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Simultaneous determination of sample thickness, tilt, and electron mean free path using tomographic tilt images based on Beer–Lambert law

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ABSTRACT

Cryo-electron tomography (cryo-ET) is an emerging technique that can elucidate the architecture of macromolecular complexes and cellular ultrastructure in a near-native state. Some important sample parameters, such as thickness and tilt, are needed for 3-D reconstruction. However, these parameters can currently only be determined using trial 3-D reconstructions. Accurate electron mean free path plays a significant role in modeling image formation process essential for simulation of electron microscopy images and model-based iterative 3-D reconstruction methods; however, their values are voltage and sample dependent and have only been experimentally measured for a limited number of sample conditions. Here, we report a computational method, *tomoThickness*, based on the Beer–Lambert law, to simultaneously determine the sample thickness, tilt and electron inelastic mean free path by solving an overdetermined nonlinear least square optimization problem utilizing the strong constraints of tilt relationships. The method has been extensively tested with both stained and cryo datasets. The fitted electron mean free paths are consistent with reported experimental measurements. The accurate thickness estimation eliminates the need for a generous assignment of *Z*-dimension size of the tomogram. Interestingly, we have also found that nearly all samples are a few degrees tilted relative to the electron beam. Compensation of the intrinsic sample tilt can result in horizontal structure and reduced *Z*-dimension of tomograms. Our fast, pre-reconstruction method can thus provide important sample parameters that can help improve performance of tomographic reconstruction of a wide range of samples.

1. Introduction

In recent years, cryo-electron tomography (cryo-ET) has emerged as a powerful imaging technique that allows 3D visualization of sub-cellular architecture and macromolecular organization in a near-native and fully frozen-hydrated state. Cryo-ET circumvents the deleterious effects from fixation, dehydration or staining (Frank, 2006; van Heel et al., 2000). This technique bridges the gap of knowledge between cellular architecture revealed by low resolution light microscopy and high resolution structures of macromolecular complexes by single particle cryo-EM.

To prepare samples for cryo-ET, a commonly used technique is plunge freezing in which the sample solution is deposited onto a holey-carbon coated grid, blotted with filter paper, and vitrified by rapidly plunging into a cryogen (e.g. liquid ethane) cooled by liquid nitrogen (Adrian et al., 1984; Dubochet et al., 1988). However, this method has poor control of thickness of the resulted vitreous ice. Another technique for cryo-sample preparation is cryo-sectioning in which the high pressure frozen thick specimen is trimmed using a diamond knife. Unfortunately, previous studies have shown that there is considerable variation in section thickness, especially for thin sections (Luther, 2006). Focused ion beam (FIB) milling has gained considerable acceptance in recent years as a precision section preparation method. However, slice thickness by FIB still has unignorable variations due to differential thermal expansion (Boergens and Denk, 2013), charging (Jones et al., 2014; Schaffer et al., 2007), stage movements (Korte et al., 2011) or ion beam instabilities (Jones et al., 2014).

To obtain a high quality tomogram, it is essential to use volumes with sufficiently large *Z*-dimension to contain the entire sample. Due to lack of both thickness control during sample preparation and a method for reliable estimation of sample thickness, it

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is a common practice to use a large Z-dimension for 3-D reconstruction. Alternatively, a trial reconstruction of a small region can be used to first determine the thickness before a full reconstruction is performed. However, the poor image contrast and the significant smearing along Z-axis due to missing wedge often render the sample boundaries hard to detect. It is thus desirable to have a method to reliably determine sample thickness to allow 3-D reconstruction with optimal Z-dimension, which will not only eliminate the need for trial reconstruction but also improve the speed of reconstruction by reducing the tomogram volume to the smallest possible Z-dimension. Current methods for thickness measurement include measuring the shift of top/bottom surface projections of a small cylindrical hole through sample in a tilt pair image (Cheng et al., 2006), image contrast between open and sample area (Cheng et al., 2006), and electron energy loss spectroscopy (Cho et al., 2013; Malis et al., 1988). The first method is destructive to the sample while the last two methods rely on an accurately known electron mean free path (Zhang et al., 2012). Unfortunately, the mean free path is dependent on accelerating voltage and sample types, and can vary significantly as shown by drastically different values obtained by experimental measurements for a few sample conditions (Feja and Aebi, 1999; Grimm et al., 1996; Vulovic et al., 2013). Different elemental compositions of the sample, for example, samples stained with heavy metals vs cryo biological samples mostly composed of low atomic number elements, can lead to a large change of mean free path (Langmore and Smith, 1992; Wall et al., 1974). It is desirable to have a convenient method that can reliably estimate the mean free path of every sample.

