Scanning Microscopy

Volume 7 | Number 1 Article 37

11-30-1992

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Recommended Citation
Hermann, René and Müller, Martin (1992) "Progress in Scanning Electron Microscopy of Frozen-Hydrated Biological Specimens," *Scanning Microscopy*: Vol. 7 : No. 1 , Article 37.
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Scanning Microscopy, Vol. 7, No. 1, 1993 (Pages 343-350) 0891-7035/93$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

PROGRESS IN SCANNING ELECTRON MICROSCOPY OF FROZEN-HYDRATED BIOLOGICAL SPECIMENS

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(Received for publication September 14, 1992, and in revised form November 30, 1992)

Abstract

Modern scanning electron microscopy yields structural information down to 2 to 5 nm from thin, beam transparent biological specimens. This paper examines the possibilities of garnering this level of structural information from bulk, frozen-hydrated samples.

Freeze-fractured, frozen-hydrated yeast cells, frequently taken as a yardstick to monitor progress in low-temperature scanning electron microscopy, have been used to optimize both metal shadowing methods and observation parameters (e.g. accelerating voltage, electron beam irradiation of the specimen).

Uncoated frozen-hydrated yeast cells do not charge electrically at an accelerating voltage of 30 kV. Increasing charging effects are however observed with decreasing accelerating voltages. Very thin metal films are therefore used for specimen coating to localize and enhance the specific secondary electron signal. Planar-magnetron sputtering of a 1 nm metal layer provides high resolution secondary electron images, at 30 kV, of freeze-fractured, frozen-hydrated yeast cells in an in-lens field-emission scanning electron microscope. Structural information comparable to that of transmission electron microscopy of freeze-fractures is attained. Planar-magnetron sputtering of either chromium, tungsten or platinum results in essentially the same information density (smallest visible significant structural detail). Frozen-hydrated samples are very beam sensitive and have to be observed under minimum dose conditions.

Key words: Beam damage, frozen-hydrated, high-resolution scanning electron microscopy, low-temperature scanning electron microscopy, yeast, planar-magnetron sputtering, chromium, platinum, tungsten.

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Introduction

The major task of biological high resolution scanning electron microscopy ((SEM), and transmission electron microscopy (TEM)) is to provide information with which one may correlate structure and function. It is the only methodology with the inherent power of visualizing structures down to molecular dimensions within the context of complex biological systems. Specimen preparation, modern instrumentation, signal generation and signal detection have to be interactively optimized, with a minimum of compromises, to finally reach this goal.

Freezing preserves biological structures most closely related to the living state. Cryoimmobilized bulk samples, frozen-hydrated or partially freeze-dried (etched), are frequently examined by TEM freeze-fracture replica techniques, or by low-temperature scanning electron microscopy (LTSEM). The latter technique eliminates the tedious task of digesting the biological material away, which often results in fractionation of the heavy metal replica. The observation of frozen-hydrated biological samples permits their processing in the native environment. Possible artefacts of drying procedures such as shrinkage and collapse (Kellenberger, 1987), are avoided.

LTSEM is now a routine method for structural description of biological surfaces and fracture-faces at low to medium magnification (up to approximately 10,000 x). The structural information of the TEM freeze-fracture technique has so far not been achieved by LTSEM (see Read and Jeffree, 1991), even after combining most sophisticated cryo-attachments with conventional field-emission SEM's (Walther et al., 1990).

Distortion of fine structures by inadequate freezing, too thick metal coatings, and the use of inappropriate instrumentation (insufficient vacuum conditions in the preparation chamber or in the microscope, insufficiently stable cryostages at higher magnifications, scanning electron microscopes with low instrumental resolution) are among the major reasons for this difference.

The goal of our study is to demonstrate that LTSEM can provide structural information that parallels that of TEM freeze-fracture techniques, provided that adequate specimen preparation procedures and an in-lens field-emission SEM are available. Baker's yeast was employed as a model system. Freeze-fractured frozen-hydrated or partially freeze-dried yeast cells have been frequently used to monitor the progress in TEM freeze-fracturing and in low-temperature scanning electron microscopy (Moor and Mühlethaler, 1963; Gross et al., 1978a; Pawley et al., 1978 (glutaraldehyde fixed and cryoprotected yeasts); Walther et al., 1990; Wergin and Erbe, 1992).

A large amount of micrographs of freeze-fractured yeast is therefore available, permitting the validation of structural information achieved during our investigation.

Structural information of 2-5 nm on thin, beam transparent biological test specimens (freeze-dried T4 polyhead mutants; Hermann and Müller, 1991a) is achieved with in-lens field-emission scanning electron microscopes (at high accelerating voltage; 30 kV), in conjunction with cryopreparative techniques. The high resolution secondary electron (SE) signal produced by these specimens is very little perturbed by SE's that are generated by backscattered electrons, unlike in bulk samples. Pawley (1984) and Joy (1991) suggested that this problem may be overcome by working with low accelerating voltage where the respective excitation volumes of the SE I and SE II signal nearly coincide (~ 1 to 2 kV; Joy, 1991). The drawback, under these conditions, is the increase in electron beam diameter due to reduced accelerating voltage (4 nm at 1 kV in the Hitachi S-900 high resolution SEM, as compared to ~ 0.7 nm at 30 kV; Nagatani et al., 1987).

