Supporting Text

Constrained Cross-Correlation Function. The CCF of a reference A in an orientation (φ,ψ,θ) and a subtomogram V was introduced in ref. 1:

$$CCF_{xyz;\varphi\phi\theta} = \frac{\sum_{x'y'z'} V_{x'y'z'} \cdot (A_{x+x',y+y',z+z';\varphi\phi\theta} \otimes PSF_{x+x',y+y',z+z'})}{\sqrt{\sum_{xyz} (A_{xyz;\varphi\phi\theta} \otimes PSF_{xyz})^2} \cdot \sqrt{\sum_{xyz} V_{xyz}^{\ 2}}}. \qquad \textbf{[1]}$$

The pointspread function PSF is a function that corresponds to the tilt range of the tomogram. In Fourier space, the convolution with the PSF corresponds to multiplication with a wedge function. The denominator of Eq. **1** ensures normalization of the reference signal to the sampling range of the tomogram. Omitting this normalization can cause common alignment of the subtomograms with respect to the missing wedge for low SNRs. This normally erroneous orientation maximizes the common energy of different particles and can outweigh the fact that the resulting average is not coherent. Especially for membrane-associated complexes, the normalization of Eq. **1** is necessary because the signal is typically highly anisotropic for those objects. Furthermore, masking of the subtomograms containing the macromolecule of interest is performed, because surrounding features such as the inhomogeneous membrane tend to distort the CCF. The significance of the peaks derived from the CCF is further improved by applying bandpass filtering. However, the computation of the above CCF is time-demanding, effectively prohibiting an exhaustive scanning of the Eulerian angles. Therefore, the evaluation of the above CCF is restricted to the vicinity of an initial orientation within every iteration.

Polar Alignment. The alignment procedure with respect to the polar angle φ uses the rotational correlation function RCF as it is often used in 2D (e.g. ref. 2). The 3D formulation is:

$$RCF(\varphi) = \sum_{z}\sum_{\rho} \rho \mathsf{F}_{\varphi}^{-1}(\mathsf{F}_{\varphi} A_{z\rho\varphi} \cdot (\mathsf{F}_{\varphi} V_{z\rho\varphi})^{*} \quad . \qquad \textbf{[2]}$$

The average A and subtomogram V are transformed to cylindrical coordinates z, ρ, and φ prior to computing their Fourier transformed F_{φ} with respect to φ. The coordinate transformation is done by means of trilinear interpolation. By applying the Fourier transform in φ, exhaustive scanning can be achieved at moderate computational expense in Eq. **2**. However, the RCF in Eq. **2** does not account for the missing wedge, which favors the CCF of Eq. **1** for fine alignment.

Fitting. The electrostatic potential was convoluted with an effective CTF consisting of two factors: (*i*) A CTF considering the electron-optics inside the transmission EM assuming pure phase contrast. The CTF is computed for 300 kV and 4 µm defocus. (*ii*) A mutual transfer function (MTF) that describes the imaging properties of the charge-coupled device camera. For the MTF it is assumed that it retains 15% of the incoming signal at 0.5 of the Nyquist frequency.

1. Frangakis, A. S., Böhm, J., Förster, F., Nickell, S., Nicastro, D., Typke, D., Hegerl, R. & Baumeister, W. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 14153-14158.
2. Penczek, P., Radermacher, M. & Frank, J. (1992) *Ultramicroscopy* **40,** 33-53.