Choosing AFM Probes for Biological Applications

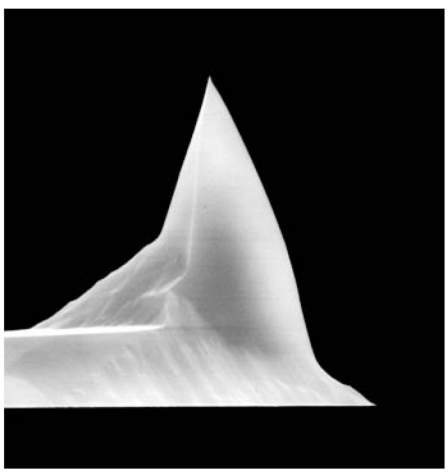

Figure 1. TappingMode Etched, Silicon Probe (TESP) used for imaging biological samples in air.

Introduction

AFM has proven to be a powerful tool for biological studies. Applications include imaging molecules[1-4], cells[5-7] tissues[8,9] biomaterials[10-12], and measuring forces[13-18]. Relative to such techniques as electron microscopy, the main advantage of working on biological samples with AFM is the ability to operate in liquids. Lateral resolution of better than one nanometer can be achieved[19] and a variety of sample sizes ranging from a few nanometers up to several micrometers can be imaged. Advances in sample preparation techniques, greater control of tip-sample interactions, and improved probes are allowing better resolution, study of more sophisticated samples, and even analysis of dynamic events[20].

The appropriate choice of AFM probe is crucial for optimal results when imaging biological samples. This application note provides an overview of probes available for biological applications, along with an explanation of the parameters to consider in the choice of the correct probe.

Probe Chemistry

The most common probe materials are silicon and silicon nitride because of their easy micromachining. The tip-sample interactions depend on the nature of the materials present. When working in liquid, the interaction also depends on the pH and the electrolytes in solution. For example, in physiological conditions (aqueous solution of electrolytes, pH around 7) silica (SiO_2) is negatively charged[26]. Repulsive forces may therefore occur when imaging a negatively charged sample.

For some applications, it is necessary to have a specific surface charge on the tip or to change its hydration properties. This can be achieved by silanization or plasma treatment. Also, increased hydrophobicity of the tip reduces the applied force by reducing hydration and capillary forces for imaging in air. The same process also reduces the wear of the tip when imaging in liquid.

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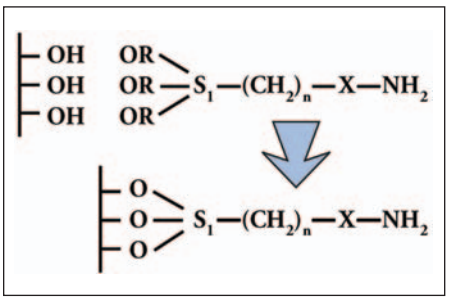

Figure 2. Schematic representation of the silanization process.

Plasma treatment

Plasma processing can change the hydration properties of the tip. Glow discharge in a hexafluoro–propene–atmosphere, resulting in a Teflon-like coating of the tip, renders silicon nitride cantilevers hydrophobic due to the hydration properties of Teflon[27]. One minute glow discharge in air renders silicon nitride probes hydrophilic[23,28] by "cleaning" the organically contaminated surface.

Silanization

Silanization (Figure 2) is another possibility for changing the wetting properties and the surface charge of a tip. Organochloro- or organoalkoxy-silanes are chemically bound to the probe surface. Non-reactive groups such as alkyls provide hydrophobicity. For example, trimethoxysilylpropyl-diethylene- triamine (DETA) renders a silicon oxide surface (oxide-sharpened cantilevers) positively charged and hydrophobic[29]. Lubricant coating – N-3(3- triethoxysilylpropyl)perfluoro (polyisopropoxy 2-methylacethyl) amide for example – can also be used to render silicon nitride tips hydrophobic and, thus, reduce adhesion forces that might damage the sample[30].

Note that Silanization is also useful for modifying the surface chemistry of the substrates used for sample support in biology, for example to promote the binding of molecules to the substrate. A typical example is the binding of DNA molecules onto a mica surface treated with APTES (Aminopropytriethoxysilane), which renders the surface positively charged[31].

Tip Functionalization

Functionalization of tips by coating them with molecules has opened a new research area for studying specific interactions on a molecular level, e.g. ligand-receptor pairs or cell-cell-interactions (Figure 3).

- *Chemical coating* of probes is mainly done by silanization or by functionalized thiols and is often a first step before biological functionalization.
- *Biological coating* has been mostly used for mapping the distribution of binding partners on samples[32-34], as well as for force measurements[13,35,36]. It becomes therefore possible to investigate molecular forces, like:
  – forces between a receptor and a ligand[13,37], including antigen-antibody-pairs[38].
  – forces between molecules and cells.
  – forces between cells[36,39].

