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High resolution X-ray micrography of live *Candida albicans* using laser plasma pulsed point X-ray sources

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Electron microscopy is still the most frequently used method for visualization of subcellular structures in spite of limitations due to the preparation required to visualize the specimen. High resolution X-ray microscopy is a relatively new technique, still under development and restricted to a few large synchrotron X-ray sources. We utilized a single-shot laser (nanosecond) plasma to generate X-rays similar to synchrotron facilities to image live cells of *Candida albicans*. The emission spectrum was tuned for optimal absorption by carbon-rich material. The photoresist was then scanned by an atomic force microscope to give a differential X-ray absorption pattern. Using this technique, with a sample image time of 90 min, we have visualized a distinct 152·24 nm thick consistent ring structure around cells of *C. albicans* representing the cell wall, and distinct 'craters' inside, one of 570·90 nm diameter and three smaller ones, each 400 nm in diameter. This technique deserves further exploration concerning its application in the ultrastructural study of live, hydrated microbiological samples and of macromolecules.

Keywords: *Candida albicans*, X-ray micrography, high resolution, ultrastructure, laser plasmas

INTRODUCTION

The detection and study of microbial cells is performed by low magnification optical microscopy, and direct and indirect labelling techniques. In bacteriology, cationic (methylene blue, crystal violet, safranin) or anionic (eosin, acid fuchsin, congo red) colour dyes are used for resolution at the 1–100 μm scale and radioactively labelled substrate uptake is measured for very slow growing mycobacteria (Chapin-Robertson & Edberg, 1991). In virology, for nm-scale detection, secondary-conjugated fluorescent antibodies are applied. Visual ultrastructural studies on subcellular organelles are possible with variations of electron microscopy (thin section, scanning and freeze fracture) although specimen preparation steps such as fixation, dehydration, resin embedding, ultra-thin sectioning, coating and staining are very technical, extensive and may introduce artefacts in the original sample. Although electron microscopy can be used at the nm resolution level, the sample preparation steps involved limit its use for routine studies of microbial cells (Kay, 1976; Lichfeld, 1976).

X-ray microscopy is a relatively new technique that has not been applied to any significant extent for biological specimens (DeMeis, 1996). It eliminates specimen preparation which may alter the target, has resolution at the 100 Å level (Ohnesorge & Binning, 1993) and is able to probe the internal structures of *in vivo* assemblies, enabling the observation of complex features in their natural, live state (Feder *et al.*, 1985; Hoh *et al.*, 1992). Most X-ray microscope development has been made so far using large synchrotron sources (Neiman, 1992), thus limiting X-ray microscopy as a research tool to where such facilities are available. The use of a laser plasma X-ray source simplifies the generation of the X-ray source, specimen handling and overall ease of applicability for fragile biological specimens (Shinohara, 1990; Richardson *et al.*, 1992; Rajyaguru *et al.*, 1995, 1996a, b; Kado *et al.*, 1996). The potential advantages of a laser plasma X-ray source stem from its compactness and flexibility. The spectral brightness of laser plasma X-ray sources is comparable to the brightest available synchrotron and can be generated under modest vacuum

Abbreviations: AFM, atomic force microscopy; PMMA, poly(methyl methacrylate).

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requirements. The X-ray emission spectrum of laser plasmas is rich in bright broad (Planckian) continuum emission and in narrow atomic emission lines, and can be easily varied to suit specific microscopy needs. There is also selectivity in X-ray wavelengths to facilitate the elemental analysis of features within biological structures by difference-imaging with emissions at two different wavelengths (Cheng *et al.*, 1989). Laser plasmas are point sources with high reproducibility and therefore fulfil the requirements of the extremely high alignment specifications of precision X-ray optical systems.

