Zoological Studies 34, Supplement I (1995)

Zoological Studies

3-D Electron Microscopy of Cells and Organelles by High-voltage EM, Tomography, and Stereopair Analysis

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MICROSCOPY

3-D electron microscopy is greatly facilitated by the high-voltage EM (HVEM, ca. 1000kV) and the intermediate-voltage EM (IVEM, ca. 200-500kV). Compared to the conventional transmission EM (CTEM, ca. 100kV), the increased penetration of the specimen by the high-energy beam allows the use of thicker specimens without loss of resolution. Thus, there is the potential for more depth information in the images.

For effective 3-D microscopy using stereoscopy or tomography, the thickness of the specimen must be an appreciable fraction of the width of the image field. In the case of CTEM, the specimen thickness is limited to not much more than 100 nm. This means that a final magnification of at least 80-100,000X is required, otherwise the stereo view or tomographic volume has little depth. At such high magnifications, only quite small volumes can be examined. For IVEM, specimens as thick as 300-1000 nm can be used, so magnifications as low as 15-30,000X will provide reasonably deep volumes. For HVEM, specimens of 1000 nm are commonly used, but the instrument is capable of good imaging up to about 10 micrometers, in certain cases. Thus, quite low magnifications can be used with good 3-D results. Clearly, with higher voltage the range of usable thickness and magnification increases.

RECONSTRUCTION METHODS

There are three basic methods by which the 3-D structure of cells and organelles can be obtained using EM: (1) observation of stereopairs, (2) making serial-section reconstructions, and (3) making tomographic reconstructions.

1. *Stereopairs*

Stereopairs can be used for critical observation and analysis when the specimen is not excessively crowded with detail, and when a useful amount of the specimen volume is present within the thickness of the section. Stereopairs are created by tilting the specimen and recording two images. This is still the most common procedure used in HVEM. Within the limits mentioned above, optimum imaging requires that the specimen tilt angle be chosen for the section thickness and magnification used (Beeston 1973). Since the structures seen in EM stereopairs are semi-transparent, excessive over-lapping details limit the useable section thickness for some specimens. For sections much over 1 micrometer in thickness, selective staining is often required.

Stereopairs not only provide a 3-D view, but can be used to make reconstructions and measurements of objects in the specimen. We have developed a computer system which includes provision for drawing parallel depth contours, and 3-D lines, within the 3-D volume of a stereoscopic image. The contours are stored in a database, and used to determine statistics such as length, volume, and surface area. The contours can also be used to create surface-rendered reconstructions. This system is called Sterecon (Marko et al. 1988, 1992).

2. *Serial-section reconstructions*

Serial-section reconstruction is an appropriate technique when the volume of the specimen to be reconstructed is too large to be contained within the thickness of a single section. Although serial sections of 50-100nm thickness can be used with the CTEM, there are advantages to using serial-

section stereopairs of "thick" (about 0.25 to 1.5 micrometers) sections, recorded using the IVEM or HVEM. Fewer but thicker sections can be used, reducing microtomy and microscopy time. The use of Sterecon avoids loss of z-resolution associated with fewer sections, since tracing takes place on several planes within each section (Fig. 1a). The major advantage of using thicker sections is that the 3-D structure of the specimen can be appreciated during tracing. This makes decisions about branching and connectivity of structures much easier and more certain.

For correct serial-section reconstruction, the actual section thickness during microscopy has to be measured and compared to the pre-irradiation thickness. The actual thickness is measured by Sterecon, and the original thickness by interference microscopy or a well-calibrated microtome. The difference is due to mass loss from electron irradiation (Luther 1992). Any incidental tilt of the specimen is measured and corrected by Sterecon during tracing. Alignment between sections is aided by comparison of the contours of the top plane of one section with the structures on the bottom plane of the succeeding section. Fiducial marks independent of the specimen (Bron et al. 1990) are needed for alignment if it is important to find the exact overall shape of the specimen. The contours from all of the sections are combined and grouped to make a reconstruction of the specimen, and to find any desired morphometric parameter.

