Ultramicroscopy 110 (2010) 254–258

Contents lists available at ScienceDirect

Ultramicroscopy

journal homepage: www.elsevier.com/locate/ultramic

Immobilization method of yeast cells for intermittent contact mode imaging using the atomic force microscope

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ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form
15 November 2009
Accepted 1 December 2009

Keywords:
Live cells
Yeast
Intermittent contact mode
Atomic force microscopy

ABSTRACT

The atomic force microscope (AFM) is widely used for studying the surface morphology and growth of live cells. There are relatively fewer reports on the AFM imaging of yeast cells [1] (Kasas and Ikai, 1995), [2] (Gad and Ikai, 1995). Yeasts have thick and mechanically strong cell walls and are therefore difficult to attach to a solid substrate. In this report, a new immobilization technique for the height mode imaging of living yeast cells in solid media using AFM is presented. The proposed technique allows the cell surface to be almost completely exposed to the environment and studied using AFM. Apart from the new immobilization protocol, for the first time, height mode imaging of live yeast cell surface in intermittent contact mode is presented in this report. Stable and reproducible imaging over a 10-h time span is observed. A significant improvement in operational stability will facilitate the investigation of growth patterns and surface patterns of yeast cells.

1. Introduction

Cell walls, as the interface between intracellular material and the environment, play a crucial role in the biophysical processes in cells. In general, genetic and chemical methods are used to provide insights into the formation, control, and function of cell walls [3]. However, the cell wall has a highly dynamic structure which changes constantly during cell division and growth. Study of cell wall properties in native condition can provide new information about the evolution of cell wall morphology. *Saccharomyces cerevisiae* has been established as a convenient organism for studying plant cell walls due to its robustness, short half-life, and well-characterized collection of mutants. AFM is an enabling technology for single-cell investigation, with high spatial resolution and direct measurement of surface material properties. Compared to typical methods of cell wall characterization (for instance: chemical analysis of cell wall extracts, binding studies, digesting by enzymes, and electrophoretic measurements), AFM studies do not require prior cell manipulation or averaging over an ensemble of cells. AFM provides complementary information to traditional invasive and statistical methods by enabling study in a native environment at the single-cell level.

Even though *S. cerevisiae* is only one species of a 1500-strong family of yeasts, it is the most common one, and in subsequent sections, we have used the terms *S. cerevisiae* and yeast interchangeably.

2. Role of AFM in cell wall characterization

Deciphering functions of cell surfaces requires knowledge of their structural and physical properties. Electron microscopy has been the key tool for understanding cell surface ultrastructure. However, the AFM proves to be a powerful tool for the investigation of cell surfaces with nanometer resolution. AFM provides images of the surface ultrastructure under physiological conditions.

The ability of an AFM to image live cells at nanometer resolution has generated new possibilities for gathering microscopic physiochemical properties of cell surfaces [4]. AFM can be used to measure cell volume change and relate topological changes of the cell surface at nanoscale with cellular stress physiology [5]. Besides metrology, AFM probes can be utilized as external mechanical indenters, and models for the measurement of cell membrane elasticity and internal osmotic pressure can be found in [6]. Also, AFM studies have demonstrated the effect of osmolarity and sodium azide concentration on cell viability and cell surface elasticity [7]. The atomic resolution of AFMs has assisted scientists in studying the ultrastructures of live yeast which are not visible with optical microscopes. AFMs have been used to observe the phenomenon that individual pores of the hexagonally packed intermediate surface layers switched from

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doi:10.1016/j.ultramic.2009.12.003

one state to the other [8]. The need for stable and repeatable methods of anchoring yeast cells on a hard surface was emphasized in these studies.

2.1. Existing methods and challenges of cell immobilization for AFM imaging

The first successful attempt of immobilizing live yeast cells was reported in 1995 [2]. Cells were gently immobilized on 3% agar and submerged in a culture medium. These cells were imaged in contact mode with an AFM for 6–7 h. We may term the proposed method as "reverse agar-plating", because in this method, a drop of molten agar is placed on yeast solution, and as the agar solidifies, yeast cells get trapped in the agar. Confocal microscopy confirmed that most of the cells do not travel deep in the agar surface and hence, the agar plate can be flipped upside down and trapped cells may be imaged by AFM. However, this method poses a few challenges for proper imaging: molten agar introduces a heat shock for yeast, since below 30 °C, the viscosity of agar gel quickly increases, leading to an uneven spread, or flow, from the pipette. Also, only a very small area of yeast surface gets exposed to the AFM tip and nutrients. Contact mode imaging causes easy tip contamination and sometimes displaces cells which are not tethered well to the surface (e.g., budding cells).

