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Cryo-electron tomography of microtubule-kinesin motor complexes

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Abstract

Microtubules complexed with molecular motors of the kinesin family or non-motor microtubule associated proteins (MAPs) such as tau or EB1 have been the subject of cryo-electron microscopy based 3-D studies for several years. Most of these studies that targeted complexes with intact microtubules have been carried out by helical 3-D reconstruction, while few were analyzed by single particle approaches or from 2-D crystalline arrays. Helical reconstruction of microtubule-MAP or motor complexes has been extremely successful but by definition, all helical 3-D reconstruction attempts require perfectly helical assemblies, which present a serious limitation and confine the attempts to 15- or 16-protofilament microtubules, microtubule configurations that are very rare in nature. The rise of cryo-electron tomography within the last few years has now opened a new avenue towards solving 3-D structures of microtubule-MAP complexes that do not form helical assemblies, most importantly for the subject here, all microtubules that exhibit a lattice seam. In addition, not all motor domains or MAPs decorate the microtubule surface regularly enough to match the underlying microtubule lattice, or they adopt conformations that deviate from helical symmetry. Here we demonstrate the power and limitation of cryo-electron tomography using two kinesin motor domains, the monomeric Eg5 motor domain, and the heterodimeric Kar3Vik1 motor. We show here that tomography does not exclude the possibility of post-tomographic averaging when identical subvolumes can be extracted from tomograms and in both cases we were able to reconstruct 3-D maps of conformations that are not possible to obtain using helical or other averaging-based methods.

Keywords

Cryo-electron microscopy; Cryo-electron tomography; Kinesin; Microtubules; Helical 3-D reconstruction; Eg5; Kar3Vik1

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Introduction

Until now, most cryo-electron microscopy 3-D data on microtubules and microtubule-kinesin complexes have been produced by so-called averaging-based methods, mainly helical averaging (DeRosier & Klug, 1970; for tubulin examples see: Sosa et al., 1997; Beuron & Hoenger, 2001; Wendt et al., 2002; Skiniotis et al., 2003; Kikkawa et al., 2000; Hirose et al., 2006; Sindelar et al., 2007), but also by single particle approaches (Li et al., 2008) and 2-D crystallography (e.g. to solve the structure of the $\alpha\beta$ -tubulin dimer itself: Nogales et al., 1998). Common to all these cases, 2-D projections of identical particles at various angles are collected, averaged and back-projected into 3-D space. Though this could be done in real space, it is often carried out in Fourier space where phases and amplitudes can be assessed separately. The most important prerequisites for averaging-based 3-D reconstructions are **A**: the averaged particle must be identical in shape and conformation, and **B**: their projection angles relative to each other (so-called Euler angles) must be determined accurately. If this is achievable, averaging-based methods are very powerful for elucidating molecular structures and may yield close to atomic detail (tubulin: Nogales et al., 1998).

In most biological applications, however, many structures, even small macromolecular assemblies are often too flexible and dynamic for averaging procedures. For these cases, the currently most promising alternative to averaging-based 3-D reconstruction methods is cryo-electron tomography. Initially, tomographic 3-D reconstruction does not rely on averaging and therefore can be applied to any specimen that can be imaged in an electron microscope, no matter how complex its structure may be (reviewed in Lucic et al., 2005; Hoenger & McIntosh, 2009). However, once a tomogram is completed, subvolumes containing identical particles may be extracted and further processed by averaging (Walz et al., 1997; Taylor et al., 2007; Förster et al., 2007; Bartesaghi et al., 2008). To this end, we have developed our own software called **PEET** (**P**article **E**stimation for **E**lectron **T**omography: Mastronarde et al., in preparation, for example see Nicastro et al., 2006). Here we illustrate the advantages and disadvantages of both helical reconstruction and volume averaging methods for microtubule-kinesin motor domain complexes.

