Forces positioning the mitotic spindle: Theories, and now experiments

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The position of the spindle determines the position of the cleavage plane, and is thus crucial for cell division. Although spindle positioning has been extensively studied, the underlying forces ultimately responsible for moving the spindle remain poorly understood. A recent pioneering study by Garzon-Coral et al. uses magnetic tweezers to perform the first direct measurements of the forces involved in positioning the mitotic spindle. Combining this with molecular perturbations and geometrical effects, they use their data to argue that the forces that keep the spindle in its proper position for cell division arise from astral microtubules growing and pushing against the cell's cortex. Here, we review these ground-breaking experiments, the various biomechanical models for spindle positioning that they seek to differentiate, and discuss new questions raised by these measurements.

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Abbreviations:
A-P, anterior-posterior; **CRISPR,** clustered regularly interspaced short palindromic repeats; **DIC,** differential interference contrast; **MT,** microtubule; **NCC,** nuclear centrosome complex; **PAR,** partitioning-defective; **RNAi,** RNA interference.

Introduction

The spindle is a self-organized structure at the heart of cell division in eukaryotes, which both segregates chromosomes and positions the division plane. The spindle is a bipolar structure, consisting of chromosomes, abundant microtubules, and a large collection of associated proteins. At the start of cell division, the spindle assembles with the chromosomes arranged at its center. The spindle then elongates and separates sister chromosomes. Later on, the cell cleaves at the spindle equatorial plane, perpendicular to and at the midpoint of the connecting line between the two spindle poles. If the spindle is located off center, or if cellular components are not equally distributed on the two sides of the spindle, then the division is said to be "asymmetric," and the subsequent daughter cells will be different from each other. Thus, the proper positioning and orientation of the spindle is of great developmental import, because it both determines the relative locations of the daughter cells and the partitioning of cellular materials. The positioning of spindles and microtubule asters has been studied in diverse systems [1–4], and it is still unclear to what extent similar mechanisms are dominant in these different contexts. In this short review, we focus on work on *Caenorhabditis elegans*.

The early embryo of the nematode *C. elegans* is an excellent model system for studying asymmetric cell division. Its rapid and stereotyped cell division is well suited for comparing the phenotypes of wild-type animals and experimentally perturbed ones [5]. Detailed studies of molecular functions have been enabled by *C. elegans'* excellent genetics, the ease of RNA inference (RNAi) [6], and, increasingly, CRISPR genome editing. The *C. elegans* embryo is elliptical in shape with anterior-posterior (A-P) polarity established along its long axis. The first mitotic division is asymmetric, generating two daughter cells with different cell fates and sizes: The larger anterior cell, named the AB cell, develops into purely somatic tissues, while the smaller posterior cell, named the P_1 cell, gives rise to the germline and other somatic precursor cells [7]. Despite extensive progress it remains poorly understood how underlying mechanical and biochemical processes control spindle position and orientation to ensure the correct daughter cell sizes, locations, and partitioning of cell-fate determinants.

Positioning of the pronuclei and spindle in *C. elegans*

The events leading to asymmetric cell division in *C. elegans* embryos begin at the earliest stages of development. Entry of sperm into the oocyte induces the completion of meiosis, extrusion of a polar body, and synthesis of the eggshell [8]. The centrosome, which enters with the sperm, activates contractions in the actomyosin cortex, which cause the cell periphery to ruffle [9] and facilitates the A-P polarity establishment [10, 11], giving rise to the spatial organization of the partitioning-defective (PAR) proteins on the cell cortex [8]. The localized cortical accumulation of anterior PARs (PAR-3, PAR-6) and posterior PARs (PAR-1, PAR-2) further drives the polarized distribution, along the A-P axis, of downstream factors, including cell fate determinants [7]. Upon completion of meiosis, two pronuclei form: one containing the paternal genome, the other, the maternal genome (Fig. 1A). The paternal pronucleus, with its associated duplicated centrosomes, and the maternal pronucleus, move toward each other, meeting in the embryo posterior to form the nuclear centrosome complex (NCC). Then the NCC migrates to the center of the embryo while rotating to align with the A-P axis (the processes called pronuclear migration and rotation). Subsequently, the nuclear envelope breaks down and the mitotic spindle forms and starts to elongate [12]. In anaphase, the spindle elongates while keeping its anterior pole relatively fixed, causing its center to move toward the cell's posterior. During elongation along the A-P axis, the spindle oscillates in the transverse direction. These motions eventually cease and the spindle elicits a cleavage plane running through its center, creating a larger anterior cell and a smaller posterior cell (Fig. 1B).

