Chapter 2

Automated high-throughput electron tomography by pre-calibration of image shifts

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Summary

Electron tomography is a versatile method for obtaining three-dimensional (3D) images with transmission electron microscopy (TEM). The technique is suitable to investigate cell organelles and tissue sections (100–500 nm thick) with 4–20 nm resolution. 3D reconstructions are obtained by processing a series of images acquired with the samples tilted over different angles. While tilting the sample, image shifts and defocus changes of several µm can occur. The current generation of automated acquisition software detects and corrects for these changes with a procedure that incorporates switching the electron optical magnification. We developed a novel method for data collection based on the measurement of shifts prior to data acquisition, which results in a five-fold increase in speed, enabling the acquisition of 151 images in less than 20 min. The method will enhance the quality of a tilt series by minimizing the amount of required focus-change compensation by aligning the optical axis to the tilt axis of the specimen stage. The alignment is achieved by invoking an amount of image shift as deduced from the mathematical model describing the effect of specimen tilt. As examples for the application in biological and materials sciences 3D reconstructions of a mitochondrion and a zeolite crystal are presented.

Electron tomography is a technique for imaging relatively large (100–500 nm) variable structures (e.g. cell organelles, whole cells, tissue sections) in 3D with moderate to high resolution (4–20 nm) (Frank, 1992 and 1995; Koster et al., 1997; Baumeister et al., 1999; McEwen and Marko, 2001). In contrast to stereomicroscopy, where two tilted electron microscope recordings are used for 3D analysis of microscope specimens (Starink et al., 1995), with electron tomography TEM images of a specimen tilted over different tilt angles (a tilt series) are combined to compute a 3D image (reconstruction) of the specimen density. Inherent to the approach are two key assumptions: (1) that the TEM images are in good approximation 2D projections of the mass density of the imaged sample; and (2) that the specimen does not change during data collection.

In spite of the early recognition of electron tomography as a high-resolution 3D imaging tool (Hart, 1968; Hoppe et al., 1968; Crowther et al., 1970; DeRosier and Klug, 1968), routine application of the technique has been prevented by considerable technical obstacles during the last few decades. A major problem lay in reconciling two conflicting requirements of the technique. To obtain a high-resolution (detailed) 3D reconstruction, a tilt series must be recorded that covers as wide an angular tilt interval as possible (to minimize the missing amount of information) with tilt increments as small as possible (to maximize the resolution by fine angular sampling) (McEwen and Marko, 1999; Baumeister and Steven, 2000). At the same time, the specimen must not change during data collection and, consequently, the electron dose used to record a tilt series must be as low as possible (Grimm et al., 1998).

Early on, a solution to these contradicting requirements was proposed: to partition a fixed amount of (allowable) electron dose over the number of projections that are required to attain a certain (desirable) resolution (Hegerl and Hoppe, 1976; McEwen et al., 1995). Although low-dose imaging techniques can be carried out to distribute the allowable dose over the number of images, practical problems are aggravated due to the fact that when a specimen holder is tilted, image shifts and defocus changes of several µm can occur while tilting the sample. Therefore, the acquisition of more than a hundred images with 1° tilt increment is a demanding task as defocus changes and
image shifts need to be corrected after every tilt increment. As a consequence, the application of high-resolution electron tomography remained restricted to a limited group of practitioners of the technique.

During the last decade, the application of electron tomography has gained pace. With the advent of computer-controlled microscopes, large-scale digital cameras and high-performance desktop computing power, it became possible to implement automated procedures for the acquisition of tilt series containing hundreds of views that are recorded under very low-dose conditions (Dierksen et al., 1992, 1993, 1995; Koster et al., 1992; Braunfeld et al., 1994; Grimm et al., 1997; Koster et al., 1997; Rath et al., 1997; Dierksen et al., 1995). Simultaneously, several computer program packages for alignment, reconstruction and manipulation of large volumetric data sets matured and became available to the scientific community (Frank et al., 1996; Fung et al., 1996; van Heel et al., 1996; Kremer et al., 1996; Schroeter et al., 1996; Hegerl and Altbauer, 1982).

With automated data collection several problems that limited the practical application of electron tomography are overcome. Automated tomography enables the image acquisition with a (digital) CCD camera, which implies that changes in image position and defocus can be detected by on-line image processing and immediately be corrected for by computer control of the microscope. In addition, the tilt series are directly available in digital format for subsequent processing and reconstruction. Typically, carrying out an electron tomography experiment with the first generation of automated systems will take a day, and the actual data collection 2–4 hours (Koster et al., 1997).

In this article we discuss the possibilities and the implementation of a second generation automated data collection procedure that significantly increases data collection speed and widens the applicability of the technique. The basic idea of the novel method is that the image movement is calibrated prior to data collection. The movement of the stage is measured both in the $xy$-plane (image shifts) and the $z$-direction (defocus change) for the range of tilt angles needed to acquire a tilt series. It appears to be sufficient to record these measurements at low magnification with $5^\circ$ tilt increments and to interpolate the values of image shift and defocus change in between when the actual data set is acquired with smaller tilt increments.
and at higher magnifications. In addition, it also appears possible to model the observations of image shifts and focus changes. Using the mathematical model it becomes possible to predict the overall image movements after a few measurements of image shift and defocus change. It is also possible to create novel strategies in collecting tilt series to optimize data collection to specific requirements such as the desired field of view, resolution or electron dose.

