Entropy Regularized Deconvolution of Cellular Cryo-Transmission Electron Tomograms

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Cryo-electron tomography (cryo-ET) allows for the high resolution visualization of biological macromolecules. However, the technique is limited by a low signal-to-noise ratio (SNR) and variance in contrast at different frequencies, as well as reduced Z resolution. Here, we applied entropy regularized deconvolution (ER DC) to cryo-electron tomography data generated from transmission electron microscopy (TEM) and reconstructed using weighted back projection (WBP). We applied DC to several *in situ* cryo-ET data sets, and assess the results by Fourier analysis and subtomogram analysis (STA).

Cryo-Electron Tomography | Deconvolution | Subtomogram Analysis | Structural Biology | Missing Wedge

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Recent advances in cryo-electron tomography (cryo-ET), most notably the ability to thin cryo-preserved specimens using a focused ion beam (FIB), have opened windows for the direct visualization of the cell interior at nanometer-scale resolution (1–9). Cells are rapidly frozen to achieve a vitreous form of ice that preserves biological molecules in a near-native state. They are then cryo-FIB milled to a suitable thickness of 100-350 nm for imaging with transmission electron microscopy (TEM). A series of projection images is acquired, typically with 1-5 degree increments and then reconstructed into a 3D volume (10). This 3D reconstruction is rendered for display and analysis, which may entail segmentation to highlight extended structures or averaging of sub-volumes for enhancement of molecular-scale resolution (11, 12).

While cryo-ET offers unparalleled resolution of cellular interiors, it is challenging for a number of reasons. First, vitrified biological samples are highly sensitive to damage by the electron irradiation required for imaging. Constraints on the permissible exposure result in limited contrast and a low signal to noise ratio (13). Second, the modality of wide-field TEM depends on defocus to generate useful phase contrast, but with a non-trivial dependence on spatial frequency that is expressed in a contrast transfer function (CTF). Contrast is lost at low spatial frequencies and oscillates at high spatial frequencies, meaning that material density could be represented as intensity either darker or lighter than background (14–16). Post-processing is applied to correct this representation in the image intensities. The correction is inherently approximate, and is especially challenging in tomography where the defocus varies across the field of view (17). Third, the available raw data are never sufficient to produce an unambiguous reconstruction. The tilt range is restricted by the slab geometry, typically to about 120° around the vertical. The projected thickness of a slab also increases with tilt angle, resulting in degraded contrast and resolution from these contributions to the reconstruction. The missing information is best recognized in Fourier space, where it is known as the missing wedge. The gaps between discrete tilt angles also leave small missing wedges as seen in Fig. 1. Since the reconstruction is equivalent to an inversion in Fourier space, it is obvious that some interpolation is required and that the data are incomplete. As such, it is not surprising that different algorithms can generate somewhat different reconstructions from the same data. Commonly recognized artifacts are elongation along the Z direction and streaks projecting from high contrast points into neighboring planes in the volume.

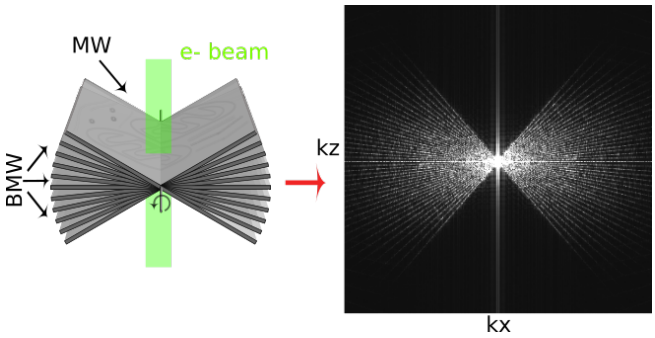

Fig. 1. Tilt series collection and the missing wedge issue. Left: Schematic of tilt-series collection scheme. Sample projections are acquired over a range of tilt angles, typically from -60° to +60° . Right: Middle slice of the kxkz plane shows the missing wedge (MW) and baby missing wedges (BMW) of information visualized in Fourier space.

In addition to the missing wedges, TEM images require a significant defocus to get adequate contrast. For *in situ* cryo-ET data, a typical defocus of at least 5 μm is used. Finally, the process of reconstruction by weighted back projection (WBP) introduces well-known problems. These include significant intensity above and below the sample volume, where we expect vacuum with no signal. This is due to cross-terms in the WBP coming from the tilt wedges, as well as distortions in the WBP arising from the missing wedge. Because of these issues with cryo-ET data, filters to improve contrast and compensate for the missing wedge are an area of ongoing research (18). These techniques include non-linear anisotropic diffusion (NAD), convolutional neural networks based on de-

tector noise models, wavelet based filtering methods, different implementations of deconvolution, and model based iterative reconstruction (MBIR) (19–28). Here, we present a deconvolution approach to achieve both enhanced SNR and missing wedge compensation.

