Towards high-resolution three-dimensional imaging of native mammalian tissue: Electron tomography of frozen-hydrated rat liver sections

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Abstract

Cryo-electron tomography of frozen-hydrated specimens holds considerable promise for high-resolution three-dimensional imaging of organelles and macromolecular complexes in their native cellular environment. While the technique has been successfully used with small, plunge-frozen cells and organelles, application to bulk mammalian tissue has proven to be difficult. We report progress with cryo-electron tomography of frozen-hydrated sections of rat liver prepared by high-pressure freezing and cryo-ultramicrotomy. Improvements include identification of suitable grids for mounting sections for tomography, reduction of surface artifacts on the sections, improved image quality by the use of energy filtering, and more rapid tissue excision using a biopsy needle. Tomographic reconstructions of frozen-hydrated liver sections reveal the native structure of such cellular components as mitochondria, endoplasmic reticulum, and ribosomes, without the selective attenuation or enhancement of ultrastructural details associated with the osmication and post-staining used with freeze-substitution.

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1. Introduction and background

A major challenge for structural biology is the understanding of macromolecular interactions in their cellular context. In order to achieve this, the cell must be imaged in three dimensions in its native hydrated state and at sufficient resolution (3–10 nm) so that macromolecular complexes may be recognized. At present, cryo-electron tomography is the only available technique for this task. Considerable success with in situ identification of macromolecular complexes has been obtained with plunge-frozen specimens such as liposomes containing large protein complexes, isolated organelles, and small cells (Förster et al., 2005; Frangakis and Förster, 2004; Grünewald et al., 2002; Medalia et al., 2002; Rath et al., 2003; Wagenknecht et al., 2002). Larger cells and bulk tissue present a problem, since specimen thickness needs to be limited to 100–200 nm to achieve resolution suitable for macromolecular identification by tomography. Thicker specimens are useful for understanding long-range 3-D cellular organization; however, even using zero-loss energy filtering, and high (300–400 kV) accelerating voltage, specimen thickness is limited to about 1 μm, because the electron dose needed to provide a sufficient number of singly elastically scattered electrons for phase-contrast imaging becomes excessive. As a result,
2. Methods

2.1. High-pressure freezing

Tissue was prepared by high-pressure freezing (Moor, 1987; Shimoni and Müller, 1998; Studer et al., 1989), the method of choice for specimens in the 100- to 300-nm thickness range. Both EM-PACT (Leica, Vienna, Austria; Studer et al., 2001) and HPM 010 (Bal-Tec, Balzers, Liechtenstein) high-pressure freezers were used, with equally good results. The sample carrier used for the HPM 010 was a 3-mm aluminum platelet with 2-mm cavity diameter, 0.3-mm depth, and 0.5-mm total thickness. The sample carrier for the EM-PACT was a flat platelet 3 mm in diameter and 0.6 mm in thickness, with a central slot 0.3 mm wide and 1.2 mm long.

Specimens of liver tissue were obtained from white Sprague-Dawley rats shortly after sacrificing. Tissue to be freeze-substituted was excised and diced in Ringer’s buffer, then placed in a specimen carrier for high-pressure freezing that had been pre-filled with 1-hexadecene. The first specimen was frozen about 1 min after excision. Early specimens used for cryo-ultramicrotomy were prepared in the same way, but the best results were obtained by needle biopsy using kits from Leica (Vanhecke et al., 2003) and Bal-Tec (Hohenberg et al., 1996). When using needle biopsy, tissue could be transferred to the specimen carrier and frozen within 40 s of cessation of blood flow. For the recent work with frozen-hydrated sections, the free space in the specimen carrier was filled with 20% dextran (Sartori et al., 1993) in mammalian Ringer’s solution. We found that the dextran-containing buffer provided superior results with frozen-hydrated sections.

2.2. Freeze-substitution and preparation of plastic sections

For comparison with frozen-hydrated sections, we avoided specialized freeze-substitution procedures, and instead employed a simple, straightforward procedure (Hohenberg et al., 1994; Van Harreveld and Crowell, 1964). Tissue was removed from the high-pressure freezing carriers under liquid nitrogen and was freeze-substituted in 2% OsO4 in acetone as follows: 8 h at −90 °C, 8 h at −60 °C, 8 h at −30 °C, and 1 h at 0 °C. This was followed by three 15-min rinses in acetone at 0 °C, followed by infiltration of graded Epon-Araldite at 30, 70, and 100%, at 4 °C, 12 h for each step. Polymerization was at 60 °C. The procedure was carried out with a Bal-Tec FSU 010 automated freeze-substitution device.