3. *Electron tomography*

For an important class of objects, such as some cell organelles, HVEM tomography provides the ideal means of high-resolution 3-D study. Any object which can be contained within a single section not thicker than about 2-3 micrometers is a candidate for HVEM tomography. Tomography is required when there is too much information in a stereopair view of the specimen to make a reconstruction by stereoscopic tracing. A tomographic reconstruction (Frank 1992) is created by combining a series of tilted views. The number of views, and the maximum tilt angle, are important resolution-limiting factors (Crowther et al. 1970).

A 3-D resolution of about 6nm is readily obtained by HVEM tomography. The same 3-D resolution cannot be obtained by serial ultrathin sections. The depth resolution of a conventional serial-section reconstruction is limited by the minimum thickness it is possible to cut, and the loss of material between sections due to microtomy and electron irradiation. Thus, depth resolution better than 30-40nm would not be expected. Stereoscopic tracing within serial sections can provide better resolution than this (although not as good as tomography), but only if the specimen is not too complex.

In common practice, a tilt series is recorded around a single axis. It is not possible to tilt sections to 90 degrees (the maximum tilt is often around 70 degrees), so there is a loss of resolution due to incomplete filling of Fourier space during the reconstruction process. We have developed methods to combine tilt series around two axes, thus reducing the missing information (Marko et al. 1994). Compared to a single-tilt reconstruction--even if the same total number of tilt images are recorded in both cases--the 3-D resolution and faithfulness of the reconstruction is increased, and artifacts are reduced (Penczek et al. 1995). We have found that the key to obtaining a good reconstruction is accurate alignment of the tilt-series images, which is only possible if specimen distortion during imaging is controlled or corrected (Marko et al. 1995).

VISUALIZATION METHODS

Visualization of the results of either contour-traced or tomographic reconstruction methods often requires more effort than the reconstruction itself. Reconstructions based on contours are often visualized by surface rendering. The first step is "tiling", in which the points on contour lines at adjacent depth levels are connected to form polygons (often triangles). In the second step, these triangular tiles are shaded and smoothed to produce a realistic surface. Since the contour database provides for grouping contours into objects, display scenes with all objects in the specimen, or only selected objects, can easily be created (Fig. 1b)

Tomographic reconstructions can be volume-rendered by applying voxel density thresholds in an attempt to select or "segment" objects of interest. The opacity of the volume can also be adjusted to make internal structures visible. An example of volume rendering is seen in Fig. 1c. Often, volume-rendering methods work poorly on EM specimens because density segmentation fails due to low contrast. In those cases, we use Sterecon to trace 3-D contours within successive depth regions of the tomographic volume (Fig. 1a). The resulting contours are surface-rendered as above (Fig. 1b). Alternatively, the contours can be used to create a 3-D mask to apply to the volume

