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A CRITICAL COMPARISON BETWEEN THE TWO CURRENT METHODS OF VIEWING FROZEN, LIVE CELLS IN THE ELECTRON MICROSCOPE: CRYO-ELECTRON MICROSCOPIC TOMOGRAPHY *VERSUS* "DEEP-ETCH" ELECTRON MICROSCOPY

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The ultimate goal of cell biology is to understand how all of the many different sorts of molecular machines in the living cell operate in situ, *and ultimately, to understand how the concerted activity of all these machines is coordinated or integrated into a whole, to create the behavior of the living cell. Electron microscopy can help to answer these questions, if it can reach the level of resolving individual macromolecules in whole cells, and if it can reach this level of resolution* without *at the same time introducing gross distortions in the arrangement of these molecules or in the architecture of the cell's cytoplasm, in general. The aim of this article is to compare and contrast the two major approaches that electron microscopists are using today, in their efforts to achieve this broad, ambitious goal.* ***Biomed Rev 2001; 12: 11-29.***

INTRODUCTION

The goals of electron microscopy, as applied to problems in cell biology

Cell dynamics or cell "life" is an emergent product of the activities of a vast array of different molecular machines, hundreds if not thousands of different machines, all operating in concert. The ultimate goal of cell biologists is, of course, to understand how these molecular machines operate within the context of the cell, and specifically, to understand how they operate individually in the midst of all the other machines, and then, how their individual activities are coordinated or integrated into the functioning of the whole (cf: 1).

Reflection: The cell is indeed a fragile construct, a constellation of improbable parts, none of which can function on its own, but only in conjunction with all the others... indeed, in concert with all the others, The cell as a whole is a singular, unique entity, seemingly independent in its wholeness, yet dependent on each of its components working together perfectly and flawlessly. If any one part fails, the whole can be sabotaged; if any one molecule malfunctions, the whole cell can abruptly collapse. Any weak link can break the cell's whole fragile chain of existence. (For "the cell", one could substitute here the phrase: "the person", "the society", etc.)

Arguably, the greatest challenge facing electron microscopy (EM) today is to understand the basic structure or architecture of the cytoplasm, itself. This has long been one of EM's great challenges, if not its *greatest* challenge. To finally understand the basic structure or architecture of the cyto-

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plasm, the microscopist must ultimately determine to what extent the proteins of the cytoplasm are free in solution, and to what extent are they are bound to each other (and if bound, HOW they are bound and in what FORM they are bound to each other). The basic problem in determining this, however, is in learning how to *preserve* the architecture of the cytoplasm on the time-scale of molecular diffusion. Any technique that might be imagined for immobilizing the proteins of the cytoplasm long enough to acquire an EM image of them - whether it be by freezing or by chemical fixation of any sort - can only proceed, inevitably, at the same rate as molecular diffusion, itself. Hence, it would seem that the microscopist cannot possibly hope to capture an accurate image of the architecture of the cytoplasm, without profoundly distorting it in the very act of capture.....the microscopist's own "uncertainty principle"!

THE NEED TO AVOID CHEMICAL FIXATION IN EM

In fact, to see the natural relationships of molecules *in situ*, in their natural environment within the living cell, it will certainly be necessary to totally eliminate chemical fixation as one step in the protocol of sample preparation for EM. This is because fixation inevitably involves "gluing" molecules to each other. That is of course it's basic purpose: to rigidify and convert what was originally a living, flowing substance - within which molecules were more or less free to diffuse - into an inert solid, capable of withstanding the rigors of dehydration and "embedding", in preparation for some sort of thin-sectioning of the cell. However, decades of firsthand experience of many microscopists has now firmly established that the arrangement of macromolecules in the cell is invariably destroyed by all sorts of chemical fixation, dehydration, and plastic embedding - or at least, is degraded to such an extent that little useful, realistic information can be obtained about cytoplasmic or macromolecular architecture *in situ* (7, 15). Indeed, this degradation occurs even during the fixation and dehydration protocols that are applied after cells are frozen (the procedure called "freeze-substitution" (cf: 8.9.10).

Over the decades, it has become abundantly clear that the only way to retain meaningful molecular architecture within cells at the macromolecular level is to freeze them directly from life and keep them frozen while they are being imaged in the EM (2, 3,12,43-45). This means that after freezing, cells must either be cryo-sectioned and the resultant thin sections must be imaged while they are still frozen (38), or else the frozen cells must be cryo-fractured open and some sort of surface-imaging of their fractured surfaces must be done while they are still frozen (e.g., cryo-SEM, cryo-AFM, or as we do, cryo-replication followed by TEM imaging of the resultant replica (16-20,27-32)).

In practical terms, only the lattermost procedure turns out to be technically feasible at the present time. Thin sectioning of frozen but unfixed cells has been tried many times over the years, but has never really succeeded (38,44,45). (The standard technique of cryo-thin-sectioning for EM immunocytochemistry uses cells that have been fixed and soaked in concentrated solutions of sucrose before freezing. The frozen sucrose is what makes their sectioning possible; without it, cryo-thin sectioning has proven to be well nigh impossible.) Cryo-SEM does not provide adequate resolution to see macromolecules and is plagued by severe electron-charging of the frozen surface of the cell, because it is not coated with any conductive metal. And to date, cryo-AFM is wildly impractical. Hence, the only technically feasible way to image the surface of a cryo-fractured cell, these days, is to replicate it with an evaporated metal film before imaging. By great good fortune, this can be done without melting or otherwise degrading the fractured cell surface. And by equally good fortune, the resultant metal replicas are impervious to subsequent change, can be imaged by the simplest of TEM, and can provide resolution down into the molecular level (~2 nm) (21,26,41).

SOME OF THE MAIN REASONS FOR WANTING TO ACHIEVE HIGH-RESOLUTION EM IMAGING OF WHOLE CELLS

If one could succeed, by any method, to image a live cell properly and at high enough resolution, one could answer at least three fundamental questions about the nature of cytoplasm, in particular. First, what proportion of the proteins present in the cytoplasm are at any one time crosslinked to each other, and what proportion are in free solution? Second, considering only the proteins that are insoluble: what structure do they create, what patterns do they assume, what specific protein-protein interactions do they manifest? Third, what contribution(s) do these transient and fleeting protein-protein interactions make to various cellular processes and to the basic physical properties of the cytoplasm, and in particular, to its shape-dynamics (e.g., to the "push-pull" behavior by cytoplasm)?

As stressed above, the answers to these fundamental questions will only come from images of cells that have been frozen directly from life, without having had any prior chemical fixation or staining applied to them, and that have been imaged while they remain frozen and still encased in the ice that was created in and around them at the moment of freezing. Moreover, that encasement of ice must either be completely amorphous (e.g., totally glass-like, in the form of an inert, supercooled liquid) or else must be so finely microcrystalline that its ice crystals are smaller than the natural interstices that exist between the proteins in the living cytosol, so that they do not significantly distort the cytoplasm.

Unfortunately, this goal is well-nigh impossible. Even the latest generation of cold-stage, high resolution tomographic EM cannot ever do better than imaging viruses (4,5,35) or

imaging one organelle at a time (43). They simply don't have the penetrating-power (or the imaging capabilities) to look through a sample as thick as a whole frozen cell. To date, they have been most successful at imaging frozen thin films of dispersed macromolecules, or at the most, squashed prokaryotes or semi-thin cryosections of frozen cells. On the other hand, the techniques that involve cracking-open frozen cells and making metal replicas of its inner surfaces are plagued by the problem of recrystallization of the ice encasing the cell, which creates ice crystals large enough to distort the architecture of the cytoplasm. Unfortunately, a frozen cell cannot be totally freeze-dried or "freeze-substituted" without allowing this damaging recrystallization to occur. That is because it is "part-and-parcel" of the increase in molecular mobility needed to get water molecules out of the cell. That increase in mobility, achieved by warming the frozen cell to approximately minus 80 degrees Celsius, is needed to get water to sublime from the cell (or to allow its "substitution" with organic solvents). In practice, we have found by long and sometimes bitter experience, that to optimally image a cryofractured cell in the EM, we are restricted to simply "deep-etching" its cracked-open surface, e.g., to quickly removing just enough ice from this surface, by very brief vacuum-sublimation (only a minute or two at about minus 100 degrees Celsius), to reveal the superficial layer of proteins immediately beneath this surface. Still, despite this restriction, we can still succeed in this way to obtain fascinating "bas-relief" images of cytoplasmic structure, in a relatively undistorted condition.

AN ADMISSION OF THIS AUTHOR'S PERSONAL BIAS

Personally, I feel like relic from another era - the last of the "old-school" of classical electron microscopists. Yet at the same time I feel that I have a responsibility to "finish a job" and to demonstrate that "deep-etch" EM remains vitally important to EM, and in some instances remains superior to other methods of specimen preparation and imaging for EM. It seems to have fallen to me to insure that this technique is transmitted to the next generation, and to prevent its loss and decay. In this effort, I apparently have to make a concerted effort to prevent "deep-etch" EM from being totally supplanted by the rush into EM tomography that is occurring today.

It has been said that many scientists would "rather use each other's toothbrushes than their techniques." I think this is because scientists typically seek to establish their own uniqueness and superiority; hence, they avoid using another's techniques out of uncertainty (and sometimes out of envy?), rather than from the security of knowing that their own technique is actually superior. Of course, such knowledge can only be obtained by a direct comparison between techniques. In the case of EM, such a comparison is completely straightforward: one only needs to prepare and image the same sample by various different methods, and see which method yields the best looking and the most informative images. This will be the approach used below, in comparing the appearance of a prototypical prokaryote by "deep-etch" EM *versus* EM tomography.

