

As emphasized by Srinivasan<sup>21</sup>, efforts to kindle interest in these neglected areas may have to be intensified without further delay if classical microbiology has to retain its rightful place in future developments in biotechnology. The fact that classical biology is still the bedrock from which modern developments can spring must be firmly established and the important role that microbial biodiversity shall play in achieving this development needs to be recognized fully.

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## Design and capabilities of scanning tunnelling microscope operating in air medium

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*Scanning tunnelling microscope has a variety of uses in physics, chemistry, material and biological sciences. A couple of years back, one team from CSIO has designed and developed an STM which has added features over those available commercially in addition to cost factor. Scans of graphite and gold gave atomic resolution. Biological macromolecules were scanned in their native state. STM study on keratin gave information regarding the confirmation of this protein.*

THE scanning tunnelling microscope (STM) is now a well-established tool for investigating the surface structures of metals and semiconductors at atomic-level resolution<sup>1-3</sup>. One of the fast-spreading areas of application of the STM is surface studies of biological macromolecules<sup>4-7</sup> in their native form.

For a long time, transmission (TEM) and scanning (SEM) electron microscopes have been used extensively for structural studies of biological samples at the molecular level. The limitations of such studies on biological samples in TEM and SEM, which result from their inherent nature and the associated sample preparation requirements, are well known. These considerations now motivate the applicability of STM and related techniques<sup>8</sup> to the study of biological samples.

In India, a programme to develop STM was started at the Central Scientific Instruments Organization (CSIO),

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Chandigarh, in 1989, in collaboration with the Naval Research Laboratory (NRL), Washington, USA, under the United States India Fund (USIF). A prototype of the tunnelling microscope capable of operation in air medium was developed in phase I of the project, which was completed towards the end of 1991. In the second phase, an ultrahigh vacuum (UHV)-compatible STM is in an advanced stage of integration and testing.

We report here the details on the design and construction of an STM. This microscope is operated in air medium. Its applications in biology and materials sciences have also been presented.

### Configuration and capabilities of the microscope

STM has its theoretical basis in electron tunnelling, which is a quantum-mechanical phenomenon. Tunneling in STM is initiated by positioning the scanning tip (a conductor) within a few angstroms ( $\text{\AA}$ ) of the sample surface, which should also be a conductor. A bias voltage when applied between the tip and the sample causes the electrons to tunnel from one to the other.

#### *STM mechanics*

The STM mechanics consists of the sample, the scanning tip and the precision positioning (coarse as well as fine) mechanisms for bringing the tip and the sample within the tunnelling distance, which is typically in the range 4–20  $\text{\AA}$ . Development of the STM fetched Binnig and Rohrer the Nobel prize in 1986. The theory underlying STM was not new. Their accomplishment was the experimental realization of what was considered to be theoretically possible. Developing a functional STM which solved problems in measurement, micro-positioning and vibration isolation, all together, had never been successfully accomplished earlier.

The STM developed at CSIO, Chandigarh, contains a 'Fully Computerized STM Mechanics' (Figure 1) in which both coarse and fine positioning movements are actuated and controlled by software. In contrast to most designs, the CSIO STM mechanics does not contain any springs, levers or micrometers and is, therefore, more compact and simple. Coarse positioning of the tip is performed by moving the sample closer to the tip. The movement of the sample towards or away from the tip is achieved by rotating the sample holder through the outer three piezo tubes, each provided with a steel ball for support. The surface of the sample holder, which remains in contact with the steel balls, is provided with three grooves, each having a ramp profile.

Fine positioning for final placement of the tip in the tunnelling zone was achieved by expanding the central piezo tube. For minimizing vibrational effects, the instrument size was kept very small, and components

