Quantitative biological measurement in Transmission Electron Tomography

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Abstract. It has been known for some time that biological sections shrink in the transmission electron microscope from exposure to the electron beam. This phenomenon is especially important in Electron Tomography (ET). The effect on shrinkage of parameters such as embedding medium or sample type is less well understood. In addition anisotropic area shrinkage has largely been ignored. The intention of this study is to explore the shrinkage on a number of samples ranging in thickness from 200 nm to 500 nm. A protocol was developed to determine the shrinkage in area and thickness using the gold fiducials used in electron tomography. In brief: Using low dose philosophy on the section, a focus area was used prior to a separate virgin study area for a series of known exposures on a tilted sample. The shrinkage was determined by measurements on the gold beads from both sides of the section as determined by a confirmatory tomogram. It was found that the shrinkage in area (approximately to 90-95% of the original) and the thickness (approximately 65% of the original at most) agreed with previous authors, but that almost all the shrinkage was in the first minute and that although the direction of the in-plane shrinkage (in x and y) was sometimes uneven the end result was consistent. It was observed, in general, that thinner samples showed more percentage shrinkage than thicker ones. In conclusion, if direct quantitative measurements are required then the protocol described should be used for all areas studied.

1. Introduction

The often-observed effect of “bleaching” of biological material in the electron microscope (where the observed area appears lighter than the surrounding area) can be attributed to local shrinkage of the sample. Luther et al., [1] used the relative measurement between beads on the sample surface in tilted samples, also containing tropomyosin paracrystals of “known” spacing, to measure this collapse of plastic sections. However, images were taken on photographic plates and due to initial sample “drift”, the first images used for measurements were after some initial sample irradiation. Under their fluence rate (<50 e/nm²/s), they recorded an initial period of fairly rapid collapse followed by a longer period (40 minutes) of slower change. It has since become accepted practice to pre-irradiate the area of interest for up to an hour, before collecting a tomography tilt series [2]. However, if one would like to make a quantitative size or volume measurement of a certain feature within cellular material, then it is vital to know the initial volume, preferably at the region of interest. In this study, modern CCD
camera technology and microscope Low Dose software procedures have been applied to investigate these first moments of irradiation. A protocol was developed that can be applied to any sample prepared for tomography using fiducial markers. This has then been used to study the shrinkage in z, as well as anisotropic movement in the x,y plane, and to investigate the possible protective effect of carbon coating [2].

2. Materials and Methods

2.1 Materials
High pressure frozen, freeze substituted, Epon embedded samples of isolated rat islets of Langerhans [3] and standard resin embedded sample of equine metacarpophalangeal calcified cartilage, were cut at nominal 200, 300, 400, and 500 nm thickness on a Reichert Ultracut S microtome and collected on pioform coated, copper slot grids. 15 nm fiducial markers (Aurion, NL) were applied to both sides. The sections were examined in a Tecnai 20 LaB6 TEM (FEI Company), operating at 200 kV with images captured on a 4k x 4k Eagle CCD camera (FEI Company). One of the 300 nm sections was subsequently carbon coated in an Edwards Auto 306 Evaporation system and re-examined. The electron fluence rate was measured via the Low Dose software.

2.2 Microscope protocol
The specimen was initially imaged at low magnification and beam settings to find an area of cellular material, as in a typical Low Dose procedure, with an electron fluence rate of less than 10 e/nm²/s. The sample was then tilted to 45°. The Low Dose Focus mode was used to align and focus an adjacent area 10 µm further along the tilt axis from the area to be exposed. The electron fluence rate was now increased to around 300 e/nm²/s, typical of our beam settings for tomography. In the Exposure mode, a series of 60 exposures was then recorded at 1 second intervals, using the pre-specimen shutter between exposures. Subsequently a quick tomography series between 45° and -45° at 5° increments was recorded to identify those fiducials above/below each other on the top and bottom surfaces. This tilt series was used to calculate the “final” tomogram thickness, using IMOD tomography software [4], following what was a further 15 minutes of electron irradiation and a total electron fluence of approximately 3x10⁵ e/nm². Finally a reference image at zero tilt was taken.