The image distortions resulting from the incomplete tilt series and CTF can be characterized in terms of a single sample point of the data. This model is referred to as the point spread function (PSF), of which the hour-glass PSF in light microscopy is a classical example (29–31). Formally, the PSF is convolved with all points in the specimen function to form what is recorded in the image (32). If the PSF is well defined, it becomes possible to partially reverse the process of convolution to obtain an improved reconstruction. This reversal is referred to as deconvolution, which is a mathematical/computational iterative inversion processing procedure, extensively utilized in astronomy, spectroscopy, and light microscopy to partially restore data distorted by the imaging process (32). The deconvolution process is constrained. The most common constraint is the imposition of positivity of the deconvolved data (32). Other stabilizing constraints may include smoothing in real space to suppress high-frequency oscillations. DC is also very sensitive to noise, and most DC algorithms include regularization parameters whose values are difficult to evaluate theoretically. Additionally, in most cases the DC algorithms will diverge with increasing iterations, building up mottle and noise that obscure the interpretation of the final DC image. Finally, most DC implementations do not have a practical estimate of the error in the converged solution.

Entropy-regularized deconvolution ER-DC (33) is formulated to handle data with a weak signal to noise ratio, with a regularization term that exploits certain characteristics specific to images originating from cell organelles. Specifically, in cellular images, high intensities and high second-order derivatives exhibit certain sparse distribution, and this property is exploited by the custom regularization used in ER-DC. This regularization was originally designed for fluorescence images, and this approach was taken recently for processing of STEM cryo-tomography (CSTET) reconstructions (26). It is similar to deconvolution applied to fluorescence microscopy, where out of focus light creates a haze, but differs in that the artifacts to be removed originate primarily in the reconstruction rather than the optics. Whereas the individual 2D image is treated as a bona fide 2D projection, the kernel of the deconvolution was taken as the sum of the illumination profiles used in the tilt series. However, since TEM is currently the dominant modality for biological 3-D imaging of cells (34) and its CTF is complex, this deserves a separate study, which is the focus of this paper. The major distinction is that the contrast inversions, which were absent in the STEM data as acquired for tomography, should be accommodated in construction of the 3D PSF for TEM tomography.

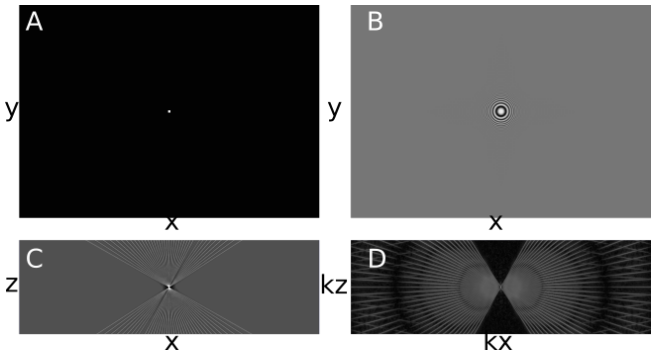

Fig. 2. Generating the TEM Point Spread Function. (A) Synthetic tilt series of a centered point source. (B) Point source tilt series convolved with CTF. (C) Slice of the weighted back projection tomogram of convolved CTF-point source (PSF), xz view. (D) 3D FFT of tomogram showed in C, xz slice.

Results

Electron Tomography Point Spread Function. The key to a meaningful deconvolution is that the synthetic PSF should represent as closely as possible the 3D image of an ideal point source. In the case of TEM, this requires an accounting for the defocus imposed in the image acquisition, which is customarily expressed in terms of a contrast transfer function (CTF). The 3D PSF for deconvolution was computed from simulated projections of a point source with the same dimensions and pixel spacing as the aligned tilt series (Fig. 2A). The CTF was first convolved with a projected point-source (Fig. 2B), and then a synthetic tilt series was reconstructed to the same dimensions as the original tomogram using the tilt angles represented in the corresponding reconstruction (Fig. 2C). This is the real-space PSF, whose 3D FFT serves as the optical transfer function, or kernel, for the deconvolution (Fig 2D). In principle, the 2D original CTFs vary with the gradient of defocus of the reconstructed volume. For simplicity, in this first demonstration we limited the analysis to a single nominal defocus and a spatially-invariant deconvolution kernel. A flow diagram for the PSF process is shown in (SI Appendix Fig. S1).

Tomogram Deconvolution. As a first demonstration of the TEM deconvolution we used a HEK cell cultured on-grid that had been FIB-milled to 150 nm thickness. The reconstructed volume contains membranes, microtubules, and a prominent crystalline protein array. The cells were overexpressing human Parkinson's related protein LRRK2-I2020T (35), and the observed repetitive structure is likely an autophagosome, given its double lipid bilayer structure (36). Contrast is sharp in slices through the XY plane of the tomogram, as expected (blue plane-mid structure, Fig. 3B), but contrast and resolution in the Z direction, seen in a slice through the XZ plane (orthogonal green plane in mid structure, Figure 3C) are severely compromised. Furthermore, the reconstructed volume displays a signal both above and below the specimen when observed in the XZ plane. Since the milled slab of material is finite in the z direction, and the sample is imaged in a vacuum, there should be negligible intensity outside it in the reconstructed data. This is a known artifact of back projection. These image distortions in real space can also be char-

acterized in Fourier space, where the real space dimensions (x, y, z) correspond to the Fourier dimensions (K_x, K_y, K_z). The protein array in the real space XY plane appears as a lattice of calculated diffraction spots in the plane (K_x, K_y), as expected (Figure 3E). In the XZ plane, the lattice of spots is sharply truncated at the Fourier planes normal to the limits of the acquired tilts. In summary, back projection suffers from major distortions visible in both real and Fourier space.