The pre-calibration data acquisition approach shows a substantial increase—five-fold—in data collection speed compared to the first generation of automated data acquisition methods. One of the reasons for this increase in speed is that, in contrast to the first generation of automated systems, it is not required to switch the electron optical magnification of the TEM back and forth during data collection.

The pre-calibration approach will also enhance the quality of a tilt series if the amount of required focus-change compensation during data acquisition is minimized by aligning the optical axis to the tilt axis of the specimen stage. The alignment is achieved by invoking an amount of image shift as deduced from the mathematical model describing the effect of specimen tilt.

Another advantage of the pre-calibration approach is that the required computer-controlled steps for tracking image shifts and defocus changes are uncoupled from the acquisition of the tilt series. The uncoupling provides possibilities for acquisition modes other than bright-field imaging and provides opportunities to use detectors other than CCD/film. For instance, a tilt series can be taken in STEM mode (Beorchia et al., 1993; Midgley et al., 2001) and, simultaneously, (3D) element-specific information of the sample could be collected with an energy dispersive X-ray (EDX) detector. Especially for applications in the field of materials sciences, which just recently started to apply the technique (Koster et al., 2000; Janssen et al., 2001), as well as for automated procedures to localize in 3D high-density labels within a specimen, 3D elemental map information could be very useful.

We evaluated the pre-calibration method under different stage and imaging conditions. A model describing the overall image movement when tilting the stage is derived and the reliability of determining relative shifts is shown to be 10 nm. In addition, we describe the current software implementation and present 3D
reconstructions obtained by the methodology in the fields of both biological and materials sciences.

Materials

Experiments were performed on a 200-kV Tecnai 20 (S) transmission electron microscope (FEI/Philips Electron Optics, Eindhoven, The Netherlands) at a nominal magnification of 5000× (screen up) and a defocus of −10 µm. The Tecnai PC is equipped with a dual 350 MHz Pentium II processor, which contains 512 Mb of RAM and 4 Gb of disk space. Images were recorded with a bottom-mounted slow-scan CCD camera (TemCam F214, Tietz Video and Image Processing Systems GmbH, Gauting, Germany). The post-magnification factor from the film-plane to the CCD chip is 1.8×. The CCD array is composed of 2048 × 2048 square pixels of 14 µm. At a magnification of 5000× the images represented a specimen area of 3186 nm × 3186 nm. For the experiments, images of 512 × 512 pixels with 6.2 nm/pixel were collected (binning 4). The readout rate of the CCD images is 2 Mb/s (12 bits/pixel). The replica of a 2160 lines/mm waffle-pattern diffraction grating (Electron Microscopy Sciences, Washington, PA) was used as a calibration standard and tilted from −60° to +50°/55° and vice versa either in a FEI/Philips standard specimen holder or a Gatan model 670 ultra-high-tilt holder.

Automation procedures for calibration of image shift and autofocus, and detection of these shifts and changes, were implemented in JavaScript as a component (macro) in TIA (Tecnai Imaging and Analysis, Emispec Systems Inc., Tempe, AZ). TIA is an addition to the Tecnai software, interfacing with detectors on the microscope (e.g. CCD camera, STEM detector). At every tilt angle CCD images of the sample were recorded to detect image shifts and defocus changes. Image shifts were measured by cross-correlating images acquired before and after a specimen tilt increment. Defocus changes were measured using the beam-tilt method (Koster et al., 1987 and 1989). The beam-tilt method makes use of the fact that an image shift will occur when the illuminating beam is tilted and the microscope is not in focus. The amount of image shift is linearly related to the amount of defocus.
Off-line image processing has been carried out on a UNIX workstation (Octane dual-processor 2 × 250 MHz R10000 (IP30), Silicon Graphics Inc., Mountain View, CA). The IMOD program (Kremer et al., 1996) was used for the detection of the specimen-tilt axis, image alignment and 3D reconstruction. The IVE program (Fung et al., 1996) was used for general 3D volume handling and processing. The Huygens and FluVR programs (Scientific Volume Imaging BV, Hilversum, The Netherlands) for visualization. Modeling computations were carried out on a Windows 98 PC (Microsoft Inc., Redmond, WA) with the program Excel (Microsoft Inc.).

Methods and Results

A. Acquisition of a stage calibration curve

The magnification of the microscope was calibrated with a cross-grating and the proper functioning and accuracy of the computer-control procedures for determining and correcting image shifts and focus changes were determined once before the whole study.

Stage calibration curves were acquired by measuring specimen movement (i.e. image shifts in the xy-plane and defocus changes in the z-direction) as a function of specimen tilt.

1. First, to ensure proper cross-correlation between images recorded before and after an induced specimen tilt, an imperfection (a specific, non-repeating) feature on the cross-grating grid was selected.

2. Next, the sample was set manually to eucentric height.

3. The sample was tilted to its initial tilt angle.

4. The initial ImageBeamShift and/or the z-height of the specimen relative to the manually chosen eucentric height were varied in the range +3 µm to −3 µm for testing different experimental situations.

5. The image was defocused to −10 µm (automatically).
6. The feature on the cross-grating was centered on the CCD camera image using the image-shift coils (automated).

7. To minimize mechanical hysteresis effects, and therefore minimize initial image shifts, a tilt angle setting was always approached from the same direction. For instance, if the initial tilt angle was $-60^\circ$, the stage was first set to $-62^\circ$ and then back to $-60^\circ$ to make sure that even the first tilt angle in the measurement was approached from the negative side. The image feature was then automatically re-centered and re-focused.

