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SCANNING TUNNELING MICROSCOPY STUDIES OF CHLOROPLASTS IN SOLUTION

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Abstract

Previous work has shown that it is possible to image whole uncoated chloroplasts using scanning tunneling microscopy (STM), provided this is done in solution using tunnelling currents below about 100 pA.

More recent images include some which suggest that the STM is sensitive to dynamic processes occurring on chloroplast surfaces. Current-versus-distance curves and dI/ds measurements are consistent with tunnelling between the tip and sample surface, and relatively small deformations of the sample surface due to tip-sample forces. Attempts to use the same imaging conditions on bacteria were unsuccessful.

Key words: Scanning tunneling microscopy (STM), biological applications of STM, chloroplast, chloroplast envelope membrane, STM in liquids.

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Introduction

Biological applications of scanning probe microscopes such as the scanning force microscope (SFM) and scanning tunnelling microscope (STM) are of considerable interest. One particularly exciting application is *in situ* imaging of surfaces of large intact biological objects, such as cells and organelles. SFM has been chosen for a number of studies, because there is no requirement for sample conductivity. For example, Hörber *et al.* (1992) imaged dynamic processes on the surfaces of living cells. However, STM also remains of interest, because of the hope that it will yield higher resolution.

Some of the well-known problems associated with biological STM are: (a) Forces between the STM tip and the sample can deform a sample due to the soft nature of biological materials. (b) Samples must be supported by a solid, conducting substrate. Finding a suitable substrate and means of bonding the sample to it is often problematic. (c) Uncoated biological materials are normally not expected to be good electrical conductors, and it is difficult to see how tunnel current from the STM tip can be conducted through the sample to the substrate. Although the conduction mechanisms involved remain poorly understood, sample hydration can play an important role (Guckenberger *et al.*, 1989). In spite of these problems with biological STM, images of, for example, cells (Ruppersberg *et al.*, 1989; Dai *et al.*, 1991; Ito *et al.*, 1991; Garcia *et al.*, 1993) and chloroplasts (Mainsbridge and Thundat, 1991, Dahn *et al.*, 1992) have been reported.

Chloroplasts, the photosynthetic organelles found in the cells of green plants, are a few micrometers in size and are bounded by an outer envelope of two membranes. In the interior of the chloroplast is a third (thylakoid) membrane, distinct from those of the envelope, that is folded into stacked vesicles (grana). By studying the chloroplast envelope with STM, we hope to eventually obtain new information about its structure and transport mechanisms. More importantly at this stage, however, the chloroplast is unusual since it is one of relatively few micrometer-scale biological objects to have

been imaged with STM. Our results should, therefore, help to delimit the types of samples for which STM can be used and to contribute to an understanding of the physical processes involved in biological STM.

Sample Preparation and Imaging

Using the protocol of Dowling *et al.* (1990), chloroplasts were isolated from two to three week old radish and corn sprouts raised from commercially available seeds. From observations of the preparation under a phase-contrast microscope (Halliwell, 1984), the chloroplasts appeared to be mostly unbroken. However, we cannot be certain that the outer membranes, which are particularly prone to disruption by mechanical forces (Tribe and Whittaker, 1972), have remained intact. Such disruption would lead to uncharacteristic shapes of the chloroplast and leakage of stromal components.

To ensure that chloroplast aggregates were loosened, the chloroplast suspension was mixed with a pipet for 2 to 6 minutes, diluted with TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA)], deposited on a substrate of freshly cleaved highly oriented pyrolytic graphite (HOPG) for 10 to 15 minutes, and removed with filter paper. The dilution and deposition time were varied as required to ensure that the chloroplasts were an average distance of 10 to 20 micrometers apart as determined by an optical microscope. Next, a drop of buffer solution was placed on the sample for a few seconds as a rinse, and blotted away. It is important to rinse, since it ensures that any loose chloroplasts are removed and eliminates the potential risk of chloroplasts adhering to the STM tip. To prevent dehydration, another drop of solution was placed on the sample. Optical microscope observations showed that chloroplasts readily adhere to HOPG and remain in place even after rinsing.

