Method for Immobilizing Microbial Cells on Gel Surface for Dynamic AFM Studies

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ABSTRACT The processes of cell growth and budding of the yeast cells *Saccharomyces cerevisiae*, which were gently immobilized on 3% agar and submerged in culture medium, were successfully imaged with an atomic force microscope for 6–7 h. Similar experiments on chemically fixed cells did not detect any appreciable change in their appearance except in a few scannings at the very beginning, indicating that the dissolution of agar and/or scraping of its surface by the scanning tip, if any, did not significantly interfere with the images taken thereafter. The increment in the height of many of the untreated cells, accompanied by their lateral enlargement, was taken as an indication of successful imaging of the growth process of yeast cells, together with an image of a growing daughter cell attached to its mother cell.

INTRODUCTION

The atomic force microscope (AFM), invented by Binnig and co-workers (Binnig et al., 1986), is a member of the scanning probe microscope family. It is a high-resolution imaging device that has several advantages over previous ones, the most significant of which are the ability to give real-time three-dimensional images (Binnig et al., 1986) and to do so under fluids (Drake et al, 1989; Hansma et al., 1992; Henderson et al., 1992). In addition, it can be used as a force measurement device to measure tip-surface interactions (Weisenhorn et al., 1989; Burnham et al., 1990), atomic scale friction (Mate et al., 1987; Erlandsson et al., 1988), and magnetic and electric interactions (Martin et al., 1988; Stern et al., 1988). It has also been used as a nanoindentor to measure the microelastic properties of various materials, including biological materials (Burnham and Colton, 1989; Pethica and Oliver, 1987; Tao et al., 1992; Shroff et al., 1994).

This technology was recognized by biologists as an outstanding tool for dynamic studies at the cellular as well as the molecular level with high resolution. Pioneering studies were made in this field, and each one of them proposed a suitable technique to treat the sample being investigated. Drake et al. (1989) presented the first dynamic study by AFM for the clotting process of the human blood protein fibrinogen that clearly showed the potential of AFM in the study of in situ biological processes. However, there have always been some limitations that restricted the use of AFM in the investigation of biological processes in real time under physiological conditions. First, a properly fixed sample is a prerequisite for successful imaging. Second, a sample having a maximum height less than 1 μm is recommended. And third, but not finally, tip sample affinity should not exceed a certain limit for a stable measurement.

Although immobilizing the sample has always been the major difficulty for imaging globular and nonadhesive samples under fluids, many successful methods were developed to modify the substrates to allow the sample to adsorb to them either physically or chemically. Many of these methods have been proved to be suitable for biological applications (Allison et al., 1992; Benzallina et al., 1993; Butt et al., 1990a; Hansma et al., 1993; Hegner et al., 1993; Thundat et al., 1992; Weisenhorn et al., 1990; Yang et al., 1994; Henderson, 1994). Most of the cellular level studies using AFM have been done on animal cells rather than plant cells. This is because of the capability of some animal cell lines to form monolayers on culturing. This makes them particularly suited for AFM imaging (Kasas et al., 1993). An elegant method was introduced by Häberle et al. (1992) of investigating the process of viral infection of a single cell while holding it with a micropipette. Fritz and co-workers reported the dynamics of granula motion and membrane spreading during the activation of human platelets (Fritz et al., 1994). They made use of the tendency of platelets to adhere to glass surfaces to immobilize them.

Only a very few cases of imaging of live plant cells, however, have so far been reported (Butt et al., 1990b; Hörber et al., 1992; Kasas and Ikai, 1995). This is probably because they present problems of proper fixation on the substrate and/or because of the maintenance of stable scanning over tall objects. Plant cells usually have very rigid and chemically rather inert walls that give them their shapes and protect them from rupturing. As a result, there is no tendency for them to spread on the substrate as animal cells do, and the contact area between a plant cell and the substrate is thus much smaller than in the case of animal cells. Such situations make it more difficult for them to withstand the lateral forces exerted by the AFM tip during its scanning operation. There is another possibility that they might detach themselves from the substrate, even if they are immobilized by being covalently attached to it while growing,

Received for publication 15 May 1995 and in final form 5 September 1995.
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0006-3495/95/12/2226/08 $2.00

because of the fact that the surface layer of the cell wall, in some microbial cells, peels off the cell surface as the cell expands (Cooper, 1991). Recently, Kasas and Ikai developed a method for anchoring round cells in a Millipore filter for AFM imaging. Although they imaged the diploid form of the yeast *Saccaromyces cerevisiae* under the liquid culture medium, no growth activity was noticed. This might be due to the rigidity of the filter that anchored the cells in a hole of limited diameter.

In this context, we present a method for immobilizing living haploid cells of *Saccharomyces cerevisiae* on agar surface without using any chemicals, which we expected to give them more freedom to grow during AFM scanning. This method has an advantage over the already published one by Kasas and Ikai (1995), as it enabled us to image actively growing cells held in a flexible matrix. Although the cells were immobilized on a seemingly soft gel surface, they did not rotate significantly, nor were they pushed deeper into the gel during imaging by the scanning tip.

