Commentary

MITOSIS : SPINDLE EVOLUTION AND THE MATRIX MODEL

by

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SUMMARY

Current spindle models explain "anaphase A" (movement of chromosomes to the poles) in terms of a motility system based solely on microtubules (MTs) and that functions in a manner unique to mitosis. We find both these propositions unlikely. An evolutionary perspective suggests that when the spindle evolved, it should have come to share not only components (e.g., microtubules) of the interphase cell but also the primitive motility systems available, including those using actin and myosin. Other systems also came to be involved in the additional types of motility that now accompany mitosis in extant spindles. The resultant functional redundancy built reliability into this critical and complex process. Such multiple mechanisms are also confusing to those who seek to understand how chromosomes move. Narrowing this commentary down to just anaphase A, we argue that the spindle matrix participates with MTs in anaphase A and that this matrix may contain actin and myosin. The diatom spindle illustrates how such a system could function. This matrix may be motile and work in association with the MT cytoskeleton, as it does with the actin cytoskeleton during cell ruffling and amoeboid movement. Instead of pulling the chromosome polewards, the kinetochore fibre's role might be to slow polewards movement to allow correct chromosome attachment to the spindle.

Perhaps the earliest eukaryotic cell was a cytoplast organized around a radial MT cytoskeleton. For cell division, it separated into two cytoplasts via a spindle of overlapping MTs. Cytokinesis was actin-based cleavage. As chromosomes evolved into individual entities, their interaction with the dividing cytoplast developed into attachment of the kinetochore to radial (cytoplast) MTs. We think it most likely that cytoplasmic motility systems participated in these events.

Introduction. The discovery of mitosis by Flemming and his contemporaries in the 1870s and 1880s (Hughes, 1959; Wilson, 1928) provided us with one of the greatest and most exciting insights in cell biology. The drastic morphological changes that cells undergo during mitosis were soon understood as the means by which genetic continuity was maintained between successive generations of cells. How the cell generates this elaborate choreography of the chromosomes has turned out to be extraordinarily subtle. Many generations of biologists have enthusiastically plunged into research on the mechanisms of mitosis, only to retreat frustrated. Here is how Schrader (1944) summarised this situation more than 60 years ago: "*Since about 1870, there has been a succession of periods in which triumph seemed to stand on the threshold as first, observers of the living cell, then students of the morphology of the fixed cell, and lastly, the physiologists, marshalled the evidence furnished by their different attacks … each of these periods had a corresponding aftermath of disillusion, always accompanied by a new appreciation of the difficulties of the problem*." In spite of the vast amount of new data acquired since Schrader's book, we believe the situation is little changed, and that present models of spindle function remain deficient.

Why has it been so difficult to explain how the spindle works? One reason is that spindles have functional redundancies. It has been clear for many years (e.g., Pickett-Heaps and Bajer, 1978) that a number of different mechanisms, used to varying extents in different organisms, collectively contribute to the separation of chromosomes achieved by the spindle. For example, "anaphase B" (elongation of the spindle by separation of the poles) is accomplished by elongation of the two half spindles, by

sliding apart of the two half spindles and/or by pulling forces generated in the reforming asters. Numerous observations suggest that a similar range of other mechanisms can bring about anaphase A (e.g., Pickett-Heaps and Forer, 2001; Fabian and Forer, 2005). This is obviously important in evaluating the results of experiments, and in generalizing on the nature of spindle motility.

Another reason is that straightforward and reasonable interpretations of morphological and experimental data have turned out to be consistently wrong. For example, in considering how the spindle sets itself up, established wisdom in the 1960s through late 1970's was that kinetochores (the mitotically active site on the chromosome) function as Microtubule Organizing Centres growing the kinetochore-fibres (k-fibres) that connect them to the pole. Although this conclusion was supported by numerous experimental investigations, the concept was wrong. Later work showed that kinetochores capture microtubules (MTs) that originate from the poles. Recent data now indicate that kinetochore MTs can originate either from the pole or from the kinetochore (Wadsworth and Khodjakov, 2004; Rieder, 2005). In addition, new data turned what initially seemed a simple phenomenon into an increasingly complex one. In explaining how MTs might produce the force that moves chromosomes, in the 1960s it was generally attributed to interstitial depolymerisation of MTs. Next, MTs were thought to depolymerise at the poles and act like a traction fibre. Then it was suggested that they might function via cross-bridges. More recent suggestions are that the spindle generates force by MT-depolymerisation at the kinetochore end of the k-fibre (the "PacMan" model), then that assembly/disassembly at either end leads to fluxing of MT-subunits through the fibre. Now combinations of the above are proposed plus enzyme action at poles and kinetochore (e.g., Inoué, 1964; Margolis et al., 1978; Gorbsky et al., 1988; Koshland et al., 1988; LaFountain et al., 2001, 2004; Rogers et al., 2004, 2005; Buster et al., 2007). All these models are based on the assumption that MTs and their associated motors are the prime if not the only actors in organising the spindle and moving chromosomes. Accordingly, MT-motors have now become major targets of researchers. This reductionistic approach has not clarified how chromosomes move. While the lists of potential mitotic motors continues to grow, the bottom line is that we still don't know which of them are involved, how they work, or, often, where they are located. We think it too simplistic to concentrate solely on MTs and their motors, and that other components need be considered.

