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Review

Limiting factors in atomic resolution cryo electron microscopy: No simple tricks

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ABSTRACT

To bring cryo electron microscopy (cryoEM) of large biological complexes to atomic resolution, several factors – in both cryoEM image acquisition and 3D reconstruction – that may be neglected at low resolution become significantly limiting. Here we present thorough analyses of four limiting factors: (a) electron-beam tilt, (b) inaccurate determination of defocus values, (c) focus gradient through particles, and (d) particularly for large particles, dynamic (multiple) scattering of electrons. We also propose strategies to cope with these factors: (a) the divergence and direction tilt components of electron-beam tilt could be reduced by maintaining parallel illumination and by using a coma-free alignment procedure, respectively. Moreover, the effect of all beam tilt components, including spiral tilt, could be eliminated by use of a spherical aberration corrector. (b) More accurate measurement of defocus value could be obtained by imaging areas adjacent to the target area at high electron dose and by measuring the image shift induced by tilting the electron beam. (c) Each known Fourier coefficient in the Fourier transform of a cryoEM image is the sum of two Fourier coefficients of the 3D structure, one on each of two curved 'characteristic surfaces' in 3D Fourier space. We describe a simple model-based iterative method that could recover these two Fourier coefficients on the two characteristic surfaces. (d) The effect of dynamic scattering could be corrected by deconvolution of a transfer function. These analyses and our proposed strategies offer useful guidance for future experimental designs targeting atomic resolution cryoEM reconstruction.

1. Introduction

Single particle cryo electron microscopy (cryoEM) can provide the three-dimensional (3D) structure of a biological complex in its native environment. Recent progress has demonstrated that this technique is capable of determining 3D structures to near atomic resolution, allowing the building of backbones or even full atom models of biological complexes that include multi-subunit proteins (Ludtke et al., 2008; Cong et al., 2010; Zhang et al., 2010a), icosahedral viruses (Jiang et al., 2008; Yu et al., 2008, 2011; Zhang et al., 2008, 2010c; Chen et al., 2009; Liu et al., 2010; Wolf et al., 2010; Cheng et al., 2011), helical viruses (and portions of nucleic acid) (Ge and Zhou, 2011). Nevertheless, the current resolution of cryoEM is rarely sufficient to build atomic models of complexes, identify small molecules in the complexes (e.g., tightly bound water molecules or small ligands), resolve carboxyl oxygen atoms of the backbone of amino acids to determine peptide planes, differentiate amino acids with small side-chains, or determine the B-factor of the structures. Such detailed information is critical for studying and understanding the functional mechanisms of biological complexes.

For two-dimensional (2D) crystalline samples, electron crystallography has successfully achieved resolution better than 3 Å for aquaporin (Yonekura et al., 2003; Gonen et al., 2005). In contrast, for non-crystalline samples, for which single particle cryoEM must be used, the resolution of published structures has not reached a comparable level. Some of the limitations of the single-particle cryoEM technique have already been addressed by optimization of sample preparation (Grassucci et al., 2007; Zhou, 2008), use of an electron beam with better coherence (Zhou and Chiu, 1993), minimization of the magnification variation due to defocus during data acquisition (van Duinen et al., 2005), calibration of the magnification by using a standard sample (Olson and Baker, 1989), detection and correction for distortion of electron lens (Capitani et al., 2006), and improvement of particle alignment during image processing (Grigorieff, 2007). Nevertheless, as resolution of single particle cryoEM approaches atomic level, several other limiting factors that are related to the fundamental physics and optics of electron image formation begin to emerge.

Recent advances in high resolution single-particle cryoEM have been reviewed elsewhere (Grigorieff and Harrison, 2011; Zhou, 2011). This paper focuses on theoretical considerations about

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several of the most significant limiting factors at present for achieving even finer resolution in single particle cryoEM. These factors include the effects of beam tilt, inaccurate determination of defocus, defocus gradient across the depth of a specimen, and dynamic scattering.

2. Results and discussion

2.1. Electron beam tilt

2.1.1. Limitation imposed by beam tilt

To obtain high resolution reconstruction with cryoEM, the incident electron beam should be perfectly parallel to the optical axis, as any beam tilt introduces phase shift to images (Fig. 1A) (Smith et al., 1983). Beam tilt can originate from three sources in electron microscopy: the overall beam tilt relative to the optical axis ('direction tilt') (Fig. 1B), beam divergence by over-focus of the C2 condenser lens ('divergence tilt') (Fig. 1C), and the spiraling trajectory of electrons in the inhomogeneous magnetic lens ('spiral tilt') (Fig. 1D) (Loretto, 1984) [the latter two tilts are also known as local beam tilts since they are position-dependent (Christenson and Eades, 1988; Eyidi et al., 2006)]. Indeed, the 'total beam tilt' of images is the vector sum of direction tilt, divergence tilt and spiral tilt. The phase shift ($\Delta\varphi$) induced by beam tilt ($\vec{b}$) can be expressed as a function of spatial frequency (s) (Smith et al., 1983).

$$\Delta\phi = -2\pi(k^2 - D)\vec{k}\cdot\vec{k}_0 \quad (1)$$

where $\Delta\varphi$ is the phase shift in radians. The variables k, D, $\vec{k}$ and $\vec{k}_0$ (see Supplementary material) are defined in (Hawkes, 1980). As discussed in detail by Henderson et al. (1986), the phase shift in equation 1 for images includes two terms (Eqs. (S1)–(S8), see detail in Supplementary material). The first part corresponds directly to the position shift of the whole image in real space, which depends on defocus level (Δf) and the beam tilt ($\vec{b}$). In practice, boxing a particle in an image nulls this shift. The magnitude of the observable phase shift is (see Supplementary material):

$$\Delta\phi = -2\pi C_s\lambda^2 s^3\theta\cos\omega \quad (2)$$

where ω is the azimuth angle between vectors $\vec{b}$ and $\vec{s}$ (Fig. 1A). The cubic term determines the effective phase shift – independent of defocus level – due to beam tilt (Fig. 1E–G).

