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Commentary

Atomic force microscopy as a novel pharmacological tool

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Abstract

With the advent of the atomic force microscope (AFM), the study of biological samples has become more realistic because, in most cases, samples are not covered or fixed, which makes it possible to observe them while the cells are alive. This advantage of the AFM allowed the advent of a new invention: nanobiosensors using the cantilever (probe) of the AFM and, in this case, it is possible to observe the entering or exiting of specific molecules (including medications) from living cells. This is the smallest biosensor in the world, measuring about 100 μm long (about the width of a hair). Beyond sensing the area of interest with this biosensor, it is also possible to see the area and exactly what is occurring on it, in real time. This new tool will be very useful for several areas: molecular pharmacology, enzymology, physiology, molecular biology, biotechnology, biophysics, physical chemistry, analytical chemistry, and organic chemistry. This article discusses, mainly, the applications of this new technique to the field of pharmacology.

Keywords: Atomic force microscopy; Biosensors; Electrodes; Penicillin; Drugs; Drug delivery; Calcium blockers; Nifedipine derivatives

1. How the atomic force microscope works

The AFM works like a profilometer works: by moving a commercial microfabricated tip across the sample while recording the X, Y, and Z coordinates of the preparation being scanned. This tip is held at the end of a thin, flexible, cantilever (100 μm long; about the width of a hair) comprised of two layers: a gold mirror and a silicon layer (Fig. 1) [1–8]. The Z coordinate is calculated by having a laser beam reflect off the surface of the cantilever. The reflected laser light reaches the surface of a split photodiode that transforms this reflected laser beam into electrical pulses. These pulses are sent to a computer that records changes in the position with high precision, producing an image on the monitor screen [1–7,9]. For example, in Fig. 2, we can see the scanning of a sample by the cantilever; the sample topography makes the laser beam change its direction. Changes in direction produce a variation of the electrical pulses sent to the computer by the photodiode. The computer transforms this information into dark and light regions, producing a contrasted and three-dimensional image of the sample.

2. Advantage of the AFM in relation to the scanning electron microscope

For a very long time, SEMs have been used to obtain high resolution visualizations of the surfaces of biological samples. In the SEM, the image of the specimen surface is formed by recording secondary electrons or backscattered electrons emitted from the area irradiated by the scanning electron probe. This is done with an electron detector placed sideways from the specimen. The surface of the specimen should be electron conducting, which, in most cases, requires that the specimen surface be covered by a conducting metal layer obtained by sputtering. The contrast is due mainly to the difference in the electron collection efficiency depending on the angle of emission and surface relief, but it also depends on the atomic number of the elements [10].

An SEM gives a high depth of field and high contrast three-dimensional images of specimen surfaces where cells appear shaded [10].

Normally, in order to scan samples of *Saccharomyces cerevisiae* cells with an SEM, each preparation is coated with a film of evaporated gold (approximately 20 nm in thickness [2,11]). The application of this conductive

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Abbreviations: AFM, atomic force microscope; SEMs, scanning electron microscopes; and SNP, single nucleotide polymorphism.

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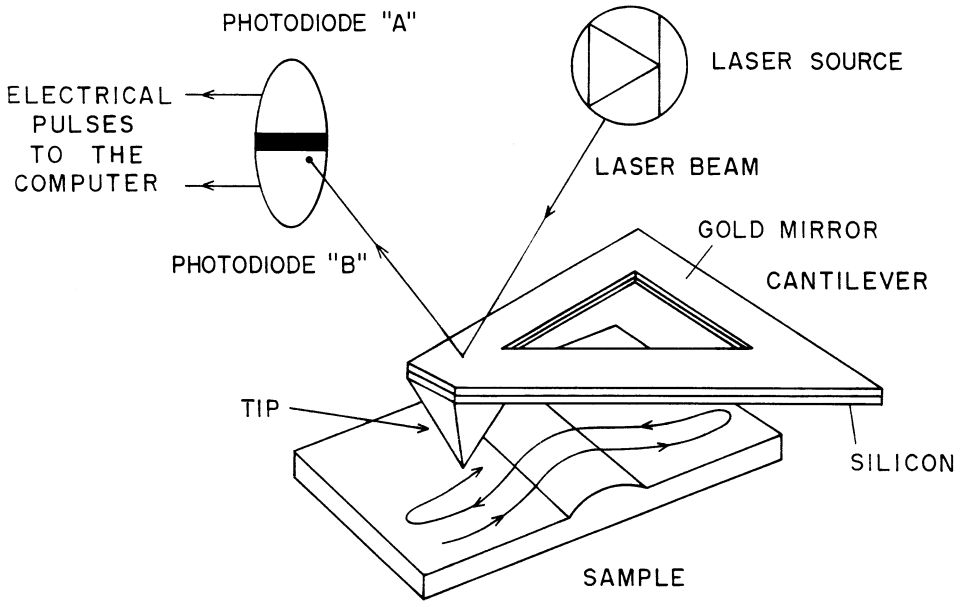


Fig. 1. Working principle of the atomic force microscope (AFM).

coating to the surface of the sample masks important structures that exist on the cell wall [1,2], and the cells are killed during the preparation. With the advent of the AFM, this fact changed, and the cells now are visualized uncoated and alive, at high resolution, producing excellent and reproducible images. Some years ago, my research group generated the world's first micrographs of different industrial strains of *S. cerevisiae* using AFM equipment [1], and we could observe structures, on the cell wall, never described before [1].

