Biophysics of chromosome segregation: New evidence for a pushing body model of chromosome segregation

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Biophysics of chromosome segregation:

New evidence for a pushing body model of chromosome segregation

A dissertation presented
by
Che-Hang Yu
to
The John A. Paulson School of Engineering and Applied Sciences

in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the subject of Applied Physics

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Biophysics of chromosome segregation:

New evidence for a pushing body model of chromosome segregation

Abstract

Chromosome segregation is the essential process during cell division. In all eukaryotes, chromosome segregation is carried out by the spindle, which consists of microtubules and associated proteins. Despite being studied for over a century, the manner by which forces are generated by the spindle and move chromosomes remains poorly understood. In the past few decades, a canonical model of chromosome segregation has emerged and posited that chromosome motion in anaphase is the sum of two independent, mechanistically distinct processes. However, spindles which have been studied in detail are often found to exhibit behaviors that deviate from the predictions of this canonical model. This situation reveals the inadequacy of the canonical model as a general basis for explaining chromosome segregation. To dissect the mechanism, understanding the spatial organization of microtubule polarity and its interplay with protein localization are thought to be crucial, but the mechanism remains poorly understood, in part due to the difficulty of measuring microtubule polarity in spindles.
In this dissertation, we developed a quantitative method to nonperturbatively measure microtubule polarity throughout spindles using a combination of second harmonic generation and two-photon fluorescence. We validated this method using computer simulations and comparison to structural data on spindles with known polarity. We used this method to measure microtubule polarity throughout the first mitotic spindle in C. elegans embryos. We believe that this method should provide a powerful tool for studying spindle organization and function, and may be applicable for investigating microtubule polarity in other systems.

Furthermore, we have investigated the mechanism of chromosome segregation in C. elegans mitotic spindles, human mitotic spindles, and C. elegans female meiotic spindles. We found that these spindles all contain microtubules with both ends between segregating sister chromosomes. Even as chromosomes move towards spindle poles, these inter-chromosomal microtubules slide apart at the same speed as chromosomes. Perturbing inter-chromosomal microtubules causes chromosome motion to immediately cease. Our results are inconsistent with the canonical model, and support a pushing body model proposed ∼100 years ago, arguing that the extension of the inter-chromosomal array of microtubules is the solely primary driver of chromosome segregation in diverse systems.
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Chapter 1

Introduction

1.1 Mechanism of chromosome segregation

Chromosome segregation is the essential process during cell division, in which chromosomes are partitioned into the two daughter cells. In eukaryotes, chromosome segregation is carried out by the spindle. In many organisms, while the chromosomes separate, the spindle elongates but at a slower speed, such that chromosomes move closer to spindle poles over the course of anaphase. The movement of the chromosomes towards spindle poles is referred to as anaphase A; spindle elongation (i.e. the separation of spindle poles) is referred to as anaphase B. Despite intensive work, the mechanism of chromosome segregation remains unclear. Most proposals center around what has become the canonical model, which postulates that anaphase A and anaphase B are two mechanistically distinct processes whose combination produces net chromosome motion in anaphase (Ris, 1949). In this canonical model, chromosome motion contributing to anaphase A is typically attributed to depolymerization of kinetochore microtubules (Asbury, 2017), while anaphase B is typically proposed to contribute to chromosome motion by chromosomes being dragged apart via their attachment to the separating spindle poles, with pole separation driven by either external pulling forces or internal pushing forces (Scholey et al., 2016). Different spindles
display different extents of anaphase A and anaphase B. If the canonical model is correct, this would imply that the mechanism of chromosome motion in anaphase is different in these different spindles.

While many lines of evidence are consistent with the canonical model (McIntosh et al., 2012, Scholey et al., 2016, Asbury, 2017), there are diverse findings that are not easily explained by it: in many systems, chromosome segregation can proceed without attachment to spindle poles (Nahaboo et al., 2015, Khodjakov et al., 2004, Nicklas, 1989, Forer et al., 2015, Elting et al., 2014, Sikirzhytski et al., 2014, Vukusic et al., 2017, Laband et al., 2017); in some systems, microtubules continuously polymerize at kinetochores during anaphase A (LaFountain et al., 2004); while in others, chromosome segregation can proceed without kinetochores (Deng et al., 2009, Dumont et al., 2010); long microtubules connecting spindle poles and the center of the spindle have not been observed in metazoan spindles (Mastronarde et al., 1993, Redemann et al., 2017). Apparent discrepancies with the canonical model such as these are often attributed to the peculiarities of particular systems, an explanation contingent on the hypothesis that chromosome motion in anaphase is driven by different processes in different spindles. However, an alternative hypothesis is that the canonical model itself is not correct.

The canonical model was developed by Hans Ris (Ris, 1949) in opposition to an earlier model championed by Karl Bělař (Belar, 1929). Bělař had postulated that chromosome segregation is driven by the elongation of a “Stemmkörper” (pushing body) between chromosomes that pushed them apart. Bělař’s pushing body model is fundamentally different from the canonical model (Figure 1.1). The canonical model and the pushing body model propose different structures for the spindle: the canonical model postulates that “interpolar” microtubules extend from spindles poles to the center of the spindle (Figure 1.1A, left). In a
pushing body model, there is no such direct connection between poles and the center of the spindle; rather, this model implies the existence of “inter-chromosomal” microtubules, which form the pushing body (Figure 1.1A, right). Moreover, different populations of microtubules are proposed to drive chromosome segregation in the canonical model versus the pushing body model: the canonical model postulates that chromosome motion results from the combination of two mechanistically distinct processes: 1) chromosome-to-pole motion (i.e. anaphase A), driven by microtubules between chromosomes and poles (Figure 1.1B, upper left); and 2) chromosomes dragged apart by pole separation (i.e. anaphase B), driven by interpolar and/or astral microtubules (Figure 1.1B, lower left). In contrast, in the pushing body model, the inter-chromosomal microtubules that form the pushing body are the primary driver of chromosome segregation, even when chromosomes move closer to spindle poles (Figure 1.1B, right). For spindles simultaneously undergoing anaphase A and anaphase B, the canonical model and the pushing body model postulate different relative movements of microtubules and chromosomes: the canonical model implies that chromosomes move faster than a fiducial mark on microtubules near the middle of the spindle (Figure 1.1C, left). In contrast, in a pushing body model driven by expansion at the spindle center, chromosomes move at the same speed as a fiducial mark on inter-chromosomal microtubules (Figure 1.1C, right).
Figure 1.1 Canonical model versus pushing body model of anaphase chromosome motion. (A) Structural difference of spindle organization. (B) Different microtubules responsible for moving the chromosomes. (C) Different predictions on the relative movement of microtubules and chromosomes.
Ris originally rejected the pushing body model, and formulated the canonical model, because (Ris, 1949): 1) application of a chemical agent, chloralhydrate, to grasshopper spermatocytes caused an equal reduction of the movement of poles and chromosomes in anaphase (and hence no change in chromosome-to-pole motion), which he took to imply that anaphase A and anaphase B are mechanistically distinct processes; 2) based on light microscopy observation, he argued that the structure of the spindle is incompatible with the pushing body model (Figure 1.1A, left). However, these results are not entirely conclusive because of the complex cell biological effect of chloralhydrate (Lee et al., 1987) and the limited resolution of optical microscopy. Despite the extensive additional data that has been obtained since that time (McIntosh et al., 2012, Scholey et al., 2016, Asbury, 2017), it is still challenging to determine which model is a better representation of the behavior of spindles. For example, it has been shown that *C. elegans* mitotic spindles can segregate chromosomes after the central spindle is destroyed by laser ablation (Grill et al., 2001), which appears to argue against a pushing body model, but *C. elegans* mitotic spindles can also segregate chromosomes after the centrosomes are destroyed by laser ablation (Nahaboo et al., 2015), which appears to argue for a pushing body model. The speed of chromosome movement after laser ablation was very different from controls in both those experiments, making it difficult to determine which (if either) interpretation is correct. Therefore, we sought to further investigate the mechanism of chromosome movement in anaphase using genetic perturbation, laser ablation, quantitative light microscopy, and serial-section electron tomographic reconstruction.

1.2 Measurement of microtubule polarity
Microtubules are crystalline lattices of α- and β-tubulin heterodimers and, since the two ends of the tubulin subunits are different, microtubules are intrinsically polar polymers. The polarity of microtubules has important biological consequences. The two microtubules ends polymerize with different dynamics (Desai and Mitchison, 1997) and the intrinsic directionality of the microtubule lattice allows molecular motors to move along them in a directed fashion (Goldstein and Philp, 1999, Hirokawa, 1998). Microtubules are often organized into arrays in vivo (Li and Gundersen, 2008, Baas and Lin, 2011, Harumoto et al., 2010, Viktorinova and Dahmann, 2013, Heisler et al., 2010) and the polarity of such a collection of microtubules can be defined as the vector sum of the normalized polarity of all the constituent microtubules. A bundle of parallel microtubules all oriented in the same direction has a polarity of magnitude one, while a bundle of anti-parallel microtubules has a polarity of zero, and is thus a non-polar structure. The polarity of microtubule arrays is thought to be crucial for the organization and function of many cell types (Li and Gundersen, 2008), including neurons (Baas and Lin, 2011), epidermal cells (Harumoto et al., 2010), egg chambers in Drosophila (Viktorinova and Dahmann, 2013), and shoot apical meristem cells (Heisler et al., 2010).