8. Next, change of tilt angle. The tilt increment was $\pm 5^\circ$.

9. Detection of defocus change using the beam-tilt method. With the beam-tilt method the position of the maximum value (i.e. peak) after cross-correlating images recorded with ‘+’ and ‘−’ beam tilt is a direct measure of defocus. Defocus change was only measured, not corrected for.

10. Detection and correction of image shifts. Image shift was measured by cross-correlating two images before and after specimen tilt. The centering of the feature provided an immediate feedback to the operator if the detected shifts and compensations were carried out correctly.

11. The absolute image shifts and defocus changes that occurred relative to the conditions of the first tilt angle were calculated and saved to a file.

12. The measurements as listed in steps 8–11 were repeated until the final tilt angle was reached.
B. Model for stage calibration curves

The actual shape of the acquired calibration curve depends on:

- a displacement of the tilt axis of the goniometer from the optical axis;
- the fact that the specimen holder was removed/inserted;
- the x/y of the stage position;
- an x/y/z change in stage position;
- the z-height of an image feature;
- image-shift settings.

Only the last two parameters can be adjusted by the user, who is interested in an image feature at a certain stage position. Note, however, that the first parameter (the displacement of the tilt axis of the goniometer from the optical axis) is influenced by the last parameter (the image-shift settings). Fig. 1 shows x- and z-calibration curves acquired at a single stage position under different z-height and image-shift settings. With the best settings (Fig. 1E) there will be hardly any shifts; otherwise shifts can be positive or negative and can take values up to several µm.

We postulate that all observed calibration curves can be explained by modeling the specimen tilt as a rotation of a rigid body around an axis \( A \) of the coordinate system (Fig. 2). A difference of just 2° between the tilt axis (i.e. the direction along the specimen holder) and the y-axis of the CCD images was determined from the images subsequently acquired during the calibration measurements. Therefore, the tilt axis could be identified to a good approximation with the y-axis. At 0° tilt the sample point \( P \) has coordinates \( x_0, y_0 \) and \( z_0 \). When the sample is tilted by \( \alpha \) the new coordinates will be:

\[
x(\alpha, x_0, y_0, z_0) = z_0 \cdot \sin(\alpha) + x_0 \cdot \cos(\alpha)
\]

\[
y(\alpha, x_0, y_0, z_0) = y_0
\]

\[
z(\alpha, x_0, y_0, z_0) = z_0 \cdot \cos(\alpha) - x_0 \cdot \sin(\alpha)
\] (1)
Fig. 1. Absolute $x$-image shifts (●) and defocus changes (■) that an image feature undergoes after the sample is tilted to a certain angle ($0^\circ$–$60^\circ$), under various image-shift and $z$-height settings at a single stage position. From left to right the initial image shift and from bottom to top the $z$-height was varied from about $-3\ \mu m$ to $+3\ \mu m$.

So, the measured image shifts correspond to the changes in the $x$- and $y$-coordinates and the defocus change corresponds to the change in the $z$-coordinate. The parameter $z_0$ can be identified as the $z$-height of the feature of interest and $x_0$ describes a composite effect of the other influences that displace the center of rotation.
from the optical axis. $x_0$ can take values of up to several µm even if the image shift was set to zero before starting the experiment.

C. Least-squares fitting of calibration curves

For showing that the measured image shifts and defocus changes in a TEM actually describe a rotation of the image feature around the $y$-axis of the image, we have applied statistical parameter estimation techniques to two sets of calibration curves.

The first set corresponds to a situation were $x_0$ is close to zero (recorded at stage position $x = 3.49$ µm, $y = -12.48$ µm); in the second set $x_0$ was about 9 µm ($x = -198.47$ µm, $y = 11.03$ µm; Fig. 3). Before taking each data set the sample was set to eucentricity. In one set the $z$-height of the holder was varied from eucentricity $+3$ µm to eucentricity $-3$ µm, in the other from $+3$ µm to $-4$ µm (both with increments of 1 µm). Note especially the large change in defocus for the second data set. We could not find a direct connection between stage position and difference in the $x_0$ parameter and we conclude that the difference was mainly due to the (unregistered) initial image-shift settings. Therefore pre-calibration curves acquired for a certain stage position cannot be applied directly to the acquisition of a tilt series at another stage position if the initial image-shift settings are not identical.
Fig. 3. Calibration curves acquired from −60° to +50° at two stage positions. (A) and (C) are absolute $x$-image shifts, (B) and (D) are absolute defocus changes. The left set corresponds to a situation were $x_0$ is close to zero (recorded at stage position $x = 3.49$, $y = −12.48$ µm), in the right set $x_0$ was about 9 µm ($x = −198.47$, $y = 11.03$ µm). Each set consists of several curves that were recorded with different $z$-height settings of the goniometer. The nominal values from top to bottom as indicated by the Tecnai user interface were $z = 2.98, 1.97, 0.98, 0.02, −0.94, −1.94, −2.96$ (left) and $z = 2.98, 2.07, 1.04, 0.05, −0.94, −1.94, −2.95, −3.93$ (right) µm.