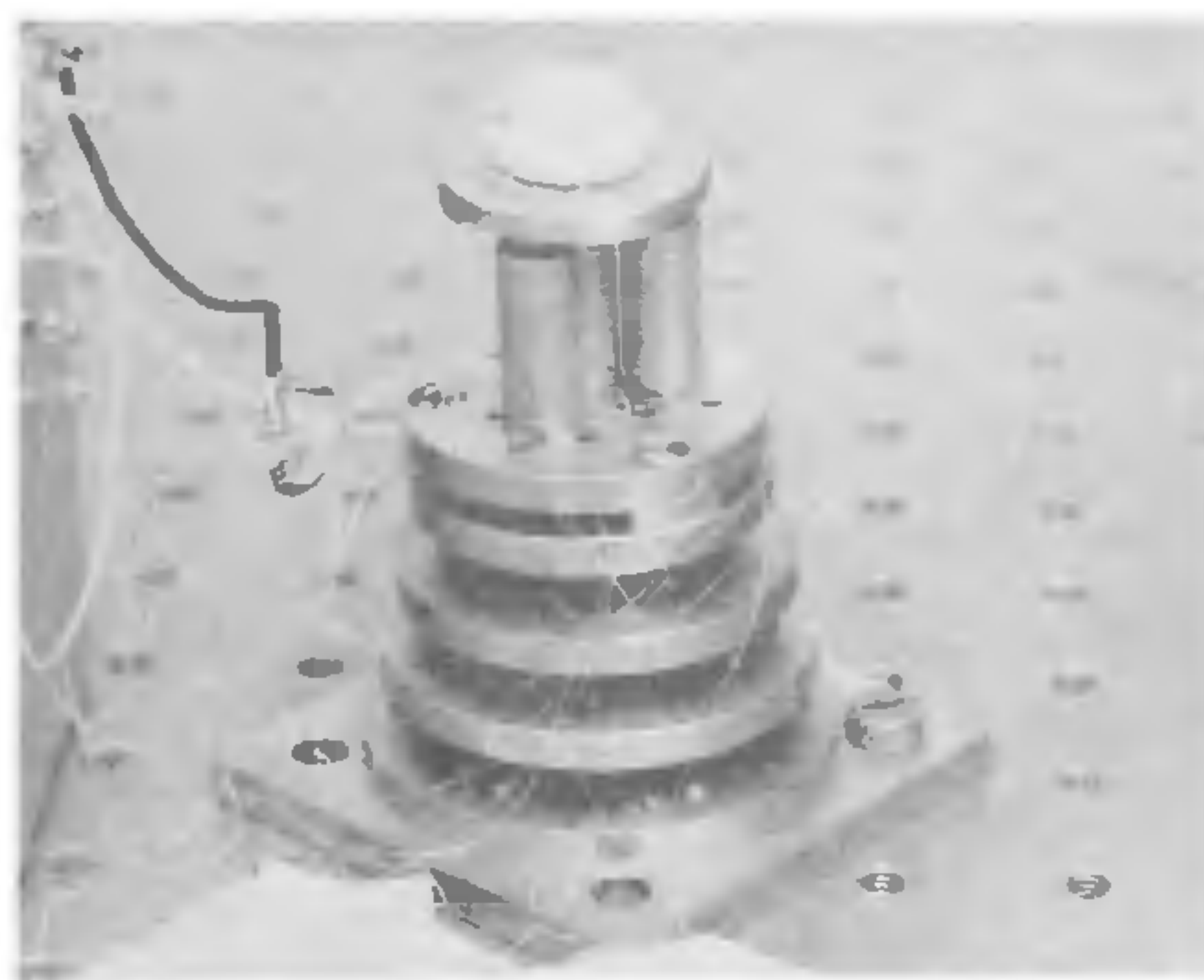


Figure 1. Fully computerized mechanics in the CSIO STM

having high resonant frequencies were used. The inherent rigidity of the piezo tubes makes them well-suited for this purpose. A set of steel plates separated by viton 'O' rings provide additional isolation from vibrations.