What are the forces involved in these motions? And how are the forces produced and regulated to rotate and translocate the spindle? Extensive evidence demonstrates that these motions are microtubule dependent. Microtubules, the most prevalent component of the spindle, are tubular filaments made of heterodimers of alpha and beta-tubulin. Microtubules are polar polymers and their two ends are distinct: one end, called the plus end, terminates with beta tubulin, whereas, the other, called the minus end, terminates with alpha tubulin. Many microtubules radiate outward from the centrosome, with their plus ends predominantly pointing toward the cell cortex. The plus ends of these so called astral microtubules are highly dynamic, causing these microtubules to continuously and rapidly polymerize and depolymerize (Fig. 1C). Experimental manipulations curtailing astral microtubules yield abnormal positioning or rotation of the NCC or spindle, arguing for their great importance in these processes [13–17]. In addition to relying on astral microtubules, spindle positioning is also dependent on cortical polarity: perturbing some PAR proteins leads to defective NCC rotation and symmetric spindle positioning [18–20]. This argues that the PAR proteins are responsible for establishing the asymmetric distribution of force generators which act on astral microtubules to move spindles asymmetrically in anaphase. Furthermore, several experiments have demonstrated that the minus end directed motor dynein is critical for pronuclear migration, rotation and spindle displacement [21, 22]. Previous studies have identified many other genes which can influence NCC and spindle motion, including factors that change microtubules dynamics, affect motor activity, and alter the function and localization of motor-associated proteins [19, 23–29]. However, the nature of the forces acting on astral microtubules in vivo, and hence, on the NCC and the spindle, remain unclear. Nor is it known how these forces combine to center and rotate the NCC or position the spindle. A recent study by Garzon-Coral et al. [30] performed the first measurement of the forces responsible for centering the spindle, and thus provides great insight into these issues.

Forces proposed to act on pronuclei and spindles

Three types of mechanisms have been proposed for pronuclear centering and maintenance of metaphase spindle position, all of which involve forces exerted through astral microtubules to the centrosomes. In *cortical pushing models*, the growth of astral microtubules against the cell cortex induces repulsive forces on microtubules [31–33]. In a *cortical pulling model*, microtubules impinging on the cortex are pulled upon by dynein motors bound to the plasma membrane [34]. Finally, in a *cytoplasmic pulling model*, dynein drags cargo along microtubules through the cytoplasm, and hence, pulls upon the microtubules as they move toward the centrosomes [35, 36]. There is good evidence that all of these force mechanisms are operative in both pronuclear migration, and in maintenance of metaphase spindle position. One question is which of these mechanisms, if any, is the dominant one. We review each of these in turn below. The study of Garzon-Coral et al. uses force microscopy, and various perturbations, to probe this question. The authors argue that their results are well-explained in terms of cortical pushing by microtubules.