Atomic force microscopy (AFM) is derived from the scanning tunnelling microscope (Binning *et al.*, 1982) and provides detailed topographic maps of sample surfaces (Tillmann *et al.*, 1992). The AFM maps surfaces by raster-scanning a fine tip gently over the sample surface, resulting in a 3-dimensional profile of the surface at a resolution that can be as high as atomic resolution on hard flat surfaces (Binning *et al.*, 1987; Panessa-Warren & Warren, 1980). Several DNA–protein complexes have been imaged with an AFM, including DNA complexes with *Escherichia coli* RNA polymerase (Hansma *et al.*, 1993; Zenhausern *et al.*, 1992; Rees *et al.*, 1993), DNA polymerase (Yang *et al.*, 1992), the *Eco*RI restriction enzyme (Nui *et al.*, 1993) and the large T-antigen of SV-40. Biological membranes containing phospholipid mono-, bi- and multilayers (Zasadzinski *et al.*, 1991), individual reconstituted proteins, e.g. fibrinogen (Wigren *et al.*, 1991), immunoglobulins (Hansma *et al.*, 1991; Ill *et al.*, 1993), phosphorylase kinase complexes (Edstrom *et al.*, 1990) and α-macroglobulin (Arakawa *et al.*, 1992), as well as protein arrays in lipid membranes, have also been investigated (Arnsdorf & Lal, 1992; Meyer-Isle *et al.*, 1992; Schabert *et al.*, 1995) with AFM.

In collaboration with the Center for Research and Education in Optics and Lasers (CREOL) at the University of Central Florida, Orlando, Florida, USA, we performed a pilot study to test the ultramicrography technique described above to obtain high resolution images of *Candida albicans*.

METHODS

Whole cells. An overnight culture of *C. albicans* ATCC 14053 grown at 35 °C on Sabouraud dextrose agar was suspended directly into 0·9% PBS, pH 7·4 (Sigma), at a density of 1×10^5 c.f.u. ml^{-1} and vortexed vigorously. A 5 µl drop of the suspension was streaked onto the poly(methyl methacrylate) (PMMA) pre-coated photoresist (Fastec Fabrications) and covered with a silicon nitride window (Fastec Fabrications) before exposure to X-rays. The dimensions of the photoresist and the window were both 5 mm × 5 mm.

Exposure to X-rays. PMMA acts as a photographic film negative, absorbing the X-ray absorption profile of the specimen placed on it. This photoresist was placed at room temperature under vacuum at a distance of 2·5 cm from a silicon target disc (Fig. 1). The silicon plasma produced by a 10 ns burst of laser energy at 20 J radiated strong emission lines in the region of 300 eV. The soft X-rays generated were in a parallel beam, 1–10 nm wavelength and perpendicular to the resist surface. The intensity of X-rays that passed through the specimen and reached the surface of the photoresist was inversely proportional to the amount absorbed by the specimen. The difference in intensities was recorded on the photoresist as radiation damage. The resulting uneven structure on the photoresist was then recorded with an atomic force microscope.

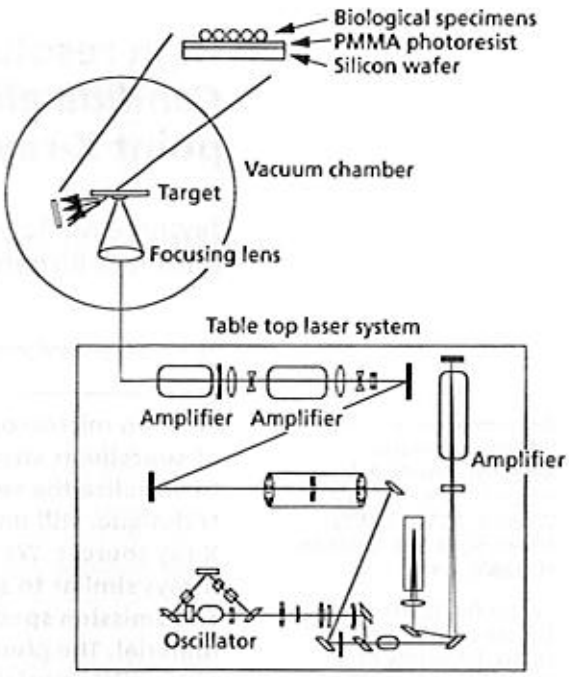

Fig. 1. Laser plasma facility for X-ray microscopy. Characteristics of laser plasma source: laser wavelength, 1·06 µm; laser energy, 10–20 J; pulse width, 10–20 ns; focusing diameter on targets, 100 µm; laser intensity, 5×10^{12} W cm^{-2}; plasma temperature, 300 eV; plasma density, 1×10^{21} cm^{-3}; X-ray conversion ratio, 5–20%; total X-ray intensity, 1×10^5–4×10^5 W.

