A new method reveals microtubule minus ends throughout the meiotic spindle

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Introduction

In this study, we investigate the localization of microtubule (MT) minus ends within the Xenopus laevis meiotic spindle. This localization is important because it may reflect the location of MT nucleation within the spindle. Since the discovery of centrosomes, models for the assembly and maintenance of mitotic and meiotic spindles have included a dominant role for spindle poles as MT nucleation centers (Wilson, 1937; Brinkley, 1985). The “search-and-capture” model suggested that poles dominate spindle morphogenesis, anchoring the minus ends of MTs, whereas plus ends polymerize and depolymerize until some are stabilized by kinetochores (Kirschner and Mitchison, 1986). Later models proposed that MTs could be stabilized “at a distance” by chromosomes, presumably via diffusible factors such as RanGTP (Dogterom et al., 1996; Hyman and Karsenti, 1996; Carazo-Salas and Karsenti, 2003).

In anastral spindles, which are typified by oocyte/egg meiotic spindles, centrosomes are unnecessary for spindle morphogenesis (Heald et al., 1996). Spindles assemble in an “inside-out” manner, with initial formation of MTs near chromatin, followed by condensation of minus ends into poles (Matthies et al., 1996; Gaglio et al., 1997; Endow and Komma, 1998; Sköld et al., 2005). In some meiotic spindles, density tapers off toward the poles in a manner suggesting that many MTs terminate before reaching the poles (Theurkauf and Hawley, 1992). Studies in X. laevis egg extracts, which recapitulate assembly of the anastral meiois II spindle, show that chromosomes trigger an exchange of GTP on Ran, promoting MT nucleation in the absence of centrosomes, thereby probably explaining early steps in spindle assembly (for review see Gruss and Vernos, 2004). Continued production of RanGTP is also required for maintenance of the metaphase steady-state in anastral spindles (Mitchison et al., 2004; unpublished data), but it is unknown whether this is caused by stabilization or nucleation activity downstream. Steady-state anastral spindles might be dominated by nucleation at chromatin, like during assembly, or at poles assembled in response to Ran activation (Gruss et al., 2001; Nachury et al., 2001). Knowing the localization of nucleating sites is, thus, central to understanding spindle morphogenesis. The search-and-capture picture is based on spatial separation between nucleating and stabilizing centers, and new models would be required to account for morphogenesis by other mechanisms. To this end, we sought to measure the localization of minus ends within the spindle.

Previous work localized minus and plus ends using serial-section electron microscopy (McDonald et al., 1992; Ding et al., 1993; Mastronarde et al., 1993), but this method is difficult to apply to large spindles and lacks reliable markers for end polarity. MT ends nearest to centrosomes were assumed to be minus ends (McIntosh et al., 1979; Mastronarde et al., 1993; O’Toole et al., 2003), which is an unreliable criterion if MTs are nucleated throughout the spindle. “Hook decoration” (McIntosh and Euteneuer, 1984; Heald et al., 1997) allows
identification of polarity, but is unsuitable for localizing ends because MTs elongate under the hook decoration conditions. γ-Tubulin complex is probably involved in nucleation, but our knowledge of its function is limited, so we cannot equate its localization with that of minus ends. NuMA and other spindle pole proteins probably move to the most distal minus ends in the spindle via dynein-mediated transport (Merdes et al., 2000). Instead of using any of these to locate minus ends, we developed a quantitative optical method combining analysis of oriented MT distributions with localization of plus ends by tubulin incorporation. Our analysis shows that MT minus ends are present everywhere in the spindle, with a minimum density near the chromosomes.

Results and discussion

Our method to calculate the density of plus and minus ends at a single location within a X. laevis extract spindle is shown in Fig. 1. Although we could not directly measure the density of minus ends, we could calculate the density of plus ends and the difference between the densities of plus and minus ends. The sum of these two quantities was the density of minus ends.