Materials and Methods

Cryoimmobilization

Commercially available Baker's yeast (*Saccharomyces cerevisiae*) was suspended in distilled water for one day and then frozen either in a propane jet (Müller et al., 1980) or by high pressure freezing (Moor, 1987). Uncoated 300 mesh EM copper grids (3 mm diameter, 12 µm thick) were immersed into the yeast suspension and sandwiched between copper platelets for propane jet freezing (Müller and Moor, 1984). A cellulose capillary tube (200 µm diameter, 2 mm length) was filled with the yeast suspension and sandwiched between two aluminum platelets, one of which had a cavity of 200 µm, for high pressure freezing (Hohenberg et al., in preparation). High pressure freezing was performed according to Studer et al. (1989).

Coating and cryotransfer of frozen-hydrated yeast

Propane jet-frozen copper sandwiches were fractured under liquid nitrogen; the EM copper grid, serving as a spacer, was mounted under liquid nitrogen into a brass specimen capsule that fits into the Gatan cryoholder. The high pressure frozen cellulose tubes were mounted into the brass specimen capsules and fractured under liquid nitrogen with a razor blade. The brass capsules were fixed in the Gatan cryoholder (model 626) by a press-stud mechanism. The insertion of the specimen capsules into the holder is made under liquid nitrogen in a Gatan loading station. This also permits the transfer of the cryoholder, with the specimen under liquid nitrogen, from the high vacuum coating device to the microscope. The specimen was protected from contamination immediately after mounting the sample by closing the moveable cryoshield of the cryoholder.

The cryoholder was withdrawn from the loading station and inserted against a slight overpressure of purified nitrogen gas into a Balzers MED 010 table-top high vacuum coating device equipped for sputtering at low temperatures (Hermann and Müller, 1991a; HR 010, Baltec, Balzers, FL). After having reached a vacuum < 5 x 10^{-7} mbar, the pressure was increased to 2 x 10^{-2} mbar by high-purity argon gas for planar-magnetron sputtering. An approximately 1 nm thick layer of tungsten, platinum, or chromium was applied to the specimen at a specimen temperature < -155° C. Metal film thickness was estimated by a quartz crystal device. The cryoshield of the cryoholder was opened after a frequency change of 50 - 100 hertz had been registered by the quartz crystal monitor and closed again to interrupt metal coating of the specimen at the desired metal thickness. High vacuum was then re-established by closing the argon valve. The coating unit was then vented by purified gaseous nitrogen. The cryoholder was withdrawn and inserted as soon as possible into the loading station filled with liquid nitrogen.

SEM observation

The loading station with the cryoholder was transfered to the Hitachi S-900 in-lens field-emission SEM and the cryoholder inserted into the microscope. After having reached temperature equilibrium at about -155° C (~ 20 minutes), the cryoshield of the cryoholder was opened and the specimen examined.

Specimen areas were pre-selected at low magnification (≤10,000 x). High resolution micrographs were then taken immediately at 100,000 x. Final focussing was performed during the first lines of the exposure (2000 lines/40 sec total exposure) at an accelerating voltage of 30 kV, unless otherwise stated. Under these conditions, the electron impact onto the examined specimen surface is roughly 3500 electrons/nm^2 with a beam current of 10 pA.

Results

Freeze-fractured yeast cells were first observed without coating in order to find adequate accelerating voltages for examination of bulk, frozen-hydrated samples by secondary electrons. Figure 1 shows a comparison of secondary electron images of the uncoated protoplasmic fracture faces (PF) of yeast cell membranes, recorded at different accelerating voltages at a primary magnification of 40,000 times: No charging occurs when uncoated frozen-hydrated samples are observed at high accelerating voltage. Except for the presence of invaginations, little structural detail is visible on the PF (Fig. 1 a, at 30 kV). Accelerating voltages below 10 kV result in dramatic charging. The hexagonally arranged particles become visible in areas of the frozen-hydrated fracture faces not obscured by charging effects (Fig. 1 b, at 6 kV).

Planar-magnetron sputtering of a 1 nm thick tungsten layer at -155° C yields excellent structural information of frozen-hydrated freeze-fractured yeast cells in the Hitachi S-900 in-lens field emission scanning electron microscope operating at 30 kV accelerating voltage (Figs. 2 a and 3 a, b). Observation at lower accelerating voltages, however, results in charging even after metal coating (Fig. 2 b, at 5 kV). The structural information attained by secondary electron images of frozen-hydrated yeast membranes equals the information of TEM freeze-fracture replicas (Gross et al, 1978a).

Planar-magnetron sputtered 1 nm thick platinum (Fig. 4 a) or chromium coatings (Fig. 4 b) result in nearly identical structural information in the SEM, as demonstrated by micrographs of the PF of yeast plasmalemma. Chromium planar-magnetron sputtered samples, however, result in less crisp images (Fig. 4 b).