The result of 3D deconvolution processing is shown alongside the reconstruction in Figure 3. Full details appear in the supporting information. All processing was performed using PRIISM image processing software (37). Briefly, the entropy-regularized deconvolution algorithm from PRIISM was applied using the simulated PSF. In addition to imposing a penalty on negative intensities (positivity constraint), a spatial constraint was added to the error function on each iteration in order to penalize for spurious intensity reconstructed above and below the specimen volume. While contrast is enhanced in the XY plane, the more striking improvement is seen in the XZ plane (Fig. 3J), in comparison with the back projection (Fig. 3C). In the deconvolved tomogram, two lipid bilayers are visible (arrow) across the entire sample along Z, as is the crystalline array (Fig. 3J). The restoration of information along Z in real space can also be seen in the 3D Fourier transform of the deconvolved volume, which shows increased signal in the previously empty regions corresponding to the missing wedges (Fig. 3F,L).

A very effective way to observe the results of DC is to study a small volume of the WBP and/or DC in a dynamic interacting display module, typically a video of the rotating volume. Stereo pairs with additional rotated views are shown for the WBP and DC (SI Appendix videos 1 and 2). These may be rocked with a cursor bar, as described in supporting information, in order to gain an impression in 3D. Distortions along the Z axis associated with the WBP are largely removed after DC processing, with a visual improvement in resolution along Z. **Resolution in the deconvolution can be estimated by analysis of the Fourier transform as shown by the data (Figure S2).** Significant information appears in the power spectrum appears beyond a spatial frequency of approximately 2.5 nm, which corresponds nominally to the second zero in the CTF for 6 micron defocus.

A Second Deconvolution Example. For a second example, we applied ER-DC to a tomogram of a relatively thick lamella of *S. cerevisiae* cells (370 nm). Besides the thickness, cryo-electron tomography data of nuclei are challenging samples to interpret as nuclei are densely packed, and lack high-contrast features like membranes and cytoskeletal elements. As with the DC of mammalian cells, DC provided increased contrast in XY and an improved ability to visually interpret information along Z compared to the back projection. The nuclear envelope is clearly visible in the XY slices of the WBP and the two DCs (Fig. 4 A, E, I). In XZ however, no clear structure can be followed in the BP(Fig. 4C), but can be more easily followed in the DC (Fig. 4 G, K)). Additionally, the missing wedge seen in Fourier space is filled in by the DC process (Fig. 4 H, L)). By utilizing rotating angle stereo-pair renderings of the volume (RAPSs), one can compare the WBP and DC volumes in 3D (SI Appendix videos 3 and 4). In the BP, there is little distinguishable structure as the volume rotates. In contrast, fine features can be identified at every angle, such as the nuclear envelope, as well as densities that could correspond to chromatin and nucleosomes. The 3D-FFT of the DC (Fig. 4), KxKz view, shows the missing wedges being filled in, indicating that the DC process helps correct for these artifacts, even in challenging samples.

Deconvolution and Subtomogram Analysis. Subtomogram analysis is an approach to protein structure determination *in situ* (11, 38–52). Similarly to single particle analysis, of which it is an extension to 3D, averaging multiple examples of identical images serves to reduce noise. 3D averaging can also be used to compensate for the missing wedge in a given tomogram acquisition if the molecules lie in random orientations (11). The crystalline-like body seen in Fig. 3 provided an interesting test case for averaging where orientations were determined uniformly by translational symmetry in the crystal (Fig. 5). Therefore only select orientations are available for view. First, we attempted to align the crystal subunits over 360^0 in θ and ϕ on the WBP reconstruction. This resulted in an average that was dominated by the missing wedge, a common pitfall in sub-tomogram averaging, and produced a structure that was strongly elongated in the view direction (Fig. 5A). Second, we used the same particles, but this time from the deconvolved data set, aligned and averaged them with a resulting structure that resembled much better the unit of the crystalline array in the original tomogram (Fig. 5E). Third, we averaged the WBP particles using the DC alignment transformations. In this last case, we obtained a structure similar to the one obtained from DC-aligned and DC-averaged particles (SI Appendix Fig. 4B). This is a very practical realization of the improved axial resolution in the DC, or equivalently the suppression of the missing wedge artifact in the WBP data, demonstrating that the alignment of subtomograms is improved by DC. This tomogram was acquired from a HEK cell overexpressing human LRRK2 (35). While the identity of the molecules forming the crystalline-like array was not specifically established (e.g., by CLEM) and the number of particles in this tomogram is severely limited (82), the DC average resembles the cryo-EM structures of LRRK2 determined both *in situ* bound to microtubules (35) and *in vitro* (53).

Fourier shell correlation (FSC) is widely used in single particle cryo-EM (54), as a metric of the resolution of a molecular structure. It is a quantitative measure of similarity, typically implemented in cryo-EM by comparing two structures, each generated from a half dataset. Standard FSC compares global similarities, giving a single curve for the entire structure, showing the correlation score as a function of spatial frequency. Resolution is then quoted as the inverse spatial frequency where the correlation drops below an accepted threshold. Directional FSC (dFSC) is a variant in which all Euler angles are explored for frequency comparison, and provides a representation of resolution in all directions (55). dFSC was applied to two half-map averages of the crystalline