Sometimes, the practitioners of EM tomography seem to me like those "toothbrush" scientists. They adamantly maintain that their technique is totally superior; while in fact, the only high-resolution images of frozen cells they have obtained, to date, have come from exceedingly thin regions of them, or from thin films of frozen-hydrated prokaryotes. Yet, the technique of "deep-etching" can provide entirely comparable "thin-views", simply by limiting the etching to very shallow depths of relief (say, <100 nm). Furthermore, "deep-etch" EM allows proper freezing of large 3-dimensional volumes in the first place, as well as for straightforward imaging of thin slices through these volumes, without requiring the microscopist to cryo-section the entire volume. Additionally, "deep-etching" provides images that look exactly like what they, in fact, are: surface-renderings. As such, they are immediately recognizable and interpretable, since humans are used to looking at the surfaces of things. In contrast, EM tomography provides only translucent images, and these are exceedingly hard to interpret because they display an overlap of structure that can be horrendously confusing.

OVERVIEW OF THE TECHNIQUE OF "DEEP-ETCH" EM

The term "deep-etching" refers specifically to vacuum-sublimation of ice from a quick-frozen, unfixed sample that has been frozen by "slamming" it against a metal block cooled to minus hundreds of degrees, then typically freeze-fractured at an equally low temperature (16-20). The etching is allowed to progress "deeply" enough to reveal a significant amount of surface-topology at the fractured surface of the sample. The term "deep-etching" was originally intended to differentiate our technique from the more generic term "freeze-etching", which generally referred to an earlier procedure that involved the use of fixed and glycerinated cells that were "quench"-frozen by plunging them into a cryogenic liquid (which freezes much slower than "slamming" them against ultracold metal), and then, after they were freeze-fractured, were subjected to very brief period of etching that, in fact, did not progress to any significant extent because of all the glycerol that was present, but simply made non-membrane areas look a bit "rougher" than fractured membranes, and thereby allowed the two to be discriminated from each other. (Of course, if "deep-etching" were continued for a very long time, it would result in a completely freeze-dried sample; but this we avoid, since it creates too much topology and leads to the recrystallization artifacts mentioned above.)

One finds in the literature a wide variety of acronyms given to the process of freezing against a cold metal block, which is

still the only way to achieve cooling rates high enough to obviate the need for cryoprotectants and to create samples suitable for "deep-etching". These acronyms include "fast-freezing", "rapid-freezing", "flash-freezing", and our favorite "quick-freezing"; but most people simply use the term that actually describes the operating-sound of all metal-block freezing machines: "slamming". In fact, we abhor the use of this term, since "slamming" commonly implies the use of extreme force, with a strong likelihood of physical damage to the sample. In fact, with metal-block freezing, surface regions of the samples that are to be "deep-etched" generally freeze before they have a chance to become compressed. As well as we can determine, freezing proceeds into the sample at nearly the speed of sound! Hence, only the deeper layers of the sample, which of course freeze much more slowly, show any compression-artifacts. Thus, we greatly prefer to call our machine a "freezing press" or "Cryopress", rather than a "slammer".

Admittedly, the biggest problem with "deep-etching" is that it invariably requires the production of a metal replica of the surface of the frozen sample. As mentioned above, a frozen sample cannot simply be mounted directly in a TEM, SEM or AFM and viewed without any metal on it. On the other hand, a metal replica can be visualized with any of the above microscopes. In particular, it is most easily viewed with standard TEM's, which are universally available and extremely simple to operate (especially when compared to the extraordinarily expensive and tricky TEM's necessary for EM tomography!). Nevertheless, the fundamental problem with the platinum replicas produced during "deep-etching" is that they are not truly *amorphous* films that fully and faithfully represent the surface of the frozen sample in every minute detail. Rather, they are actually microcrystalline, sub-confluent monolayers of platinum grains that assume a characteristic size and thickness (roughly 2 nm). As a result, they actually represent a "frosting' on the surface of the sample; and in that sense, they inevitably distort the proportions of the underlying biological structures, especially the smaller structures. Still, despite this fundamental limitation, platinum replicas continue to provide - due to their very uniformity and reproducibility - the most straightforward way to image biological structures across the full range of sample-sizes, from whole cells and tissues, all the way down to individual macromolecules and their component parts (6,13,21-26,42,46).

OVERVIEW OF THE TEN BASIC STEPS INVOLVED IN EM TOMOGRAPHY

This section can best be understood by following along step-by-step with the abbreviated "Box" that is appended to this article, on pages 24, 25. This will allow the reader to garner a proper appreciation of the several important differences between EM tomography and "deep-etch" EM.

Electron microscopic tomography, the latest technique for using a transmission EM to obtain 3-D views of whole cell volumes, including whole cell organelles, involves the following 10 sequential steps. First the cell must be fixed or frozen to immobilize and stabilize its ultrastructure (7). Second, it must be prepared for EM and mounted in the microscope in a manner that is consonant with how it was first fixed or frozen. For example, if it was frozen, it must either be "freeze-substituted" (which basically involves sub-zero fixation and dehydration) in preparation for plastic embedding and thin sectioning (8-10), or it must be directly thin sectioned while still frozen (e.g., cryo-sectioned, cf: 38). Alternatively, if the sample was thin enough to start with, and was grown on a transparent substrate, it can be mounted directly in the EM and viewed as a whole mount (2.3.12). Finally, if it was originally fixed by traditional methods (glutaraldehyde and osmium) rather than frozen, it can be dehydrated, embedded in plastic, and thin sectioned as has always been done for TEM. The only difference here is that EM tomography permits viewing of slightly thicker sections, up to a quarter micron thick, compared to the old days when earlier TEM required sections one-tenth of a micron thick or less in order to obtain sharp, 2-D images (cf: 14,33,34,36).

Third, the cell must be imaged in the TEM, which is of course the critical step. Here, EM tomography differs drastically from earlier procedures that relied on single views, or at most on pairs of views to create stereo images. Tomography requires a whole set of images of the same field, viewed from as many different angles (and is over as wide a range of angles) as possible. Practically speaking, this generates the need for highly accurate "goniometric" (e.g., centerable) tilt-stages for holding the cell stably in place while it is being photographed from these many different angles. And not only must this stage be eucentric and mechanically stable, but it must be capable of being cooled with liquid nitrogen, to keep frozen samples well frozen during the imaging (and even to keep plastic-embedded samples from suffering from too much heating and radiation damage from the electron beam).

Other special requirements generated by the need to take many dozens or even many hundreds of images of the same field, are that the EM used for tomography must have a "low dose" illumination system. This allows the electron beam to be accurately displaced back and forth between a region of the cell that can be illuminated brightly enough to permit focusing of the image, and an adjacent region that is only briefly illuminated and receives just enough electrons to create an adequate image for each tilt angle. Additionally, the microscope needs to have a quality digital camera rather than a film camera for capturing the images, since as mentioned above, the tomographic sets run into hundreds of views, at which point classical film recording becomes totally unwieldy.

Finally, for proper imaging of relatively thick samples, especially frozen samples that are unusually sensitive to radiation damage, an "energy filter" is generally needed for the