Our technique required three steps. First, to obtain the end densities, we observed the flow of MTs in a portion of the spindle (Fig. 1 A, i [dashed box]). The amounts of leftward and rightward flow were proportional to the local numbers of MTs with their minus ends toward each pole. Second, we looked at how the numbers of MTs varied in space to find the difference between the local densities of minus versus plus ends (Fig. 1 B). Third, we measured the local density of plus ends by observing incorporation of labeled tubulin into the spindle (Fig. 1 C). We summed the results of steps two and three to find the local density of minus ends. The process was repeated at many locations on the spindle-pole axis to find the spatial distributions of plus and minus ends.

Definitions

Throughout this paper, we rotate all spindles to be horizontal, then use “x” to denote position along the spindle-pole axis and “y” for the perpendicular direction.

We call MTs with plus ends to the left “left-pointing” and define “right-pointing” analogously. The “MT number,” N_{L}(x) or N_{R}(x), is the number of left- or right-pointing MTs passing through a cross section of the spindle at point x. We can only measure N_{L}(x) and N_{R}(x) up to an unknown proportionally constant c. The “plus end density at x” is the number of plus ends present in a 1-μm-wide window around x. The minus end density is similarly defined. The “fractional plus end density,” e_{L}(x), is the fraction of MTs passing x which have plus ends present in a 1-μm-wide window around x, i.e., the plus end density divided by (N_{L}(x) + N_{R}(x)). The fractional minus end density, e_{R}(x), is similarly defined. We will show that we can calculate fractional densities with no unknown proportionality constants.

Measurement of the number of left- and right-pointing MTs

We first needed to find the number of left- and right-pointing MTs that passed through a spindle cross section at position x (Fig. 1 A). We determined orientation by using the fact that all
MTs in *X. laevis* extract spindles are thought to slide continuously in the direction of their minus ends as they move poleward during metaphase. Leftward MT flow could thus be attributed to right-pointing MTs. We used speckle microscopy to visualize the sliding of MTs (Fig. 1 A, i) and cross-correlation (Westerweel, 1997; Miyamoto et al., 2004) to quantify the sliding in each direction (Fig. 1 A, ii). Thus, we obtained the number of left- and right-pointing MTs sliding through the window, up to an unknown proportionality constant, which was \( c \) \( N_L(x) \) and \( c \) \( N_R(x) \), respectively (Fig. 1 A, iii).

Fig. 2 shows examples of the number distributions of right- (solid lines) and left-pointing (dotted lines) MTs, which were plotted as functions of position along the spindle pole axis. These data represent 14 spindles from five extracts. The detailed distributions varied from spindle to spindle, reflecting the well-known variability in spindle morphology in the extract system, but the overall shapes were similar. The distributions agree with those calculated using the more precise, but computationally demanding, method of tracking and counting individual speckles (Vallotton et al., 2004; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200511112/DC1).

**Calculation of the difference between the densities of plus and minus ends**

We used the results from the previous step to find the difference in the local fractional densities of minus versus plus ends. We extracted this information from the spatial variations in the numbers of left- and right-pointing MTs (Fig. 1 B). To visualize how this was done, consider two adjacent windows in the spindle, and the number of right-pointing MTs in those windows (Fig. 1 B, i). A MT that does not terminate between the windows extends through both, thus, giving rise to no change in MT number. A minus end implies an increase in MT number moving from left to right, whereas a plus end implies a decrease. These effects are additive, so the MT number increases with \( x \) when minus ends outnumber plus ends. Thus, we found the difference in the densities of minus versus plus ends on right-pointing MTs by measuring their change in number from one window to the next. A similar analysis of the left-pointing MT numbers gave the difference in end densities for left-pointing MTs. The sum of these quantities, divided by the total number of MTs present, was the difference in fractional end densities for all MTs, \( e_-(x) - e_+(x) \) (Fig. 1 B, ii). Mathematically, it is written as follows:

\[
e_-(x) - e_+(x) = \frac{d}{dt}(cN_L(x)) - \frac{d}{dt}(cN_R(x)) \Bigg|_{t=0} \left( \frac{cN_L(x) + cN_R(x)}{cN_L(x)} \right) \quad [1]
\]

The unknown proportionality constant \( c \) cancels, so the fractional density difference is obtained in absolute units.