Photoresist development. After exposure to X-rays, the photoresist was developed briefly by one wash (5 min) in sodium hypochlorite, and for 2 min in 4-methyl-2-pentanone to develop the image. Over-development of the image was prevented by rinsing in 2-propanol. The photoresist was then examined by AFM to obtain a digital image of the absorption profile.

AFM. Scanning probe microscope TMX-2000 from Topometrix was used with TMXSPM software. A 200-µm-long cantilever silicon nitride tip was employed to obtain X-ray images from the developed photoresist.

RESULTS

Fig. 2 shows an atomic force image of a whole cell of *C. albicans* ATCC 14053 in PBS. This image is an X-ray absorption profile of the chemical constituents of the cell. The parameters for the laser energy output were set for optimal absorption by carbon-rich materials, which is suitable for living matter. Therefore, the image in Fig. 2 represents the absorption, and the difference in absorption of the X-ray energy corresponding to the cell constituents. We speculate that the ring-like structure

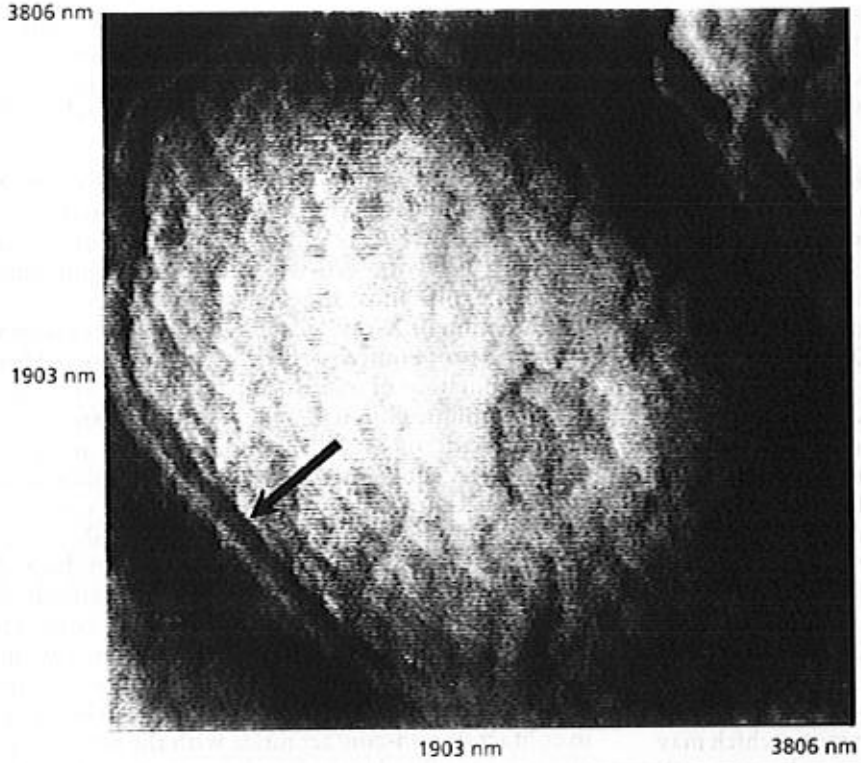

Fig. 2. X-ray micrograph of live *C. albicans* ATCC 14053 in PBS. The arrow points to the cell wall. The roughly circular structure in the lower right quadrant may be the nucleus.

