AFM Nanotools for Surgery of Biological Cells

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Abstract. Using a method of electron-beam induced deposition, we have been able to fabricate specialized AFM probes with application as “nanotools” for the manipulation of biological structures (“nanosurgery”). We describe several such tools, including a “nanoscalpel”, “nanoneedles” for probing intracellular structures, and a “nanotome” which can separate surface layers from a biological structure. These applications are demonstrated by performing nanomanipulation on corneocyte cells from the outer layer of human skin.

1. Introduction

There is increasing interest among biologists in the ability to manipulate both single cells and smaller intracellular components at the micro- and nano-scale. This manipulation is often described as “nanosurgery”. In recent years, technologies for the manipulation of nanoscale structures, including optical tweezers [1], laser microscalpels [2], and the atomic force microscope [3], have allowed nanosurgery to be applied in a variety of different ways. Nanosurgery has been used to isolate intracellular organelles [1] or single cells [4], for the dissection of viruses [5], collagen fibrils [6], plasmids [7], chromosomes [3], and for in situ dissection of bacterial cell walls [8] to image their internal structure. It has also been used to cut single cytoskeleton filaments [2] and to create incisions in cell membranes [4].

Of the techniques available to biologists, the atomic force microscope (AFM) can manipulate structures with the highest precision. AFM probe tips are typically ~5-10 nm in radius and can be positioned with nanometre accuracy, allowing for very precise control of the manipulation combined with high resolution 3D imaging of the sample. [9] However, standard AFM probes are not ideal for manipulation. They are typically pyramidal in shape with a low aspect ratio, decreasing the precision of nanomanipulation as the tip penetrates deeper into a sample, and are usually fabricated from crystalline silicon or silicon nitride, which are relatively brittle materials that can easily be damaged during manipulation. High lateral precision is desirable in nanosurgery; just as in macroscopic surgery and dissection, low precision can cause great damage to the tissue. Several approaches have been used to improve the aspect ratio of AFM tips, including the attachment of carbon nanotubes to the AFM probe tip [10,11,12,13] and the fabrication of a needle-like structure on the tip apex using focused ion beam milling. Needles fabricated by this technique have been used for microinjection of biomolecules (including DNA) into cells and specific organelles. [14,15,16,17,18,19]

Another technique which has been widely applied for the modification of AFM probes is electron beam induced deposition (EBID). [20,21,22,23,24,25] EBID is a highly versatile technique for the
fabrication of two-dimensional and three-dimensional nanostructures. [26,27,28,29,30,31,32,33] To fabricate a structure using EBID, a high-energy focused electron beam in an electron microscope is used to “write” the structure. The electron beam causes the emission of lower-energy secondary electrons from the atoms of the substrate, which are able to decompose hydrocarbon precursor molecules adsorbed onto the substrate surface. The sources of these hydrocarbon molecules are the trace gases and normal contaminants present in the vacuum system of the electron microscope [23,25,34], although some groups have performed EBID by deliberately introducing precursor molecules using a gas source. The decomposition of these molecules creates a deposit of carbon atoms on the substrate surface, creating a nanoscale structure from amorphous carbon.

We have used EBID to fabricate a variety of different structures on standard AFM probes for different nanomanipulation applications, including “nanoscalpel” blades which can be used to cut and dissect biological structures as well as for mechanical nanolithography of inorganic materials. [35] In this paper, we describe a number of different “nanotools” which can be applied to different nanosurgical applications, including high-aspect ratio “nanoneedles” for microinjection into cells, extraction of material from target sites on the surface of a cell, and indentation on cells to determine their mechanical properties. Other nanotools which we have fabricated include a “nanotome” for removing material from the surface of cells to image the underlying structures in situ. In future, these tools could be used for sophisticated manipulation of biological cells, offering a new approach to the investigation of the properties of cells.

We have also investigated the mechanical properties of these probes by bending the EBID carbon blades using AFM manipulation. In the process we discovered that the deposited structures are extremely flexible, capable of recovering elastically from deformations of up to ~75% of their total length. [36] This indicates that they are highly robust, and able to withstand and recover from large applied forces during nanomanipulation.

2. Fabrication of Nanotools
Fabrication of a nanostructure on an AFM probe was performed using a scanning electron microscope (Hitachi SE-4300) equipped with a Raith electron beam lithography (EBL) system. Typically, a beam energy of 20kV and a beam current of 0.1nA were used. In our experiments, the pressure within the SEM system was ~10^-3 Pa. The electron beam was moved laterally from the AFM tip apex, depositing a line of carbon which extends beyond the tip as a self-supporting structure (see Figure 1a). [37] The dimensions and geometry of the nanostructure depend on the velocity at which the beam spot is moved over the surface. At low beam spot velocities (1-6nm/s), the deep penetration of the electron beam into the substrate and deposited structure means that it grows as a plate-like or blade-like structure in the
plane of the electron beam (see Figure 1 b-c). The blade-like shape of this structure makes it ideal as a “nanoscalpel” for making fine incisions in the surface of a sample such as a cell, thereby acting as a dissection tool.