3. Use of the AFM as a heat detector

An apparatus for measuring variation of temperature is the thermo-optical detector. A water-containing reaction cell, whose bottom is coated with an enzyme immobilized on a thin gold film, is immersed into CCl_4 through which a probe beam is passed. The heat produced from the reaction between the enzyme and its substrate in the water phase is transferred to the gold film and the external CCl_4 phase. This generates a temperature gradient in the CCl_4 phase,

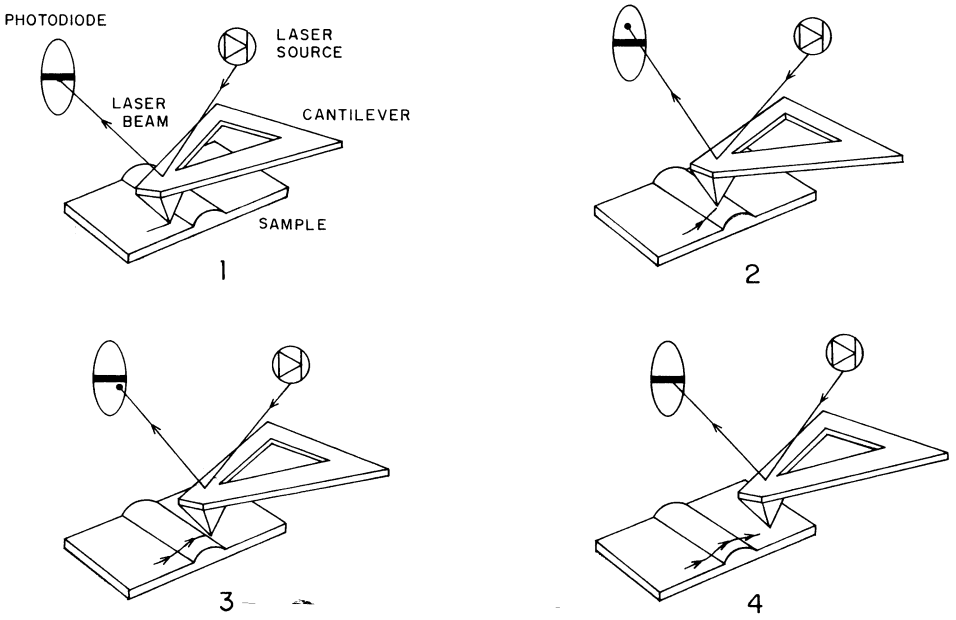


Fig. 2. Steps to explain the formation of an image by the AFM.

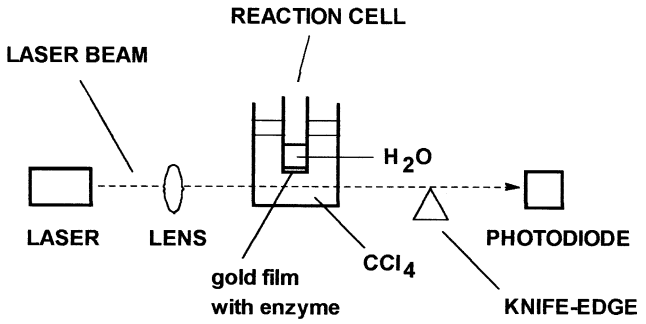


Fig. 3. Working principle of the thermo-optical detector. Reprinted with permission from FEBS Lett 2000;475:43–46. Copyright [2000] Federation of European Biochemical Societies. [Ref. 3].