The kinesin superfamily comprises a large and diverse group of molecular motors that interact with microtubules to perform a wide variety of essential functions in the cell including roles in intracellular transport and cell division (reviewed in: Hirokawa et al., 2009). Mitotic kinesins are a group of kinesins that are required for cell division, playing a crucial role in assembly, maintenance and function of the mitotic spindle (reviewed in: Wittmann et al., 2001). As such, they now constitute a new focus for studies on anti-cancer drugs and therapies targeted specifically to kinesin motor function, either directly towards the motor domains (e.g., Monastrol: Maliga et al., 2002 & 2006; Cochran et al., 2004; Crevel et al., 2004; Yan et al., 2004; Cochran & Gilbert, 2005; Krzysiak *et al.*, 2006), or to other functional parts such as cargo domains and receptors. This report focuses on the motor domains of two structurally very different kinesins with important roles in mitosis: the plus-end directed kinesin-5 Eg5 (Kashina et al., 1996; X-ray structure: Turner et al., 2001) as a monomeric motor domain, and the minus-end directed kinesin-14 Kar3 (Meluh & Rose, 1990) as a functional heterodimer with Vik1 (Manning et al., 1999; Barrett et al., 2000).

Eg5 is a highly conserved homotetrameric kinesin with an essential role in formation of the bipolar microtubule spindle and separation of the chromosomes during mitosis (Blangy et al., 1995). Due to its bipolar structure, it is currently believed that Eg5 motors translocate to the plus-ends of microtubules at the onset of mitosis and begin to organize microtubules by cross-linking leading to the formation of the stable bipolar spindle seen in metaphase (Kapitein et al., 2005). Once the spindle is in place, Eg5 provides structural support and

contributes to poleward flux by sliding the microtubules apart towards the centrosomes (Valentine et al., 2006). Structural studies are of interest to elucidate the mechanical properties of this homotetrameric kinesin and its mode of action in spindle maintenance.

Kar3Vik1 is an unusual heterodimeric kinesin found in *Saccharomyces cerevisiae*. Both components of the heterodimer have been individually crystallized and solved to about 2.5Å resolution (Kar3: Gulick et al., 1998; Vik1: Allingham et al., 2007). Kar3Vik1 is unusual because it has only one motor domain, Kar3, whose localization and activity is regulated through its association with a non-motor protein, Vik1 (Manning et al., 1999). Kar3Vik1 is active during vegetative growth where it localizes predominantly at the spindle poles and is thought to cross-link microtubules, contributing to mitotic spindle assembly and stabilization (Manning & Snyder 2000, Gardner et al., 2008). Interestingly, the recently solved crystal structure of the C-terminal globular domain of Vik1 revealed that it has a fold remarkably similar to a kinesin motor domain, but does not have an active site for ATP hydrolysis (Allingham et al., 2007). Despite being unable to hydrolyze ATP, Vik1 binds tightly to microtubules in vitro supporting a model where both Kar3 and Vik1 interact with the microtubule during its walking cycle. Structural studies on Kar3Vik1 thus aim to identify a novel kinesin-microtubule interaction and may also provide insight into the mechanisms of movement of other heterodimeric kinesins that have thus far been difficult to study (Woehlke & Schliwa 2007).

Tomography is not a new method for electron microscopy, but recent technical advances on hardware and software have since strongly boosted its popularity (Frank, 2005). In particular, tomography on vitrified specimens is now realistic due to sensitive detectors and automated low-dose recording software. Here we focus exclusively on cryo-electron tomography since only frozen-hydrated specimens have the structural preservation required for molecular analysis. By using cryo-electron tomography we could resolve molecular details in kinesin-microtubule complexes that would be impossible to image in 3-D by helical averaging. However, even by volume averaging approaches the interpretable molecular details of cryo-tomography compare to the lower-resolution end of helically averaged maps. Hence, both methods are very complementary. Helical averaging wins the resolution race but sometimes loses important details due to averaging and symmetry enforcement.

Results & Discussion

Cryo-electron tomography versus helical 3-D reconstruction on kinesin-microtubule complexes

The first structural analyses of microtubules date back to Amos and Klug (1974) who also produced the first 3-D reconstruction of tubulin protofilaments and model of a microtubule (Amos and Baker, 1979) from zinc-induced 2-D crystalline tubulin sheets. However, 3-D cryo-EM on microtubules picked up only after the developments of cryo-EM methods (Dubochet et al., 1988) and the discovery that microtubules composed of 15 protofilaments may form helical polymers (Fig. 1E–I; Wade & Chretien, 1993; Arnal et al., 1996; Beuron & Hoenger, 2001) while most others carry a so-called lattice seam (Fig. 1A & C; Mandelkow et al., 1986; Kikkawa et al., 1994; Sandblad et al., 2006) that interrupts helical symmetry. The second boost came from the X-ray crystal structures of monomeric and dimeric ncd (Sablin et al., 1996 & 1998) as well as monomeric (Kull et al., 1996) and dimeric kinesin-1 (Kozielski et al., 1997). With these data at hand, molecular docking became popular for kinesin-microtubule structures (Sosa et al., 1997) and many other macromolecular assemblies after automated procedures became available (Volkmann & Hanein, 1999; Wriggers et al., 1999). Today the best resolved 3-D data on kinesin-

