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Current Opinion in Structural Biology

Challenges and opportunities in the high-resolution cryo-EM visualization of microtubules and their binding partners

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As non-crystallizable polymers, microtubules have been the target of cryo-electron microscopy (cryo-EM) studies since the technique was first established. Over the years, image processing strategies have been developed that take care of the unique, pseudo-helical symmetry of the microtubule. With recent progress in data quality and data processing, cryo-EM reconstructions are now reaching resolutions that allow the generation of atomic models of microtubules and the factors that bind them. These include cellular partners that contribute to microtubule cellular functions, or small ligands that interfere with those functions in the treatment of cancer. The stage is set to generate a family portrait for all identified microtubule interacting proteins and to use cryo-EM as a drug development tool in the targeting of tubulin.

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Current Opinion in Structural Biology 2017, **46**:65–70

This review comes from a themed issue on **Cryo-electron microscopy**

Edited by **Wah Chiu** and **Kenneth Downing**

http://dx.doi.org/10.1016/j.sbi.2017.06.003

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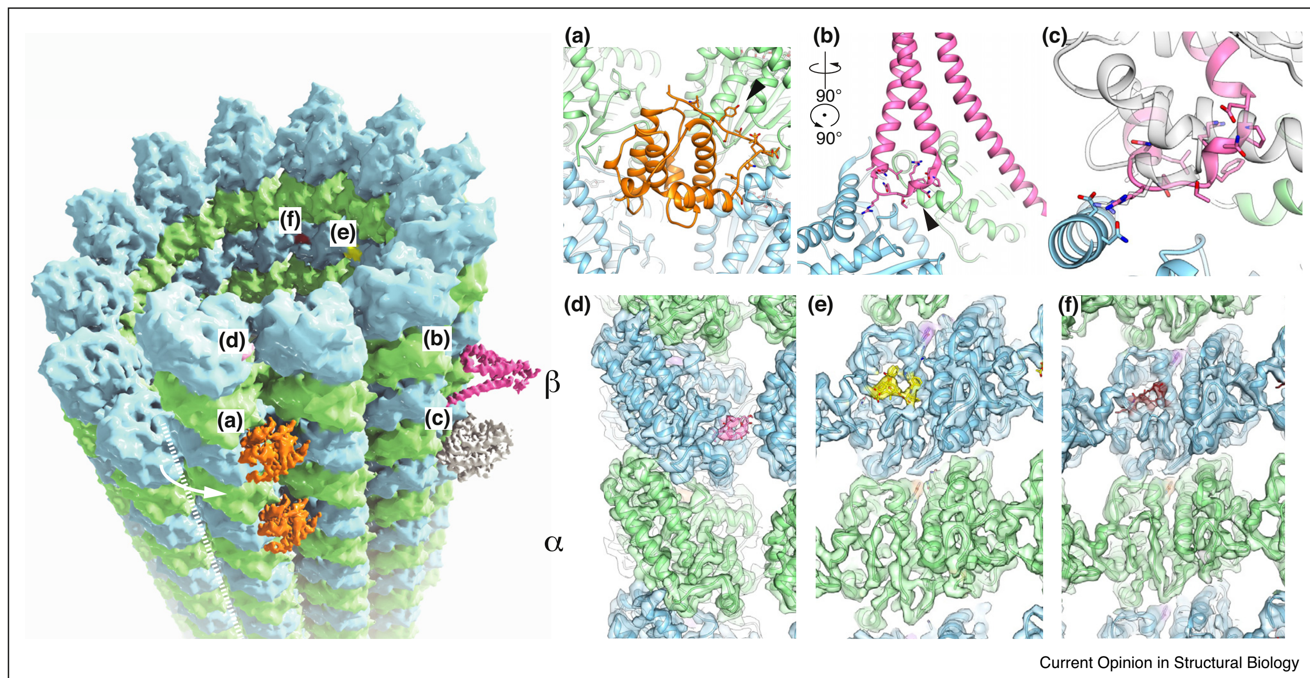
Tubulin organization within the microtubule: symmetry or no symmetry?