An alternative trapping method, using polycarbonate membranes, was introduced in 1995 [1]. In this method yeast solution was filtered via a porous polycarbonate membrane under negative pressure. If the cell diameter matches the pore diameter, then the cell gets trapped in the pore. The trapped cells are imaged using AFM. However, the yield of trapping is significantly low. Not all pores are of equal size and cell sizes do vary considerably. Only a good match between the cell and pore sizes ensured proper trapping. Even when cells are trapped, they may get pushed out of the pore due to the vertical force applied by the probe. Also, stiff trapping hinders the lateral growth of cells, and younger cells, which are significantly smaller than the pore size, cannot be trapped, giving inaccurate results where the trapped cells are not representative of the actual cell population.

Several other different methods of immobilization exist: notably, air drying [5]; chemical immobilization [9]; and adsorption on silica surface [9]. However, all of these methods have irreversible effects on cell morphology, viability, and metabolism. Hence, authors think, these methods are not suitable for the investigation of cells in their native environment.

3. New protocol for immobilization of live *S. cerevisiae* cells for AFM imaging

In this work, we have developed a new immobilization method where we rely on the natural process of growing yeast culture on solid media. When yeasts are grown on solid media with nutrients, newly grown cells are well anchored to the media, and so are the mature live cells which absorb nutrients from the media. Experimental data show that this method is well suited for AFM imaging and provides stable anchoring of live cells. Also, we obtained images of *S. cerevisiae* cells in intermittent mode imaging, which is an enabling step toward noninvasive imaging of live cells.

3.1. Preparation of yeast sample

3.1.1. Preparation of yeast cells

A budding yeast sample, *S. cerevisiae* (YM4271, Clontech Laboratories, USA), was used in this study. An overnight saturated culture of YM4271 cells was diluted 10 times and grown at 30 °C under continuous shaking. When the cells reached the logarithmic phase of growth, 60 ml yeast solution was collected. Cells were collected by centrifugation at 1000*g* for 5 min. Then the collected cells were resuspended in 1.25 ml distilled water.

3.1.2. Preparation of yeast–peptone–dextrose (YPD) media

YPD media is a blend of peptone, dextrose and agar in optimal proportion to facilitate growth of yeast cells. Yeast extract and peptone provide the growth nutrients and dextrose provides the energy source for yeast growth.

3.1.3. Composition of YPD media

Ingredients	g/l
Yeast extract	10.0
Peptone	20.0
Dextrose	20.0
Agar	15.0

A 6.5 g of the above mixture was boiled in 100 ml distilled water to prepare the YPD media with 1.5% agar. About 100 µl of