A least squares fit to the modelled image movement can be found with:

$$\text{measurement}(\alpha, x_0, z_0) = x_0 \cos(\alpha) + z_0 \sin(\alpha) + c$$

$$\begin{bmatrix} x_0 \\ z_0 \\ c \end{bmatrix} = \left( \begin{bmatrix} \sum \cos(\alpha)^2 & \sum \sin(\alpha) \cos(\alpha) & \sum \cos(\alpha) \\ \sum \sin(\alpha) \cos(\alpha) & \sum \sin(\alpha)^2 & \sum \sin(\alpha) \\ \sum \cos(\alpha) & \sum \sin(\alpha) & \text{number measurements} \end{bmatrix} \right)^{-1} \left( \begin{bmatrix} \sum \cos(\alpha) \text{measurement} \\ \sum \sin(\alpha) \text{measurement} \\ \text{number measurements} \end{bmatrix} \right)$$
Table 1

<table>
<thead>
<tr>
<th>Estimates (µm) from all data</th>
<th>Data set one</th>
<th>Data set two</th>
</tr>
</thead>
<tbody>
<tr>
<td>( x_0 )</td>
<td>0.08, 0.18, 0.25, 0.48, 0.40, 0.46, 0.54</td>
<td>8.98, 8.67, 8.79, 8.71, 8.98, 9.01, 9.03, 9.16</td>
</tr>
<tr>
<td>( z_0 )</td>
<td>2.43, 1.54, 0.7, -0.17, -1.28, -2.20, -3.03</td>
<td>2.71, 1.62, 0.7, -0.22, -1.12, -2.01, -2.91, -3.82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Estimates (µm) from 3 points</th>
<th>Data set one</th>
<th>Data set two</th>
</tr>
</thead>
<tbody>
<tr>
<td>( x_0 )</td>
<td>0.09, 0.35, 0.40, 0.70, 0.51, 0.63, 0.84</td>
<td>9.20, 9.13, 9.25, 9.27, 9.32, 9.52, 9.64, 9.76</td>
</tr>
<tr>
<td>( z_0 )</td>
<td>2.47, 1.58, 0.73, -0.14, -1.25, -2.15, -2.97</td>
<td>2.78, 1.72, 0.80, -0.12, -1.04, -1.89, -2.80, -3.70</td>
</tr>
</tbody>
</table>

Table 1 shows the parameters that were calculated from the measured \( x \)-shifts for the first and second data set. Note that the value for \( x_0 \) was found to vary slightly between measurements at different \( z \)-heights.

Substituting the estimated values of \( x_0 \) and \( z_0 \) in the mathematical model, we can compute the difference between the measured curves and the estimated curves. As differences are small, we have displayed the relative shifts (changes between images of successive tilt angles) rather than the absolute \( x \)-shifts. Fig. 4 shows the measured data (top), the modelled data (middle) and the difference (bottom) for the two data sets. Note that the difference is just in the order of tens of nm, indicating that the stage does rotate in a predictable manner and that the movement is highly accurate. The difference is probably caused mainly by the characteristic properties of the stage.

The statistical estimation of parameters can be exploited in different ways. When an estimate of the parameters (the distance from eucentric height, the initial image shift) has been performed after a first measurement, it would be possible to adapt those parameters on the microscope. The goal would be that less image movement and less focus change will occur when the specimen is tilted.
Fig. 4. (A) and (D) Relative x-image shifts between tilt angles, extracted from the curves shown in Fig. 3. (B) and (E) Relative shifts, calculated from a mathematical model whose parameters were given by least squares fitting of the measurement. (C) and (F) Mean value and standard deviation of differences between measured and modelled data.
Finally, as it was shown, the parameters $x_0$ and $z_0$ can even be estimated quite well from just three measurements. When we define $\Delta x(+\alpha)$ as the $x$-shift occurring between angles $+\alpha$ and $0^\circ$ and $\Delta x(-\alpha)$ the $x$-shift between angles $-\alpha$ and $0^\circ$, then $x_0$ and $z_0$ can be calculated from:

$$x(-\alpha, x_0, z_0) = z_0 \cdot \sin(-\alpha) + x_0 \cdot \cos(-\alpha)$$

$$x(0, x_0, z_0) = x_0$$

$$x(+\alpha, x_0, z_0) = z_0 \cdot \sin(+\alpha) + x_0 \cdot \cos(+\alpha)$$

$$\Delta x(-\alpha) = x(0, x_0, z_0) - x(-\alpha, x_0, z_0)$$

$$\Delta x(+\alpha) = x(0, x_0, z_0) - x(+\alpha, x_0, z_0)$$

$$x_0 = 0.5 \cdot (1 - \cos(\alpha)) \cdot (\Delta x(-\alpha) + \Delta x(+\alpha))$$

$$z_0 = 0.5 \cdot \sin(\alpha) \cdot (\Delta x(-\alpha) - \Delta x(+\alpha))$$

Table 1 shows the values that were calculated from the measured image shifts between $0^\circ$ and $\pm 30^\circ$ for the first and second data set mentioned above.

In principle, the parameters $x_0$ and $z_0$ can also be calculated from the defocus changes and in the general case, where the tilt axis is not identical to the $y$-axis, image shifts and defocus changes have to be used simultaneously to find the best fit to the measurement.