Plastic sections were cut at 80- to 250-nm thickness with an Ultracut-S ultramicrotome (Reichert, Vienna, Austria) and a 45° diamond knife (Diatome, Biel, Switzerland). The sections were picked up on 200-mesh copper grids coated with a 60-nm-thick Formvar film and a 10-nm-thick carbon film, to which a suspension of 10-nm colloidal gold particles was applied to serve as alignment fiducial markers for tomographic tilt series. Sections were stained with 2% aque-
ous uranyl acetate for 4–10 min and Reynolds lead citrate for 2–5 min, both at room temperature.

2.3. Cryo-ultramicrotomy

The method used for routine sectioning is very similar to that described earlier (Hsieh et al., 2002). Frozen-hydrated sections are cut with a UCT ultramicrotome with an EM-FCS cryo kit (Leica, Vienna, Austria). Within the cryo-ultramicrotome chamber, the two halves of the high-pressure freezing specimen carrier are separated, exposing the tissue. The carrier is clamped, on edge, directly in a frosted microscope slide. A 45° trimming diamond knife (Diatome) is used to cut away portions of the carrier metal, and to trim the block face to approximately 100 × 100 μm for sectioning. If the block face does not appear perfectly smooth (indicating reasonably good freezing), the block is trimmed more deeply, or discarded. Sections are cut at −150 or −160 °C with a microtome feed setting of 40–200 nm, with a 35° or 25° “dry” cryo-diamond knife (Diatome). The knife clearance angle is 6°, the cutting speed 0.2–0.6 mm/s, and the return cycle 30 mm/s. An adjustable Static Line Ionizer II (Haug, Biel, Switzerland) is located 30 mm from the knife, and is used only during sectioning. We avoided high cutting speeds, recently recommended for reduction of chatter (Al-Amoudi et al., 2005), because of rapid knife damage when cutting thick sections.

2.4. Mounting of frozen-hydrated sections for tomography

Two types of copper specimen grids are used. For long-term storage in liquid nitrogen, 200-mesh hexagonal folding grids are used, as previously described (Hsieh et al., 2002). In our more current practice, when sections are examined within a week of microtomy, sections are collected on 200-mesh R3.5/1 Quantifoil grids (Quantifoil Microtools, Jena, Germany). The grids are coated with a 10-nm-thick continuous carbon film, and a mixture of 10- and 20-nm colloidal gold particles is then applied to the carbon film.

A short ribbon consisting of about eight sections is cut, and is left attached to the knife edge. The knife is then moved laterally, and another ribbon is cut. After a number of sections or ribbons have accumulated on the knife edge, the static ionizer is turned off, and the sections are transferred to the grids using a white hair from a Dalmatian dog (Forbes, 1986). This type of hair resists breakage at low temperature.

To facilitate collection of the sections on grids, we fabricated a polished metal shelf that is installed just adjacent to, and at the same level as, the knife edge. Freshly glow-discharged grids, prepared as described above, are placed on a fresh strip of 0.127-mm-thick indium foil (Alfa Inorganics, Beverly; Buchanan et al., 1993). Sections are placed as close as possible to the center of the grid, and are also centered on the grid squares, so as to maximize the tilt range during tomography. About four ribbons of sections are collected on each grid.

The grids are then pressed into the soft indium foil using the glass screw-press tool supplied with the microtome. During this process, the indium foil comes in contact with the back side of the Quantifoil support film, and supports the film as the sections are pressed down. The screw-press tool is preferred over the polished metal rod press tool, also supplied with the microtome, because section damage and frost accumulation is less. The grids are then placed in carriers and transferred to the cryo-transfer specimen holder workstation, or to storage under liquid nitrogen. If grids are stored, rather than used on the same day as cut, the grids are pressed again in the microtome cryo-chamber before transfer to the specimen holder workstation.

2.5. Electron tomography

Images were recorded at 400 kV acceleration voltage using a JEM-4000FX electron microscope (JEOL, Tokyo, Japan) equipped with a GIF2002 imaging energy filter (Gatan, Pleasanton, CA). The energy filter is equipped with a 2048 × 2048-pixel CCD camera that is fiber-optically coupled to a high-sensitivity phosphor scintillator. The CCD element size is 30 μm, and all images are binned to 1024 × 1024 pixels, thereby doubling the effective CCD pixel size and compensating for the point-spread function of the thick scintillator. The image pixel size relative to the specimen was either 1.8 or 1.0 nm. All images were recorded using zero-loss filtering with a 15-eV slit. The objective aperture angle was 10 mrad.