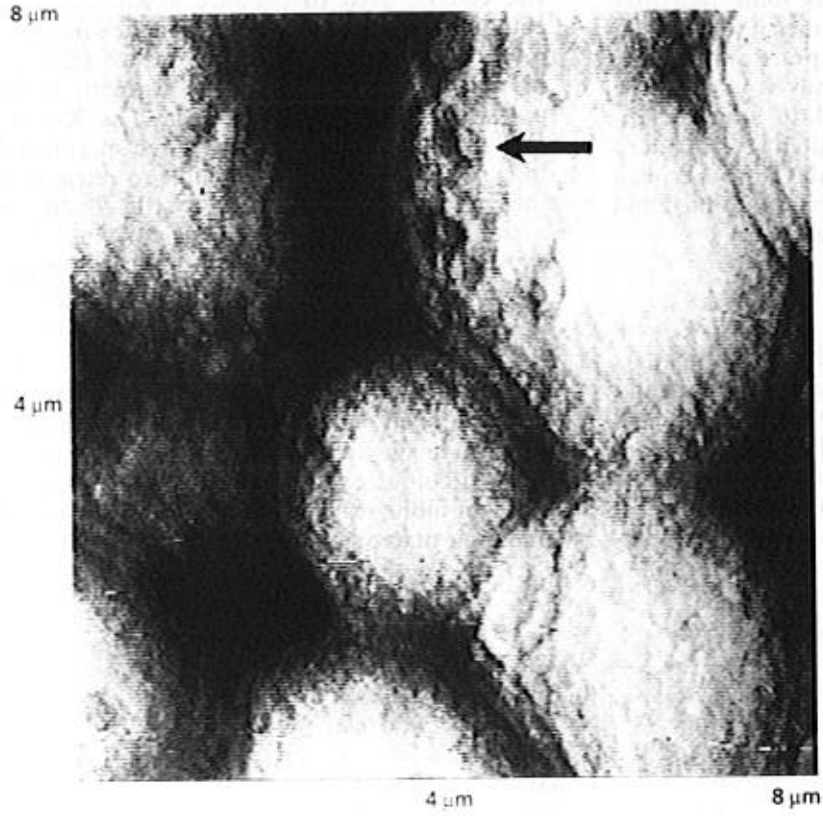

Fig. 3. X-ray micrograph of live *C. albicans* ATCC 14053 in PBS. The arrow points to structures which may be vacuoles.

seen around the cell represents the cell wall, which is characteristic of yeasts such as *Candida*. The width of the cell wall from this image was measured at 152·4 nm. Other investigators have estimated the thickness of the yeast cell wall to range from 150 to 400 nm, depending on the culture conditions (Robinow & Johnson, 1991).

The circular structure seen inside the cell (570·9 nm) may represent the nucleus packed with condensed DNA which would absorb relatively more X-rays than the rest of the surrounding, relatively sparsely distributed cytoplasm.

In Fig. 3 the cell wall is distinctly visible. Three circular crater-like areas which measure 400 nm in diameter can also be seen in Fig. 3. These latter structures may be vacuoles because (1) there is more than one of them in the same cell, and (2) they are much smaller than the nucleus seen in Fig. 2. Again these structures in both X-ray images represent a difference in X-ray absorption. Thin section electron micrographs of yeasts cells clearly show the presence of one large nucleus and other smaller circular areas representing vacuoles (Robinow & Johnson, 1991). In our experiments, the structures shown were reproducibly present in all the cells scanned from the same resist, and also in independent experiments. The overall usually round shape of *C. albicans* is slightly distorted due to the high density of the cells in these images. The symmetrical appearance and apparent subunits seen on the cell surface (Figs 2 and 3) may be an artefact from the grain pattern of the PMMA ridges on the resist. The *C. albicans* cell wall is made up of long symmetrical cross-linked mannose polymers, which may have an ultrastructural repetitive pattern, but no such differentiation within the cell wall structure has been reported in the literature. If there is some repetitive structure within the cell wall material then it would be at the resolution limit of this technique (practically 50 nm) due to the use of the resist, and thus would not be imaged. AFM alone has been used to scan macromolecules such as DNA, protein and lipid arrays, but to scan live, whole hydrated cells with AFM, the specimen needs to be anchored to the surface being scanned and this would definitely distort a live specimen.