The model of the sample movement can further be used to predict the maximal image shift and can indicate up to what magnification the approach can be used. To ensure that a shift between images acquired before and after a tilt increment can be measured, the magnification has to be chosen such that the image size is four times (at best) larger than the shift to be measured. The maximal image shift for $x_0=10 \ \mu$m (corresponding to the curves with the largest shifts measured) for a tilt series starting at $-70^\circ$ and tilt-angle increments of $5/1^\circ$ is $0.8/0.16 \ \mu$m for $z_0=0 \ \mu$m. For $z_0=3 \ \mu$m the maximal shift is $0.9/0.18 \ \mu$m.
Table 2

<table>
<thead>
<tr>
<th>Magnification (kx)</th>
<th>Image size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCD camera</td>
</tr>
<tr>
<td>5</td>
<td>3.19</td>
</tr>
<tr>
<td>11.5</td>
<td>1.39</td>
</tr>
<tr>
<td>19</td>
<td>0.84</td>
</tr>
<tr>
<td>29</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Table 2 shows the CCD image size at different magnifications (2048 × 2048 CCD pixels of 14 µm and post-magnification factor 1.8×). The values show that when the z-height of the sample is kept within 3 µm of eucentricity, pre-calibration curves with a tilt-angle increment of 5° can be acquired up to a magnification of 5000×. According to the mathematical model it would not be necessary to acquire a pre-calibration curve up to a magnification of 19000× because the maximal x-shifts could still be detected at that magnification provided that the tilt increment is not larger than 1°.

D. Reproducibility of calibration curves

It has been shown that the movement and defocus change that an image feature undergoes while being tilted from one angle to another can be explained by a simple rotation and that consequently the displacement of the image feature from the center of the rotation in x- and z-directions can be determined and corrected for from these changes.

The applicability of this approach is closely related to the accuracy that is expected of the pre-calibration method. This depends on the envisioned magnification and if the aim is to use the method merely to avoid having to determine the shifts at low magnification or to avoid any measurements at the acquisition magnification during data acquisition.

To determine the fine structure and reproducibility of calibration curves, measurements were performed without initial image shift and close to eucentricity.
Eight measurements were recorded at a single stage position, four with increasing and four with decreasing tilt-angle increment for the normal and high-tilt holder (Fig. 5). $x_0$ and $z_0$ values were calculated from the measurements but have to be taken with care, because least squares fitting becomes less meaningful as the additional shifts due to unique stage characteristics reach the same order of magnitude as that imposed by the rotational movement.

For the tilt direction with increasing angles $x_0$ values of 0.9 and 0.8 µm were calculated for both the standard and high-tilt holder. This indicates that the displacement of the center of rotation is about 1 µm for the two holders tested and thus probably the accuracy to which the stage was assembled. $z_0$ has been determined to 0 and 0.2 µm. As already noticed during other measurements, the same parameters calculated from the defocus changes yielded slightly different values, 1.8 and 1.1 µm for $x_0$ and 0.9 and 0.5 µm for $z_0$. Thus the $z$-change of an image feature seems to follow a rotation around a slightly different center. This implies furthermore that either image $x$-shift or defocus change could be minimized by accordingly adjusting initial image shift and $z$-height. During further experiments it was shown that by doing so the maximal relative and absolute $x$-image shift could be reduced to about 20 and 200 nm, respectively, while the absolute defocus change still reached 2 µm.

Fig. 5 displays the relative shifts of the 16 measurements. The relative shifts in $x$-direction were less than 100 nm and would have been even smaller if $x_0$ had been corrected before the measurement. The deviation in values was 10 nm, indicating to what accuracy the pre-calibration curves could be applied during the final tilt series for image acquisition. The similarity of the curves for the normal and high-tilt holders further implies that the shifts are a characteristic of the stage rather than of the holders. The difference in these curves could be ascribed to slightly different values in $x_0$ and $z_0$—the particular loading of the holder into the stage—rather than to the holders themselves. Possibly, one could extract the stage characteristics to avoid the necessity for any pre-calibration measurement.

Relative image $y$-shifts are generally insignificant (also error bars were too small to show), except for a sudden jump of 100 nm at 15° tilt angle (increasing tilt angles) or 0° (decreasing tilt angles). Note that this increase happens in the same $y$-direction for
Fig. 5. Mean value and standard deviation of eight calibration curves recorded with the normal (■) and high-tilt (◆) holder, when the image feature was close to eucentricity and with no initial image shift. The shifts/changes are relative values between tilt angles, which were effectively measured and need to be corrected for. (A)–(C) recorded from −65° to +65° (high-tilt holder) and −60° to +55° (normal holder). (D)–(F) Curves recorded for the same angular range but with opposite tilt direction. Values have been inversed to facilitate comparison.
both tilt directions. Thus there is an absolute displacement of an image feature of about 500 nm after first tilting the sample in one direction and then going back, if these shifts are not corrected. The relative defocus changes vary around 0.1 µm for all measurements. Further conclusions cannot be drawn, as the accuracy of defocus measurement was not better than about 200 nm due to the small magnification.

The overall conclusions from these measurements are that the pre-calibration of image shifts and defocus changes should be applied in two steps. First, to predict and correct $x_0$ and $z_0$ at a low magnification from a few measurements. Second, to record the changes in detail to an accuracy of 10 nm (if this accuracy is needed) at the envisioned magnification for data acquisition (mainly to accurately determine defocus changes). The remaining task for the software during the acquisition of a tilt series would possibly be to correct for drift, which may well be present when a cryo-holder is used.

E. Software implementation

The current generation of transmission electron microscopes from FEI/Philips Electron Optics (Tecnai series of TEM) allows a much higher degree of remote control than previous TEM models. This is caused by the fact that the whole logistics of the microscope (e.g. control of currents) are performed by the Temserver, a software program (service) that runs on a PC under the Windows NT operating system (at the time of writing). The procedure for operating the microscope consists of logging in to the PC and starting the Tecnai User Interface, a software program (application) that presents functionality to the microscope operator and that communicates with the Temserver. In addition, the most often accessed microscope functions, such as change of magnification or defocus, are controllable through buttons and knobs on panels left and right of the column. Furthermore, the Windows Scripting Host, which is part of the operating system, can act as a means to glue various programs or components (COM objects) together. The Tecnai User Interface, for instance, is a COM object that communicates with the Temserver. Another one is Tecnai Scripting, which interfaces between scripting languages and the Temserver. This software set-up implies that it is possible to build a separate COM object to control the microscope in some particular manner. For instance,
Pre-calibration electron tomography

**Fig. 6A.** Hardware and software set-up utilized in our laboratory to implement pre-calibration electron tomography. Contributions from different parties are displayed in different shades of gray. Our contribution comes in form of a JavaScript program (see label Utrecht).