Anaphase A in an Evolutionary Context. Eukaryotic cells arose more than 2 billion years ago. A major feature of their evolution was the appearance of the membrane-bound nucleus, and how the nucleus arose has been the subject of some speculation (e.g., Pickett-Heaps, 1974). The ability of this nucleus to divide reliably during cell division was a vital accomplishment of the primordial eukaryotic cell.

Cellular processes, like everything else in the cell, undergo adaptation and diversification. Yet a consistent feature of the MT-based models of anaphase-A movement so far proposed is that the mechanism(s) invoked function exclusively in mitosis; we have not seen any suggestion that the various PacMan, treadmill and MT-fluxing models of force production are used for other activities during interphase. This exclusivity implies that these systems have not, over the two billion years that the eukaryotic cell has been evolving, been put to any other uses and become integrated with the rest of the cell's activities. No other motility system we know of is used exclusively for only one aspect of cell motility. In contrast, MT and actin motors function individually and jointly in a variety of cellular

motilities (Rodriguez et al. 2003), so we expect motility systems to be shared, to be used by a variety of cellular processes.

Sharing of useful abilities by interphase and mitotic cells has indeed happened. For example, the ability of cells to transport objects over the surface of MTs is a basic mechanism for generating intracellular motility in interphase. This type of MT-based motility also generates prometaphase movements of chromosomes by their kinetochores sliding over polar MTs (Tippit et al., 1980; Rieder and Alexander, 1990). Likewise, MTs are cross-linked by a wide variety of motor proteins in other systems (e.g., cilia, cytoplasmic MTs, protistan axonemes) as they are also in the spindle. Thus we would expect spindle motile systems to have utilized components from cytoplasmic motile systems. Models that invoke a "spindle matrix" satisfy this criterion.

Matrix Models of the Spindle. We have championed the proposition that non-MT components within the spindle (the "spindle matrix") might be centrally involved in generating anaphase A, and others, too, have suggested matrix involvement (discussed in Johansen and Johansen, 2007). In most cells, MTs attached to each kinetochore constitute the fibre that structurally connects it to either pole of the spindle. It is assumed from such morphological observations on living and fixed cells, that the k-fibre pulls the chromosome to the pole. We have no doubt that MTs, the major structural component of spindle fibres, are central to mitotic movement, and specifically anaphase A. But what if the spindle contains a motor system utilizing agents separate from kinetochore-MTs, but working in concert with them?

Our interest in this possibility was stimulated by experiments which showed that when its k-fibre is cut, a chromosome can continue anaphase A (Figure 1), even accelerate; furthermore, the cut stub of the k-fibre can grow polewards while its chromosome is moving polewards (Pickett-Heaps et al., 1996; Spurck et al., 1997). How, then, could the shortening of the k-fibre in normal cells generate poleward movement of a chromosome? An alternative interpretation is that the rigid k-fibre MTs that extend the full distance to the pole actually inhibit polewards movement, and their controlled disassembly (or their experimental cutting) permits chromosomes to move (Forer et al., 2007, 2008). We think it likely that some other system, the spindle matrix, does the pulling and that the force acting on the fibre (i.e., the compression or tension generated by this second structural system) controls the fibre's assembly and disassembly.

Our suggestions of matrix involvement have been based primarily on physiological/experimental evidences (Pickett-Heaps et al. 1996; Forer et al., 2003, 2008). The basic thesis is that MTs are primarily cytoskeletal elements (as they are during interphase), and that force is generated by k-MTs interacting with the spindle matrix, most likely via motor proteins. MTs are the structural elements which organise the matrix and determine how the chromosomes are arranged and directed to the poles, but they are not direct producers of force.

As reviewed in a recent "*News*" article in Science (Travis, 2007), most workers in the field show little or no enthusiasm for such matrix models. This is not surprising as the morphology, nature and properties of such matrix components remain to be convincingly demonstrated. However, immunofluorescence and molecular techniques have identified a range of proteins which could

constitute components of a spindle matrix (e.g., Johansen and Johansen, 2007), although how they might function is not clear. We now describe a cell division which illustrates directly how a spindle matrix might work.