Beam damage is a major problem that occurs during observation of frozen-hydrated samples. The second scan of an area that has been once previously scanned at a higher primary magnification already shows deformation of the membrane (Fig. 5). Beam damage to the specimen can be reduced by pre-selecting areas of interest at low magnification (< 10,000 x) and then taking the micrographs at 100,000 x. Final focussing must be performed during the first lines of the exposure (2000 lines/40 sec total exposure). The first scan causes immediate visible beam damage at primary magnifications exceeding 100,000 x. Uncoated frozen-hydrated specimens can be observed at 30 kV accelerating voltage up to 40,000 x without visible beam damage during the first scan.

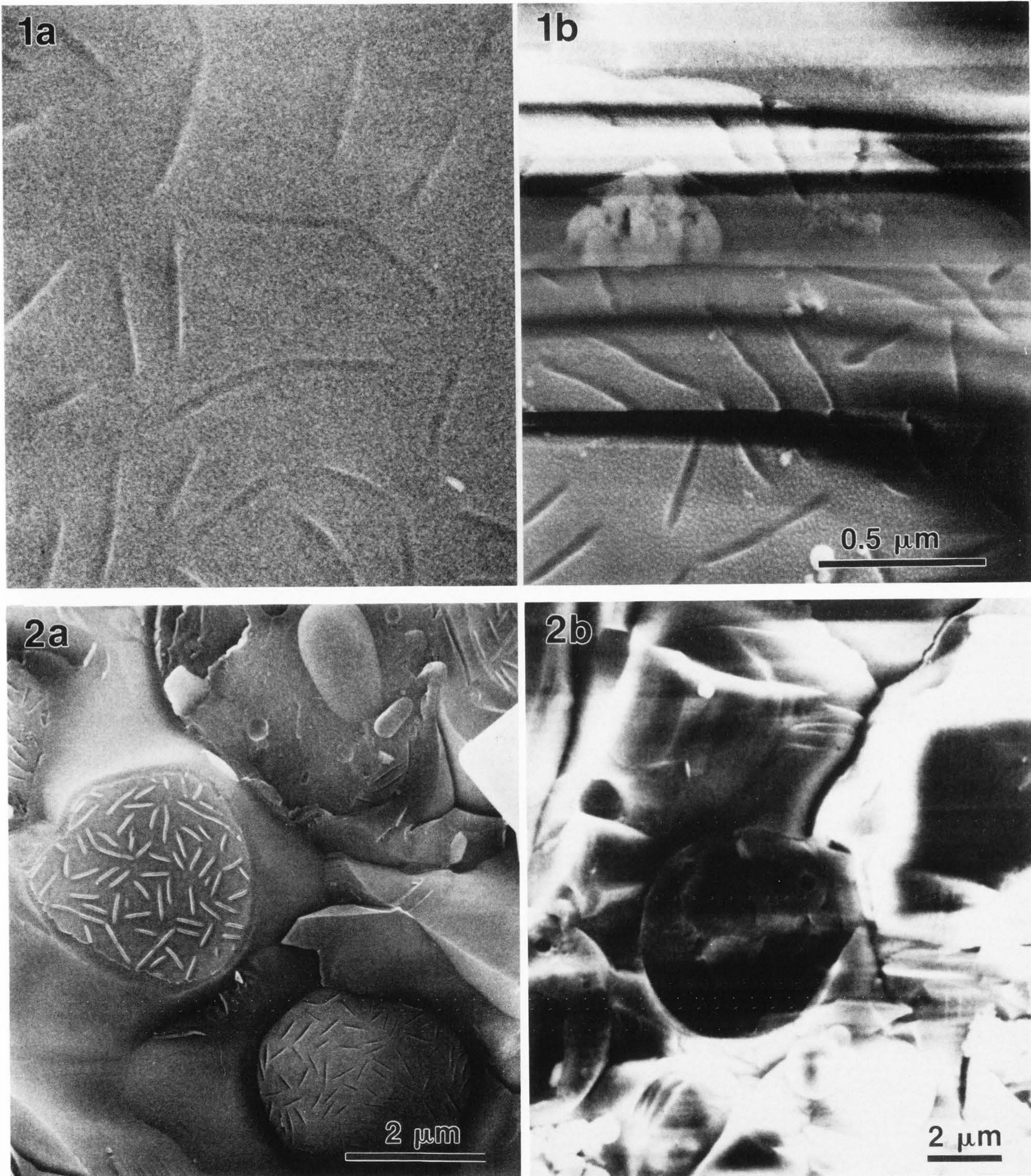

Fig. 1. SE images of uncoated protoplasmic fracture faces of frozen-hydrated Baker's yeast cell membranes, recorded at different accelerating voltages (Fig. 1 a at 30 kV, Fig. 1 b at 6 kV).
The samples do not charge at 30 kV accelerating voltage. They display heavy charging at 6 kV, paracrystalline areas are nevertheless temporarily visible during scanning.