A common element for each mechanism is the required balance of any applied forces – external forces, motor-induced forces, polymerization forces, etc. – with the drag forces created by moving the spindle and its components through the cytoplasm. Here, are some simple relevant estimates and comparisons: (i) the drag force on a single straight microtubule of length L, and moving at speed U along its axis, is $D_{MT} = 4\pi\mu LU/\left|\ln(e\varepsilon^2)\right|$ where μ is the cytoplasmic viscosity and $\varepsilon = 1$ is the microtubule aspect ratio. And (ii), if the spindle shape is approximated as a prolate spheroid with its long axis a and short axis $b \approx a/2$, the drag on the spindle moving, also at speed U, orthogonally to its long axis is $D_{Sp} = 3.612\pi a U$ (for *C. elegans* spindle, $b \approx 7\mu m$). By these estimates, only 18 microtubules, each of length $L \approx b$ produce as much drag as the spindle to which they are attached. (iii) The characteristic force scale for the end-loaded buckling of a hinged Euler beam is $F_e = \pi^2 E/L^2$ where E is the bending modulus of a single microtubule (it is useful to consider microtubules as Euler elasticae). And (iv), the external pushing force that stops the polymerization reaction for microtubules is $F_s \approx 4\text{pN}$ [37]. These two forces, F_s and F_e are in balance for $L \sim 5\,\mu m$ for $E = 10^{-23}\,\text{Nm}^2$ [38].
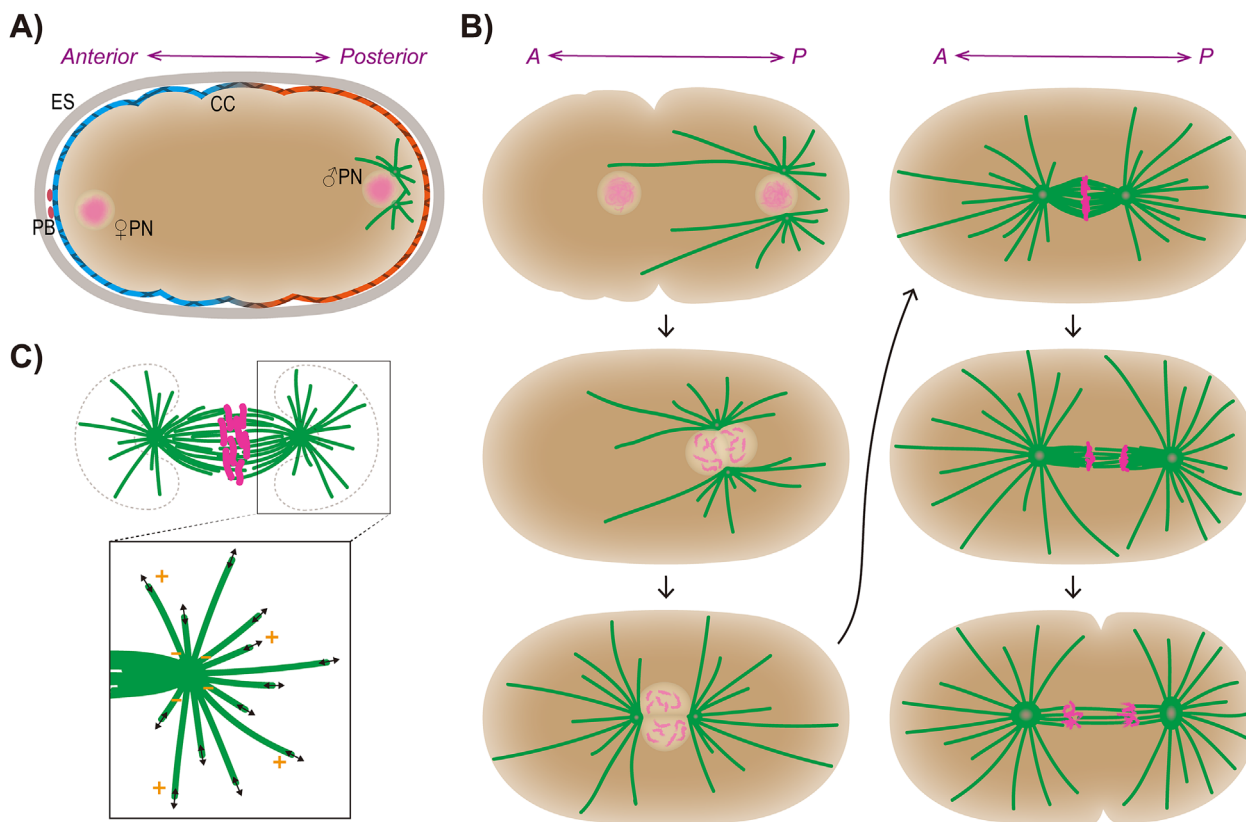
Figure 1. The *C. elegans* early embryo. **A:** The early *C. elegans* zygote. The posterior end is designated by the site of sperm entry. Fertilization first triggers the completion of meiosis, extrusion of polar bodies, and synthesis of the eggshell. Signal from the paternal-origin centrosomes leads to both the onset of anterior-posterior (A-P) polarity establishment, and also contractile cortical activity, which can help build the cortical A-P polarity. The paternal and the maternal pronuclei form upon completion of meiosis. The PAR-3/PAR-6 proteins (shown in blue) and the PAR-1/PAR-2 proteins (shown in orange) are localized to the anterior and the posterior cortex, respectively. (ES: eggshell; CC: cell cortex (actomyosin network); PS: polar body; ♂PN: paternal pronucleus; ♀PN: maternal pronucleus.) **B:** Events in first mitotic division in *C. elegans*. These events happen later than A, and the eggshell and cell cortex are not shown. The paternal and the maternal pronuclei move toward each other, and meet in the posterior region of the embryo. The two pronuclei and the centrosomes form the nuclear centrosome complex (NCC). The NCC migrates toward the cell center and rotates to align the two centrosomes along the A-P axis. After the nuclear envelope breaks down, the metaphase spindle forms near the cell center, with the spindle orientation aligned along the A-P axis. Later in anaphase, the spindle elongates and segregates the chromosomes. Meanwhile, the spindle is displaced toward the posterior end, accompanied by the transverse oscillating motions of the two centrosomes. The displacement and the oscillation eventually cease. Cytokinesis starts at the transverse plane, elicited by the spindle center, giving rise to a larger anterior and smaller posterior cell. **C:** Astral microtubules (indicated by the gray dashed line region) radiate outward from the centrosomes. Their plus ends are highly dynamic and undergo continuous and rapid polymerization and depolymerization. Astral microtubules are believed to play a very important role in spindle positioning.

