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Implementation of a cryo-electron tomography tilt-scheme optimized for high resolution subtomogram averaging

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ABSTRACT

Cryo-electron tomography (cryoET) allows 3D structural information to be obtained from cells and other biological samples in their close-to-native state. In combination with subtomogram averaging, detailed structures of repeating features can be resolved. CryoET data is collected as a series of images of the sample from different tilt angles; this is performed by physically rotating the sample in the microscope between each image. The angles at which the images are collected, and the order in which they are collected, together are called the tilt-scheme. Here we describe a "dose-symmetric tilt-scheme" that begins at low tilt and then alternates between increasingly positive and negative tilts. This tilt-scheme maximizes the amount of high-resolution information maintained in the tomogram for subsequent subtomogram averaging, and may also be advantageous for other applications. We describe implementation of the tilt-scheme in combination with further data-collection refinements including setting thresholds on acceptable drift and improving focus accuracy. Requirements for microscope set-up are introduced, and a macro is provided which automates the application of the tilt-scheme within SerialEM.

1. Cryo-electron tomography and subtomogram averaging

In cryoET (Frank, 2008), a plunge frozen specimen is tilted using a rotating specimen stage inside a transmission electron microscope (TEM) and projection images are acquired onto a camera at discrete tilt angles. This series of tilted images can then be computationally reconstructed into a tomogram, i.e. the three-dimensional representation of the field of view. Collection of a tilt series is performed by iterative tracking, focusing and imaging steps for each tilt. Free and commercial software packages are available that allow fully automated acquisition of multiple tilt series (Mastronarde, 2005; Suloway et al., 2009; Zheng et al., 2010). Tomogram reconstruction involves tilt image alignment and 3D reconstruction. Tilt image alignment includes refinement of tilt-axis angle and tilt angles, determination of image shifts, and accounting for beam-induced sample deformation. These steps are performed computationally using software such as IMOD (Mastronarde, 1997).

The interpretable information in a cryo tomogram is limited by poor signal-to-noise ratio (SNR). However, objects present in multiple copies within the tomogram can be further analyzed by extracting them as sub-volumes. These sub-volumes can then be iteratively aligned and averaged to obtain reconstructions with improved SNR and more interpretable high-resolution features. This technique is called subtomogram averaging (for reviews see e.g. (Briggs, 2013; Förster and Hegerl, 2007; Schmid, 2011; Wan and Briggs, 2016)). Recent optimizations of data acquisition and processing have allowed the structures of protein complexes to be determined at subnanometer resolutions using subtomogram averaging (Bharat et al., 2015; Pfeffer et al., 2015; Schur et al., 2015a,b, 2013), and further optimization can be expected to lead to further improvements in the attainable resolution.

2. Tilt-schemes for cryo-electron tomography

According to the central slice theorem, the Fourier transform (FT) of each 2D projection image in the tilt series corresponds to a slice through the 3D FT of the volume being imaged. The thickness of these Fourier slices is inversely proportional to the thickness of the object in real space. Tomographic reconstruction can therefore be envisaged as filling Fourier space with a series of slices. The slab geometry of the sample limits the range of tilts that can be collected, often to ±60°. This limited range leads to a "missing wedge" of information in Fourier space; the real space effect is a deformation of structures parallel to the axis of missing information. Fourier space is also incomplete at higher resolutions because the tilt increment is not infinitely small; the Fourier slices may not

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have sufficient thickness to fill Fourier space at higher resolutions. The resolution to which the information is complete is related to the number of uniformly-distributed tilt-images by the Crowther criterion $m = \pi * D/d$ with number of tilt images m, particle diameter D and resolution d (Crowther et al., 1970).

In practice the distribution of information in Fourier space is complicated by three other factors. Firstly, at higher tilts the slab-shaped sample is thicker, leading to a larger number of non-elastic scattering events and a lower SNR. Secondly, frozen hydrated samples are sensitive to electron radiation damage. Higher resolution features are lost in images collected later in the tilt series due to accumulated electron dose. Thirdly, the sample may distort, bend or move as a result of exposure to the electron beam, such that the object imaged late in the tilt series is not truly identical to that imaged early in the tilt series. These factors mean that the distribution of information in Fourier space is dependent on the order and increment of angles at which the tilted images are collected, referred to as the tilt-scheme.

The ideal distribution of information in Fourier space, and therefore the preferred tilt-scheme, depends on whether the tomogram will be used for subsequent subtomogram averaging or not. If the tomogram is to be directly interpreted, it is preferable to distribute the information at the interpreted resolution as evenly as possible in Fourier space. If the tomogram is to be used for subtomogram averaging, Fourier space will be filled in the final reconstruction by averaging subtomograms that have different orientations relative to the electron beam. In this case it is usually possible to tolerate a larger missing wedge, and a larger tilt increment. Therefore, the aim when collecting data for subtomogram averaging is to maintain the maximum amount of high-resolution information while maintaining sufficiently complete low-resolution information to allow accurate 3D alignment of the subtomograms.

