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Re-examination of the Polarity of Microtubules and Sheets Decorated with Kinesin Motor Domain

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Electron microscope images of microtubules and tubulin sheets decorated with kinesin head domains have shown the main mass of the kinesin head domain to be superimposed on one subunit of each tubulin dimer. We have polymerized brain tubulin extensions on to the ends of flagellar axonemes under varied conditions, in order to check the polarity of the tubulin–kinesin head complex. Since the polarity of axonemes incubated with normal brain tubulin may be ambiguous, we also tried 50% *N*-ethylmaleimide-treated tubulin which specifically blocks minus ends. Our conclusion, which conflicts with recently published results, is that the main mass of the kinesin head is associated with the tubulin subunit closer to the plus end of a microtubule.

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Kinesin is a molecular motor molecule that moves along microtubules towards their plus ends, defined by their faster rate of assembly (Brady, 1985; Scholey *et al.*, 1985; Vale *et al.*, 1985). The N-terminal 30 to 40 kDa part of the heavy chain, the motor domain, binds to microtubules in a nucleotide-dependent manner. In electron microscope images, microtubules decorated with motor domain show a longitudinal periodicity of 8 nm, equivalent to the spacing of the tubulin dimer (Harrison *et al.*, 1993; Song & Mandelkow, 1993; Huang & Hackney, 1994; Kikkawa *et al.*, 1994).

The kinesin–tubulin complex has a polar appearance that is clearly recognized in electron microscopy (EM) images when open-out microtubules (sheets) are decorated with kinesin heads. It is important to know how this polar appearance of kinesin decoration is related to the polarity of the underlying microtubules, in order to relate the structure of kinesin to its direction of movement. One way of determining the polarity of microtubules is to grow them from the ends of flagellar microtubule seeds, since the faster-growing plus ends are distinguishable by longer extensions. Using this method, Song & Mandelkow (1995) reported that the main mass of the motor domain is superimposed on the monomer occupying the minus end of the tubulin heterodimer. We have repeated the experiments, preparing the

decorated specimens under a range of conditions, and have come to a different conclusion.

Three different procedures were used for preparing decorated specimens. In method 1, axonemes prepared from sea urchin sperm (Gibbons & Fronk, 1979) were mixed with tubulin (0.8 mg/PC tubulin/ml, prepared from porcine brain tissue (Mandelkow *et al.*, 1985)) in assembly buffer (80 mM K-Pipes, 1 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT, 0.5 mM GTP (pH 6.8)) and incubated at 37°C. The axonemes with polymerized microtubules were diluted with Hepes buffer (20 mM Hepes, 1 mM EGTA, 2 mM magnesium acetate, 40 mM potassium acetate, 1 mM DTT (pH 7.2)) containing bacterially-expressed rat kinesin motor domain KΔ340 (Lockhart *et al.*, 1995) and 0.5 to 1.0 mM AMP-PNP, were incubated at room temperature for 5 to 10 minutes, and then were put on to carbon-coated grids to be negatively stained. Some axonemes showed assembly at both ends (see Figure 1C), while others had additional microtubules at only one end (Figure 1D). Some of the ends without additional microtubules seemed to be blocked by debris.