Intramolecular forces have also been measured with great success[40].

Many protocols can be performed to attach proteins to a tip. Sometimes non-specific binding is enough, but more complicated protocols may be required. Most of them use a spacer to covalently bind the proteins. The advantage is that it is possible to orient the protein in order to expose specific site(s) of the proteins (enzymatic site for example). Here are a few examples:

- Polyethyleneglycol (PEG) is a common spacer. A terminal thiol group can be first attached to PEG and this thiol group can bind to a gold coated silicon nitride tip. An amine group at the other end of the PEG molecule attaches proteins (antibodies for example) via a covalent bond[41].
- To coat the tip with biotin, it is possible to first coat the tip with bovine serum albumin (BSA), which allows the attachment of biotin.
- Oligonucleotides can be covalently attached to the tip and to probe sequence specific interactions.
- Cells can be grown onto tipless cantilevers or onto cantilevers with small beads at the end. They can also be chemically attached via polyethyleneimine (PEI) interactions for example[42].

Important probe Parameters

Two fundamental parameters of an AFM probe are tip shape and mechanical parameters (the cantilever spring constant, resonance frequency and quality factor "Q").

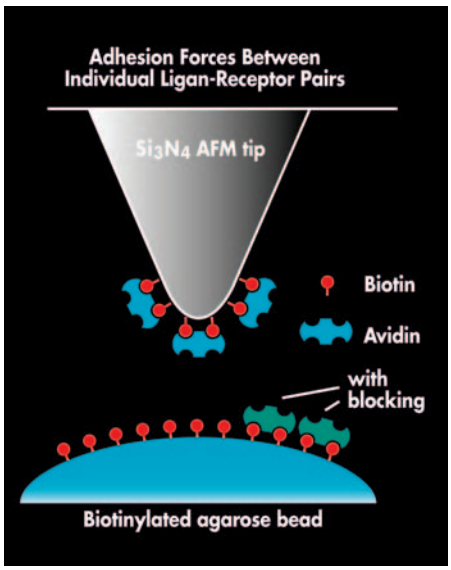

Figure 3. Functionalized cantilever for force measurements on ligand-receptor pairs[14].

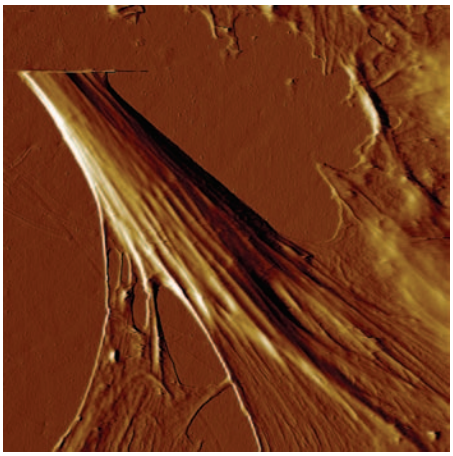

Figure 4. Dissecting a cell membrane locally by applying too high forces.

Tip shape

The radius of curvature of the tip, or tip sharpness, determines the lateral resolution. An AFM image is always a 'combination' between the tip shape and the sample topography. Therefore the duller the tip is, the wider the topography appears ("tip broadening" effect). Most of the time, optimal resolution on biomolecules requires the minimum possible tip radius[43]. Nevertheless, it is sometimes preferable to use dull tips rather than sharp tips, when imaging cells for example, because the pressure exerted on the sample is less[44]. A sharp tip can poke through the membrane and damage the cell[45,46] (Figure.4).

To help quantify the tip broadening it may be useful to do a quality assessment of the probe. Knowing the tip shape is then essential to restore the sample surface. The following methods help for studying the tip shape and restoring the true sample surface:

- Imaging and measuring the tips with electron microscopy[47], or with field ion emission microscopy.
- It is also possible to estimate the tip shape by scanning a sample of known topography such as a synthetic calibration standard like colloidal gold particles[48], latex beads[49] and calibration arrays, or by scanning well known biomolecules like DNA[50]. Note that the Tip Evaluation Option available on the Digital Instruments NanoScope® software contains a calibration standard to estimate tip shape.
- The sample topography can also be calculated by estimating the tip shape with mathematical morphology operations without using a known standard topography ("blind" mathematical restoration)[51-55]. These non-linear mathematical operations (dilation and erosion) consist in an over- and under-estimation of the tip broadening effect (Figure 5).

Due to variations in etching during production, double tips can sometimes be generated, yielding images in which the topography appears repeated (Figure 6). This is a very common artifact, and any user should be able to recognize it.