MATERIALS AND METHODS

Cell culture

A haploid strain of *Saccharomyces cerevisiae* X2180-1A from the Yeast Genetic Stock Center, Berkeley, was used in this study. They were grown in 10 ml of a medium containing 1% yeast extract (Difco), 2% polypeptone, and 2% glucose at 25°C with reciprocal shaking at 150 strokes/min.

Sample preparation

Cells were harvested in the logarithmic phase of growth by centrifugation at 2150 × *g* for 5 min, washed once with distilled water at 20°C, and suspended in 0.25 ml of the same fluid. Five microliters of a highly concentrated cell suspension was deposited on a clean circular cover glass 15 mm in diameter. About 200 μl of 3% molten agar at 45–50°C was dropped over the deposited cells by a micropipette. Another piece of cover glass was quickly put on the agar before it hardened to create a flat surface almost parallel to the lower one. On adding the molten agar, the droplet of the cell suspension spread almost homogeneously over the cover glass. The agar was allowed to completely solidify for 30 min, and then the sample was turned upside down. The lower cover glass was gently pulled parallel to the agar surface, while the agar disc was held between the index finger and thumb and bent slowly away from the coverslip facing the cells. Most of the yeast cells were found to be localized on that side of the agar disc because the high viscosity of molten agar prevented them from diffusing deep into the agar before it solidified. Weakly captured or uncaptured cells were washed off with deionized water.

A control sample was prepared by fixing the yeast cells in 2% gluteraldehyde for 30 min, washing them with distilled water, and immobilizing them on agar as described above.

Atomic force microscopy

The cell-free side of the agar disc was dried carefully on a sheet of Kimwipe tissue (Jujokimberly, Tokyo) and then was put on the metal disc of a Nanoscope III atomic force microscope without using glue. The liquid cell of AFM was set up without the usual rubber O-ring. A limited volume of liquid culture medium was injected into the liquid cell so that a thin water interface was formed between the agar surface and the liquid cell. Images were obtained by contact mode Nanoscope III AFM (Digital Instruments, Santa Barbara, CA). We used sharpened silicon nitride tips supplied by Digital Instruments with a spring constant of 0.06 N/m and a J scanner (165 μm maximum scan size). It was possible to image the same area continuously for more than 7 h, during which the time lapse between subsequent images was 6 min. This is because additional culture medium was injected at intervals while scanning to replace the evaporating water. Room temperature was kept at 22°C during imaging. Scanning parameters were as follows; scan size, 20 μm; scan angle, 35°; scan rate, 0.702 Hz; integral gain, 0.266; proportional gain, 0.318; set point, −1.95 V.

We recorded the error signal images to show the fine details on the surface of the cells (Putman et al., 1992; Neagu et al., 1994; Fritz et al., 1994). In this mode the feedback loop compensates the large cantilever deflections by keeping the sample height constant, resulting in the height image. The remaining deflection signal is amplified and recorded, giving the error signal image.

RESULTS AND DISCUSSION

Fig. 1 shows the distribution of immobilized cells over the agar surface after staining them with 1% crystal violet for 1 min, then imaging them with a confocal laser microscope (MRC-500 BIORAD Cambridge). Several optical sections made at different depths showed that cells were captured in high density on the very top of the gel surface, almost in a monolayer form. This result verified that the cells were accessible to the AFM tip.

The viability of the immobilized cells prepared as described above was checked by incubating some of the agar discs overnight in the same culture broth mentioned above. We noticed a thick cell coverage on the agar surface after incubation. In addition, on examining the sample surface after imaging for several hours it was possible to observe the formation of minute colonies.

Typical results showing real-time images of live yeast cells are shown in Fig. 2. It can be seen that some of these cells are increasing in height and width, e.g., cell number 1 and 3. The corresponding height mode images are shown in Fig. 3.

It was possible to ascertain that the rapid height changes as noted in Fig. 2 and Fig. 3 probably resulted from the growth activity of the immobilized cells and are not due to the slow dissolution and/or the mechanical scraping of the

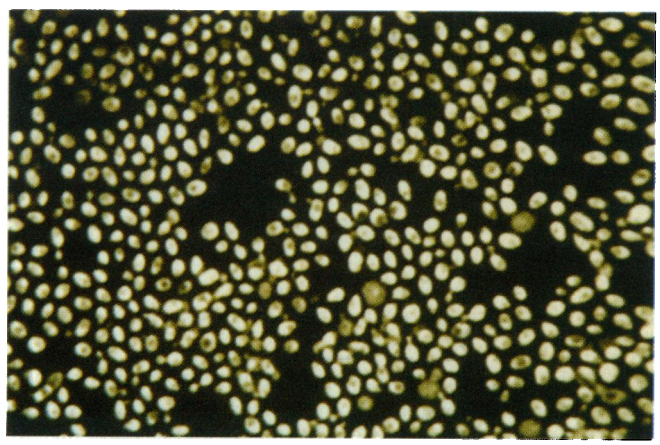

FIGURE 1 A confocal laser microscope image of a stained sample of *Saccharomyces cerevisiae* showing the cell distribution on the agar surface.