A homebuilt STM was used for this work (Dahn *et al.*, 1992). A preamplifier employing an AD549 electrometer op-amp (Analog Devices) allows imaging at currents as low as 1 pA. Tips were made by ac etching of tungsten wire in NaOH solution. For work in aqueous solutions, the tips were coated with Apiezon wax (Nagahara *et al.*, 1989).

STM imaging involved constant current scans in an aqueous environment. To do so, a glass cell was placed over the sample and filled with TE buffer solution. The tip bias was set to the value which minimized faradaic leakage currents, as measured with the tip in the solution but withdrawn a few hundred nanometers from the sample. This electrochemical rest potential was in the range -.2 to -.4 V relative to the sample for all of our images. Tunnel currents in the range 20 to 100 pA were used during STM scanning.

For several samples, we recorded curves of tunnel current as a function of tip position as the tip approached and was withdrawn from the sample during a time period of a few seconds. A simple theory of tunnelling through a planar barrier indicates that for a fixed bias,

$$I \propto e^{-2\kappa s} \tag{1}$$

where I is the tunnel current, s is the barrier width, and κ is given by

$$\kappa = \hbar^{-1}\sqrt{2m\phi} \tag{2}$$

where $\hbar$ is the Planck's constant, m is the electron mass, and ϕ is the barrier energy (work function). Using these equations, values for ϕ can be estimated from I versus s curves or from measurements of dI/ds.

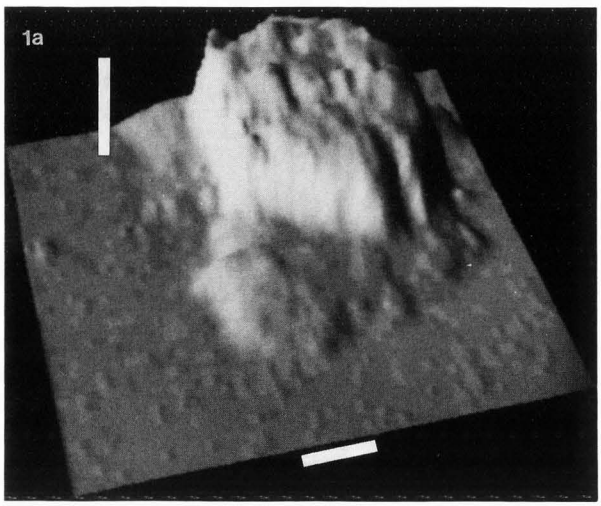
Results

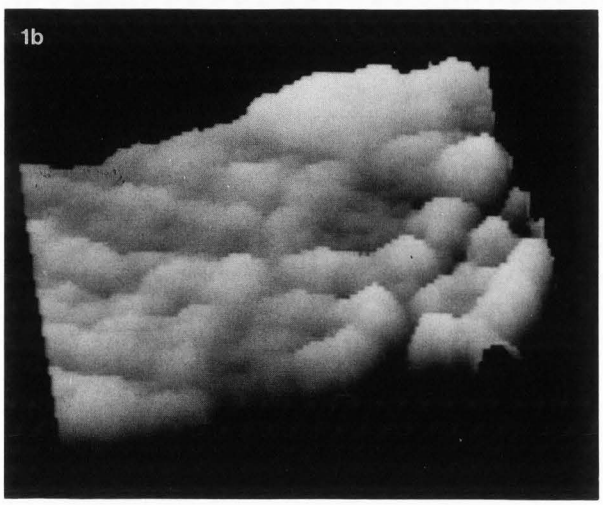
Figure 1a shows a typical corn chloroplast in TE buffer solution. The general appearance of the chloroplasts is similar to those seen in our previous large-area STM images of coated chloroplasts in air, and bare chloroplasts in distilled water (Dahn *et al.*, 1992). Higher magnification images on top of the chloroplasts typically show wavelike features. Molecular-resolution images sometimes exhibit a nearly periodic array with a period of about 4 nm as in Figure 1b. Similar structures were seen on chloroplast surfaces in distilled water (Dahn *et al.*, 1992).