This analysis does not give the fractional densities of plus and minus ends separately, only the difference between them. Specifically, it cannot distinguish between a spindle made up of many short MTs, which would have a large number of both plus and minus ends, and a spindle with a smaller number of long MTs.

**Measurement of the fractional plus end density**

We needed an independent measurement of the fractional plus end density. We localized plus ends by pulsing labeled tubulin into extract and measuring its incorporation into preassembled spindles (Fig. 1 C, i). We assumed that tubulin is incorporated into MTs only at growing plus ends, so the initial rate at which fluorescence intensity increases is constant and proportional to the local density of plus ends (Supplemental materials and methods, available at http://www.jcb.org/cgi/content/full/jcb.200511112/DC1). From the rate of increase, we determined the fractional plus end density, \( e_+(x) \). To visualize how this was done, imagine that the fractional plus end density is 0.2 ends per micrometer per MT and that 75% of all plus ends are growing. Thus, 15% of the MTs in a 1-μm-wide window will have plus ends. Assuming growth at the published rate of 10 μm/min (Verde et al., 1992), each of these ends will grow through the window in 6 s. Eventually, all MTs will be fully labeled. After 6 s, then, the intensity in the window will be 15% of its final value. We thereby calculated the 6-s fractional intensity increase to find the fraction of MTs with growing plus ends in the window. We divided this by the estimated ratio of growing to total plus ends, 0.75, to find the fractional density of all plus ends, growing or shrinking. Mathematically, it is written as follows:

\[
e_+(x) = \frac{d}{dt} \left( I(x,t) \right) \Bigg|_{t=0} \left( \frac{v_g f_6 I(x,t)}{I_0} \right). \quad [2]
\]

**Figure 2. Oriented MT number distributions.** Plots for 14 spindles showing oriented MT number distribution, in arbitrary units, versus position along the spindle-pole axis. Distributions for right-pointing MTs are given in solid lines, and distributions for left-pointing MTs are given in dotted lines. Spindle-pole positions, which were manually selected by the edge of visible fluorescence in spindle images, are marked with vertical dashed lines. The distance between tick marks on the x axis is 10 μm.
where \( I(x,t) \) is the background-subtracted fluorescence intensity after summation in the \( y \) direction, at position \( x \) and time \( t \) after mixing. \( v_g \) is the plus end growth velocity, \( f_g \) is the fraction of plus ends which are growing, and \( t_f \) is the final time.

We found the densities and fractional densities of plus ends at each point along the spindle axis. Fig. 3 A (dotted lines) shows the distributions of fractional plus end densities from spindles assembled on four separate days, whereas Fig. 3 B shows the density distributions for the same spindles. Plus ends are broadly distributed throughout the center of the spindle, but their density drops sharply toward the poles.

The distributions were qualitatively similar to those obtained by imaging the tip-tracking protein EB1 (Tirnauer et al., 2004; unpublished data), but we chose to use the tubulin addition method because there was no way to calibrate the EB1 data to calculate the fractional plus end densities.

Averaging among spindles, we measured 0.08 plus ends per micrometer of MT, corresponding to an average MT length of \( \sim 14 \, \mu\text{m} \).

**Calculation of the fractional minus end density**

Our main goal in this study was to measure the localization of minus ends, for which we had no probe. Because at each position we now knew the difference between the fractional densities of plus and minus ends, and also the fractional density of plus ends from an independent measurement, we could calculate the fractional minus end density, \( e_-(x) \), as the sum of these two numbers (Fig. 1 C, ii). The density was then given by \( e_-(x) \) times the number of MTs.

In Fig. 3 (A and B), we show a gallery of minus end fractional density distributions (A, solid lines) and density distributions (B, solid lines). For each spindle, the minus end density was low at the equator and increased to broad peaks near the poles. The fractional density at the equator went as low as zero in spindles where antiparallel overlap was small, but was typically \( \sim 0.1 \) minus end per micrometer per MT, rising to 0.2 minus ends per micrometer per MT at the peaks.