Fig. 2. Overviews of freeze-fractured, frozen-hydrated yeast cells planar-magnetron sputter coated with 1 nm tungsten. Fig. 2 a is taken at 30 kV accelerating voltage, Figure 2 b at 5 kV accelerating voltage.
The 1 nm metal coating does not prevent the samples from charging at the lower accelerating voltage.

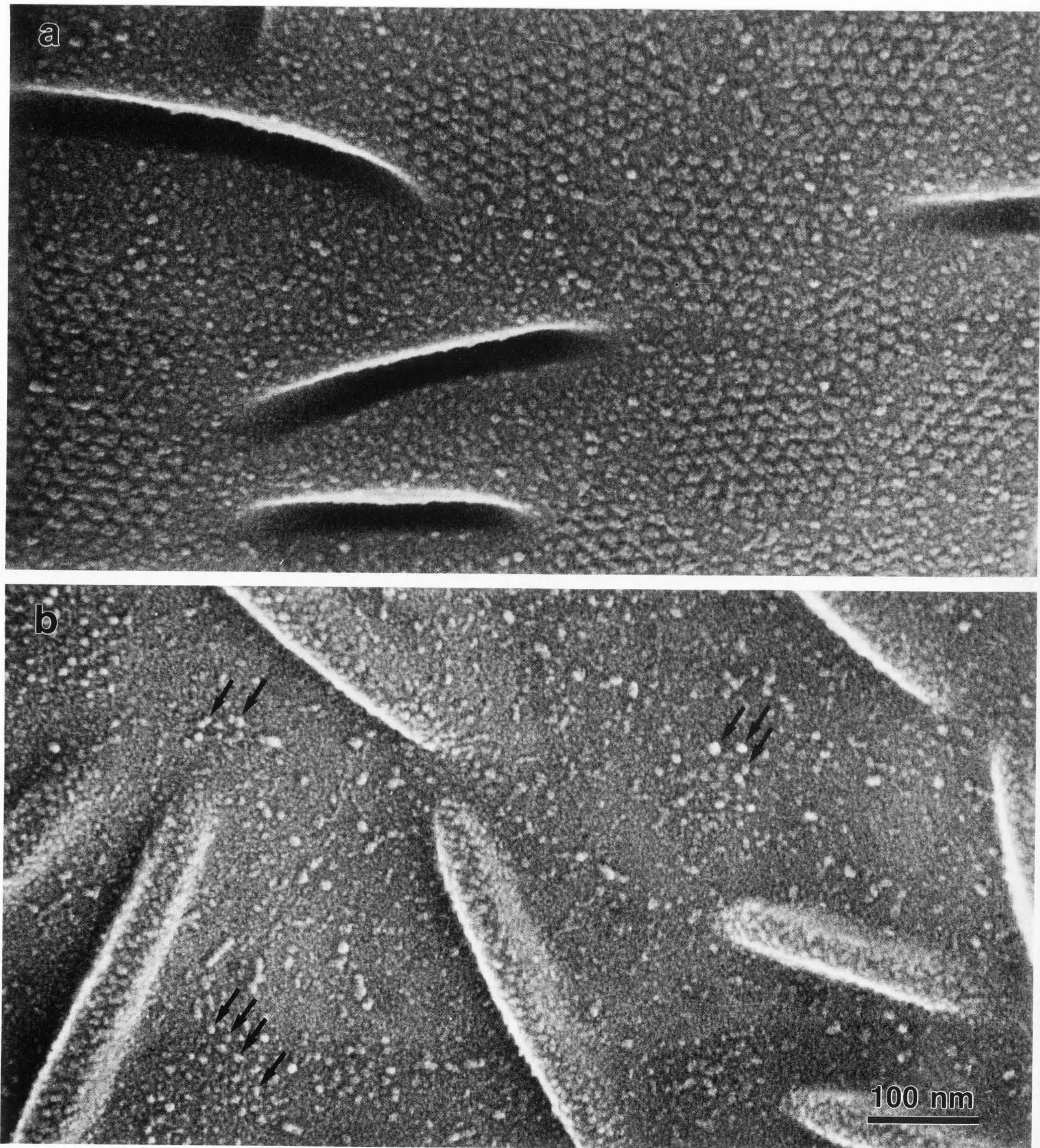

Fig. 3. Fracture faces of frozen-hydrated yeast plasmalemma, planar-magnetron sputter coated with 1 nm tungsten and observed at 30 kV accelerating voltage. Fig. 3 a shows the protoplasmic fracture face, Fig. 3 b the exoplasmic fracture face. Arrows point to contamination by small ice crystals.