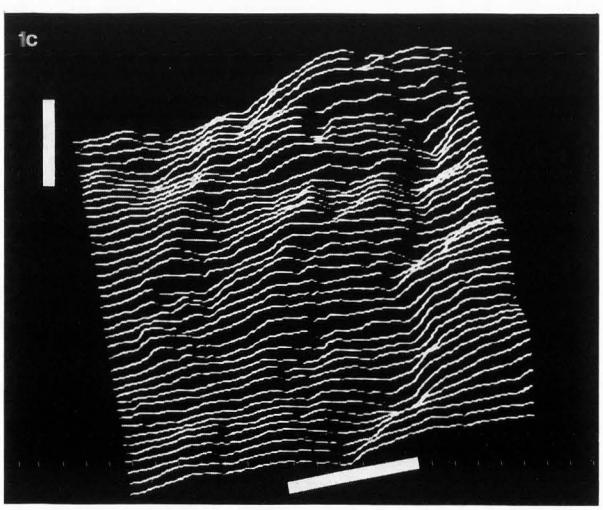
More typical are images, such as Figure 1c, which are dominated by larger-scale wavelike features. However, if the large features are removed by two-dimensional Fourier transform high-pass filtering, there is again some evidence of the 4 nm period (Fig. 1d).

Some images appear to contain anomalously high sharp features, such as the spikes in Figure 2a, and the spikes and ridges in Figure 2b. These are clearly not accurate representations of static surface structures since if such sharp high structures really existed, they would be broadened by tip shape effects. Scanning electron microscope examination of STM tips similar to the ones used here normally give tip radii of 50 nm or more. The tips are not sharp enough to give true profiles of such sharp features. As discussed below, sample deformation during scanning and/or local variability of electrical properties may explain these features.

Also on some images are what we call noisy regions. For example, the high spikes are clustered in three distinct spots in Figure 2a. When repeat scans are made, noisy regions generally again appear noisy, but the detailed noise pattern is different. This is seen in Figures 2b and 2c, in which a region containing a large







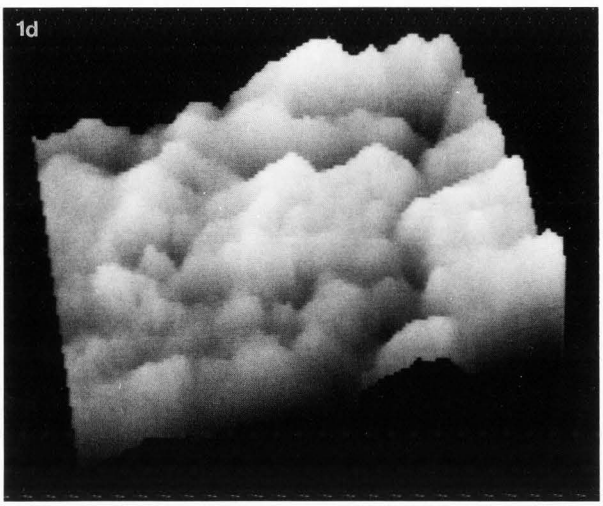


Figure 1. Typical images of chloroplast surfaces in TE buffer solution. (a) Large area scan showing an entire corn chloroplast. Scale bars 1 μm (x), 0.5 μm (z); tip bias -.47 V, current 20 pA. (b) Periodic molecular-scale structure observed in some places on chloroplast surfaces. The scan area is 32 x 16 nm (radish, -.25 V, 40 pA) (c) Original image of an area on a corn chloroplast (-.49 V, 80 pA); scale bars 10 nm (x), 5 nm (z). (d) Same data after Fourier transform spatial filtering using two-dimensional FFT methods. The filter used was a first-order high-pass with a cutoff frequency of 3 cycles per frame.