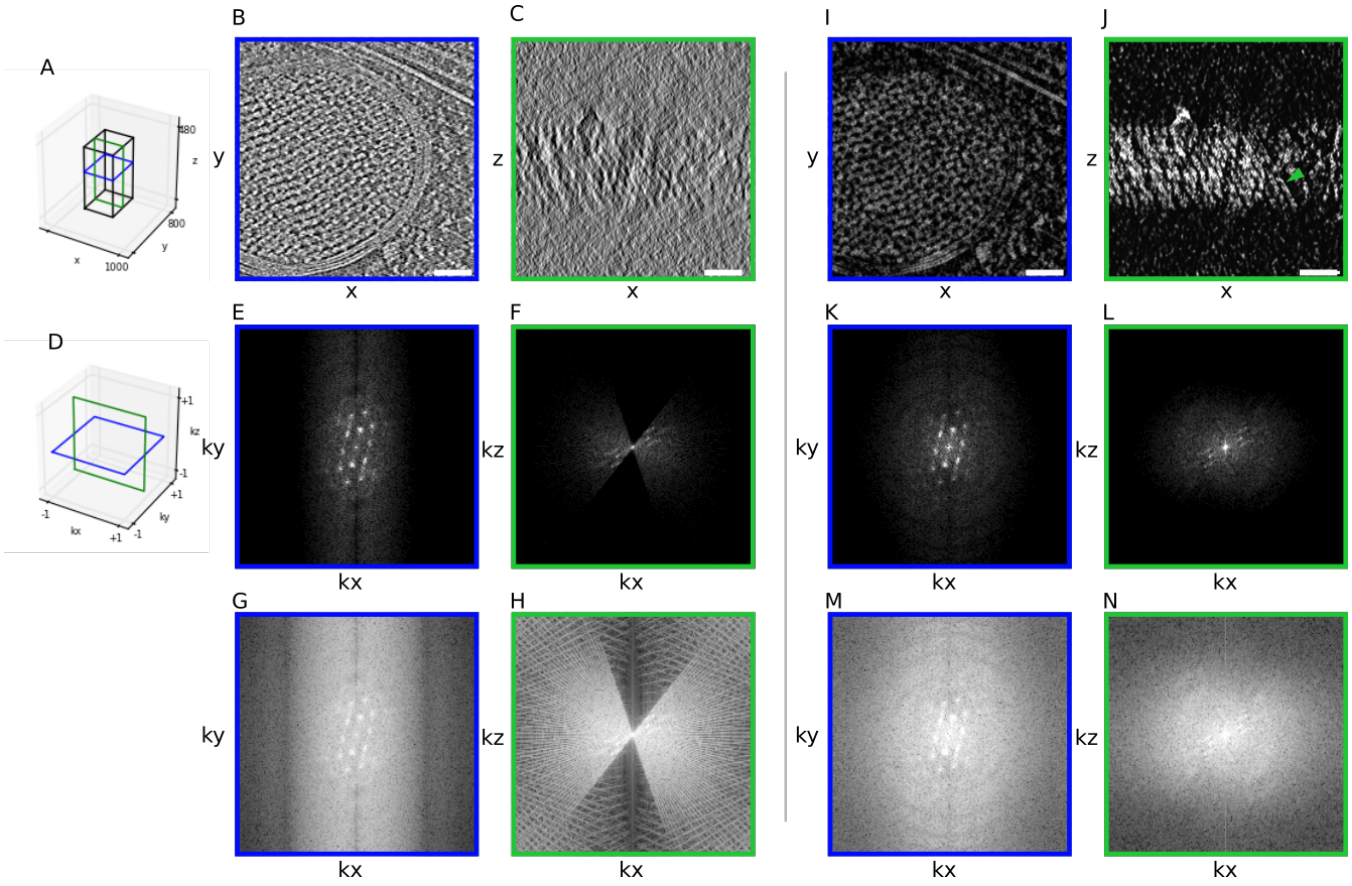

Fig. 3. Filling of Missing Wedge by Deconvolution (A) Schematic of slices used to generate panels B, C, I and J. (B)XY Slice of a tomogram of a HEK cell reconstructed using back projection. Throughout this work, white intensities correspond to high density values. (C) XZ slice of the same tomogram. (D) Schematic to show slices through Fourier space used to generate E,G,K,M in blue and F,H,M,L,M in green. (E,G) Slice through 3D FFT of the back projection corresponding to XY shown with a different distribution of voxel intensities . (F,H) Slice through 3D FFT of the back projection corresponding to XZ shown at two different intensity scales. (I-N) Corresponding results for the tomogram after deconvolution including a spatial constraint. Scale bars: 100 nm.

arrays from the WBP, and then to the DC averages to assess any change in resolution in any direction between the WBP (Fig. 5 C) and DC (Fig. 5G). Both averaged correlation curves drop to near zero by a frequency of 1/.02 $Å^{-1}$), implying a resolution on the order of 5 nm. However there is a striking difference in the degree of anisotropy as a function of direction in the WBP; in the DC curves most of the directional correlation lines run nearly parallel. This is also reflected in the isosurfaces shown in Figure 5D and H.