**Conclusions**

Our method provides the first way to optically localize MT ends of each polarity in an anastral spindle. It is currently the only way, as there is no reliable marker known for minus ends. Our analysis relies on two important assumptions; that all MTs moving left have their minus ends to the left, and that only plus ends incorporate new tubulin. The former is strongly expected from models in which motor proteins drive anastral spindle morphogenesis (Walczak et al., 1998; Miyamoto et al., 2004), but it has not been independently validated; if it is invalid, our method is not reliable. The latter assumption is supported by many observations of tubulin polymerization in cells, but also has not been independently validated for extract spindles. Our method has important limitations. It does not address kinetochore MTs, because these are very small in number compared with interpolar MTs, and all our measurements are bulk observations, not single MT data. For a more in-depth discussion of limitations, see the Supplemental materials and methods.

Because it depends on all spindle MTs sliding poleward, on speckle imaging, and on tubulin pulse labeling, our method cannot be applied to live somatic cells or eggs. However, *X. laevis* extract spindles provide a useful model for spindle assembly in general, and anastral morphogenesis in particular. Although their exact relevance to intact cell spindles can be debated, the mechanisms they have revealed have proven generally relevant.
We find that minus ends are localized in a distinctive manner within the spindle, with a deep trough near chromosomes, rising to a broad peak nearer the poles, then decreasing at or slightly before the poles (Fig. 3, A and B). This picture of a steady-state spindle with plus and minus ends distributed throughout has not been considered in any theoretical models. It is inconsistent with search-and-capture models (Kirschner and Mitchison, 1986) and computational models based on as- ters and motors (Nédélec, 2002) because minus ends are not located in discrete nucleating structures at poles.

The continued presence of minus ends throughout a spindle where all MTs are moving poleward implies either that minus ends are continuously produced in the center of the spindle by nucleation or severing, and then moved poleward, or that minus ends are static in position and depolymerize continuously.

distinguishing between these alternatives requires a method for dynamic imaging of minus ends. We currently favor the moving end hypothesis for several reasons: TPX2, a Ran target implicated in MT nucleation, moves continually poleward at the flux rate, possibly in association with minus ends (Mitchison et al., 2004); Minus ends produced in S2 cell kinetochore fibers that are not attached to poles move toward the pole and only begin depolymerizing when they reach it (Maiato et al., 2005); and the Ran pathway, which can trigger MT nucleation, continues to operate in steady-state metaphase spindles, and there is no known reason nucleation near chromosomes should cease after spindles are assembled.

We favor a model in which maintenance of the metaphase steady-state in anastral spindles depends on continuous nucleation of MTs in a wide region around the chromosomes, followed by sorting and movement toward poles with minus ends neither polymerizing nor depolymerizing (Fig. 4). As existing minus ends are moved outward, they are joined at each point in the nucleation region by newly created ends, so their density increases from a minimum at the chromosomes. Other mechanisms, such as nucleation from the poles, may coexist with this process. The continuous nucleation proposed in this work is consistent with proposed functions of RanGTP (Carazo-Salas et al., 1999, 2001), but our data go a step further, emphasizing that Ran-driven nucleation is probably central to maintenance of the metaphase steady-state, as well as to initial spindle assembly. It is also consistent with recent observation of diffuse nucleation in S2 cells (Mahoney et al., 2006).

Our model makes testable predictions for the behavior of minus ends; because a single round of dynamic instability for a MT lasts, on average, ~1 min, while reaching the pole from the chromosomes takes ~8 min at the flux rate, minus ends would have to last for several cycles of dynamic instability. Our model then suggests a factor that stabilizes minus ends as they travel, possibly the same as the nucleator. To test this prediction, we need a reliable marker for minus ends that can be visualized optically, together with biochemical information on how MTs are nucleated—and how minus ends are transiently stabilized, if indeed they are—by the Ran pathway.

