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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.

Keywords: Cryo-ultramicrotomy; Cryosections; High-pressure freezing; Vitrification; Freeze-substitution; Rat liver; Mitochondria; Ribosomes; Endoplasmic reticulum

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,

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tomographic imaging of plunge-frozen large cells, such as those of the slime mold *Dictyostelium* (Medalia et al., 2002), is usually limited to cellular margins or flattened regions.

Recent progress in the field of cryo-ultramicrotomy has made it possible to prepare frozen-hydrated sections from a variety of biological specimens, thus overcoming the above thickness limitation. Key technical contributions have come from several laboratories, including those of Dubochet (Al-Amoudi et al., 2005; Chang et al., 1983), Mueller (Michel et al., 1991, 1992), Lepault (Erk et al., 1998), Somlyo (Somlyo et al., 1977, 1985), and Leapman (Shi et al., 1996). This work is detailed in a comprehensive review by Sitte (1996). Cryo-electron microscopy of frozen-hydrated sections has reached a stage of development that allows its application to several types of biological specimens. Recent successful applications of cryo-ultramicrotomy include DNA and chromatin (Leforestier et al., 2001, 2005; Sartori-Blanc et al., 2001), rat liver (Hsieh et al., 2002), bacteria (Al-Amoudi et al., 2004; Matias et al., 2003; Zhang et al., 2004), yeast (Schwartz et al., 2003), human skin (Norlén et al., 2003; Norlén and Al-Amoudi, 2004), algae (Leis et al., 2005), and isolated chloroplasts (Nicastro et al., 2005).

We are pursuing electron tomography of frozen-hydrated mammalian tissue using two model systems of greatly differing metabolic activity and water content, mouse skin (Hsieh et al., 2004) and rat liver (Hsieh et al., 2002). The present report focuses on the latter tissue, which presents special challenges for cryo-preservation and cryo-ultramicrotomy. In a previous report on rat liver (Hsieh et al., 2002), we described the first successful application of electron tomography to frozen-hydrated mammalian tissue. Artifacts associated with cryo-ultramicrotomy include knife marks, crevasses, chatter, and compression (Al-Amoudi et al., 2005; Chang et al., 1983; Frederik et al., 1982, 1984; Michel et al., 1991, 1992; Richter et al., 1991; Richter, 1994; Zierold, 1994). Our tomographic analysis revealed that the first two types of defects were predominantly confined to the section surface, and that there was considerable useful ultrastructural information in the interior of the sections (Hsieh et al., 2002).

In the present study, we report further progress in the development of techniques for tomographic imaging of frozen-hydrated mammalian tissue. (1) We demonstrate that imaging frozen-hydrated sections using zero-loss energy filtering is effective in increasing contrast without an increase in electron dose. (2) We identify conditions under which crevasses on the surface of the section may be reduced or eliminated, even for sections thicker than 150 nm. (3) We show that Quantifoil grids afford several advantages for tomography of frozen-hydrated sections. (4) We employ the needle-biopsy technique for rapid excision of tissue for high-pressure freezing.

In addition, we compare tomographic images of rat liver tissue recorded from frozen-hydrated sections, with those recorded from plastic sections of freeze-substituted material. The structural preservation is equally good in both cases, but complications in structural interpretation caused by metal staining are avoided with frozen-hydrated material.

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% OsO_4 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-