2.3 Measurement protocol
Changes in thickness in the z direction were calculated using the geometry shown in Figure 1. At each sample position on a grid (3 positions analysed per grid, or sometimes on separate grids), measurements were made through the image stack between at least three pairs of fiducials on top and bottom surfaces. Fiji software, a version of ImageJ,[5]was used for this. This is then one dataset.

\[
\delta x_0 = B_{T0} - B_{B0} \\
\delta x_\theta = \delta r + \delta x_0 \cos \theta \\
\text{hence} \\
B_{T0} - B_{B0} = T \sin \theta + (B_{T0} - B_{B0}) \cos \theta \\
T = ((B_{T0} - B_{B0}) - (B_{T0} - B_{B0}) \cos \theta)/\sin \theta
\]
These were averaged and normalised to the thickness calculated from the image taken at zero tilt, immediately after the exposure series. Changes in shape in the x, y plane were also investigated using relative shifts between images of fiducials on one surface, taken from each quadrant of all images. These were again averaged and normalised.

3. Results
Initially measurements on 200 and 300 nm sections, which are our standard thicknesses for ET, were used to test our protocol extensively. As data appeared consistent, the protocol was then applied to samples of increased thickness. Averaged results for all the different sample thicknesses, with number of data sets analysed from different positions given in brackets, are shown in Figures 2 and 3. The initial collapse of the sections is extremely rapid at this fluence rate. It was also observed that in three out of four cases, the thinner samples shrink proportionately more than thicker samples for the same exposure. There was no intuitive reason why the 400 nm sections behaved differently. Measurements of thickness at the end of the first minute were then compared with the “final” thickness after the tomogram collection and presented in Table 1. Data from the 300 nm section which was subsequently carbon coated is also presented here as well as provisional data from sample resin, in which no biological material was contained. A complete data set could be collected and analysed in a few hours.

![Figure 2](image)

**Figure 2.** Proportional change in thickness in z-direction over time. The figure in brackets gives the number of different sample data sets analysed.

![Figure 3](image)

**Figure 3.** Proportional in-plane shrinkage in x,y direction over time. The figure in brackets gives the number of different sample data sets analysed.
Table 1. Thicknesses at start, end and after completed tomogram for different samples

| Sample and thickness | Range of thickness in nm ± 10 nm at 1 s. Fluence: 300e/nm² | Range of thickness in nm ± 10 nm at 60 s. Fluence: 1.8 x10⁵ e/nm² | Thickness from tomogram ±10 nm. Fluence : 3x10⁵ e/nm² |
|----------------------|-------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------------------|
| 200 nm (3)           | 184 - 219                                                   | 139 - 179                                                     | 116 - 142                                           |
| 200 nm resin         | 169                                                         | 159                                                          | 132                                                |
| 300nm (3)            | 333 - 375                                                   | 243 - 293                                                     | 246 - 264                                           |
| 300nm C Coated (1)   | 267                                                         | 266                                                          | 248                                                |
| 400nm (3)            | 322 - 349                                                   | 304 - 340                                                     | 335 - 340                                           |
| 400nm resin (1)      | 360                                                         | 329                                                          | 318                                                |
| 500nm (1)            | 476                                                         | 417                                                          | 414                                                |

4. Discussion and conclusions

A review of shrinkage data and possible mechanisms involved, as well as inhibiting factors, is available in Luther’s contributed chapter to the Electron Tomography Methods book [2] and is beyond the scope of this paper. The x,y movement, although displaying initial variations, showed a consistent change of about 1.5–3 %. The greatest z shrinkage (to 65% of the original) was measured in the thinnest samples: the 300 nm sections of rat islets and the 200 nm calcified cartilage material. The maximum z shrinkages were similar to the results from Luther et al for 80 nm sections, though as their first measurements correspond to our data after 3-4 s, we would expect that some shrinkage had already taken place on both the paracrystals and the section. The greatest change happens in the first few seconds and appears to follow a first order exponential decay model. Although further small shrinkage was measured after collection of the tomogram (see Table 1), we would suggest that in this type of biological sample, even when quantitative measurements are not required, further pre-irradiation of samples beyond setting up the tomography experiment, is not necessary. Our initial data on carbon coating supports that it does indeed reduce shrinkage. However results also suggest some pre-shrinkage occurring either in the microscope vacuum or during the coating process. Interferometry might be useful here to measure sample thickness at different stages in the analysis and is planned for future studies. It was also found that resins alone can behave differently to areas where the resin contains the biological material. As sample behavior is individual, it is our conclusion that this quick and simple protocol should be applied to any sample if volume measurements of cellular components within cellular material are desired. The authors would be happy to supply a template spreadsheet to this end. The protocol could also be used to investigate further the influence of other parameters on shrinkage: parameters such as specimen cooling, electron microscope operating voltage, and other differences due to sample preparation methods.

References

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