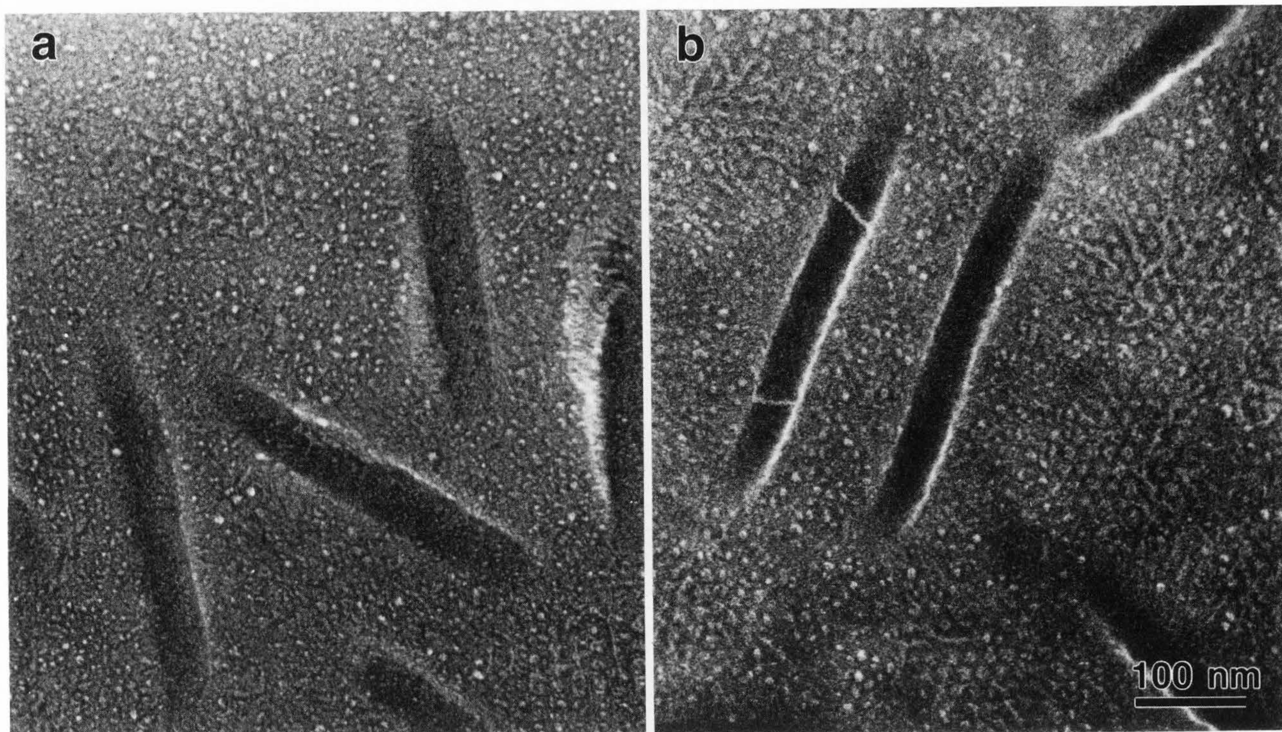

Fig. 4. Protoplasmic fracture-faces of frozen-hydrated yeast plasmalemma (at 30 kV), planar-magnetron sputter coated with 1 nm platinum (Fig. 4 a), or 1 nm chromium (Fig. 4 b). The SE images show nearly identical structural information to that shown by tungsten coated samples (Fig. 2 a and 3).