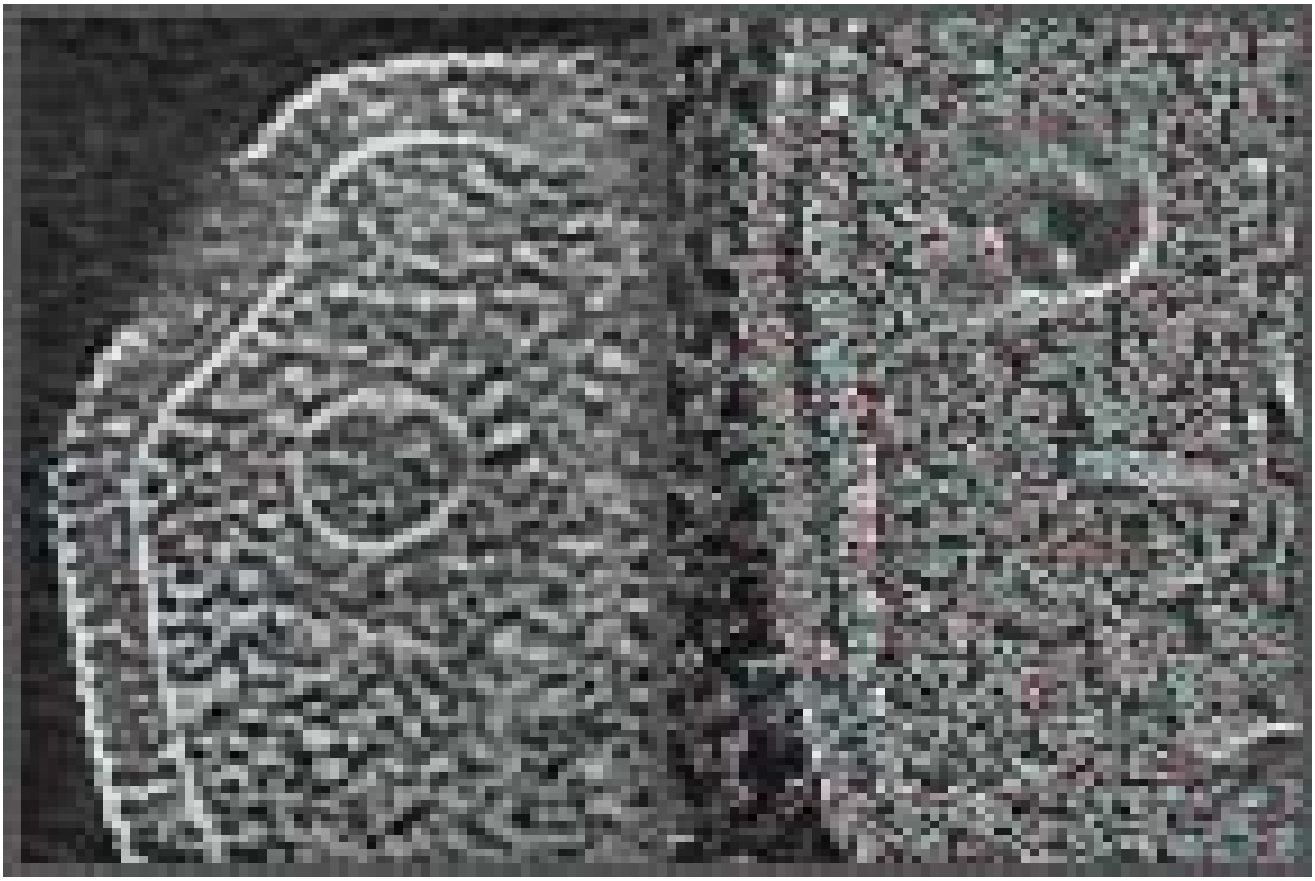


Figure 1.
Left panel: *EM tomogram of a whole, frozen Archaebacterium,* Pyrodictium abyssi, *taken from Fig. 1 of Reference 3. To present these author's flat images into this "anaglyph" stereo image, we took the two 2-nm slices through the reconstructed volume these authors chose to present, and offset them by a reasonable distance, to give the impression that they lie at different planes. (The original distance between the two slices was not defined, so the amount of missing information in the center of the anaglyph is unknown.) The authors described this image as follows: "Plasma membrane and intracellular vesicles are clearly recognizable. The vesicles are surrounded by dark protein masses, probably macromolecular assemblies involved in exo- or endocytosis"(3). The authors claimed that the resolution of this tomographic data-set was about 8 nm (3).*

Right panel: *Comparable "anaglyph"-stereo "deep-etch" EM image of a related Archaebacterium,* Haloferax volcanii, *prepared by our standard procedures. As in the tomogram, a distinct ~ 40 nm space can easily be seen between the plasma membrane immediately adjacent to the freeze-fractured cytoplasm (to the right) and the surface crystalline-protein or "S layer" lying outside it. However, unlike the smooth and featureless membrane seen in the tomogram, the plasma membrane in the deep-etching looks granular and filled with proteins, as expected. (In contrast, the membrane around the internal vesicle in the freeze-fracture does look smooth and featureless, implying that it possesses few proteins, which serves as an important "control".) Many other macromolecules and formed elements can be seen directly in the "deep-etching", as compared to the amorphously granular image of cytoplasm in the tomogram. Both panels:* x *250,000.*

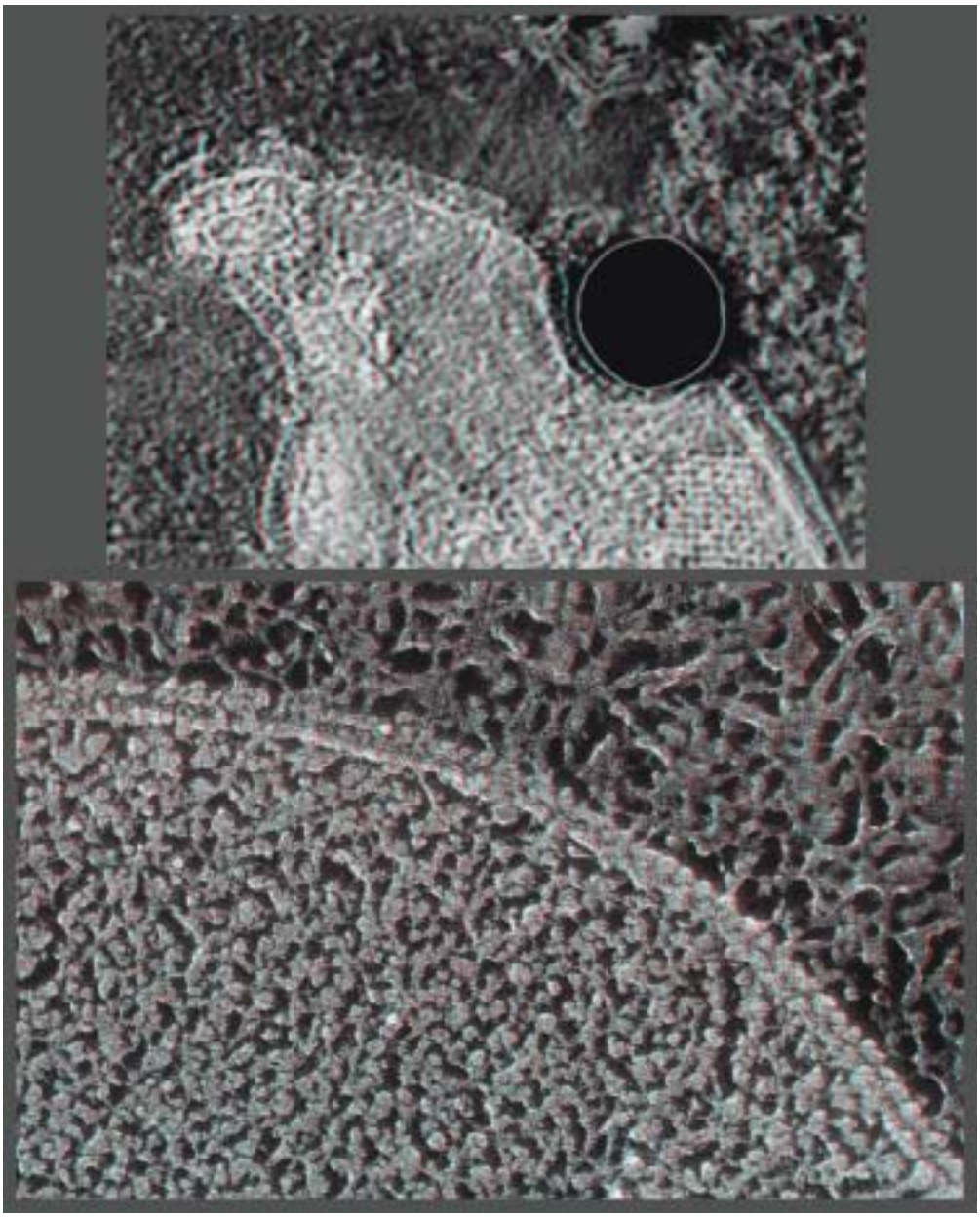
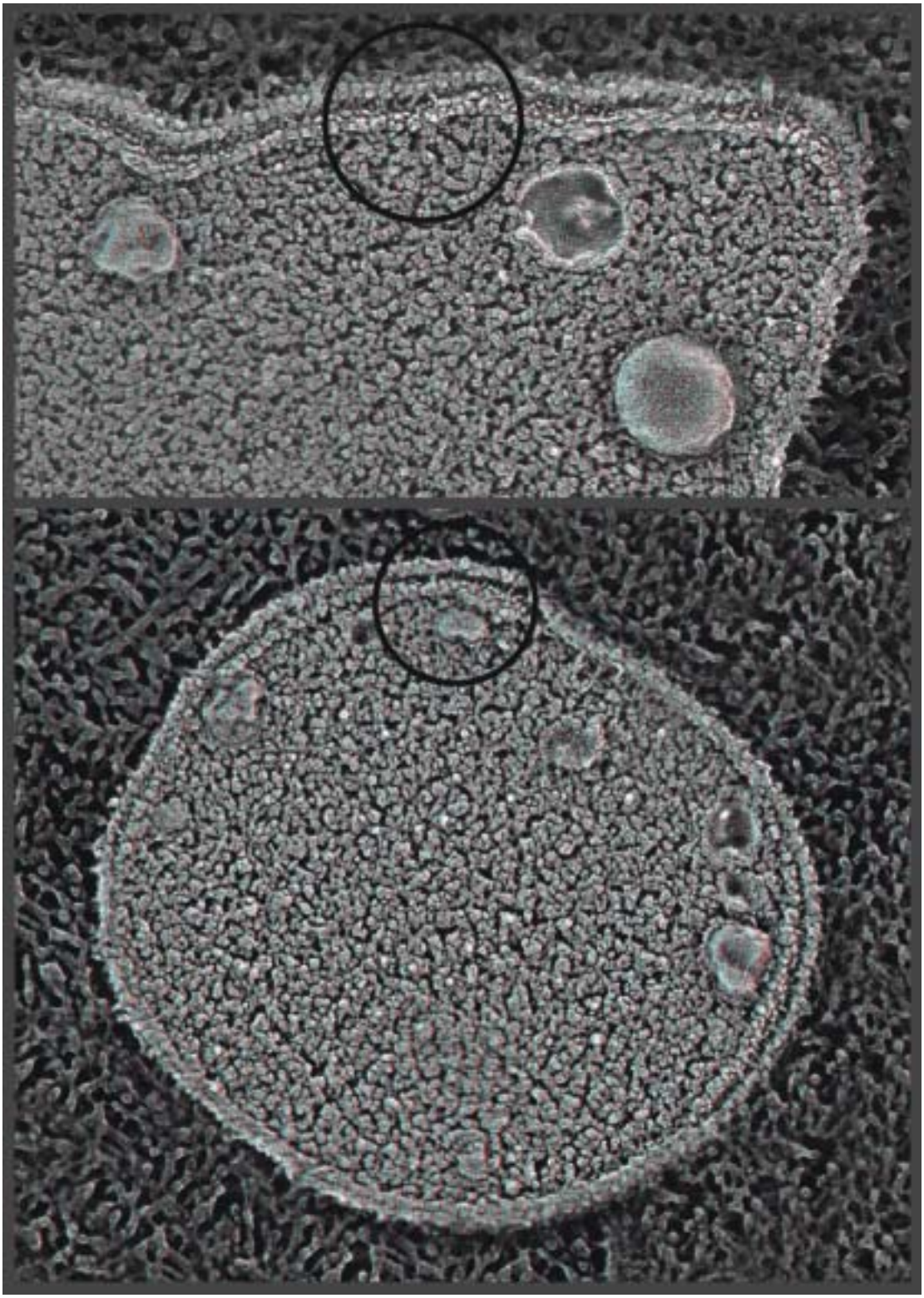