The spindle, which segregates chromosomes during cell division, is a microtubule array with spatially varying polarity: Microtubules in the center of the spindle are anti-parallel, while microtubules near the spindle poles are predominantly parallel. The polarity of microtubules has long been thought to play a key role in spindle assembly (McIntosh et al., 1969, Heald et al., 1996, Sharp et al., 2000, Wittmann et al., 2001, Gadde and Heald, 2004, Walczak and Heald, 2008). Proteins that localize to the anti-parallel microtubules in the center of the spindle are believed to drive the elongation of the spindle in anaphase and to direct the location of the cleavage plan in cytokinesis (Cande and Hogan, 1989, Hogan and Cande, 1990, Brust-Mascher
et al., 2004, Glotzer, 2009, Roostalu et al., 2010, Brust-Mascher and Scholey, 2011, Fededa and Gerlich, 2012, Glotzer, 2004). However, the interplay between protein localization, microtubule polarity, and spindle behavior remain poorly understood, largely because of the difficulty measuring microtubule polarity in spindles.

Several methods have been used to study microtubule polarity. Electron microscopy can directly visualize the polarity of microtubules, but this technique requires the sample to be fixed and is highly labor intensive (Heidemann and McIntosh, 1980, McIntosh and Euteneuer, 1984). Microtubule polarity can be estimated in live cells by imaging fluorescence-labeled plus-end-tracking, but this method does not provide information on shrinking microtubules or stabilized microtubules (Stepanova et al., 2003). Laser ablation can also be used to infer microtubule polarity, but this technique is perturbative (Brugues et al., 2012). Kwan, et al. recently demonstrated that the polarity of microtubules arrays in dendrites and axons can be measured with second harmonic generation (SHG) microscopy (Kwan et al., 2008). As SHG is a noninvasive optical technique, it is a promising method for studying microtubule polarity in other structures such as spindles.

SHG is a nonlinear optical process in which highly polarizable, noncentrosymmetric materials emit photons with half the wavelength of incident light. The resulting emitted light is the coherent sum of the electromagnetic fields generated by the SHG emitters, and thus constructive and destructive interference between emitters can occur, depending on their positions and orientations. Few biological molecules produce detectable SHG signals (Freund et al., 1986, Guo et al., 1997, Campagnola et al., 2002, Chu et al., 2001, Chu et al., 2002). Individual microtubules are very weak SHG emitters, but the constructive interference between aligned microtubules in arrays produce appreciable signals that have been used to visualize
microtubules in mitotic spindles (Campagnola et al., 2002, Mohler et al., 2003, Hsieh et al., 2008a, Chu et al., 2003, Chen et al., 2006, Hsieh et al., 2008b, Olivier et al., 2010, Dombeck et al., 2003), neurons (Kwan et al., 2008, Dombeck et al., 2003, Mertz, 2004, Stoothoff et al., 2008, Kwan et al., 2009, Psilodimitrakopoulos et al., 2009, Barnes et al., 2010), cilia (Dombeck et al., 2003), axonemes (Odin et al., 2009), and astroglial filaments in spinal tissues (Fu et al., 2007).

Kwan et al. used experiments and simulations to demonstrate that the polarity of microtubules in highly ordered arrays inside neurons can be determined by measuring the ratio of forward propagating and backward propagating SHG signals (Kwan et al., 2008). Building on approaches that have been pioneered in neuroscience by Kwan et al. (Kwan et al., 2008), we sought to develop a quantitative method using non-linear optical microscopy – second harmonic generation and two-photon fluorescence – to map microtubule polarity throughout spindles.

1.3 Scope of this dissertation

This dissertation focuses on addressing two questions: 1) developing a quantitative method to measure microtubule polarity in the spindles of living cells; 2) investigating the mechanism of chromosome segregation in eukaryotes. Chapter 1 provides a brief overview on the caveat of the canonical model of chromosome segregation and the need for measuring microtubule polarity in living cells. Chapter 2 presents a method to nonperturbatively and quantitatively measure microtubule polarity throughout spindles using a combination of SHG and two-photon (TP) fluorescence. We first used numerical simulations to investigate the expected SHG signal from microtubule arrays with similar disorder to that found in spindles. These simulations, validated by comparison to electron tomography reconstructions and SHG images of spindles, led us to
propose a method to combine the SHG and TP signals to measure microtubule polarity throughout spindles. We confirmed that the polarity in meiotic spindles in Xenopus laevis egg extracts measured with this technique agrees with the polarity in those spindles previously determined by laser ablation (Brugues et al., 2012). Finally, we used this method to measure microtubule polarity throughout the first mitotic spindle in C. elegans embryos, a spindle whose polarity has not previously been studied. Chapter 3 presents the investigation on the mechanism of chromosome segregation in C. elegans mitotic spindles, human mitotic spindles, and C. elegans female meiotic spindles using genetic perturbations, laser ablation, electron tomography, and quantitative optical microscopy. We characterize the behaviors of these spindles in the structural, functional, and dynamical aspects. All of the results are inconsistent with the canonical model of chromosomes segregation. Rather, our results all conform to the predictions of the pushing body model, as proposed nearly a century ago. Furthermore, the pushing body model can also naturally account for the diverse previous findings that are not easily explained by the canonical model, suggesting that this mechanism is widely conserved.
Chapter 2

Measuring Microtubule Polarity in Spindles with Second Harmonic Generation

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2.1 Introduction

The spatial organization of microtubule polarity, and the interplay between microtubule polarity and protein localization, is thought to be crucial for spindle assembly, anaphase, and cytokinesis, but these phenomena remain poorly understood, in part due to the difficulty of measuring microtubule polarity in spindles. We develop and implement a method to nonperturbatively and quantitatively measure microtubule polarity throughout spindles using a combination of second harmonic generation and two-photon fluorescence. We validate this method using computer simulations and comparison to structural data on spindles obtained from electron tomography.
and laser ablation. This method should provide a powerful tool for studying spindle organization and function, and may be applicable for investigating microtubule polarity in other systems.

2.2 Result

Numerical simulation of SHG

SHG arises from the nonlinear polarization of molecules by light, given in general by:

\[
P = P_0 + \alpha \mathbf{E} + \beta \mathbf{E} \mathbf{E} + \gamma \mathbf{E} \mathbf{E} \mathbf{E} + \ldots
\]  

(Equation 2.1)

where the dipole moment \( P \) is the permanent dipole moment \( P_0 \) plus an induced polarization given by a Taylor expansion in powers of the electric field. \( \alpha \) is the linear polarizability, \( \beta \) and \( \gamma \) are the second- and third-order electric susceptibility tensors. \( \mathbf{E} \) is the total electric field, but will be approximated as the field from laser excitation only, because even linear polarization is very small compared to the very intense laser pulses used in biological nonlinear microscopy. For SHG from a cylindrically symmetrical source such as a microtubule, the number of independent, non-zero elements of the second order polarizability \( \beta_{ijk} \) is greatly reduced. Further assuming that the nonlinear polarization of tubulin arises from individual molecular bonds polarized along only a single axis, only two elements remain: \( \beta_{zzz} = n \) and \( \beta_{zxx} = \beta_{zyy} = \beta_{xzx} = \beta_{yyz} = \beta_{yzy} = m \) (Plotnikov et al., 2006). The ratio \( n/m \) has been measured by polarization analysis of SHG from microtubules both in axonemes (Odin et al., 2009) and primary cortical neurons (Psilodimitrakopoulos et al., 2009), and is here set at \( n/m = 4 \), intermediate between the two measurements. Considered in the coordinate frame of the microtubule shown in Figure 2.1 (from here on, given by \( x' \), \( y' \), and \( z' \)), this gives a second-order polarization of:
\( P_x^{(2)} = 2mE_x'E_z. \)
\( P_y^{(2)} = 2mE_y'E_z. \)
\( P_z^{(2)} = nE_z^2 + m(E_{x'}^2 + E_{y'}^2). \)

(Equation 2.2)

Figure 2.1 A schematic of the simulation of SHG, showing one microtubule and one possible direction of collected SHG. The amplitude and phase of the applied electric field depend on the lab coordinates \((x, y, z)\), while the polarization of each microtubule is calculated in its coordinate frame \((x', y', z')\) and transformed back to lab coordinates for summation. The phasor addition of SHG emission from different points in the sample depends on the path difference, a function of the direction of observation \((\theta^*, \phi^*)\).

We model the light as being circularly polarized, as used experimentally, and we assume that the polarization distortion introduced by the high NA objective is negligible in either the lateral direction or the axial direction. Kang et al. have reported that the maximum polarization distortion induced by a high NA (we use a NA 1.25 objective) objective is less than 1% in the lateral direction of the focal volume and ~15% in the axial direction (Kang et al., 2010). These levels of distortion occur in the very periphery of the point spread function where the nonlinear SHG excitation efficiency is extremely weak. In addition, any axial component of the electric
field will be largely perpendicular to spindle microtubules which are observed, via electron microscopy (Mastronarde et al., 1993) and birefringence measurements (Inoue, 1953), to be closely parallel to the spindle pole-to-pole axis. As a result, there is negligible SHG contribution from the axial component of the electric field.