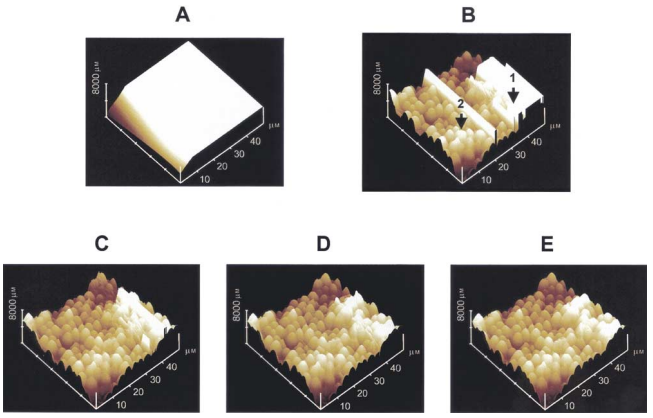


Fig. 4. Scanning of the surface of *S. cerevisiae* cells by the nanobiosensor (glucose oxidase immobilized on the cantilever surface). (A) The presence of high concentrations of glucose molecules induces a great number of deflections, impeding the visualization of the cells. (B) After 3 hr, some of the glucose molecules were absorbed by the cells and, since the number of deflections is lower, it became possible to see, partially, the images of the cells. Arrow 1 indicates the deflections of the cantilever due to the presence of glucose molecules, and arrow 2 indicates an *S. cerevisiae* cell. (C) Four hours after the first scanning (A), more glucose molecules were absorbed, and the image of the cells is nearly totally visible. (D and E) Five hours after the first scanning (A), it is possible to observe the image of the entire group of cells, indicating that nearly all of the glucose molecules have been absorbed by the *S. cerevisae* cells. Reprinted with permission from FEBS Lett 2000;475:43–46. Copyright [2000] Federation of European Biochemical Societies. [Ref. 3].

which induces a deflection of the probe beam, and the results are recorded as a graph [2–16].

Since the components of the AFM are similar to those of the thermo-optical detector (laser beam, lens, and photodiode) (Fig. 3), if an enzyme was immobilized on the cantilever, one would expect that the temperature gradient generated by the heat of reaction (between the enzyme and its substrate) would induce a deflection of the cantilever, transforming the AFM into an apparatus that could reveal the presence of specific molecules.

As mentioned before, with the AFM it is possible to visualize living cells, and, with this enzyme-coated cantilever (nanobiosensor), it should be possible to detect specific molecules being absorbed by living cells, together with the image of the cells. These assumptions were shown to be valid, as demonstrated by experiments made with the first nanobiosensor. The nanobiosensor was made in order to detect absorption of glucose molecules by *S. cerevisiae* cells. A suspension containing glucose and *S. cerevisiae* cells in ultra-pure water was scanned by this nanobiosensor. In the initial scannings, cell images failed to appear due to successive deflections of the cantilever, indicating the presence of glucose molecules in all the scanned areas (Fig. 4A). After 3 hr of incubation, part of this glucose was absorbed by living cells, and the image of the cells appeared in the point where there was no glucose (Fig. 4B). This image of cells was gradually completed after all the glucose molecules were absorbed (Fig. 4, C–E) [3].

The deflection of the cantilever is explained by a physical phenomenon when two different metal blades (with, of course, different coefficients of dilation) are bonded together. This is called a bimetallic system, and the presence of heat makes the blade bend. One practical example is the working of the tail lights of automobiles: a bimetallic blade is heated which bends, producing a braking light. The cantilever of the AFM is not a bimetallic blade but can work as one because it has two different materials (a metal and a semi-metal) with different dilation coefficients: gold and silicon. If a biochemical (or chemical) reaction takes place near this "bimetallic blade," it is possible to produce a deflection in the cantilever. This latter produces an enormous deviation of the direction of the laser beam, which is recorded in real time by the computer (via a photodiode) (Fig. 5).

When a cantilever without immobilized enzymes was used, no deflection was observed during the scanning process (Fig. 6). [3].

Beyond observing the absorption of glucose molecules by living cells, it was possible to visualize the opposite phenomenon: the release of specific molecules from living cells. In this case, the enzyme alcohol dehydrogenase type II, which transforms ethyl alcohol into glutaraldehyde and releases heat in the process, was immobilized on the cantilever surface (Pereira *et al.*, unpublished results) in order to detect ethyl alcohol molecules produced by *S. cerevisiae* cells. In the first 15 min, it was possible to see living *S. cerevisiae* cells (Fig. 7A). After a few minutes of incubation, the cells began to produce and release ethyl alcohol to the extracellular medium. This is observed in panels B–E of Fig. 7. It can be seen that ethyl alcohol molecules are produced gradually until they reach a saturation point (Fig. 7E) where it is impossible to visualize the image of the cells. At this point, orthophenothroline (a zinc chelator), which inhibits the activity of the enzyme alcohol dehydrogenase type II, was added. Then the inhibition of the enzyme stopped the deflections of the cantilever, indicating that the presence of the active enzyme is necessary to produce the deflections (Fig. 7F). This nanobiosensor will be very useful for organic chemists who work with synthetic chiral drugs [17].