Spring constant

The spring constant k is defined as the ratio between the applied force F and the cantilever deflection Δd:

$$k = \frac{F}{\Delta d} \quad \textit{(Hooke's Law)}$$

For force measurements it is crucial to know the value of the spring constant. The accuracy of the force measurement is determined by the error of the force constant and by any errors in the detection system.

Different methods have been proposed to determine the spring constant:

- Acquiring the thermal vibration spectrum of a cantilever and deducing the spring constant of the cantilever from the equipartition theorem[56,57].
- Measuring the change of the resonance frequency while loading the cantilever with small weights[58].
- Measuring the resonance frequency and calculating the spring constant from the geometrical dimensions of the cantilever[59] or from its quality factor, Q[60].
- Measuring the deflection of the cantilever when loading with small weights[61] or while exerting a force with another cantilever of known force constant[62].

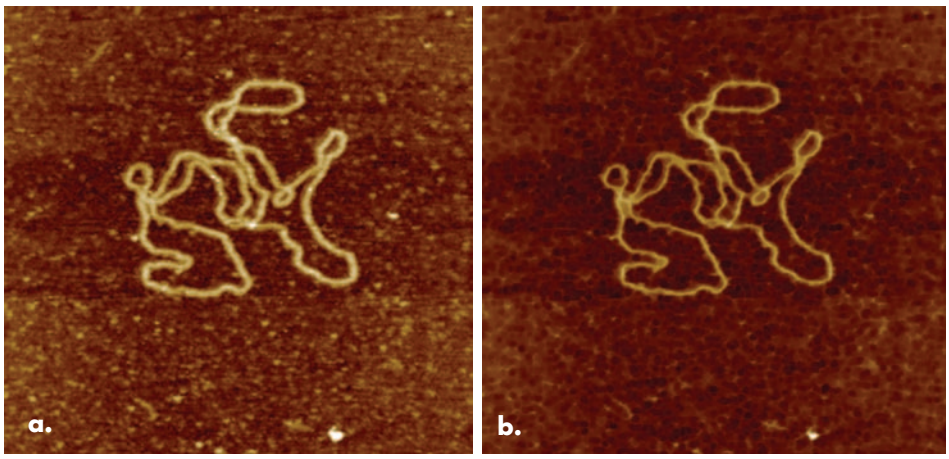

Figure 5. Morphological filter applied to an AFM image (a) to reconstruct the DNA shape (b).

Resonance frequency and quality factor

The resonance frequency is the first natural mode of vibration of a cantilever and is determined by the material, geometry and the environment (air or liquid). TappingMode is performed with a cantilever oscillating preferentially close to its first natural mode of vibration. In air, typical values of resonance frequencies are 900Hz to 88kHz for silicon nitride cantilevers and 60kHz to 400kHz for silicon cantilevers, depending upon the cantilever geometry. In liquid, with silicon nitrides probes (100µm long, narrow legs, spring constant = 0.06N/m), we recommend choosing a frequency between 8 and 10kHz. Note that it is important to tune the frequency very close to the surface because there can be a frequency shift as the tip approaches the surface.

The quality factor Q of the cantilever is another parameter of interest affecting the scan speed and the sensitivity. Q is defined as $Q = \frac{2\pi m F_0}{b}$ or $Q = \frac{F_0}{\Delta F'}$ where:

m = cantilever mass
F_0 = resonance frequency
ΔF = width of the resonance peak
b = damping factor

Q is a measure of the dissipation mechanisms that damp the oscillation of the cantilever. A high Q is desirable for TappingMode to optimize the sensitivity[63]. Typical values of Q for cantilevers in air are 100 to 300, and around 1 in water due to hydrodynamic damping[64,65].

Problems of Tip Contamination

Tips can easily become contaminated during the scanning process. This is especially the case for biological samples that can easily detach from the surface or from the sample itself, like proteins. This can result in double tip images (Figure 6) and/or reduction of the lateral resolution. Imaging tips with electron microscopy after imaging with an AFM can show the contamination of the tip[47]. A change in the image during a scan should always alert the user about an alteration of tip quality.

Cleaning tips

Cleaning a tip is a good way to decrease contamination from the fabrication process or storage. This precaution can increase the image resolution. The most popular cleaning method is UV-light treatment that produces ozone and removes organic debris[66]. Another possibility is to incubate the cantilevers in a piranha solution (mixture of sulfuric acid and hydrogen peroxide) for 30 minutes. This procedure is used to clean silicon and silicon nitride surfaces in the electronics industry and also removes the silicone oil contamination often introduced from the cantilever packing material[67].

Plasma ashing/etching, also derived from the electronics industry, is known to remove organic contaminants. Here, hydrogen, oxygen and argon plasma react with carbon compounds or oxides on the surface[68]. For example, exposing cantilevers to argon plasma (80W) for 30 seconds seems to sufficiently clean probes for tip functionalization[28]. CO_2 snow also removes organic debris from the surface because of a transient formation of liquid CO_2[69].