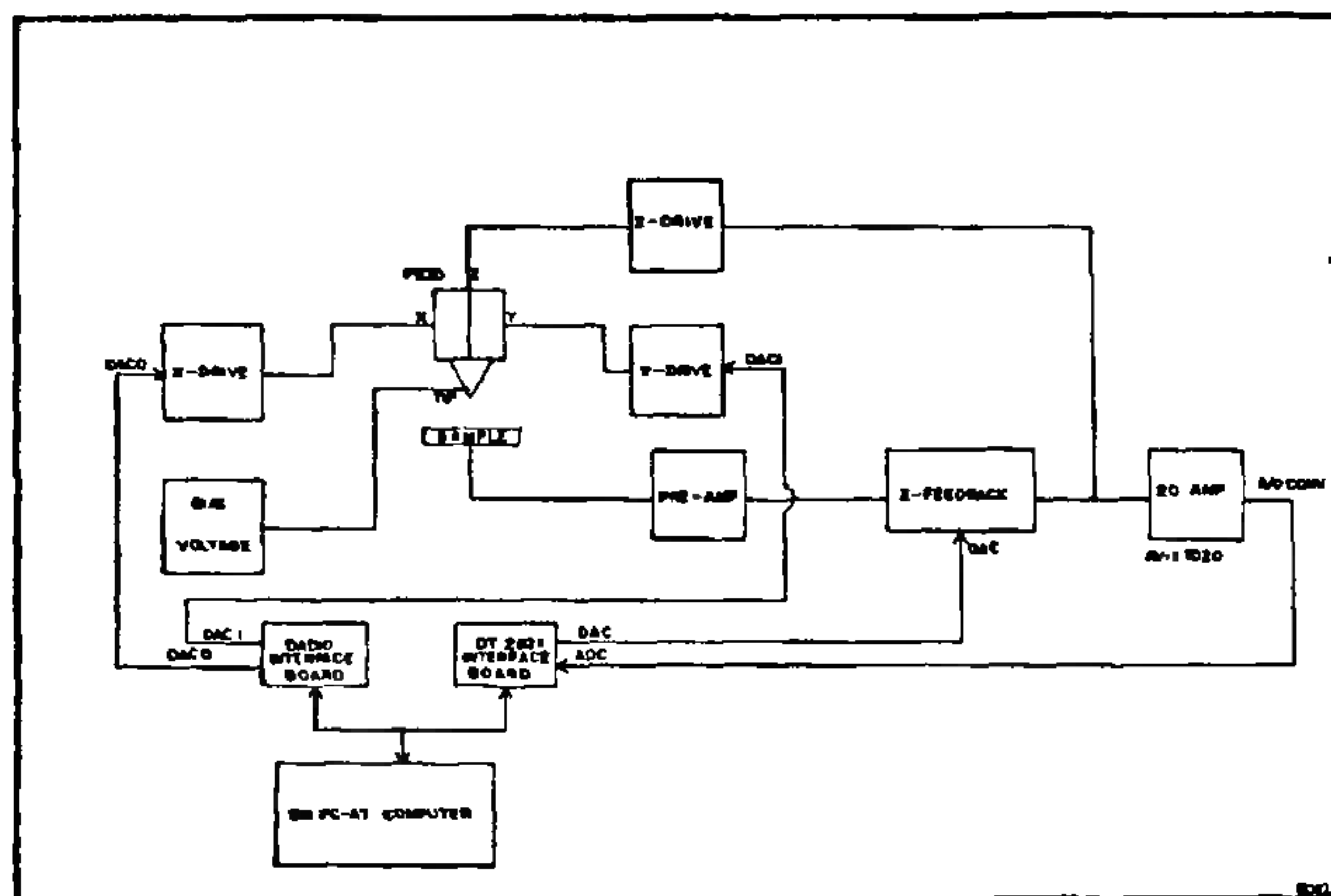
Thermal stability was achieved, once again, by minimizing the size of the components of the STM mechanics and also by providing symmetry to thermal responses. Primarily for this reason, a four-piezo-tube arrangement was incorporated wherein the outer three tubes support the sample and the central tube holds the tip for scanning purposes. Since all these tubes are of the same physical size and material, thermal expansion or contraction has no effect on the tip-sample gap.

#### *Control and acquisition unit*

The CSIO STM is based on the block diagram shown in Figure 2. The control and acquisition unit controls and provides data on the tip position. The block diagram shows how the tip positioning controls loop functions for X, Y and Z axes. The feedback for the control loop is the tunnelling current. Control loop input circuitry amplifies the tunnelling current signal and thereby provides a value corresponding to the actual tip height. Circuitry then compares this analog value with the reference value for the desired height. Based on the result of this comparison, control loop adjusts the Z piezo driver output to move the tip in or out as required. The correcting Z voltage is simultaneously digitized and stored in the computer for final image display.

In order to scan across the surface (X or Y direction) while changing the tip height, the control loop must have a fast response. As a general rule, the control loop bandwidth should be more than 1 kHz and, therefore, in CSIO STM control loop, we have provided a bandwidth of 1.5 kHz. The loop is connected to high-speed A/D and D/A converters.





**Figure 2. STM block diagram**

Data acquisition is performed by storing the data acquired from the control loop. For constant-current scanning, the STM stores successive values used for correcting the tip height. For constant-height scanning, the STM disables the feedback loop; the instrument then simply stores the measured tunnelling current values in this mode.

### *Image display and image processing*

**An efficient STM must have high scan speed, real-time operation and latest capabilities for processing the acquired image.**

For acquiring and displaying images in real time, software must provide a method for easy entering and changing of the scanning parameters in the STM. It should also provide flexibility to users to control these operating variables based on the type of sample, resolution, data needed, the overall operating environment and response of the instrument. Menu-driven screens provide useful 'templates' for user entry of desired values for tunnelling, image control, etc. All these parameters have been incorporated into the CSIO STM.