In method 2, a similar mixture of the proteins and the same buffers were used, but the whole procedure was performed on an EM grid in order to avoid breakage of the microtubule–axoneme complex by pipetting. Also, taxol was added after assembly but before decoration, to prevent depolymerization of the extended microtubules. Tubulin and axonemes in the assembly buffer were mixed on a carbon-coated EM grid, which was incubated in a humid chamber at 37°C for 5 to 10 minutes to polymerize tubulin. The
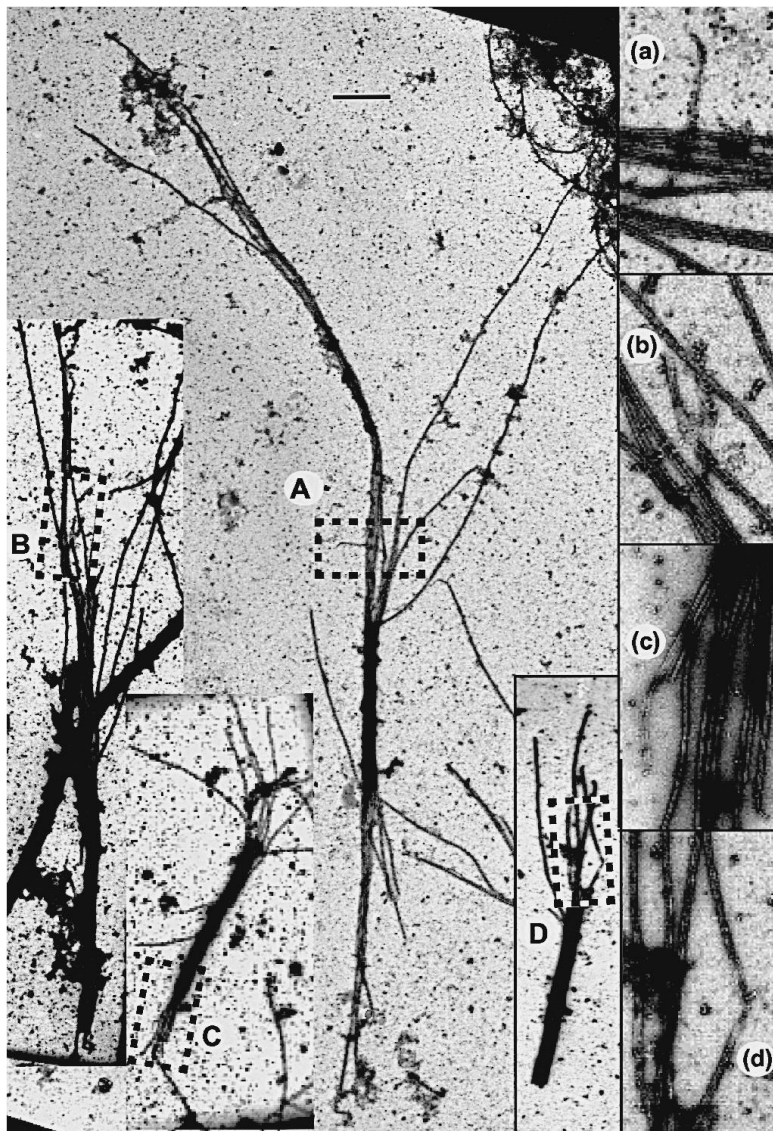

Figure 1. Low magnification electron micrographs of negatively stained sea urchin flagellar axonemes with brain tubulin assembled on to their ends. The main image, A, is typical of the results obtained by incubating axonemes on the EM grids with normal tubulin followed by careful washing, fixation with taxol and finally incubation with KΔ340 and AMP-PNP, a non-hydrolysable ATP analogue (method 2). Axoneme B, shows the effect of using a 1:1 mixture of normal and NEM-treated tubulin (method 3). Axonemes C and D, are typical products of method 1, in which KΔ340 was added to axonemes that had been incubated in solution with brain tubulin and then pipetted on to EM grids. The scale bar represents 2 μm on the main image. The areas enclosed by dashed boxes include opened out tubulin sheets and are shown at successively higher magnifications on the right (a) to (d) and in Figure 2.

grids were rinsed by adding Hepes buffer containing 10 μM taxol onto the grid and carefully blotting off excess solution. KΔ340 (final concentration 2 μM) and AMP-PNP were then added, and the grid was incubated for 5 to 10 minutes before negative staining. When decorated samples were prepared using this procedure, the proportion of axonemes lacking brain extensions at one end was greatly decreased. A majority of the axonemes showed assembly at both ends, with one end growing significantly longer than the other (Figure 1A).