All tilt series were recorded semi-automatically at a uniform 1° or 2° interval from −60 to +60°, using software written in-house (M. Marko and A. Leith, unpublished). The software, which is controlled by a webpage user interface, integrates an Emispec ESVision imaging system (FEI, Tempe, AZ) and the JEOL stage- and EM-control computers, as well as the Gatan Digital Micrograph software. In addition to controlling the EM, the stage, and the CCD camera of the energy filter, the software automatically adjusts the energy offset to keep the zero-loss peak centered in the slit during the tilt series. The acquisition software also integrates a FastScan 114 frame-transfer CCD camera (TVIPS, Gauting, Germany). This camera is mounted next to the energy filter and is used for reduced-magnification re-focusing and tracking after each tilt. These operations are done manually and on-axis, at an electron dose 1/100th of the exposure dose. The FastScan camera is also used for low-dose surveying of the specimen and for recording of electron diffraction patterns to verify the state of the ice.

Images from frozen-hydrated sections were recorded at 15 μm underfocus, which corresponds to a spacing of 5 nm at the first minimum of the contrast transfer function. The average electron dose per image was scaled down from 0.7 e−/Å² for a 2°-interval tilt series to 0.35 e−/Å² for a 1° interval, in order to keep the total dose to about 50 e−/Å².
Images from plastic sections were recorded at 1 μm under-focus and a dose of 10 e⁻/Å² per image. For a 1°-interval tilt series, the total electron dose was 1300 e⁻/Å², which included pre-irradiation to stabilize the section.

A cryo-transfer specimen holder (Model 626; Gatan, Warrington, PA) was used for both types of specimen, but the plastic sections were imaged at room temperature, while the frozen-hydrated sections were imaged at −176 to −179 °C. In both cases, dual liquid-nitrogen anticontaminators were used.

Alignment of the tilt series images was done using fiducial markers (Penczek et al., 1995), and the 3-D reconstructions were made by weighted back-projection using SPIDER (Frank et al., 1996). In single-tilt reconstructions, resolution varies along the three axes, as a function of the image sampling size and microscope defocus (y-axis), the specimen thickness and number of projections (x-axis; Crowther et al., 1970), and the maximum tilt angle (z-axis; Radermacher and Hoppe, 1980). In the current reconstructions, the predicted resolution limits were, typically, 5 nm along the tilt axis direction (y), 10–12 nm (depending on section thickness) along the x-axis, and 15–18 nm along the z-axis.

The thickness (t) of frozen-hydrated sections was measured both directly from the tomograms and by electron energy-loss imaging, using the relationship \( t/\lambda = \ln(I/I_0) \), where \( \lambda \) is the mean-free path of 400 keV electrons in vitreous ice containing biological material, \( I_0 \) was taken as the mean gain-normalized CCD counts with the 15 eV slit in, and \( I \) the same with the slit out. The value for \( \lambda \) was determined to be 380 nm, based on comparison with thickness determined from tomograms (results to be reported elsewhere).

3. Results

3.1. Contrast gain from zero-loss energy-filtered imaging

The important advantage provided by energy filtration when imaging sections of unstained, frozen-hydrated tissue is demonstrated in Fig. 1 for a 220-nm-thick liver section. A zero-loss filtered image and an unfiltered image from the same field, recorded using the same incident electron dose, 1 e⁻/Å², are presented in Figs. 1A and C, respectively. Line scans in identical locations on both images indicate that the zero-loss image has about half the background intensity as the unfiltered image, while retaining similar signal intensity. For example, the mean signal-to-background ratio along the indicated line was 0.41 in the filtered image and 0.24 in the unfiltered image, representing an increase by a factor of 1.7. Another measure of the contrast gain can be made by comparing the relative intensities of large, uniformly textured areas of differing density in the two images, using the relationship \( C = (I_2 - I_1)/I_1 \), where \( C \) is contrast and \( I_1 \) and \( I_2 \) are the mean CCD counts within boxes at the two areas. In the example, the contrast measured between the cytoplasm and the mitochondrial matrix in the filtered image is \( C_f = 0.091 \), and in the unfiltered image, \( C_u = 0.030 \). The contrast increase is \( C_f/C_u = 3 \).

Note that these methods of contrast and signal-to-background estimation depend on the inherent contrast of the specimen features, as well imaging conditions such as the defocus setting. These results are consistent with an improvement in contrast, demonstrated using cross-correlation methods, using a variety of specimens (although not including frozen-hydrated specimens) imaged at 120 kV (Grimm et al., 1998).