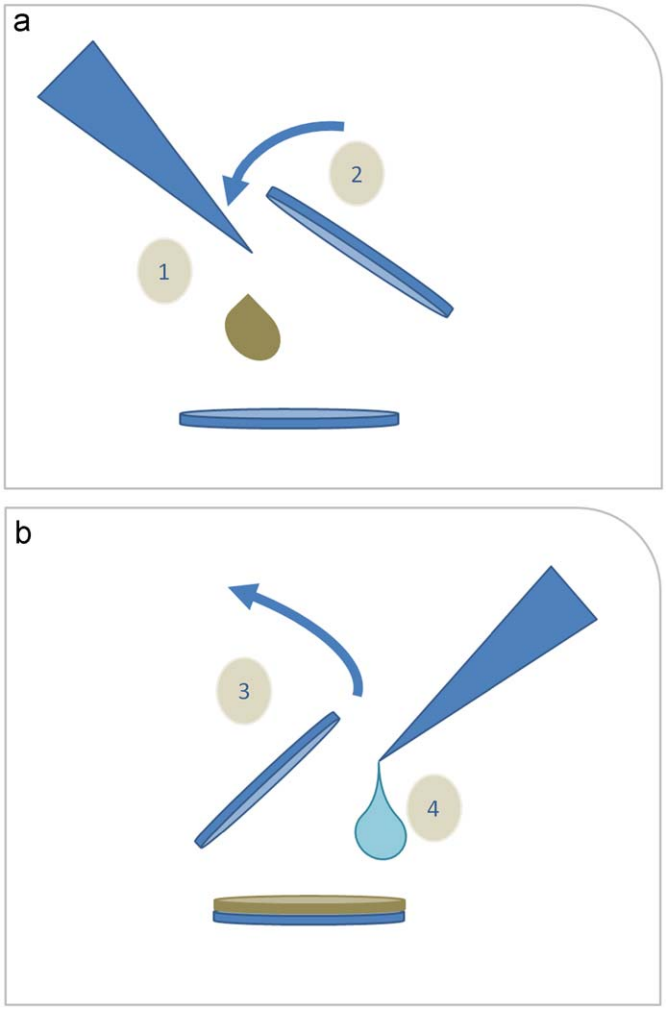

Fig. 1. Immobilization method of yeast cells. Step 1: 100 µl molten agar with nutrients is dropped on a cover slip. Step 2: another cover slip is placed on the agar droplet. Step 3: top cover slip is removed. Step 4: a drop of yeast solution is spotted on the agar disc. (a) Preparation of nutrient rich agar disc. (b) Spotting of yeast solution on the agar disc.

molten YPD media containing 1.5% agar was dropped over a #1-thickness cover slip (Fig. 1(a), step 1). Another cover slip was placed over the agar surface and the agar allowed to solidify (Fig. 1(a), step 2). The top cover slip was gently removed (Fig. 1(b), step 3) and 5 μl of yeast suspension was dropped on the agar surface (Fig. 1(b), step 4). Once the yeast cells were dropped on the agar surface, the cover slip with agar disc and yeast solution was placed on a petri dish, and the petri dish was sealed with paraffin film to prevent desiccation of cells. The petri dish was placed in an incubator at 30 °C for 30–45 min. However, it was necessary to increase incubation time up to 3 h in some cases (when cells were growing slowly; e.g., after treating with inhibitors like sodium azide). After incubation was completed, the cover slips with yeast-spotted agar discs were placed under airflow for a brief amount of time (typically 10 min or less) to remove surface moisture. At the end of this process, the sample was then ready for AFM imaging.

3.2. Prepare immobilized cells for AFM imaging

The yeast samples were imaged with MFP–3D in intermittent contact mode. MFP–3D is an AFM manufactured by Asylum Research, Santa Barbara, CA. The sample and the AFM were placed in an enclosed environment (Herzan isolation chamber). We used AC240 silicon cantilevers, manufactured by Olympus, with approximate spring constant of 1 N/m and quality factor of 160. Typically, AFM integral gain and proportional gains were chosen to be in the ranges of 16–20 and 0–4, respectively. Typical free oscillation amplitude of the cantilever was in the range of 150–200 nm and set point was set at 80% of free oscillation amplitude.

Initially, 90 μm square area was imaged and multiple cells in different phases of growth were found. Then we zoomed in a two-cell-cluster which showed interesting cell structures, like bud scars. For second set of images, imaged area was approximately 18 μm square. All images were captured at 512 × 512 resolution, with tip velocity in the range of 2.5 to 5 μm/s. The nanopositioning stage was moved in the *X* and *Y* directions by a piezo-actuated flexure in a feedback-controlled closed loop with sub-nanometer lateral resolution. As the nanopositioning stage moved the sample for raster scan, the vertical feedback loop extended and contracted the vertical piezo to keep the amplitude of oscillation constant [10].

4. Results and discussion

With the above-mentioned immobilization method and AFM settings, *S. cerevisiae* samples were imaged for 10 h without any degradation in image quality. Even though some cells stopped