Figure 2.
Upper panel:
EM tomogram of a whole, frozen Archaebacterium, Pyrodictium abyssi, *taken from Fig. 4 of Reference 2. Here, we again converted into one "anaglyph" stereo view the two 2-nm computer-generated "slices" through the reconstructed volume these authors chose to present. However, the original distance between the two slices was stated to be ~90 nm (2), so there is a lot of "missing information" in the center of the anaglyph. The authors described this image as follows: "The regular surface layer, which forms a porous canopy 40 nm above the surface of the plasma membrane, is clearly visible at the periphery of the cell." They added: "The cell appears to be in the process of engulfing a 250 nm latex bead" (the black circle) and: "inside the cell, a small protein crystal and several vesicles are clearly visible. Some of these vesicles are docked to the plasma membrane and have recruited an array of particles on their outer surface." They concluded: "The existence of such vesicles and of vesicle traffic across the cell wall were quite unexpected findings."*

Lower Panel: *Comparable "deep-etch" EM image, again of the Archaebacterium* Haloferax volcanii *prepared by our standard procedures. It shows how much more clearly one can discern the macromolecular organization in the cytoplasm, in the surface membranes and even in the extracellular matrix of "deep-etchings," compared to EM tomograms. Furthermore, the vastly greater yield of useful images and information provided by deep-etching has allowed us to conclude that in the Archaebactera, vesicle traffic across the cell wall is not simply "quite unexpected finding" but a completely artifactual one, probably generated by shrinkage and other distortions introduced during the mounting of whole cells in thin films of ice, as is required for EM tomography. Both panels:* x *150,000.*



Figures 3.
Typical "crossfractures" through the interiors of two Haloferax volcanii *Archaebacteria, illustrating the distinct space that exists between the plasma membrane* per se *and the external "S layer" that characterizes this phylum (circled). This space is comparable most likely to the "periplasm" between the two membranes of the higher gram-negative bacteria. Also readily apparent are variously-shaped organelles that lie immediately under the surface of the cell. These are clearly composed of membranes, because they fracture over their surfaces in the manner typical for membrane bilayers. However, their membranes appear to be very poor in protein. It would be most interesting to determine the function of these newly-discovered structures in such a primitive prokaryote. They may be evolutionary precursors of the membranous organelles that support the life of all the eukaryotic cells we know today.*
Upper panel: x *120,000.*
Lower survey panel: x *65,000.*

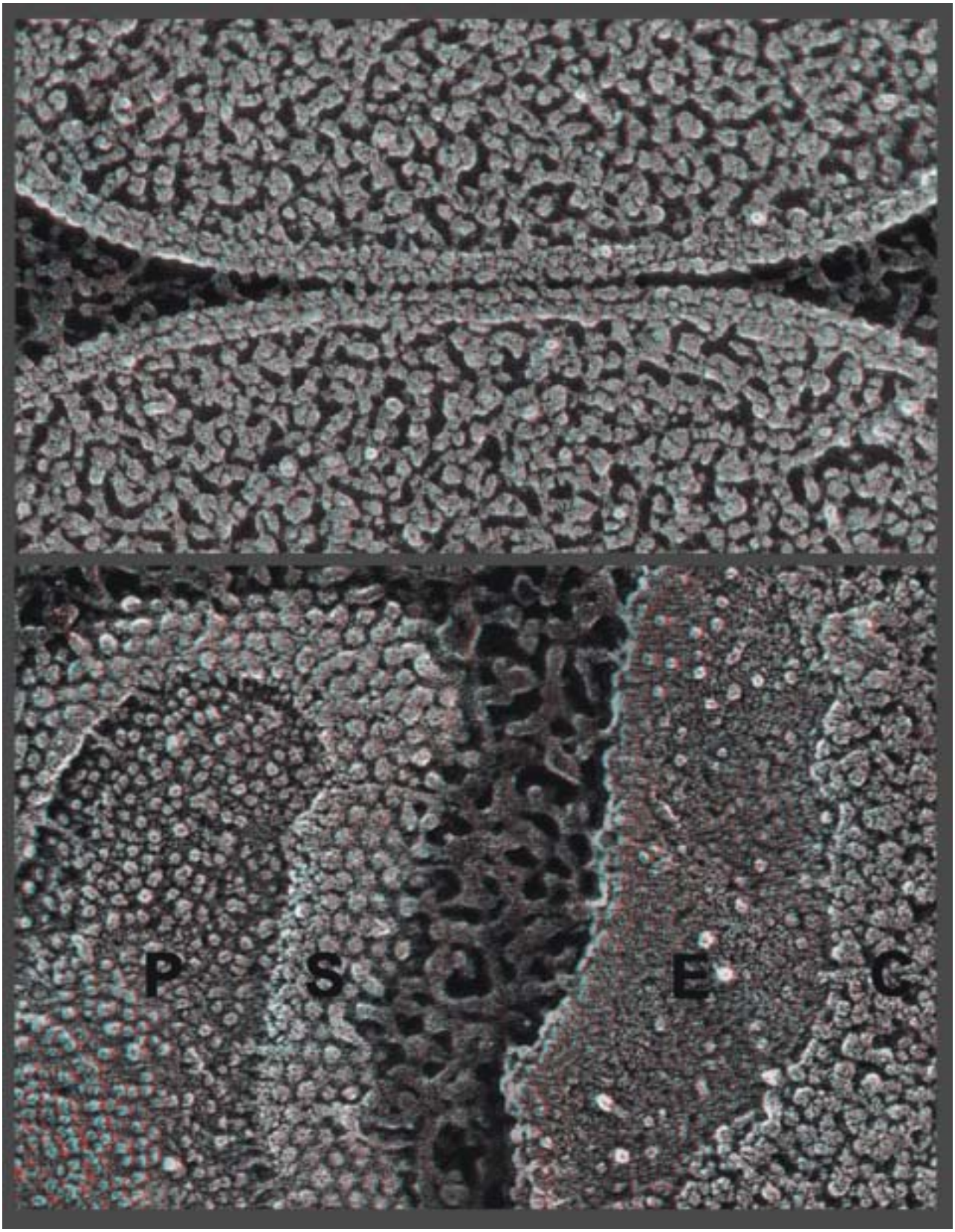

P
S
E
G

Figure 4. *Higher magnification views of crossfractures (above) versus oblique fractures (below) through the plasmalemma and surface crystalline layer (or "S-layer") of* Haloferax volcanii *Archaebacteria. The crossfractures illustrate that the space between these two layers is often very attenuated or obliterated entirely. Despite this, the oblique fractures yield a wealth of information about the internal architecture of the plasmalemma, shown in its two complementary fracture-faces in the adjacent organisms, below. ("P" classically refers to the internal membrane surface that faces outward from the cell and is underlain by cytoplasm or "protoplasm" (hence "P"), while "E" refers to the internal surface of the membrane that would normally face into the cell, and would be back onto the extracellular world (hence "E").) Note that "deep-etching" has revealed two additional surfaces not visible in classical freeze-fractures: namely the true outer surface of the beautifully crystalline "S layer" (labeled "S"), and the granular cytoplasm underlying the plasmalemma of the Archaebacterium on the right (labeled "C"). Both panels:* x *250,000.*