Active cortical pushing

It is now understood that cytoskeletal filaments can generate pushing forces by their polymerization-driven growth against a barrier [37, 39, 40]. The motility of a keratocyte cell is believed to be driven by the polymerization of actin against its membrane, and microtubule polymerization forces are thought to be involved in nuclear centering during interphase in fission yeast *Schizosaccharomyces pombe*. There have been in vitro measurements of microtubule polymerization forces. Dogterom and Yurke [37] used the shape and length of microtubules buckled against barriers to estimate the functional relation between the polymerization growth velocity V_g and the associated polymerization force F_p. Of the several theories proposed to predict this relation [39–43], that of Mogilner and Oster [43] gives a good accounting of the Dogterom and Yurke's data.

Active pushing via microtubule polymerization could underlie the positioning of the spindle. There are different regimes to be considered, but the easiest to understand is when, $F_e >> F_s$ meaning that microtubules are not easily bent by polymerization forces. Being straight, we can assume for simplicity that each microtubule at the cortex transmits a force near, F_s to its centrosome. The resultant total force on the posterior or anterior centrosome, $F_{P\,or\,A}$, respectively, scales with the number of its microtubules in cortical contact: $F_{P\,or\,A}$: $N_{P\,or\,A}F_s$. If the spindle is displaced to the posterior, as in Fig. 2A, there is a force imbalance as more microtubules now contact the posterior cortex. Under various assumptions, Howard [44] shows that the spindle experiences a spring-like restoring force with a spring constant K proportional to F_s.
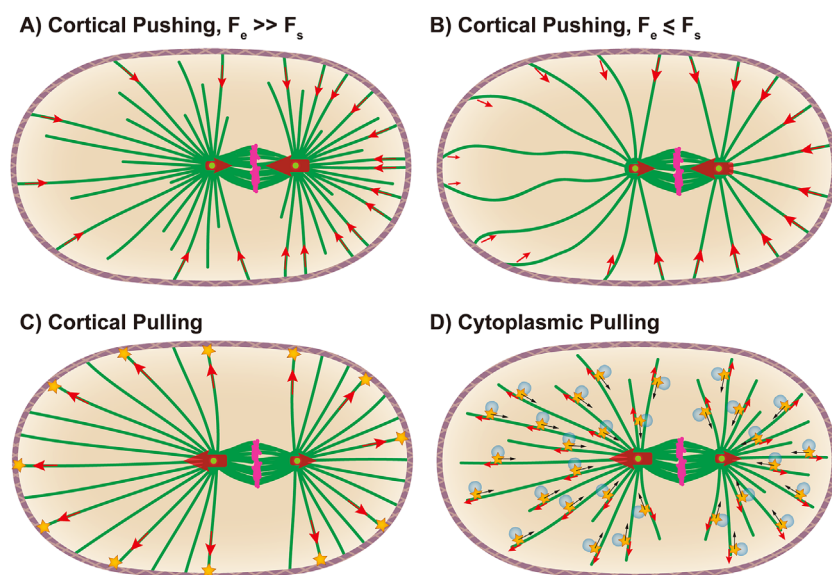
Figure 2. Schematics of different spindle centering mechanisms. (Forces are specified by red arrows.) **A** and **B:** In the cortical pushing mechanism, pushing forces from microtubule polymerization maintain the spindle position. **A:** When the elastic forces are much larger than the polymerization stall force, microtubules growing against the cortex remain straight and each microtubule generates a force roughly equal to the stall force, $F_P \sim Fs$. A posterior-wise displacement generates an anterior-wise restoring force, as more posterior microtubules now contact the cortex than do anterior microtubules. **B:** When the elastic forces are comparable to the stall force, growing microtubules bend or buckle against the cortex. A posterior-wise displacement of the spindle again gives a restoring force, as the length of posterior microtubules is shorter than anterior microtubules leading to larger elastic forces, $F_P \sim Fs \sim E/L^2$ on the posterior side. **C:** In the cortical pulling mechanism positioning is maintained by pulling on microtubules by cortically bound motors (shown by yellow stars). When the number of bound motors is substantially smaller than the number of microtubules near the cortex, a posterior-wise displacement of the spindle decreases the number of attached posterior microtubules pulled by bound motors, while the number of attached and