Mitosis in Diatoms. The diatom spindle, a model system rarely acknowledged in the current literature on mitosis, demonstrates how a spindle matrix attaches chromosomes to the spindle and then moves them polewards (Pickett-Heaps, 1991). In three major structural features, the diatom spindle resembles conventional spindles (Figure 2A). *First*, it contains a "central spindle" composed of two interdigitated half-spindles; a similar arrangement of MTs creates the pole-to-pole fibres in more conventional spindles. *Second*, numerous polar MTs radiate outwards from the poles, amongst the chromosomes. *Third,* kinetochores attach laterally to these polar MTs, sliding along them during prometaphase alignment of chromosomes.

However, the diatom spindle is, at first sight, different from conventional spindles in four other ways, and these differences allow us to see the matrix at work. *First*, in metaphase and anaphase polar MTs do *not* terminate in kinetochores; they run past them (Tippit et al., 1980), ensheathing stretched chromatin strands (Fig. 2A). This morphology eliminates models which require MT kinetics to power anaphase (e.g., Koshland et al., 1988; Rogers et al., 2005). *Second*, a dense matrix (the "collar") extends between kinetochores and poles (Pickett-Heaps, 1991). *Third*, kinetochores are attached to this matrix. This is particularly evident when spindles are exposed to anti-MT drugs which rapidly break down the radiating polar MTs, leaving the more stable central spindle intact (Fig. 2B). In vivo, this selective destruction of the kinetochore-associated MTs results in stretched bipolar metaphase chromosomes releasing randomly from either pole and springing to the other pole with their kinetochores still attached to the collar which now forms an aggregated mass around each pole (Figs. 2B-D, as summarised in the diagrams of Figure 3). *Finally*, the metaphase spindle appears to be spring loaded, presumably by the collar (the matrix), with chromosomes springing to the pole when the chromatids split (Pickett-Heaps and Pickett-Heaps, 2002). Since MTs do not terminate in these kinetochores, it is a reasonable conclusion that the matrix is under tension and effects chromosome movement.

We agree that such a matrix component is rarely morphologically distinct in more conventional spindles. Nonetheless, we submit that the unusual features of the diatom spindle allow a clearer vision of a non-MT spindle matrix, with important functions during conventional mitosis. The alternative is that diatoms have evolved a unique structural basis for spindle function, a view for which there is no evidence – nor need.

To deal with the evolutionary criteria that we started with, we now need to ask: what non-mitotic systems might share properties with the spindle matrix? The cell's other major cytoskeletal component, actin, is part of this sort of matrix in interphase cells. Many vertebrate interphase cells generate "actin cycling", a slow steady movement of an actin matrix "polewards" (i.e., toward the cell center or centrosome), which, as discussed below, resembles "polar transport" in asters. In actin cycling, actin is polymerised at the periphery of the cell, transported inwards and then disassembled and transported back again (e.g., Wittmann and Waterman-Storer, 2001). There are many suggestions that this cycling is affected by the cell's MT cytoskeleton (Rodriguez et al., 2003), just as a spindle matrix might interact with spindle MTs. Further, Rodriguez et al. review situations where the actin cytoskeleton directly

affects MT assembly and disassembly – precisely how we envisage interactions between the spindle matrix and spindle microtubules during assembly of the spindle (Fabian et al., 2007b). Cytoplasmic streaming in lower forms such as myxomycetes does not involve cycling in the same form; rather streaming regularly reverses directions, with a period of about 2 minutes, associated with bundles that contain actin filaments and myosin and that aggregate and disaggregate in the 'contraction' and 'relaxation' phases (Kamiya, 1968; Nagai et al., 1978; Ishigami et al., 1987). This is reminiscent of the regular fast oscillatory movements of kinetochores (to and from the pole) in *Mesostoma* spermatocytes that occur throughout prometaphase, with a period of about 2 minutes (Fuge, 1989).

Is there any evidence that mitotic spindles utilise such actin/myosin systems?