Materials and methods
Preparation and imaging of X. laevis extracts
We prepared X. laevis egg extracts and assembled spindles after one cycle of DNA replication (Desai et al., 1999). We performed fluorescence speckle microscopy (Waterman-Storer et al., 1998) using X-rhodamine–labeled tubulin (Invitrogen) at 25 μg/ml. Images were acquired at 20°C on a microscope (either EB00 or 90i; Nikon) with 60×/1.4 NA or 100×/1.4 NA objectives (Plan Apo DIC; Nikon), immersion oil (Delta Vision), and a cooled charge-coupled device camera (MicroMAX; Princeton Instruments [or ORCA-ER; Hamamatsu]) using Metamorph imaging software (Universal Imaging Corp.). 4–5 μl of spindle reactions were squashed under 18 mm coverslips and imaged by wide-field microscopy, with the focal plane in the middle of each spindle. We typically acquired 18 frames per spindle at 5-s intervals and 400-ms exposures.

Calculation of oriented MT number distributions
Each spindle was rotated to align its pole–pole axis with the x axis. Cross-correlations were calculated between sequential frames as a function of the x and y displacements ∆x and ∆y. These were averaged over the temporal sequence, as described in Miyamoto et al. [2004; Fig. 1 A, i]. A profile of the resultant surface was calculated along a line, near parallel with the
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Δx axis, passing through the two peaks that represented leftward and rightward flow. To estimate the volumes of the two peaks, this profile was fit using Matlab (Mathworks) to a sum of two Gaussians plus a background term. The numbers of left- and right-pointing MTs were obtained from the integrated intensities under the peaks, up to an unknown proportionality constant [see Supplemental materials and methods].

Cross-correlations were found for windows 22 pixels wide (≈3 μm), which were spaced every 1 μm along the length of the spindle, to obtain distributions of oriented MT number as a function of position (Fig. 2).

Calculation of fractional end and fractional density differences Oriented MT number distributions were smoothed in Matlab using a 20-pixel-wide moving-average filter. Left- and right-end number differences were obtained from the derivatives, computed moving left to right for right-pointing MTs and right to left for left-pointing MTs. The left and right end number differences were summed and then divided by the total MT number at each point to obtain the fractional end density difference. We focused on the midplane of each spindle, where the mean angle of MTs in the z direction was minimal, to minimize the effects of MTs entering or departing the plane of focus.

Plus end localization 3 μl of preformed spindles in extract that had been assembled with speckle-level X-rhodamine–labeled (red) tubulin were mixed on the slide with 2 μl of extract preequilibrated with 50 μg/ml green Alexa Fluor 488–labeled (blue) tubulin, squashed under a coverslip, and imaged as soon as possible (within 10–30 s) using a dry 40×/0.95 NA lens and ORCA-ER camera. After observation of incorporation of the green tubulin until near steady state (≈3 min), the objective was switched to a 60×/1.4 NA oil lens, and a speckle sequence of the same spindle was recorded for use in calculating oriented MT distributions. The median intensity, calculated in a region outside the spindle, was subtracted from each frame. New tubulin incorporation was measured as the intensity, recorded as a function of position along the spindle-pole axis (x), and the time elapsed after mixing (t). Incorporation was measured as the intensity, recorded as a function of position along the spindle-pole axis (x) and the time elapsed after mixing (t). In order to calculate the density, the intensity, recorded as a function of position along the spindle-pole axis (x), and the time elapsed after mixing (t). We would like to thank Ge Yang and Gaudenz Danuser for use of their speckle-tracking software and discussions about measurement of speckle flow, and Ryoma Ohi for suggesting the plus-end localization technique.

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References


Supplemental materials and methods

Dynamic cross-correlations
To measure the flow of MTs within the spindle, we calculated the cross-correlation between images in a time sequence with fixed time differences, \( \Delta t \), averaged over time, \( t \), and over \( y \), which is the direction perpendicular to the spindle axis. The resulting correlations were thus a function of \( x \), the position along the spindle-pole axis (in practice, averaged over windows in \( x \)), and the displacements \( \Delta x \) and \( \Delta y \). At each position in \( x \), the cross-correlation surface as a function of \( \Delta x \) and \( \Delta y \) exhibited two peaks, corresponding to flow in the direction of the two poles. We rotated and shifted each surface slightly so that the two peaks were on the \( \Delta x \) axis. We measured the sizes of the two peaks by analyzing the profile of the cross-correlation along the \( \Delta x \) axis (i.e., at \( \Delta y = 0 \)).