microtubule complexes were obtained by helical averaging and show 8–9Å detail (Hirose et al., 2006; Sindelar et al., 2007; Bodey et al., 2009).

The major advantages of helical reconstruction of microtubule-MAP complexes can be summarized as follows:

- A.** Helical averaging produces low-noise data with isotropic resolution, sometimes to near-atomic detail. The averaging power is very high as a large number of unit cells (50,000 and more) can be included rapidly, yielding an excellent signal to noise ratio. Diffraction (Fig. 1G) and Fourier filtering (Fig. 1D, E & G) allow rapid assessments of the data quality.
- B.** Raw-data are obtained from single, untilted projections which strongly facilitates corrections for the contrast transfer function (CTF) and allows the application of a high electron dose (~40–100 electrons/Å²; Fig. 1F) at each image since each individual specimen is only recorded once.

The major constraints of helical averaging are:

- A.** By definition, helical 3-D reconstruction requires a perfectly helical assembly (Fig. 1E–I) that is often either highly artificial (e.g. in the case of kinesin-microtubule complexes the motor to tubulin ratio is much higher than under in vivo conditions (Fig. 1I)), does not reproduce native conformations, or is simply impossible to obtain (structures too flexible and dynamic).
- B.** Single molecular events such as lattice seams (Fig. 1A & C) or missing individual particles (Fig. 3B) cannot be observed in 3-D. If these events occur, they will be lost by being averaged over all other events during the 3-D reconstruction process, potentially interfering with the accuracy of the resulting 3-D maps.

The emerging alternative method to study macromolecular assemblies without the constraints associated with helical reconstruction is cryo-electron tomography (Figs. 1A, 3A, 3B, 4A & 5A). As illustrated in Figure 1A, kinesin-microtubule complexes can be reconstructed in 3-D independent of the polymer composition and can be analyzed for structural detail (such as the lattice seam or handedness of the helical path at distinct locations) that would not be observable by a helical averaging approach. By focusing on thin slices through the tomogram, single motor domains, and their empty spots when missing can be observed (Fig. 3B). Such single events would be difficult to detect on regular cryo-micrographs that superimpose all the densities along the projection. 3-D data from cooperative decoration events (Figs. 2A & 3B) or bridging motor domains between two adjacent microtubules (Fig. 4) cannot be obtained by helical averaging since they do not follow any helical symmetry.

However, although no averaging is done with the raw data and/or in the tomographic 3-D reconstruction procedure, averaging may still be applied as a post-tomographic procedure, further enhancing the signal to noise ratio for particles which can be identified within a tomogram at various orientations, but which are otherwise structurally reproducible enough to be averaged as entire volumes (Walz et al. 1997; Taylor et al. 2007; Förster et al., 2007; Bartesaghi et al., 2008). This is much more computationally demanding and only 10–15 years ago would not have been realistic without the Linux clusters or multi-core computers that we use today. We are developing a dedicated software suite for averaging volumes selected from tomograms called PEET (**P**article **E**stimation for **E**lectron **T**omography: Mastronarde et al., in preparation, for example see Nicastro et al., 2006). With this software we produced the averages presented in figures 1B&C, 3C, 4B, and 5A–F. Each average requires between 10–20 CPU hours split up over 35 processors on our Linux cluster. Typically the number of particles that go into an average is much smaller than for a helical

approach. This reduces the averaging power, but 3-D particles contain more structural information than 2-D projections, which facilitates accurate alignments.