To investigate the effects of DC in the alignament of the particles and the improvement of the average due to the missing wedge separately, we chose to use microtubules, since their structure is well established, as are the pipelines for subtomogram analysis. We analyzed a tomogram of reconstituted microtubules decorated by the Parkinson's related protein $LRRK2^{RCKW}$(53). In the tomogram, it is evident that the deconvolution process increased the contrast between the microtubules and the surrounding media, and we again see a reduction in XZ distortions (Fig. 6A,B,D,E), as well as a corresponding filling of information in the missing wedge in Fourier space (Fig. 6C, F). Microtubule subtomograms were extracted from both the WBP and DC volumes using the filament tracing function in Dynamo(56). The subtomograms were independently aligned and averaged as described in (35) (Fig. 7 A, B). Note that the contrast between protofilaments is distinctly sharper for the DC data. However, this method of alignment includes an azimuthal randomization that is specifically designed to cancel out the missing wedge in the final average. To assess the effect of DC specifically on the missing wedge, we also ran the alignment on particles without this randomization step. Compared to the WBP, the DC-processed average shows more distinctly visible protofilaments in the direction of the missing wedge (Fig. 7E). Last, we used the DC alignment parameters to average the WBP particles (Fig. 7F). Here, the average still shows a prominent missing wedge. Thus, the DC improves both the alignment and the averaging steps of subtomogram analysis.

Discussion

We have successfully applied ER DC to cryo-electron tomograms, and demonstrated enhanced contrast compared to the back projection reconstructions, as well as less distorted structures along the Z axis. In real space, one can follow membranes in the XZ plane of the deconvolved volume, that were hardly visible in the back projection. In Fourier space, it is clear that portions of the missing wedge are filled in, and the distribution of voxel intensities changes significantly as a result of deconvolution. However, there are still several considerations for TEM deconvolution, and these are further