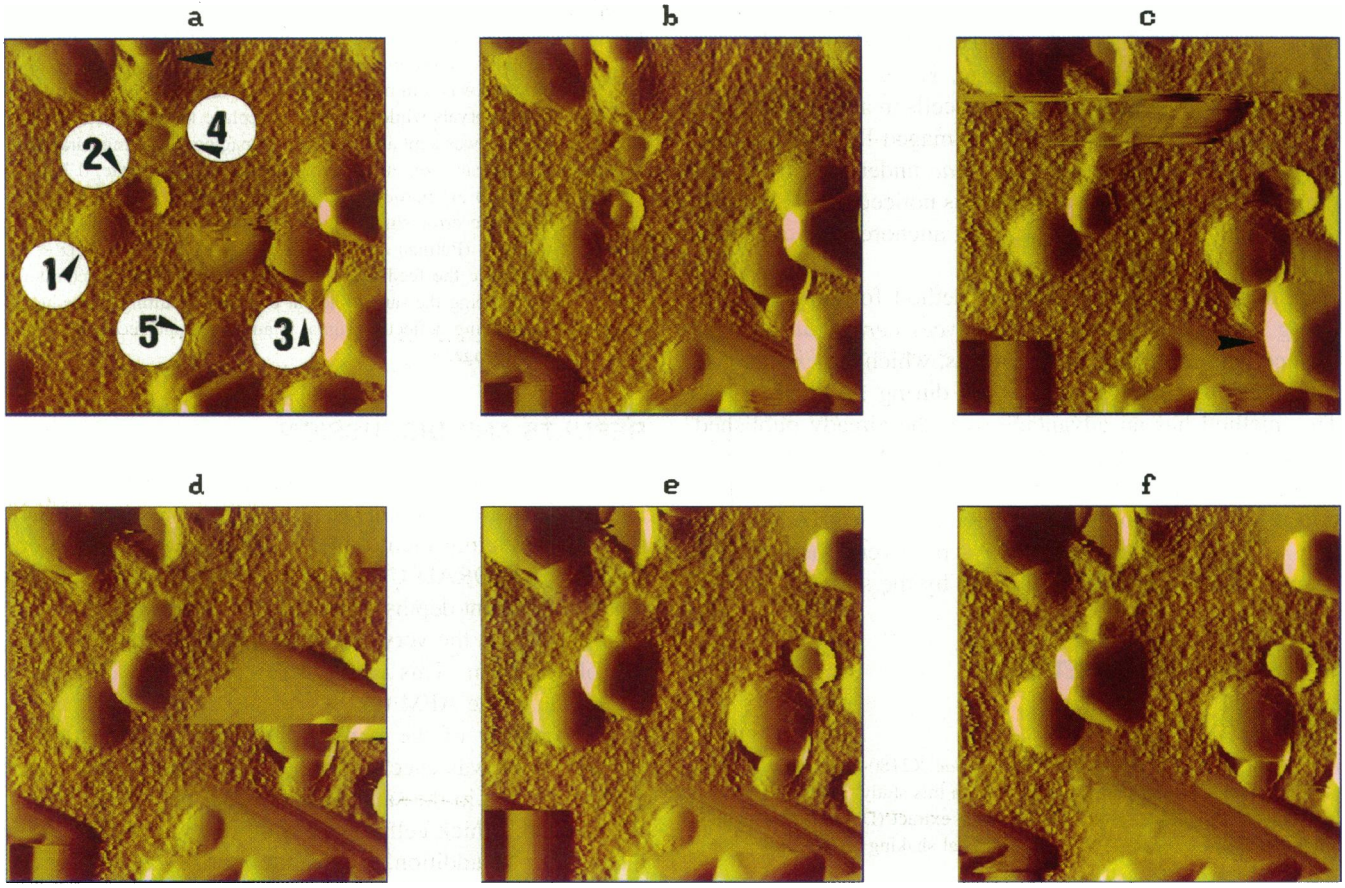

FIGURE 2 Deflection mode real-time images of yeast cell growth and budding. The time lapse between each subsequent image is 6 min. Notice the removal of the growing cell in *c* (shown by an *arrowhead*) when it reaches a critical height in *d*.