The major advantages of tomographic reconstruction of microtubule-MAP complexes can be summarized as follows:

- A. Cryo-tomographic 3-D reconstruction is applicable to any random structure and requires neither symmetry nor any structurally reproducible sub-particles (unless post-tomographic averaging should be applied). Cryo-tomography works well on plunge-frozen isolated macromolecular assemblies (as presented here) as well as on vitrified sections (Norlén et al., 2009).
- B. Extraction of thin slices from tomograms reveals relatively noise-free data that omits the convolution of projected data with lower and upper structures (see Figs. 3B, 5B&C; the result is somewhat similar to a confocal recording in light microscopy).
- C. Single molecular events may be observed and analyzed (e.g. missing particles in an otherwise regular array; Fig. 3B).
- D. Post-tomographic data processing such as sub-volume averaging is facilitated by starting off with 3-D data rather than 2-D projections and if successful, yields high signal to noise 3-D data (see Figs. 1B&C, 3C, 4B, 5B–F).

The major constraints of tomographic 3-D reconstruction are:

- A. Cryo-tomograms are composed of tilt-series (typically 80–100 projections) recorded on the very same object. The resolution in tomograms depends on the accuracy of alignment and back-projection of each projection. Deviations from the assumed tilt angles, focus variations, and technical limitations such as bending support films reduce the resolution in the final tomograms.
- B. The design of electron microscope stages and grid holder geometries limit the recording range of tilt-series to approximately -70 and $+70$ degrees (rather than a full 180 degree rotation), leaving a missing wedge of data that results in anisotropic resolution (see Figs. 1B, 3C, 4B, 5E and 6A&B) that is usually lower than what is achieved with a helical approach.
- C. Cryo-tomograms have to be collected under strict low-dose conditions and therefore individual projections are extremely noisy, which may interfere with the alignment and back-projection process.
- D. While not impossible, CTF correction on tilt series is demanding due to the various focus values in images from tilted specimens (see: Xiong et al., 2009).

Cryo-EM on Kar3Vik1-microtubule complexes reveals a highly cooperative binding pattern and a one-head-down and one-head-up dimer-binding configuration

Kar3Vik1 is an unusual kinesin in the sense that it comprises a typical kinesin-14 motor domain (Kar3-MD) heterodimerized with a non-motor protein, the Vik1 motor homology domain (Vik1-MHD) by a coiled-coil interaction. Conventional cryo-electron microscopy reveals a Kar3Vik1 binding property that is dominated by a cooperative process (Fig. 2A). This can be seen directly as uneven decoration along microtubules where one side of some microtubules is completely free of motor decoration while the other side appears fully decorated. Figure 3 shows a cryo-tomographic 3-D reconstruction of a partially decorated microtubule. Slices through the microtubule framed in Figure 3A reveal the top portion to be void of motors, while the bottom portion shows full decoration. The center part reveals some

missing individual motors along otherwise fully decorated protofilaments (circles in Figure 3B).

Due to the partial decoration in the framed area in Figure 2A and the optimal side-view of the motors, the overall shape of the dimeric Kar3Vik1 construct is clearly visualized. Figure 2B shows a contrast-enhanced magnification of the framed region in Figure 2A. Here, the three domains of the tubulin dimer, the Kar3-MD, and the Vik1-MHD are well separated. The tomographic analysis shown in Figure 5B confirms this finding. The motors have been flushed with AMP-PNP, a non-hydrolyzable ATP analogue used to mimic an ATP binding state. Under these conditions we found isolated Kar3-MD to be strongly bound to microtubules while Vik1-MHD's bound with much lower affinity. Accordingly, we assume that the domain in contact with the microtubule surface is the Kar3-MD while the tethered domain is Vik1-MHD. However, this remains to be determined experimentally.

Both features of Kar3Vik1, the cooperative binding process (Wendt et al., 2002), and the binding conformation with one-head-down, one-head-up is highly reminiscent of dimeric ncd constructs (Sosa et al., 1997; Hirose et al., 1998; Wendt et al., 2002; Endres et al., 2006) suggesting a common pattern for minus-end directed motors and the members of the kinesin-14 family. Other dimeric kinesin MD's prefer to bind with both heads simultaneously to the microtubule surface and do not display the strong cooperativity seen here and with dimeric ncd (Kinesin-1; Hoenger et al., 2000; Eg5; Krzysiak et al., 2006).