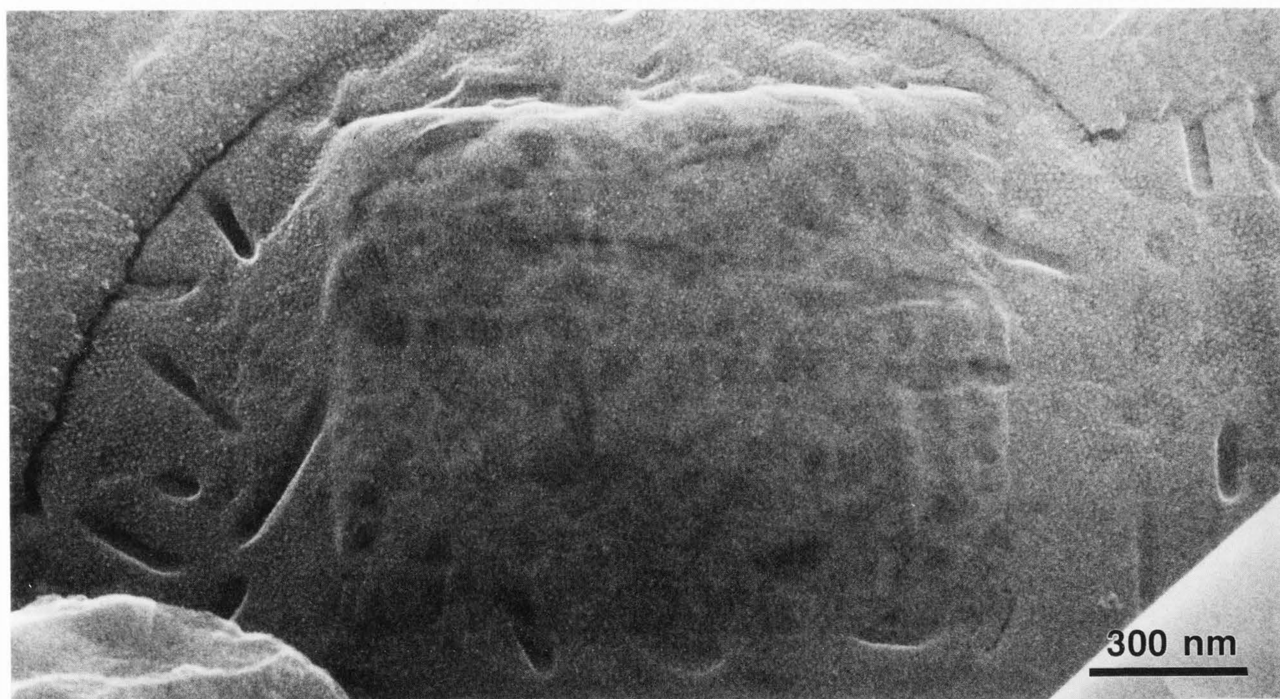

Fig. 5. The micrograph shows the second scan over an area that has been previously recorded at a higher primary magnification (at 30 kV). The previously scanned area exhibits heavy beam damage.

Discussion

Uncoated frozen-hydrated yeast cells do not exhibit charging at high accelerating voltages (30 kV; Fig. 1 a). The results show that with a thin 1 nm metal coat, a high resolution signal can be localized at the surface of frozen-hydrated specimens, similar to thin, beam transparent freeze-dried specimens (Hermann and Müller, 1991a). The use of thin metal coatings to optimize the high resolution information has been initially proposed by Peters for critical point or air-dried samples (1982).

The 1 nm thick metal films are not conductive enough to prevent the samples from charging at low accelerating voltages (Fig. 2 b), nor are they able to stabilize the surface structures at high accelerating voltage, as demonstrated by the heavily deformed surface detected in the second scan of an area (Fig. 5). The metal layer permits a dramatic increase of the signal to noise ratio (Fig. 1 a, uncoated samples, as compared with Figs. 3 and 4, metal coated samples) and result in a structural description of the yeast PF that equals TEM freeze-fracture replicas. The hexagonally arranged particles on the PF are clearly visible. They consist of volcano-like intramembraneous particles with diameters of approximately 10 nm and a center to center spacing of 16.5 nm (TEM freeze-fracture study by Gross et al, 1978a).

All three metal coatings tested (tungsten, platinum and chromium) yield similar structural information. Chromium, however, the only light metal film examined, results in slightly less crisp images (Fig. 4 b). This observation may be due to the fact that 1 nm thick chromium films generate less backscattered electrons (Hermann et al., 1988), in contrast to platinum or tungsten coated specimens. Heavy metal coatings therefore provide a higher SE-signal (SE I; and SE II generated by precisely localized backscattered electrons) which is less obscured by the background signal produced within the frozen-hydrated sample.

The exoplasmic fracture face (EF) of yeast plasmalemma exhibits depressions, corresponding to the hexagonally arranged particles on the PF, as revealed by TEM freeze-fracture replicas at ultrahigh vacuum conditions (Gross et al, 1978a). The secondary electron images of these areas, however, exhibit clustered, protruding particles (Fig. 3 b, arrows), which represent small ice crystals (Gross et al., 1978b). Specific water condensation may have occured during fracturing of the yeast cells under liquid nitrogen, or during transfer of the specimen through air on the cryo-holder to the sputter device (the sample was covered during transfer, however, by a cryo-shield; see materials and methods).

Beam damage of the frozen-hydrated samples is severe (Fig. 5), as can be expected from samples containing water (see e.g. reviews by Talmon, 1987; or Echlin, 1991). Structural description of coated, frozen-hydrated samples at a 100,000x primary magnification is achieved by recording the first scan over an area. The total electron impact onto the specimen surface is then roughly 3500 electrons/nm^2 with 10 pA beam current. This value is, however, difficult to interprete, since important parameters such as the exact spot size of the primary electron beam and the excitation volume of the electrons in the frozen-hydrated sample are unknown. Higher primary magnifications (i.e. higher beam doses) result in visible destruction of the specimen surface during the first scan. This occurs too when uncoated frozen-hydrated samples are observed at magnifications > 40,000 x. Alternative coating procedures might permit work at higher primary magnifications, e.g. unidirectional heavy metal shadowing followed by carbon backing and subsequent observation in the backscattered electron mode (Walther and Hentschel, 1989).

Observation of uncoated, frozen-hydrated bulk samples, at accelerating voltages up to 30 kV, without charging, has already been reported by Echlin and coworkers (1970; also cited in detail in Echlin, 1978). Beam currents similar to those used in our experiments were employed (5-15 pA). Echlin explains the absence of charging phenomena under these working conditions by the presence of ions and electrolytes in the frozen tissue fluids that provide a sufficiently conductive path for any charge build up to leak to ground.

In LTSEM, there is however a tendency to work at low to medium accelerating voltages for the observation of frozen-hydrated or partially freeze-dried specimens (see the review by Read and Jeffree, 1991). A comparison of the present observations with published results employing low accelerating voltage is difficult due to the different preparation procedures and working conditions (e.g. much thicker metal coatings and low to medium magnification).