agar surface or further caused by shifting of the positions of cells within the agar matrix. One line of evidence supporting the above assertion came from the study of vertical sections of the cells and measuring their height change using Nanoscope III software. In Fig. 4 the height change of five representative cells (numbered from 1 to 5 in Fig. 3 *a*) were plotted against time. The results in Fig. 4 show that whereas the heights of cells numbered from 1 to 3 increased by about 440 nm to more than 1 μm, those of cells 4 and 5 remained almost constant throughout the scanning period of 36 min. The slight increase in the height of cell number 4 might have been induced by the growth of the neighboring cell, which could be either the upper cell shown by an arrowhead in Fig. 3 *a*, or cell number 2, which emerged from the hole shown in the same figure. We do not know whether cells 4 and 5 were dormant or dead, but the very fact that there were such cells that did not show any noticeable increase in their heights attested to our tentative conclusion that the agar surface was stable against the force exerted by the scanning tip and that there was no significant, continuous dissolution of agar during the scanning period. This conclusion will be augmented later in this paper by the experiment performed on chemically fixed cells for which there was no possibility of growth. In addition, it can be clearly seen in Fig. 3 that cell number 2 grew faster than the adjacent cell number 1, although the agar matrix surrounding them was exposed to the same scanning force.

Another important observation that supported that what we observed is cellular growth is that the cells were becoming taller compared to the agar level at different rates (Fig. 4).

The result of the control experiment performed on chemically fixed cells as described in the previous section was quite distinct from the result on live cells given above. We imaged the fixed cells with Nanoscope III for several hours. Throughout the scanning time, none of the imaged cells showed rapid change in their height or width or detached from the substrate, except for the initial 90 min, when there was a very slow increase in their heights. The total height change during this 90-min period was roughly equivalent to that observed within one scan of 6 min in the case of live cells. This may be due to the removal of the very fine agar layer covering the periphery of the exposed cell surface. Another possibility that may expain this phenomenon is the shrinkage of the agar matrix as a result of the increasing osmotic pressure due to the evaporation of water. Furthermore, we tried to scrape the agar surface by increasing the set point of the AFM tip, and hence the friction force, to the maximum setting. Surprisingly, none of the cells detached from the agar surface, indicating the strength of adhesive