Actin and myosin in the spindle. We and our colleagues have intensively investigated whether actin and myosin could be involved in mitosis (Forer, 1988; Czaban and Forer, 1994; Sampson et al., 1996; Forer and Pickett-Heaps, 1998; Sampson and Pickett-Heaps, 2001; Forer et al., 2003; Fabian and Forer, 2005, 2007; Fabian et al., 2007a; Forer et al., 2007b, 2008). In brief, actin, myosin and other "muscle" proteins are present in spindles (e.g., Fig. 4), in many cell types, from insects to vertebrates to algae to plants, and anti-actin and anti-myosin treatments have marked effects on mitosis *in vivo* in many cells (Forer et al., 2003). However, the localisations of components and the effects of drugs often are different in different cell types (Sampson et al. 1996; Forer and Pickett-Heaps, 1998; Fabian and Forer, 2007) and often the effects are not easy to interpret (perhaps because multiple mechanisms operate during anaphase). Two systems we have studied intensively, however, *Oedogonium* (a green alga) and crane-fly spermatocytes, respond to anti-actin agents with a rapid and reversible inhibition of mitosis. Furthermore, in *Oedogonium*, appropriate fixation shows microfilaments from the kinetochore lying amongst the MTs of the k-fibre (Schibler and Pickett-Heaps, 1980). Destruction of the k-fibre MTs with anti-MT drugs leaves a fibrous matrix extending from the kinetochore (Pickett-Heaps and Carpenter, 1993), and actin has been localised in these k-fibres using flouorescent staining techniques (Sampson et al., 1996; Sampson and Pickett-Heaps, 2001). Similar, though perhaps less extensive, results have been obtained in other cells. For example, in vertebrate cells actin and myosin are present in spindles (e.g., Guerriero et al., 1981; Dulyaninova et al., 2004; Woolner et al., 2008; Azoury et al., 2008). Making myosin's regulatory light chain unphosphorylatable (Komatsu et al., 2000) or blocking its phosphorylation (Dulyaninova et al., 2004) or rendering it unregulated (Fishkind et al., 1991) delays and/or disrupts anaphase onset, and, depending on the experimental protocol, arrests anaphase and causes alterations to spindle MTs (Dulyaninova et al., 2004) . In sea urchin zygotes, phosphorylated myosin is present in mitotic spindles, and blocking phosphorylation blocks anaphase onset (Uehara et al., 2008). In *Xenopus* cells, removing myosin-10 or actin filaments causes abnormal spindles and abnormal mitoses (Woolner et al., 2008). Thus from such observations and experiments, we argue that actin and myosin may be functional components of the spindle matrix.

The role of the matrix: using an interphase motility system to move chromosomes. Recent considerations of the possible roles of a spindle matrix focus on two possibilities (e.g., Johansen and Johansen, 2007; Travis, 2007), that the matrix is:

i) essentially *static*, functioning by pushing/pulling on kinetochore-MTs via motor proteins;

or ii), that the matrix is *contractile* (e.g., Lenart et al., 2005).

An interesting alternative is that the matrix is *motile* and that the cell uses this motility to drive chromosomal movement by the matrix interacting with kinetochore-MTs. Why invoke a motile matrix? Two phenomena support this possibility. *First* is the well known phenomenon of "polar transport", the slow steady movement of non-attached pieces of chromosomes or of large or small cytoplasmic particles to spindle poles or to the centres of asters throughout mitosis (Bajer, 1958, 1967; Rebhun, 1964; Nicklas and Koch, 1972; Mole-Bajer et al., 1974; LaFountain et al., 2001, 2002). The origin and mechanism of this transport are not known. S*econd*, the slow steady actin cycling discussed above is essentially directed "polewards" in that it is directed toward the centrosome; poleward transport and actin cycling may be manifestations of the same underlying phenomena. A provocative observation is that poleward transport is at about the same speed that chromosomes move polewards during anaphase. Ostergren et al., (1960: p. 393) used an elegant metaphor to describe this phenomenon: *"….the chromosomes are propelled in very much the same way as sailboats. The pumping activity …* (i.e., polar transport)… *is the wind, and the chromosomal spindle fibres are the sails of the chromosomes"*. We find it difficult to avoid the obvious conclusion: that if a slow, steady polewards transport system exists in the half spindles, then the cell would surely have found a way to harness it for polewards chromosome transport.

A new possibility for kinetochore function. These considerations lead to another, unexpected conclusion. Since chromosomes that are not attached to kinetochore-MTs move poleward nonetheless, by "polar transport", as described above, the basic function of the kinetochore/fibre cooperation is not to get the chromosomes to the pole. A prime function of the attachment to the rigid k-fibre would be to slow or prevent premature polewards movement (anaphase), thereby allowing the chromosomes time to orient to opposite poles and thus to subsequently divide equally into daughter nuclei [1]. If this view is correct, then the function of the kinetochore and its fibre is the opposite to what we have all held to be self evident. It does *not* primarily pull the attached chromatid; rather, it prevents the metaphase chromosome from moving polewards until the proper orientation is achieved, after which the chromosome and its fibre are slowly moved polewards by the underlying polar transport mechanism (the " molecular wind"). The ability of cells to attach chromatids to the spindle correctly is of overwhelming importance to the long term survival of cells, and we believe this might be the main function of kinetochores.