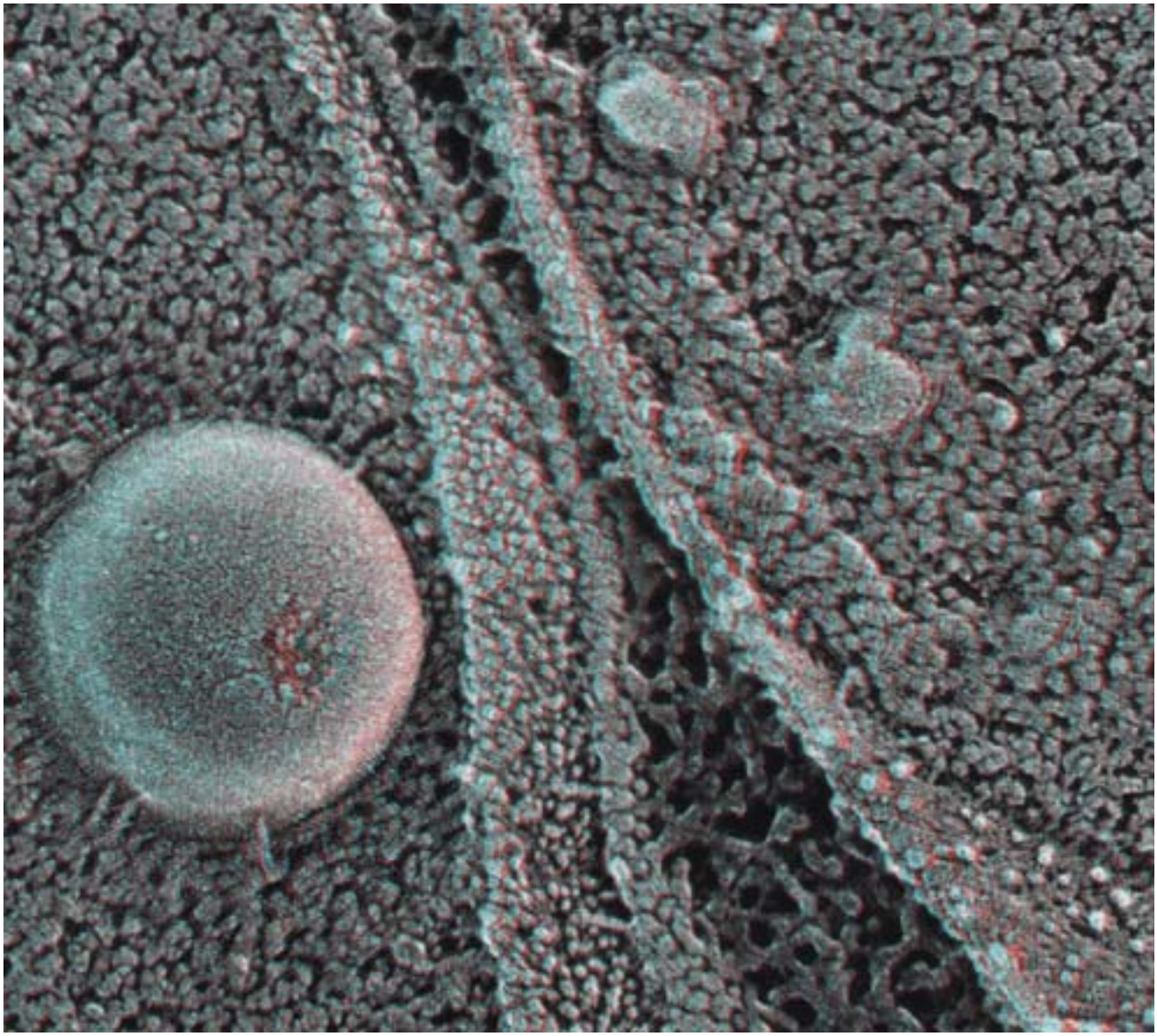


Figure 5. *Still higher magnification view of two Archaebacteria that lie immediately adjacent to each other, to show more clearly the amazing wealth of three-dimensional structural detail that is available in "deep-etch" EM's. Note especially the very large (~150 nm) membranous vesicle in the left cell, showing many exceedingly fine fibrillar connections with the surrounding cytoplasm, all around its circumference. Contrast this turgid sphere with the tiny, limp compartments seen in the cell to the right. One cannot but wonder if these are images of different stages of filling of a common organelle, perhaps a gas vacuole or some form of food-storage organelle, which have only been dimly glimpsed in the Archaebacteria heretofore. This image is presented at the magnification that we use for presenting "deep-etch" EM's of individual molecules in our related "molecules on mica" technique (6,13,21,24,46), namely* x *350,000.*

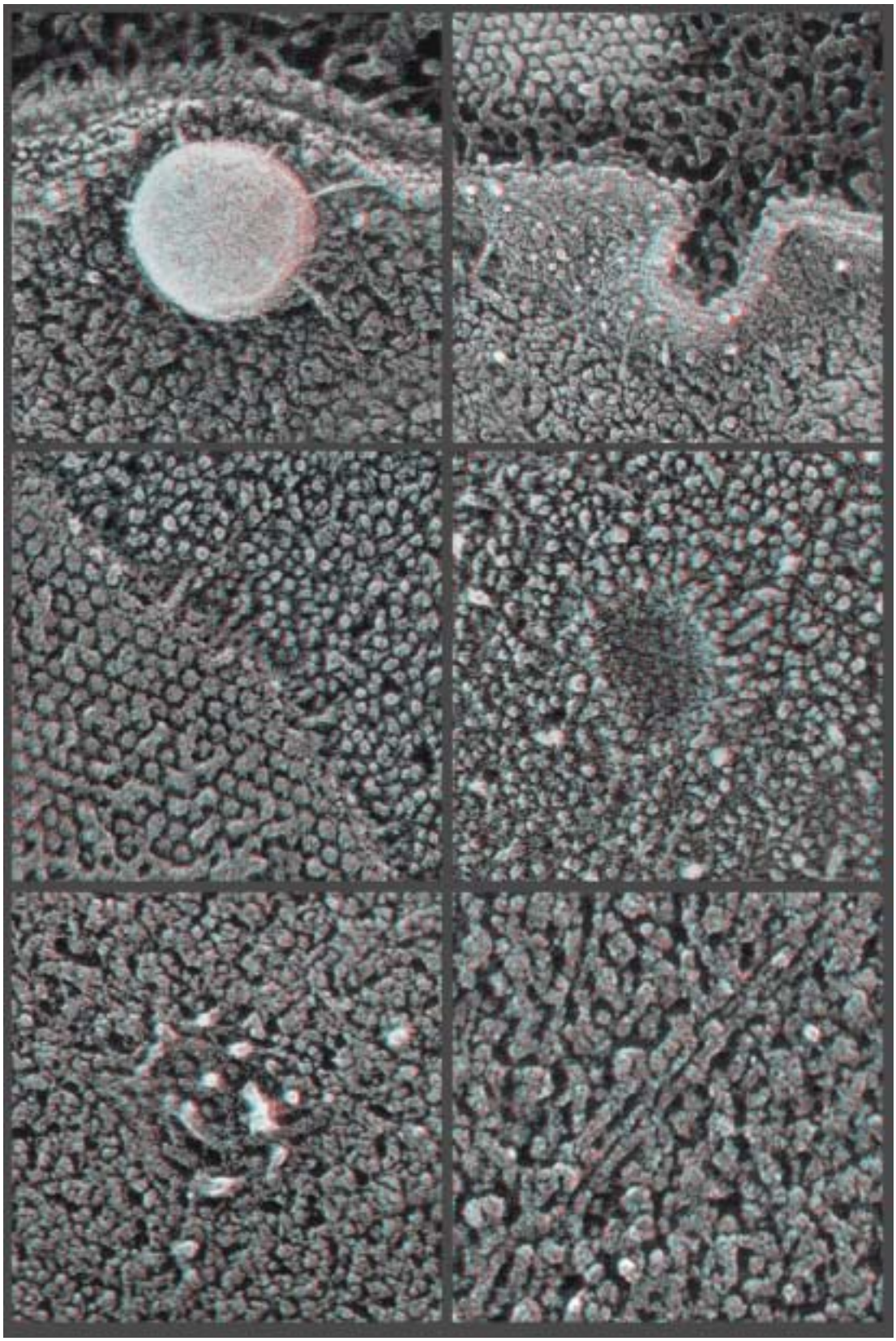


Figure 6.
Survey of some to the interesting and new structural differentiations observed in the course of this comparative study of the Archaebacterium Haloferax volcanii. ***Upper left panel:*** *a large (~120 nm) membranous vesicle, showing around its circumference many fine fibrillar connections into the surrounding cytoplasm (or to the plasmalemma), much like the vesicle in Fig. 5.* ***Upper right panel:*** *a cuplike involution of the plasmalemma that could conceivably be a precursor of some sort of exo- or endocytotic event (see, however, our comments in Fig 2, above).* ***Middle left panel:*** *optimal en face view of the interface between surface "S layer" and the "P" face of the plasmalemma, beneath. Note the fibrillar attachments between the two, possibly plastic deformation artifacts introduced during freeze-fracturing or possibly real structures.* ***Middle right panel:*** *An en face view of a cuplike depression in the plasmalemma, comparable to the cup seen on edge just above this panel, illustrating that the rich complement of bumpy "intramembrane particles' that is normally seen on the "P" face of the plasmalemma is entirely lacking in this specialized domain, indicating a fundamental difference in membrane organization there.* ***Lower left panel:*** *deep in the center of a crossfractured cell appears a whorled bundle of cytoplasmic filaments that have been cut across (and stretched) during freeze-fracture. The existence of such cytoplasmic filaments has not heretofore been recognized in the Archaebacteria.* ***Lower right panel:*** *Also deep in the center of a crossfractured cell appears a longitudinally fractured bundle of cytoplasmic filaments that have the lateral dimensions (~7 nm) and the faint cross-striations (q.5.2 nm) characteristic of the actin filaments that form the cytoskeletons of eukaryotic cells. Perhaps this will be the first demonstration of an Archaebacterial cytoskeleton.*

TEMs that make tomograms. These energy filters reject all electrons that suffer multiple collision-events within the sample, or for other reasons are not completely scattered out of the objective lens's focusing range. The proportion of such electrons that contaminate the final image increases exponentially with section thickness, and contributes to a severe blurring of the image, due essentially to the inevitably high chromatic aberration of electron microscopic lenses. Indeed, energy filters are preferable not just for frozen cells, but for all samples when they are viewed at high tilt-angles, since at that point the electrons must traverse them very obliquely and hence have a much-extended path-length. (For instance, at 70° of tilt, the cosine-relationship dictates that the electron path-length will be almost 2 times greater than the sections' actual thickness.)