Fig. 1. Contrast gain provided by zero-loss energy filtering when imaging a 220-nm-thick frozen-hydrated section of rat liver. Comparison of filtered (A) and unfiltered (C) images taken at the same incident dose, 1 e⁻/Å². Mean CCD counts are 1127 for the filtered image and 1966 for the unfiltered image. Boxes show regions of the mitochondrial matrix (M) and cytoplasm used to measure contrast gain, as described in the text. Intensity profiles (B and D) across mitochondrial inner and outer membranes are along the 30-pixel (= 30 nm) lines indicated in (A) and (C), respectively. Bar = 100 nm.
3.2. Correlation between crevasse formation and quality of high-pressure freezing

Images of typical thick sections of frozen-hydrated rat liver, recorded at 400 kV with zero-loss energy filtering, are presented in Figs. 2 and 3. Knife marks, which indicate the cutting direction, extend diagonally from lower left to upper right in Figs. 2 and 3C, and from upper left to lower right in Fig. 3A. The images show considerable variability in the extent of crevasse formation. Crevasses are seen as narrow grooves, about 20 nm wide and 50–200 nm apart, running normal to the cutting direction. Crevasses, when they occur, may be reduced by cutting thinner sections, and the depth of crevasses may be decreased by high (50–100 mm/sec) cutting speeds, although this increases the number of crevasses (Al-Amoudi et al., 2005). The sections in Figs. 2 and 3C are nearly free of crevasses, and the mitochondria and endoplasmic reticulum (ER) are easily recognized. In contrast, the crevasses are so severe in Fig. 3A that those organelles are barely discernible. Using tomographic analysis, we previously showed that crevasses typically extend only 20–50 nm into the section, and only from the surface that was the block face before the section was cut (Hsieh et al., 2002). However, significant crevasse formation can impair the ability of the microscopist to select regions of interest in sections for tomographic analysis.

We have found that the severity of crevasse formation tends to vary with the quality of tissue preservation. When tissue is not optimally preserved, as characterized primarily by scalloped membranes (Fig. 4), sections display moderate to severe crevasses and are more difficult to cut in the cryo-ultramicrotome. Conversely, sections that show excellent ultrastructural preservation are generally easier to cut, and tend to have very few crevasses (e.g., Figs. 2 and 3C), even when sections 200 nm or thicker are cut. The tissue shown in Fig. 4 was prepared using our previous technique, mincing followed by freezing in 1-hexadecene, while all the other examples of frozen-hydrated sections are from material prepared using needle biopsy followed by freezing in 20% dextran. Although crevasse-free sections are more often obtained using the latter technique, severe crevasses can still occasionally be observed (Fig. 3A).

**Fig. 2.** Projection image of a 200-nm-thick frozen-hydrated section of rat liver tissue. Note the smooth membranes and the ribosomes studding the endoplasmic reticulum (arrowheads). Electron dense beads in this and the following figures are colloidal gold particles used as fiducial markers for alignment of the tomographic tilt series. Bar = 200 nm.

**Fig. 3.** Images and electron diffraction patterns of frozen-hydrated sections of rat liver. (A) 180-nm-thick section marked by numerous crevasses (examples at arrows). (B) Electron diffraction pattern corresponding to (A). (C) Much smoother section, with the same thickness as in (A). (D) Electron diffraction pattern from the specimen in (C). (E) Electron diffraction pattern from a 100-nm-thick film of plunge-frozen pure water. Diffuse bands at spacings of 0.214 and 0.370 nm correspond to vitreous ice. Bars = 200 nm.
It is known that smooth sections are not obtained from frozen-hydrated specimens containing crystalline ice (e.g., Chang et al., 1983; Erk et al., 1998; Frederik et al., 1984). Specimens that cannot be completely vitri\textit{W}ed by high-pressure freezing contain ice microcrystals that range in size from 2 to 30 nm (Echlin, 1992; Michel et al., 1991; Moor, 1987; Sartori et al., 1993). Therefore, it is reasonable to consider that crevasse formation might be due to sub-optimal freezing, which would also be reflected in poor ultrastructural preservation. To test this hypothesis, we used electron diffraction to characterize the state of the ice in frozen-hydrated liver sections that have drastically different degrees of crevasse formation, as shown in Figs. 3A and B. Both sections were found to contain predominantly vitreous ice, as indicated by the presence of two diffuse bands in the patterns (Figs. 3B and D) at spacings characteristic of vitreous ice (Dubochet et al., 1988). For comparison, the diffraction pattern of a 100-nm-thick layer of plunge-frozen pure water is shown in Fig. 3E. Thus, we were not able to correlate severe crevasses in the liver sections with a microcrystalline ice content that is high enough to be detected as sharp rings or spots in the diffraction patterns.