In method 3, we carried out experiments in the same way as in method 2, but including NEM-treated tubulin. The latter, when mixed in a 1:1 ratio with normal tubulin, has been reported to specifically inhibit addition to the minus ends of microtubules (Huitorel & Kirschner, 1988; Hyman *et al.*, 1991). We found that most axonemes treated in this way had extensions at only one end (Figure 1B).

We found opened-out sheets growing from the ends of the brain microtubule extensions under all conditions (Figures 1(a) to (d)). In higher magnification views (Figure 2), we saw the 8 nm periodicity due to kinesin decoration, as reported by other researchers, with a polar pattern consisting of a line of strong black stain next to a thin white line of high protein density (see asterisks in Figure 2), followed by a region of intermediate density. This pattern is shown more clearly in filtered images (also shown in Figure 2), produced by calculating two-dimensional Fourier transforms of the digitized images and masking off non-periodic components. With much of the background noise removed in this way, the reconstructed images clearly show the extra protein density associated with alternate tubulin subunits. To demonstrate further the polarity of each sheet, we also made plots of the density profiles along the protofilaments. The results (see Figure 2), which mostly show pairs of peaks, one higher than the other and separated by shallower minima than those between different pairs, are similar to those published by Song & Mandelkow (1995). Images of well-ordered extreme ends, such as those shown in Figure 3(a), and in Figure 10 of Song & Mandelkow (1995), support the idea that the deeper minima separate different tubulin dimers, while the
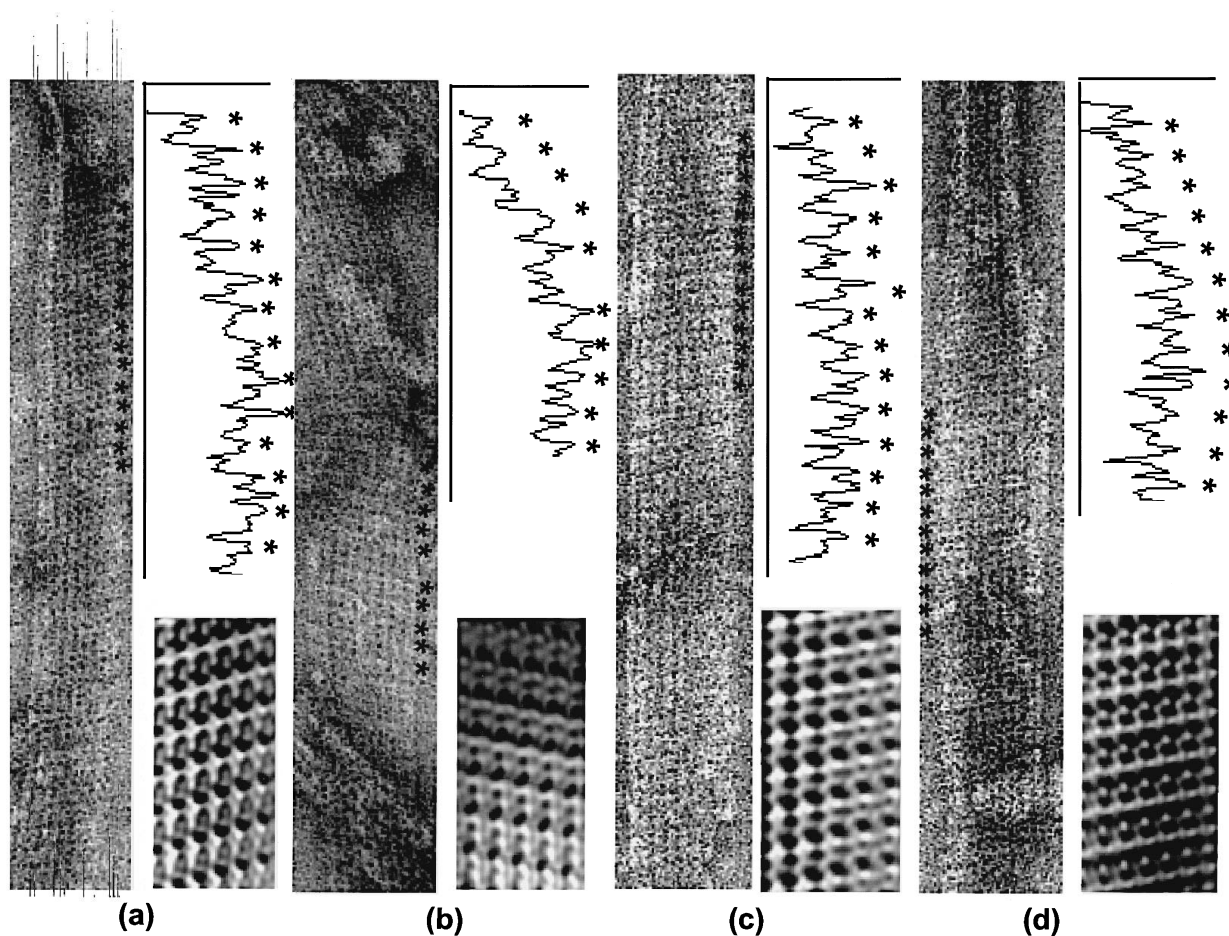
Figure 2. Electron microscope images of tubulin sheets decorated with KΔ340 that come from the four selected regions of Figure 1. Alongside each image is a profile of the density variation, averaged over six to eight adjacent protofilaments; there are peaks with an average spacing of 4 nm, corresponding to the spacing of tubulin monomers along the protofilaments. Alternate peaks in protein density (marked by asterisks) are mostly higher, showing the presence of kinesin heads, which are also marked by 8 nm-spaced asterisks next to the strong white lines in the images. Part of an enlarged filtered image is also shown for each sheet. In (a) to (c) each strong white line lies just below a strong black line. The slope of the lines in (b) is opposite to that in (a) and (c) because the sheets are lying on opposite surfaces. In contrast, the longitudinal reversal of both the pattern and the density profile in (d) indicates that the orientation of axoneme D in Figure 1 is inconsistent with the others.