A computer is typically used to gather, process and display the STM results. Normally, a high-resolution colour monitor is preferred for image display. The image display includes the three-dimensional view, line scan view and false-colour top view (or gray-scale view). Image-processing facilities such as plane subtraction for removal of a slope, various types of filters, fast Fourier transform (FFT) feature and smoothing in  $X$  and  $Y$  directions have also been incorporated into the STM.

### Sample/tip preparation

### *Nonbiological sample surfaces*

The CSIO STM has been extensively used for studying the topography of the surface structure of gold evaporated onto glass, highly oriented pyrolytic graphite (HOPG) and single crystals of silver prepared in the laboratory.

### Biological macromolecules

Hydrated biomolecules are conductive and since the CSIO STM can work in air medium, there is no need of



**Figure 3. Set-up for etching tungsten tips**



special sample preparation except that the molecules should be spread in uniform thickness on a flat surface. This can be achieved by diluting the sample in distilled water at a suitable concentration. Keratin filaments isolated from rat vaginal epithelial cells<sup>9</sup> were suspended in distilled water, spread on freshly cleaved HOPG, air-dried and scanned in the STM.

### Tip preparation

At present, for STM work in CSIO, two types of tips are being used, namely gold and etched tungsten. The etching set-up for tungsten tips has been specially developed at CSIO. Etching set-up (Figure 3) gives a high yield of good-quality tips and is able to produce tips with a diameter around 500 Å. This set-up produces tips with high aspect ratio (approx. 1.0), to minimize vibration during scanning, and with minimum whiskers, to lower the junction noise. Tungsten tips are cleaned with acetone before use.

### Images by STM

Details of scanning tunnelling microscopic images obtained on samples from each of the categories mentioned earlier (biological and nonbiological) are presented here. Figures 4*a, b* show the line scan and gray-scale images of gold evaporated onto a glass surface. The images give a beautiful display of clusters of gold atoms. Hexagonal symmetry of freshly cleaved highly oriented pyrolytic graphite surface is clearly exhibited in the three-dimensional view in Figure 5. The spacing between the two neighbours is 2.46 Å and compares well with the theoretical calculations.

Keratin fibre, which is 10 nm thick, shows a regular helical structure (Figures 6 and 7) in three-dimensional

view. These structures compare well with atomic models of the protein.

### New dimensions

The operation of STM in air or even water medium opens new possibilities regarding the types of samples which can be studied. The application of STM to biological specimens is being attempted to overcome some of the notorious limitations of TEM and SEM, such as the requirement of exposing the specimen to vacuum. The exposure of biological material to vacuum causes dehydration, which induces gross distortion of the structures. There are various techniques to avoid dehydration during sample examination in TEM or SEM; however, it is difficult to achieve atomic resolution in such preparations.

Unlike metal or semiconductor surfaces, biological specimens are nonhomogeneous and areas of interest are sparsely distributed over the specimen support. For working with a reasonable rate of success, it is therefore mandatory to combine STM with a search system that helps to identify and localize areas of interest. Such a requirement led to the development of a variety of combinations including optical, scanning or scanning transmission electron microscopy with STM. The CSIO STM can also easily be fitted with such combinations if required. For scanning of biological molecules, a search system using an optical microscope should be preferred. The use of SEM or STEM with STM for the search system is not only expensive but also lacks the advantage of examining the biological sample in its natural form.

The STM has a small field of view (1–2 μm) which is good enough for studying surface structures of metals and semiconductors because of their homogeneity;