Having obtained an extensive series of tilt images of one region of the cell, presumably without destroying it in the process by irradiating it excessively with electrons, the images are next subjected to step four of the procedure: alignment of all of the images so that they will be interpretable by a computer (11,35,37,39,40). (This is a step not usually necessary in medical tomography, since the patient is usually held very still and the recording device is rotated accurately around them, while in EM the recording situation is *vice versa*.) However, since the goniometer stage in the microscope is not mechanically perfect and because the sample changes with time during its irradiation in the microscope, the images must be transformed in complex ways, including adjusting their relative sizes, proportions, "skews", etc., as well as positioning them in space so that they properly align (11,35,37,39,40). Here, "fiducial markers" (gold particles adsorbed to the surface of the sample before mounting it in the microscope) are what the computer actually uses for alignment, since the images at this stage are so pale and noisy that the computer cannot actually "see" anything in them (since, remember, they are taken at far under-focus and "low dose" conditions).

Next, the fifth and critical step is finally to construct the 3-D tomogram of the region of the cell that was photographed so many times. This involves standard methods of computer "back projection" (actually, what is called "weighted" back projection, to take account of how dark the high-tilt EM images actually are), which is pretty much exactly like computing a medical CAT-scan. The computer algorithm for doing these back-projections is far beyond the scope of this article to explain in any detail. Suffice it to say, the result is indeed like a translucent "x-ray" of the cell volume in question. However, for just this reason, the tomogram still requires many more steps of processing before it is interpretable. This is because its very translucency creates much too much overlap of structure, to allow the microscopist to resolve or discriminate anything in it by direct visual inspection.

Instead, to begin to view it, the next step, step six, is to use special computer algorithms to artificially "thin section" it all over again. (At first, this may seem like "going around in circles"; and one may well ask why the microscopist didn't just create serial thin sections of their sample to begin with; but that is not for us to question here. Here, we are merely reporting what is actually done in EM tomography - never mind its laboriousness.) In fact, the artificial, computer-generated thin sections can be made as thin as one wants, to the limit where they are extraordinarily pale and noisy and low in information content; but typically applied at this step are additional computer manipulations to "de-noise" the sections (in common parlance, to "blur" them.) Here, various labs around the world differ in their approach: some use relatively straightforward "wavelet transform" programs, while others

advocate complex, non-intuitive "anisotropic diffusion" programs that somehow progressively wash away the noise (and if applied too harshly, wash away all the image data as well, ultimately "throwing out the baby with the bath water").

Once one has an interpretable image on a computer screen that effectively represents an artificial thin section through the tomographic volume, the observer is ready for the next step, step seven, which in computer-ese is called "segmentation". What this means in common parlance, is to select something out of the jumble of structures in the image. Generally this is done just by tracing manually around that something, with one of those special pens that a computer can recognize when it touches the screen. In essence, the microscopist is thereby subjectively defining the boundaries of the objects he/she is interested in, within the image. This selection or "segmentation" is not entirely subjective, however, since many laboratories are developing yet more computer algorithms that "spruce up" the observer's selection by applying "grey-scale neighborhood-search" schemes to the initial tracings, thereby allowing the microscopist to apply more objective selection criteria. These also help the microscopist to properly propagate their tracings throughout all the adjacent sections and "to keep them honest".

Once the computer has thereby been told with the tracing-pen just "what is what", it is ready to perform the next step, step eight, of creating a "model" of the subject of interest, usually in the form of a surface-rendering or volume-rendering. Today, these sorts of renderings can be made by lots of different, commercial software packages. The only thing unique about the models generated in EM tomography is that they do not represent solid organs and tissues, the way that medical tomographs do; but rather, they represent 3-D renditions of surfaces (defined by the operator's tracings) that had a particular grey-scale in the original EM image (equivalent, presumably, to "isopotential lines" of constant stain density in the original biological sample).

The last two steps in EM tomography involve working around the computer-generated surface or volume renderings obtained in step eight, to get more information from the original tomograms. In particular, much effort is going today into developing new programs for automatically picking out macromolecules within the tomographs, even if they were not recognized and traced by hand at earlier stages (2,3,12). This can be considered step nine: developing and applying various computer-automated forms of "feature-extraction" or "pattern-recognition" algorithms to the EM tomograms. In essence, these computer operations involve complex sorts of "cross-correlations" between various discernible features in the computer tomographic volume, and previously defined 3-D computer images of "reference molecules" that are generally called "templates". However, exactly how this is done is beyond the scope of this review (or the comprehension of this observer). In any case, some potential pluses and minuses of this step are discussed in more detail in footnote 1, below.

The final step in EM tomography, step ten, is still a bit of a 'pipe dream', but should be considered essential to any scientific inquiry: namely, to quantitatively analyze the model(s) generated in step eight. While this step has never actually been performed on any tomographic reconstruction (at least that this reporter is aware of), it would presumably involve some sort of an application of a spatial-density algorithm to the EM tomographic volume, to calculate a quantitative "something" out of it. After all, despite all the computer-manipulations made of it, the EM tomogram will always remain a translucent "x-ray" sort of an image of a cell volume, in which differing greyscales are the actual data (and at some level, represent the molecules and organelles contained within it - however, ghostily).

THE FIRST PROBLEM WITH EM TOMOGRAPHY: HOW THE CELLS IT IMAGES ARE INITIALLY FROZEN

The technique of the EM tomography involves several initial steps that are sub-optimal, to say the least, in terms of sample preparation and sample scrutiny, steps which we consider to be the "lifeblood" of EM. First, it "skimps" on the methods for freezing samples, because the samples must remain (or must become, via the very difficult and still wholly unsatisfactory procedure of cryo-thin-sectioning of quick-frozen, unfixed tissues), semi-transparent to the electron beam. That is, the electron beam must be able to penetrate them to create the stack of images needed for 3-D tomography.

Furthermore, even if cryo-thin-sectioning of unfixed and unembedded samples could someday be made to work with some degree of success, for cellular preparations to be made amenable to this approach, they still would have to be frozen by one of the expensive and cumbersome "high-pressure" freezers. This is simply because cryo-microtomy requires a several-micron thick zone of high-quality freezing, simply in order to be cut-able. This immediately rules out our preferred technique of freezing cells by "slamming" them against an ultra-cold block of metal, since this procedure invariably yields only a few microns of good freezing on the immediate surface of the sample. Cryo-microtomy is difficult enough, without adding the requirement of hitting just this very thin, very superficial zone of optimal freezing. (Moreover, nobody has any idea about how this zone would cut in a cryo-ultramicrotome, even if they could hit it on their first approach to the microtome knife.)

We should add here that in "deep-etch" EM we do not face this problem, since the microtome we use for freeze-fracture of our "slammed," quick-frozen samples is intrinsically a "first-approach" instrument, designed to provide direct visual and mechanical control of the microtome knife so that its approach

to the sample can be closely monitored and a "first-cut" through the most superficial few microns of optimal freezing can be assured. Furthermore, no further cuts need to be made and no delicate thin-sections ever need to be produced, since the first cut is intended simply to scrape away the inevitably air-dried film on the surface of the sample and arrive at the best frozen zone inside the top layer of cells. As described above, a surface-view of this zone is next made by vacuum evaporation of a metal onto it, thereby creating a "replica" of it. It is never subjected to the harsh conditions of trans-illumination in the EM, nor subjected to the indignity of cryo-transfer from the microtome to the microscope, as must be done for cryo-sections. This latter step is exceedingly tricky, in and of itself.

The only alternative "first step" for cryo EM tomography is to avoid high pressure freezing and cryo-microtomy entirely, by converting the cell into a ultra-thin film on an electron-transparent plastic substrate before freezing, either by culturing it on the plastic (or theoretically, by squashing it against the plastic and then freezing its extruded parts). Here, the freezing of such ultrathin cellular films for EM tomography could theoretically follow the traditional procedure of rapid "quenching", wherein an electron microscopic grid containing a thin film of an unfrozen sample mounted on a "holey" plastic film is abruptly "plunged" into a cryogenic liquid like liquid ethane (-150° C). Dubochet established, years ago, that this procedure could achieve near-perfect freezing (e.g., it could truly "vitrify") samples in the sub-micron range of thickness (7). However, for thicker samples even as small as prokaryotes, the ability of this freezing technique to achieve vitrification remains doubtful. Rather, our experience suggests that one cannot adequately freeze, by "quenching," even the full thickness of a sample as small as a prokaryote, let alone an entire cultured mammalian cell. This we conclude from having spent decades fracturing at various depths into samples frozen by "slamming", a heat-extraction procedure proven to be at least 10 times faster than "quenching" (simply due to the much lower temperature of the metal versus the cryogenic "quenching" liquid, as well as to the metal's astronomically higher thermal conductivity and diffusivity).