For the sake of clarity, we treat only a single frequency component \( \omega \) of the incident laser, though in practice a pulsed laser is required to obtain sufficient peak power for nonlinear microscopy. Therefore, the electric field in lab coordinates is

\[
E_x = E_0(x, y, z) \cos(\omega t - \delta(z)) \\
E_y = E_0(x, y, z) \sin(\omega t - \delta(z)) \\
E_z = 0
\]

(Equation 2.3)

where \( E_0(x, y, z) \) is the amplitude distribution of the laser and \( \delta(z) \) is the spatial component of the phase (for example, \( E_0(x, y, z) \) is constant and \( \delta(z) = kz \) for a plane wave). For a microtubule with its axis of symmetry \( z' \) at inclination \( \theta \) from the \( z \)-axis and azimuth \( \phi \) in the \( xy \)-plane, we may arbitrarily choose the \( x' \) axis purely in the \( xy \)-plane, and transforming the electric field, obtaining

\[
E_{x'} = E_0(x, y, z) \sin(\omega t - \phi - \delta(z)) \\
E_{y'} = -E_0(x, y, z) \cos \theta \cos(\omega t - \phi - \delta(z)) \\
E_{z'} = E_0(x, y, z) \sin \theta \cos(\omega t - \phi - \delta(z))
\]

(Equation 2.4)

Substituting this into Equation 2.2 gives the polarization for the microtubule:
\[ P_{x}^{(2)} = mE_{0}^{2}(x, y, z)\sin \theta \sin(2\alpha x - 2\phi - 2\delta(z)) \]
\[ P_{y}^{(2)} = -\frac{1}{2} mE_{0}^{2}(x, y, z)\sin 2\theta \cos(2\alpha x - 2\phi - 2\delta(z)) - \frac{1}{2} mE_{0}^{2}(x, y, z)\sin 2\theta \]
\[ P_{z}^{(2)} = \frac{1}{2} (n - m)E_{0}^{2}(x, y, z)\sin^{2} \theta \cos(2\alpha x - 2\phi - 2\delta(z)) - \frac{1}{2} (n - m)E_{0}^{2}(x, y, z)\cos^{2} \theta + \frac{1}{2} (n + m)E_{0}^{2}(x, y, z) \]

(Equation 2.5)

Only the time-dependent terms in Equation 2.5 produce radiation, so the constant terms (representing optical rectification) can be dropped for consideration of SHG.

Now turning to the problem of the total SHG signal emitted from many dipoles, we take advantage of the fact that radiation is collected and observed very far from the sample compared to the size of the focal volume. Differences in position within the sample will affect the phase of the radiated electric field but neither the amplitude nor the direction of radiation for a given orientation of dipole. As a result, we can treat the sample as a single effective dipole, with the amplitude determined by the superposition of dipoles using phasor addition rather than simple addition. However, the relative phase radiated from each dipole depends on the differences in path length to a specific point of detection, so the phasor addition must be performed for each direction at which radiation could be observed. The difference in path length relative to a dipole at the origin is given by

\[ \Delta r(\theta^{*},\phi^{*}) = x\cos(\phi^{*})\sin(\theta^{*}) + y\sin(\phi^{*})\sin(\theta^{*}) + z\cos(\theta^{*}) \]

(Equation 2.6)

where x, y, and z are the coordinates of the microtubule and \( \theta^{*} \) and \( \phi^{*} \) are the inclination from z and azimuth in the xy-plane of the far-field point of observation.

The previous equations could apply to circularly polarized incident light with any amplitude or phase distribution, but following previous simulations of SHG (Moreaux et al.,
we use a 3D Gaussian approximation for the amplitude of the electric field from a tightly-focused laser, with the phase differing from a plane wave by the Gouy phase shift, representing the loss of axial momentum due to focusing (Feng and Winful, 2001). Thus, the laser electric field is given by

\[ E_0(x, y, z) = E_I \cdot \exp \left( \frac{-x^2 + y^2}{w_{xy}^2} - \frac{z^2}{w_z^2} \right) \cdot \delta(z) = k_0 z - \arctan \left( \frac{z}{z_R} \right) \]  

(Equation 2.7)

Where \( E_I \) is the peak magnitude of the electric field, \( \omega \) is the angular frequency of the laser light (850 nm in all simulations), \( k_0 = 2\pi n_0/\lambda \) is the wave vector of the fundamental in a sample with refractive index \( n_0 \) (chosen as 1.36 for cells), \( z_R = \frac{1}{2}k_0 w_{xy}^2 \) is the Rayleigh length of the focused beam, and \( w_{xy} \) and \( w_z \) are the 1/e lateral and axial beam waists. Values of \( w_{xy} \) and \( w_z \) were determined empirically by two-photon fluorescence correlation spectroscopy of Alexa 488 dye (Invitrogen, Carlsbad CA), following a published calibration protocol (Kim et al., 2007).

Simulations of SHG signal from microtubules were carried out in MATLAB (The MathWorks, Natick MA). For each simulated microtubule configuration, microtubules were broken up into individual harmonophores each representing a single ring of tubulin dimers. This length scale is a coarse-graining with minimal loss of accuracy, as a ring of tubulin dimers is 8 nm long and 25 nm in diameter, small enough compared to the SHG wavelength of 425 nm to be considered an approximate point source. This choice follows Kwan et al., who compared this approximation to simulations with a separate point harmonophore for each tubulin dimer in a ring and found no significant difference (Kwan et al., 2008). The amplitude and phase of each component of polarization was calculated in the microtubule coordinate frame according to Equation 2.5, then transformed into the lab frame. Each lab frame component of the total
polarization was summed using phasor addition for each angle collected by the condenser, and the total power radiated at each point, with the final SHG signal determined by integrating over all collected angles, which can be chosen to simulate microscope condensers of varying NA. Combining all these results, the time-averaged total collected power of SHG is

\[
SHG = \sum_{\theta^*} \sum_{\phi^*} \Delta \theta^* \cdot \Delta \phi^* \cdot \sin(\theta^*) \cdot \left( \frac{(2\omega)^4}{(4\pi\varepsilon_0 c^2)^2} \right) \left| \sum_{i} R_i(\theta, \phi) P_i^{(2)}(x, y, z, \theta, \phi, \theta^*, \phi^*) \right|^2 \cdot \sin^2(\psi)
\]

(Equation 2.8)

where \(\theta^*\) and \(\phi^*\) are the coordinates of a solid angle in the lab space, discretized over all those collected by the condenser, the sum over \(i\) is over all microtubule harmonophores, \(R_i\) is the rotation matrix from microtubule to lab coordinates for the dipole \(i\), \(P_i^{(2)}\) is the polarization of harmonophore \(i\) in the microtubule coordinate frame, and \(\psi\) is the angle between the summed lab-frame vector \(\sum R_i P_i^{(2)}\) and the direction \((\theta^*, \phi^*)\). Incorporating the phase shifts due to microtubule rotation, phase of the laser at an individual harmonophore’s position, and the path difference in the direction \((\theta^*, \phi^*)\), we have

\[
P_i^{(2)}(x, y, z, \theta, \phi, \theta^*, \phi^*) = E_i^2 \cdot \exp \left[ -\frac{2x^2 + 2y^2}{w_{xy}^2} - \frac{2z^2}{w_z^2} \right] \cdot \left\{ \begin{array}{l}
m \sin(\theta) \sin \left[ 2\omega_t - k_2 \Delta r(x, y, z, \phi^*, \theta^*) - 2\phi_i - 2k_w z_i + 2 \arctan \left( \frac{z}{z_R} \right) \right] \\
- \frac{1}{2} m \sin(2\theta) \cos \left[ 2\omega_t - k_2 \Delta r(x, y, z, \phi^*, \theta^*) - 2\phi_i - 2k_w z_i + 2 \arctan \left( \frac{z}{z_R} \right) \right] \\
\frac{1}{2} \left( n - m \right) \sin^2(\theta) \cos \left[ 2\omega_t - k_2 \Delta r(x, y, z, \phi^*, \theta^*) - 2\phi_i - 2k_w z_i + 2 \arctan \left( \frac{z}{z_R} \right) \right]
\end{array} \right\}
\]

(Equation 2.9) and
\[
R(\theta, \phi) = \begin{pmatrix}
-\sin \phi & -\cos \theta \cos \phi & \sin \theta \cos \phi \\
\cos \phi & -\cos \theta \sin \phi & \sin \theta \sin \phi \\
0 & \sin \theta_i & \cos \theta_i
\end{pmatrix}
\] (Equation 2.10)

**SHG imaging of spindles**

We imaged the first mitotic division of *C. elegans* embryos with SHG and were able to visualize the spindle (Figure 2.2). The middle of the spindle appears dark, as previously observed by Campagnola et al. (Campagnola et al., 2002). Such a dark region could result from a low density of microtubules, an anti-parallel alignment of microtubules, or a combination of both those effects, but SHG alone cannot distinguish between these possibilities. Campagnola et al. (Campagnola et al., 2002) sought to resolve this ambiguity by simultaneously imaging spindles with SHG and TP of GFP-tubulin. As the TP image provides a measure of the density of microtubules, it may be possible to use this signal to correct the SHG signal for spatial variations in microtubule density, and thus obtain a measure of microtubule polarity throughout spindles.

To apply this procedure, it is necessary to have an understanding of how the SHG signal depends on microtubule density, polarity, and other parameters.
Figure 2.2 An SHG image of the first mitotic division of a *C. elegans* embryo. The mitotic spindle is clearly visible and displays a dark region in the middle where the microtubules are predominantly anti-parallel. 10 μm scale bar.