A few lines of the JavaScript programming language placed in a simple HTML page and viewed with a web browser will be sufficient.

A full-featured tomography data acquisition program has to be able to carry out several functions that are not mere microscope control functions and (so far) not available by the FEI/Philips software. The functions are: control of CCD cameras, display of images, image processing and creation of user interface elements. To have access to this additional functionality, we have chosen to implement tomography as a macro (component) in TIA (Tecnai Imaging and Analysis, Emispec), a program that provides these missing functions. Furthermore, TIA is able to host COM objects and script macros and can thus execute JavaScript programs. We have chosen to write the component in the JavaScript language, widely used and easy to learn. Fig. 6A shows an overview of the software architecture.

A first version of the tomography component for TIA is available for download from our web site (http://www.bio.uu.nl/mcb/3dem > Scripting > OpenTomography > Download the latest JavaScript code). We have named it OpenTomography, indicating that the source code of the software is freely available and with the possibility to modify it. OpenTomography is available under the GNU Public License and is distributed by the 3D EM group at Utrecht University. It contains essential parts for collecting a tilt series on a FEI/Philips Tecnai TEM. The current version was tested at various microscope set-ups, at Utrecht University (a Tecnai 20 LaB₆ with TVIPS 2k × 2k slow-scan CCD camera) and the FEI/Philips research
laboratories (e.g. a Tecnai 12 BioTwin with GIF, a Tecnai 20 FEG with GIF).

In our opinion, the ideal open source code for the further development of electron tomography should be based on as few commercial software components as possible. The code should therefore be as generic as possible to enable the end-user to decide which non-commercial (or commercial) software to use to deliver CCD control, image display and user interface. It is therefore perfectly feasible to imagine a future version of open source code tomography to use a straightforward web page as user interface and a dedicated mathematical package (e.g. MRC libraries) for image processing.

Fig. 6B gives a comparison between the conventional data acquisition approach and the pre-calibration approach. The left-most column shows the timing for the conventional approach. Acquiring a data set will take 103 min, of which 50 are needed for switching the magnification. For the pre-calibration approach, the timing for four different data collection modes is shown. The column second from the left shows the timing when additional focusing and tracking is carried out (for the highest accuracy). The total time will be 60 min: for taking a pre-calibration curve (6 min), for the actual tilt series (18 min) and for the additional focusing and tracking during data collection (36 min). The middle column shows the timing when additional tracking is carried out. The total time will be 36 min: for taking a pre-calibration curve (6 min), for the actual tilt series (18 min) and for the tracking (12 min). The column second from the right shows the timing for taking a tilt series, together with a calibration curve. The total time will be 24 min: for taking a pre-calibration curve (6 min) and for the actual tilt series (18 min). The right-most column shows the timing for taking the tilt series only. The total time will be 18 min, 5.9 times faster than the conventional approach. In the comparison it is assumed that the hardware used to carry out the experiments is identical. A tilt series is supposed to consist of 151 images (with 1° tilt increment) and that 31 images are needed for the calibration curve images (with 5° tilt increments). Furthermore, it is assumed that a magnification switch requires 10 s of idle time before all currents in the lenses are stable and that 3 s are needed before the specimen is sufficiently stable after a specimen tilt-angle increment. Also, it is assumed that 1 s exposure time is required for the images of the tilt series (the data) as well as for the images of microscope control (focusing,
Fig. 6B. Comparison between the conventional data acquisition approach and the pre-calibration approach. Currently, our implementation of pre-calibration tomography acquires a tomographic data series with 151 images in 20 min (current version of software and PC). This is a six-fold increase in the speed of the tracking step compared to the conventional implementation where switching forth and back to a small magnification is necessary to detect large image shifts.

tracking). It is assumed that 3 s are needed to transfer and display each individual image and that a cross-correlation will take 1 s.

F. Example Reconstructions

To illustrate the application of pre-calibration electron tomography we next discuss the reconstruction of two tilt series that were acquired with the described set-up. In these early applications we did not yet fully exploit the potential of the method, but merely took advantage of the possibility to eliminate magnification switches.

As one experiment in a series to elucidate the formation of cavities in zeolite crystals under different preparation methods (Janssen et al., 2001; also see the addendum to this chapter), 151 projections (-75° to +75°) of a crystal were acquired. The understanding of the growth mechanism is important as cavities influence the catalytic activity of zeolite crystals. A slice through the reconstructed volume clearly displays cavities of different sizes in the structure (Fig. 7A). Fig. 7B shows a surface rendered view of the zeolite.