pulled anterior microtubules is increased, yielding a net anterior-wise force. **D:** In the cytoplasmic pulling mechanism, dyneins (shown by yellow stars) drag cargo along microtubules through the cytoplasm (the black arrows indicate the transportation directions), and hence, pull upon microtubules as they move polewards. If the number of walking dyneins is proportional to microtubule length, a posterior-wise displacement results in longer anterior-wise microtubules, and larger anterior-wise pulling forces. In this mechanism, unlike the other two, the cargo transport induces cytoplasmic streaming flows toward the centrosomes [50, 52].

However, it could also plausibly be that $F_e \leq F_s$ so that microtubules are easily buckled or bent by polymerization forces. This also gives a restoring mechanism. If the polymerization force now scales with the elastic force F_e then for a posteriorly displaced spindle, a larger net pushing force is applied to the posterior centrosome as it has (more and) shorter astral microtubules in contact with the cortex (Fig. 2B). Both of these mechanisms can be operative, depending on biophysical details such as catastrophe rates and buckling growth-rates. Finally, the elastic energy stored

in a buckled or bent microtubule can also provide a restoring force as it is released through the microtubule's evolution back to being straight [45].

Cortical pulling

In this mechanism, astral microtubules impinging upon the cortex are pulled upon by dynein motors that are attached to the plasma membrane, in particular by association to the protein complex formed by the Gα subunits, GPR-1/2, and LIN-5. Experiments using laser ablation [28, 46–48] and RNAi against *grp-1/2* [23, 29] suggest the importance of cortical pulling forces during anaphase spindle elongation and oscillation. The importance is less clear to pronuclear migration and metaphase spindle positioning.

In one model for pronuclear positioning, an enrichment of LET-99 proteins on the posterior cortex near the division plane results in inactivation of cortical dynein there. This yields larger anterior microtubule pulling forces, so the NCC moves to the center and rotates [19, 49], where, once in proper position, the forces on the spindle are presumably in balance. The overall behavior − centering, rotation, time-scales − is quite sensitive to choices of biophysical parameters [49, 50]. In another model [51], metaphase spindle positioning relies instead on having the number of potential attachment and pulling sites be much smaller than the number of astral microtubules that reach the cortex. If the spindle were displaced, say posterior-wise, it would have more microtubules impinging on the posterior but over a smaller area and so the number of actual attachments decreases (Fig. 2C). The situation is reversed on the anterior cortex, so the spindle is pulled back to center. If instead there were a large number of attachment sites so that the number of attached microtubules scaled with the total number of astral microtubules, then displacement of the spindle from the center could result in "anti-centering" [30], that is, its further motion toward the cortex.