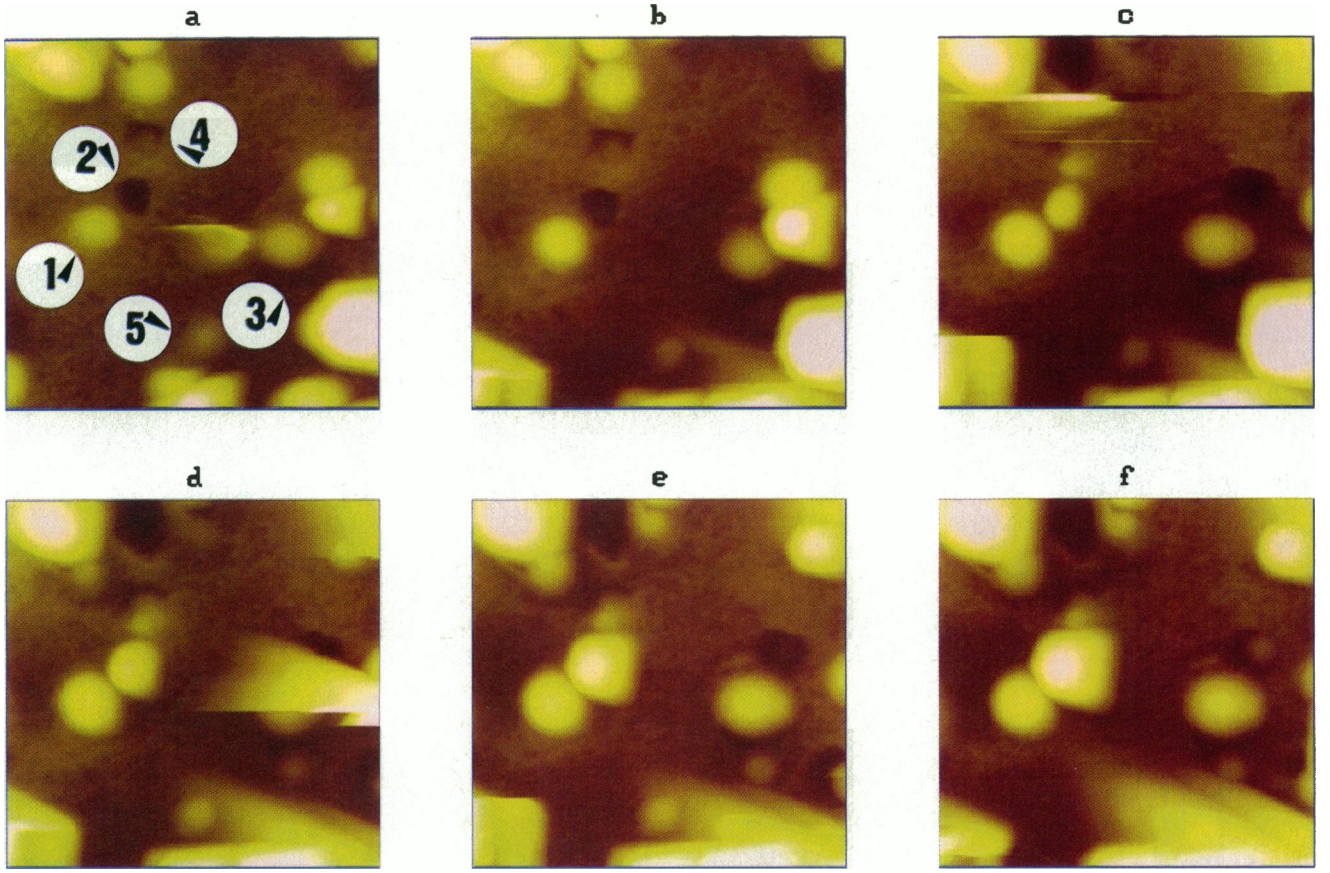

FIGURE 3 The corresponding height mode images of Fig. 2.

force between agar and yeast cells when they were not alive. Hence, we chose ten representative cells from among the chemically fixed ones and monitored their height changes, as given in Figs. 5 and 6, which clearly showed that indeed there was a small increase in the height of the cells during the long scanning period, but the rate of increase was very much smaller.

Once the stability and firmness of the agar surface were established, the conspicuous increase in heights of cells 1 to 3 was interpreted to represent their growth and not a mechanical exposure of the cells as a result of gradual loss of the surrounding agar. Even among the cells mentioned above there was a wide range of difference in the rate of their height change, indicating again that there was no uniform loss of agar from their surroundings. The growing cells showed not only a change in the height but also an increase in their apparent width. The rapid increase of the cellular dimensions observed for cells 1 to 3 by AFM was not at all an exceptional case but rather the norm in a number of experiments repeated in a similar manner. We believe that most, if not all, of such changes reflected the activities of living yeast cells, including their growth and, in some rare cases, budding of daughter cells from mother cells.

In the case of such live cell experiments, we noticed that almost all of the cells were actively changing their appearances during AFM scanning, which we hereafter refer to as growth activities. The shape of the imaged cells depended mainly on their orientation within the agar matrix. Three typical types were noticed as in Fig. 2 *a* for the cells

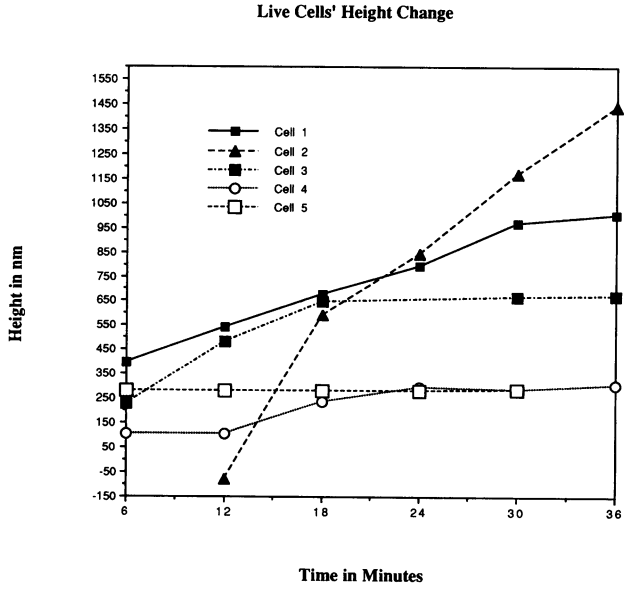

FIGURE 4 A line graph showing the height changes of living cells against time. The cell numbers indicated correspond to the representative cells shown in Figs. 2 and 3.