The power of tomography in combination with volume averaging

Although tomographic 3-D reconstruction does not rely on any kind of averaging procedure, post-tomographic averaging constitutes a powerful tool to further enhance the signal to noise ratio in some parts of the tomogram. However, as in every averaging approach, the structures subjected to averaging have to be identical else corrupted 3-D maps prone to misinterpretations and false conclusions will be obtained. The advantage of post-tomographic averaging lies in the fact that 3-D structures are generally more easily assessed than 2-D projections and often show more recognizable features. The difficulty with post-tomographic averaging, however, comes from the missing wedge of data that reduces the resolution particularly in the Z-direction and the resulting elongation of structures in Z potentially interferes with the 3-D alignment process. Our data on volume-averaging presented here has been exclusively produced by our software suite PEET (initial procedure described in Nicastro et al., 2006; Mastronarde et al., in preparation). The mathematical and computational details of the averaging process with PEET will be published elsewhere (Mastronarde et al., in preparation).

In the case of kinesin-microtubule complexes the tomogram allows us to carefully inspect a microtubule for lattice defects and other irregularities. Once approved, we divide the microtubule into overlapping axial segments about 130 nm long, at intervals of 8 nm, making sure to include a full $\alpha\beta$ -tubulin repeat. While the lattice seams interrupt any helical symmetry, the sequence of circular segments along the microtubule axis and each individual protofilament are still identical. Once the segments have been selected, they are aligned according to the microtubule supertwist (if there is one) and averaged. The results are shown for a microtubule complexed with monomeric Eg5 (Fig. 1B & 1C; a total of 218 particles were averaged), a microtubule partially decorated with dimeric KarVik1 (Fig. 3C; 146 particles), Kar3Vik1 dimers bridging between two adjacent, parallel microtubules (Fig. 4B; 44 particles) and a microtubule fully decorated with Kar3Vik1 dimers (Figs. 5A–E and 6A; 99 particles).

Two factors are of utmost importance here: i) the usable tilt-range, and ii) the number of different angular views integrated. Even with a relatively large group of particles as shown

in Figures 1B and 1C where 218 particles have been averaged, some missing wedge effects remain visible such as a smeared-out density wall of badly separated protofilaments along the Z-axis, while the X and Y axes show very clear separations. The same is visible in Figures 3C, 5E and 6A. This is an intrinsic effect of any single-tilt tomogram. For microtubule reconstructions that focus on 3-D data surrounding the lattice seams this means no rotational averaging around the tubular axis can be done (Fig. 1B & 1C) and the only rotation that partially weakens the missing wedge effect comes from an axial supertwist (see Fig. 1E & 1G). That supertwist is completely absent in 13-protofilament microtubules (see Fig. 1D). The microtubule in Figures 5 and 6, however, is a helical 16-protofilament microtubule where rotational averaging due to the helical symmetry can be applied in addition to averaging axial segments (Figs. 5F, 6D&E). We have done such averaging not by imposing rotational symmetry on the original average, but by separately aligning each segment of the microtubule in 16 different orientations. The same rotational averaging could be done with any tomographic reconstruction of a microtubule that does not possess a seam, but only where features along protofilaments are identical such as fully decorated motor-microtubule complexes. As shown in Figures 5 and 6, rotational averaging immediately increases the number of asymmetric units by the number of protofilaments (i.e. $16 \times 99 = 1584$). The asymmetric unit is now reduced from an entire tubular slice to a single $\alpha\beta$ -tubulin-motor domain complex. Accordingly, the Fourier shell correlation graphs reveal a significantly increased signal to noise ratio, and an interpretable resolution (i.e. visible detail not obscured by noise) improvement from 3.8nm to 3.2nm (Fig. 6C&F).

Molecular details in cryo-electron tomograms

Here we demonstrate that cryo-electron tomography and helical 3-D approaches (or any other averaging-based methods) are highly complementary. We can conclude that cryo-electron tomography is an excellent method for any kind of assembly that lacks apparent symmetry and that precludes helical, single-particle, or 2-D crystalline averaging, such as the examples shown here in Figures 1, 3 and 4. Even without post-tomographic averaging, structural details can be interpreted to about 4nm resolution. For example, individual protofilaments are spaced 5nm laterally and 4nm axially, features that are clearly visible in tomographic slices (see Fig. 3B), at least when not obscured by the missing wedge effect. Other structural features such as lattice angles and missing domains can be assessed reliably. However, molecular docking of small molecules such as kinesin motor domains remains difficult at the current resolution limits attainable by cryo-electron tomography. Volume averaging, where applicable currently pushes the interpretable resolution close to 3nm detail and may reach beyond 2nm in the future. Here, features such as the two domains of the $\alpha\beta$ -tubulin dimer and the binding geometry of motor domains to microtubules can be reliably visualized (see Fig. 5C; and after rotational averaging: Figs. 5F, 6D&E). However, the theoretical resolution attainable by post-tomographic averaging ultimately depends on the initial resolution that can be preserved from the raw images by tomographic reconstruction, which is the limiting factor in this case. Therefore, even for highly symmetric assemblies such as the helical 16-protofilament motor-microtubule complex shown in Figures 5 & 6, reaching better than 2nm isotropic resolution via a cryo-electron tomography approach may remain out of reach for a while longer.