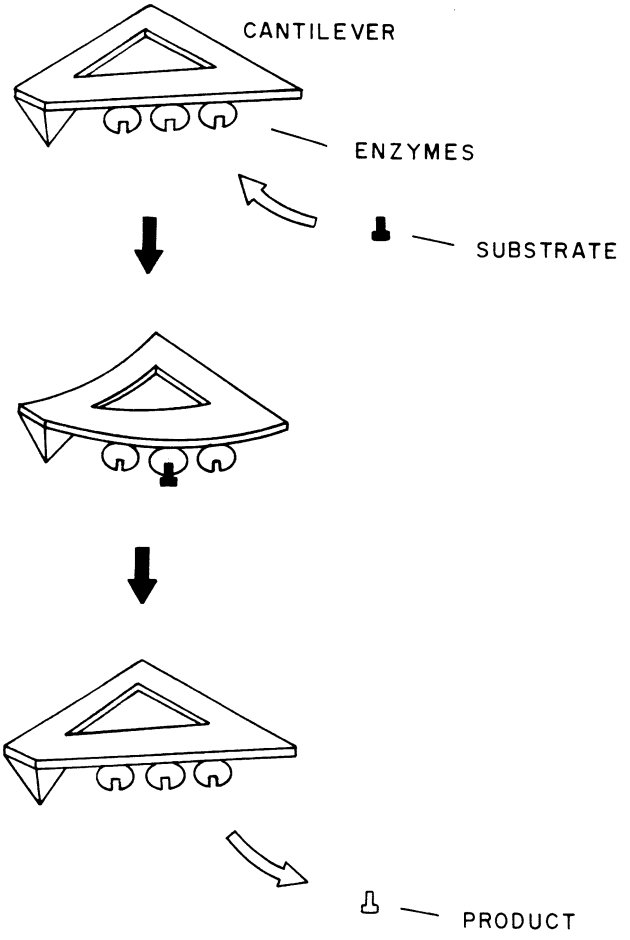


Fig. 5. Enzyme molecules immobilized on the cantilever surface in order to produce a nanobiosensor.

Other biosensors were made using the enzymes superoxide dismutase and catalase (Pereira RS, unpublished results). Fig. 8A shows the results obtained with a nanobiosensor containing immobilized superoxide dismutase when used to detect superoxide ion production in the walls of *S. cerevisiae* cells. A few narrow peaks were captured by the computer, indicating that these cells produce the superoxide ion, and that it is possible to monitor this using this method. In Fig. 8B, a nanobiosensor with immobilized catalase was used to monitor the production of hydrogen peroxide by living cells. In this case, the peaks are wide, indicating that this reactive oxygen species is more stable and abundant than the superoxide ion (Pereira RS, unpublished results).

4. Use of this technique for pharmacological studies

In terms of drugs, my current project is to immobilize, via silanization [18,19], calcium blockers on the tip of the AFM to identify calcium channels on the surface of living cells. The aim of this project is to check different areas of the cell surface (including the pores) with a cantilever containing a calcium blocker immobilized on its tip, and observe if the force-distance graph will change upon detection of a calcium channel. The cell surface pores may possibly be the main areas where calcium channels are found.

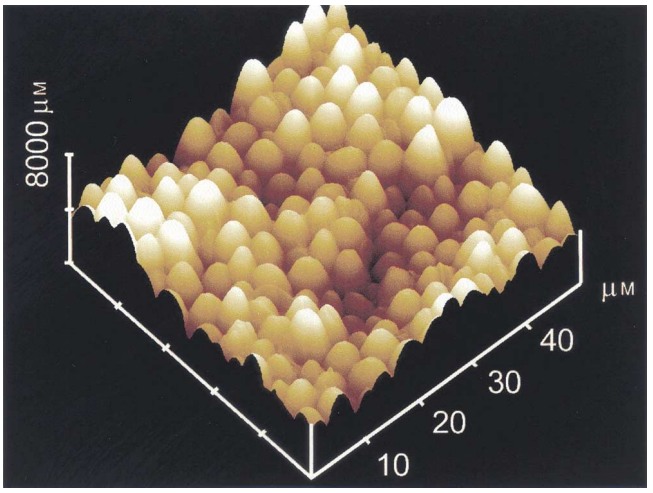
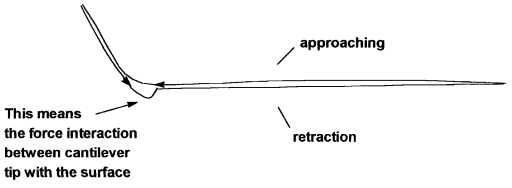
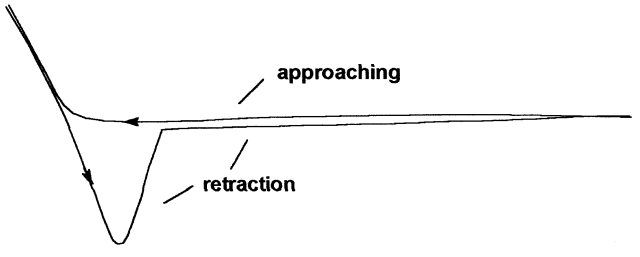


Fig. 6. Scanning of the surface of *S. cerevisiae* cells by a cantilever without immobilized enzyme. No deflection was detected, indicating that the presence of the enzyme is necessary to produce deflections. During 5 hr of scanning, the same image was obtained. Reprinted with permission from FEBS Lett 2000;475:43–46. Copyright [2000] Federation of European Biochemical Societies. [Ref. 3].