DISCUSSION

High resolution imaging of fixed non-living specimens is routinely performed in the field of materials science and optical physics under extremely clean and controlled conditions. However, in spite of the need for such technology, analysis and study of biological specimens have predominantly been restricted to various electron microscopy techniques. The extensive specimen preparation and specialized handling of fixed, dehydrated cells or macromolecules is time consuming and thus relatively expensive, even though the materials required for this established technique are now relatively inexpensive.

We have studied the potential for the use of an X-ray optical system (Fig. 1) to image live hydrated microbial cells. We chose *C. albicans* as a unicellular, easy to handle, eukaryotic cell which is also of contemporary clinical significance, especially in AIDS patients. The combination of X-rays generated from laser plasma and scanning probe microscopy to scan images is ideal for the examination of microbial cells and macromolecules. This technique eliminates the sample preparation steps characteristic of established microscopic analysis. No colour dyes, conjugated fluorescent antibodies or radioactive labels are necessary due to the high resolution of the scanning probe microscope. The short (10 ns) exposure to X-rays is too short to introduce dehydration, physical sample degradation, plasmolysis for whole cells or enzymic degradation of cell constituents. Thus the absorption profile of the specimen would be unaltered by this technique. The X-ray image on the PMMA-coated resist is permanent and can be examined in contact or non-contact mode with the scanning probe microscope. For large samples, e.g. *C. albicans*, and other eukaryotic cells in the 5–100 μm range, a larger tip size can be used to produce a whole cell image, in combination with a fine tip to scan the same image at nm resolution. At nm resolution, surface features such as the cell wall of *C. albicans* are distinctly visible with a precise depth-of-field parameter. The X-rays are certainly strong enough to damage the specimen, but with the present system we are unable to retrieve enough of the irradiated specimen under sterile conditions to start a culture and, hence, we have not studied any post-exposure effects on the organism. However, we speculate a higher mutation rate in the irradiated cells due to nucleic acid damage, similar to UV damage.

Laser plasma X-ray and scanning probe microscopy is unmatched in its efficiency, cost, resolution, depth of field, minimal sample preparation and handling, permanent image record, dimensional detail, computer-assisted colour coding and ease of applicability to the study of biological specimens, especially microbial cells and their macromolecules (Table 1). X-ray microscopy,

Table 1. Comparison of microscopy techniques

Microscope	Wavelength (nm)	Numerical aperture	Resolution (nm)	Depth of field (nm)
Optical	500	0·7	435	1244
		1·4	218	311
X-ray	3·2	0·02	90	8200
	2·5	0·6	20	520
STEM* (100 kV)	–	0·005	0·5	0·37

*STEM, scanning transmission electron microscopy.

as studied in this brief report, suggests a new and exciting field that requires the establishment of a library of reference images to serve as a baseline for further, more complex interaction visual studies at the molecular level. There is great potential to discover new natural features in hydrated cells and molecules directly in their dynamic state due to minimal sample handling, treatment and labelling requirements. The implications for the direct visual study of new and existing drug interactions with target sites, such as enzyme inhibition by competition, receptor blockage and receptor stimulation in various disease states, are outstanding. The relatively short period of time (30 min) required to obtain high resolution images from natural hydrated samples simplifies the study of a variety of fungi, protozoa, bacteria and viruses which need not be present in large numbers in the specimen. Heavy-metal- or latex-microsphere-conjugated monoclonal antibodies can also be used to localize specific markers in the cell.

Finally, pulsed laser plasmas introduce the time domain element into X-ray microscopy. Exposure times for microscopy with synchrotrons are measured in seconds; the laser plasmas utilized here can provide X-ray emission in pulses ranging from several nanoseconds to less than one picosecond. This introduces the possibility of capturing kinetic, chemical or morphological snapshots of biological ultrastructures in time-frames of interest to enable the understanding of complex biological processes. No other type of microscopy offers this potential.