A total of 121 projections (-69° to +51°) of a semi-thick section of high-pressure frozen and freeze-substituted murine dentritic cells were acquired. Slices through a part of the reconstructed volume display the organization of the lamellar compartments of a mitochondrion (Fig. 7C, top xz-, bottom xy- and right zy-slice). Fig. 7D shows a surface rendered view.
Discussion and conclusions

While tilting samples in our Tecnai 20 transmission electron microscope, we measured relative image shifts between images recorded at different tilt angles of up to about 1 µm and absolute image shifts up to about 6 µm for tilt series from −60° to +60°, 5° angle increments and a z-position of the specimen holder 3 µm away from eucentricity. The absolute defocus changes reached values up to about 20 µm. This measurement was not done under accurately specified initial image-shift conditions. When no image shift was induced prior to the experiment and for a z-position close to eucentricity the relative and absolute image shifts were less than 100 nm and 500 nm, respectively. The shifts can further be decreased to about 20 nm and 200 nm, respectively, by applying a compensating initial image shift. We investigated shifts and defocus changes under different conditions to determine if they can reliably be detected by a pre-calibration measurement. It was shown that the measured calibration curves are different for different distances from eucentricity, different for different imaging conditions (image-shift settings), reproducible to an accuracy of about 10 nm for the relative image $x$-shift between tilt angles if acquired under the same conditions. These observations indicate that changes can reliably be detected by a pre-calibration measurement, but that a pre-calibration measurement cannot be directly used to predict the movements under other (unknown) conditions.

It was also shown that the overall shape of measured calibration curves could be explained mathematically by a rotation of the specimen holder around a fixed axis. This implies that the measured image shifts of an image feature will be the changes in the $x$- and $y$-coordinate and that its defocus change corresponds to the change in the $z$-coordinate. Consequently, the unknown parameters of the movement can be approximated from the image shifts between a few tilt angles by least squares fitting and in most cases even three tilt angles would be sufficient. The differences between measured and mathematically predicted calibration curves were small, in the order of tens of nm, and can be regarded a characteristic of the stage, as similar results were obtained for a normal and an ultra-high-tilt sample holder.

One could envision several scenarios for the implementation of pre-calibration measurements. (A) For the best performance regarding accuracy (and the worst
Pre-calibration electron tomography

Fig. 7. Two reconstructions calculated from images acquired with pre-calibration electron tomography. (A) and (B) a zeolite crystal. (A) Slice through the volume, scale bar 100 nm. (B) Surface rendered view. (C) and (D) part of a mitochondrion in a semi-thick section (150 nm) of high-pressure frozen and freeze-substituted murine dendritic cells. (C) Slices through the volume (top xz-, bottom xy- and right zy-plane). (D) Surface rendered view.

regarding acquisition speed) the parameters of the rotational movement should be estimated from a few measurements (e.g. $-30^\circ$, $0^\circ$, $+30^\circ$ at low magnification) and consequently be corrected for. Next, a full pre-calibration curve can be measured (e.g. with $5^\circ$ increments at high magnification) to detect remaining image shifts of a few tens of nm with an accuracy of about 10 nm. Finally, during data acquisition (e.g. with $1^\circ$ increment at high magnification), additional cross-correlation of images can ensure high accuracy in case of e.g. drift. (B) If relatively large image shifts during data acquisition do not occur because, e.g. the absence of drift, a single
pre-calibration measurement (e.g. with 5° increments at low magnification) together with a moderate number of drift corrections should be sufficient (and will take a few minutes less) to ensure proper data acquisition. (C) In a lot of cases at, e.g. moderate magnifications, the image feature of interest set close to eucentricity and the knowledge that the distance of the tilt to the optical axis is small, it will be possible to bypass the measurement of a pre-calibration curve and estimate (and adapt) the unknown parameters of the mathematical model while collecting a tilt series (‘on the go’, which would save some more minutes).

We implemented a first version of pre-calibration electron tomography as a JavaScript macro (available for download, see section E) in the Tecnai Image and Analysis software (TIA). In the current version of pre-calibration electron tomography software, we did not implement procedures suitable for cryo electron tomography applications. Nevertheless, the pre-calibration approach can be extended with steps, which (1) compensate for disturbing effects due to image drift (which often occur with a cryo holder) and (2) that ultra-low-dose data collection can be carried out by taking a different area on the sample for recording a tilt series from the area for measuring the pre-calibration curve.

High-throughput collection of tilt series could be combined with advanced automated relocation approaches (Potter et al., 1999; Pulokas et al., 1999; Carragher et al., 2000): (1) specific structures are identified on a microscope grid (manually or automatically) of which tilt series are to be required; (2) the specified structures on the grid are automatically relocated and centered by (xy-) stage-movements; (3) measurement and compensation of image shifts and defocus changes by pre-calibration; (4) from each structure a tilt series is collected.

Three main advantages of our novel method of data collection for automated electron tomography based upon pre-calibration of image shifts and defocus changes are:

1. Identification of the displacement of the tilt axis from the optical axis and thus the possibility for compensation of any image shifts and defocus changes that are caused by this misalignment. Remaining shifts due to intrinsic characteristics of stage/specimen-holders can also be pre-measured and pre-corrected, enabling high-throughput during data acquisition, which allows users to concentrate on
sample preparation, setting up the (biological) essay and the discussion of results. At the time of writing, the microscope hardware (i.e. the Tecnai PC) could acquire 151 images (with 1° increment) in less than 20 min. On the same hardware, data collection with the conventional approach takes more than 100 min.

2. The quality of a tilt series can be significantly enhanced, because the amount of required focus-change compensation during data acquisition can be minimized. The alignment of the optical axis to the tilt axis of the specimen stage can be achieved by invoking an amount of image shift (as deduced from the mathematical model describing the effect of specimen tilt). Less focus change during data collection will result in (1) less image rotation and (2) less magnification change. As a consequence, practical implementation of ‘on-the-fly’ automated data acquisition, alignment and 3D reconstruction procedures (which do not depend on the presence of fiducial markers in the images to correct for magnification changes and image rotation due to focus changes) has become within reach.