Some thin cells, especially prokaryotes, have already been prepared for cryo-EM tomography by "quenching", but the resultant images have shown that they have been severely shrunken and flattened during the "blotting" step that removes nearly all overlying liquid from the sample in the last moments before the plunge into the cryogen (2,3,12). That is, their thickness turns out to be less than 1/2 of their breadth, and the apparent concentration of their cytoplasm at least 2 times greater than expected: 30-40% solid *versus* the 10-15 % solids actually present in the living cell, as has been monitored by various biophysical and biochemical studies of living protozoa. Hence, such preparations are relatively well frozen only because (as the authors of these studies readily admit), they have been shrunken and made grossly hypertonic - a condition known to provide good "anti-freeze" properties, but not a one recommended for yielding clear, representative views of a cell's interior.

Some practitioners of "plunge"-freezing or high-pressure freezing of whole cells have even "cheated" by applying extracellular cryoprotectants like polyethylene glycol or polyvinyl pyrrolidinone to their cells immediately before freezing. This assures that their cells will be shrunken and unrepresentative, even if they were adequately frozen! This we conclude because we find that applying such extracellular cryoprotectants to cells before freezing them with our "slam-freezer" creates a zone of superb freezing that is dozens of microns thick; but we find no useful cell architecture remaining in this zone. Rather, subsequent freeze-fracture through any part of it shows that osmotic shrinkage prior to (or during) freezing has severely compacted the cytoplasm of the cells and grossly distorted their architecture.

One virtue of the "blotting & quenching" technique of freezing whole cells mounted on holey films is that it is one and the same as the technique traditionally used for freezing suspensions of molecules and for doing EM tomography or other forms of EM reconstruction on them, so many investigators are comfortable doing it and have learned how to do it successfully (4,5,35). However, we feel that there are several problems with this approach, even for freezing molecules, that have been "swept under the rug" since it became standard. For instance, we find that even individual molecules are subjected to severe osmotic stresses and air-drying at either (or both!) surfaces of the thin film in which they are "quenched". This creates all sorts of artifacts, ranging from mild tendencies toward preferential orientation and adhesion of amphipathic molecules at the air-water interface, to severe tendencies to tear molecules apart, due to the severe surface-tension forces operating at that interface. Indeed, our "first-cut" procedure of freeze-fracture intentionally scrapes away this air/water interface, in order to exclude regions of molecular architecture that are grossly distorted and misleading.

The above reasons are why we feel that the freezing of samples in preparation for EM tomography falls far short of our method of "slam-freezing" and produces samples of dubious quality. Indeed, we suspect that even "high-pressure" freezing itself will turn out to be prone to serious artifact, since the pressure is on for such a long time, and the freezing is relatively so slow, that severe changes are likely to occur (changes such as the complete destruction of DNA's helical signature in the nucleus; cf: 7), even if it does prevent the formation of grossly damaging ice-crystals. Furthermore, "high-pressure" freezing is much more difficult and dangerous (and requires absurdly expensive instruments) than freezing with our metal-block "Cryopress," and continues to be

BOX SUMMARY

EM Tomography

Step 1: Freezing:
Approach 1:
Thin cells or organelle/molecule suspensions are applied as droplets to holey grids, blotted down to thin film and "quenched" by plunging into a cryogenic liquid such as liquid ethane or liquid propane (-150° C)

Approach 2:
Sandwich the sample as a tiny droplet mounted in between two gold "hats" and insert it into a "high-pressure" freezer.
Interjection: Afterwards, remove frozen pellet from gold hats, mount on a "stub" for cryo-microtomy, and cut frozen thin sections with a cryo-ultramicrotome

Step 2: Mounting in the EM:
Mount EM grid in a "cryo-transfer" device and transfer it from the freezing-bath or cryomicrotome to the cold-stage of a cryo-EM

Step 3: Photograph the sample in the EM:
Obtain a whole series of images at different degrees of tilt and rotation using a cryo-tilt/rotation goniometer stage.

Maximize contrast of the images, (since the frozen samples are unstained), by automated underfocus of the microscope and the use of a field-emission electron gun to obtain a more coherent beam of electrons.

Minimize electron beam damage to the sample by recording images under low dose conditions.

Maximize clarity of the image by removing "inelastically", or multiply-scattered electrons, using a post-objective lens energy filter (these electrons become a serious problem, because cryo-samples are relatively *thick*).

Capture images with a high-resolution digital camera mounted inside the EM or fiber-optically coupled to it, since the numbers of images exceed a film-camera's capacity and film would be too expensive.

Step 4: Transform and align the resultant images:
Since the images of each field run into the hundreds, and are digital, this requires computer-based procedures that operate only on SGI workstations.

"Deep-etch" EM

Step 1: Freezing:
Big or small samples, and thick or thin samples, Are all mounted identically on a spongy cushion of fixed lung Sitting open on a on thin aluminum "planchette" (which will be their support for storage in LN2 and subsequent transfer to the freeze-etch apparatus) then driven hard with a "Cryopress" onto a pure block of copper cooled with liquid helium to 4 deg Kelvin.

Interjection: Before mounting in the EM, The frozen sample must be transferred to a Balzers freeze-etch apparatus, wherein it is freeze-fractured, "deep-etched" and platinum-replicated. Then the replica needs to be separated from the tissue, To be cleaned, and to be mounted on an EM grid.

Step 2: Mounting in the EM:
An EM grid with a replica on it is stable at any temperature And impervious to all atmospheric conditions, so it will last for decades and it requires no special transfer devices or cold stages in the EM.

Step 3: Photograph the sample in the EM:
Obtain just two images of each field of interest at something like +/- 10 degrees of tilt, in order to generate simple 3-D images using the simplest of side-entry goniometer stages

Because metal replicas naturally provide the ideal contrast for imaging in a standard TEM, they are imaged at true focus, not underfocussed. This makes them look relatively "soft" by eye, but provides the highest EM resolution.) Hence, replicas require *no* automated underfocussing and *no* use of a field-emission electron gun

Recording images under low-dose conditions is also completely unnecessary with platinum replicas, since they are totally thermo-stable, and therefore, not subject to any electron-beam damage.

Because platinum replicas are exceedingly thin (~2 nm) they generate very few "inelastically"-scattered electrons, hence their image-clarity does not need to be enhanced by using a post-objective lens energy filter.

Images of replicas can be captured with any device that is available: anything from standard EM film, which is still the most convenient (and still provides, by far the highest possible resolution) to the simplest or the fanciest digital camera that one might have.

Step 4: Transform and align the resultant images:
Since there were only two images made of each field, these are most easily made into stereo-pairs or into "anaglyphs" (two-colored 3-D images) "by hand," using the simple transformations and alignment-procedures built into Adobe Photoshop®.

BOX SUMMARY

EM Tomography	"Deep-etch" EM
Step 5. Construct the 3-D tomogram: This involves standard computer-based algorithms for "weighted" back-projection of the aligned images from Step 4.	**Step 5. Constructing a 3-D tomogram:** Tomograms are, of course, not needed for the imaging of replicas. (However, in a few recent studies 3-D reconstructions of replicas have been generated, specifically to determine the "inner envelope" of the replica, which should represent the true contours of the biological structures that lay beneath it.)
Step 6. View the final tomogram: By computer-generation of artificial, pseudo-"thin section" images of it are generated. (To actually see anything in these images, they first need to be "de-noised" by additional, highly complex computer algorithms.)	**Step 6. Viewing of "deep-etch" replicas.** This is done simply by direct visual inspection of the stereo-pairs or direct visual inspection of the "anaglyphs" generated in step 4. No "de-noising" of these images is necessary.
Step 7: "Segment" the tomogram: In preparation for "modeling" the tomographic volume, one views the pseudo-thin sections generated in Step 6 on an interactive computer screen and traces around whatever objects are of interest, thereby giving the computer information about where the things of interest are located.	**Step 7: "Selecting" objects from the above 3-D views of replicas.** This could also be done on an interactive computer screen, again, by tracing around interesting structures in the replicas; but it is generally unnecessary, since the locations and shapes of most structures in replicas are immediately apparent.
Step 8: Generate "models" of the objects of interest: Develop computer-algorithms that will generate surface-renderings or volume-renderings of the of the hand-tracings generated in step 7.	**Step 8: Generate "models" of the objects of interest:** Algorithms that generate surface-renderings or volume-renderings of any tracings made above could be used for interpreting replicas, as well; but replicas are already surface-renderings and have essentially no 3-D volume. So such model-building is generally unnecessary.
Step 9: Locate new structures in the tomographic volumes: By developing and applying complex, computer-based pattern-recognition algorithms to untraced tomographic data files, observers hope to resolve individual macromolecules *in situ*.	**Step 9: Locate new structures in the tomographic volumes:** Discerning individual macromolecules *in situ* can be done simply by *direct visual inspection* of the replicas. No special computer-manipulations are needed.
Step 10: Quantitative analysis: The "dream" of all EM approaches, to bring them from the realm of "stamp collecting" into the real scientific world, where data takes the form of something measured and quantitated. (As yet undeveloped.)	**Step 10: Quantitative analysis:** The dream of accomplishing this final step remains just as much a "pipe-dream" in the field of deep-etch EM, as it does in EM tomography
Total cost for equipment (underlined) ***~ $2.5 million dollars***	**Total cost for equipment (underlined)** ***~ $ 0.5 million dollars***
Time from live sample till obtaining final image ***~ 3 months***	**Time from live sample till obtaining final image** ***< 1 day***

more expensive to operate, due to its huge consumption of cryogens, compared to the modest amount of liquid helium or liquid nitrogen needed to operate our device.