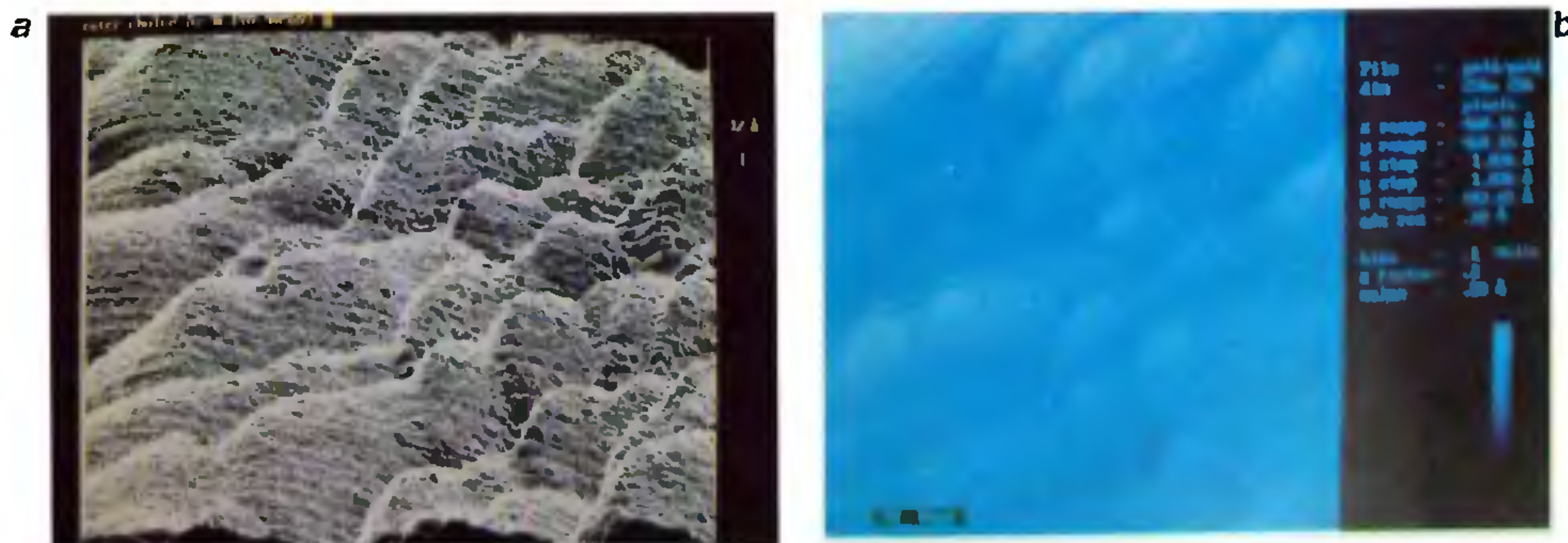
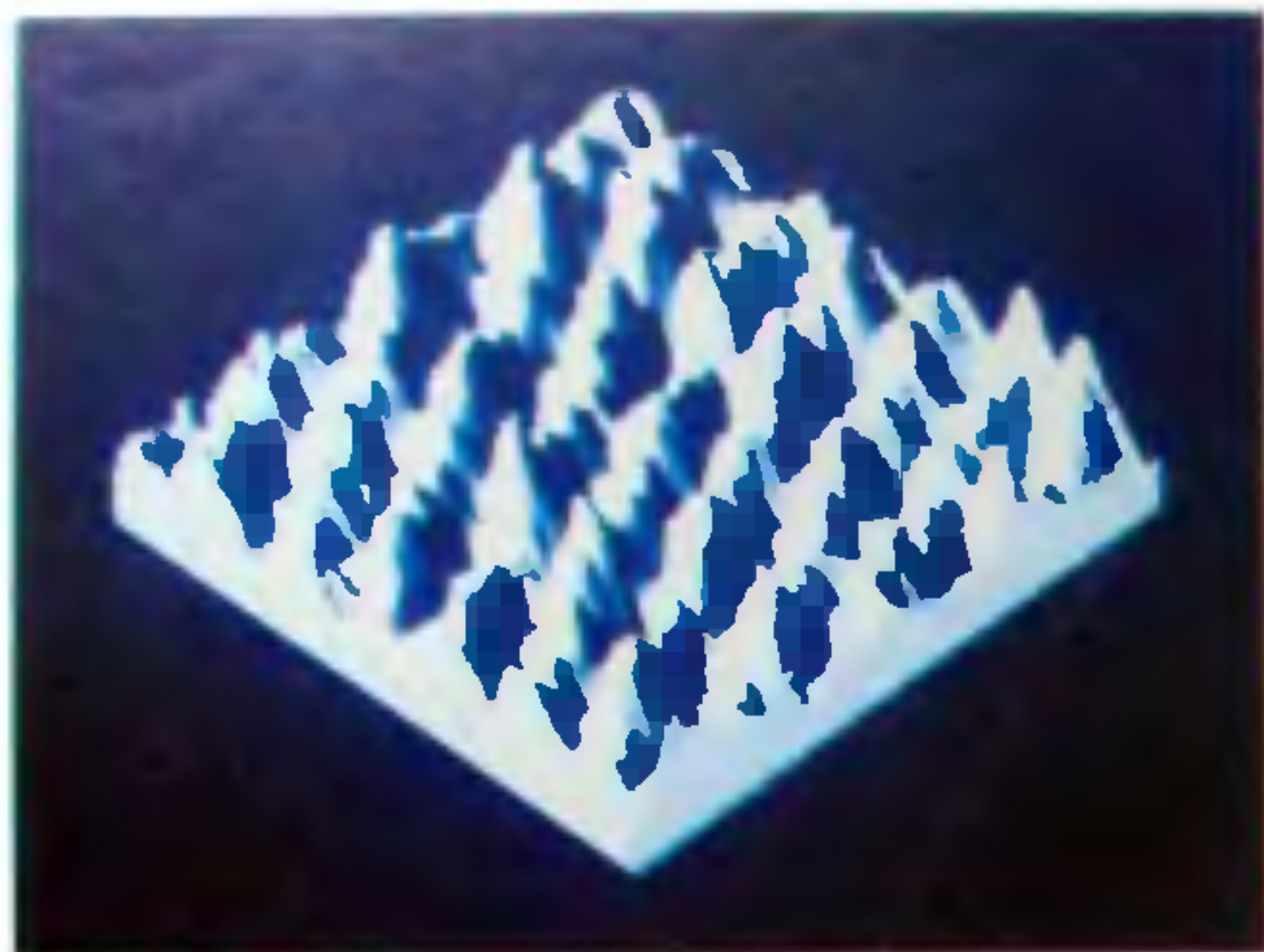