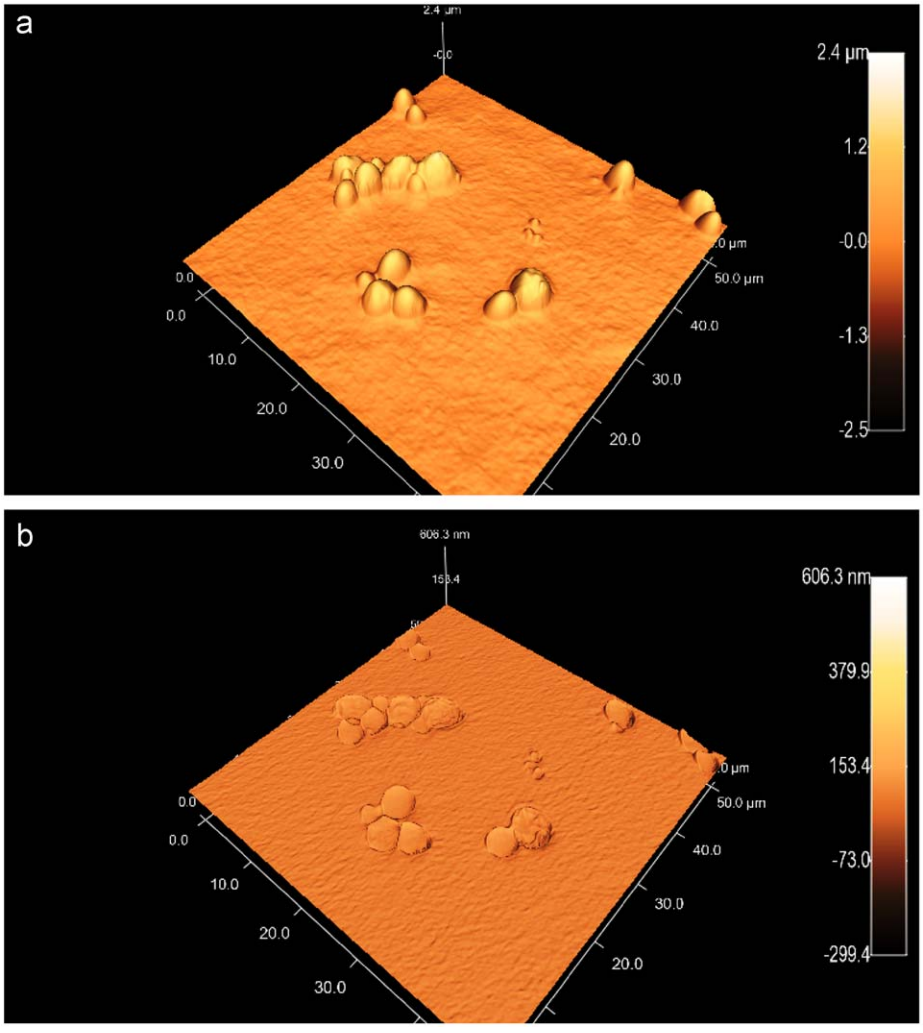

Fig. 2. Multiple *Saccharomyces cerevisiae* cells in nutrient-rich media. A large collection of yeast cells is imaged in intermittent contact mode at a fidelity similar to a two-cell cluster. (a) Height image. (b) Error signal image.

growing after 6–8 h, anchoring was firm enough to continue imaging for 10 h.

Fig. 2(a) shows the image of live yeasts after 6 h of imaging. Figs. 2(b) and 3(b) show the so-called "error signal", which corresponds to the difference between the set point amplitude and the real amplitude of the cantilever oscillation. This type of image allows to showing the sharp edges of the yeast surface. Furthermore, in Fig. 3(a), the bud scar on the larger cell is evident. It is worth noting that, in Fig. 3(b), error mode imaging shows almost a flat profile (excluding at the edges of the cells). This shows that the feedback loop has kept the amplitude of oscillation close to the set point through out the imaged area. Maintaining the amplitude set point has a positive impact on the fidelity of the image and non-invasiveness of the probe. When set point is closely maintained, the cantilever does not encounter non-linearity during imaging and the height mode image represents the true height of the cells.

The new immobilization method has enabled trapping of live cells in a non-invasive way where a large part of the cell gets exposed to the environment. This helps in more efficient absorption of nutrients and inhibitors as compared to existing methods. Since cells are grown on nutrient-rich agar media, the new method of immobilization is essentially the same as the streaking of yeast stocks on agar and very close to the natural growth environment for yeast colonies. The immobilization method developed in the paper produces a uniform cell density over the agar surface where cells of all ages, sizes, and shapes are accessible to the AFM probe. In contrast to membrane-based immobilization, the new method also allows the investigation of the daughter cells, which have significantly different physiological properties as compared to the mother cells. As observed in Fig. 2(a), cells of different sizes are imaged simultaneously, which is not possible with membrane-based immobilization.

The immobilization method developed in this paper also offers advantages over "reverse agar-plating". In the new method, a large fraction of the cell surface is accessible to the probe, and therefore, a more complete image of the cell is obtained. In "reverse agar-plating", the growth of neighboring cells affects the height image of a cell due to the stretching of agar film—a problem which is eliminated in the new method.