The simplest approximation is to assume that all microtubules produce equally scattering, with no phase shift due to their relative positions, resulting in an SHG signal proportional to the square of the vector sum of all the constituent microtubules. If the microtubules only point in either the +x (ϕ=0) or –x (ϕ=π) direction, then, in this approximation, Equation 2.8 reduces to:

\[
SHG = (N_+ - N_-)^2 |P_i^{(2)}|^2 \sum_{\theta^*} \sum_{\phi^*} |\Delta \theta^* \cdot \Delta \phi^* \cdot \sin(\theta^*)| \cdot \frac{(2\omega)^4}{(4\pi\varepsilon_0 c^2)^2} \cdot \sin^2(\psi)
\]

\[
SHG \propto (N_+ - N_-)^2 \quad \text{(Equation 2.11)}
\]

\[
SHG \propto (D_{MT} \cdot P_{MT})^2
\]
where \( N_+ \) is the number of MTs with plus ends pointing in the \(+x\) direction and \( N_- \) is the number of MTs with plus ends pointing in the \(-x\) direction. \( D_{MT} = N_+ + N_- \) is the density of microtubules, and \( P_{MT} \) is the polarity of microtubules, defined by \( P_{MT} = \frac{N_+ - N_-}{N_+ + N_-} \). If this model were valid, the polarity could be measured, up to a multiplicative factor, by taking the square root of the SHG signal (which would be proportional to microtubule density times microtubule polarity) and dividing by the two-photon signal (which is proportional to the density of microtubules). Kwan et al. performed simulations of ordered arrays of microtubules and found that this simple quadratic relationship with polarity did fit the forward SHG signal quite well (Kwan et al., 2008). However, it is not clear if Equation 2.11 will be valid for more disordered arrays of microtubules as found in spindles since SHG is a coherent process, so, in general, the generated signal depends on the detailed position and orientation of microtubules, not just their average density and polarity. We performed a series of simulations of the expected SHG signal from arrays of microtubules in configurations similar to those found in the spindle to better address this issue.

**Simulation of SHG**

Previous work has relied on simulation of SHG for interpreting SHG microscopy of membrane-embedded dyes (Moreaux et al., 2000), collagen (Williams et al., 2005), and neuronal microtubule bundles (Kwan et al., 2008). We adopted similar methods to analyze spindle microtubules, with some extension to account for dipoles with any possible orientation in three dimensions (see 2.4 Methods and materials). By providing the simulation with different possibilities for the exact position of every microtubule, we can determine how the confounding variables of MT spacing and orientation effect the SHG signal and how polarity information can
be extracted from images of real spindles where the underlying microtubule configurations are unknown.

We first considered bundles of microtubules randomly positioned in the xy-plane, with their plus ends oriented parallel or antiparallel to the x-axis. This model represents an upper bound on the possible effect of positional disorder on the SHG signal. For a fixed microtubule density, our simulations of these positionally disordered arrays show an increase in SHG signal with increasing microtubule polarity, but the simulated SHG does not go to zero when the polarity is zero, even though the microtubules are antiparallel (Figure 2.3A). The non-negligible SHG signal from antiparallel microtubules shows that Equation 2.11 is not applicable for disordered arrays of microtubules.

To better understand why the simulations show deviations from Equation 2.11, we performed simulations over a broad range of polarities and microtubule densities. While the average SHG signal from parallel microtubules approaches quadratic scaling with density at high density, SHG from antiparallel microtubule arrays scales only linearly with microtubule density (Figure 2.3B). This result suggests that there is a polarity-independent component of SHG which is linear with microtubule density, in addition to the expected polarity-dependent component which scales quadratically with both density and polarity.
Figure 2.3 Computer simulation of SHG from arrays of microtubules (MTs). (A) SHG dependence on polarity, at density 50 MTs/µm². Mean SHG increases quadratically with polarity, but with large variance. (B) SHG dependence on density, on a log-log scale, for microtubules with antiparallel (green), parallel (red), and intermediate polarity (blue). Lines are simultaneous fits to the model given in Equation 2.13. (C) SHG signal from microtubules with the angular deviation from parallel drawn to a half-normal distribution, for microtubules with antiparallel (green), parallel (red), and intermediate polarity (blue). SHG decreases at very high skew but is minimally affected at the level of angular disorder found in the spindle. Error bars in (A)-(C) show standard deviation. (D) Histograms of simulated SHG at low (blue) and high (red) polarity. Distributions have positive skew but are close to Gaussian, more so at higher SHG signal.
In these simulations, the breakdown of Equation 2.11 results from the positional disorder of microtubules giving rise to local fluctuations in microtubule density and polarity, which are significant due to the random positioning of microtubules and the small effective focal volume, chosen to represent a nonlinear microscope. We were able to construct a simple theory to explain these simulations results: We assume that Equation 2.11 holds for any given configuration of microtubules on the length scale of a single microscope focal volume, a micron-scale region with a particular density and polarity. Then, the average SHG generated from a collection of focal volumes with a particular average microtubule density and polarity will be a weighted average of Equation 2.11 over the different focal volumes. Equation 2.11 shows that the SHG signal is proportional to the square of the number of excess parallel microtubules, but the average of the squared number of excess parallel microtubules is not simply the square of the average of the excess parallel microtubules. Thus, the result of averaging Equation 2.11 cannot be obtained by substituting in the average microtubule polarity and density into Equation 2.11. Rather,

$$\langle \text{SHG} \rangle \propto \langle (N_+ - N_-)^2 \rangle = \langle N_+ - N_- \rangle^2 + \text{Var}(N_+ - N_-) = (D_{MT} \cdot P_{MT})^2 + \text{Var}[D_{MT} \cdot P_{MT}]$$

(Equation 2.12)

Where the first term after the final equality results because the average of number of excessive parallel microtubules is equal to the density of microtubules times the polarity. The variance term depends on the distribution of +x and –x facing microtubules, but in general

$$\text{Var}(N_+ - N_-) = \text{Var}(N_+) + \text{Var}(N_-) - 2\text{Cov}(N_+, N_-).$$

Since antiparallel microtubules will be growing from minus ends located in different parts of the spindle, it is reasonable to assume that their distributions in a given focal will be independent and the covariance term can be dropped. Assuming each microtubule is positioned approximately independently, the distribution of
effective $N_+$ and $N_-$ in each focal volume is the sum over the effective contribution from each microtubule to the focal volume, so $\text{Var}(N_+)$ and $\text{Var}(N_-)$ will scale with $N_+$ and $N_-$, respectively. Therefore, $\text{Var}(N_+ - N_-) = D_{MT}(1+P_{MT})/2 + D_{MT}(1-P_{MT})/2 = D_{MT}$ and we arrive at a contribution to SHG signal depending linearly on the density of microtubules alone, independent of polarity. Substituting into Equation 2.12 gives

$$\langle SHG \rangle = A\langle D_{MT} \rangle^2 \langle P_{MT} \rangle^2 + B\langle D_{MT} \rangle$$

(Equation 2.13)

where A and B are proportionality constants. The solid lines in Figure 2.3B are fits of Equation 2.13, showing excellent agreement for parallel, antiparallel, and mixed-polarity microtubule arrays. As an alternate perspective on this model, consider that it in Equation 2.11, we approximated the inner sum of Equation 2.8 as $D_{MT} \cdot P_{MT}$. In Equation 2.13, we alter the approximation to $D_{MT} \cdot P_{MT} + \sqrt{D_{MT}}$, making a reasonable assumption, confirmed by our simulation results, that the deviations from perfect cancellation of antiparallel microtubules are likely to scale with the square root of microtubule density.

Microtubules in spindles are not arranged in perfectly parallel or antiparallel arrays, but have some degree of angular disorder. To test if this will be significant in interpreting SHG microscopy, we simulated microtubule bundles with varying degrees of angular disorder (Figure 2.3C). Simulated SHG does decrease with increasing angular disorder, but the effect is small except with levels of disorder far greater than found in spindles (based on electron microscopy (Mastronarde et al., 1993) and birefringence measurements (Inoue, 1953)). Thus, angular disorder of microtubules in spindles is not expected to significantly effect SHG measurements.
In all of our simulations, there is considerable variance around the average SHG signal at a given density and polarity due to the many different configurations of microtubules that are consistent with a particular average polarity and density, and the sensitive dependence of SHG on microtubule spacing. The distributions of simulated SHG are close to normal (Figure 2.3D), suggesting that if the signal from multiple locations are averaged together, the uncertainty in polarity will be close to the standard error of the mean, assuming each location is sampled from the same underlying normal distribution. In the spindle, we are primarily interested in how polarity varies along the axis between the two poles, and can thus calculate a profile of SHG at each position using an average over a line of pixels perpendicular to this pole-to-pole spindle axis.

**Analysis of spindles**

Our simulations of SHG from microtubule arrays involved a number of simplifications and approximations, so we sought to test if they were realistic enough to apply to the analysis of experimental results by comparing the predicted and measured SHG signal from microtubule arrays of known structure. To this end, we studied centrosomes from the first mitotic spindle in *C. elegans* embryos as a model system. We measured the density, orientation, and location of microtubules emanating from these centrosomes in electron tomography reconstructions (provided by Thomas Müller-Reichert (O'Toole et al., 2012)) and simulated SHG signals from microtubules with these distributions. Experimentally obtained SHG images of these centrosomes appear quite similar to the simulated versions (compare Figure 2.4A and 2.4B). The close agreement between the average measured radial SHG profile and the simulated radial profile (Figure 2.4C) demonstrates that the simulations accurately describes SHG from
microtubules in spindles. This suggests that the SHG signal generated from microtubules in spindles should be described by Equation 2.13.