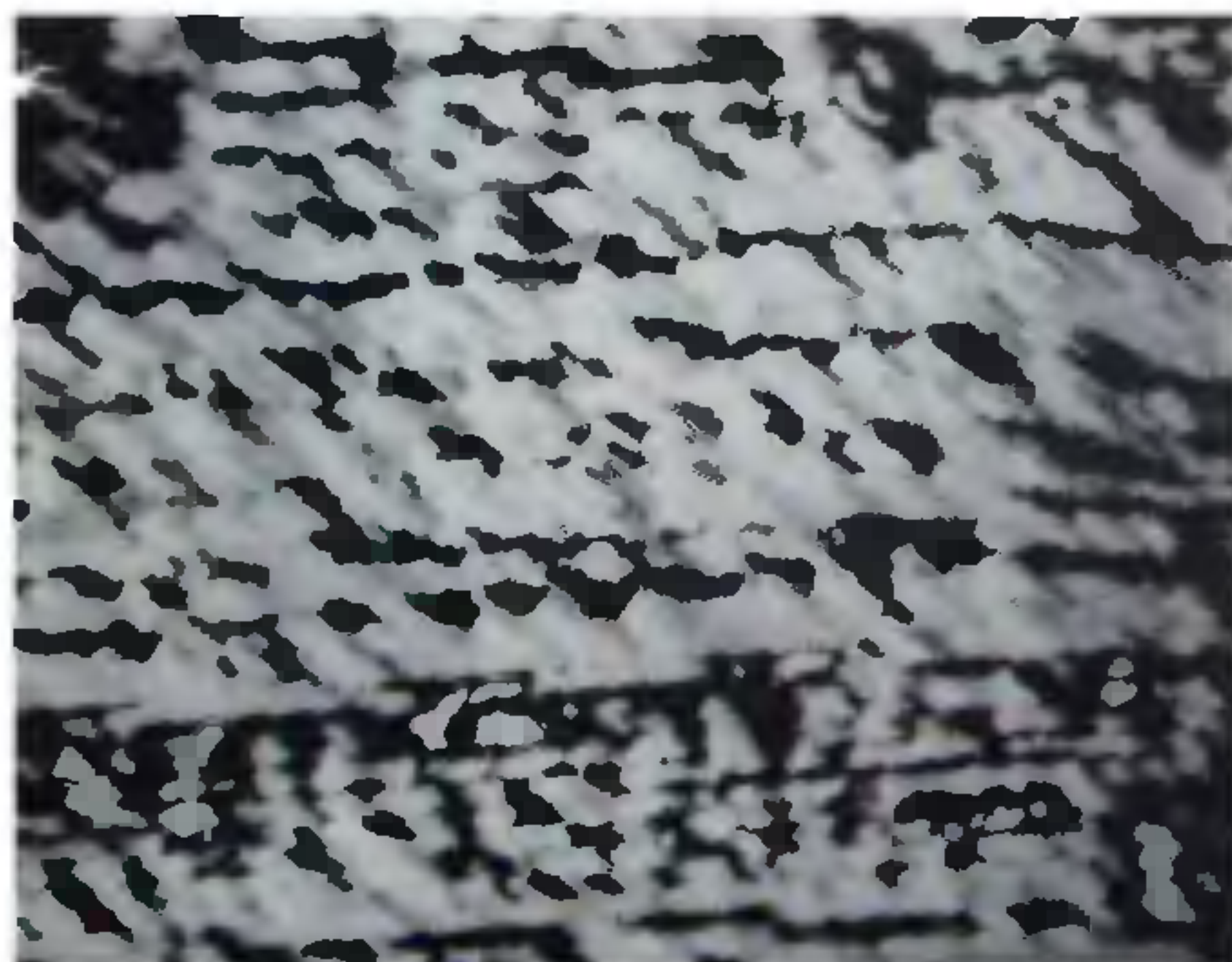