Cytoplasmic pulling

Both optical microscopy and RNAi studies suggest that cytoplasmic dynein is involved in the active transport of organelles along astral microtubules toward the centrosomes [36, 52]. As a consequence of Newton's third law, the force applied by organelle-bound dynein on microtubules must be equal and opposite to the force required to move the cargo through the viscous cytoplasm [52]. Such a force (which is tensile) on astral microtubules will be transferred to the centrosomes, thereby pulling upon the NCC or spindle. Given that longer microtubules would carry more payloads, the pronuclear complex moves in the direction of its longer microtubules (Fig. 2D). This was first investigated theoretically
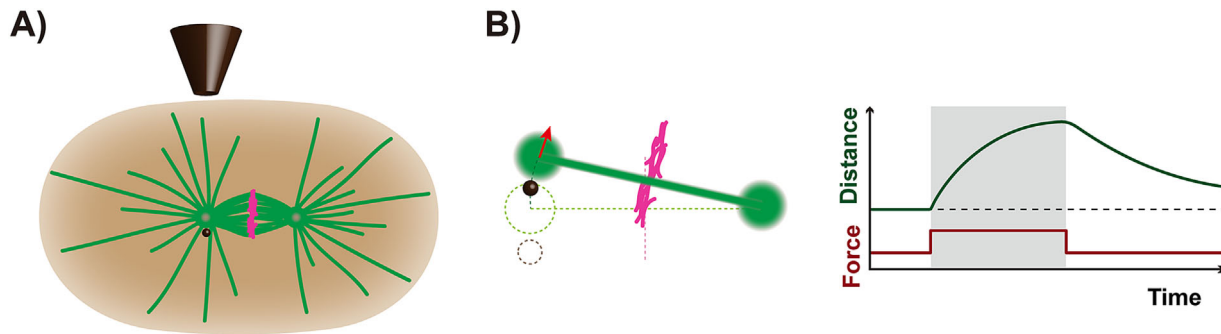
Figure 3. The magnetic tweezers experiments by Garzon-Coral et al. **A:** A magnetic bead inside a *C.elegans* embryo with the magnetic tweezers outside the cell. The authors can apply calibrated forces on the anterior centrosome through the bead. **B:** After the magnetic force (red arrow) is turned on, the centrosome deviates away from the A-P axis. The green and brown dashed circles indicate the original locations of the centrosome and the bead. Distance (dark green line) of the centrosome away from the A-P axis and the force (dark red line) exerted by the magnetic bead are plotted over time on the right. The centrosome moves rapidly initially and then slows down in response to the constant external force. When the force is turned off, the centrosome gradually relaxes back toward its original position.

by Kimura and Onami [35] who used a length-dependent pulling force to drag the NCC to the cell center. At the center, the posterior and anterior directed microtubules yield the same, balanced force. Their simple model, however, did not account for cytoplasmic flows induced by the motion of the NCC, or those produced by the forces exerted by cargo transport along microtubules (via Newton's third law). Shinar et al. [52] accounted for both of these effects (as well as the effect of cellular confinement) in a full hydrodynamic simulation of NCC migration, based upon the immersed boundary method [53]. They were able to capture the observed streaming flows along microtubules toward the centrosomes, and showed that cytoplasmic pulling yielded both centering and rotation to proper position. In this model, the elongated cell makes proper spindle alignment along the A-P axis a mechanically stable state of rest.

While DIC microscopy showed organelle transport along microtubules [52], RNAi of *dyrb-1*, a dyein light subunit, substantially reduced organelle transport and yielded slower and less successful centration [36]. Note that, like the cortical pushing model, this mechanism will not yield rotation to proper alignment in a spherical eggshell. That such rotation nonetheless is observed [13, 19] tells us that other positioning mechanisms are at play.