![Image of SHG signals from C. elegans centrosomes](image)

**Figure 2.4** Comparison of experimental and simulated SHG from *C. elegans* centrosomes at the first mitosis. (A) An example of experimental SHG images of *C. elegans* centrosomes. (B) Simulated SHG signals based on a *C. elegans* centrosome in electron tomography reconstructions (provided by Thomas Müller-Reichert, (O'Toole et al., 2012)). Image size is 3.3 X 2.3 µm. (C) Profile of SHG signal as a function of distance from the center of imaged and simulated centrosomes. Profiles from individual imaged centrosomes are in light gray, with the mean ± standard deviation in dark gray and the scaled simulated SHG profile in red.

Equation 2.13 relates the SHG signal to the density and polarity of microtubules. TP microscopy of fluorescently labeled tubulin should produce a signal in the spindle that is proportional to the density of microtubules (after background subtraction). Using the proportionality between two photon fluorescence and microtubule density with Equation 2.13 gives a formula for polarity, $P_{MT}$:
\[ P_{MT} = \pm \sqrt{\frac{(\langle SHG \rangle - \beta \langle TP \rangle)}{\alpha \langle TP \rangle^2}} \]  
(Equation 2.14)

where \( \alpha \) and \( \beta \) are constants that depend on the statistics of microtubule spacing, the level of tubulin labeling, laser power, and other factors. Predicting \( \alpha \) and \( \beta \) is not feasible, but they can be experimentally measured in one image of a spindle if the polarity is known at two locations. The polarity in the middle of spindles is zero, so if other methods can be used to establish polarity at another location, then \( \alpha \) and \( \beta \) can be measured and the magnitude of polarity at all other locations can be determined using Equation 2.14. There is also ambiguity in the sign of the measured polarity, but we can take advantage of the a priori knowledge that the spindle is bipolar, with plus ends pointing predominantly from the poles towards the equator and a plane of reflection symmetry in the middle. Therefore, the physically correct choice is the positive square root between the left-hand pole and the center of the spindle, and the negative square root on the other side.

Next, we tested this procedure for measuring polarity in spindles by applying it to meiotic spindles in *Xenopus laevis* egg extracts, whose polarity has previously been measured with laser ablation (Brugues et al., 2012). Simultaneously acquired SHG images (Figure 2.5A) and TP images (Figure 2.5B) of these spindles, were background subtracted and analyzed in a direction parallel to the spindle axis by averaging perpendicularly in a region of interest. The lowest value of SHG intensity, SHG_{mid}, was found near the middle of the SHG profile, where the two-photon intensity, TP_{mid}, was recorded as well. As the polarity in the center of the spindle is zero, Equation 2.14 gives, \( \beta = \frac{\text{SHG}_{\text{mid}}}{\text{TP}_{\text{mid}}} \). We determined \( \alpha \) using Brugues et al.’s measurement of microtubule polarity (Brugues et al., 2012) at a distance of \( \sim 14 \) \( \mu \)m from the spindle center.
With values for $\alpha$ and $\beta$ established, we used Equation 2.14 in conjunction with the SHG and TP data to determine how polarity varies along the spindle (Figure 2.5C). The agreement of the polarity profile measured from SHG and TP with the one determined from laser ablation argues for the validity of the proposed procedure.

Having verified our analysis method, we next thought to apply this approach to study a spindle whose polarity has not been previously characterized. We studied the first mitotic division of *C. elegans*: a widely used model system for cell division. We acquired simultaneous SHG and TP images of the first mitotic spindle in a *C. elegans* embryo (Figure 2.6A and 2.6B). We determined the value of $\beta$ from the intensity of the SHG and TP signals in the middle of the spindle as described above. To determine a value for $\alpha$, we need to know the polarity at another location in the spindle. We used electron tomography reconstructions of centrosomes in these spindles (O'Toole et al., 2012) to calculate a polarity of 0.95 near the pole. We calculated the profile of polarity through the spindle using the measured values of $\alpha$ and $\beta$ (Figure 2.6C). The observed MT polarity pattern varies nearly linearly from pole to pole in metaphase. This system can record successive images in a single embryo, showing the potential to measure the temporal evolution of microtubule polarity in spindles throughout the course of cell division.
Figure 2.5 Analysis of MT polarity of a spindle assembled in Xenopus laevis egg extracts. (A) SHG image, (B) TP image of the spindle. The yellow rectangle specifies the region of interest. 10 μm scale bar. (C) Polarity pattern extracted from SHG and TP images based on our proposed model in blue (mean ± standard errors of the mean (SEM)). Polarity pattern previously measured by laser ablation in red (mean ± standard errors of the mean (SEM)) (Brugues et al., 2012).
Figure 2.6 Analysis of MT polarity in the first mitotic spindle in a *C. elegans* embryo. (A) SHG image, (B) TP image of the spindle. The yellow rectangle specifies the region of interest. 5 μm scale bar. (C) Polarity profile extracted from SHG and TP images based on the proposed model in blue (mean ± standard errors of the mean (SEM)).

This procedure for measuring microtubule polarity from SHG and TP imaging requires an independent measure of microtubule polarity in the spindle. While this is not a serious limitation for the many model systems that have already been studied with electron microscopy, if such information is not known, it is possible to use an alternative approach to measure microtubule polarity with SHG and TP. This alternative approach requires independent calibrations of the SHG and TP signals. In Equation 2.13, the constants A and B depend on the
intrinsic SHG emission of microtubules and details of the experimental setup such as the excitation laser conditions, optical layout, and the detector sensitivity. These constants can be measured for a particular optical system using a sample with a known polarity and density of microtubules. The C. elegans spindle provides one such possibility (Figure 2.4) or in vitro arrays of microtubules are another option. TP measurements can be calibrated by 1) measuring the molecular brightness of labelled proteins with fluorescence correlation spectroscopy (Kim et al., 2007) and 2) measuring the fraction of labeled tubulin molecules with western blot or other techniques. Once both SHG and TP are calibrated, then a TP image of a spindle can be converted into a measure of microtubule density, and Equation 2.13 can be used to measure microtubule polarity. We used this procedure on spindles in HeLa cells expressing GFP-tubulin, and found microtubule densities similar to that reported by electron microscopy (McIntosh and Landis, 1971) and polarity ranging continuously from near -1 to 1 at the poles (data not shown). This result demonstrated the feasibility of this alternative approach, but it is technically more challenging than the previously described method, introduces more uncertainty due to possible experimental errors during calibration, and requires a new calibration when changing the excitation laser power, the laser pulse-width, the laser wavelength, the optics configuration, or any other aspect of the experimental setup.

2.3 Discussion

In this chapter, we have shown that the combination of SHG and TP microscopy can be used to quantitatively measure the polarity of microtubules throughout spindles. The polarity of microtubules in the spindle has been proposed to play roles in spindle assembly, protein localization, force generation, and cleavage furrow positioning, but it has been challenging to rigorously test these models due to difficulties measuring microtubule polarity in situ. Our
method should greatly enhance the study of these phenomena by allowing real-time measurement of polarity under genetic, biochemical, and physical perturbation.

SHG and TP microscopy can be directly combined to measure spatial variations in microtubule polarity in spindles, up to an unknown constant of proportionality. We described two related approaches to determine this constant of proportionality and thus provide absolute measurements of microtubule polarity throughout spindles. The first approach requires independent measurements of microtubule polarity at two locations. As all spindles that have been examined have a polarity of zero in their center, it is necessary to determine polarity at only one additional location. This information can be obtained from either laser ablation (Brugues et al., 2012) or electron microscopy (O'Toole et al., 2012). If the polarity at a second location cannot be determined, then the alternative, second approach can be used, in which SHG and TP are independently calibrated.

It will be interesting to explore the extent to which our approach can be adopted to measure microtubule polarity in other systems such as neuronal processes (Baas and Lin, 2011, Sakakibara et al., 2013), epidermal cells (Harumoto et al., 2010), egg chambers in Drosophila (Viktorinova and Dahmann, 2013), and shoot apical meristem cells (Heisler et al., 2010). A promising future direction would be to extend our setup to include dual-color, two-photon imaging, allowing simultaneous visualization of microtubule polarity and protein localization. Such a system would be a powerful tool for testing the proposed role of anti-parallel microtubules in recruiting proteins that are thought to drive central spindle assembly, anaphase B and midbody formation. Another potential modification would be to measure microtubule density with quantitative polarized light microscopy (Oldenbourg, 2007) instead of fluorescence microscopy. With that modification, the system could provide a label free method of measuring...
microtubule density and polarity, which could be applied to study non-model organisms, or in medical imaging.