Figure 4. Micrograph of evaporated gold particles showing clusters of atoms. *a*, line scan mode. *b*, gray-scale mode.





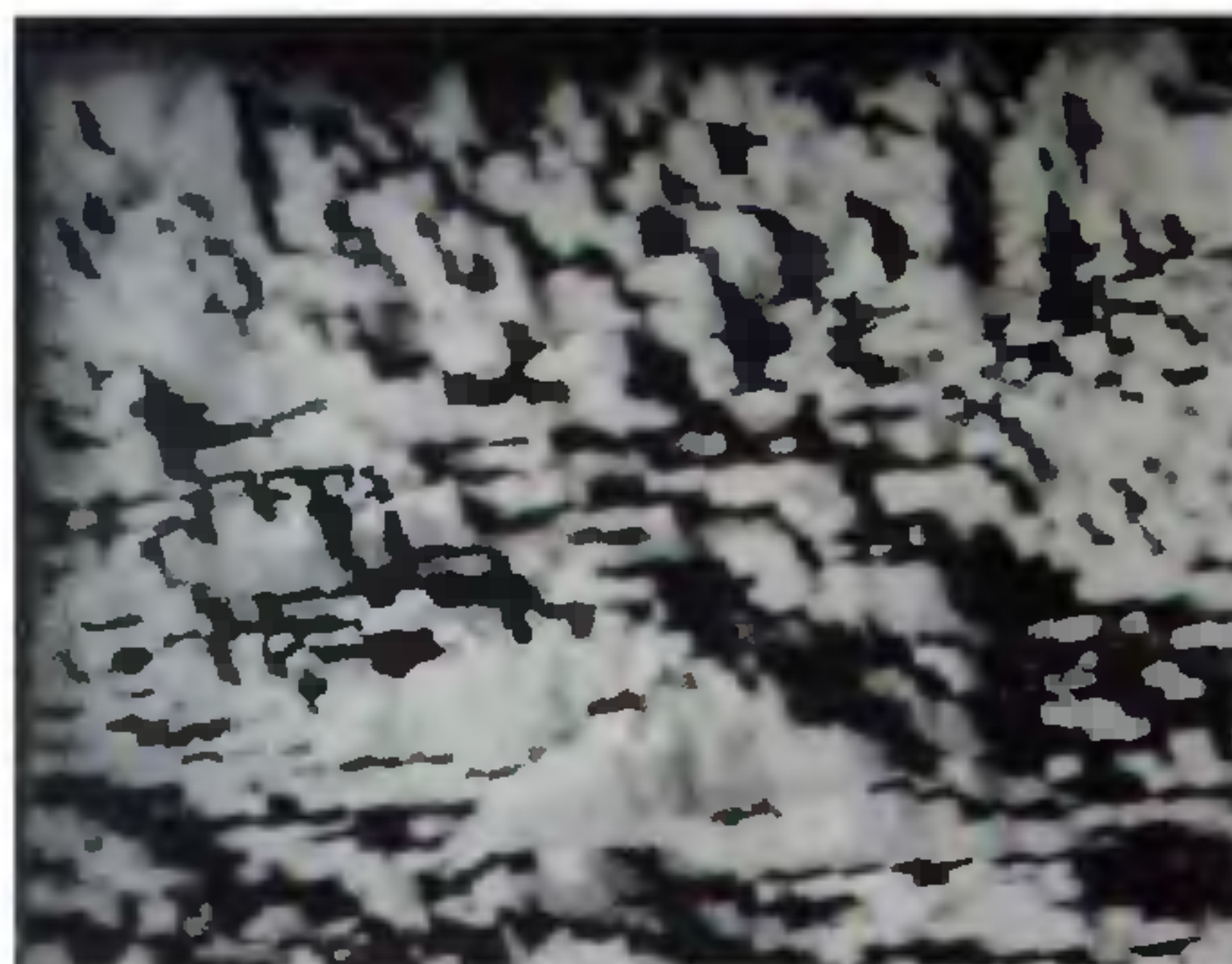
**Figure 5.** Three-dimensional view of a highly oriented pyrolytic graphite surface showing arrangement of atoms. The line scan signals were processed and the 3D view was produced through a software.