Since the phase shift induced by beam tilt increases dramatically with spatial frequency (Eq. (2)) (Fig. 1E–G), correcting its effect computationally or experimentally is critical for achieving atomic resolution by single particle cryoEM. Thus, for example, a beam tilt of 0.05° degrees (0.873 mrad) would introduce an unacceptable phase shift of 90.4° for structure factors at 3 Å resolution with C_s equal to 2 mm, λ equal to 0.0197 Å (as would be obtained with a 300 kV accelerating voltage), and ω equal to 0° (Eq. (1)) (Tables 1 and 2). If a phase shift of 45° were acceptable, the allowed beam tilt would be just 0.025° (0.436 mrad) for 3 Å resolution or 0.0074° (0.129 mrad) for 2 Å resolution. Therefore, to reach atomic resolution, the beam tilt must be reduced to such low values.

Eq. (2) also shows that the phase shift induced by beam tilt depends on the square of wavelength. Thus, a higher accelerating voltage (e.g., an increase from 200 kV to 300 kV) can reduce the wavelength of the electron from 0.0251 to 0.0197 Å and in turn reduce the phase shift due to beam tilt for each structure factor by 36%. However, in practice, higher accelerating voltage has one disadvantage of reducing image contrast.

2.2. Methods to overcome the effect of total beam tilt

Methods to reduce beam tilt include use of beam-deflecting coils to physically align the beam to the optical axis and use of a collimating lens to produce a parallel beam. In theory, it is also possible to compensate for the effect of beam tilt with image processing software (Henderson et al., 1986), but the efficacy of this approach is limited to some extent by the low signal-to-noise ratio (SNR) of high resolution cryoEM images. Most significantly, the effect of total beam tilt can be completely eliminated by use of a hardware device, a spherical aberration corrector (i.e., $C_s = 0$ in Eq. (2)). Each of these methods is described below.

2.1.2. Minimizing direction tilt and divergence tilt

Aligning the direction of the electron beam parallel to the optical axis is usually accomplished by adjustment of the rotation center or current center of the objective lens. However, these adjustments are only good enough for medium resolution structural studies, since the remaining direction tilt is typically more than 1 milliradian (1 mrad = 0.0573°) (Henderson et al., 1986; Zemlin, 1989; Koster and de Ruijter, 1992). The 'coma-free alignment procedure' provides a more accurate method of reducing direction tilt (Zemlin, 1978; Smith et al., 1983; Koster and de Ruijter, 1992). For experienced users, assisted by visual or computational comparison of power spectra – the aim being equally compromised power spectra for left and right tilts of the beam in the x-direction and up and down tilts of the beam in the y-direction – the error margin of coma-free alignment can be as little as 0.2 mrads (~0.011°), which corresponds to phase shifts of 18.1° at 3 Å and 61° at 2 Å resolution (Table 1) (Zemlin, 1979; Koster and de Ruijter, 1992). For users less experienced in the visual comparison, the residual beam tilt of this procedure could be up to 2 mrads (~0.11°), corresponding to a phase error of 238° at 3 Å resolution for a 300 kV microscope with $C_s = 2$ mm (Smith et al., 1985; Overwijk et al., 1997). Nevertheless, the coma-free alignment procedure can only minimize direction tilt.

The divergence tilt occurs when the specimen is illuminated with a conical beam instead of a parallel beam (Fig. 1C). The effects of this tilt component could be alleviated by underfocusing the beam with the C2 condenser lens, which however would sacrifice beam coherence (Christenson and Eades, 1988; Zemlin, 1992). Therefore, a better solution is to use a parallel beam in the first place, which can be achieved by addition of a collimating lens like the C3 condenser lens in the FEI Titan Krios microscope (Zhang et al., 2010c). In the absence of such C3 condenser lens, parallel beam can also be achieved. There are two lenses between the C2 condenser lens and specimen: the one just above specimen is the pre-objective lens and the another one above the pre-objective lens is mini-condenser lens. The C2 condenser lens can be carefully coupled to the mini-condenser lens and the pre-objective lens to generate a parallel beam on specimen.

2.1.3. Compensating spiral tilt effect by image processing

Spiral tilt originates from the spiral trajectory of electrons in the magnetic field of the pre-objective lens. The spiral tilt angle of the electron beam with respect to optical axis depends on both the strength of the magnetic field of the pre-objective lens and the distance of individual electrons to the optical axis (Eyidi et al., 2006). For example, the spiral tilt angle is about 0.033°/μm for the Tecnai TF20 TEM (Eyidi et al., 2006), resulting in a phase shift of ~64° at 3 Å resolution or 200° at 2 Å resolution for particles that are 1 μm from the optical axis, about the distance for electrons near the edge of typical micrographs. Although the effect becomes significant only at atomic resolution (e.g., better than 3 Å), spiral tilt cannot be corrected either by instrument alignment or by use of a parallel beam.

Instead, image processing may correct the effect of the total beam tilt vector $\vec{b}$, specifically its magnitude (θ) and its direction angle (β) (Fig. 1A). However, since particles at different positions have distinctive spiral and divergence tilts (Eq. (S4)) – though