Supplementary Material

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

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S1. Effect of electron beam tilt

Below, we explain in greater detail the effects of beam tilt and the ways to prevent it or correct for it. The combination of the three sources of beam tilt introduces a phase shift $\Delta\phi$ in equation 1 (Smith et al., 1983). Through a series of substitutions and approximations (equations S2-8), the phase shift can be approximated by a cubic function of spatial frequency (*s*) (equation S8) (Henderson et al., 1986).

in more detail,

$$\Delta\phi = -2\pi(k^2 - D)\vec{k}\cdot\vec{k}_0 \qquad \text{(S1)}$$

where $\Delta\phi$ is the phase shift in radians. The variables k , D, $\vec{k}$ and $\vec{k}_0$ are defined in (Hawkes, 1980) and given by equations 2, 3 and 4 therein:

$$D = \Delta f C_s^{-\frac{1}{2}} \lambda^{-\frac{1}{2}} \qquad \text{(S2)}$$

$$\vec{k} = \vec{s} C_s^{\frac{1}{4}} \lambda^{\frac{3}{4}} \qquad \text{(S3)}$$

$$\vec{k}_0 = \vec{b} C_s^{\frac{1}{4}} \lambda^{\frac{3}{4}} \qquad \text{(S4)}$$

We use equations S2, S3 and S4 to substitute for D, the vector $\vec{k}$ and the vector $\vec{k}_0$ in equation S1 to produce equation S5, which expresses the phase shift $\Delta\phi$ of the spatial frequency $\vec{s}$ induced by the total beam tilt $\vec{b}$ vector (with a magnitude of θ and a direction angle of β (Fig. 1A)).

$$\Delta\phi = -2\pi\left(C_s^{\frac{1}{2}} \lambda^{\frac{3}{2}} s^2 - \Delta f C_s^{-\frac{1}{2}} \lambda^{-\frac{1}{2}}\right) C_s^{\frac{1}{2}} \lambda^{\frac{3}{2}} \cdot \vec{b} \cdot \vec{s} \qquad \text{(S5)}$$

where *s* is the magnitude (spatial frequency) of the spatial frequency $\vec{s}$, C_s is the spherical aberration coefficient, λ is the wavelength of the electron, Δf is the defocus value, and $\vec{b}$ is the beam tilt vector, also defined in (Hawkes, 1980).

The phase shift in radians (Eq. S5) may be simplified to equation S6:

$$\Delta\phi = 2\pi\left(\Delta f \lambda \vec{b} - C_s \lambda^3 s^2 \vec{b}\right)\cdot\vec{s} \qquad \text{(S6)}$$

The overall effect of beam tilt is to add phase shifts $\Delta\phi$ to the intrinsic phases of the structure factors of specimen images as a function of spatial frequency ($\vec{s}$), defocus (Δf), and the beam tilt ($\vec{b}$) (Eq. S6).

With $\Delta\phi = \frac{\Delta\phi}{2\pi\vec{s}}$, equation S7 expresses the phase shift as a displacement of the position shift in real space.

$$\Delta x = \Delta f\lambda\vec{b} - C_s\lambda^3 s^2\vec{b} \quad \text{(S7)}$$

As discussed in detail by Henderson et al (1986), the phase shift in equation S6 includes two terms. The first term ($\Delta f\lambda\vec{b}$) corresponds directly to the first term in equation S7, 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.

Taking the dot product of the second term in equation S6 provides the magnitude of the observable phase shift:

$$\Delta\phi = -2\pi C_s\lambda^2 s^3\theta\cos\omega \quad \text{(S8)}$$

where ω is the azimuth angle between vectors $\vec{b}$ and $\vec{s}$ (Fig.1A). This second term is a cubic function of spatial frequency that cannot be corrected by boxing a particle. Therefore, the cubic term determines the effective phase shift – independent of defocus level – due to beam tilt (Fig.1E-G).