**Figure 6.** Three-dimensional STM micrograph of single fibres of keratin running parallel to each other showing coil-coiled arrangement. Thickness of a single fibre is 10 nm.

however, for nonhomogeneous biological samples, a small scanning area is not useful and, therefore, STMs are now being provided with low-magnification mode of scanning as well, wherein a larger field of view up to 11–12  $\mu\text{m}$  is available. To some extent, this will be helpful while working with biological samples.

Binnig and Rohrer soon realized that the application of STM is limited to the study of electrically conductive surfaces only and, therefore, they immediately came forward with the development of atomic force microscope (AFM) for the topographic study of non-conductive surfaces through force measurement. Since most of the biological samples in dehydrated form are nonconductive in nature, the use of AFM will eliminate the need for depositing a conductive coating on the



**Figure 7.** Three-dimensional STM micrograph of adjoining keratin fibres in various orientations.

biological molecules if they do not respond to tunnelling in STM.

The successful achievements of STM technology have initiated a surge of research and engineering activity. This has brought about rapid advances leading to the introduction of related types of instruments such as atomic, laser, magnetic and electrostatic force microscopes, including near-field optical scanning and scanning ion-conductive microscopes. This list is not complete and new instruments are being added to it very fast.

## Conclusions

Progress in achieving atomic and sub-atomic resolutions in materials and biological sciences is proceeding at a rapid rate. The new class of analytical tools having proximal probes has enabled this progress. The field is still very young. It promises not only new insights into the traditional technological areas but also provides a more realistic approach for examining the biological phenomenon at a scale not attempted before.

The STM developed at CSIO based on 'fully computerized mechanics' has many advantages over similar microscopes available abroad. The design can be easily adapted to work in air or water medium or in ultrahigh vacuum.

The CSIO STM can further be combined to work with an optical microscope for providing a good search system and with the AFM head for studying non-conductive surfaces, thus making it an effective system for studying biological and technological surfaces.

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## RESEARCH ARTICLE

# Expression of microinjected foreign DNA in silkworm, *Bombyx mori*

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As a prelude to achieving transgenesis in *Bombyx mori*, conditions have been established for successful microinjection of cloned foreign genes into the silk worm eggs. A sharpened metallic needle is used to pierce the thick chorion layer of the eggshell, approaching through a droplet of DNA solution deposited on its surface. The microinjection is carried out within 2-2.5 h after oviposition and the injected eggs show 3-5% hatchability and 80-90% survival. Such larvae continuously expressed the microinjected cloned reporter gene,  $\beta$ -galactosidase, placed under the control of a constitutively expressed cytoplasmic actin A3 gene promoter from *B. mori*. The expression is seen in different tissues, viz. the fat body, tracheae and the silk glands, till the late larval instars. The microinjected DNA sequences are retained in the adult  $G_0$  moths.

THE successful development of transgenesis methodologies has made the germ line transformation of metazoa a reality. This technique permits the modification of traits in the recipients through the introduction of foreign DNA sequences in their germ line and thus offers a great potential for the improvement of desired traits in animals and plants. Transgenesis techniques

have been developed for a variety of animals ranging from insects such as *Drosophila*<sup>1</sup> and mosquitos<sup>2</sup> to mammalian species<sup>3-5</sup> and aquatic organisms or amphibians<sup>6-9</sup>.

The microinjection of cloned DNA has also opened up new concepts in basic molecular biology for the analysis of gene expression *in vivo*, related to the study of tissue- and development-stage-specificity. In the absence of any well-documented methodologies for transgenesis in *Bombyx mori*<sup>10</sup>, the *Drosophila* system has, in fact, been exploited as a model for *in vivo* regulation of the expression of silk fibroin and related genes. Microinjection of cloned foreign DNA sequences into *B. mori* eggs was first attempted by Nikolaev *et al.*<sup>11</sup> but they had examined only the survival of the injected embryos and not the expression of the injected genes. Subsequently, Tamura *et al.*<sup>12</sup> reported the microinjection into *B. mori* eggs of a cloned reporter gene *CAT* and its transient expression in the early stages of embryonic development. Most recently, Coulon-Bublex *et al.*<sup>13</sup> have demonstrated the expression of a microinjected  $\beta$ -galactosidase gene almost exclusively in the vitellophages of *B. mori* during the early embryonic stages. Here we report the introduction of the cloned foreign gene into the silk worm eggs and its expression in the tissues and organs of the larvae until the late stages of development.

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