It is important to point out here that one "hybrid" approach used by some EM tomography laboratories is to go ahead and use a "high-pressure" freezing device to freeze supramolecular samples such as prokaryotes or eukaryotic cells and tissues, and then "freeze-substitute" these samples by thawing them in acetone/OsO4, prior to embedding them in standard plastics in preparation for standard thin-sectioning (14,33,34,36). This "hybrid" procedure is relatively simple, and may well yield better cellular architecture than standard room-temperature chemical fixation and dehydration. However, is not a technique under consideration here, since the object of this review is to "critique" current EM tomographic efforts to locate and map molecules in whole cells frozen directly from life.... cells that have never been subjected to any kind of chemical fixation or dehydration. As discussed in the introduction to this essay, there is absolutely no hope of ever learning anything about the natural condition of molecules *in situ*, by observing chemically-fixed cells. In comparison, EM tomography of "freeze-substituted" cells will be useful only for determining the overall shapes and relationships of cellular organelles, not for finer-scale analyses of molecular relationships.

On a practical note, we would add that the freezing techniques currently used for EM tomography are so cumbersome, and require the investment of so much more time for the ancillary techniques of preparing the "holey" thin films and/or doing cryo-microtomy on frozen cells, that they yield far fewer samples than our standard "deep-etch" EM techniques. Typically, only 15-30 min is needed for our routine of quick-freezing, freeze-fracture, deep-etching and platinum-replication. In comparison, sample preparation for EM tomography is so difficult that only a handful of images of whole cells have *ever* been produced by this approach - one can count the number of papers on two hands, while our own "deep-etch" EM lab, alone, has produced 10 times that number of papers on hundreds of different sorts of quick-frozen cells and molecules. Furthermore, there has also been a rich output of "deep-etch" EM images from other laboratories around the world that use roughly the same approaches as we do, and typically use our freezing machine (and often, indeed, have been trained by us). Compared with this outpouring, the information-content of the few EM tomograms of cells obtained by EM tomography seems very meager, indeed.

(See here the attached 3-D "deep-etch" EM images, presented in "anaglyph" stereo views, of the Archaebacterium *Haloferax volcanii*. This test-sample was kindly provided by Joe Lutkenhaus of the University of Kansas, in order to allow us to compare our procedures with various tomographic EM's of other Archaebacteria.)

THE SECOND PROBLEM WITH EM TOMOGRAPHY: HAVING TO "SHOOT BLINDLY" IN THE EM

The second fundamental problem with EM tomography, besides its sub-optimal start due to its use of dubious freezing techniques, is that it is ultimately and fundamentally a totally "blind" procedure - one that does not permit direct observation of the field of interest while it is being imaged. This is because samples that are kept frozen in the EM are so prone to electron beam damage that they can only be imaged by "low dose" techniques. That is, only regions in the cell that are adjacent to the regions that are viewed directly can ever be converted into EM tomograms. To understand this, one must realize that "low dose" EM involves automatically dimming and deflecting the electron beam from regions where focusing is done, over to the actual regions that are to be imaged for the tomograms. Any longer imaging would "fry" these regions completely; they can never be viewed directly. Thus, the microscopist can only hope that the same structures or the same molecular architecture will be present in them, as in the region of focus; but there is no assurance of this, nor is there any way to be sure about it until the entire laborious process of EM tomography has been completed, days or weeks later. This is roughly equivalent to asking Ansel Adams to produce his beautiful photos of Yosemite Falls by standing with his back to the Valley at midnight, suddenly wheeling his heavy box camera around roughly 180 degrees in the dark, and popping a flashbulb at his intended subject. Had he followed this approach, the likelihood that his photos would have come out as beautiful as they were (let alone be correctly framed, focused, and exposed) would have been very small, indeed.

Furthermore, since cryo-EM tomography offers no special advantages at all, if it does not avoid the more usual techniques of fixation and staining of cells by metal-impregnation, the samples it uses for imaging have essentially zero contrast, so nothing is visible to the eye when they are viewed in the EM anyway. In fact, the "low-dose" method of focusing on regions adjacent to the region-of-interest, described above, is generally not done by direct visual inspection, but rather, by observing electron diffraction-patterns from the focusable regions. This permits correct generation of the substantial degree of underfocus needed to create enough phase-effects in the cryo-EM, which pulls a little bit of contrast out of the unfixed and unstained samples.

What renders all of these 'blind' or "shot-in-the dark" techniques of microscopy so problematic in practical daily use, (and here we would fault atomic force microscopy as well, which provides no preliminary images before an area must be blindly scraped or tapped to death, and a computer image of its surface is generated some many minutes later), is that they totally subvert the microscopist's fundamental role of being

'an explorer of inner space'. He or she is no longer able to search for a particular region of interest in a cell and choose an area to photograph, nor can he or she search for a particularly well-frozen and well-spread field of molecules to photograph - two critical activities of the true explorer! Lacking this, EM becomes instead a deadly "hit or miss" affair, with little or no direct intellectual or aesthetic feedback - a really dreary activity, in our opinion. In fact, we do not consider EM tomography to really be microscopy at all, in the fundamental sense of the word. Rather, it is a sort of remote-controlled, computer operation of a horrendously expensive "spy camera" (the cryo-EM), which is pointed almost randomly at any scene within its range. In this sense, EM becomes a battle against the mind's eye, rather than an extension of it.

THE THIRD PROBLEM WITH EM TOMOGRAPHY: ITS DEPENDENCE ON COMPUTERS FOR IMAGE-RECOGNITION

The fundamental goal of studying whole cells with the EM is, of course, to image their internal molecular machines *in situ*. This first requires that one is able to find the macromolecules in properly prepared cells. This locating-activity is commonly glorified by the term "mapping the distribution of the molecules" within the cell. Of course, to be able to find molecules within a cell, one must first be able to recognize them: to pick them out from the background of everything else that is crowded around them. This boils down to the old problem of finding the "needle in a haystack." Of course, the human act of recognition, in general, basically involves scanning a new scene for objects or patterns that match one's preconceived mental image of whatever one is seeking. One must have a clear preconceived notion - a clear "mental image" of the needle - if one is ever going to pick it out within a confusing tangle of straws of hay.

Unfortunately, current EM-tomography has not succeeded in creating images wherein this simple act of human recognition can operate. The tomographs of whole cells obtained to date are simply incomprehensible, due to their thickness and due to the horrendous mount of molecular-overlap they manifest. For instance, if the molecular complex or small membranous organelle one wished to see is, say, 12.5 nm in size - a typical dimension for one of the many different types of molecular "machines" that are expected to exist in the living cell - there could be as many as 20 of them piled on top of each other in the typical quarter-micron thickness imaged by tomography! And worse still, the molecule of interest could exist in only one or a few copies, lost somewhere amongst 20 other sorts of molecules piled on top of each other in that quarter-micron thickness. Furthermore, in addition to suffering from too much overlap of structure, current EM tomograms are too translucent, too noisy, and too low in contrast to permit direct visual inspection.

Being thus unable to perform the simple act of human recognition on their 3-D tomograms, the practitioners of this technique have turned their entire attention toward the development of computer-based "object-identification" or "pattern-recognition" algorithms (2,3,12,39,40). These are being designed to operate much like human perception, in that they involve a comparison (or in computer jargon, a "cross-correlation") between previously generated computer-images of purified molecules (analogous to a human being's mental image of the needle) and various features in the computed EM tomogram (say, all of the particular features that look like thin straws of hay).

The computer generated images of pure molecules are called "templates" in computer-ese; and since one must assume that molecules *in situ* are likely to be randomly oriented, one must repeatedly rotate these "templates" and recalculate the "cross-correlation coefficients" for each rotation. This is horrendously expensive and time-consuming, in terms of computer operation. The Martinsried group predicts that 2,000 days of operation of a SGI R10000 processor would be needed to search for one type of molecule in one quarter-micron cube of tomographically-reconstructed cytoplasmic volume. To look for a handful of different types of molecules, as would be need to determine their interrelationships (or at least to determine their relative positions in that volume) would take, on such a computer, they admit, many centuries! To reduce their computing time to a reasonable level, this group contracted with the Research Center of the city of Garching to use their Cray T3E-600 supercomputer - 784 processors working in parallel - to search a one tenth-micron square tomographic reconstruction; and after a week or so of continuous processing, they achieved what they concluded was a "satisfactory" level of detection (>90 % detection of one sort of molecule within that volume, the volume itself being represented by a relatively gargantuan ~2GB file).

CONCLUSION

To this observer, at least, it therefore seems useful to continue to use EM-imaging techniques that are not wholly dependent on present or future sorts of computer-based "feature-extraction" algorithms, but instead sustain the opportunity for a living human being to determine the *in situ* spatial relationships of many different sorts of molecules, in many different orientations, by direct visual inspection, using his or her personal mental images of what the different molecules should look like - those images having been acquired "in his or her mind's eye" by previously imaging and recognizing different sorts of purified molecules prepared in exactly the same way and observed in exactly the same way; namely, also by direct visual inspection.

In any case, the ultimate goal remains the same for all elec-

tron microscopists: to learn what molecules look like *in situ*, what structural changes they undergo as they function *in situ*, and what functionally relevant interactions and associations they establish with each other (e.g., what sort of "architecture" they produce in the cytoplasm), all while inside the living cell.

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