Conclusion. When considering mitosis from an evolutionary perspective, one expects the mechanisms used for moving chromosomes to share components and abilities used in other cellular motile systems. The matrix model accommodates this scenario: the spindle uses an existing matrix that is part of the interphase cell's motility systems and that is capable of movement in the right direction. Actin-MT interactions are highly conserved while being utilised in a variety of motile and morphogenetic systems (Rodriguez et al., 2003): why should the mitotic spindle be exempt from this principle? An obvious – but, in the current climate, radical - conclusion is that the smooth flowing anaphase A transport system in the half spindles (the "molecular wind") is a modified form of interphase

[1] In the interests of brevity, we ignore the complication of "polar ejection forces" which push larger inclusions *away* from the poles (Ostergren et al., 1960: p. 397), apparently by elongation of polar MTs (Rieder and Salmon, 1994; LaFountain et al, 2002).

actin cycling from the cell periphery to the cell centre. The transition between interphase and mitosis would require relatively minor structural modifications of the actin and MT cytoskeleton. We have argued previously that mitosis is brought about by redundant motility systems, i.e., that there are various mechanisms by which chromosomes are moved to the poles (Pickett-Heaps and Bajer, 1978; Pickett-Heaps and Forer, 2001; Fabian and Forer, 2005), and we do not expect all cells to have ended up with the same mechanisms from the palette of possibilities. Nor do we think the spindle matrix functions in all cells as it does in diatoms. Nonetheless, we feel that the primordial eukaryote cell utilised a matrix, and that there is physiological and cytological evidence that a spindle matrix functions during mitosis.

The origin of spindles in eukaryotic cells. To broaden the context of this discussion, we finally raise a "chicken-and-egg" question: which came first in the primordial eukaryotic cell, the nucleus or the spindle? We guess the spindle. We visualise the most primitive eukaryotic cell as a simple cytoplast organised around a radial MT cytoskeleton focussed on a cell centre (centrosome), before the membrane-bounded nucleus had evolved. Primordial cell division would have started with the two replicated cell centres being pushed apart by a continuous spindle of overlapping MTs (Pickett-Heaps, 1974), separating them into two cytoplasts ("cytoplasmic domains"), and division would finish with cytokinesis via cleavage. In this scenario, we see the nucleus appearing later, and indeed anaphase and cytokinesis can take place even when there are no chromosomes associated with the spindle (Zhang and Nicklas, 1996; Bucciarelli et al., 2003). A major feature of spindle evolution would be the attachment of chromosomes to the MTs of the replicating cytoplasts (Pickett-Heaps et al., 1999). The mechanism used to separate chromosomes (anaphase A) would incorporate the pre-existing motility systems of the cell – and surely would not have needed the evolution of new MT- based systems that were subsequently used exclusively for mitosis. The later inclusion of additional mechanisms (functional redundancy) for the promoting of accuracy and reliability of distributing chromosomes were developments perfected by evolution over unimaginable numbers of cellular replications.

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Captions for Figures.

Fig. 1. Crane fly spermatocyte in meiosis-I anaphase. One kinetochore fibre (bottom right) was severed with a UV microbeam and the chromosome attached to the kinetochore-fibre stub (arrowhead) continued to move poleward at normal speed. After 3 minutes, the cell was fixed and prepared for immunofluorescence microscopy. Total tubulin is green and acetylated tubulin red; co-localised regions (e.g., the kinetochore fibres) appear yellow. The arrow points to the severed end of the kinetochore stub. Since no MTs were detectable poleward from the end of the kinetochore stub, the chromosome had continued to move without any MT connection with the pole. Bar = 5μm.

Fig. 2. Metaphase spindles of the diatom *Hantzschia amphioxys*. **Fig, 2A**. Paired chromatids are stretched over a massive central spindle composed of two sets of interdigitated MTs. Polar MTs, radiating amongst the chromosomes, ensheathing the strands of chromatin, are densely stained near the poles by the collar (i.e., the spindle matrix; arrows). **Fig. 2B.** This cell was treated with 0.1% colchicine for 20 mins. before fixation. The central spindle is resistant to disassembly but breakdown of the polar MTs has allowed the chromosomes to release their tension by randomly releasing from either pole and springing to the other. The collar (c) material is aggregated at the poles. **Figs. 2C, D.** Detail of the cell in Fig 2B, showing kinetochores (arrows) clustered near the poles and attached to the aggregated collar material, labelled c. Bars = 1μm. Figs. 2B-D are adapted from the European Journal of Cell Biology 28: 77-82 (1982).