shallower minima separate the two monomers of each dimer.

The polarity of the pattern of kinesin decoration was compared with the axoneme polarity suggested by the relative amounts of brain tubulin assembled from the two ends. For all axonemes with longer microtubules at one end than the other (e.g. Figures 1A and C), the pattern of decoration was orientated as shown in Figures 2(a) and (c), that is, with the strong white line (or the higher peak in the density profile) at the plus end of a dimer (pattern ''b'' in Table 1). When the NEM-tubulin mixture was used, the pattern on all sheets had the same orientation (Figure 2(b), Table 1). However, without NEM-treated tubulin, axonemes with brain microtubules at only one end, which were common using method 1, gave conflicting results if that end was assumed to be the plus end (Table 1). The example shown in Figure 1(D) gives the reverse pattern (''a'' in Table 1), as shown in Figure 2(d). We conclude from the results of method 1 that unmodified brain

tubulin extensions growing from a single end are not reliable indicators of polarity. However, it should not be necessary to use NEM-tubulin, provided unmodified tubulin is used under experimental conditions that extend axonemes at both ends.

The results of methods 2 and 3 suggest that the main mass of kinesin head is superimposed on the tubulin subunit closer to the plus end of a microtubule (see Figure 3(c)). An independent check of the polarities of a few specimens (not shown) was obtained from the successively unequal spacings of radial spoke triplets sticking out from the flagellar doublet tubules (e.g. see Goodenough & Heuser, 1985) and agreed with this conclusion. It is not really clear why our results contradict those of Song & Mandelkow (1995), since the conditions used in their experiments are not specified. None of the conditions we have tried favours extensions only from what we identify as the minus end, though Table 1 shows that the number of axonemes with single extensions can depend on their treatment during decoration.
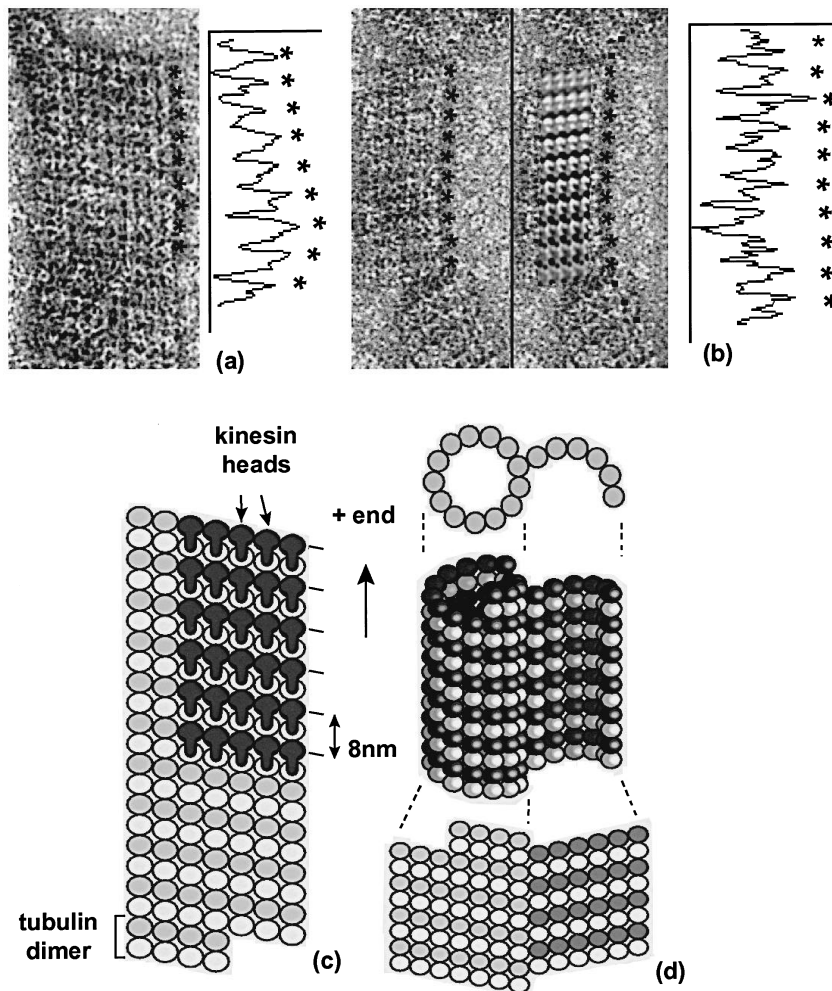
Figure 3. (a) Electron microscope image (outside view) of a sheet with a clear end, which our results indicate is a plus end. The density profile alongside is averaged over six protofilaments. The profile of the top dimer suggests that the end of the sheet is slightly curled; nevertheless, it is clear that the strong white lines (indicated by asterisks) are associated with the top monomer of each dimer. (b) Inside view of a sheet with two breaks, which are assumed to occur between rows of heterodimers. The central panel includes the filtered image of one segment of sheet; the pattern of kinesin decoration is most clearly seen in the middle (possibly the ends are disordered) but the phasing of the strong white lines relative to both ends is unambiguous. The density profile is from four protofilaments in the unfiltered image. (c) Our interpretation of the images of KΔ340-decorated sheets. The main mass of the kinesin head domain is associated with the monomer at the plus end of a tubulin dimer but, to account for the asymmetric density profile (higher peak, shallower minimum; lower peak, deeper minimum seen in (a)), part of the head extends on to the other monomer. (d) How a curved tubulin sheet apparently associates *via* one edge with a microtubule, to form a "hook" with the observed orientation (redrawn from McIntosh & Euteneuer, 1984). A cross-sectional view (top), an off-front view (middle), and a corresponding sheet (bottom). The attachment of a hook to a microtubule has essentially the same configuration as the outer junction of a flagellar doublet tubule. Below, two sheets associating with one another *via* both of their "sticky" edges produce a chevron pattern, which is predicted to point towards the minus ends of the sheets.