A force-distance graph measures the interactions of the Van der Waals forces between molecules, and it has the following appearance:



I think that when a calcium channel is found, this graph will change, indicating more interaction between the calcium blocker (on the cantilever tip) and a possible receptor on the calcium channel. Then, this graph will have a new conformation as depicted below:



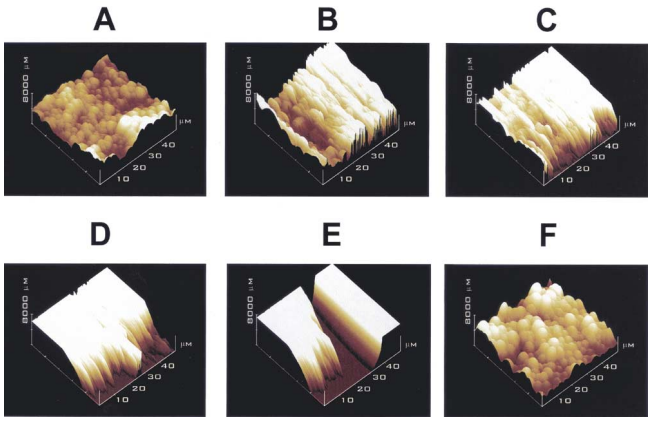


Fig. 7. Scanning of the surface of *S. cerevisiae* cells by the nanobiosensor (alcohol dehydrogenase type II). Continuous detection, by the nanobiosensor, of the production of ethyl alcohol by *S. cerevisiae* cells. (A) In the first 15 min, the cells are not producing ethyl alcohol. (B) After 30 min of incubation, the *S. cerevisiae* cells begin to produce ethyl alcohol and make the nanobiosensor deflect. These deflections are visualized clearly in the picture. (C) After 45 min of incubation, the frequency of peaks increased, and the image of the cells started to disappear. (D) After 60 min of incubation, the image of the cells disappeared completely due to the increase of the cantilever deflections. (E) After 75 min of incubation, the presence of ethyl alcohol produced a great number of deflections of the cantilever, which reached a "plateau," impeding the visualization of the cells. Probably, these cells reached the maximum production of ethyl alcohol. (F) After addition of orthophenothroline.

After this, it is possible to visualize the area with the AFM and see the three-dimensional conformation of the receptor of the calcium blocker.

Fig. 9 shows an explanation of the production of a force-distance graph [20].

5. Identifying defective genes

Tiny variations in the genetic code are what make us unique. Changes in one chemical unit in the sequence of a gene, called SNPs, can influence an individual's risk for disease. Comparing variations will help identify defective genes and may lead to better diagnosis and treatments.

If a cantilever is coated with DNA strands, it is possible to detect genetic variations or SNPs, when an individual's DNA is tested against them.

In a recent report, scientists from IBM demonstrated that when DNA strands are immobilized on a cantilever surface, it is possible to identify the complementary strand of the DNA in solution (Fig. 10) [8]. When these strands (immobilized and complementary) match, the heat evolved from the process makes the cantilever bend.

This nanomechanical property transforms the cantilever into a DNA-chip, very useful in identifying mutations [8].

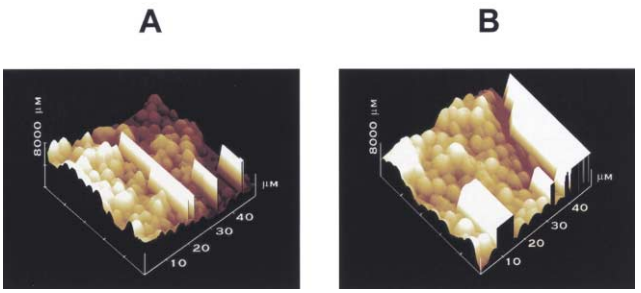


Fig. 8. Scanning of the surface of *S. cerevisiae* cells by the nanobiosensors. (A) Superoxide dismutase; and (B) catalase.

6. Possibilities for the future

6.1. Stress and depression

In our current research, some analyses of samples from the human body, using AFM, are useful to detect stress or depression conditions due to molecular modifications on the topography of the samples (Pereira *et al.*, data not shown).

6.2. Drug delivery

A test medication can be put on the cantilever surface (without immobilization) and delivered to a chosen area. The mechanical movement of the AFM makes the releasing of the test drug slow, and the changes of the cell surface can be observed on the monitor of the AFM in real time. With this method it is possible to deliver a drug to only one cell or to a specific area of a cell.

6.3. Genetic therapy

If an RNAase or a DNAase were immobilized on the cantilever, the resulting nanobiosensor would be a useful tool to monitor the uptake of DNA or RNA during genetic therapy.

6.4. Penicillin

If the enzyme penicillinase is immobilized on the cantilever, we could see penicillin being released from *Penicillium notatum* or being absorbed by penicillin-sensitive cells. In both cases, the time of release or absorption can be monitored in several types of cells (including those from human beings). This method can also be used to separate the strains of *P. notatum* that produce higher quantities of penicillin.