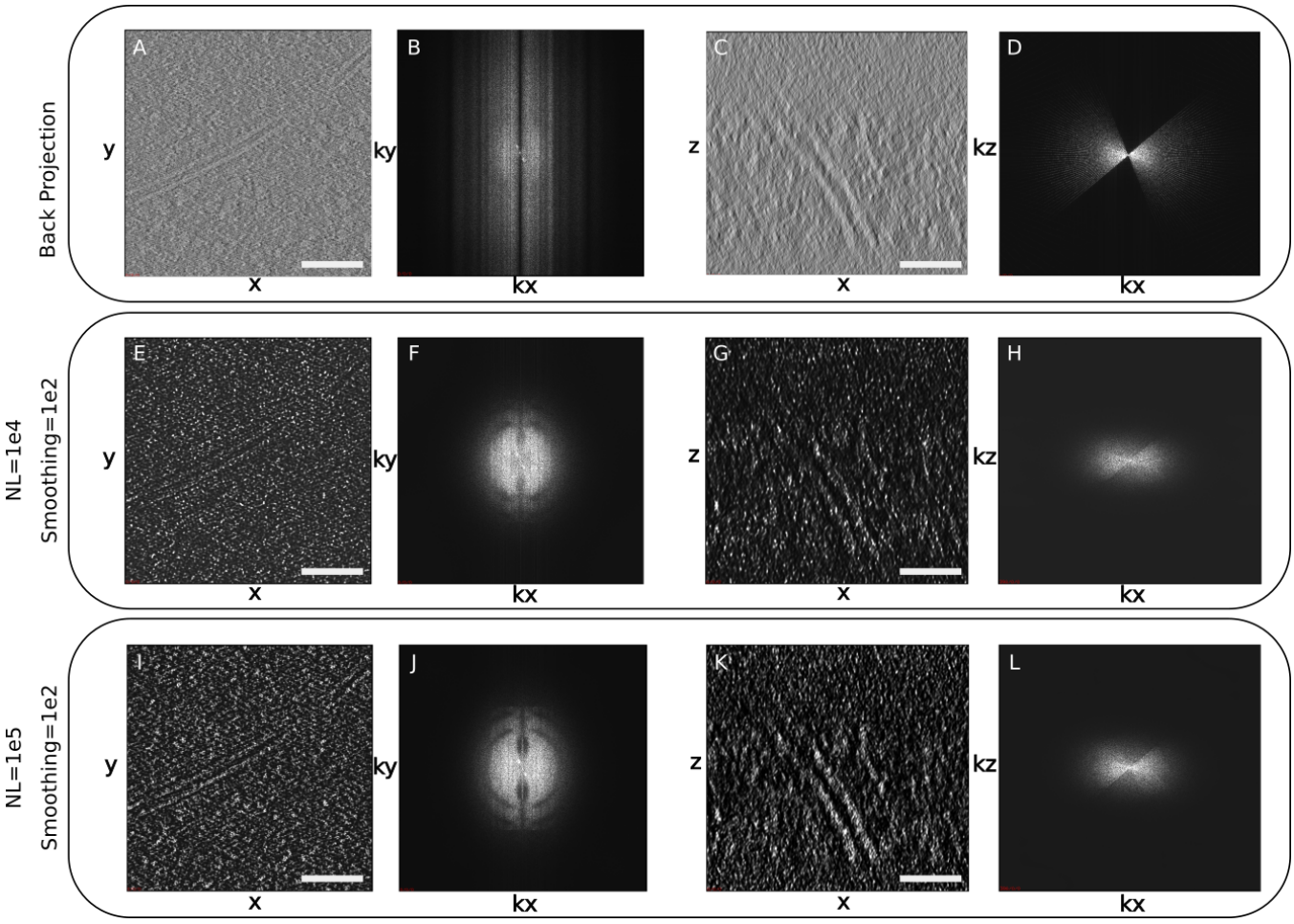

Fig. 4. Deconvolution of a Tomogram of the Nuclear Periphery of a Yeast Cell. (A) Central slice (10.62 nm thick) of the XY plane of a WBP tomogram of the nuclear periphery of a *S. cerevisiae* cell. (B) Fourier transform of A. (C) Central slice of the XZ plane of a WBP tomogram. (D) Fourier transform of C. (E) Central slice of the XY plane of a the tomogram from A, deconvolved with a smoothing parameter of 1e2 and a non-linearity factor of 10,000. (F)Fourier transform of E. (G) Central slice of the XZ plane of the deconvolved tomogram from E. (H) Fourier transform of G.(I) 10.6 nm slice of the XY plane of a the tomogram from A, deconvolved with a smoothing parameter of 1e2 and a non-linearity factor of 100,000. (J)Fourier transform of I. (K) 10.6 nm slice of the XZ plane of the deconvolved tomogram from I. (L) Fourier transform of K. Scale bars: 100 nm

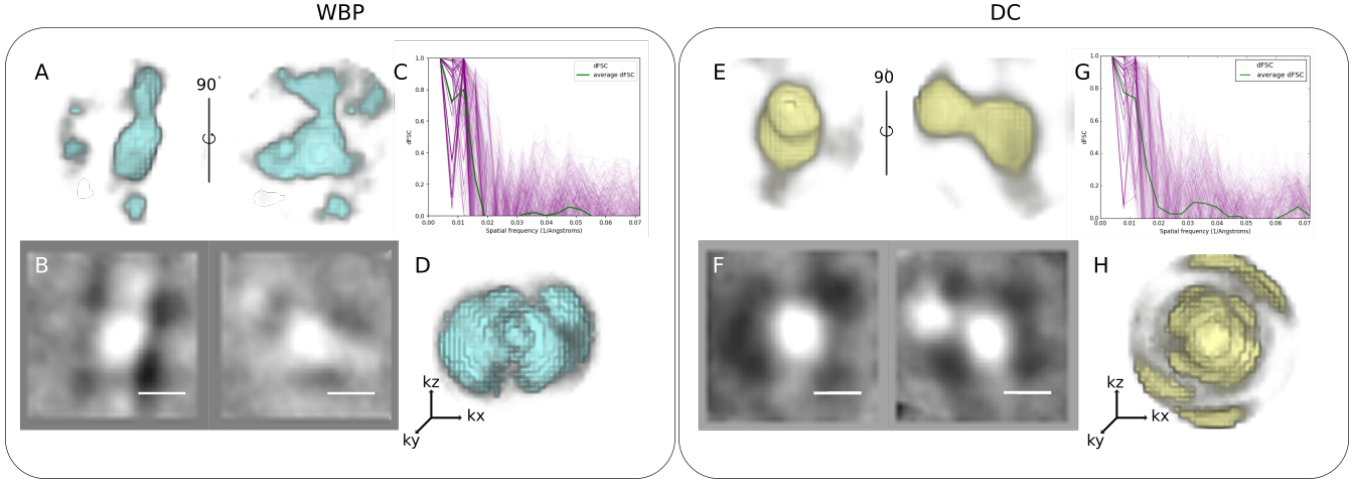

Fig. 5. Back projection vs deconvolution crystal body averages. (A) Two views of the crystal body average of WBP subtomograms. (B) Central 3.5nm slice of WBP crystal body subtomogram average. (C) 3D-FSC curves generated from two half-map averages of the WBP crystal body subtomogram average. Green line is the average, pink lines are individual directional FSCs. D) 3D render of directional FSC curves. (E-H) corresponding averages and FSCs derived from the DC volume. Scale bars: 10 nm

discussed in the supporting information.

First, the reality is that DC acts as a filter for the data. The intensity of each voxel is modified in some fashion, and care must be taken in interpreting the DC volume. DC has two parameters, for non-linearity and smoothness, and the optimal values must be determined experimentally by systematically varying the parameters over several orders of magnitude; the parameter search quickly settles into basic conver-

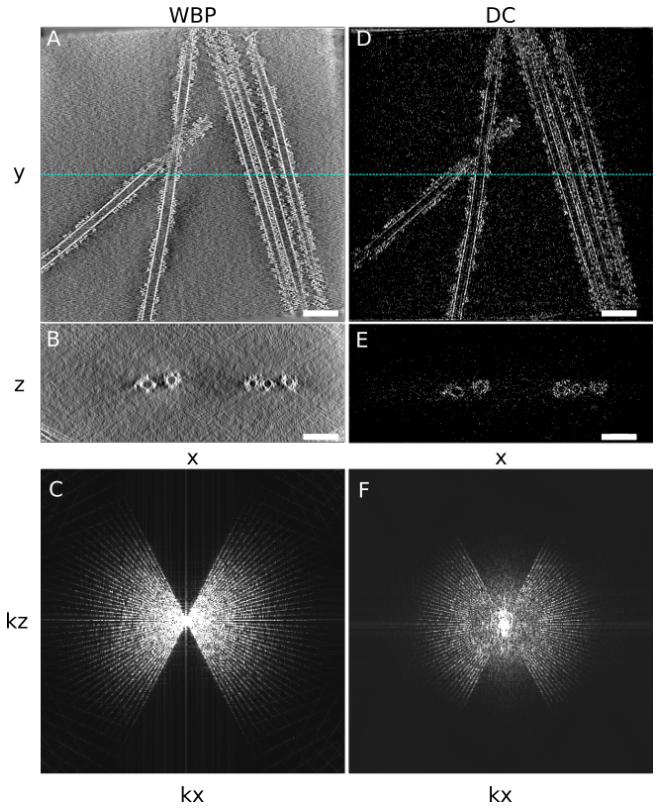

Fig. 6. Deconvolution of a tomogram of reconstituted microtubules. (A) XY slice of the WBP-reconstructed tomogram containing microtubules. (B) XZ view of the tomogram in A; blue dashed line in A corresponds to the slice shown. (C) kxkz view showing the missing wedge. (D) XY of the deconvolved tomogram. (E) XZ of deconvolved microtubule tomogram in D; blue dashed line in D corresponds to the slice shown. (F) kxkz of the deconvolved tomogram. Scale bar = 50 nm