Materials & Methods

Expression and purification of Eg5 and Kar3Vik1

Eg5 monomer—The monomeric motor domain of Eg5 was expressed and purified as described by Cochran et al. (2004).

Kar3Vik1 heterodimer—Heterodimers of Kar3Vik1 were expressed and purified as described by Allingham et al. (2006).

Microtubule polymerization

Microtubules were polymerized in vitro from 45 μ M bovine tubulin (Cytoskeleton, Inc, Denver CO) with BRB80 (80mM PIPES pH 6.8, 1mM MgCl₂, 1mM EGTA) in the presence of 1mM GTP, 10 μ M paclitaxel and 8% (v/v) DMSO for 30 minutes at 37°C, and allowed to stabilize overnight at room temperature.

Sample preparation for cryo-electron microscopy

Eg5-microtubule complexes were formed in solution in the presence of 2mM AMP-PNP (Sigma-Aldrich Corp., St Louis, MO), at a final tubulin concentration of 4.5 μ M and a final Eg5 concentration of 18 μ M (see also Krzysiak et al., 2006). Complexes were adsorbed to holey-carbon C-flat grids (Protochips, Inc, Raleigh, NC) for 45 seconds and subsequently plunge-frozen into liquid ethane using a homemade plunge-freezing device.

Kar3Vik1-microtubule complexes were formed directly on holey-carbon C-flat grids to prevent bundling of the microtubules. Microtubules at a concentration of 4.5 μ M were adsorbed onto grids for 45 seconds and excess liquid was blotted away. Kar3Vik1 at concentrations ranging from 3.5 μ M–4.5 μ M was incubated with the microtubules for 2 minutes in the presence of 2mM AMP-PNP and plunge-frozen as described above. 10nm colloidal gold was added to all samples before plunging for use as fiducial markers.

Electron microscopy data collection

Samples were transferred under liquid nitrogen to a Gatan-626 cryo-holder (Gatan, Inc, Pleasanton, CA). Cryo-electron microscopy data was collected on an FEI Tecnai F20 FEG transmission electron microscope (FEI-Company, Eindhoven, The Netherlands) operating at 200kV. Images and tilt-series were collected at a nominal magnification of 29 000x and a defocus of -2.5μ m (Kar3Vik1-microtubules complexes) or -6μ m (Eg5-microtubule complexes). Single frame images (Fig. 2A) were taken with an electron dose of 15electrons/ $\text{\AA}^2$. Tilt series consisting of about 80 low dose images, were collected by tilting the specimen between $-60^\circ/+60^\circ$ (Figs. 1A, 3A and 4A), or $-70^\circ/+70^\circ$ (Fig. 5A), imaging at 1.5° increments. Images at higher tilts were recorded with a higher dose to compensate for the increase in sample thickness. SerialEM software (Mastronarde, 2005) was used to automate the data acquisition and minimize exposure of the specimen to the electron beam. The total dose for each tilt series was about 100electrons/ $\text{\AA}^2$. Images were recorded binned by two on a 4K $\times$ 4K Gatan Ultrascan 895 CCD camera (Gatan, Inc). With this camera, at a nominal microscope magnification of 29,000x the resulting pixel size corresponds to 7.6 $\text{\AA}$ on the specimen.

Tomogram reconstruction and sub-tomogram averaging

Tomograms were reconstructed from tilt series data by weighted back-projection using the IMOD software package (Kremer et al., 1996). 10nm colloidal gold beads were tracked as fiducial markers to align the stack of tilted images.