Note that a "cleaned" probe is generally hydrophilic because of the removal of the organic contaminants that are generally present in ambient air.

Figure 6. "Double tip" image of two DNA molecules.

Choosing Probes

Table 1 is a guide to choosing probes for various and typical biological samples as deduced from a wealth of user experience. As previously mentioned, the main choice of probes is between two families — silicon and silicon nitride probes, differing primarily by their spring constant. The table indicates the probe as a function of sample type, as well as the preferred imaging mode (TappingMode vs. Contact Mode). Unless otherwise noted, when discussing TappingMode in liquid, we are referring to the indirect drive fluid cell. Magnetic actuated TappingMode is discussed separately. For more detail about these and other probes, please visit www.veecoprobes.com.

SAMPLE TYPE	PROBE FAMILY/MODEL		EXPERIMENT		AFM MODE	
			LIQUID	AIR	TAPPING	CONTACT
Biomolecules (Nucleic Acids, Proteins, Lipids, Carbohydrates, etc.)	Silicon	OTESPA	—	x	x	—
		RTESP	—	x	x	—
		TESP	—	x	x	—
Biomolecules (Nucleic Acids, Proteins, Lipids, Carbohydrates, etc.)	Silicon Nitride	(D)NP-S	x	—	x	x
		NP-STT	x	—	x	x
		OTR4	x	—	x	x
Cells	Silicon Nitride	(D)NP	x	—	x	x
Tissues	Silicon	TESP	—	x	x	—
Tissues	Silicon Nitride	(D)NP	x	—	x	—
		(D)NP-S	x	—	x	—
Biomaterials	Silicon	FESP	—	x	x	—
		OTESPA	—	x	x	—
		TESP	—	x	x	—
Biomaterials	Silicon Nitride	(D)NP-S	x	—	x	—
Force Measurements	Silicon Nitride	(D)NP	x	—	—	x
Force Measurements		MSCT	x	—	—	—

Table 1. Recommended probe types for specific biological samples and imaging modes.

Key to Probe Model Names

Silicon Probes:

OTESPA: TappingMode Etched Silicon Probe. General purpose, air, TappingMode probe with backside coating for increased laser reflectivity.

RTESP: TappingMode Etched Silicon Probe. General purpose, air, TappingMode probe with the tip rotated to optimize sidewall angle symmetry in the fast scan direction. While advantageous on some samples with tall, sharp step features, this feature is not generally important on most biological samples.

TESP: TappingMode Etched Silicon Probe. General purpose, air, TappingMode probe, interchangeable with RTESP in most applications.

FESP: Force Modulation Etched Silicon Probe. Originally intended for force modulation experiments, this probe also works well for general purpose TappingMode in air in case where its lower spring constant is advantageous (i.e. soft samples).

Silicon Nitride Probes:

(D)NP-S: Oxide-Sharpened Silicon Nitride Probe. General purpose probe for both contact mode and TappingMode in fluid. Four different cantilevers on each substrate allow an appropriate choice of spring constant for different experiments. Optional "D" prefix indicates low stress cantilevers recommended for Digital Instruments Dimension-series scanners.

(D)NP: Silicon Nitride Probe. Same as (D)NP-S probes, except that the tip is not oxide-sharpened, resulting in a less sharp tip. This is advantageous on some samples, especially cells, where a sharp tip may damage the sample. Optional "D" prefix indicates low stress cantilevers recommended for Digital Instruments Dimension-series scanners.

NP-STT: Oxide-Sharpened Silicon Nitride Probe Twin Tip. These probes are very similar to the NP-S probes except their tips are formed by a process that produces a considerably sharper tip. However, a second, shorter tip is also formed near the main tip as result of the process. The second tip is not intended for imaging and can result in "double tip" artifacts if it contacts the sample. For this reason the NP-STT probes are restricted to samples with small vertical features (typically less than 100 nm).

OTR4: Oxide-Sharpened Silicon Nitride Probes. General purpose probe for both contact mode and TappingMode in fluid. Two different cantilever spring constants. Generally interchangeable with NP-S probes.

MSCT: Sharpened Contact MicroLevers with Backside Gold Coating, oxide sharpened tip. General purpose probe for both contact mode and TappingMode in fluid. Six different cantilevers offer a wide range of spring constants. These are particularly popular for piconewton-scale for measurements as some of the spring constants are very low. Also unique in that one of the levers is rectangular, rather than triangular like most silicon nitride levers.

Conclusion

Choosing the correct probe is a crucial part of working on biological samples. The chemical and physical parameters of the probe greatly affect AFM measurements and can reduce resolution. A large number of commercially available probes already enable many different applications and skilled users are increasingly modifying these probes to achieve their specific goals.

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