Figure 2: (a) Thin nanoneedle probe for high-resolution imaging. (b) Needle after exposure for ~26 mins in SEM, depositing additional carbon to stabilize needle against bending.

The growth of the structure in the lateral direction is restricted by the much smaller penetration of the secondary electrons through the deposited material. The width of this blade-like structure decreases with increasing beam spot velocity. For higher velocities (6-9 nm/s) the blade width and thickness are approximately equal (~20-30nm), resulting in a cylindrical, needle-like structure.

The thin needles fabricated by this technique can be used as high-aspect ratio AFM probes for the profiling of samples with steep, high surface features or deep trenches. An example of a nanoneedle is shown in Figure 2a. However, these thin structures are flexible and this limits the force which can be applied. [36] To stabilize the needle, it can be imaged at high magnification (concentrating the electron beam into a smaller area and so increasing the beam flux), depositing additional carbon onto the needle and increasing its diameter. Typically, imaging for ~30 minutes at a magnification of 250,000x can increase the diameter of a needle from about 20 to 90 nm. During this process, the growth rate is even on all sides of the nanoneedle; it therefore retains a well-defined geometry with a circular cross-section and a smooth, hemispherical tip. An example of a tip stabilized by thickening the needle using this process is shown in Figure 2b. Control of the geometry is important if the needle is to be used as an indenter to determine the mechanical properties of a biological structure because its shape determines the dynamics of its interaction with the sample during indentation. The simultaneous SEM imaging of the nanoneedle during the stabilization process allows control of the diameter and shape of the needle.

The EBID process is highly versatile and can be used to create complex structures. This allows the fabrication of highly specialized nanotools on AFM probes for manipulation of samples. For example, a “nanotome” device was fabricated which can be used to remove thin layers from the surface of a biological structure using a single pass of the AFM probe, in a manner similar to a vegetable peeler. This consists of two parallel nanoscalpel blades with a suspended filament between the blade tips. Examples of these structures are shown in Figure 3a-c. To fabricate this structure, the parallel blades were tilted after EBID fabrication to expose only the blade tips to the beam. This prevents the growth of multiple filaments, which often occur in depositions extending from the edge of thick substrates. Since the primary electron beam can penetrate deep into the substrate, the generated secondary electrons can cause deposition from multiple sites at the edge of a thick substrate, producing multiple separate filaments. [38] In the case of the nanotome fabrication, tilting the blades to deposit a filament
across their tips allowed the successful deposition of a single carbon filament supported by the nanoscalpel blades. The growth process is illustrated in Figure 3d.

![Figure 3](image)

Figure 3: (a) Suspended filament between nanoscalpel blades. (b) Image from above showing single filament. (c) Similar structure deposited on an AFM tip, creating a “nanotome”. (d) Process of fabrication of a single filament across tips of parallel blades.

3. Cell Sample Preparation

To test the capabilities of these probes, we performed investigations on corneocytes. These cells form the outermost layer of mammalian skin, the stratum corneum, which forms a barrier against water loss from the body. The cells were isolated using an adhesive stripping technique from the upper skin layers of healthy human subjects. Commercial hair removal wax, heated to 65ºC on glass slides, was used as an adhesive to remove the cells from the skin surface. This method is an adaptation of a commonly used tape-stripping technique. [39]

Corneocytes were chosen because they are easily harvested and prepared, and have a comparatively rigid structure compared to other cell types (making them easier to manipulate). They can also be imaged using AFM in air without the need for dehydration, fixation or other complex sample preparation techniques, unlike most other mammalian cell types which must be imaged in liquid if not fixed. We were able to image the corneocytes in air using AFM; examples of AFM images of corneocytes are shown in Figure 4.

4. Manipulation of Cells

We successfully showed that nanoscalpel probes could be used to make incisions of controlled depth on the surface of the corneocytes. To control the force on the AFM probe during an incision, a feedback loop was implemented to hold the AFM cantilever deflection at a fixed setpoint value during the cut. The force exerted on the sample is proportionate to the cantilever deflection and so this
feedback loop holds the applied force at a constant value. This allows the applied force and hence the cut depth to be controlled. It also prevents a large tip-sample force from breaking the nanoscalpel or detaching it from the AFM tip. Incisions using a nanoscalpel probe on cells and on inorganic structures have recently been described in detail [35].

Figure 4: (a) AFM image of corneocytes. (b) Image of a small area of a corneocyte surface, showing many small, round structures which are probably protein clusters.

Manipulation and imaging using the nanotool probes was carried out using a Veeco Multimode IIIa AFM running Nanoscope software. The manipulation program was written such that the AFM probe would first profile the sample surface along the cut path in tapping mode. The probe would then return to the start of the cut path, and the tip-sample force would be increased until the cantilever deflection reached the chosen setpoint, then moved along the cut path to create a cut. An example of an incision on the surface of a corneocyte is shown in Figure 5.