S2. Limitation imposed by variation of defocus values

CryoEM images of biological samples are recorded at under-focus conditions, and the images are modulated by the objective lens' contrast transfer function (CTF) (Eq. 3 in the main text). Variation in determination of the under-focus values is a limiting factor for retrieving precise structural information from the cryoEM images. Assuming the determined defocus value is (Δf-ε), in which ε is the variation in the defocus value, ~500 Å in this case, then the wave aberration function in equation 4 becomes the one in equation S9:

$$\chi = 0.5\pi C_s \lambda^3 s^4 - \pi\lambda(\Delta f - \varepsilon)s^2$$

$$= (0.5\pi C_s \lambda^3 s^4 - \pi\lambda\Delta f s^2) + \pi\lambda\varepsilon s^2 \quad \text{(S9)}$$

a difference

$$\Delta\chi \text{ of } \pi\lambda\varepsilon s^2, \quad \text{(S10)}$$

and the CTF becomes

$$CTF(\Delta f - \varepsilon) = \sin[(0.5\pi C_s \lambda^3 s^4 - \pi\lambda\Delta f s^2) + \pi\lambda\varepsilon s^2] \quad \text{(S11)}$$

The *CTF* varies as the sine of spatial frequency *s* (Eq. S11) (Fig. 2B-C), and variation of defocus value ε may erroneously change the sign of structure factors in areas where the CTF changes sign. To estimate the resolution limitation caused by the variation of ε, we assume that the acceptable shift ($\Delta\chi$) (Eq. S10) of the wave aberration function is $\pi/2$, that is, $\Delta\chi \le \pi/2$. Thus, for a certain spatial frequency, the allowable defocus variation ε_{max} is given by equation S12.

$$\pi\lambda\varepsilon_{\max} s^2 \le \frac{\pi}{2} \quad \Rightarrow \quad \varepsilon_{\max} = \frac{d^2}{2\lambda} \quad \text{(S12)}$$

S3. Proof of the Characteristic Surfaces Theorem

Below, we derive the Characteristic Surfaces Theorem. Under the framework of weak phase object (WPO) approximation (Spence, 1988), particle $\varphi(x, y, z)$ can be regarded as a composite of multiple thin slices in real space, with each slice at its own focus level and independently forms its own image, $\varphi_z(x, y)$. The final projection image $\varphi_{obs}(x, y)$ of the particle is therefore the integral of all of these images:

$$\varphi_{obs}(x, y) = \int \varphi_z(x, y)dz \quad \text{(S13)}$$

When each particle is small, all of the slices can be approximated as being at the same height z, and we can use the Central Projection Theorem (Crowther, 1971). In the presence of defocus, the Fourier transform of the projection image $\varphi_{obs}(x, y)$ is multiplied by the CTF, as described in the prior section. Performing a 2D (i.e., x, y) Fourier transform on both sides of equation S13, we get:

$$F_{obs}(X,Y) = \int F_z(X,Y) \cdot CTF(X,Y)dz \qquad \text{(S14)}$$

That is, the Fourier transform of the projection image, $F_{obs}(X,Y)$, with X and Y in reciprocal space, is the integral of the 2D structure factors $F_z(X,Y)$ of the individual slices of the particle. Here, the CTF is described in equations 3 and 4 with $s = \sqrt{X^2 + Y^2}$ (see Figure 3A) denotes spatial frequency, and Δf denotes the defocus value of each slice $\varphi_z(x, y)$.

The 3D Fourier transform of the 3D particle is:

$$F(X,Y,Z) = \iiint \varphi(x, y, z)e^{2\pi i(xX+yY+zZ)}dxdydz = \iiint \varphi_z(x, y)e^{2\pi i(xX+yY+zZ)}dxdydz \qquad \text{(S15)}$$