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REFERENCES

Arakawa, H., Umemura, K. & Ikai, A. (1992). Protein images obtained by STM, AFM and TEM. *Nature* 358, 171–173.

Arnsdorf, M. F. & Lal, R. (1992). Recent progress with atomic force microscopy in biology: molecular resolution of cell membranes, constituent biomolecules, and microcrystals. *SPIE* 1778, 112–116.

Binning, G., Rohrer, H., Gerber, C. & Weibel, E. (1982). Surface studies by scanning tunneling microscopy. *Phys Rev Lett* 49, 57–61.

Binning, G., Gerber, C., Stoll, E., Albrecht, R. T. & Quate, C. F. (1987). Atomic resolution with atomic force microscope. *Europhys Lett* 3, 1281–1286.

Chapin-Robertson, K. & Edberg, S. C. (1991). Microscopy. In *Manual of Clinical Microbiology*, 5th edn, pp. 29–35. Edited by A. Ballows, W. J. Hausler, Jr, K. L. Herrman, H. D. Isenberg & H. J. Shadomy. Washington, DC: American Society for Microbiology.

Cheng, P. C., Newberry, S. P., Kim, H. G. & Wittman, M. D. (1989). X-ray contact microradiography and shadow projection X-ray microscopy. *Eur J Cell Biol* 48 (suppl. 25), 169.

DeMeis, R. (1996). X-ray imaging. Laser plasma 'X-rays' living organisms. *Laser Focus World* June, 37–38.

Edstrom, R. D., Meinke, M. H., Yang, X. R., Yang, R., Elings, V. & Evans, D. F. (1990). Direct visualization of phosphorylase-kinase complexes by scanning tunneling and atomic force microscopy. *Biophys J* 58, 1437–1448.

Feder, R., Banton, V., Sayre, D., Costa, J., Baldini, M. & Kim, B. (1985). Direct imaging of live human platelets by flash X-ray microscopy. *Science* 227, 63–64.

Hansma, H. G., Weisenhorn, A. L., Edmundson, A. B., Gaub, H. E. & Hansma, P. K. (1991). Atomic force microscopy: seeing molecules of lipid and immunoglobulin. *Clin Chem* 37, 1497–1501.

Hansma, H. G., Bezanilla, M., Zenhausern, F., Adrian, M. & Sinsheimer, R. L. (1993). Atomic force microscopy of DNA in aqueous solutions. *Nucleic Acids Res* 21, 505–512.

Hoh, J. H. & Hansma, P. K. (1992). Atomic Force Microscopy for high resolution imaging in cell biology. *Trends Cell Biol* 7, 208–213.

Ill, C. R., Keivens, V. M., Hale, J. E., Nakamura, K. K., Jue, R. A., Cheng, S., Melcher, E. D., Drake, B. & Smith, M. C. (1993). A COOH-terminal peptide confers regiospecific orientation and facilitates atomic force microscopy of an IgG/sub 1. *Biophys J* 64, 919–924.

Kado, M., Nekula, K., Richardson, M. C., Yamamoto, Y. & Friedman, H. (1996). Direct ultrastructural imaging of macrophages using a novel X-ray contact microscopy. *FASEB J* 10, A824.

Kay, D. (1976). Electron microscopy of small particles, macromolecular structures and nucleic acids. *Methods Microbiol* 9, 177–215.

Lichfeld, K. G. (1976). Transmission electron microscopy of bacteria. *Methods Microbiol* 9, 127–176.

Meyer-Ilse, W., Gutmann, P. A., Thieme, J., Rudolph, D., Schmahl, G., Anderson, E., Batson, P., Attwood, D., Isklander, N. & Kern, D. (1992). Experimental characterization of zone plates for high resolution X-ray microscopy. In *X-ray Microscopy III*, pp. 284–289. Edited by A. G. Michette, G. R. Morrison & C. J. Buckley. Berlin: Springer.