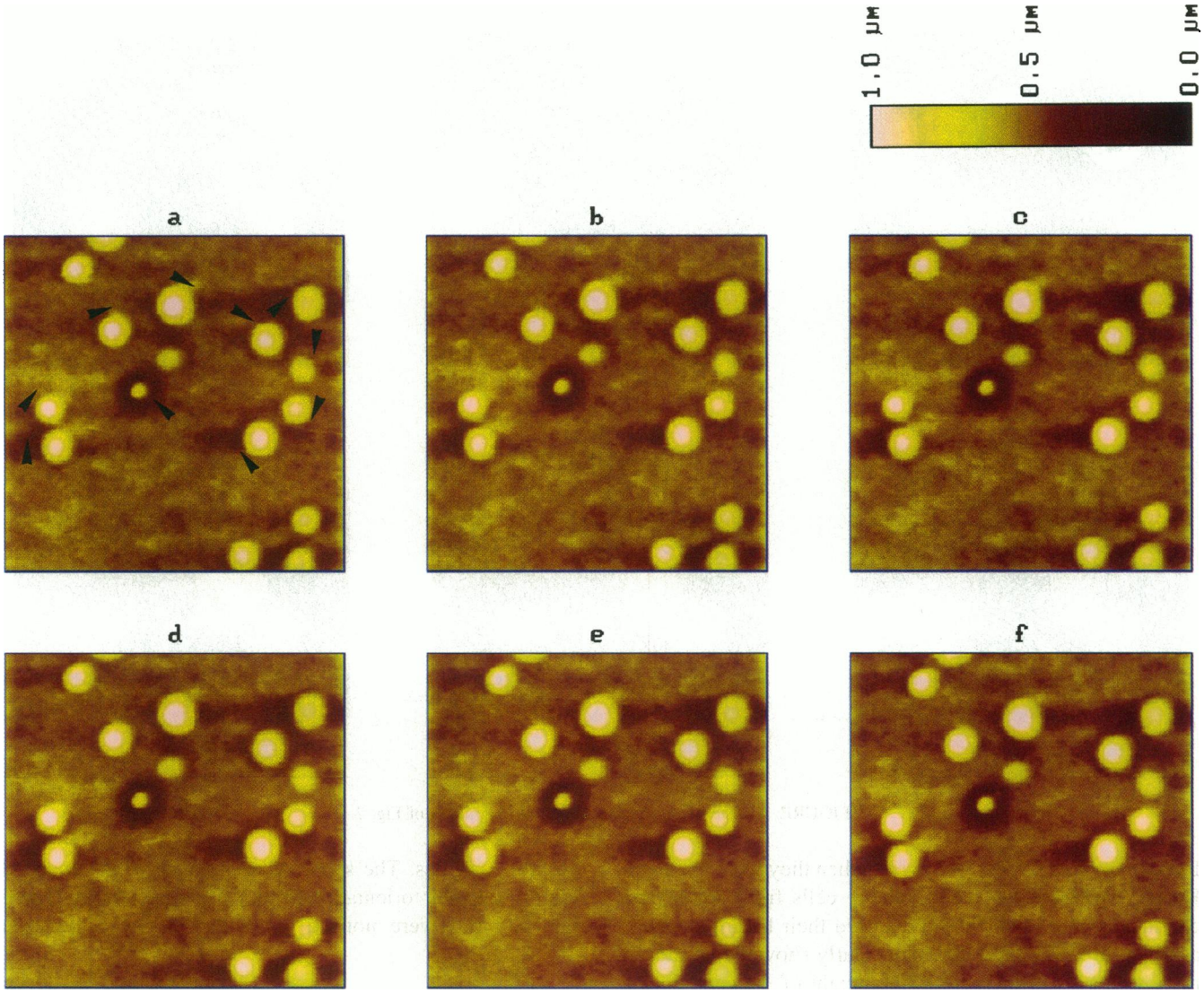

FIGURE 5 Height mode images of fixed yeast cells. The time lapse between each subsequent image is 6 min. The cells adjacent to the arrows are the representative cells on which we carried out the section study.

numbered 1 to 3. Type 1 image represents an almost submerged or deeply embedded cell. The cell growth of this type can be followed in the subsequent images as it increases in size and height (notice the height change in the corresponding height image Fig. 3). On the other hand, type 2 image in the same figure shows a vacant hole that represents an impression of a detached cell. By following the subsequent images at the bottom of the hole we detected the emergence of a new cell, which most probably represents a bud of a totally embedded cell under the agar surface. At a certain height the cell topography is turned into a pyramidal shape due to the tip convolution effect. Type 3 image represents a partially embedded cell budding from within the agar matrix.

An interesting feature was noticed by following the budding process of a type 3 cell. The seemingly growing bud looked to be strongly pushing out of the agar while it expanded. This produced certain strain on the agar that was imaged clearly as shown in Fig. 2. Tkacz and Lampen (1972) showed with fluorescein-labeled concanavalin A that the newly synthesized mannan is inserted at the tip of the bud in a hybrid *Saccharomyces cerevisiae.* The apical growth of the yeast bud was reconfirmed by autoradiographic studies (Biely et al., 1973; Farkas et al., 1974). This fact could be observed from the mechanical behavior of the bud in the AFM images. We noticed that, as the bud expanded a slight bending of the cell axis occurred, normally followed by straightening of the axis while the bud forced its way out; notice a small change in the angle between the mother cell and the emerging bud observed during this process. The time lapse for a complete scan was 6 min. In the future we should be able to obtain new quantitative data for the force by which the bud pushes the gel. This can be calculated by studying the mechanical properties of the suitable gel matrix by the force modulation mode.