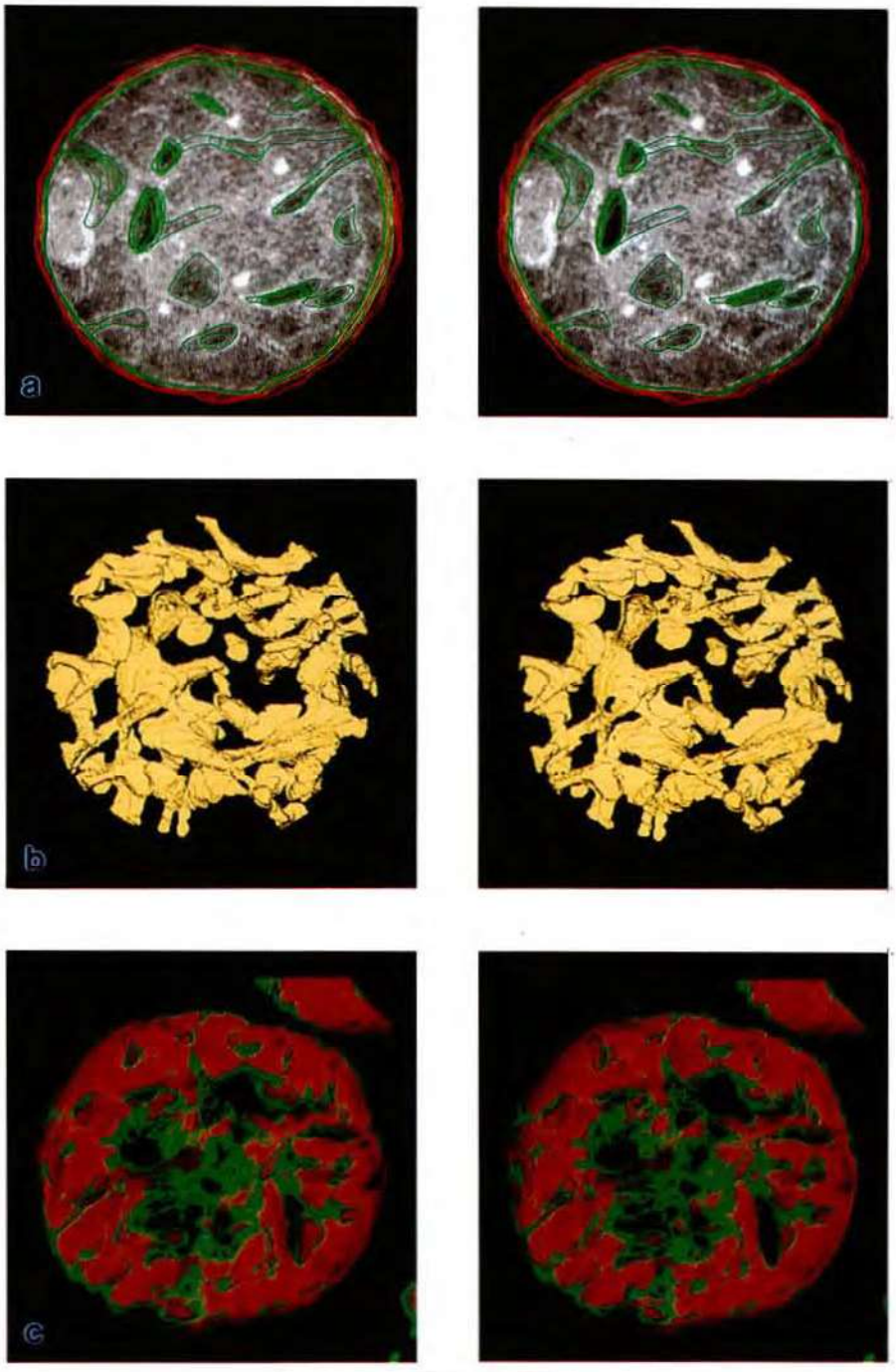

Fig. 1. Stereopair examples of segmentation and rendering techniques, using HVEM tomographic volumes from 0.5-0.7-micrometer thick sections of isolated rat-liver mitochondria (work in progress, Mannella et al. 1995). Scale bar = 1 micrometer, applies to all figures. (a)Segmentation of membranes by contour tracing using Sterecon. A portion of a tomographic volume, with contour lines drawn during stereoscopic viewing. Outer membrane (red) and inner membrane and cristae (green) traced on five depth planes. Same technique used with HVEM stereopairs for serial thick sections. (b)Surface rendering of cristae made from contours traced within a tomographic volume, of which (a) is a portion. About one-half the thickness of the full volume is shown. Inner and outer membranes removed. (c)Volume rendering of a portion of a mitochondrion in the "condensed" state (based on results in Mannella et al. 1994). Membranes (green) and matrix material (red) segmented by setting two density ranges; opacity adjusted to show interior structure. Outer membrane not shown.

for segmentation purposes. The segmented objects can then be volume rendered.

The Albany HVEM is part of the Biological Microscopy and Image Reconstruction Resource, supported in part by NIH PHS grant R01219 (P.I. Dr. Conly Rieder). The Sterecon software, and that used for tomography, a subset of SPIDER, were developed in association with our image-processing lab, supported in part by NSF grant BIR 921 9043 (P.I. Dr. Joachim Frank). The mitochondrial work is supported in part by NSF grant MCB 921 9043 (P.I. Dr. Carmen Mannella).

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