This profile was fit to a function of the form

\[
h_{x}(x)e^{c_{x}(x)x^{2}/w_{x}(x)^{2}} + h_{y}(x)e^{c_{y}(x)y^{2}/w_{y}(x)^{2}} + b(x) + d(x) \left| f(x) - x \right|,
\]

where \( \Delta x \) is displacement along the profile line, and the parameters were all functions of the position \( x \) along the spindle axis, with \( h_{x}(x) \), \( c_{x}(x) \), and \( w_{x}(x) \) the height, center position, and width of the left Gaussian, respectively, and \( h_{y}(x) \), \( c_{y}(x) \), and \( w_{y}(x) \) similarly defined for the right Gaussian. The correlations provided information on the intensity and speed of flux through a cross section of the spindle at \( x \), with \( c_{R}(x) - c_{L}(x) \) equal to twice the local flux velocity times \( \Delta t \). The parameter, \( b(x) \), corresponded to constant correlation caused by background fluorescence. The last term was a very small correction (\( d(x) \) was typically >1,000 times smaller than \( h_{x}(x) \) and \( h_{y}(x) \) for enhanced correlation at small displacements between different speckles on the same MT, with \( f(x) \) correcting for possible (small) drift of the spindle over the time interval \( \Delta t \).

Because the dependence of the background on the transverse displacement, \( \Delta y \), it was difficult to accurately measure the widths of the peaks in this direction via an automated fit. However, visual inspection showed that the ratio between the widths in \( \Delta y \) and \( \Delta x \) did not vary significantly as a function of position within the spindle; indeed, in the regions near the peaks, the profiles were generally close to radially symmetric. As these regions accounted for most of the integrated intensity of the peaks, we therefore used the width calculated in \( \Delta x \) as a proxy for the \( \Delta y \) width. The total integrated intensities of the two peaks in the cross-correlation were thus proportional to \( h_{x}(x)w_{x}(x)^{2} \) and \( h_{y}(x)w_{y}(x)^{2} \), respectively.

Plus end density measurements
Several aspects of the inference of plus end density profiles from the incorporation of new tubulin need further explanation.

Occasionally, at the initiation of imaging, the labeled tubulin could be seen to have diffused only partially into the spindle structure, leaving the bulk darker than the surrounding background; in these cases, we began our analysis of the time dependence of the newly incorporated tubulin once mixing was complete.

We expected the fluorescence intensity in a window to initially increase linearly after mixing. This is because as the plus ends, which were present in a window at the time of labeled tubulin addition, move out of the window, new ones grow into it to take their place. For a spindle at steady-state, the number of growing plus ends in a window should remain constant in time. Because each growing plus end presumably incorporates labeled tubulin at the same rate, the initial incorporation rate should be constant. Eventually, as labeled tubulin begins to saturate the spindle, other effects will affect the rate of intensity increase. These include depolymerization of already labeled MTs, contributing to a decrease in fluorescence intensity, and sliding of labeled MTs in and out of the window caused by flux. Data from experiments (Fig. S1 D) and simulations confirm that the fluorescent increase is linear in time for approximately the first 60 s after mixing. This linear increase meant that the short delay (at most 30 s) between addition of labeled tubulin and the initiation of imaging did not substantially affect the inferred plus end densities. The effects of such delays would, at worst, lead us to somewhat underestimate the number of minus ends at any given point in the spindle, strengthening our main conclusion that minus ends are present in the central spindle.