The microtubule (MT) is a highly conserved and essential cytoskeletal polymer built of αβ-tubulin that undergoes remarkable dynamics powered by the energy of GTP hydrolysis. In the MT, tubulin dimers add head-to-tail making linear protofilaments (PFs), which themselves interact in parallel, with a certain lateral curvature and stagger, giving rise to the cylindrical shape of the MT. The contacts between PFs involve homotypic interactions (α-tubulin interacting with α-tubulin and β-tubulin with β-tubulin), except at a special site, referred to as the seam, in which heterotypic contacts are required to close the lattice of the most common MT assembly, containing 13 PFs (Figure 1). This seam breaks true helical symmetry, although the MT can still be considered a helix of tubulin monomers if the differences between α- and β-tubulin are disregarded. Indeed, given the high similarity in the structure of the two tubulin subunits [1], MTs appear helical at low resolution, with an axial repeat of ~40 Å corresponding to the size of each tubulin protein (Figure 1). This apparent symmetry is immediately broken when a MT-binding protein that recognizes specific sites on the αβ-tubulin dimer is bound to the MT surface (a couple of rare exceptions will be mentioned later). These geometrical considerations are important in structural studies of MTs and their interaction with binding partners. In this review, we briefly summarize the technical advances that have led to the progressive improvement in the resolution of cryo-EM structures of MTs that now allow the generation of atomic models, and then concentrate on the biological insights provided by recent MT structures.

Brief historical overview and how we have coped with the MT seam

As a polymer, the MT is not amenable to crystallization for X-ray studies and thus its structure has been pursued over the years using electron microscopy, both *in vivo* and *in vitro*. With the advent of cryo-EM to visualize frozen-hydrated samples, well preserved MTs became an obvious sample for structural characterization by this methodology. Many of the early cryo-EM MT studies involved the interaction of the motor protein kinesin with MTs. As helical Fourier methods were an obvious choice at the time, and given the problem caused by the seam mentioned above, many of such studies relied on the presence of a small percentage of MTs that *in vitro* are composed of 15 PFs organized in a 4-start helix [2–4], instead of the most common 3-start helix, 13-PF or 14-PF lattices. Such MTs do not have a seam and thus are true helical arrays of αβ-tubulin dimers. Given their scarcity, a typical reconstruction of these helical MTs used only one or a few MT images and was limited to a resolution of 20–25 Å. Downing and Li, with the use of a high-end (400 keV) electron microscope, the average of many more images, and ignoring the seam in the study of unbound MTs (and thus averaging α- and β-tubulin), dramatically improved the resolution to better than 10 Å [5]. After that sharp

Figure 1



Cryo-EM visualization of drug and protein binding on the microtubule surface.
The MT lattice is shown (left) along with the location of associated MAPS: EB3 **(a)**, PRC1 **(b)**, kinesin **(c)**, and small molecules: peloruside **(d)**, Taxol **(e)**, and zampanolide **(f)**. The seam is indicated with a white dashed line and the arrow indicates the heterotypic (β/blue – α/green) interactions across the seam. Insets displayed on the right correspond to experimental cryo-EM maps at the site of interaction of each factor with tubulin.

resolution jump, the field stagnated for over a decade in terms of resolution improvement, with structures limited to 8 Å in the best of cases, although not in terms of new biological insights. The sub-nanometer resolution proved very useful in hybrid methodologies that involved docking crystal structures in a significant number of studies concerning the interaction of different cellular factors bound to MTs [6–8]. Through the last decade, image analysis strategies shifted from Fourier Bessel reconstruction to real space-based approaches, most significantly the Iterative Helical Real Space Reconstruction (IHRSR) method developed by Ed Egelman [9,10].

Our lab (*i.e.*, Gregory Alushin and Gabriel Lander) implemented a pipeline for MT cryo-EM reconstruction that built on the IHRSR and in the use of non-helical averaging strategies as originally developed by Sindelar and Downing [11], to finally break the previous resolution barrier for the MT, obtaining structures at about 5 Å [12]. To reach that resolution required that α- and β-tubulin be distinguished and the seam located in order to align small, overlapping fragments of MTs (that ultimately corresponded to a total MT length of ~6 mm!). To be able to accomplish this in the context of the noisy images obtained by cryo-EM, and given the similarity between α- and β-tubulin, we used a kinesin motor domain as a fiducial for alignment of the tubulin dimer and the seam. The resulting ~5 Å structures were interpreted at the atomic level through the use of Rosetta modeling by using the fit to the cryo-EM map as an additional term in the energy function for minimization [13,14]. With the advent of direct electron detectors for data collection, it suddenly became possible to obtain higher resolution with significantly less data. Together with improvements to the computational scheme, including a method for accurately determining the position of the seam in our MT images (by Rui Zhang) [15], we have now reported a number of structures in the 4 to 3.5 Å resolution regime that allowed the generation of atomic models from the cryo-EM density maps. These studies have included MTs that were bound by different MT-associated proteins (MAPs), thus eliminating the need of kinesin as a fiducial. Although smaller than the kinesin head, these MAPs still provide sufficient signal for alignment of the seam, thus avoiding mixing of α- and β-tubulin. Our studies have most recently included the description of the effect of MT stabilizing drugs on MT structure, contributing to our understanding of how they exert their function. The resolution of our drug-bound MTs allowed the direct visualization of the binding site and thus supports the promise of cryo-EM for use in drug discovery/optimization.

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