6.5. Study of anti-virus drugs

(a) The use of the enzyme reverse transcriptase from the AIDS virus transforms the AFM cantilever into a very

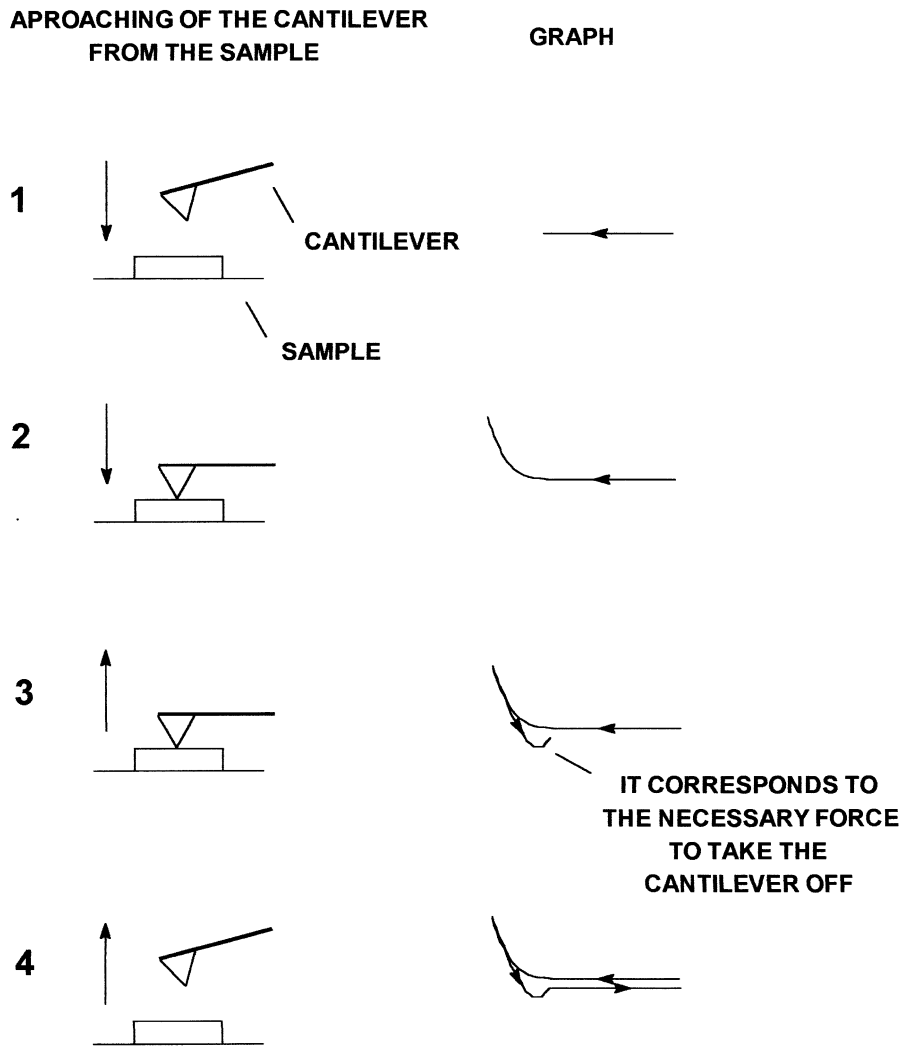


Fig. 9. Production of a force-distance curve by AFM. (1) The cantilever going down towards the sample; (2) when the cantilever touches the sample, the direction of the laser light is changed and, as a consequence, the graph is modified; (3) taking the cantilever off the sample. The interpenetration between the electron orbitals of the sample atoms and the cantilever atoms produces a "belly" in the line of retraction, which corresponds to the necessary force to take the cantilever off; and (4) the cantilever going up and the completion of the force-distance graph.

powerful tool to test new medications capable of inhibiting this enzyme and inactivating the action of the virus [21–24].

(b) If one virus particle is immobilized on the tip of the cantilever (Fig. 11), it would be possible to measure the force necessary for this virus to infect a single cell [25]. In this way, a test medication that could weaken these Van der Waals forces could be a potent anti-virus drug and could avoid virus infection.

6.6. Clinical analysis

The immobilization of *para*-nitrophenylphosphate (PNPP) on the cantilever can be used to identify cancer in the bones, prostate gland, and ovaries. PNPP is the substrate of the acid phosphatase enzyme, and it is currently used in clinical analysis [26–29]. The enzyme is released by tumor cells to the extracellular medium.