### 3.3. Attachment of frozen-hydrated sections to EM grids

Another important characteristic of frozen-hydrated sections, from the perspective of tomographic data collection, is that they generally are not flat (e.g., Richter et al., 1991). The sections may show a complicated curvature, often with several undulations roughly along the cutting direction. Cross-sections through three different tomo\textit{gr}ams of frozen-hydrated rat liver, as shown in Fig. 5, illustrate the problem caused by lack of section flatness. Due to section curvature, the areas of contact between the section and the support film are few, and in many places there is a gap between the two. This defect has two consequences: poor attachment of the section to the grid, causing instability in the electron beam during tilt-series collection (see Fig. 5 in Frank et al., 2002), and reduced alignment accuracy, due to the large distance between the section and the carbon film which carries the colloidal gold particles that are used as fiducial markers. In the latter case, residual errors in alignment of the tilt series, present in the plane of..
the gold particles, are magnified in the section when it is some distance from the plane of the markers. In an attempt to improve section flatness and induce attachment, we routinely press sections onto grids with a glass section press (see Section 2) or a metal rod (Frederik et al., 1982; McDowell et al., 1983). While these procedures may flatten the sections to some degree, results are not consistent. Also, extensive pressing of the sections may cause damage, such as deep vertical fractures sometimes observed in tomograms of thicker sections, usually along membrane surfaces. We find that, in general, thin sections attach better to the grid after pressing than do sections thicker than 150 nm.

We find that Quantifoil grids offer several advantages for electron tomography of frozen-hydrated sections, compared to the folding grids that we previously recommended (Hsieh et al., 2002). The local attachment of the section to the support film may be easily assessed at low magnification by observing the amount of movement of the specimen relative to the array of holes as the stage is tilted. We routinely record low-magnification on-axis images at 0° and 30° tilt. If there is excessive displacement of the specimen relative to the Quantifoil film, a tilt series is not usually attempted. Since the thick holey carbon provides the main specimen support, only a thin (10 nm) carbon coat is needed for carrying the colloidal gold particles over the holes. Indeed, for the highest-resolution work, tilt series may be collected from sections over uncoated holes, relying on gold particles on the edge of the hole, or a markerless alignment method. The array of regular holes makes it easy to re-locate the specimen after in-plane rotation for collection of double-tilt series. Since the grids are single-thickness, tilt series may be collected closer to the grid bars without occlusion at high tilt. The disadvantage of single grids such as Quantifoil over double (folding) grids is that the sections tend to detach during long-term storage in liquid nitrogen, and they are more likely to accumulate frost during handling since they cannot safely be wrapped in indium foil. However, the advantages far outweigh the potential loss of sections for experiments that are not limited by specimen availability.

3.4. Tomographic reconstruction of liver: morphology of mitochondrial cristae

A tomographic reconstruction of a small field from a frozen-hydrated rat liver section is presented in Fig. 4. Crevasses in this case were moderate (Fig. 4A) and structural details in the field were easily recognized (unlike Fig. 3A). A slice from the tomogram (Fig. 4B) reveals that the tissue in the section has undergone noticeable compression in the cutting direction. Compression is a common characteristic of frozen-hydrated sections prepared by cryo-ultramicrotomy. The compression of the specimen of Fig. 4 is readily apparent in spacings between mitochondrial outer and inner membranes. The distance between these membranes is generally smaller when the membranes are aligned normal to the cutting direction (arrowhead in Fig. 4B; also visible in Fig. 3C). In a few places, membranes display local step-like dislocations (Figs. 4C and D; see also Matias et al., 2003). Fortunately, such severe membrane defects tend to be localized, and occur at low frequency in well-frozen tissue.

The overall structural preservation of the tissue in Fig. 4 was good enough to warrant closer examination of the 3-D morphology of the membranes in the mitochondrion that occupies most of the field. The infoldings of the mitochondrial inner membrane are called cristae, which until recently were represented as random baffle-like folds in the inner membrane. However, tomographic reconstructions of plastic-embedded specimens, first of rat liver mitochondria (Mannella et al., 1994, 1997; Penczek et al., 1995), and later of a wide variety of mitochondria (reviewed in Frey and Mannella, 2000), have conclusively shown that the cristae are normally connected by narrow openings to the peripheral region of the inner membrane. This structural principle was later confirmed for intact, plunge-frozen mitochondria isolated from rat liver (Mannella et al., 2001), as well as from fungi (Nicastro et al., 2000). Close examination of several cristae in the tomographic reconstruction of Fig. 4, as well as examples from a tomogram of another sample of liver tissue, confirms that the cristae in mitochondria in frozen-hydrated liver have the same circular or small slit-like openings (Fig. 6).