gent DC images that look biologically reasonable (e.g. membrane bilayers are visible, ribosomes are distinct, etc.) At the end of the DC process, one can usually settle on a few DC images coming from a close smoothness parameter. These different DC images are studied side-by-side comparing 3-dimensional volumes for details. The side-by-side images are very similar to one another, but subtle features between them exist. Crucial are the orthogonal Z image planes for judging smoothness parameters and structure. There are a number of considerations for the DC process, discussed in the supporting information.

How does one know if the DC structure is credible? In addition to the side-by-side study of several smoothness DC images, a control raw WBP image must be studied alongside the DC images, at several intensity scalings of the WBP data. Any feature uncovered in the DC data would be searched for in the raw WBP data control, and would have to be present in the WBP control. However, in our experience, the DC process has never been observed to invent a structure that is not present in the WBP raw data control. (26).

This study makes the statement that the missing wedge of information is substantially filled by DC. Visually and in Fourier space representation this is the case; however, this statement needs caution. We do not know if DC will improve Z resolution for certain kinds of data, intensities, or different structures *e.g.*, of various sizes. It is possible that spaced periodic structures position on top of one another along Z in a tomogram are not resolved correctly in the DC data.

A second point in the DC discussion centers on what mathematics allows unobserved data to propagate from areas of observed data, into their correct structural space. Since all image information can be decomposed into 3-dimensional Fourier representation, one is, in essence, saying that there is information in one region of Fourier space that can be extrapolated correctly into other regions of Fourier space by the DC process. There are two examples from the inverse problems literature to reassure that such extrapolated information can indeed be real. The first one is called "Analytical Continuation" ((57), and references therein), which is known in optics literature. The Analytical Continuation (AC) conjecture, taken from (57), states: All image information can be decomposed into a Fourier transform, and a spatially bounded region of Fourier space can be expressed as an analytical function. The analytical function can be exactly known for a small region, and if there is no noise, the entire analytical function can be determined/extrapolated by AC. The extension can continue indefinitely, and this is a hallmark of AC (57). Noise is critical and the analytical values become small as iterations progress as the function get extended to higher resolution regions of Fourier space, reasons AC is little used (but see (57)). In the case of ER DC, noise is heavily suppressed and resolution extensions required are modest, suggesting that AC might work.

The second one is called compressive sensing reconstruction, used in modalities such asmagnetic resonance imaging (58) and tomography (59). It involves high-quality reconstruction from highly under-sampled Fourier data and tomographic projections with a limited set of angles with regularization constructed using derivatives. Because of the way the derivative operator is related to the measurement operator (tomographic projection or Fourier transformation), high quality reconstruction becomes possible from sparse Fourier samples or from tomographic projections from a limited set of angles. Although these theories are not directly extensible to our recovery problem, these theories reassure that exten-

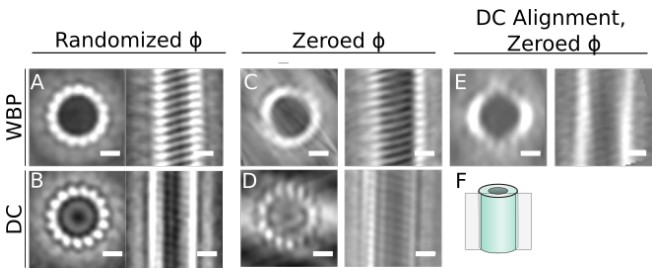

Fig. 7. Subtomogram analysis of WBP and DC-processed microtubule. In all panels, top and side views of the average are shown for the microtubule average obtained under the specified conditions (A) Average from the WBP tomogram, using a randomized azimuth-angle (ϕ) averaging approach to compensate for the missing wedge (35). (B) Average from the DC tomogram using the same randomized ϕ scheme. (C) Average from the WBP tomogram, with initial constant ϕ angles for all particles, allowing the missing wedge to affect the average. (D) Average from the DC tomogram using the same constant $]phi$ scheme. (E) Average generated by applying the alignment parameters from the DC uniform starting azimuth/restricted rotation alignment to the the WBP particles. (F) Schematic showing the location of the slice on the right-hand side image in each panel. Scale bars: 10 nm.

sions in Fourier space are possible, and hence the filled-in missing wedges may be trusted if the resultant structures in the real space appear plausible.

Another independent argument supporting why the missing wedges could correctly be filled can be given from a statistical viewpoint. Recall that the regularization used in the ER-DC enforces certain hypothesized joint distribution of intensity and second-order derivative magnitude. It turns out that the back projected images deviate significantly from this joint distribution. Hence, the minimization involved in ER-DC brings in a proper filling on the wedges such that: 1) the resulting real space image is consistent with the measured projections, and 2) the resulting real space image better matches with hypothesized distribution.