We estimated the fraction of growing plus ends within the spindle by comparing the previously measured speeds of MT polymerization in the spindle, 10 \( \mu \)m/min, and of depolymerization, 30 \( \mu \)m/min (Timauer et al., 2004). This implies that in steady-state, about three quarters of the MTs are growing. However, if plus ends in the spindle spend a significant amount of time paused—or if this growing fraction is an overestimate for any other reason—the densities of plus and minus ends we calculated from these data, together with the end imbalance data, will both be underestimated by the same amount. Fig. S2 E shows the effects of errors in the estimation of this value on simulated data.
For inferring the fractional plus end density (i.e., plus ends per micrometer per MT) it is necessary that certain aspects of the noise in the measurements roughly cancel. One type of noise that we expected to make a significant contribution to our measurements was that caused by out-of-plane fluorescence. Because of the nonconstant shape of the spindle, we could expect this noise to contribute different amounts at different points along the spindle pole axis. However, both out-of-plane fluorescence and the signal were expected to increase via the same process, labeled tubulin incorporation. If the out-of-focus planes in the spindle have the same fractional density of plus ends as the in-focus plane, this noise would increase at the same relative rate as the signal. For example, if out-of-plane fluorescence doubles the recorded intensity at one position for a given time point, we expect it to double the recorded intensity at every time point. This means that the rate of fractional intensity increase, which we measure to obtain the plus end density, is unchanged by this type of noise.

To test the method for computing plus end density, we made a one-dimensional simulation of a collection of MTs in a spindle using MatLab. For each MT at each time point, we recorded the locations of the plus and minus ends, the orientation, whether the MT was growing or shrinking, and intensities in “green” and “red” channels for each point along its length. MTs were nucleated in regions around the center of the spindle and were initially growing. With each iteration, every MT had a fixed probability of changing to a shrinking state. With every iteration, the length of each growing MT was increased and the length of shrinking MTs was decreased, and MTs were moved in the directions of their minus ends to simulate flux. When a MT shrank to zero length, it was re-
moved and a new one was created in the central spindle to replace it. To simulate speckle labeling, MTs were assigned red intensities given by a Poisson distribution along their length. Initially, all green intensities were zero. The simulation, using 10,000 MTs, was allowed to run for 10,000 5-s iterations to assure that the spindle achieved steady-state. From that point, to simulate the addition of green tubulin, all new MT growth was given a green intensity of one. We produced images from the green and red channels for 100 5-s iterations after addition of the “green tubulin.” Noise was added to the images to simulate out-of-focus fluorescence, with the amount added at each point proportional to a Gaussian centered at the middle of the spindle and to the fluorescent MT density.

We found the oriented MT distributions, and used these along with the green channel images to calculate the plus and minus end distributions with the same script used in the real data analysis. We then compared these with the actual distributions from the simulations. By changing the patterns of MT nucleation, we simulated three types of spindle: one which approximated the spindles we saw (Fig. S2 A), one in which minus ends were clustered in the central spindle (Fig. S2 B), and one in which minus ends were confined to the poles (Fig. S2 C). The inferred end densities were accurate in all three cases, and easily allowed us to distinguish between the different types of spindles.

Next, we simulated the effects of delays before initiation of green imaging (Fig. S2 D). Generally, the effect of such delays was to underestimate the plus end population, because the rate of intensity increase decreased as the spindles approached steady-state. With parameters similar to the real data, this led to only a slight underestimation of minus-end densities, as discussed in the previous paragraphs. Finally, we studied the effects of errors in the estimated fraction of growing MTs (Fig. S2 E).
Internal consistency checks, and inconsistencies if minus ends are at poles

The main conclusion of this paper is that a substantial number of minus ends are found in the central spindle, away from the poles. How plausible is it that biases in our measurements or invalid assumptions in our inferences could have falsely produced this result? In particular, could the data be consistent with the conventional picture of minus ends concentrated near the poles?