To select subtomograms for averaging motor-decorated microtubules (Figs. 1, 3, 5 & 6), the center of the microtubule and the trajectory along one of its protofilaments were modeled by hand every 15–30nm. A program was run to fill in model points every 8nm along the microtubule axis (representing the length of an $\alpha\beta$ -tubulin dimer repeat), and to determine the twist of the microtubule to produce a set of initial orientations for an alignment search in

the first round of averaging. The PEET software package was used to align and average repeating subvolumes.

To average the Kar3Vik1 heterodimers in between two microtubules (Fig. 4), subvolumes each containing one Kar3Vik1 ‘bridge’ were modeled by hand and input into PEET for alignment and averaging.

Isosurface rendering of the subvolume averages was carried out using IMOD (Figs. 5 & 6) or UCSF-Chimera (Figs. 1, 3 & 4) (Pettersen et al., 2004). A low pass filter was applied to all averages to reduce noise before rendering.

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Figure 1. Comparison between cryo-electron tomography and helical 3-D reconstruction of frozen-hydrated microtubules decorated with monomeric kinesin (Eg5) motor domains

(A) A 4.5nm slice from a tomogram of microtubules decorated with monomeric Eg5 motors. Microtubules which are slightly tilted to the image plane expose a sequence of their top, lumen and bottom regions as they cut through the slice. The B-lattice pattern emphasized by the motor decoration shows a different angle along the Bessel -2 helix according to the sketch. Since this is a left-handed helix, red lines show the angular orientation of the helical path according to the top view (towards the observer), while the green lines indicate the course of the helix at the bottom of the microtubule. Occasionally a lattice seam, typical for non-helical microtubules with B-lattices, can be seen directly. The inset magnifies the framed area and reveals a bottom view of the seam, here made visible by the motor decoration. The seam is visible due to the shift of one protofilament by 4nm in the axial direction relative to the other (interruptions in green lines). (B) Cross-section through a 9nm projection of a volume-average obtained by PEET combining 218 particles along one microtubule. This microtubule is composed of 14 protofilaments, which can be clearly seen despite the obvious loss of resolution along the Z-axis (here top to bottom) due to anisotropy caused by the missing wedge of data. (C) The surface rendering of the average clearly shows the helical path of the microtubule as well as the lattice seam typical for a 14-protofilament microtubule. (D–F): Helical averaging of microtubules is only possible if they are truly helical. This is the case for a 15-protofilament microtubule (red frame in F) but not for the more common 13-protofilament microtubules (yellow frame in F). Fourier filtering (D & E) of the microtubules marked by frames reveals strikingly different 2-D projection patterns. 13-protofilament microtubules (D) are perfectly aligned with the tubular axis while 15- (E–I), or 16-protofilament microtubules (Figs. 5 & 6) reveals a super-twisted arrangement. The supertwist is visible in the 3-D reconstruction in F as a deviation of the protofilaments (dotted green line) from the microtubule axis (solid green line). Kinesin motor decoration reveals a Fourier filtered image as shown in G. The helical diffraction pattern (H) shows the helical layerline pattern where the layerlines around Bessel -2 (axial motor-tubulin dimer repeat) and -4 (axial α - β - α - β tubulin repeat) form a cluster of 3–4

strong layerlines due to the convolution of the -2 and -4 helices with the protofilament supertwist. **(I)** The 3-D reconstruction reveals the added kinesin heads as yellow densities, marking each $\alpha\beta$ -tubulin dimer.

Figure 2. Cryo-electron microscopy of microtubules decorated with Kar3Vik1 heterodimers
(A) Frozen-hydrated microtubules decorated with a dimeric Kar3Vik1 construct reveals obvious cooperative microtubule-binding properties. The cooperative binding creates a heterogeneous decoration pattern where some microtubules show full decoration on one side while the opposing side is free of motor decoration (red frame). **(Insets in A)** Crystal structures of the Vik1 motor-homology domain (left), and the Kar3 motor domain (right) are shown for comparison. Some structural similarities such as the central β -sheet surrounded by three α helices on each side are obvious. However, despite the similarity, Vik1 lacks a nucleotide-binding site. **(B)** Magnified and contrast enhanced region of the frame in **A**. The picture contrast has been inverted and boosted by an “embossing” filter to emphasize the visibility of the tubulin-Kar3Vik1 dimer complexes. Since the motors have been flushed with AMP-PNP, we believe that the domain in contact with the microtubule surface is the Kar3-MD while the tethered domain is Vik1-MHD. However, this remains to be determined experimentally