From this equation, we obtain the central section $F(X,Y,0)$ of the 3D Fourier transform as:

$$F(X,Y,0) = \int \{\iint \varphi_z(x, y)e^{2\pi i(xX+yY)}dxdy\}dz$$

$$= \int F_z(X,Y) \cdot CTF(X,Y)dz$$

$$= CTF(X,Y)\int F_z(X,Y)dz \qquad \text{(S16)}$$

If a single defocus value were valid for all of the slices in real space -- as required by the Central Projection Theorem -- Equation S17 shows that the central section in 3D Fourier space [$F(X,Y,C)_{C=0}$] would be the integral of the 2D Fourier transforms of all of the slice images. Based on Eqs. S14 and S16,

$$F_{obs}(X,Y) = F(X,Y,0) \qquad \text{(S17)}$$

That is, if the defocus values among the slices were so close that they could be regarded as the same, the Central Projection Theorem would hold. Therefore, for a 2D image or a projection image of a thin specimen, $\varphi_{obs}(x, y)$, we define its Fourier transform as:

$$F_{obs}(X,Y) = F_{obs}(X,Y,C)_{C=0} \text{ (note: } C\text{=0 represents the central section)} \qquad \text{(S18)}$$

However, for a large particle, the focus gradient cannot be neglected, and the Central Projection Theorem is invalid. In this case, the final projection image $\varphi_{obs}(x, y)$ of the particle is still the integral of the images of all the particle slices $\varphi_z(x, y)$ (Eq. S13), but the Fourier transform of the image of each slice at height (lower case) z is modified by a different CTF_z, each with its own defocus value (*Δf+z*) (Wan et al., 2004), where *Δf* is the same for all of the slices of a single particle. Taking a Fourier transform of both sides of equation S13, using equation S18 for the left side, and using a different CTF for each slice at height ***z***, hence CTF_z for the right side, we get:

$$F_{obs}(X,Y,C)_{C=0} = \int F_z(X,Y) \cdot CTF_z(X,Y)dz \tag{S19}$$

This treatment is similar to the case for particles centered at different heights z in ice (Section 2.1 in the main text), but here lower case z refers to the heights of different slices within a single particle.

One can convert equation S19 to a form that can be solved by using the convolution theorem (Wan et al., 2004). One starts by multiplying both sides of equation S19 by $e^{-2\pi i z C}$, which equals one because C=0. (Physically, C=0 corresponds to the central section in reciprocal space, the plane in Figure 3A. Since each individual slice of the particle is thin and its image obeys the Central Projection Theorem, the Fourier transform of the image of each slice at height *z* in real space is a central section in reciprocal space (upper case *C*=0). Later, we will show that this *C*=0 plane is the sum of the two curved surfaces in Figure 3A.) Because the $e^{-2\pi i z C}$ term is a constant, we can place it on the left or even ignore it; on the right, we can place it inside the integral. The result is equation S20.

$$F_{obs}(X,Y,C)_{C=0} = \int F_z(X,Y) \cdot CTF_z(X,Y)e^{-2\pi i z C}dz \quad (note: C=0) \tag{S20}$$

$$= \xi_z\{F_z(X,Y) \cdot CTF_z(X,Y)\} \mid \qquad (C=0) \tag{S21}$$

where ξ_z denotes the 1D Fourier transform over height *z* (in real space), whereas *$F_z(X,Y)$* represents the 2D Fourier transform (in reciprocal space) over *x* and *y* of a slice (in real space) at height *z*.

According to the convolution theorem, we can further reorganize equation S21:

$$F_{obs}(X,Y,C)_{C=0} = \xi_z\left[F_z(X,Y)\right] \otimes \xi_z\left[CTF_z(X,Y)\right] \quad \text{(S22)}$$

$$= F(X,Y,\mathrm{C})_{C=0} \otimes \xi[CTF(X,Y,C)_{C=0}] \quad \text{(S23)}$$

$$F_{obs}(X,Y,C)_{C=0} = \int F(X,Y,C-\mathrm{Z})_{C=0} \cdot \xi[CTF(X,Y,Z)]\, dZ \quad \text{(S24)}$$

where "$\otimes$" denotes the convolution operator. Here, the convolution operation in equation S23 introduces a new variable *Z* for the 3D structure factor *F* of the object in the right side of equation S24, while *C* is a variable of the Fourier cofficient F_{obs} of the recorded image in the left side of equation S24 and *C* is always zero, corresponding to the central section in reciprocal space.