3.5. Comparison of frozen-hydrated and freeze-substituted rat liver tissue

It is informative to compare “typical best” images of high-pressure frozen rat liver in frozen-hydrated sections, and in plastic sections following freeze-substitution. The latter is generally considered to be the best technique widely available for preserving tissue in a close-to-native state for electron microscopic imaging (Dahl and Staehelin, 1989; Giddings, 2003; McDonald, 1994, 1999; McDonald and Müller-Reichert, 2002; Studer et al., 1989).

In general, images of frozen-hydrated and freeze-substituted liver sections present comparable views of overall cellular architecture, in terms of the morphology and distribution of components such as mitochondria, ER, ribosomes, and nuclear pores (Figs. 7 and 8). In well-frozen material, the membranes of cells and organelles are smooth, not scalloped, in both frozen-hydrated and freeze-substituted tissue. In the uncompressed direction, the center-to-center spacings between the peripheral (outer and inner) membranes and adjacent internal (cristae) membranes of mitochondria in the frozen-hydrated tissue fall in the range of 7–12 nm. These membrane spacings are the same or slightly wider than those observed in freeze-substituted tissue. Note that the membranes in the latter specimens tend to be negatively stained, appearing white against a dark background. While negatively stained membranes are not always found in high-pressure frozen, freeze-substituted material (e.g., Giddings, 2003), it is believed that this appearance indicates optimal preservation. With optimal
freezing, the membrane proteins are intact, protecting the polar head-groups, and forming a barrier to osmium during freeze-substitution, such that subsequent post-stains are not taken up. While with freeze-substitution using 2% osmium in acetone, this optimal preservation is only rarely observed (M. Mueller, personal communication), recent work (Matsko and Mueller, 2005) has shown that specimens stabilized by freeze-substitution in a mixture of epon/araldite in acetone reliably exhibit excellent preservation, with negative staining of membranes. However, this negative staining of membranes in freeze-substituted specimens tends to make the membranes less distinct, especially

Fig. 6. Morphology of crista openings in mitochondrial inner membranes. (A, D, and G) 1.8-nm-thick slices from tomograms of frozen-hydrated sections of rat liver showing the attachment of cristae to the inner boundary membrane. (B, C, E, F, H, and I) Surface renderings of the cristae showing the inner membrane junctions. In each case, the opening is a small circle or slit. (A–C) and (D–F) are from the tomogram of Fig. 4; (G–I) is from a tomogram of another sample. Bar = 50 nm.

Fig. 7. Comparison of projection images of frozen-hydrated (A and C) and freeze-substituted (B and D) rat liver sections. Section thickness in both cases is 100 nm. (A and B) Fields containing mitochondria and adjacent rough endoplasmic reticulum. Note in (B) that membranes are negatively stained, while the mitochondrial matrix and ribosomes are strongly positively stained. Arrowheads indicate individual examples of ribosomes lining the endoplasmic reticulum. (C and D) Fields that each contain the edge of a nucleus (lower right) and adjacent cytoplasm. Arrows indicate nuclear pores. In the nucleus in (C), note areas of heterochromatin outlined by dots, while in (D) heterochromatin (+) is heavily stained. The white patches in the cytoplasm in (D) are unstained glycogen deposits. Bars (A and B) = 100 nm; (C and D) = 150 nm.
against a dense background. In this case, the crista membranes in images of frozen-hydrated liver sections are more visible against the mitochondrial matrix than those in the freeze-substituted sections.

The osmication and post-staining of proteins and nucleic acids in the freeze-substituted tissue strongly contrast the mitochondrial matrix and the heterochromatin, while clusters of glycogen are visible as stain-excluding plaques in the cytosol. Glycogen granules cannot be unambiguously identified in the frozen-hydrated tissue, suggesting the occurrence of contrast matching with the cytosol. In the frozen-hydrated tissue, the density of the mitochondrial matrix is similar to that in plunge-frozen isolated rat liver mitochondria (Mannella et al., 2001). Heterochromatin, obvious in freeze-substituted cells, is seen in frozen-hydrated cells as regions of comparable size and distribution that appear with considerably less contrast relative to the rest of the nucleoplasm. Ribosomes are clearly visible in both cases.