The high aspect ratio of the nanoscalpel allows penetration into the cell to large depths without loss of lateral precision, creating a narrow incision in the surface. The penetration into the cell can be calculated by comparing the topographic profile taken before the cut with the path taken by the nanoscalpel tip during the cut. The depth of the cut was shown by this method to increase in proportion to the applied force; this could be used to control the depth of penetration of the scalpel blade. This point is illustrated in Figure 6.

EBID nanoneedle probes could be used for the accurate imaging of the corneocyte surface, and for indentation on the surface to probe mechanical properties of the cell. The penetration of the needle can be measured by examination of the AFM force curve; the deformation of the cell can be measured by comparing the deflection of the AFM cantilever recorded during an indent on the cell surface to that on a rigid sample (e.g., a clean silicon wafer) which the nanoneedle cannot penetrate. This process is illustrated in Figure 7a. The high aspect ratio cylindrical needle shape allows it to penetrate the sample to a large depth without loss of lateral precision. An example of the indent remaining in the corneocyte surface is shown in Figure 7b.

Nanoneedles thickened to ~75 nm diameter were able to exert forces of up to ~1.5 µN on a sample without detectable damage or deformation of the needle structure. The thickened nanoneedles can be used for imaging cell surfaces and for indentation studies. During these experiments, we found that indenting on a protein cluster on the surface of a corneocyte cell apparently led to its removal, leaving a pore in the surface of the cell. Indentation a second time appeared to replace an object at approximately the same site; it is likely that this object was the same protein cluster. This indicates that the structure had adhered to the nanoneedle, and could be transplanted to a desired site using the
nanoneedle tip. This shows that AFM nanotools may be used for the extraction of samples from cells, and for the manipulation of subcellular structures. This process is shown in Figure 8.

It was also shown that nanotome probes can be used to separate the outer layers of a biological structure from the underlying layers. The two nanoscalpel blades create parallel cuts in the cell surface, while the suspended filament cuts beneath the surface layer to separate it from the underlying material. This is illustrated diagrammatically in Figure 9a.

A slight asymmetry in the length of the parallel nanoscalpel blades meant that only one blade tip touched the sample surface during normal imaging, allowing the nanotome probe to be used to capture reasonably accurate images of the sample surface. This also permitted the alignment of the nanotome over a target site on the surface. Increasing the tip-sample force and making an incision using the same manipulation program as was used for the nanoscalpel brought both blades into contact, creating two parallel cuts. After this manipulation, the probe was passed across the cuts in a perpendicular direction. This moved aside the surface layer between the parallel cuts, indicating that it had been separated from the underlying structures by the suspended filament. After displacing the upper layer in this way, an unmodified sharp AFM probe could be used to image the structures which had previously been concealed beneath the outer layer of the corneocyte, as shown in Figure 9b.

5. Conclusions

A number of different AFM tools for the manipulation of biological structures have been developed using electron-beam induced deposition (EBID). These include a “nanoscalpel” capable of making incisions to a controlled depth in the surface of biological objects, and “nanoneedle” probes which can be used as high-aspect ratio probes for the imaging of samples with steep surface gradients. Thickening of these nanoneedles by deposition of additional carbon onto the needle creates a stiffer structure which can exert large forces on a sample; these stabilized needles can be used to indent on the surface of a biological object, penetrating it to a large depth without loss of lateral precision. This could be used for nanoindentation experiments to determine the mechanical properties of intracellular structures, and for the microinjection of materials (e.g., DNA) into the cell. The needle can also be used for the extraction of a sample from the cell, as shown by the experiment in which a protein cluster was removed from the surface of a cell, and then replaced, by indentation using a nanoneedle.

Suspended filaments were successfully fabricated between the tips of parallel nanoscalpel blades using EBID. This created a “nanotome” tool which can be used to remove part of the outer layer of corneocyte cells by AFM manipulation, exposing structures beneath for AFM imaging in situ.

These nanotools provide a new route to the specialized nanomanipulation and nanodissection of biological structures. Combined with the precision and force sensitivity of AFM, they can be used to manipulate cells at the scale of individual organelles and subcellular structures. In future, these tools could open up novel methods of investigation of the structure and function of biological systems at the nanoscale.
Figure 6: (a) Comparison of undamaged surface topography of a corneocyte with the path of a nanoscalpel during cut. (b) Depth of cut as a function of applied cutting force; data compiled from incisions on 2 different corneocyte cells.

Figure 7: (a) Force curve recorded during an indent using a nanoneedle; the deformation of the cell and the remaining indentation depth can be determined from the curve by comparison to a calibration curve recorded on a rigid substrate. (b) AFM image of a nanoneedle indent on the corneocyte surface. (1.0x0.5µm area).

Figure 8: Removal of a protein cluster using a nanoneedle. (a) AFM image of corneocyte surface before indent. (b) Surface after indent showing pore created in surface by nanoneedle tip. (c) Image after indentation was performed a second time at the same site. The protein cluster has been deposited back onto the surface of the cell after adhering to the needle during the first indent. Imaged area is 1.0x0.75µm.
Figure 9: (a) Use of a nanotome to remove an area of the cell sample surface. (b) Area of corneocyte surface which has had a section of surface removed using the nanotome. Imaged area is 1.0x1.0 µm.

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