Fig. 3: Diagrammatic interpretations of diatom spindle structure. MTs are represented by solid black lines, chromosomes are brown, and the collar (spindle matrix) is blue. Compare with the electron microscopic images in Figure 2. **Fig. 3A. Metaphase**. The central spindle consists of two interdigitated half spindles. Polar MTs radiate amongst and past the bipolarly attached chromosomes, ensheathing their kinetochores which are stretched to the poles by collar material (spindle matrix). **Fig. 3B. Telophase**. Once chromosomes split at anaphase, accurate segregation of chromatids is achieved by their immediate elastic movement to each pole as the collar material condenses around the pole. Later the half spindles slide apart (generating anaphase B by spindle elongation). **Fig. 3C. Metaphase,** the same as Fig 3A, for side-by-side comparison with Fig 3D. **Fig. 3D. Metaphase** cell after 20 mins. in 0.1% colchicine. The central spindle resists breakdown, but the polar MTs have disappeared. Breakdown of these spindle fibres results in bipolarly attached chromosomes detaching randomly from one pole and springing to the other as the attached collar material condenses to the pole. Kinetochores remain attached to the collar at either pole. Anaphase B will still occur slowly. Diagrams by Ianina Altshuler.

Fig. 4: Illustrations of actin and myosin in crane fly spermatocytes. **Figs. 4A and 4B**. A living cell irradiated with an ultraviolet microbeam was processed for immunofluorescence a few minutes later. Actin and MTs are not present in the irradiated region. [From experiments described in detail in Forer et al., 2003.] **Fig. 4C**. A spermatocyte stained with antibodies against

phosphorylated (and hence active) myosin, illustrating that active myosin is associated with kinetochore spindle fibres. Adapted from an illustration in Fabian et al. (2007a).