We need the expression for the *CTF(X,Y,Z)* before we can take its Fourier transform. This expression is obtained by extending, from two dimensions into three dimensions, Equations 3 and 4 in the main text:

$$CTF(X,Y,Z) = \sin[0.5\pi C_s \lambda^3 s^4 - \pi\lambda(\Delta f + z)s^2]$$

$$= \sin(\chi - \pi\lambda z s^2)$$

$$= \sin(\chi - 2\pi z a_s) \quad \text{(S25)}$$

where we take advantage of the expression for spatial frequency $s = \sqrt{X^2 + Y^2 + Z^2}$ (see Fig. 3A), and $a_s = \frac{1}{2}\lambda s^2$.

Then, to obtain the Fourier transform of the *CTF(X,Y,Z),*

$$\xi[CTF(X,Y,Z)] = \xi_{\mathrm{z}}\left[\sin(\chi - 2\pi z a_s)\right] = \xi_{\mathrm{z}}\left\{-\frac{1}{2}i[e^{i(\chi-2\pi z a_s)} - e^{-i(\chi-2\pi z a_s)}]\right\}$$

$$= -\frac{1}{2}\{e^{-i(\chi+0.5\pi)}\delta(\mathrm{Z}-a_s) + e^{i(\chi+0.5\pi)}\delta(Z+a_s)\} \quad \text{(S26)}$$

Substituting equation S26 into equation S24, we obtain

$$F_{obs}(X,Y,0) = -\frac{1}{2}\int F(X,Y,-\mathrm{Z}) \cdot \left[e^{-i(\chi+0.5\pi)}\delta(\mathrm{Z}-a_s) + e^{i(\chi+0.5\pi)}\delta(Z+a_s)\right] dZ$$

$$= -\frac{1}{2}\left[e^{i(\chi+0.5\pi)}F(X,Y,a_s) + e^{-i(\chi+0.5\pi)}F(X,Y,-a_s)\right] \tag{S27}$$

Equation S27 shows that the observed Fourier coefficient $F_{obs}(X,Y,0)$ is a mixture of two unknown structure factors (*F*): $F(X,Y,a_s)$ (F_{a_s}) and $F(X,Y,-a_s)$ (F_{-a_s})(Fig. 3A).

The circle in the upper left inset in Figure 3A, a vertical slice through the entire upper characteristic surface in Figure 3A, has radius 1/ λ, hence diameter 2/λ. T on the characteristic surface is equal to height a_s, which varies with the spatial frequency *s* of each structure factor. Of course, various aberrations prevent us from reaching s = 1/ λ (=0.0197 Å for 300 kV electrons), so we operate with spatial frequency *s* much closer to the origin of Ewald sphere (and of the Fourier space) than the *s* shown in the inset.

Two special cases in which the Central Projection Theorem remains valid. First, when the particle is infinitesimally thin (i.e., a 2D object), the 3D Fourier transform can be expressed as F(*X, Y*) with no variation in the "*Z*" direction. The situation of imaging a small particle is similar, in that variation per reciprocal unit distance of its Fourier coefficients along the *Z* axis is small, and F(*X, Y, a_s*) ≈ F(*X,Y, -a_s*). Second, when the resolution is low (i.e., *s*→ 0; thus, a_s → 0), the Fourier coefficients are near the origin of the Fourier space in Figure 3A. As a result, the top and bottom characteristic surfaces are both nearly flat and -- as illustrated by the upper right inset in Figure 3A -- essentially merge into the central plane.

It is worth noting that the above derivation of Characteristic Surfaces Theorem is equivalent to the formulation deduced by DeRosier based on interference between two scattered electron rays symmetrically intersecting an Ewald sphere (i.e., the sphere defined by radius of 1/λ) at two points F_R and F_L (DeRosier, 2000). This F_R on the right side of the upper characteristic surface in Figure 3A is the same as F_{a_s} (Fig. 3A). Based on Friedel's law [i.e., F(X,Y) = F* (-X,-Y)], this F_L on the left side is equal to the conjugate of F_{-a_s}. Substituting these terms into equation S27, we obtain

$$F_{obs}(X,Y) = -\frac{1}{2}[|F_{a_s}|e^{i(0.5\pi+\chi+\alpha_{a_s})} + |F_{-a_s}|e^{-i(0.5\pi+\chi-\alpha_{-a_s})}]$$