# A breakthrough: Measurements of forces in spindle positioning

The new work by Garzon-Coral and colleagues presents the first measurement of the forces involved in spindle positioning [30]. In these ground-breaking experiments, the authors microinjected magnetic beads into the worm's

gonad, waited for an embryo to form with a magnetic bead in it, dissected out the embryo and placed it on a microscope with calibrated magnetic tweezers, visualized the dynamics of early cellular events, and, at just the right time, they applied forces and investigated the response of the spindle.

In these experiments, the authors pushed a $1\,\mu$m magnetic bead into the anterior centrosome (Fig. 3A), perpendicular to the A-P axis, with a constant applied force. In response, the pole moved, and approached to a new position with the spindle tilted off the A-P axis. To give a sense of the scales of things, they found that by applying a force of $F_{bead} \sim 16pN$ for 10 seconds, they displaced the pole by $2\,\mu$m. When the force was turned off, the pole relaxed back toward its original position in about 15 seconds (Fig. 3B). Given these observations, Garzon-Coral and colleagues modeled the spindle's response to an applied force as a simple spring and dashpot in parallel: $C\dot{x} = -Kx + F_{bead}$ where $x(t)$ is the spindle's position, centered at $x = 0$, $-Kx$ a restoring spring force, and $C\dot{x}$ the viscous "drag-force" on the spindle ($\dot{x} = \frac{\mathrm{d}x}{\mathrm{d}t}$ denotes velocity). From this force balance equation, $\tau = C/K$ is the characteristic time-scale of displacement, $x_{\infty} = F_{bead}/K$ and is the maximal displacement of the pole. Hence, knowing
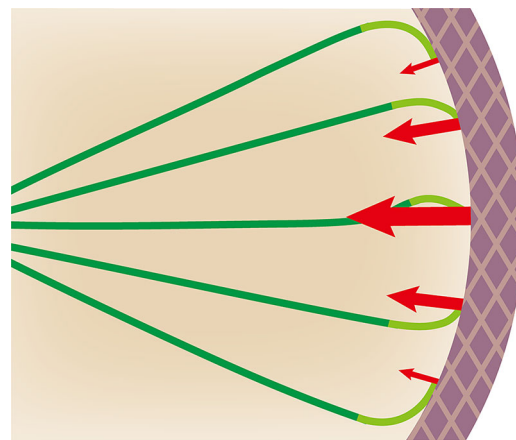
Figure 4. Astral microtubules impinge on the cortex at a variety of angles. Those that are nearly orthogonal to the cortex provide the largest forces (before buckling). The pushing forces are expected to reduce as the microtubules become more aligned with the cortical surface.
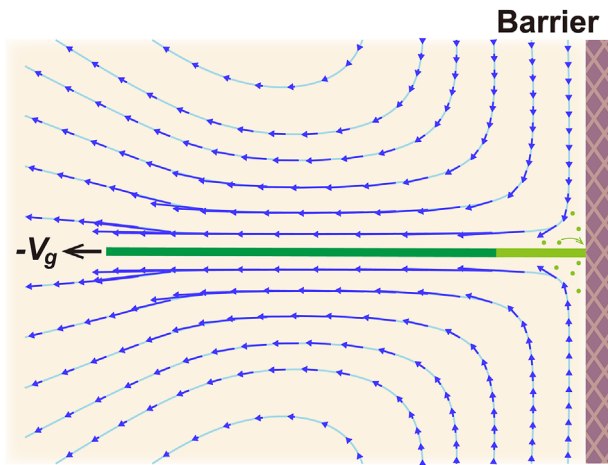
Figure 5. Schematic of cytoplasmic flow induced by the polymerization of a single microtubule against a barrier. The 3D flow is shown in the plane of the microtubule. The light blue solid lines are the flow streamlines and the dark blue arrows show the cytoplasmic fluid velocity field.

the maximal displacement yields the stiffness K. These quantities τ and K are fundamental for understanding the processes which maintain the spindle at the cell center, and any proposed biophysical mechanism of spindle positioning must explain their origin.