It should be noted that there were differences in the first steps of specimen preparation for the tissue specimens that are compared here. These differences, in tissue excision and choice of filler for high-pressure freezing, are described in Section 2. They have no bearing on the issues discussed above, which relate to contrast differences caused by osmication and post-staining of the freeze-substituted specimens. There is another apparent difference between the frozen-hydrated and freeze-substituted sections of rat liver in Fig. 7, namely a somewhat greater dilation of ER cisternae in the frozen-hydrated tissue. This dilation was observed in both types of specimens to varying degrees, and likely reflects the degree of anoxia to which the different tissue specimens were subjected prior to freezing. This physiological effect may be preventable by keeping the animals alive (under anesthesia) during tissue excision (e.g., by needle biopsy), so that organs are perfused right up to excision. It is, however, worth noting that there were no indications of major cellular pathology, such as severe mitochondrial swelling, in these specimens.

4. Discussion

Considerable progress is being made in cryo-electron tomographic imaging of soft mammalian tissue such as liver. The first critical step is routine preparation of suitable sections. Success, as noted above, appears to partially depend on the quality of freezing. For sections greater than 150 nm in thickness, crevasses (when they occur) and knife marks are confined to the surface and are not major problems for analyzing the interior of the sections. The second critical step is attachment of the sections to the grid, and improvements in this area are the focus of ongoing development efforts.

4.1. Section attachment

Lack of section flatness, resulting in poor attachment of sections to the support film, constitutes the single most important obstacle to successful tomographic data collection with these specimens. We have imaged hundreds of frozen-hydrated liver tissue sections, and have thus far collected 15 tomographic tilt series. In general, more than 50%
of the sections may be successfully imaged in the electron microscope. However, fewer than 10% are suitable for electron tomography. Most of the sections are unsuitable because of an unacceptable gap between the support film and the section, or because of instability on electron irradiation. Use of Quantifoil grids aids in rapid screening for regions of good section attachment. Some sections are unusable for tomography for other reasons, such as poor distribution of gold particles, limited tilt range due to unsuitable location of the area of interest on the grid, or sub-optimal ultrastructural preservation.

Solutions to the attachment problem, such as surface modification of supporting films and the design of new types of grids, are being explored. Vonk (2000) reported that wrinkles in the carbon support film at low temperatures can be avoided through the use of molybdenum rather than copper grids, and we have preliminary evidence that this alternative helps with attachment of frozen-hydrated sections. In limited tests with molybdenum Quantifoil grids, using sections of cyanobacteria (Ting et al., 2005), we find that there are more areas of good attachment when the carbon film is flat, and that the increased stiffness of molybdenum grids compared to copper grids helps prevent distortion of the support film during handling and when pressing the sections onto the grid.

4.2. Section compression

Section compression lowers the fidelity of representation of the native state of the tissue. The degree of compression that we observed in sections of frozen-hydrated rat liver tissue cut with a 35° knife falls in the range 30–50%, with the greatest compression observed in sections thinner than 100 nm. Reports in the literature for specimens such as liver tissue, plant cells, yeast, bacteria, and skin (Al-Amoudi et al., 2005; Chang et al., 1983; Michel et al., 1992; Richter, 1994; Shi et al., 1996) indicate a similar range of compression for each of these specimens. Al-Amoudi and colleagues (2003) found that compression in frozen-hydrated sections may be reduced by the use of an oscillating diamond knife, but only for a small fraction of sections. However, the theory behind the oscillating knife is sound, as proven by success with plastic sections (Studer and Gnägi, 2000). For frozen-hydrated sections, the effect of the oscillating knife may be specimen-dependent, and the benefit may be greater for thicker sections. Thus, we feel that continued testing is warranted.

Interestingly, we have observed that some structures in frozen-hydrated sections, such as desmosomes in skin, do not display the same degree of compression-related distortion as do neighboring structures, (Hsieh et al., 2004). Likewise, ribosome profiles in rat liver are not obviously shortened in the compression direction; this was also observed by Dubochet and Sartori Blanc (2001) in plant tissue. Other examples of macromolecular assemblies not distorted by section compression include nucleosome core particles (Leforestier et al., 2001), hexagonally packed sperm chromatin (Sartori-Blanc et al., 2001), and clusters of the chemotaxis receptor Tsr in Escherichia coli (Zhang et al., 2004). Our conclusion is that the degree of compression of various cellular structures within frozen-hydrated sections is nonuniform, with the relatively more-rigid structures resisting large-scale distortion better than flexible structures such as membranes. This structure-specific characteristic may make it practically impossible to computationally correct tomograms for the effects of compression.

4.3. Crevasse formation and freezing quality

The section in Fig. 2 is 200-nm thick (as measured by electron energy-loss imaging), and the image was recorded after a total electron dose of 5 e⁻/Å², well below the dose needed to smooth out crevasses (Hsieh et al., 2002). Nevertheless, crevasses are few. However, others (e.g., Al-Amoudi et al., 2005) report that crevasse-free sections are not to be expected at thickness above 70–100 nm. We find that excellent high-pressure freezing, as evidenced by especially good preservation of the biological structure, correlates with improved sectioning quality. Unfortunately, high-pressure freezing appears to give variable results. Even when the best technique is used, only about 40% of the blocks contain optimally frozen tissue, and this can only be identified through sectioning and electron microscopy.