Whether this is due to the presence of debris at one end or to depolymerization or breakage is unclear. Possibly, under some conditions, plus ends would be lost preferentially, as plus ends can be more unstable than minus ends.

Tubulin sheets apparently have a "sticky" edge that causes them to associate, often in pairs, and, under some conditions, to attach to the outside of a complete microtubule forming a structure that appears in cross-section as a circle with a "hook" attached to it (see Figure 3(d)). Identification of the sticky edge depends on knowing the polarities of the sheets. Song & Mandelkow deduced from their results that the chevron patterns formed by side-by-side association of two sheets point towards the plus ends of the sheets and might be related to the inner junction of a doublet tubule. Since our results suggest a reversed axoneme polarity, it follows that such chevrons should point towards the minus rather than the plus end. The polarity of hooks formed on native microtubules that have been incubated with exogenous tubulin under high-salt conditions (Heidemann & McIntosh, 1980; McIntosh & Euteneuer, 1984), is consistent with our identification of the sticky edge (Figure 3(d)). This form of association between a microtubule and sheet, which is probably similar to the outer junction of a doublet microtubule, is obviously due to tubulin–tubulin interactions. The inner junction of a flagellar doublet tubule is significantly more stable than the outer junction, presumably because of the presence of specific non-tubulin proteins there, including the highly insoluble tektin filaments (Linck & Langevin, 1982).

It has previously been shown that the motor domain can be chemically cross-linked to β-tubulin (Song & Mandelkow, 1993; Kikkawa *et al.*, 1994). On the basis of their results, Song & Mandelkow (1995) postulated that the minus end of a microtubule terminates with a β-tubulin monomer, the plus end with α-tubulin. A similar argument would now suggest the plus end terminates in β-tubulin. However, electron microscope images alone cannot pinpoint where chemical cross-links between the tubulin monomers and the kinesin motor domain are likely to be formed. Moreover, although it seems very likely that the motor domain binds to the ultimate

Table 1. Labelling of brain tubulin sheets grown from sea urchin axonemes

Pattern polarity	Method 1 (no taxol)	Method 2 (on grid + taxol)	Method 3 (50% NEM-tub)
Pattern ''a''	3 (3)	1 (1)	0
Pattern ''b''	3 (2)	6 (1)	6 (6)

Numbers of sheets supporting either of the two possible assignments for the polarity of the density profile. The plus end of each axoneme was assumed to be the one with longer microtubules growing from it (axonemes with similar length microtubules at both ends were ignored). Where axonemes had microtubules growing from only one end, this was assumed to be the plus end; the numbers of sheets associated with single-ended specimens are shown in brackets. Pattern polarity ''a'' is that reported by Song & Mandelkow (1995), whereby the higher protein density at the end of the sheet shown in Figure 3(a) would indicate a minus end; pattern polarity ''b'' would make it a plus end.

subunit on the plus end of a protofilament, it may also contact the next subunit down (see Figure 3). Beads coupled to a GTP analogue bind only to the plus ends of microtubules (Mitchison, 1993), suggesting that the exchangeable GTP-binding site of β-tubulin is only accessible at this end. The latter finding supports the idea that the plus ends of microtubules may be less stable than minus ends (Mandelkow & Mandelkow, 1989) because plus ends terminate in β-tubulin, which bonds well with neighbouring protofilaments only when it has GTP, not GDP, bound to it. The non-exchangeable GTP bound to α-tubulin is never hydrolysed, so this subunit may form more stable bonds at the tip of the minus end.

Preliminary experiments show that tubulin sheets decorated with the motor domain of ncd have a polar appearance similar to that of kinesin-decorated sheets, with the main mass on the subunit closer to the plus end (Hirose *et al.*, 1995). The observations suggest that, although kinesin and ncd move in opposite directions along microtubules, their structures and the way they bind to tubulin dimers are very similar.