To differentiate between possible centering mechanisms, the authors used RNAi to investigate how the response of the spindle to force is modified by molecular perturbations. The authors conducted experiments in *gpr-1/2 (RNAi)* embryos, in which cortical force generators are inactivated, and knocked down *klp-7*, a microtubule depolymerase, to increase the lengths of astral microtubules. In both cases the stiffness parameter K increased, which they argue shows that the cortical pulling forces have an anti-centering effect. They performed experiments in the smaller AB and P_1 cells of two-cell embryos, after the first cell division. The stiffness parameter K was again increased: doubled relative to a single cell embryo. This argues against the cytoplasmic pulling model, as the restoring force should be unchanged or reduced by the smaller length of microtubules in these smaller cells. Increased stiffness in smaller cells is, however, consistent with the cortical pushing model as (i) the fraction of microtubules interacting with the cortex is larger in smaller cells; and (ii) the elastic forces associated with microtubules interacting with the cortex are also larger in smaller cells as $F_e \sim E/L^2$. In summary, Garzon-Coral et al. [30] use the increase of the stiffness coefficient in molecular and geometrical perturbations to argue against the cortical pulling and cytoplasmic pulling models, and in favor of the cortical pushing model.

Conclusion

There are some interesting questions of cellular mechanics provoked by the Garzon-Coral et al. study. One is that, they find that the time-scale of displacement τ is shorter than the

time-scale of relaxation back to center (Fig. 3B). This is not captured by the spring-dashpot model, which has a single time-scale, and may argue that a nonlinear response is being revealed by their measurements. One possible source of nonlinearity lies in the observation that the initial speed of pole displacement, $\sim$0.2 μm/s [30], is comparable to the speed of microtubule polymerization, $\sim$0.7 μm/s [54]. Further explorations of the parameter space of applied forces, and their time windows and temporal forms (e.g. oscillatory forcing might reveal a spectrum of stiffnesses and relaxation times), would give a more complete picture.

Also, do we really understand the nature of putative microtubule pushing forces? Astral microtubules impinge on the cortex at a variety of angles, given the geometries of the cell and the aster. Those microtubules that are nearly perpendicular are expected to provide the greatest pushing force, though buckling will rapidly dissipate its magnitude. Those that are at a shallow angle to the cortex may contribute little, which is shown schematically in Fig. 4. Hence, pushing forces could involve a small number of microtubules.

Another factor in the efficacy of cortical pushing is the rate of catastrophe of microtubules reaching the cortex. Pushing forces should reduce substantially with microtubule buckling and bending at the cortex, and this may make the cortical pushing mechanism less efficacious for slower rates of catastrophe. It would be interesting to understand how in vivo perturbations to this rate affect the mechanics of positioning.

Another fascinating question is the interpretation of the drag coefficient C measured from the experiments. Does it depend on the interaction of microtubules with the cortex, or does it largely reflect the drag of moving the spindle and its microtubules through the cytoplasm within the cellular confinement? Also, given that there are approximately 300 microtubules growing against the cortex [30] there should be non-negligible cytoplasmic flows generated by these microtubules being pushed backwards from the cortex, and thus dragging along cytoplasm. Are these flows part of the mechanics captured by the model drag coefficient C? Are they contributing to the stiffness? The flow induced by polymerization of a single microtubule against a barrier is illustrated in Fig. 5. These are long-ranged hydrodynamic interactions and are neglected in almost all theories of spindle positioning (see [52] and [50]). Simulation techniques that explicitly account for hydrodynamic interactions and microtubule-cortex interactions could provide a powerful way to study these questions. Other experimental techniques, such as visualizing cytoplasmic flows and laser ablation studies, would help answer these questions.

The work reported by Garzon-Coral and colleagues is a seminal advance. It is equal in importance to the 1983 landmark paper by Nicklas [55], which is the only direct measure of the forces responsible for chromosome segregation, as the Garzon-Coral study is the only direct measure of the forces responsible for spindle position. Furthermore, their observation that the spindle returns toward the cell center after the applied force ceases is the first unambiguous demonstration that forces are continually acting on the spindle to maintain its position in the cell center.

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