Figure 3. Dissection of microtubules partially decorated with Kar3Vik1 by cryo-electron tomography

(A) A 9nm slice through a tomogram of microtubules decorated with Kar3Vik1 heterodimers reveals features similar to those discussed in Figure 1. Kar3Vik1 decorates microtubules in a cooperative fashion leaving empty patches besides fully decorated areas. (B) Three individual 4.5nm sections through the top, center, and bottom of the tomogram of the microtubule boxed in A. The inset in the center panel shows an end-on view of the microtubule. While the bottom region shows strong striations every 8nm corresponding to a fully motor-decorated surface (green lines) the top region reveals empty microtubule protofilaments running axially. A striking advantage of tomographic 3-D reconstruction is observed in the center panel in that small single events such as individual missing motors (red circles) are revealed with much better clarity than in 2-D projections. (C) An isosurface representation from a PEET average of 146 particles selected from the microtubule in B gives a clear view of how the top of the microtubule is free of motor decoration while the bottom and sides of the microtubule are completely decorated by KarVik1.

Figure 4. Cryo-electron tomography of Kar3Vik1 dimers connecting between adjacent microtubules

(A) 18nm x-z (top) and x-y (bottom) slices through a tomogram showing the cross section through two adjacent microtubules (top) and the Kar3Vik1 motors extending toward each other between the two microtubules (bottom). (B) An 18nm projection and surface rendering of an average obtained by PEET from 44 subvolumes selected from the tomogram in A. The bridging motors appear to bend towards each other in a manner different from the regular radially extending conformations shown in Figures 3 and 5. Since this is a highly artificial in vitro situation, the biological significance of these bridging motors is not clear. Rather, this exemplifies the way we are able to use cryo-electron tomography and subsequent volume averaging to analyze structural details that would not be possible by other averaging methods.

Figure 5. Cryo-electron tomography and subsequent volume averaging of a fully decorated Kar3Vik1-microtubule complex

(A) 3nm thick tomographic x-y slice of a microtubule decorated with Kar3Vik1 overlaid with the averaged volume shown in B–E and Figure 6A and 6B. (B–D) 4nm x-y slices through the top and center of an average of 99 particles selected from the tomogram in A according to the planes indicated in E. The center slice in D cuts along the missing wedge, noticeable as reduced resolution compared to the x-y slice above. The central x-y slice (C) through the microtubule clearly shows densities corresponding to the α - and β -subunits of tubulin and to the two globular domains of Kar3 and Vik1 extending out from the microtubule. (E & F): End-on view (x-z) of the 3-D map before (E) and after rotational averaging over all protofilaments (F). The rotationally averaged map now looks indistinguishable from a helical reconstruction map at the same resolution. While the missing wedge effect is clearly visible before rotational averaging as the strong densities and smearing out along the z-axis, rotational averaging eliminates the missing wedge completely.

Figure 6. Rotational averaging around the tubular axis effectively eliminates the missing wedge (A & B) Surface renderings of the averaged 3-D map are shown in Figure 5A–E. The cross-section reveals the number of protofilaments of this microtubule to be 16, which produces a perfectly helical microtubule (Bessel order -2) without seams (see B). (D & E) Surface rendered 3-D map after rotational averaging of the map in A (see also Fig. 5F). The missing wedge effect is eliminated. By rotationally averaging over all 16 protofilaments the asymmetric unit changes from axial slices along the microtubule axis to one $\alpha\beta$ -tubulin–motor domain complex. Hence, the theoretical maximum number of asymmetric units included in the average is increased from 99 to 1584 (16×99). However, our average is based on the 1242 particles with the best correlation to the reference because this subset gave the highest resolution. However, we have included particles with the highest correlation scores only up to the point where the Fourier shell correlation continues to improve; thus, the number of particles used in the rotationally averaged volume is 1242. (C & F) For Fourier-shell correlation calculations, the datasets were split in half on a random basis and the two halves were correlated against each other. Fourier-shell correlation graphs obtained from the maps in A (C) and D (F) reveal resolution limits of 3.8nm and 3.2nm respectively based on the 50% correlation criterion. For FSC calculations a dataset is split in half on a random basis and correlated against each other. Accordingly the number of asymmetric units in each group is maximally 49 before rotational averaging, and 621 afterwards.