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FIGURE 1

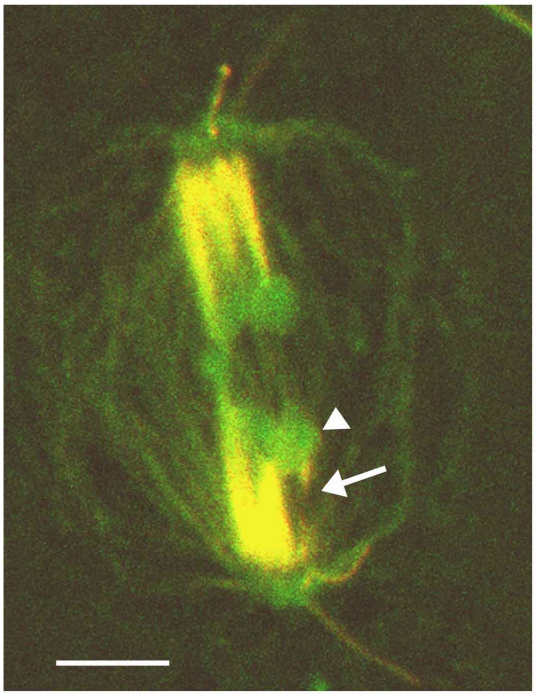


FIGURE 2

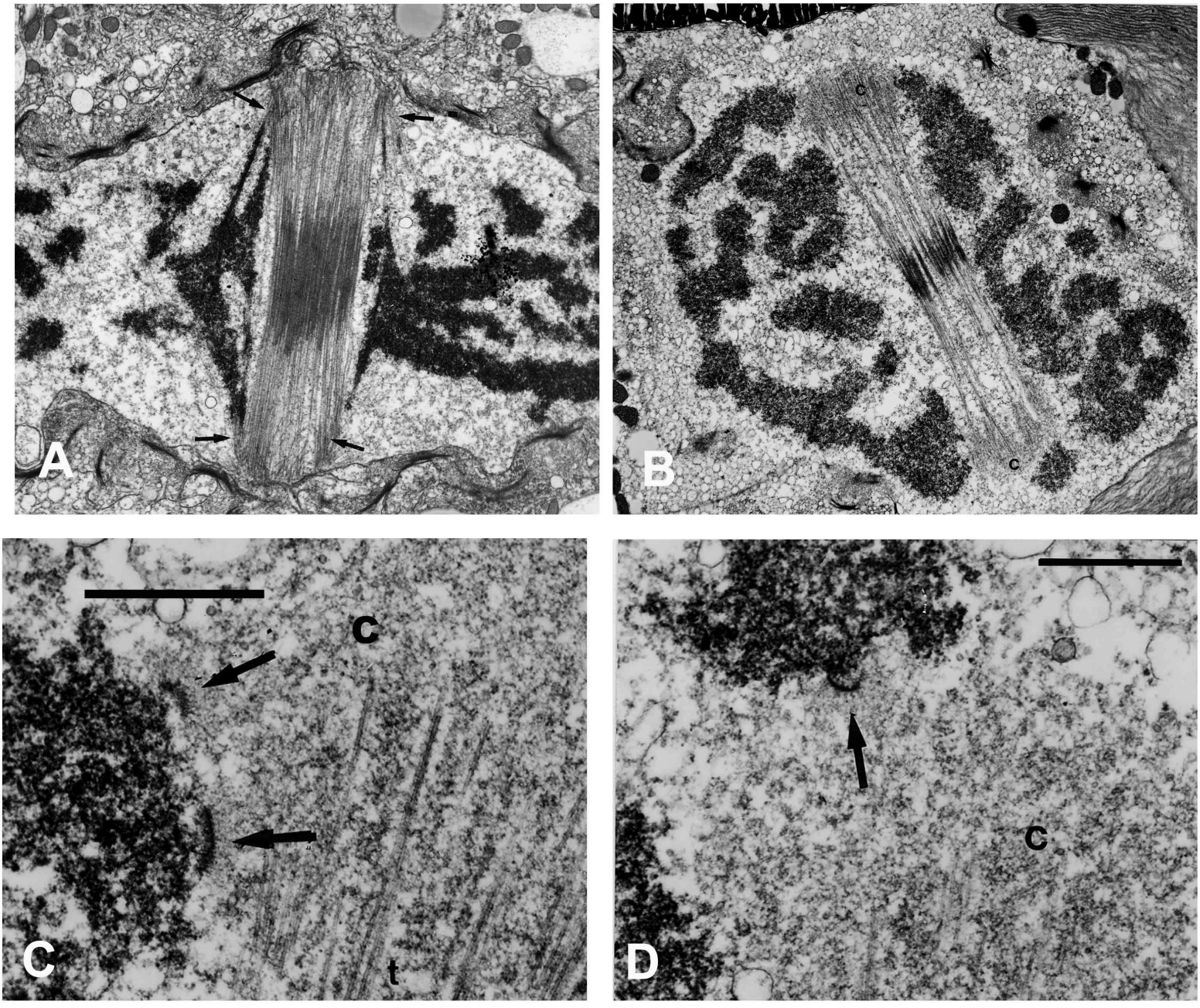
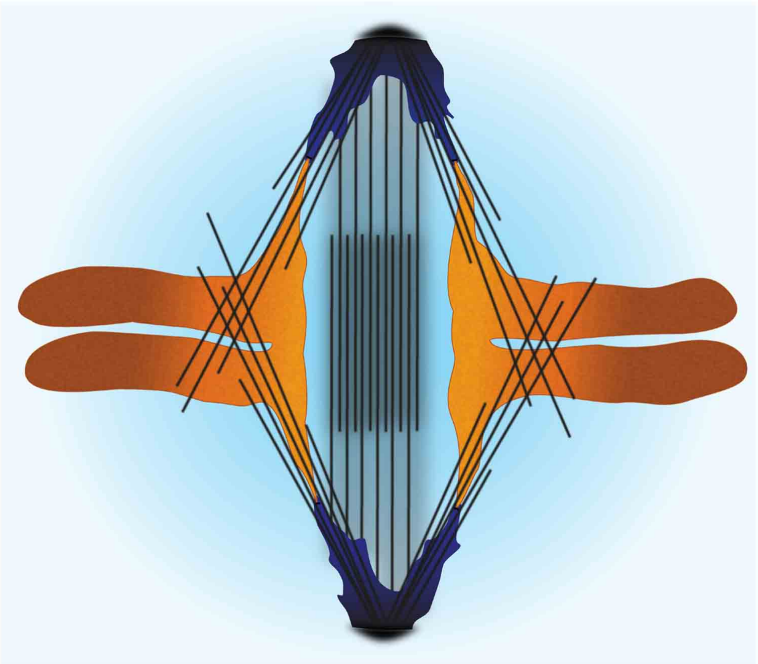


FIGURE 3



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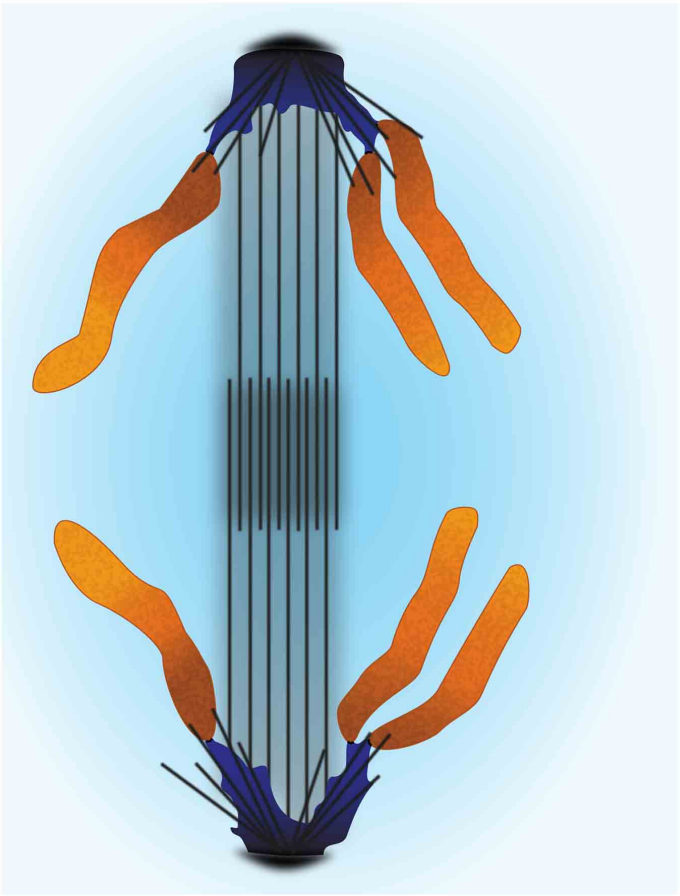