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concentration of noisy spots was imaged twice at an interval of 24 minutes. Noisy spots were not seen on all chloroplasts, perhaps due to varying amounts of damage during isolation.

I versus z curves were recorded over HOPG in air, HOPG in TE buffer, and in several different locations on chloroplasts in TE buffer, using several different tips. The range of currents used was from a few pA to 200 pA. The data are not of high quality due to noise and z drift, however, approximate κ values can be calculated (Table 1) and clear trends are seen. Apparent barrier energies on HOPG (Table 1) are higher in solution than air, in spite of effects which are expected lower the barrier in an aqueous environment (Sass *et al.*. 1991). Note, however, that the barrier energies on graphite in air are anomalously low. Anomalously low barriers can be explained in terms of the well-known elastic deformation of graphite due to tip-sample forces (Coombs and Pethica 1986; Mamin *et al.*, 1986). A contamination layer between the tip and graphite surface can transmit tip-sample forces and contribute to sample deformation. We propose that there is simply less deformation of the graphite (cleaner conditions) when it is in solution than in air.

Table 1. Approximate decay constants and barrier energies derived from I versus z data (see text).

Sample Type	Number of I-z curves	Mean $\kappa(nm^{-1})$	Standard Deviation	ϕ (eV)
HOPG in air	10	0.6	0.5	0.013
HOPG in TE buffer	8	2.1	1.0	0.16
Chloroplasts in TE	6	2.0	0.7	0.15