Acknowledgements

References

Brady, S. T. (1985). A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature*, **317**, 73–75.

Gibbons, I. R. & Fronk, E. (1979). A latent adenosine triphosphate form of dynein 1 from sea urchin sperm flagella. *J. Biol. Chem.* **254**, 187–196.

Goodenough, U. W. & Heuser, J. E. (1985). Substructure of inner dynein arms, radial spokes and the central pair-projection complex of cilia and flagella. *J. Cell. Biol.* **100**, 2008–2018.

Harrison, B. C., Marchese-Ragona, S. P., Gilbert, S. P., Cheng, N., Steven, A. C. & Johnson, K. A. (1993). Decoration of the microtubule surface by one kinesin head per tubulin heterodimer. *Nature*, **362**, 73–75.

Heidemann, S. R. & McIntosh, J. R. (1980). Visualization of the structural polarity of microtubules. *Nature*, **286**, 517–519.

Hirose, K., Lockhart, A., Cross, R. A. & Amos, L. A. (1995). Nucleotide-dependent angular change in kinesin motor domain bound to tubulin. *Nature*, **376**, 277–279.

Huang, T.-G. & Hackney, D. D. (1994). *Drosophila* kinesin minimal motor domain expressed in *Escherichia coli* – purification and kinetic characterization. *J. Biol. Chem.* **269**, 16493–16501.

Huitorel, P. & Kirschner, M. W. (1988). The polarity and stability of microtubule capture by the kinetochore. *J. Cell. Biol.* **106**, 151–159.

Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L. & Mitchison, T. (1991). Preparation of modified tubulins. *Methods Enzymol.* **196**, 478–485.

Kikkawa, M., Ishikawa, T., Nakata, T., Wakabayashi, T. & Hirokawa, N. (1994). Direct visualization of the microtubule lattice seam both in vitro and in vivo. *J. Cell Biol.* **127**, 1965–1971.

Linck, R. W. & Langevin, G. L. (1982). Structure and chemical composition of insoluble filamentous components of sperm flagellar microtubules. *J. Cell Sci.* **58**, 1–22.

Lockhart, A., Crevel, I. & Cross, R. (1995). Kinesin and ncd bind through a single head to microtubules and compete for a shared MT binding site. *J. Mol. Biol.* **246**, 763–771.

Mandelkow, E. & Mandelkow, E.-M. (1989). Tubulin, microtubules and oligomers: molecular structure and implications of assembly. *In Cell Movement* (Warner, F. D. & McIntosh, J. R., eds), vol. 2, pp. 23–45, Alan R. Liss, New York.

Mandelkow, E.-M., Herrmann, M. & Rühl, U. (1985). Tubulin domains probed by limited proteolysis and subunit-specific antibodies. *J. Mol. Biol.* **185**, 311–327.

McIntosh, J. R. & Euteneuer, U. (1984). Tubulin hooks as probes for microtubule polarity: an analysis of the method and an evaluation of data on microtubule polarity in the mitotic spindle. *J. Cell Biol.* **98**, 525–533.

Mitchison, T. J. (1993). Localization of an exchangeable GTP binding site at the plus end of microtubules. *Science*, **261**, 1044–1047.

Scholey, J. M., Porter, M. E., Grissom, P. M. & McIntosh, J. R. (1985). Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. *Nature*, **318**, 483–486.

Song, Y.-H. & Mandelkow, E. (1993). Recombinant kinesin motor domain binds to β-tubulin and decorates microtubules with a B surface lattice. *Proc. Natl. Acad. Sci., USA* **90**, 1671–1675.

Song, Y.-H. & Mandelkow, E. (1995). The anatomy of flagellar microtubules: polarity, seam, junctions, and lattice. *J. Cell Biol.* **128**, 81–94.

Vale, R. D., Schnapp, B. J., Mitchison, T., Steuer, E., Reese, T. S. & Sheetz, M. P. (1985). Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell*, **43**, 623–632.

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