To address these questions quantitatively, we consider the fractional densities of minus ends at points 5 µm on either side of the center of the spindle; these were chosen to be in the regions where the differences between our results and the conventional picture would be largest. On average, the inferred fractional minus end density at these points was 0.043 ends/µm/MT. For each spindle, we considered the “low minus end hypothesis” that the actual fractional minus end density 5 µm from the center was low, at 0.01 ends/µm/MT. We asked, given this hypothesis, what values in our measurements would have been required to produce the low minus end results and found the ratio of these to the actual results. We then calculated the ratio between the measured value and the required one. We separately considered the cases of bias in the fractional plus end density and bias in the fractional end-density difference. All values were computed for each of the 12 spindles shown in Fig. 3, and then averaged (see the following table).

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</tbody>
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What sources of bias could have caused a large overestimation of the fractional plus end density? One potential source could be the delay in measurement of fluorescence increase after tubulin addition. However, as noted in the previous section, fluorescence increase decreases over time as labeled tubulin saturates the spindle, so any delay in measurement would cause us to underestimate the fractional plus end density. The opposite bias would result from underestimating the saturation intensity (from bleaching, etc.), but, given the expected form of the time-dependent intensity, a factor of two underestimate is implausible. Another possibility is that either the fraction of plus ends that are growing or the plus end growth velocity differs in these spindles from the published values we used. But these parameters were global, and affected calculations at all positions within the spindle. If we increased these parameters to give the desired minus end densities at 5 µm from the center of the spindles, 11 of 12 spindles would show a negative fractional minus end density at the center, with an average density of –0.012, which is an unreasonable result. In contrast, with the parameters we used in our analysis, only 2 of the 12 spindles had slightly negative minus end densities at the center, and the average density there was 0.021. (We note that if, instead, the parameters we used were significantly higher than their correct value, the fractional minus end distribution would largely reflect the plus end distribution. Because we do not observe either this or substantial negative densities, we believe that the parameters we used were roughly correct.) A third possibility is that the growth velocity or the fraction of growing plus ends varies as a function of position within the spindle. But the position dependence required would have to be drastic to produce the required bias at 5 µm from the center, while preserving the positive minus end density at the center; e.g., the fraction of plus ends which are growing would have to nearly double in the 5-µm distance from the center of the spindle to the points we considered.

Is it more plausible that the fractional end density difference measurements were biased? We measured the fractional end density difference by calculating the slopes of the oriented MT number distributions, which were in turn determined from cross-correlation analysis of tubulin speckle image sequences. The low minus end hypothesis would imply that these distributions, at 5 µm from the center, were nearly twice as steep as what we measured. We do not believe that such a bias could be the result of our cross-correlation analysis, as the shapes of our oriented MT distributions were broadly similar to those produced with a different particle-tracking method (Vallotton et al. 2004; Fig. S1). Additionally, a global error of this magnitude in measuring the slopes would imply that the real MT number distributions were half as wide those we measure. This would imply that the MT density at the chromosomes was close to zero and that there is essentially no overlap of antiparallel MTs, which we consider to be unrealistic.

Both our method and that in Vallotton et al. (2004) analyzed images series taken at a single z slice (focal plane) within the spindle. Is it possible that spindle geometry might have caused us to underestimate the slope of the MT number distribution at this location? Because the spindle is wider at the center than at the poles, we would expect MTs to be more spread out in z (and y) in the central spindle. Moving toward the center of the spindle, the fraction of MTs that pass through the z section that we imaged is therefore expected to decrease. If anything, this effect would lead us to overestimate the decrease in MT number, and would not lead us to falsely conclude that minus ends were present at 5 µm from the center. We do note, however, that this effect probably makes our results more unreliable near the poles, where MTs presumably move at greater angles in z, than in the central spindle.

In summary, none of the obvious possible sources of quantitative bias in our measurements would lead us to conclude that minus ends were present in the central spindle if in fact they were not.

In addition to these inconsistencies with the pole-localized minus end picture, several internal consistency checks support our conclusion that the minus ends are located throughout the spindle. If minus ends were located only at the poles, and MTs had an exponential length distribution, we would expect to see oriented MT number distribution that were themselves exponential in nature, decreasing immediately from a peak at the poles. That the distributions we see are drastically different from exponential is already enough to make it likely that minus ends are distributed away from the poles.