2.4 Methods and materials

Microscopy

Our microscope system was constructed around an inverted microscope (Eclipse Ti, Nikon), with a Ti:sapphire pulsed laser (Mai-Tai, Spectra-Physics) for excitation (850 nm wavelength, 80 MHz repetition rate, ~70 fs pulse width), a commercial scanning system (DCS-120, Becker & Hickl GmbH), and hybrid detectors (HPM-100-40, Becker & Hickl GmbH). The maximum scan rate of the DCS-120 is ~2 frames/second for a 512×512 image. The excitation laser was collimated by a telescope to avoid power loss at the XY galvanometric mirror scanner and to fill the back aperture of a water-immersion objective (CFI Apo 40x WI, NA=1.25, Nikon). A half-wave plate (AHWP05M-980) and a quarter-wave plate (AQWP05M-980) were used in combination to achieve circular polarization at the focal plane, resulting in equal second harmonic generation of all orientations of microtubules in the plane, unbiased by the global rotation of the spindle, the spatial variation in the angle of the microtubules, and the local angular disorder of microtubules. While the use of linearly polarized light would produce a greater maximum signal, the resulting measurement would depend on the orientation of microtubules relative to the plane of polarization. The rapid rotation of the *C. elegans* spindle during anaphase would make it challenging to continually change the orientation of the polarization to match the spindle axis. We calibrated the circular polarization of our system according to the procedure reported by Chen *et al.* (Chen et al., 2012), and we restricted our scanning region to within the central ~1.6% of the viewing area to minimize polarization distortions.
Two-photon fluorescence was imaged with a non-descanned detection scheme with an emission filter appropriate for either GFP-labeled tubulin in *C. elegans* (FF01-510/42-25, Semrock) or Atto565-labeled tubulin in *Xenopus laevis* egg extract (FF01-607/36-25, Semrock). Forward propagating SHG was collected through an oil immersion condenser (1.4, Nikon) with a 425/30 nm filter (FF01-425/30-25, Semrock). Both pathways contained short pass filters (FF01-650/SP-25, Semrock) to block the fundamental laser wavelength. Image analysis was performed with MATLAB, ImageJ, and Origin 8.

**Sample Preparation**

CSF-arrested egg extracts were prepared from *Xenopus laevis* female oocytes as described previously (Hannak and Heald, 2006). Briefly, demembranated sperm and calcium were added to extracts. Nuclei formed after 45–90 min. The reactions were driven into metaphase by addition of CSF-arrested extract, and spindles formed after 1 hr at 20°C.

Strain JU1212 was used for a wild type, unlabeled *C. elegans* sample. Strain AZ244 (unc-119(ed3) III; ruIs57[unc-119(+) pie-1::GFP::tubulin]) was used as a GFP-labeled tubulin *C. elegans* sample. Both strains were cultured at 24°C and fed on OP50 bacteria on nematode growth medium (NGM) plates. Gravid *C. elegans* hermaphrodites were cut in half, and the released embryos were transferred onto a 4% agarose pad between a slide and a coverslip for imaging (Walston and Hardin, 2010).
Chapter 3

New evidence for a pushing body model of chromosome segregation

3.1 Introduction

The spindle segregates chromosomes during cell division. The canonical model postulates that chromosome segregation is a sum of two mechanistically distinct processes: one driving chromosome-to-pole motion, the other driving pole-to-pole separation. This canonical model was formulated in opposition to an older pushing body model, which proposed that chromosome segregation is driven by elongation of the central spindle. Here, we study chromosome segregation in Caenorhabditis elegans mitotic and meiotic spindles, and human mitotic spindles. We found that these spindles all contain microtubules with both ends between segregating sister chromosomes. Even as chromosomes move towards spindle poles, these inter-chromosomal microtubules slide apart at the same speed as chromosomes and perturbing them causes chromosome motion to immediately cease. Our results are inconsistent with the canonical model,
and support the pushing body model, arguing that the extension of the inter-chromosomal array of microtubules is the primary driver of chromosome segregation in diverse systems.

### 3.2 Result

**Cortical pulling forces are not the primary drivers for chromosome segregation in *C. elegans* mitosis**

We started by studying anaphase in the first mitotic division of *C. elegans*. We acquired time-lapse images of spindles for the first 3 minutes of anaphase (Figure 3.1A, upper), and averaged the traces of pole motion, chromosome motion, and chromosome-to-pole motion (Figure 3.1B, upper). The chromosome-to-pole distance stayed almost constant during this time (Figure 3.1B, upper, orange) (Oegema et al., 2001): i.e. the mean speed of chromosomes, $1.1 \pm 0.02$ µm/min, was indistinguishable from the mean speed of poles, $1.1 \pm 0.05$ µm/min (Figure 3.1C, upper, n=5, p = 0.93). Ris originally proposed the canonical model because he observed that exposing grasshopper spermatocytes to chloralhydrate equally reduced the movement of poles and chromosomes in anaphase (Ris, 1949). To determine if analogous results hold in *C. elegans* mitosis, we knocked down GPR-1/2 using RNA interference (RNAi), which leads to a great reduction of pole separation in anaphase (Grill et al., 2003). Time-lapse microscopy of *gpr-1/2(RNAi)* embryos showed that while pole motion was significantly reduced, the speed and extent of chromosome motion remained very similar to that in controls (compare Figure 3.1A upper, lower). We averaged the traces of pole motion, chromosome motion, and chromosome-to-pole motion, and found that the distance between chromosomes and poles continuously decreased over the course of anaphase (Figure 3.1B, lower, orange), as the mean speed of poles, $0.45 \pm 0.04$ µm/min, was significantly less than the mean speed of chromosomes, $0.90 \pm 0.03$ µm/min.
µm/min (Figure 3.1C, lower, n=8, p < 10^-6). Since GPR-1/2 knockdown strongly affected pole motion, and only had a very minor impact on chromosome motion, this argues that pole motion is not the primary driver of chromosome motion during anaphase in *C. elegans* mitotic spindles. More fundamentally, this result is different from the result of Ris’ classic experiment (in which a perturbation caused equal impact on chromosome motion and pole motion). Therefore, using Ris’ logic would lead one to a different conclusion from him that chromosome-to-pole motion and pole separation are not independent processes. This suggests that the canonical model might not apply to *C. elegans* mitosis, since the canonical model postulates that chromosome-to-pole motion and pole separation are two independent processes.

**Figure 3.1 Inhibiting cortical pulling forces strongly perturbs spindle elongation, but not chromosome motion, in *C. elegans* mitosis.** (A) Time-lapse images of mCherry::histone and mCherry::γ-tubulins (magenta) in control (upper) and gpr-1/2 (RNAi) spindles (lower). Time zero is the onset of anaphase. (B) Averaged pole-to-spindle center distance (blue), chromosome-to-spindle center distance (green), and chromosome-to-pole distance (orange) in control (upper, n=5) and gpr-1/2 (RNAi) (lower, n=8) embryos. (C) Scatter plots of velocities of chromosomes (green) and spindle poles (blue) in
(continued) control (upper, n=5) and gpr-1/2 (RNAi) (lower, n=8) embryos during the first 3 minutes of anaphase. Error bars are SEMs (n.s., not significant).

**Chromosome segregation in *C. elegans* mitosis is primarily driven by microtubules between chromosomes**

To further investigate the mechanism of chromosome segregation in *gpr-1/2*(RNAi) embryos, we sought to test the extent to which different populations of microtubules contribute to chromosome segregation using laser ablation. We built a custom laser ablation system utilizing an ultrafast femtosecond laser, which enables sub-diffraction-limited cuts (Vogel et al., 2005) in nearly arbitrary three-dimensional patterns. These cuts are performed in a few seconds and generates minimal collateral damage outside of the ablated region. We began by cutting a rectangular plane, 8-µm in length by 6-µm in depth, perpendicular to the spindle axis between chromosomes and poles, approximately 1.3 µm away from chromosomes (Figure 3.2A, left). Cuts were performed 30-60 seconds after the onset of anaphase when chromosomes and poles move away from the spindle center at a speed of 1.2 ± 0.1 µm/min and 0.5 ± 0.1 µm/min, respectively, so that chromosomes move toward to poles at a speed of 0.7 ± 0.1 µm/min. Thus, at this time, the chromosome-to-pole distance shrinks at a faster rate than the poles separate. Following the cut, the spindle pole detached and immediately moved away from the ablation site. After ~10 seconds, the spindle pole began moving back to its original position, presumably because the severed microtubules recovered. The motion of the pole after the cut demonstrates that microtubules were successfully ablated, but traces of the chromosome-to-chromosome distance showed no impact of this cut on chromosome motion (Figure 3.2A, right): the speed of chromosome separation after cutting these microtubules (2.5 ± 0.2 µm/min, n=6) was statistically
indistinguishable from the controls (2.4 ± 0.2 µm/min, n=6, p = 0.84). We next used laser ablation to cut a similar rectangular region between separating chromosomes (Figure 3.2B). The ablation led to an immediate cessation of chromosome segregation, reducing its speed to 0.1 ± 0.2 µm/min (n=7; indistinguishable from zero, p = 0.73). After approximately ~20 seconds, chromosome segregation resumed with a speed similar to controls, presumably due to the spontaneous repair of the damaged population of microtubules (Figure 3.2B, right). Thus, ablating microtubules between chromosomes completely stopped chromosome motion (and ablating microtubules between chromosomes and poles had no measurable impact) while, at the time of these cuts, chromosome-to-pole distance was decreasing and spindle poles were separating (see above). This result is difficult to reconcile with the canonical model (which postulates the importance of microtubules between chromosomes and poles for moving chromosomes closer to poles), and is consistent with the pushing body model, which postulates that microtubules between chromosomes are the primary driver of chromosome motion in anaphase (Figure 1.1B).
Figure 3.2 The functional, structural, and dynamical characterization of inter-chromosomal microtubules. (A) Time-lapse images of GFP::tubulin (green) and mCherry::histone (magenta) in gpr-1/2 (RNAi) C. elegans spindles (left) when microtubules are cut between chromosomes and poles. Time zero is the onset of anaphase. Dotted lines indicate the timing and location of laser ablation. Corresponding plots (right) of chromosome distance as a function of time, with an uncut spindle (grey) for reference. Example plots (right, inserts) of the change in chromosome distance after the cut from multiple spindles, aligned relative to the timing of the cut, with uncut spindles (gray) for reference. (B) Time-lapse images of GFP::tubulin (green) and mCherry::histone (magenta) in gpr-1/2 (RNAi) C. elegans spindles (left) when microtubules are cut between chromosomes. Description as in (A). (C) Electron tomographic reconstruction showing all traced microtubules (green) overlaid on a tomogram; some microtubules have both ends between chromosomes, with neither end (red) or either one (orange) contacting chromosomes; other microtubules have only one end between chromosomes (yellow, all such microtubules in a half-
The anaphase *C. elegans* mitotic spindle contains inter-chromosomal microtubules moving with chromosomes