6.7. Detection of neurotransmitters

Normally, the enzymes tyrosinase or laccase are used to build biosensors sensitive to dopamine, l-dopa, epinephrine (adrenaline), and norepinephrine (noradrenaline) [30–38]. All of these chemicals are catecholamine hormones (derivatives of catechol), and are used by neurons as neurotransmitters (Fig. 12). One of the projects of my laboratory is to immobilize tyrosinase or laccase on the cantilever surface to observe neurotransmission occurring at the synapse. In this case, it will be possible to see the neurons and the moment when neurotransmission occurs. This type of nanobiosensor

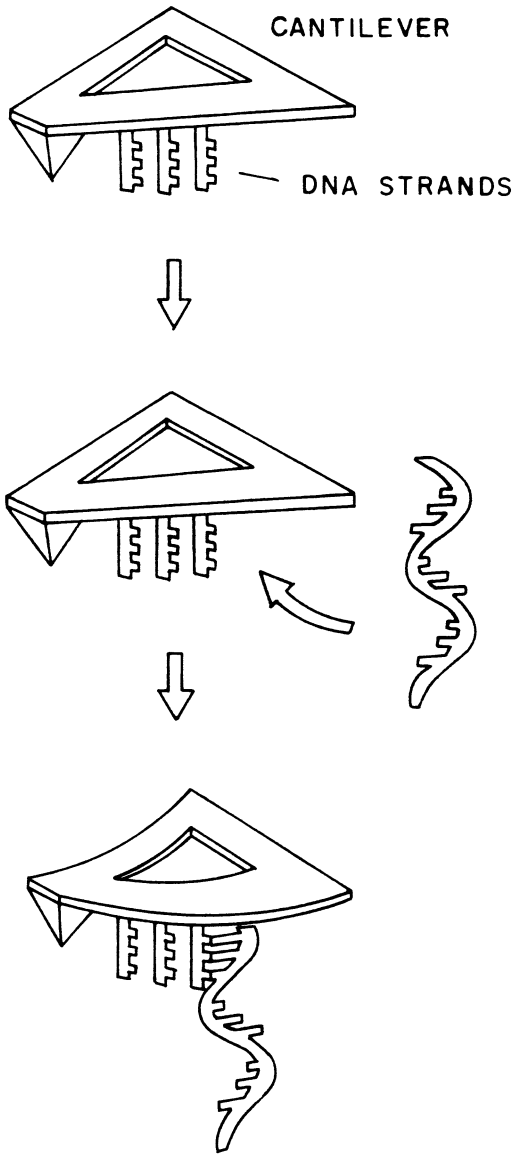


Fig. 10. Cantilever coated with DNA strands.

will be useful to study several types of drugs, for example, new antidepressants.

6.8. "Fentocalorimeter"

Calorimeters are devices for measuring heat [39–42]. In a general way, they are basically made with thermocouples, which are a pair of dissimilar conductors joined in series to form a closed circuit so as to produce a thermoelectric current when heated. According to the Thompson effect, when two different metals are bonded together and heated, a voltage appears [43]. This is the working principle of calorimeters [43]. Based on this, it is possible that a very small voltage may appear between the blades of gold and silicon, in the cantilever, which is proportional to the heat evolved from reactions of the molecules immobilized on the

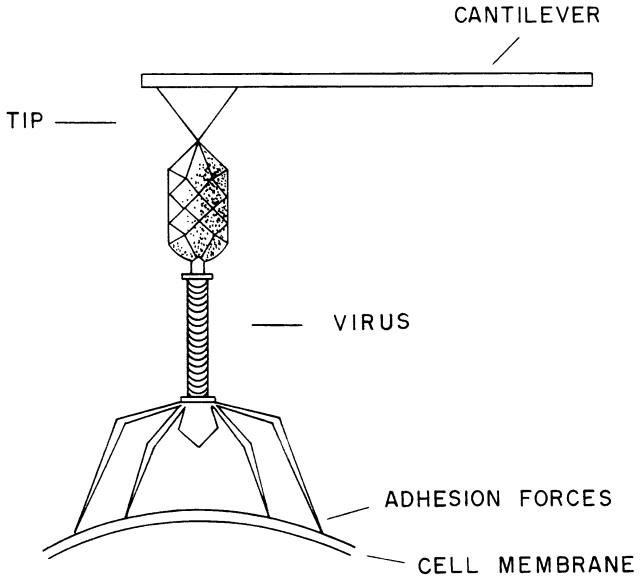


Fig. 11. A virus immobilized on the cantilever tip in order to measure the adhesion forces between the virus and a living cell.

cantilever (Fig. 13). I think it is possible to build a nanoapparatus for measuring this voltage and to make the correlation between the voltage and temperature, producing, in this way, a "fentocalorimeter" capable of measuring the heat of reaction between just a few molecules.

7. How to make a nanobiosensor

(a) Stock solution of glucose oxidase: 100 mg of glucose oxidase (or 10 mg of hexokinase) and 10 mg of BSA were dissolved in 1 mL of doubly distilled and deionized water. Five microliters of this glucose oxidase plus BSA solution was spread on a 2 cm^2 area on a coverslip.