The method presented in this paper partly solved the height problem in AFM scanning, but as the cells grew in

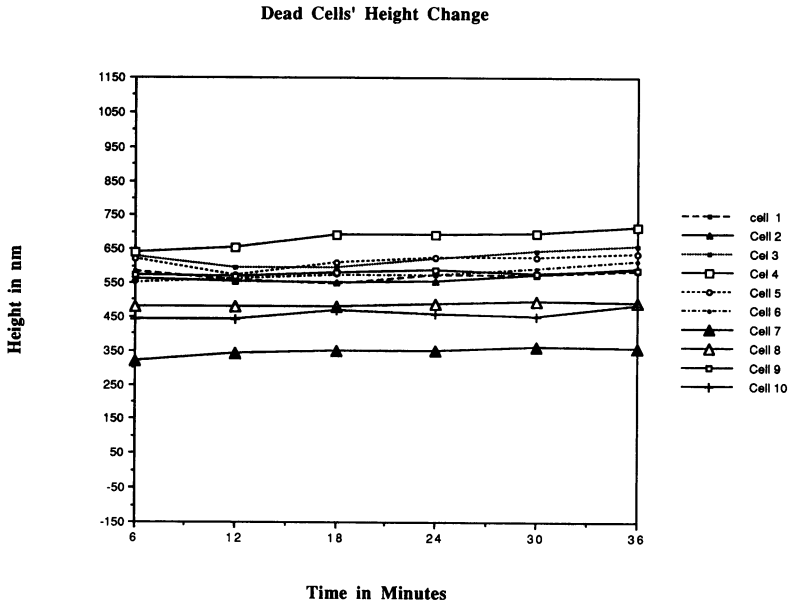

FIGURE 6 A line graph showing the height changes of fixed cells against time. The cell numbers indicated in the graph correspond the clockwise serial number of each cell according to the directions of the arrows shown in Fig. 5.

volume it became impossible to continue imaging them because of the tip-sample convolution effect, as can be seen in Fig. 7. In this figure the actual spherical shape of the cells is lost and the pyramidal artifact dominates. Such an inherent effect in AFM imaging is caused by the finite size and imperfect shape of the tip. If the shape of the probe tip is known, these distortions can be accounted for, and an undistorted image of the sample can be reconstructed (Keller and Frank, 1993).

We were successful in clearly imaging the bud scar—an ultrastructure left on the cell wall of the mother cell by budding daughter cells. This indicated that immobilizing yeast cells on gel surfaces did not limit the high-resolution imaging of definite structures on their surfaces like the bud and birth scars (shown by an arrowhead and an arrow in Fig. 8, *a* and *b*, respectively). We imaged the same cell for 5 h, and it rotated slightly, as can be noticed by comparing images *a* and *b* in the same figure. The time lapse between Fig. 8 *a* and 8 *b* is 5 h, which indicates that the cell rotation was probably not induced by the scanning tip but rather by pushes exerted by a neighboring growing cell, or probably the cell being imaged spontaneously moved when it gave rise to a bud beneath the agar surface. The regional distribution of budding sites on *S. cerevisiae*, and hence of scars, was discussed by Winge (1935) and Lindegren (1949). They noted that budding haploid cells generated their earliest

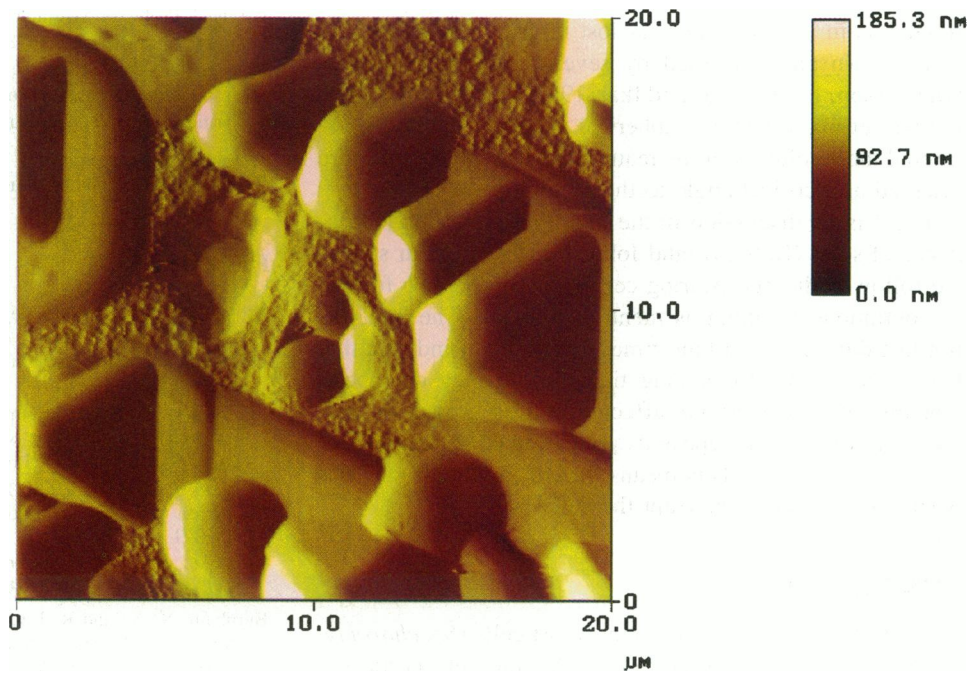

FIGURE 7 This image shows the severe limitation of clear imaging as the cells get taller while growing. The pyramidal shape represents the tip shape and not the actual topography of the sample.