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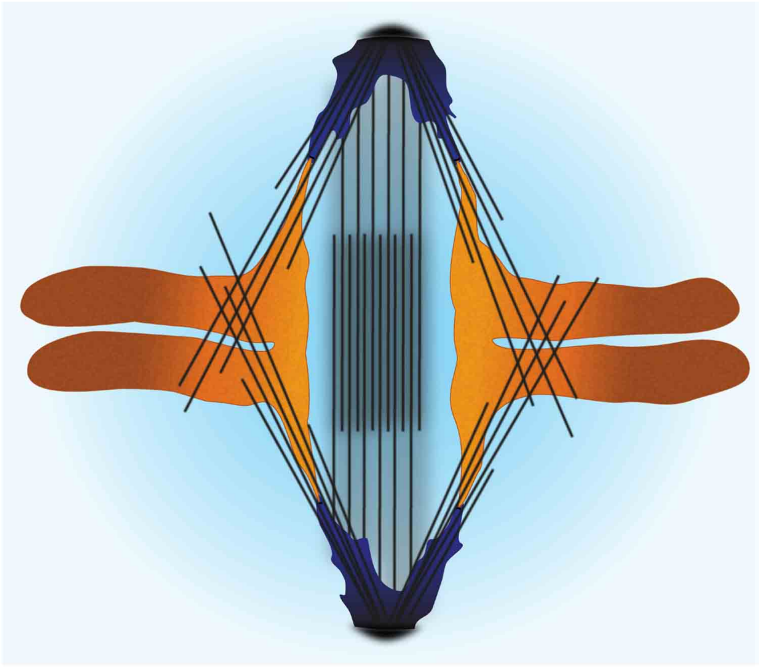

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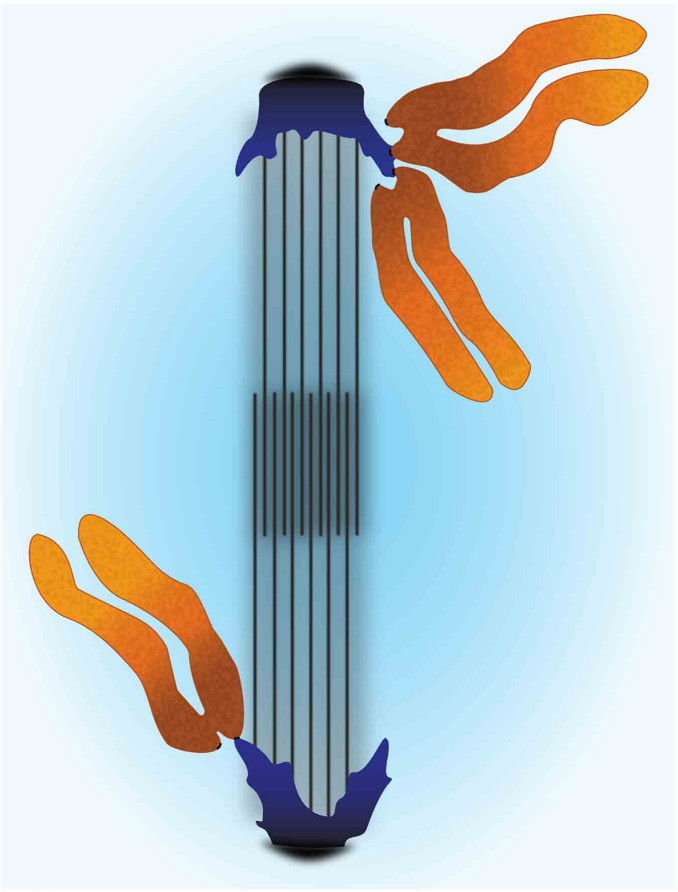

D

FIGURE 4