

Local refinement: An attempt to correct for shrinkage and distortion in electron tomography

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Abstract

A critical problem in electron tomography is the deformation of the specimen due to radiation, or “shrinkage,” which interferes with image alignment and thereby limits resolution. Here, we describe a general strategy for refining preliminary reconstructions which allows the damage due to the shrinkage of plastic-embedded thin sectioned specimens (50–80 nm) to be corrected. The basic steps of the strategy involve: (a) the partition of the preliminary reconstruction into sub-volumes; (b) the extraction of corresponding sub-areas for each sub-volume from the micrographs of the tilt series; (c) the re-projection of each sub-volume according to the orientation parameters; and (d) the refinement of these parameters by correlating each sub-area to the corresponding computed projection. We tested the strategy by refining chemical synapses reconstructed from series imaged with conical, double and single tilt geometries. The results gathered with local refinement were evaluated by visually inspecting the structure of biological membranes in the maps. In an effort to quantify these improvements, we studied the refined maps using correlation criteria and mapped the corrections applied to the orientation parameters in each sub-volume of the reconstruction. Simulation experiments complemented the data gathered by correlation analysis. Based on these criteria, we concluded that local refinement significantly improves the overall quality of the reconstructions of chemical synapses calculated from series imaged with conical and double tilt geometries.

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1. Introduction

Electron tomography is a general technique for studying the 3D structure of biological macromolecules in their cellular environments. It has been used to reconstruct macromolecular assemblies (e.g., Hart, 1968; Hoppe et al., 1974), sections of plastic-embedded tissues (e.g., He et al., 2003; McEwen et al., 1986), frozen hydrated organelles extracted

from the cell (Al-Amoudi et al., 2004; Beck et al., 2004) and large macromolecular complexes and viruses in frozen solution (Grunewald et al., 2003). In all cases, the 3D reconstructions are calculated without imposing symmetry or using averaging methods, which can be applied later, if necessary, to improve the signal to noise ratio (Liu et al., 2004; Nicastro et al., 2005; Zhu et al., 2006).

In electron tomography, the specimen is imaged many times along different directions (the “tilt series”) to produce the images used to calculate the 3D maps. Since biological specimens are comprised of elements with low atomic

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numbers, for many samples inelastic scattering predominates during image formation. Energy is transferred to the specimen and hence makes radiation damage a key limitation when calculating 3D reconstructions.

The deleterious effects of radiation damage on the chemical and structural integrity of specimens include formation of free radicals that rupture bonds, fragmentation of large macromolecules, mass loss and establishment of new cross-links between adjacent molecules in the tissue (for review, see Cosslett, 1978). After radiation damage, the specimen is highly cross-linked and bears little resemblance to the original sample. At room temperatures, these changes occur at very low electron doses ($0.5\text{--}1\text{ e/A}^2$), which make the study of the detailed structure of these specimens impossible. Since the changes depend on the temperature at which the specimen is imaged (Salih and Cosslett, 1975; Siegel, 1970), stopping mass loss and preserving the order of organic crystals necessitates imaging specimens cooled at liquid helium temperatures (Dubochet and Knapek, 1979).

Sections that are stained with heavy metal atoms and imaged at room temperatures can provide information about the boundaries between the stain and the macromolecule (the “envelope”). Since the stain is far more stable than the biological specimen, these envelopes can be obtained at *moderate* levels of resolution ($2\text{--}5\text{ nm}$). Often, however, even $2\text{--}5\text{ nm}$ resolutions are difficult to accomplish because shrinkage interferes with the alignment of the tilt series. While procedures introduced to reduce the shrinkage due to radiation during data collection (i.e., pre-irradiation), have been successful, even small changes in position of the fiduciary points used for alignment (i.e., gold particles added to the specimen’s surface) cause inconsistencies that limit resolution.

For these reasons, it is necessary to correct specimen shrinkage with computational strategies (Lawrence et al., 2006; Mastronarde, 1997). In a series collected with single-tilt geometry, correcting for magnification compensates for gross deformations while adjusting the rotation and shift parameters corrects small geometric inaccuracies that occur during image recording. These corrections improve primarily the resolution in the center of the reconstruction volume while leaving a resolution gradient from the center to the periphery. In series collected with double-tilt geometry, the effect of shrinkage becomes apparent when the fiduciary markers of reconstructions calculated from the two independent series do not properly match up. Mastronarde (1997) introduced a strategy based on the 3D layout of these reference points whereby the independent tomograms are brought into a common reference system by finding the transformation that best allows the coordinates of the fiduciary markers to superimpose. If linear transformations fail to superimpose these coordinates, non-linear corrections are used. Since this strategy, highly successful for double-tilt geometry, is deeply embedded in the reconstruction algorithm, its generalization to single and conical tilt geometries is not straightforward.

In this paper, we describe a strategy to cope with specimen shrinkage and distortions in a reconstruction from tilt series recorded with different geometries. Since shrinkage is a local phenomenon, the reconstruction is first subdivided into smaller cubes (64), processed independently and refined by projection matching (Zampighi et al., 2005). Pasting together the refined cubes reconstitutes the entire volume of the region (Lanzavecchia et al., 2005). We followed the refinement process by mapping the corrections applied to the orientation parameters of the projections of each sub-volume of the reconstruction and assessed the improvements in the refined maps using correlation criteria. Simulation experiments complemented the data gathered using correlation analysis. Based on these criteria, we concluded that local refinement significantly improves the overall quality of the reconstructions of chemical synapses calculated from series imaged with conical as well as double axis tilt geometry.

2. Material and methods

2.1. Preparation of thin sections

The preparation of the specimens has been described in Zampighi et al. (2005, 2006). In brief, three Sprague–Dawley adult rats 90–120 days of age were sacrificed and fixed by perfusion with about 1 L of 3% glutaraldehyde, 4% paraformaldehyde in 0.2 M cacodylate buffer pH 7.4. The brains were removed and sliced in a vibrotome in sections $100\text{--}150\text{ }\mu\text{m}$. Tissue from the frontal association and motor neocortices was dissected from these thick sections and processed for thin sectioning electron microscopy. After OsO_4 post-fixation and block staining, the tissue was embedded in Epon 812. Thin sections of thickness with gray-to-silver interference color ($50\text{--}80\text{ nm}$) were cut, collected on 200 mesh grids with carbon substrate and stained with solutions of uranyl and lead citrate. Gold particles, 10 nm in diameter, deposited on the surfaces of the thin sections were used for centering the conical series, estimating the thin section thickness, and calculating preliminary 3D reconstructions.

2.2. Electron microscopy

We used the Gatan 650 Single Tilt Rotating Holder in a FEI Tecnai 12 electron microscope operated at 120 kV to collect 22 tomographic tilt series. The holder allowed up to 70° tilts and controlled rotations in the azimuth. The images were collected in a $2\text{ k} \times 2\text{ k}$ CCD Gatan camera at $34,230\times$ final magnification (pixel size 0.409 nm). Imaging was carried out using a minimum-dose method: searching was done at $2700\times$ magnification with minimum illumination and the regions of interest imaged by focusing $\sim 2\text{ }\mu\text{m}$ away. The total electron dose was $\sim 800\text{ e/A}^2$, which we previously found to decrease the overall thickness of the thin section by $\sim 15\%$ (Zampighi et al., 2005).

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