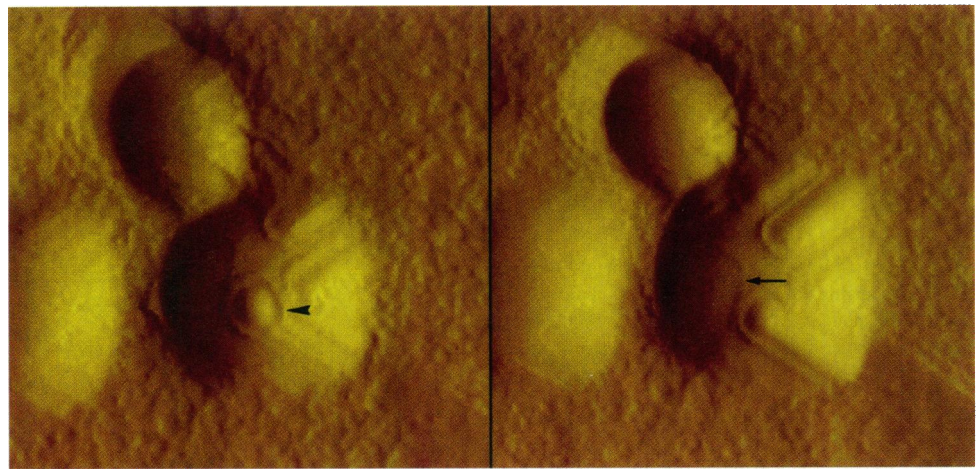

FIGURE 8 (*a*) A typical bud scar is shown by an arrowhead on the yeast cell wall. The chitin ring is seen surrounding the newly formed septum. (*b*) The same cell after a slight rotation showing a birth scar (shown by an arrow) surrounded by four or five bud scars. Notice the deformation of the bud scar that occupied the top of the cell in *a*.

buds proximal to their birth scars, forming rosettes, and thus differed from the usual distal diploid pattern. These observations were confirmed by Freifelder (1960). This is in agreement with our data shown in Fig. 8 *b*. In that figure we could image the birth scar, shown by an arrow, which is the scar left on the daughter cell when it detaches from the mother cell, as it was surrounded by four bud scars from one side. The birth scar looked larger than the bud scar (2.5 μm and 1.2 μm in diameter, respectively) in the AFM image as shown before by the electron micrograph of Gay and Martin (1971).

In Fig. 8, *a* and *b*, an artifact can be observed that distorted the typical circular shape of the bud scars that surround the birth scar. They appeared as incomplete circular features, and at the missing part of the circle a slope was formed. The position of the scars on the sloping part of the cell was the main reason for this artifact, because when one of these scars occupied the top of the cell as in Fig. 8 *a* it was imaged normally; on the other hand, when the cell rotated as in Fig. 8 *b*, the scar lost its true appearance. As well known and discussed by several early investigators (Henderson, 1994; Kasas and Ikai, 1995; Neagu et al., 1994; Putman et al., 1993), the spherical shape of the cell body limited the resolution of the features on its surface that were oriented at a critical angle to the scanning tip.

There is no discussion in the literature about the permanence of scars (Robinow and Johnson, 1991). In our study we followed the bud-bearing cell in the Fig. 8 *a, b* for 5 h of continuous scanning as mentioned above. The bud was not lost during such a long time, which might indicate that buds tend to last for a long time before they disappear. Apparently, cells were not affected in their growth or budding activities by the repeated application of scanning force during their growth. This means that the applied force was weak enough not to interrupt the cells' activity.

CONCLUSION

A method for immobilizing living yeast cells (*Saccharomyces cerevisiae*) on the surface of a flexible gel matrix was developed. It was shown that this method helped in imaging the dynamics of yeast cells that could be interpreted as their growth and budding processes, which have so far not been reported on. Thanks to the flexibility of the agar matrix, the cells were allowed to have a certain degree of freedom to expand within it. It was also possible to obtain high-resolution images for distinct structures on the cell surface (e.g., the bud and birth scars). We believe that this method can be applied to other plant cells as well as to nonliving, bulky objects.

Mechanisms controlling cell surface growth are important problems in cell biology (Schnepf, 1986). The potential of AFM to image actively growing cells at high resolution under physiological conditions opens the way to study the details of cellular development. Real-time studies of the morphological changes induced by biologically active materials will be quite possible.

We thank Professor Yoshinori Ohsumi at Tokyo University for his helpful discussions, Professor Paul K. Hansma at the University of California, Santa Barbara, for reading the manuscript and for giving useful comments, and Dr. Hideo Arakawa at Tokyo institute of Technology for suggestions and critical review of the manuscript.

This work was supported by grant-in-aid 05245102 from the Ministry of Education, Science and Culture.

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