Working at lower accelerating voltages reduces the electron interaction volume. The higher electron concentration in close proximity to the specimen surface may partially explain the charge build up on uncoated frozen-hydrated yeast fracture faces and at the same time the partially better structural information that was observed at low accelerating voltages (Fig. 1 b). Development of an unstable, pancake-shaped electron diffusion volume right below the surface of the sample caused by an internal space charge, as proposed by Brombach for uncoated ice (1975), could further explain the partially visible paracrystalline areas on uncoated, frozen-hydrated yeast at low accelerating voltages.

Echlin gave an alternative explanation for the absence of charging phenomena at high accelerating voltages (1978): The formation of a contamination layer on the frozen-hydrated surface provided a conductive coating. Formation of a contamination layer would, however, prevent structural information in the nm range (Hermann and Müller, 1991b).

Conclusions and Outlook

Thin metal coating layers deposited onto freeze-fractured specimens generate a highly localized secondary electron signal. The frozen-hydrated specimens are not charging electrically at high accelerating voltage (30 kV). The structural information obtained equals that of TEM freeze-fracture replicas. The observation of frozen-hydrated specimens by SEM is fast, compared to the TEM replica technique, and permits examination of large, intact samples within their natural context.

Observation of frozen-hydrated or partially freeze-dried (etched) samples by high-resolution scanning electron microscopy may therefore become an important tool for studying membranes and cell-components down to the (macro)molecular level, close to the native state. These observations could provide information that was unique or complementary to that obtained by TEM techniques.

This investigation used suboptimal methods to fracture and transfer the frozen-hydrated samples. The techniques employed however allowed one to work without large ice crystal contamination. Reduced contamination by nm range ice crystals (as visualized on the EF of frozen-hydrated freeze-fractured yeast; Fig. 3 b) could be expected by employing more advanced cryopreparation systems.

Acknowledgements

We thank D. Duff and M.L.Yaffee for their help with the manuscript.

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Discussion with Reviewers

N. Read: What do the authors think the ultimate spatial resolution achievable by LTSEM of bulk, frozen-hydrated samples will eventually be? How do they think this might be attained?

Authors: The structural information obtainable from bulk frozen-hydrated samples is the result of the complex interaction of specimen preparation techniques and observation parameters. We estimate the structural information achieved in our SEM investigation to be better than 5 nm (depressions of the volcano-like particles of the PF are clearly visible; Fig. 3 a). We believe having approached the limits with the present instrumentation and that any progress is governed by our own lack of knowledge in interpreting small structural details of freeze-fractured samples and not by SEM technology.

K.-R. Peters: Is the artifact by beam-damage worse if the same dose is deposited at shorter (4kx4k) or longer (1kx1k) dwell times?

K. Zierold: Can charging effects and radiation damage be reduced by specific imaging techniques e.g. low dose imaging or interlace scanning?

Authors: We don't have instrumentation for interlace scanning nor for recording TV-like scan rates. We expect interlace scanning at high scan speeds to be very effective in reducing charging. A fully digital scan generator that permits accurate work at TV scan rates should be available for corresponding experiments at high resolution.

K. Zierold: As mass loss by electron irradiation was observed to start at the interface of water and organic matrix {Talmon Y (1984). Radiation damage to organic inclusions in ice. Ultramicrosc.; 14: 305 - 316; Zierold K (1985). Contrast of biological cryosections in scanning transmission electron microscopy. J. Microsc.; 140: 65 - 71.}, do you see a chance to stabilize the specimen against radiation damage by very superficial water sublimation without damaging structural details?

Authors: No.

K.-R. Peters: Why is beam damage on frozen-hydrated and fractured yeast reduced through metal coating with a thin film?

Authors: Based upon our own experiments, the contrast exhibited by frozen-hydrated metal coated specimens is higher than that of uncoated samples. Inelastic electron scattering events can therefore be expected within the metal coat. We assume, without being able to provide quantitative data, that less energy of the primary electron beam is deposited in coated samples.

K.-R- Peters: Conventionally, in SEM low dose imaging the

first scanned images are only acceptable if the second lower magnification (and lower dose) scan does not reveal either contaminations or volume changes within the first scanned area. You demonstrate at high magnifications serious beam damage of your samples during the first low-dose scan. How may the spatial information of such cryo FESEM stand up against TEM data? Do you get partial drying, shift of metal?
Authors: The comparison of our FESEM data with TEM data of the same specimen (Gross et al., 1978a) does not suggest any spatial changes in our first scan micrographs taken at high electron dose and high magnification.

K.-R. Peters: You already find in beam damage a serious limitation when working on frozen-hydrated yeast prepared from distilled water and fractured smoothly producing a sample of relative even distribution of mass underneath the surface. Why does the SE mode produce only information at the level of TEM replicas? How promising can this technique be applied for normal biological materials which exhibit high mass density variations, are not stable in distilled water and provide natural rough surface topography?
Authors: A major goal of LTSEM is to overcome the limitations of TEM replica techniques (see introduction). Freeze-fracturing alone provides a clean natural biological surface with the structural preservation characteristic of cryoimmobilization. At present, we see no way to achieve the same level of structural preservation at the very surface of biological specimens.