The most commonly used tilt-schemes fall into two groups: continuous tilt-schemes in which the tilt angle is rotated in one direction, e.g. from +60° to −60° (Fig. 1A); and bidirectional tilt-schemes, in which the tilt series acquisition is divided into two separate tilt branches, e.g. a continuous series from 0° to +60° in 3° increments is acquired first, then a second branch from −3° to −60° is acquired (Fig. 1B). Tilt increments typically range from 0.5° to 5°. Continuous tilt-schemes are mostly used for resin embedded samples. These samples suffer from mass loss when exposed to electron beam radiation and therefore deform during data acquisition. By acquiring the data in one sweep, these deformations will be gradual and can be tolerated in the tilt series alignment step. A bidirectional tilt-scheme is better suited to automated data acquisition as it does not require tilting and tracking to locate the targeted image area at high starting tilt angle before starting acquisition. A disadvantage is that the images on either side of the starting angle (0° in the above example) have different accumulated dose, and the resulting accumulated image deformations can make it difficult to align the two tilt branches (the jump-at-start problem). This error propagates into the tomogram where it is detrimental for accurate 3D alignment of the subtomograms, and for the quality of the final average.

3. An improved tilt-scheme for subtomogram averaging

The transfer of high-resolution information can be maximized by collecting the low-tilt images (where the sample appears thin) early in the tilt series, before radiation damage has accumulated. Our optimized tilt-scheme starts at zero degrees tilt, and moves up to the highest tilt in both tilt directions simultaneously. For example, for a 3° tilt step, the order of tilt angle acquisition is 0°, +3°, −3°, −6°, +6°, +9°, −9°, −12° ... (Fig. 1C). This “dose-symmetric” tilt-scheme concentrates high-resolution information in the lower tilts where the sample is thinnest, thus providing maximum information transfer (Fig. 1D). While similar information transfer can be obtained using bidirectional tilt-schemes with a longer first branch e.g. from −21 to 60°, these schemes still suffer from the jump-at-start problem (Figs. 1D and 2).

4. The dose-symmetric tilt-scheme has a number of additional advantages

- The accumulated electron dose, and therefore the beam-induced sample deformation, varies smoothly across the tilt series, eliminating the jump-at-start problem (Fig. 2).
- It allows a higher total electron dose to be applied, because accumulated radiation damage and accumulated sample deformation at higher tilts is a lesser concern.
- The maximum tilt-range can be used, and high-tilt/late images can be discarded at a later stage if appropriate without losing “good signal”, or images can be exposure filtered (Grant and Grigorieff, 2015) according to the accumulated dose.
- No microscope optics other than beam-image shift and defocus are changed during tilt series acquisition, in contrast to bidirectional tilt-schemes in which magnification and illumination are changed for low magnification tracking between tilt branches. This makes the tilt-scheme ideal for use with phase plates, where optical stability is crucial (Fukuda et al., 2015).

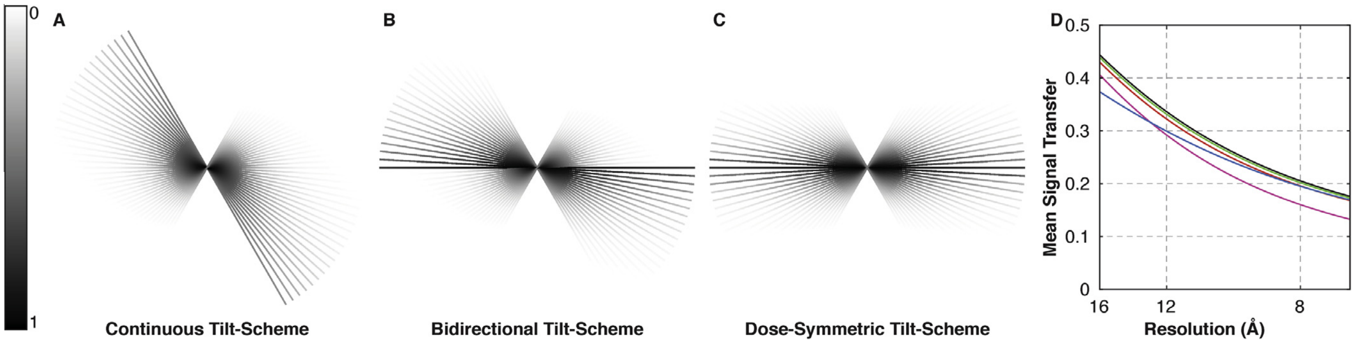

Fig. 1. Schematic showing information transfer for (A) continuous, (B) bidirectional and (C) dose-symmetric tilt-schemes. Tilts are shown from −60° to +60° in 3° increments for a total of 41 tilts. Grey values correspond to the information transfer at each tilt according to the color map shown on the left. The reduction of information transfer at high-tilts due to the increased apparent thickness of the sample is simulated by multiplication with the cosine of the tilt angle. The loss of high-resolution information due to accumulated electron dose is simulated by multiplication by low pass filters according to the measurements described in (Grant and Grigorieff, 2015) assuming constant exposure times. The dose-symmetric tilt-scheme shows optimized, near-symmetric information transfer. (D) Plot of mean signal transfer for different tilt-schemes: continuous (magenta); bidirectional starting at 0° (red); bidirectional starting at −21° (green); bidirectional starting at −21° in the case that the second branch is deleted before averaging to reduce impact of the jump-at-start problem (blue); dose-symmetric (black). Mean signal transfers are calculated as the mean of the signal transfer for all tilts within the tomogram.

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