3. The uncoupling of microscope-control calibration from data acquisition opens the field for novel acquisition modes. One could, for instance, acquire a first tilt series with large tilt increments (for e.g. a first reconstruction at reduced resolution) and then include the images at intermediate tilt angles successively. The usage of acquisition modes other than bright-field and detectors other than a CCD camera or film becomes possible. For instance, taking tilt series in STEM mode, possibly in combination with EDX or electron energy loss spectroscopy to gain element specific information of the sample, could have important applications in biological and materials sciences. In biological sciences, ultrasmall gold labels tagged to the molecular structure of interest, e.g. embedded in thick (high-pressure frozen, freeze-substituted) stained and plastic-embedded sections (Starink et al., 1995; Humbel et al, 1998) could be imaged in 3D with high contrast and possibly be distinguished from metal-labels of a different element (nickel, silver). For materials sciences, 3D structural maps, which cannot be gained by TEM bright-field imaging because of diffraction contrast, becomes possible by high angular annular dark-field Z-contrast imaging (Midgley et al, 2001) and high-resolution 3D elemental mapping by EFTEM imaging (Weyland et al, 2001).
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Addendum: Applications—Zeolites and Golgi complex

Zeolites

Electron tomography is not limited to the biological sciences. Any sample that can be prepared for transmission electron microscopy (TEM) and that satisfies the condition that the registered image contrast must be a projection of a physical characteristic of the sample can be investigated. As such, electron tomography is a useful tool to study the three-dimensional (3D) architecture and composition of inorganic solids (e.g. solid catalysts, photonic crystals and electronic devices), hundreds of nm in size at nm-scale resolution, complementing other techniques in the characterization of such materials (Koster et al., 2000).

Zeolites are key catalytic materials in applications such as hydro-isomerization of alkanes and hydrocracking of heavy petroleum fractions. One of the features of zeolites is the molecular dimension of their micropores, which are often advantageous to induce shape selectivity, but enhanced accessibility is frequently desirable to restrict mass transfer effects and/or allow catalytic conversion of larger molecules. Several approaches have been followed to enhance accessibility; i.e., the development of zeolites with intrinsically larger pores (Freyhardt et al., 1996), delaminated zeolite precursors (Corma et al., 1998), and the use of zeolite nanocrystals (Mintova et al., 1999). Besides accessibility, also the location of the catalytic active phase (e.g. metal particles) on the zeolite is important for the performance of the catalyst.

We have applied electron tomography to the study of several of these materials. For instance, we have shown that metal particles in zeolite crystals (Ag/NaY) can readily be located by electron tomography (Koster et al., 2000). As the location of the metal function inside or outside the micropores can strongly affect the selectivity of the catalyzed reaction, knowledge on the location of the metal function is crucial to steer catalyst synthesis (Maschmeyer et al., 1995; Creyghton et al., 1996). In another example we have visualized mesopores (see Figs. 7A and 7B; Koster et al., 2000; Janssen et al., 2001 and 2002) in zeolite crystals. It has been established that microporous zeolite crystals can be treated (acid leaching and/or steaming) to generate mesopores that greatly enhance accessibility and thereby catalytic activity.
and stability (Alfredsson et al., 1993; Choi-Feng et al., 1993; Sasaki et al., 1995 and 1998; Meima, 1998).

Certain inorganic solids can be unsuitable to investigate by bright-field transmission electron tomography as they show angle dependent diffraction contrast due to their crystalline structure and thus do not adhere to the condition that the recorded image contrast must be a projection of their mass density according to the tilt angle. However, it has been shown that the problem can be overcome when these materials are investigated by high angular annular dark-field (HAADF) scanning transmission electron microscopy (STEM) instead of TEM tomography (Midgley et al., 2001).

**Golgi complex**

Cells are highly dynamical and extremely complex 3D objects. Understanding how cells function requires knowledge of cellular structures and organelles in time and space. A truly 3D analysis of cellular structures with a spatial resolution in the nm-range is only possible by electron tomography. The technique is particularly useful in the analysis of polymorphous organelles that may have sizes up to several hundred nm.

One of these organelles is the Golgi complex. In the classical vesicular transport model, the transport carriers are proposed to be small vesicles (50–100 nm in diameter) that bud from donor and fuse with acceptor organelles (Rothman and Wieland, 1996). Recently however, it has become apparent that also non-vesicular structures mediate membrane transport. For example, large supramolecular cargoes move through the Golgi complex without entering carrier vesicles (Bonfanti et al. 1998), and the endoplasmic reticulum (ER) to Golgi (Fig. 8; Geerts et. al, 2002) and Golgi to plasma membrane transport carriers are large (saccular) tubular structures instead of small vesicles (Polishchuk et al., 2000; Mironov et al., 2001). A unifying vesicular paradigm for transport carriers is therefore no longer sustainable and the structure and dynamics of the transport carriers that mediate intracellular membrane traffic need to be carefully re-examined using electron tomography.
Fig. 8. (A) Electron micrograph of the Golgi complex in a 200 nm thick section of nocodazole treated normal rat kidney (NRK) cells. (B) Model gained by drawing the outlines of two cisternae (left) and the connections between the ‘vesicles’ (right) of the vesicular tubular cluster (VTC) of the 3D reconstruction of the Golgi complex shown in (A).