Since our laser ablation results support a pushing body model for anaphase chromosome motion in *C. elegans* mitosis, in which microtubules between chromosomes are the primary driver of chromosome motion, we next sought to determine if the ultrastructure of these spindles is also consistent with a pushing body model (Figure 1.1A, right). To this end, we used serial-section electron tomography to study the organization of microtubules in anaphase *C. elegans* mitotic spindles (Figure 3.2C, left). Our reconstructions revealed the presence of inter-chromosomal microtubules: some of which had two free ends between chromosomes (Figure 3.2C, red), others of which had one end near the center of the spindle with the other end contacting chromosomes (Figure 3.2C, orange). We propose that these inter-chromosomal microtubules are the pushing body, which drives chromosome motion. Forces generated by inter-chromosomal microtubules could be exerted on chromosomes through direct contact in the rear (Figure 3.2C, orange microtubules). Alternatively, the connection to chromosomes could be indirectly, by microtubules with one end extending from the inter-chromosomal region passing around chromosomes to contact kinetochore microtubules (Figure 3.2C, yellow), but only such
microtubules contacting kinetochore microtubules very near chromosomes could contribute, since cutting microtubules ~1.5 microns away from chromosomes (between chromosomes and poles) had no impact on chromosome motion (see above). Therefore, electron tomography demonstrates that inter-chromosomal microtubules exist in anaphase *C. elegans* mitotic spindles, consistent with expectations of the pushing body model for the structure of the spindle (Figure 1.1A).

We next sought to test whether the relative motion of microtubules and chromosomes in anaphase *C. elegans* mitotic spindles was consistent with the canonical model or the pushing body model (Figure 1.1C). We investigated *C. elegans* mitotic spindles in *gpr-1/2*(RNAi) embryos 40-60 seconds after the onset of anaphase, when the chromosome-to-pole speed was 0.5 ± 0.07 µm/min (n=7) and the pole-to-spindle center speed was 0.3 ± 0.05 µm/min (n=7). Thus, at this time, the chromosome-to-pole distance shrank at a faster rate than the poles separate. We photobleached GFP-labeled tubulin to create two parallel fiducial marks on microtubules near the spindle center (Figure 3.2D, left). Kymographs showed that these fiducial marks moved apart at a speed similar to the speed of chromosomes separation (Figure 3.2D, right). The movement of the photobleached marks demonstrates that that microtubules between chromosomes slide apart in anaphase. To further quantify this motion, we took line-profiles of the bleached region (Figure 3.2E), tracked their motion and the motion of chromosomes (see methods), and found that the inter-chromosomal microtubules move at a speed of 0.8 ± 0.09 µm/min (n=7), which is indistinguishable from the speed of chromosome movement (0.8 ± 0.04 µm/min, n=7, p = 0.53), and substantially greater than the speed of pole movement (0.3 ± 0.05 µm/min, n=7, p < 0.001). Thus, inter-chromosomal microtubules slide apart at the same speed as chromosomes move apart, while, at the same time, chromosome-to-pole distance was decreasing and spindle poles
were separating (see above). This result is difficult to reconcile with the canonical model (which implies that chromosomes should move faster than a fiducial mark on the microtubules between them) and is consistent with a pushing body model (which implies that chromosomes should move at the same speed as a fiducial mark on the microtubules between them) (Figure 1.1C). In summary, in *C. elegans* mitosis, the structure of the spindle, the microtubules driving chromosome segregation, and the relative motion of microtubules and chromosomes all support a pushing body model for anaphase chromosome motion over the canonical model (Figure 1.1). Therefore, we conclude that inter-chromosomal microtubules form a pushing body that drives anaphase chromosome motion in *C. elegans* mitotic spindles.

**Inter-chromosomal microtubules slide, polymerize, and maintain a fixed antiparallel overlap**

We next studied the behavior of the inter-chromosomal microtubules responsible for chromosome motion in anaphase *C. elegans* mitotic spindles. If the sliding of inter-chromosomal microtubules occurred without any polymerization, then it would be expected that the degree of anti-parallel microtubule overlap would decrease with time. Thus, we used a combination of two-photon microscopy (Figure 3.3A, upper) and second harmonic generation (SHG) microscopy (Figure 3.3A, middle), to measure microtubule polarity throughout the spindle (Yu et al., 2014). The extent of microtubule anti-parallel overlap did not change as the spindle elongated or upon knockdown of GPR-1/2 by RNAi (Figure 3.3A, lower), arguing that the polymerization of inter-chromosomal microtubules continually balances their sliding to maintain a constant anti-parallel overlap, and that this balance is internally established and unaffected by cortical pulling forces. To further investigate the polymerization of inter-chromosomal
microtubules, we imaged GFP-labeled plus-end-binding like protein (EBP-2) (Figure 3.3B, upper), which associates with growing microtubule plus-ends (Tirnauer and Bierer, 2000, Gusnowski and Srayko, 2011, Tegha-Dunghu et al., 2014). We found that EBP-2 is enriched in the anti-parallel microtubule region during anaphase as predicted, supporting the inference of continual polymerization of inter-chromosomal microtubules at that location (Figure 3.3B, lower).

Next, we measured the turnover of inter-chromosomal microtubules using fluorescence recovery after photobleaching of GFP-labeled tubulin (Figure 3.3C, left). The fluorescence intensity of GFP-tubulin in the center of the inter-chromosomal region, i.e. in the anti-parallel region, remains constant as the spindle elongates (Figure 3.3C, right, gray) and rapidly recovers when photobleached (Figure 3.3C, right), with a time scale of 12 ± 2 seconds (n=5). In contrast, the fluorescence intensity of GFP-tubulin in the inter-chromosomal region adjacent to chromosomes continually decreases over time (Figure 3.3D, gray) and does not appreciably recover when bleached (Figure 3.3D, brown). These results suggest that the polymerization of inter-chromosomal microtubules is largely confined to the central, anti-parallel region, with continual disassembly occurring outside that region. Taken together, our data in C. elegans mitosis support a pushing body model of chromosome segregation (Figure 1.1) in which inter-chromosomal microtubules assemble in anaphase, elongate from their plus ends while sliding towards their minus ends in a coupled manner, and push chromosomes apart (Figure 3.3E).
Figure 3.3 Inter-chromosomal microtubules slide, polymerize, and maintain a fixed antiparallel overlap. (A) Simultaneous two-photon fluorescence (upper), second-harmonic-generation (center) images, and calculated polarity profiles in control and gpr-1/2 (RNAi) spindles in early and late anaphase (lower). 5-μm scale bar. Error bars are SEMs. (B) Image of EBP-2::GFP in a spindle (upper) and corresponding profile obtained from averaging over an area equivalent to the annotated region in multiple spindles (lower). 5-μm scale bar. Error bars are SEMs. (C) A kymograph from fluorescence recovery after photobleaching of GFP-labeled tubulin in the center (left). Recovery profiles (right) in the bleached regions (brown) plotted along-side corresponding control regions (grey). (D) A kymograph of fluorescence recovery after photobleaching of GFP-labeled tubulin adjacent to chromosomes in the inter-chromosomal region (left). Description as in (C). (E) Model of anaphase chromosome motion in C. elegans mitosis: inter-chromosomal microtubules (dark green) assemble in anaphase, elongate from their plus ends (light green) while sliding towards their minus ends in a coupled manner (black), and pushing chromosomes apart.
Electron tomography reveals the presence of inter-chromosomal microtubules in human mitotic spindles

