfaces. (h) The unbinding of specific receptor–ligand pairs sometimes produces a stepwise return to zero deflection from the point of maximal adhesion. This is thought to be due to the sequential unbinding of multiple receptor–ligand pairs. In some experiments, the binding partners are attached at the ends of polymers and the unbinding curve is combined with the polymer-extension curve.
The saw-tooth pattern can be observed as individual polymers detach.

There are several models, such as the worm-like or freely-jointed chain models, that can be used to describe the force-distance relationships for extending polymers. Extension measurements of high molecular weight dextrans show force-distance relationships in agreement with the freely-jointed-chain model. Similar results have been described for proteoglycans.

Examining the interaction forces between specific biological receptor–ligand systems is an application that has generated enormous interest. At present, only the unbinding force (adhesion) of a receptor–ligand pair has been examined. Here, a ligand-functionalized AFM tip is brought into contact with the receptor-coated surface (or vice versa); the receptor and ligand bind, and the tip is retracted. In the simplest case, the magnitude of the adhesion ‘spike’ is a direct measure of the binding force. For a small number of discrete binding events per force curve, statistical analysis of many adhesion measurements can be used to estimate single bond strengths. This measure of bond strength is related to the depth of the potential well holding the two molecules together. In some cases, the resultant retraction force curves have a jagged appearance (Fig. 3b), which is thought to correspond to multiple pairs unbinding at different times. Controls include competition with excess free ligand/receptor to occupy all available sites and the use of nonspecific ligands.

Force-curve studies have focused on several antibody–antigen systems and complementary DNA strands. Because the effects of loading force, loading rate and tip geometry contribute to the unbinding force measured, these types of experiments are very difficult to perform and interpret, and the analytical framework for the data is still being developed.

**Special cases and artefacts in force-distance curves**

In addition to the force-curves described above, many other types of event can be observed, although it is beyond the scope of this review to cover all of them. One notable special case is the ‘punch through’ that occurs when a force curve is collected on a surfactant-covered surface. These curves have a short, smooth repulsive component, which is followed by a clear 4–5 nm downward deflection (depending on the type of surfactant) as the tip penetrates with surfactant. There is also an interesting prediction for the ‘escape’ of a polymer from beneath an AFM tip during the approach part of a force curve, where there is expected to be a smooth repulsive force as the polymer is compressed and a sharp downward deflection as the polymer escapes.

There are several types of artefact in AFM force curves. An offset between the lines of the non-contact (zero-deflection) parts of the curves usually results from viscous drag on the cantilever and can be eliminated by reducing the scan rate or the viscosity of the solution. Periodic oscillations in the non-contact portion of the curve are common and typically arise from optical interference between reflections of the diode laser from the cantilever and the substrate. It is also common to see an offset between the lines that define contact; this can have two causes.

**Isoforce imaging**

Fig. 4 Approaches to producing spatially resolved force measurements. (a) Force-volume imaging is based on collecting arrays of force curves. Individual curves are transformed into force–distance curves and all the curves are assembled into a three-dimensional force volume. (b) Isoforce imaging is based on a surface of equal force collected over or below the sample surface. By collecting a stack of such isoforce surfaces at different forces, it is in principle possible to reconstruct a force volume. However, this is not possible in practice with current instruments.

In the first case, the contact lines are separated but converge to the point at which the scanning reverses direction and starts the retract part of the curve. This form of contact-line hysteresis results from nonlinearities in the piezoelectric ceramics used to move the sample. In the second and more-common case, the contact lines are parallel and exhibit a distinct ‘jump’ at the turnaround point. This type of behaviour results from friction on the tip as it slides across a surface and the associated anomalous bending of the cantilever. Offsets in contact lines can be a serious problem because they make it difficult to determine true separation distances.

The anomalous bending of the cantilever caused by frictional effects is a special case of the general endslope problem, which results from the optical detection method used for measuring cantilever deflections. Because the deflection of the cantilever is inferred from the change of slope at one point on the cantilever, anomalous bending of the cantilever (i.e. bending in a way other than the way the cantilever bends during calibration) can give rise to erroneous measurements. This is a serious limitation of most current AFMs for quantitative force measurements.

**Force-curve feedback**

Although not yet widely applied, feedback in AFM force measurements can be extremely useful. The set point for force–curve feedback is sometimes referred to as a trigger. Two simple types of trigger are an absolute trigger, in which the tip and sample are brought
together until a predetermined cantilever deflection is reached and the tip is retracted, and a relative trigger, in which a deflection relative to the undeflected cantilever (beginning of the curve) is used as the turnaround point. The latter is particularly useful, because it compensates for cantilever drift during the course of an experiment, allows the examination of a specific range of forces and minimizes possible damage to the sample and contamination of the tip.

Spatially resolved force measurements

For a heterogeneous biological sample, the space above the sample surface can be thought of as a volume of force through which a probe moves. There are two basic approaches to examining the spatial distribution of forces within that volume using an AFM. The first is to collect an array of force curves over the surface and assemble these into a ‘force volume’ (FV) (Fig. 4a). The second is to reconstruct the same force volume by collecting a series of isoforce images, at increasing or decreasing forces, across the $x$–$y$ plane and assembling these (Fig. 4b). Because, as described below, the latter approach is currently not practical except in very limited ways, we use the term ‘force-volume imaging’ to refer to arrays of force curves.

**Force-volume imaging**

A force-volume data set (FVDS) contains an array of force curves and a so-called height image. Each force curve is collected as described above, except that the sample is also translated in the $x$–$y$ plane. The force-volume height image (FVH) is an array of $z$-piezo positions at the turnaround points (points of maximal deflection) of the force curves. Typically, the force curves making up a FV are collected using a relative deflection trigger, and so the FVH is a surface of equal force, not necessarily a height image in the sense of a topograph. Together, the FVH and the FV provide a three-dimensional, laterally resolved description of the forces over and within a sample. For a sufficiently dense collection of force curves, the lateral resolution of force-volume imaging is related to the distance dependence of the interaction. Thus, the lateral resolution varies within the volume and is not easily defined.