$$= -\frac{1}{2}[|F_R|e^{i(0.5\pi+\chi+\alpha_R)} + |F_L|e^{-i(0.5\pi+\chi+\alpha_L)}] \quad \text{(S28)}$$

where $(F = |F|e^{i\alpha})$, and “|F|” and “α” respectively denote the amplitude and phase of the structure factor “F”. Equation S28 is the same as the formula derived by DeRosier (Eq. 9 in DeRosier, 2000). Therefore, these two computational formulations for overcoming the breakdown of the Central Projection Theorem are equivalent. However, we are not done. We still need to recover F_R and F_L from F_{obs}.

S4. Dynamic scattering

From equation 6 in the Yang et al. paper (Yang et al., 2001), we obtain the following:

$$F_{obs}(X,Y) = \frac{1-e^{-i\lambda\pi s^2 D}}{4\pi^2 s^2} 4\pi F(X,Y) \quad \text{(S29)}$$

where *F(X,Y)* is the structure factor of the image without the effects of dynamic scattering, $F_{obs}(X,Y)$ is the observed structure factor of the image affected by dynamic scattering, *D* is the diameter of the particle and *s* is the spatial frequency.

Equation S29 can be rewritten as follows:

$$F_{obs}(X,Y) = \frac{\{[1-\cos(\lambda\pi s^2 D)] + i\sin(\lambda\pi s^2 D)\}}{\pi s^2} F(X,Y) \quad \text{(S30a)}$$

$$= i\frac{\sin(0.5\pi\lambda s^2 D)}{0.5\pi s^2} e^{-i0.5\pi\lambda s^2 D} F(X,Y) \quad \text{(S30b)}$$

$$= iqe^{-i0.5\pi\lambda s^2 D} F(X,Y) \quad [q = \frac{\sin(0.5\pi\lambda s^2 D)}{0.5\pi s^2} = \lambda D \,\mathrm{sinc}\,(0.5\pi\lambda s^2 D)] \quad \text{(S30c)}$$

$$= iF'(X,Y) \quad \text{(S30d)}$$

From the WPO approximation, the wave function in front of the objective lens is:

$$\gamma(x,y) = e^{i\xi[F'(X,Y)]} \approx 1 + i\xi[F'(X,Y)] \quad \text{(S31)}$$

where ξ denotes Fourier transformation. The wave function on the image plane becomes $\varphi(x, y)$, and its Fourier transform is:

$$\xi[\varphi(x, y)] = \xi[\gamma(x, y)]e^{i\chi} = \delta(X, Y) + iF'(X, Y)e^{i\chi}$$

$$= \delta(X, Y) + iqF(X, Y)e^{i(\chi - 0.5\pi\lambda s^2 D)} \tag{S32}$$

where $\chi = 0.5\pi C_s \lambda^3 s^4 - \pi\lambda(\Delta f + z)s^2$. The recorded image intensity $\psi(x, y)$ is the square of the wave function:

$$\psi(x, y) = |\varphi(x, y)|^2$$

$$= \varphi(x, y) \cdot \varphi^*(x, y) \tag{S33}$$

If one ignores the quadratic terms, one obtains:

$$\psi(x, y) \approx 1 + 2\xi[qF(X, Y)] \otimes \xi[\sin(\chi - 0.5\pi\lambda s^2 D) \tag{S34}$$

Therefore,

$$\xi[\psi(x, y)] = \delta(X, Y) + 2\frac{\sin(0.5\pi\lambda s^2 D)}{0.5\pi s^2} F(X, Y)\sin[0.5\pi Cs\lambda^3 s^4 + (\Delta f - 0.5D)\pi\lambda s^2] \tag{S35}$$

According to equation S35, the structure factor of a recorded image is modified not only by *CTF*, but also by the following dynamical transfer function (*DTF*) (Eq. S36):

$$DTF = \frac{\sin(0.5\pi\lambda s^2 D)}{0.5\pi^2 s^2} = \lambda D \mathrm{sinc}\,(0.5\pi\lambda s^2 D) \tag{S36}$$

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