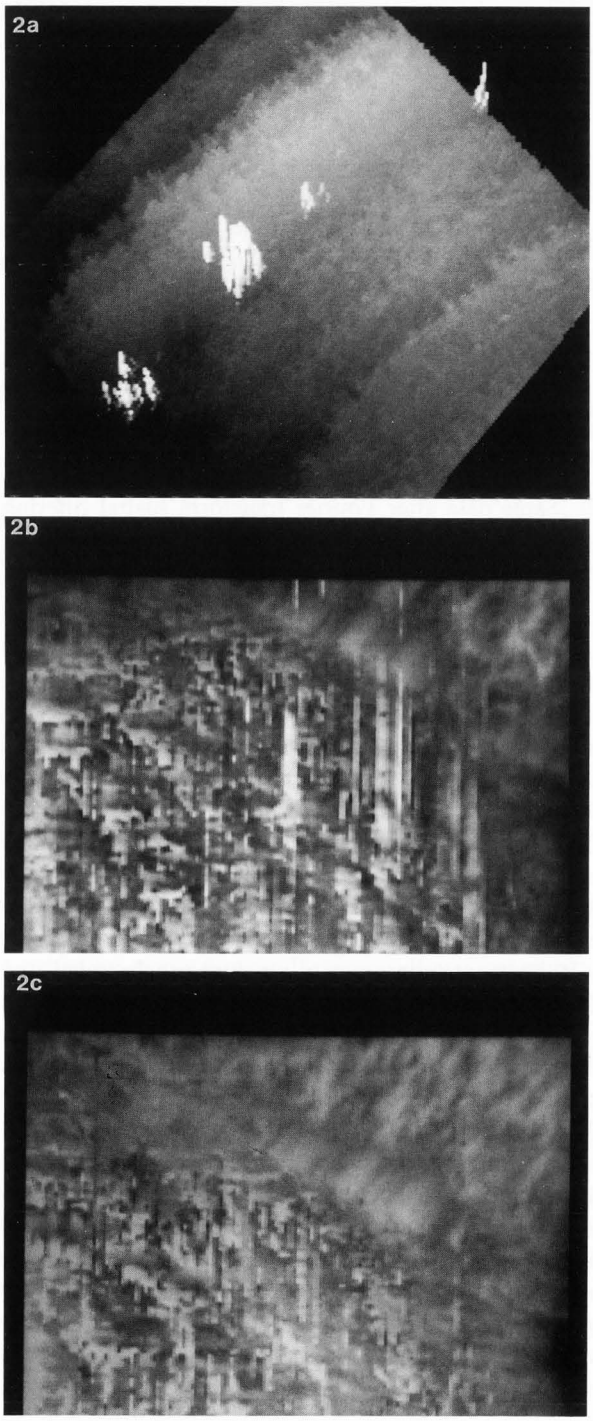

Figure 2. "Noisy regions" on chloroplast surfaces (see text). (a) 32 nm wide field of view. (b) 320 nm wide field of view. (c) Same region as b, 24 minutes later.

Barrier energies on chloroplasts are not significantly different from those on HOPG in TE buffer. This indicates rather little deformation of the chloroplast surfaces we are imaging, since such deformation would be expected to lead to low apparent barriers. The same conclusion can be drawn directly from the I - z curves. The tip usually had to move only a distance of order 1 nm toward the sample in order to increase the tunnel current from the level (about 2 pA) where it is first detectable above the electrochemical leakage, to 200 pA. This distance is the sum of the reduction in the tunnel gap plus any sample displacement which occurs. Therefore, 1 nm is a rough upper limit for the sample displacement caused by the STM tip, at least in typical areas on the chloroplast. We have not been able to acquire I vs z curves over any of the relatively rare noisy regions mentioned above, so we cannot rule out the possibility that significant sample displacements occur in those regions.

Discussion

It is possible the STM tip may be pushing through the envelope or stripping it away during scanning. If this happens, the images would almost certainly be of thylakoid membrane surfaces. There is prior evidence for a 4 nm periodic pattern in thylakoid membranes. Using transmission electron microscopy of glutaraldehyde/$KMnO_4$ fixed leaf tissue from higher plants, Weier *et al.* (1965) observed a subunit structure with core of 3.7 nm on the thylakoid membranes. Other studies, using scanning electron microscopy of freeze-etched chloroplasts with metal shading (Park and Biggins, 1964), revealed a regular arrangement on the outside surface of the thylakoid membranes of particulate units (quantosomes) made of up to four subunits that are 6-9 nm in diameter. However, it would be difficult to account for the large-scale roughness which often obscures the 4 nm periodicity.

Alternatively, the tip may be scanning the fine structure of one of the envelope membranes. Both the inner

and outer membranes of chloroplasts are typical bilayer membranes, with the exception that the inner membrane is intricately folded to form lamellae. This would not, however, account for the 4 nm periodicity. The 4 nm periodicity is something that, to our knowledge, has not previously been described for the envelope membranes. The high points may be indicative of surface transport proteins, which are found in varying numbers and types in the two envelope membranes.

Other than possible membrane penetration, STM scanning at low current in solution does not damage chloroplasts, as can be seen from the fact that they can be scanned repeatedly without changes in the images. A likely mechanism for penetration, if it occurs, would be poor conductivity of the envelope. The STM tip would descend to the thylakoids which fill much of the chloroplast interior. These may be sufficiently conductive for STM imaging, if the envelope is not.

Ruppersberg *et al.* (1989) and Garcia *et al.* (1993) have shown that the STM tip can penetrate almost completely through a cell during imaging. Based on the I-z results and the fact that the surfaces we are imaging are located several hundred nanometers above the graphite substrate, we believe the tip is probably not penetrating the chloroplast at all. Alternatively, if penetration does occur, the tip must be pushing only a short distance into the chloroplasts until it reaches the thylakoid membranes, and then they are imaged with little deformation.