Neiman, B. (1992). X-ray microscopy with the Gottingen scanning X-ray microscope at 2·4 nm. In *X-ray Microscopy III*, pp. 143–150. Edited by A. G. Michette, G. R. Morrison & C. J. Buckley. Berlin: Springer.

Nui, L., Shaiu, W.-L., Vesenka, J., Larson, D. D. & Henderson, E. (1993). Atomic force microscopy of DNA–colloidal gold and DNA–protein interactions. *SPIE* 1891, 71–77.

Ohnesorge, F. & Binning, G. (1993). True atomic resolution by atomic force microscopy through repulsive and attractive forces. *Science* 260, 1451–1456.

Panessa-Warren, B. J. & Warren, J. B. (1980). Determining biological fine structure by differential absorption of soft X-rays. *NY Acad Sci* 342, 350–367.

Rajyaguru, J. M., Muszynski, M. J., Kado, M., Richardson, M. (1995). Application of X-rays to obtain ultrastructural images of microbes and macromolecules. Abstract J9. In *Abstracts of the 95th General Meeting of the American Society for Microbiology, Washington, DC, USA.*

Rajyaguru, J. M., Kado, M., Nekula, K., Torres, D., Richardson, M. & Muszynski, M. J. (1996a). Direct high resolution (nm scale) ultrastructural X-ray imaging of live hydrated *Escherichia coli.*

Abstract J7. In *Abstracts of the 96th General Meeting of the American Society for Microbiology, New Orleans, LA, USA.*

Rajyaguru, J. M., Kado, M., Nekula, K., Torres, D., Richardson, M. & Muszynski, M. J. (1996b). Ultrastructural X-ray imaging and molecular modelling of hydrated biological specimens using laser plasma pulsed point X-ray sources. *FASEB J* 10, A824.

Rees, W. A., Keller, R. W., Vesenka, J. P., Yang, C. & Bustamante, C. (1993). Scanning force microscopy imaging of transcription complexes: evidence for DNA bending in open promoter and elongation complexes. *Science* 260, 1646–1649.

Richardson, M., Shinohara, K., Tanaka, K. A., Kinjo, Y., Ikeda, N. & Kado, M. (1992). Pulsed X-ray microscopy of biological specimens with laser plasma sources. *SPIE* 1741, 133–141.

Robinow, C. F. & Johnson, B. F. (1991). Yeast cytology: an overview. In *The Yeasts: Yeast Organelles*, vol. 4, pp. 8–120. Edited by A. H. Rose & J. S. Harrison. New York: Academic Press.

Schabert, F. A., Henn, C. & Engel, A. (1995). Native *Escherichia coli* OmpF porin surfaces probed by atomic force microscopy. *Science* 268, 92–94.

Shinohara, K. (1990). Application of X-ray microscopy to mammalian cells. In *X-ray Microscopy in Biology and Medicine*, pp. 203–212. Edited by K. Shinohara, K. Yada, H. Kihara & T. Saito. Berlin: Springer.

Tillmann, R., Radmacher, M. & Gaub, H. E. (1992). Hydrated amorphous silicon oxide surface at 3 angstrom resolution by scanning force microscopy. *Appl Phys Lett* 60, 3111–3113.

Wigren, R., Elwing, H., Erlandson, E., Welin, S. & Lundstrom, I. (1991). Structure of fibrinogen obtained by scanning force microscopy. *FEBS Lett* 280, 225–228.

Yang, J., Takeyasu, K. & Shao, Z. (1992). Atomic force microscopy of DNA molecules. *FEBS Lett* 301, 173–176.

Zasadzinski, J. A., Helm, C. A., Longo, M. L., Weisenhorn, A. L., Gould, S. A. & Hansma, P. K. (1991). Atomic force microscopy of hydrated phosphatidylethanolamine bilayers. *Biophys J* 59, 755–760.

Zenhausern, F., Adrian, M., Heggeler-Bordier, Bt., Eng, L. M. & Descouts, P. (1992). DNA and RNA polymerase/DNA complex images by scanning force microscopy: influence of molecular-scale friction. *Scanning* 14, 212–217.

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