W.P. Wergin and E.F. Erbe: In the discussion you suggest that the clustered, protruding particles in Fig. 3 b (also present, but less evident in 3 a) are due to contamination from water condensation that may have occurred either when the cells were fractured under liquid nitrogen or when the sample was transferred, through air, to the sputter coater. Have you considered the possibilities that the argon gas tank may contain water vapor as a contaminant or that the tubing that connects the tank to the coater may become contaminated with water vapour?
Authors: We have not considered these sources of contamination, though we use highly purified argon gas and stainless steel tubing.

W.P. Wergin and E.F. Erbe: Do you think that the contamination occurs prior to or after coating? Have you attempted to raise the temperature of the sample in an effort to etch or sublime the contaminant assuming that it lies on top of the coating? This procedure has worked in our laboratory {Wergin WP, Erbe EF (1991a). Increasing resolution and versatility in low temperature conventional and field emission scanning electron microscopy. Scanning Microsc. 5: 927 - 936; Wergin WP, Erbe EF. (1991b). Using high vacuum evaporation to obtain high resolution low temperature images of freeze-fractured membranes from yeast. Proc. Elect. Microsc. Soc. Amer.: 514 - 515.}.
T. Müller: On Figure 3 b (EF of yeast plasmalemma) you describe the presence of ice crystals. Can these contamination products be etched (sublimed) away? The answer could tell us whether the contamination occurred before (during fracturing) or after (during transfer) the planar-magnetron sputter coating. When looking carefully to this picture (in the higher left corner), I got the impression that even some ringlike depressions (corresponding to the hexagonally arranged particles on the PF) are partly visible. Do you judge this as an indication, that the contamination by water vapour has occurred mainly after the coating process?
Authors: We expect that the contamination observed happened prior to coating. The contaminating particles in Fig. 3 b are precisely depicted and reveal substructures. Uncoated particles in the nm range would appear blurred.

W.P. Wergin and E.F. Erbe: If we understand your procedure properly, you are fracturing under liquid nitrogen, doing a through-air transfer to the sputter coater and then doing a second through-air transfer to the microscope; as you suggest, all three steps are potential sources of contamination. To reduce the possibility of contamination, have you considered preparing a freeze-etching unit and then making one transfer to the microscope? This could be done with or without a carbon film, either by rotary or unidirectional shadowing. After the frozen sample is viewed in the SEM, the film (replica) can be retrieved and further compared and evaluated either in the SEM at room temperature or in TEM {Wergin WP, Erbe EF, Reilly TW (1989). Comparison of freeze-fractured yeast replicas using conventional TEM and low voltage field emission SEM. Proc. Elect. Microsc. Soc. Amer.: 74 - 75; Wergin WP, Erbe EF (1990). Comparison of freeze-etched membranes from yeast using low temperature SEM, conventional TEM and low voltage field emission SEM. In: Elder H.Y., Goodhew P.J. (eds.): EMAG-MICRO 89. Vol. 2; Biological Inst. of Physics Conf. Series No. 98. Proc. Inst. Physics, Electron Microscopy and Analysis Group and the Royal Microscopical Society. Bristol, England , IOP Publishing. Ltd.: 715 - 718.}. This procedure also enables individuals with conventional low-temperature SEM's to obtain high resolution images of their samples in the TEM.
Authors: We are presently building a freeze-fracture device that permits transfer to the microscope by a high vacuum cryo-transfer system. The sole goal of this paper was to demonstrate that the structural information of TEM freeze-fracture replicas can be obtained with an in-lens field-emission SEM, adequate specimen preparation techniques and adequate coating.

T. Müller: If a liquid nitrogen cooled Meissner trap was present in the vacuum chamber, can you describe in more detail its influence to the fracturing, planar-magnetron sputtering, and condensation of coating material?
Authors: We cannot provide background information. We simply observed finer granularity when sputtering was performed in a vacuum with reduced H_2O partial pressure (see Hermann and Müller, 1991a).

T. Müller: As the preparation technique is similar and the specimen and coating material is same, please compare the structural resolution of the platinum coating with the results published by Müller et al. {Müller T, Walther P, Scheidegger G, Reichelt R, Müller S, Guggenheim R (1990). Cryo-preparation and planar-magnetron sputtering for low temperature scanning electron microscopy. Scanning Microsc.; 4: 863 - 876.}.
Authors: Walther et al. (1990) and Müller et al. (1990) both presented LTSEM micrographs of freeze-fractured platinum sputtered yeast. They employed a conventional field-emission SEM (Hitachi S-800) with an attached cryopreparation unit (Balzers SCU 020) for their investigations. The results produced by their set up are far away from those achievable with TEM freeze-fracture techniques, whereas our results parallel TEM. The reasons for this difference are summarized in the introduction.