Tang *et al.* (1993) invoked a non-tunnelling electric-field-induced conduction mechanism to explain pulsing current observed during molecular-resolution STM imaging of hydroxypropylcellulose films. Garcia *et al.* (1993) have proposed that this non-tunnelling mechanism may be important in biological samples. In chloroplasts at least, the I-z data are consistent with conventional tunnelling. Also, we have never observed pulsing current like that recorded by Tang *et al.*

To summarize our understanding of the imaging mechanisms which apply to STM scans over chloroplasts in solution, we propose as a tentative working model the following:

(i) Most areas image normally, i.e., the I-z curves are consistent with ordinary tunnelling and the sample must be conducting in some way. No significant tip-induced sample displacements were detected.

(ii) In some unusual regions, the images appear to show sharp, high spikes and ridges. Sample displacements may occur during imaging in these regions. The sharp features indicate large spatial variability in tip-sample forces and/or electrical properties in these regions.

(iii) In the noisy regions, the details of the images vary from scan to scan and, in a discontinuous way, between adjacent lines in a single scan, suggesting temporal variability as well. In these regions, the STM is sensitive to dynamics of the membrane, although much additional work would be required to clarify which dynamical processes are in fact being observed, and why they are observed in only some locations on some chloroplasts. A possible mechanism to explain the noise spikes seen in Figure 2a, and perhaps similar features seen by Mainsbridge and Thundat (1991), is that when the tip passes over some locations (surface transport proteins) on the chloroplast, it may receive a pulse of current, possibly ionic. This causes the feedback system to momentarily lift the tip.

The successful use of STM with chloroplasts required the unusual combination of an aqueous solution environment, and low tunnelling current. In order to test whether these conditions can be used more generally with other micron-size biological objects, we have recently attempted to image bacteria (Bacillus subtilis). A method for adhering the bacteria to HOPG was developed, and STM images of gold-coated samples were obtained. However, repeated attempts to image uncoated bacteria in solution have not been successful.

The reason why these bacteria (for example) cannot be imaged using STM, while chloroplasts can, is presumably related to the electrical conductivity of the samples. As first suggested by Mainsbridge and Thundat (1991), the electron transport mechanism used in photosynthesis may be active during STM imaging. However, this is localized to the thylakoids, and may not explain conduction completely through a chloroplast. In our opinion, the identity and nature of the conduction mechanism remains to be clarified by future research.

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Discussion with Reviewers

R. Guckenberger: Is it possible to isolate the outer envelope and the thylakoid vesicles separately and to image them by STM?

Authors: It is possible to purify outer envelope membranes (Schnell *et al.*, 1990) and thylakoid membranes (Rock *et al.*, 1992) separately. If isolated membranes can be imaged using STM or SFM, then by checking for the 4 nm pattern we might be able to resolve the question as to whether we were imaging thylakoid or envelope structures in this study. We have not yet attempted to do this.

J.K.H. Hörber: The spikes shown in Fig. 2a are very localized, and it would be interesting whether they are still there in the next scan of the same area, similar to what is shown in Figs. 2b and 2c. For these last two images, I think it would be worthwhile filtering them with a low pass filter, as it seems that there are structures in the noisy area which are similar in both images, only drifted to the lower left corner in Fig. 2c.

Authors: Clusters of noise spikes were seen in other scans over the same area, made just before Fig. 2a. Because of drift and the lack of prominent landmarks in the area, we cannot tell if they occurred at the same exact locations on the chloroplast surface. The main reason we were convinced these spikes are due to a process localized to certain spots on the sample surface, is that spikes are seen close together on several adjacent scan lines. For example, the large cluster near the center extends over six scan lines.

In Figs. 2b and 2c, once we allow for the shift toward the lower left, several structures can indeed be seen that are common to the two images. These include the relatively smooth mounds, but also ridges, or lines of noise spikes, within the noisy areas. We have tried low-pass filtering these two images, but this gave only a marginal improvement in the visibility of these common features.