It is a common assumption in cryo-EM that the specimen plane is horizontal and thus perpendicular to the electron beam. However, unintended sample tilts have often been observed due to multiple factors, including undulation of the carbon support film (Mindell and Grigorieff, 2003), instability of the sample holder mechanics, and insufficient reproducibility of goniometer (Houben and Bar Sadan, 2011). These residual sample tilts were not detectable during data acquisition, which would result in systematic errors to the tilt angles assigned to all images in a cryo-ET tilt series. The residual tilt, if not corrected, will lead to tilted structure in the 3-D tomogram that requires larger Z-dimension to fully contain the structure and larger computing resource for reconstruction. For some specimens, for example, stained sections with gold beads coated on both surfaces, the 3-D geometric model of the fiducial markers obtained from alignment of the whole tilt series can be used to determine/correct the residual sample tilt (Kremer et al., 1996). However, this approach will not be applicable to most cryo-ET samples without markers or with fiducial markers randomly distributed in the sample solution (Hayashida et al., 2014; Winkler and Taylor, 2006).

In this study, we describe a computational approach that can simultaneously estimate sample thickness, tilt and inelastic mean free path using only the tilt images already collected for cryo-ET without need for additional data. This new approach employs a mathematical model derived from the Beer–Lambert law and estimates these parameters as a solution of a multi-variable overdetermined nonlinear least square problem with strong constraints provided by unique geometric relationships among the serial tilts of a common structure.

2. Methods

2.1. Mathematical model for thickness determination

The relationship between sample thickness and beam intensity can be represented by Eq. (1) based on the Beer–Lambert law

$$\frac{d_e}{\lambda_{\text{in}}} = \ln\frac{I_0}{I_{\text{exit}}} \tag{1}$$

where d_e represents the effective thickness which is the distance that the electron beam travels through the specimen, λ_{in} represents the mean free path for inelastic scattering, I_0 represents the intensity of the incident electron beam on the specimen and I_{exit} represents the intensity of the electron beam exiting the specimen and hitting the detector.

It is obvious that the effective thickness d_e in Eq. (1) varies when the sample is tilted. Assuming the specimen is placed in an arbitrarily tilted plane in 3D space before serial tilting, we define the residual sample tilt γ_0 as the angle between the normal vector of this plane and the Z-axis (i.e. direction of electron beam). Here, the effective thickness d_e for each tilt can be described in Eq. (2) by taking the nominal tilt angles (i.e. intended tilt angles during data collection) and residual sample tilt into consideration (Appendix)

$$d_e = \frac{d_0 \cdot \cos\theta_0}{\cos\gamma_0 \cdot \cos(\theta_0+\theta)} = d_0 \cdot \frac{\cos\theta_0 \cdot \sqrt{\tan^2\theta_0 + \tan^2\alpha_0 + 1}}{\cos(\theta_0+\theta)} \tag{2}$$

where d_0 represent the absolute geometric thickness of the specimen, θ represents the intended tilt angles around Y axis, θ_0 and α_0 represent the residual sample tilt around Y and X axis, respectively, and they can be measured via the corresponding side views of the 3D reconstruction.

Next, the exiting beam intensity I_{exit} can be expressed as Eq. (3) according to the linear relationship between I_{exit} and pixel values of images

$$I_{\text{image}} = A \cdot I_{\text{exit}} + B \tag{3}$$

where I_{image} represents the average pixel value of the targeted area in the image, A represents the gain factor of the detector. B represents the average pixel value when no electron hits the detector. Although it should always be zero, we found some corner cases in which the detector is not properly gain-normalized or the image pixel values are shifted post imaging during alignment of the tilt series. To make our method robust for all datasets, this B variable is included in our model as a nuisance parameter.

Hence, we can write our complete mathematical model for each tilt image as Eq. (4) by combining Eqs. (1)–(3).

$$\frac{d_0}{\lambda_{\text{in}}} \cdot \frac{\cos\theta_0 \cdot \sqrt{\tan^2\theta_0 + \tan^2\alpha_0 + 1}}{\cos(\theta_0+\theta)} = \ln\frac{A \cdot I_0}{I_{\text{image}} - B} \tag{4}$$

In Eq. (4), I_{image} and θ are known from tilt series. The 7 unknown parameters are I_0, d_0, θ_0, α_0, λ_{in}, A and B. The 4 parameters of interest in our study are d_0, θ_0, α_0 and λ_{in} while the remaining three are nuisance parameters. In our case, this is a vastly overdetermined system since the number of equations (the number of selected regions in each image × the number of tilt images) is much larger than the number of unknowns (7) in the model.

2.2. Parameter determination as a constrained nonlinear least square problem

In order to obtain the solution of this overdetermined least square problem, we minimize the scoring function defined in Eq. (5)

$$f(I_0, d_0, \theta_0, \alpha_0, \lambda_{\text{in}}, A, B) = \sum_{j=1}^{N}\sum_{i=1}^{M}\left[\ln\left(\frac{A \cdot I_0}{I_{\text{image}}(i,j) - B}\right) - \frac{d_0}{\lambda_{\text{in}}} \cdot \frac{\cos\theta_0 \cdot \sqrt{\tan^2\theta_0 + \tan^2\alpha_0 + 1}}{\cos(\theta_0+\theta(i))}\right]^2 \tag{5}$$