Thirdly, DC could have an impact on the electron dose required to obtain a suitable tomogram. Because only one tilt axis for the tomogram is necessary to largely fill in the missing wedges, multi-axis tilt schemes meant to minimize the missing wedge become unnecessary. The DC process might allow other dose reduction steps, such as fewer tilts and lower beam intensity. In addition, there are several aspects of the DC process that can be improved, and are described in the supporting information.

The DC process filling in the missing wedges in Fourier space allows biological structures to be followed in 3-dimensions. This resolution is adequate to see, for example, gaps between the 10 nm nucleosomes allowing a chromosome path to be followed. One imagines a two step process for cellular tomography: first, the path of a structure is followed with the architecture discerned, a process greatly improved by DC. Subsequently, once an architecture is determined, molecular features can be superimposed using averaging methods and molecular modelling (35).

Materials and Methods

Sample Preparation. Yeast *S. cerevisiae* W303a cells were grown at 30°C in YPD media (1% yeast extract, 2% bactopeptone, and 2% glucose) to mid-log phase, after which 5-μL were deposited in a glow-discharged Quantifoil grid (200-mesh copper R2/1, Electron Microscopy Sciences), followed by manual blotting and plunge freezing in a 50/50 ethane propane mix (Airgas) using a custom-built manual plunger (Max Planck Institute of Biochemistry). Human Embryonic (HEK-293T) cells transfected with LRRK2-I2020T cells were prepared as described in (35). *In vitro* reconstituted LRRK2-I2020T was prepared as described in (53). For both yeast and HEK cells, frozen cells were micromachined on a Scios or an Aquilos 2 DualBeam FIB/SEM microscope (TFS). FIB milling was done as described in (6).

Cryo-electron tomography. Tilt series were obtained on a 300 kV Tecnai G2 Polara (TFS) or Titan Krios with a field emission gun, a GIF Quantum LS energy filter (Gatan) and a K2 Summit 4k×4k pixel direct electron detector (Gatan). Tilt series were acquired between $\pm$ 50° and $\pm$70° with increments of 2° and 3°, total electron doses between 70 and 100 $e^-/Å^2$ at a target defocus of 5μm, and a pixel size of 2.2 or 3.5Å using the SerialEM software (60) in low-dose mode. Bidirectional or dose-symmetric tomography acquisition schemes were used (61), corrected for the pretilt of the lamella where appropriate. Images acquired on the K2 detector were taken in counting mode, divided into frames of 0.075 to 0.1 s.

Tomogram Reconstruction. Tilt series were aligned and dose-weighted by cumulative dose with MotionCorr2 (62). Dose-weighted tilt series were aligned and reconstructed using Etomo, part of the IMOD package (63). Patch tracking was used to define the model for fine alignment. The aligned tilt series were reconstructed using weighted back projection to generate the 3D tomograms.

Deconvolution. A set of synthetic projections was generated with x and y dimensions and pixel spacing matching the reconstructed volume that will be deconvolved. Each projection has a centered point source that is then convolved with the inverse Fourier transform of the CTF, generated using the defocus and astigmatism parameters estimated by CTFFIND4 (17). The convolved point source/CTF is then reconstructed using the same elliptically weighted back projection used to generate the target reconstructed volume. Finally, the reconstruction is cropped to the same dimensions as the volume to be deconvolved, the 3D FFT of which will be used as the final PSF. Deconvolution is then run for 100 cycles using the PSF generated. A detailed description of the deconvolution procedure can be found in the supporting information.

Subtomogram Analysis. Microtubules filaments were traced in Dynamo to define coordinates and orientation. Single particles were defined every 4 nm along the filament, and subtomograms with a side length of 66 nm were then extracted from both the back projected and the deconvolved tomograms using these coordinates. For both sets of particles, subtomograms were iteratively aligned over three rounds of two iterations each. The particles were aligned using a spherical alignment mask to minimize bias. For the first round, the alignment was constrained to a 180 degree cone aperture, with no flip allowed and 20 degrees of azimuthal rotation, corresponding to the third Euler angle. Rounds two and three used a 30 and 10 degree cone aperture, respectively, and an azimuthal search range of 10 and 2 degrees respectively. No symmetry was assumed in the alignment. For further details, see (35). To assess any compensation for the missing wedge, alignment was performed on particles with initial tables describing the particles orientation from 1) a blank table to set all particle orientations to zero, and 2) a random table assigning each particle a random orientation.

To calculate averages for the autophagosome crystal subunit in the WBP and DC tomograms, first 50 particles were identified manually in the deconvolved volume to generate an initial average. This initial average was used as a template for Dynamo's template matching functionality and used to search for similar particles. A cross correlation threshold of 0.38 was selected, below which many particles appeared

as false positives by visual inspection. Using the coordinates and putative orientations from template matching, 82 particles were cropped from both the back projected and deconvolved volumes. A global alignment was used on each dataset in two (even and odd sets) using the Dynamo subtomogram alignment function. Each half dataset was averaged and the directional Fourier shell correlation (dFSC) between the resulting half-averages. The alignment angles from the deconvolved particles were then applied to the back projection particles to create the average shown in Fig. 5A and to the relative resolution by dFSC.

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