The size of most FVs is limited by the data-acquisition times. Because the acquisition time for a single force curve is typically between 0.1 and 10 sec, the time to acquire a force volume of, say, $64 \times 64$ curves is on the order of 10 min to 10 h. There is no particular limit to data collection in the $z$ direction, although most commercial instruments have data-acquisition rates of...
<100 kHz. A FVDS contains a great deal of information about the sample and this information can be extracted and displayed in multiple ways. Distributions of forces at different z-positions, isoforce surfaces and maps of the surface and material properties of the sample can all be constructed from the information in the FVDS. Analytical methods include fitting individual force curves to appropriate force laws and mapping the differences between isoforce surfaces, force curves or properties derived from isoforce surfaces and force curves.

** Isoforce imaging**

Isoforce imaging is the main method for determining sample topography by AFM. For this application, the tip is typically in contact with the sample and the feedback loop is set to maintain a constant cantilever deflection. However, in the presence of long-range forces, it is also possible to produce isoforce surfaces in a non-contact regime. As the sample moves beneath the tip and forces of varying magnitude interact with the tip, the feedback loop repositions the stage so that the cantilever deflection remains constant. The isoforce surface is the defined by the stage's positions during the scan. By collecting a set of isoforce surfaces at different forces, it is in principle possible to describe the full force volume above the sample. However, in practice, this is essentially impossible with current technology because of cantilever drift. Because current AFM cantilevers are extremely sensitive to temperature changes, the cantilever bends slightly with time, and any drift maps would make it impossible to assemble the volume. Drift is not a problem when collecting arrays of force curves, so long as a relative trigger is used.

** Adhesion maps**

Adhesion maps are typically produced by the most negative force detected during the retraction curve as the value for adhesion and plotting that value against the x–y position of each curve. Several types of spatially resolved adhesion map have been produced. For example, Hlady and colleagues have examined the spatial distribution of adhesion in grafted-polymer systems. There has been considerable interest in using adhesion mapping to produce spatially resolved maps of the distribution of specific proteins on the surfaces of living cells and some reports of these types of experiments have appeared. This is an extremely challenging problem that will require a great deal of work before its limitations and utility are fully understood.

** Electrostatic maps**

The surface-charge density of bacteriorhodopsin (BR) membranes was measured by them by comparing force curves collected over a BR membrane with curves from the adjacent alumina substrate (which also acted as a charge-density standard). Fitting the force curves to the electrostatic part of DLVO theory and using the known surface-charge density of alumina enabled them to extract charge information about the BR membrane. Korsching and Radmacher expanded on this work by collecting a FVDS over BR adsorbed to mica and constructing maps of the surface-charge density. As noted above, fitting the force curves to DLVO theory requires that the absolute separation between tip and sample be known. However, it is sometimes desirable to minimize or prevent contact between the tip and sample in order not to damage the sample or contaminate the tip. To address this problem, an approach called D–D mapping was recently described for producing relative charge-density maps without the need for an absolute measurement of D (and thus not requiring tip–sample contact). In this approach, isoforce surfaces are collected, using FV imaging, at two different salt concentrations. The difference between these isoforce surfaces is independent of topography and can be quantitatively related to differences in local charge density (or surface potential). Using this technique, relative charge-density maps were produced of lipid bilayers and BR membranes on mica (Fig. 5).

** Polymer-brush maps**

Recently, Brown and Heh used AFM force volumes to investigate neurofilaments and found that proteinaceous projections (side arms) from the center of the filament acted as grafted polymers. These side arms gave rise to a long-range (50 nm) repulsive force that is absent on the mica substrate or on neurofilaments that lack the side arms. Isoforce difference maps were also used to show qualitatively the distribution of the long-range forces.

** Elasticity maps**

Maps of cellular mechanical properties have been produced for a range of systems. Radmacher et al. used the Hertz model to analyse FV images of platelets and to construct one of the first AFM-based maps of cellular mechanical properties. A similar approach was applied to examine the contribution of the actin cytoskeleton to the local mechanical properties of cardiac myocytes and macrophages using pharmacological agents such as cytochalasin B. The lack of vinculin was shown in a murine carcinoma cell line to reduce stiffness by approximately 20%. Laney et al. applied the FV technique to map the elastic properties of cholinergic synaptic vesicles as a function of buffer composition. One problem with fitting force curves to the Hertz model is that the tip-sample contact point must be determined, and this is extremely difficult for soft samples. The realization that the work done by the cantilever (area under the force–distance curve) can be related to the local mechanical properties has provided one way to circumvent this problem (Fig. 5). However, this approach is still subject to the limitations of the Hertz model and questions of its applicability to highly anisotropic cellular systems remain. There are a number of more-sophisticated analytical frameworks that can be applied, but it is not yet clear which of the different approaches will prove to be useful. One view is that truly quantitative measurements of stiffness that are valid for soft biological materials remain to be developed.
and what is really of interest are the spatial and temporal changes in mechanical properties. In that context, the Hertz model may well be sufficient.

Future prospects

As AFM-based force spectroscopy continues to develop, it will become increasingly sophisticated and widely used. An obviously useful near-term application is mapping the distribution of specific molecules on various surfaces. More fundamentally, the current view of biological structures as being delimited by their van der Waals surface will be expanded to include forces that surround the structures, a representation one might call an interaction surface. These interaction surfaces will provide important insights into macromolecular assembly and the use of biological structures as construction elements in novel devices.

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