(b) Building the nanobiosensor: a brand new cantilever was put in the cantilever holder of the AFM. The cantilever was engaged to a coverslip containing the glucose oxidase–BSA solution. Then 1 μL of 25% glutaraldehyde (cross-linking agent) was added to the area where the solution of

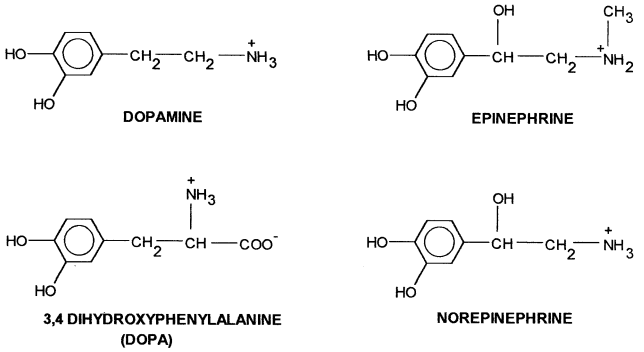


Fig. 12. Chemical structures of neurotransmitters.

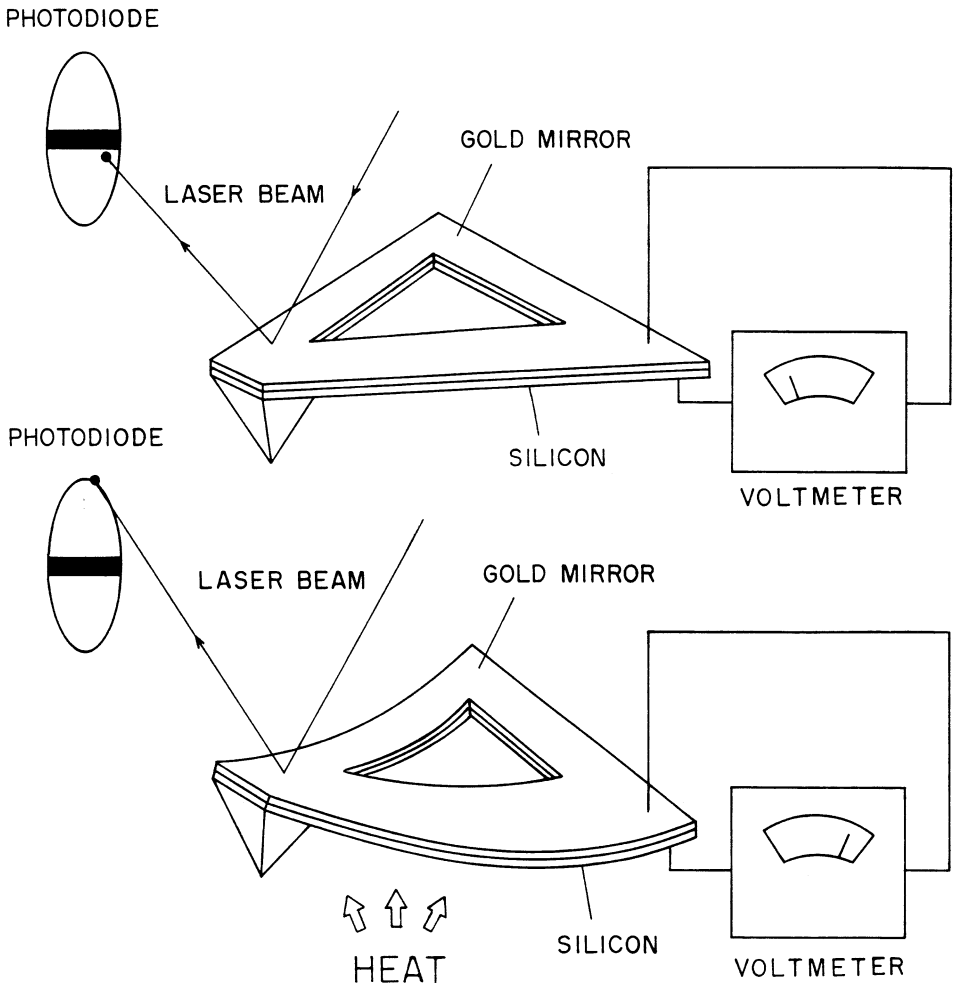


Fig. 13. Bending of the cantilever caused by heat.

glucose oxidase and BSA was spread. The resulting gel phase covering the cantilever was found to be stable in aqueous solution. After 30 sec, the cantilever was taken off the coverslip surface and dried for 3 hr at room temperature. After this, the nanobiosensor was put in a vacuum desiccator for conservation at 5°. To test the effectiveness of this methodology, a glucose solution was dropped directly on the nanobiosensor while it was scanning a glass surface; the reaction catalyzed by the immobilized glucose oxidase was monitored by the deflections of the cantilever on the AFM monitor.

The nanobiosensors should be kept in a vacuum and under refrigeration. Under these conditions they can be used for up to 4 weeks after their preparation, and the experiments with them are very reliable and reproducible [3].

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