It is known that the tendency to form ice microcrystals within high-pressure frozen material is related to the local water content, with a greater tendency for microcrystals where the water content is higher (Michel et al., 1991; Moor, 1987; Sartori et al., 1993; Studer et al., 1995). In our work, we have observed that, with good high-pressure freezing, specimens having a relatively low water content, such as yeast cells (except for their vacuoles) and mouse skin (Hsieh et al., 2004), are usually easier to section, and have less severe crevasses than do liver tissue sections. This observation is consistent with reports of high-quality frozen-hydrated sections obtained from specimens having relatively low water content, such as yeast (Al-Amoudi et al., 2003; Schwartz et al., 2003), bacteria (Matias et al., 2003; Zhang et al., 2004), and skin (Norlén et al., 2003; Norlén and Al-Amoudi, 2004). Nevertheless, with well-frozen liver tissue, we do not see a difference in crevasse formation between the mitochondrial matrix and the surrounding cytoplasm, even though there is a considerable difference in water content.

Using typical protocols, pre-fixation treatment (e.g., effects due to anoxia) should not have a major effect on the overall water content of the tissue. Therefore, the considerable variability we see in different high-pressure frozen blocks from the same experiment must be due to factors such as the geometry of the individual specimen in the carrier, the disposition of the filler, and variations in the operation of the machine from run to run. Echlin (1992) has suggested that a mixture of vitreous and microcrystalline ice may exist in aqueous specimens following high-pressure freezing. Thus, it seems reasonable that the proportion of
vitreous to microcrystalline ice might vary depending on the particular conditions during high-pressure freezing. Such a variation in the fraction of microcrystalline ice could account for observed differences in structural preservation and sectioning quality in the liver specimens. This may explain why crevasse-free thick sections are obtained only from optimally frozen tissue. While electron diffraction failed to provide evidence for microcrystalline ice in high-pressure frozen liver sections (Fig. 3), reflections from a small fraction of crystalline ice might be missed due to the strong, diffuse background scattering that results from a thick layer of ice containing embedded biological material (McDowall et al., 1983; Sartori et al., 1993). Note that the two rings of vitreous ice, seen clearly at spacings corresponding to 0.370 and 0.214 nm from a thin layer of plunge-frozen pure water in Fig. 3E, are less distinct in the diffraction patterns from the frozen-hydrated sections. Further, many of the strongest reflections from crystalline ice forms (Sartori et al., 1996) appear near the strong diffuse maxima of vitreous ice. Energy-filtered electron diffraction experiments are planned, which might better detect small amounts of crystalline ice in frozen-hydrated tissue by reducing background scattering in the diffraction patterns.

4.4. Comparison with freeze-substitution

It is clear that freeze-substitution of properly frozen tissue will continue to be a useful technique, even when sectioning of frozen-hydrated specimens becomes routine. An important advantage of freeze-substitution (in addition to ease of sectioning and ease of tomographic data collection) is the enhanced contrast that is provided by section post-staining for some cellular components, such as heterochromatin. Of course, this same characteristic also constitutes the method’s major drawback, namely, that images of stained specimens do not reflect true differences in mass thickness between cellular components. There is a degree of variability in staining of certain cellular structures, such as membranes, which sometimes stain positively, and sometimes negatively. The heavy osmication that is associated with many freeze-substitution protocols can obscure features. Mitochondrial matrix granules and membranes of the cristae, when they stain positively, often cannot be detected inside the densely stained matrix. With frozen-hydrated sections, Matias and colleagues (2003) were able to detect a discrete peptidoglycan layer in the periplasm of Gram-negative bacteria that was obscured by stain in the freeze-substituted material, while with freeze-substitution, they found that the outer membrane of Pseudomonas aeruginosa displayed a lipopolysaccharide layer, which was not seen in frozen-hydrated sections due to contrast matching with the dextran or sucrose media in which the cells had been frozen. Similarly, a comparison of desmosomes in frozen-hydrated sections of skin (Al-Amoudi et al., 2004; Hsieh et al., 2004) with the same structure in freeze-substituted material (He et al., 2003) reveals that intermediate filaments attached to the desmosomal plaque are less apparent in frozen-hydrated sections, while a low-density gap between the cell membrane and the desmosomal plaque is seen in frozen-hydrated, but not freeze-substituted sections.

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