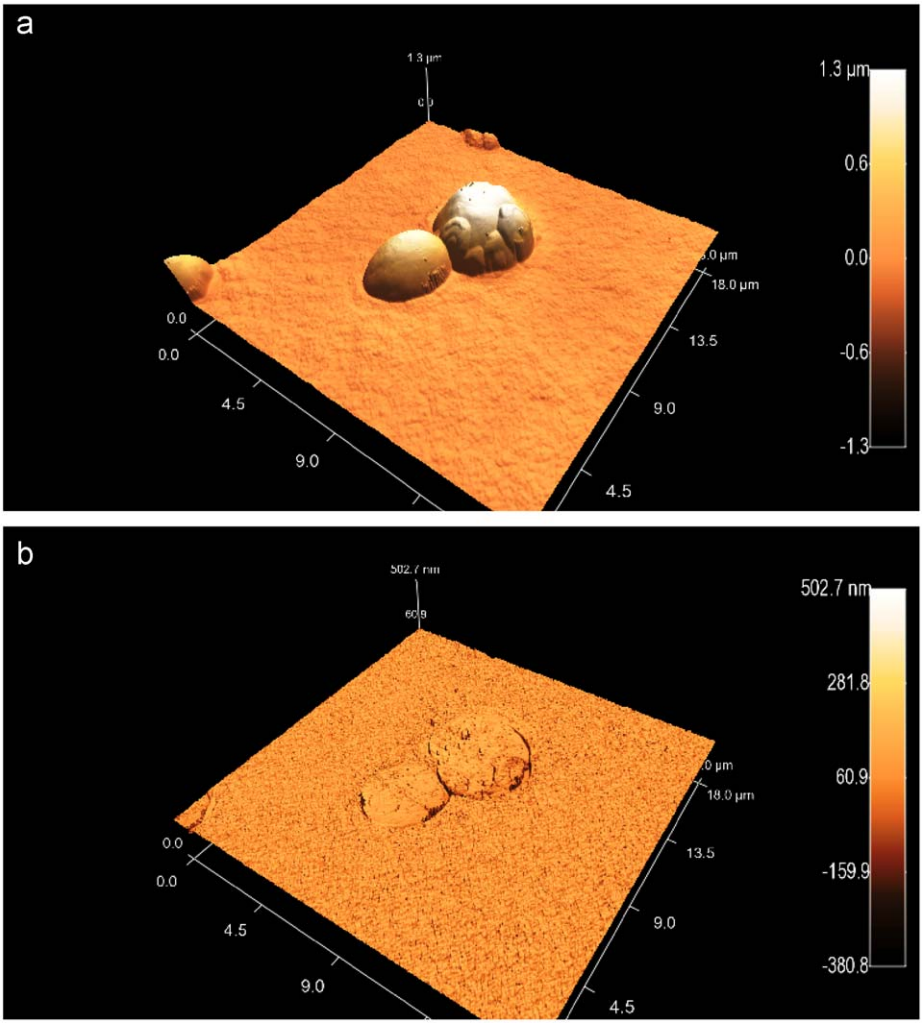

Fig. 3. *Saccharomyces cerevisiae* in nutrient-rich media. Live yeast cells are imaged in intermittent contact mode. As observed from error signal image, high-bandwidth vertical positioner can maintain the amplitude close to its set point. Since the cantilever amplitude is always close to set point, amplitude tracking error is close to constant, except at the boundaries of the cells. This allows us to analyze the height image since the cantilever operation is maintained in its linear deflection region. Also, by maintaining constant amplitude, the imaging is inherently less invasive to the sample, as compared to conventional error-mode or contact-mode imaging. (a) Height image of *Saccharomyces cerevisiae* in nutrient-rich media. (b) Error signal image of *Saccharomyces cerevisiae* in nutrient-rich media.

4.1. Significance of height-mode imaging in intermittent contact mode imaging

In this article, height-mode images of live yeast cells were obtained using the intermittent contact-mode imaging. Most of the prior arts focus on contact-mode imaging and rely on error-mode imaging for visualizing ultrastructure. Given the relatively large size of yeast cells, error-mode imaging is not suitable for topographic analysis since the probe-surface interaction is not linear and the error signal does not vary linearly with surface features. Hence, the AFM images do not represent true surface height variations. In [5], intermittent contact mode has been used for air-dried cells, but the viability of cells decreases significantly when the cells are dried on non-nutrient media (e.g., glass slide). In this report, cell wall structures are resolved in height-mode images using intermittent contact mode, which enables the analysis of cell morphology and the observation of the in situ evolution of surface features.

5. Conclusion

The proposed immobilization method is an enabling step toward the height mode imaging of live cells in intermittent contact mode. Stable anchoring and height-mode imaging opens new avenues for study of surface properties. Using the new method, cells of various sizes can be imaged for prolonged periods (up to 10 h). The method developed also allows the cells to grow freely, with free access to nutrients. Subsequent studies will include exposing the cells to growth inhibitors and comparing variations in growth rates.

Acknowledgement

This research was partially supported by NSF grants CMMI 0900113 and CMMI 0814615.

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