Having found strong evidence for a pushing body model of chromosome segregation in *C. elegans* mitosis, we next explored if a similar mechanism operates in other spindles. We thus sought to test if the canonical model or pushing body model better accounts for chromosome motion in anaphase in human mitotic spindles by investigating the predictions of these two models (Figure 1.1). We first studied the structure of human mitotic spindles in anaphase using large-scale electron tomographic reconstructions (Figure 3.4A, green). This work revealed the presence of inter-chromosomal microtubules: some of which had two free ends between chromosomes (Figure 3.4A, red), others of which had one end near the center of the spindle with the other end contacting chromosomes (Figure 3.4A, orange). Other microtubules extended from the inter-chromosomal region passed chromosomes, reaching a micron or so beyond them (Figure 3.4A, yellow). We found no microtubules, which extended all the way from the pole to the region between chromosomes, and no microtubules, which passed across the entire inter-chromosomal region, directly bridging microtubules between the two poles. The three classes of microtubules we observed are often tightly associated into bundles, which appear to connect inter-chromosomal microtubules to kinetochore-microtubules, near chromosomes (Figure 3.4B). In summary, electron tomographic reconstruction reveals the presence of inter-chromosomal microtubules in human mitotic cells in anaphase. This structure is highly reminiscent of what we observed in *C. elegans* mitotic spindles (Figure 3.2C) and consistent with the pushing body model (Figure 1.1A, right).
Chromosome segregation in human mitotic spindles is primarily driven by microtubules between chromosomes

Having found that inter-chromosomal microtubules are present in anaphase human mitotic spindles (Figure 1.1A), we next sought to determine which microtubules are primarily responsible for anaphase chromosome motion in these spindles (Figure 1.1B). We focused on studying chromosome motion 30-60 seconds after the onset of anaphase. Tracking of the motions of kinetochores and spindle poles reveals that, at this time, chromosomes move away from the center of the spindle with a speed of 2.7 ± 0.2 μm/min, while poles move away from the spindle center at a speed of 1.5 ± 0.1 μm/min, so chromosomes move toward poles at a speed of 1.2 ± 0.2 μm/min (Figure 3.4C). Thus, our experiments probe a time window in which the speed of chromosome-to-pole motion is similar to the speed of pole motion. We began by cutting a rectangular plane, 12-μm in length by 6-μm in depth, perpendicular to the spindle axis between chromosomes and poles, and approximately 2.4-μm away from chromosomes (Figure 3.4D, left). Again, we found that the detached spindle pole immediately moved away from the ablation site, and moved back toward its initial position after approximately 10 seconds, presumably because the severed microtubules recovered. The motion of the pole after the cut demonstrates that microtubules were successfully ablated. Traces of the chromosome-to-chromosome distance showed a reduction after the cut in the speed of chromosome motion, from 3.0 ± 0.2 μm/min (n=11) to 1.7 ± 0.2 μm/min (n=11; Figure 3.4D, right). We next used laser ablation to cut a similar rectangular region between separating chromosomes (Figure 3.4E; left). This ablation led to an immediate cessation of chromosome segregation, reducing its speed to 0.1 ± 0.3 μm/min (n=6; indistinguishable from zero, p = 0.83). After approximately ~20 seconds, chromosome segregation resumed with a speed similar to controls, presumably due to the spontaneous repair
of the damaged population of microtubules (Figure 3.4E, right). These results argue that microtubules between chromosomes are the primary driver of anaphase chromosome segregation in human mitotic cells, with some contribution from microtubules between chromosomes and poles. At the time of these cuts, the speed of chromosome-to-pole motion was similar to the speed of pole motion (see above). Our observation of complete cessation of chromosome segregation after cutting microtubules between separating chromosomes under these circumstances is not consistent with the canonical model, which predicts that the portion of chromosome segregation driven by chromosome-to-pole motion would precede unimpeded. However, this result is consistent with the pushing body model, which postulates that anaphase chromosome motion is primarily driven by inter-chromosomal microtubules (Figure 1.1B).

Pushing forces from inter-chromosomal microtubules might be transmitted to chromosomes directly, by microtubules contacting the rear of chromosomes (Figure 3.4A, 3.4B, red and orange), or indirectly, by microtubules which link inter-chromosomal microtubules to kinetochore microtubules (Figure 3.4A, 3.4B, yellow). The observation of a decrease in the speed of chromosome motion after cutting microtubules ~2.4 microns away from chromosomes, between chromosomes and poles, argues for a contribution from the microtubules which extend from the center of the spindle and contact kinetochore fibers near chromosomes.
Figure 3.4 Inter-chromosomal microtubules drive anaphase chromosome motion in human mitotic cells. (A) Electron tomographic reconstruction of microtubules in human mitotic spindles, showing all microtubules (green) overlaid on the tomogram. Some microtubules have both ends between chromosomes, with neither (red) or either one (orange) of their ends contacting chromosomes; other microtubules have only one end between chromosomes (yellow, all such microtubules in a half-spindle). (B) Electron tomographic reconstruction of selective microtubule bundles consisting of the three classes of microtubules (red, orange, and yellow) in (A), kinetochore microtubules (gray), and chromosomes (translucent). (C) Change of pole-to-spindle center (blue), chromosome-to-spindle center (green), and chromosome-to-pole (orange) distances during chromosome segregation. The slopes show the averaged velocity of above three quantities during the probing window (purple), in which the experiments and observations were conducted. (D) Time-lapse images of GFP::CENP-A (green) and mCherry::tubulin (magenta) human mitotic spindles with laser ablation of microtubules between chromosomes and poles. Time zero is the onset of anaphase. Dotted lines indicate the timing and location of laser ablation. Inter-
(continued) chromosome distance versus time with laser ablation of microtubules between chromosomes and poles (right) with uncut spindles (gray) for reference. (E) Time-lapse images of GFP::CENP-A (green) and mCherry::tubulin (magenta) human mitotic spindles with laser ablation of microtubules between chromosomes (left). Description as in (D). (F) Time-lapse images of GFP::CENP-A (green) and mEOS3.2::tubulin (green before photoconversion; magenta after photoconversion) human mitotic spindles with photoconverted microtubules between chromosomes. Time zero is the onset of photoconversion. (G) A kymograph of the spindle in (F), with white dashed lines illustrating that the photoconverted microtubules and chromosomes move apart at the same speed, and both are faster than poles. (H) Line-profiles of photoconverted mEOS3.2-tubulin between chromosomes after photoconversion (left) from the kymograph in (G), with arrows to indicate the split of the photoconverted region. Bar plot of velocities of microtubules (magenta), chromosomes (green) and spindle poles (blue) in human mitotic spindles 30-60 seconds after the onset of anaphase (right). Error bars are SEMs (n.s., not significant).

**Inter-chromosomal microtubules slide apart at the speed of chromosome motion in anaphase human mitotic spindles**

Our findings that inter-chromosomal microtubules are present and are the primary driver of anaphase chromosome motion argues in favor of a pushing body model, and against the canonical model, for anaphase chromosome motion in human mitotic spindles. To further test the validity of these two models, we next sought to measure the relative motion of microtubules and chromosomes in these spindles. We again focused on the period 30-60 seconds after the onset of anaphase, when the speed of chromosome-to-pole motion is similar to the speed of pole motion (see above). We generated a stable cell line expressing mEOS3.2::tubulin and GFP::CENP-A, which allowed us to photoconvert a subset of labeled microtubules between chromosomes while simultaneously tracking the motion of kinetochores and poles (Figure 3.4F). The single line of photoconverted tubulin split apart over time, demonstrating the sliding of inter-chromosomal microtubules (Figures 3.4F, magenta). Kymographs revealed that the speed of inter-
chromosomal sliding was similar to the speed of chromosome motion, and substantially faster than pole motion (Figure 3.4G). To better measure the speed of inter-chromosomal microtubules, we took line profiles and tracked the motion of photoactivated regions (Figure 3.4H), in addition to chromosomes and poles (see methods). We found that the photoconverted microtubules slide at a speed of 2.8 ± 0.1 µm/min (n=46), which is indistinguishable from the speed of chromosome motion, 2.7 ± 0.1 µm/min, (n=46, p = 0.85) and substantially greater than the speed of pole motion, 1.5 ± 0.1 µm/min (n=46, p<10^-11) (Figure 3.4H, right). At the time of these photoactivation experiments, the speed of chromosome-to-pole motion was similar to the speed of pole motion (see above). Under this condition, our observation that microtubules between chromosomes slide apart at the same speed as chromosomes segregate is not consistent with the canonical model, which predicts that chromosome-to-pole motion should lead to the speed of chromosomes being faster than the speed of microtubule sliding (Figure 1.1C, left). This result is consistent with a pushing body model which predicts that anaphase chromosome motion should occur at the same speed as the sliding of inter-chromosomal microtubules (Figure 1.1C, right), as observed. In summary, in mitotic human cells, the structure of the spindle, the microtubules driving chromosome segregation, and the relative motion of microtubules and chromosomes all support a pushing body model for anaphase chromosome motion over the canonical model (Figure 1.1). Therefore, we conclude that inter-chromosomal microtubules form a pushing body driving anaphase chromosome motion in human mitotic spindles.

**Inter-chromosomal microtubules form a pushing body that drives chromosome segregation in C. elegans meiosis**
Having found evidence for a pushing body model of anaphase chromosome segregation in both human and *C. elegans* mitotic spindles, we next sought to investigate a spindle with very different morphology and dynamics: acentrosomal *C. elegans* female meiotic spindles, whose mechanism of chromosome segregation remains controversial (Dumont et al., 2010, Laband et al., 2017, McNally et al., 2016, Muscat et al., 2015). We first generated electron tomographic reconstructions of all microtubules in *C. elegans* female meiotic spindles at various stages of anaphase (Figure 3.5A, left). Nearly all microtubules in the spindle lie between chromosomes at these stages of anaphase (Figure 3.5A, left). The organization of inter-chromosomal microtubules in *C. elegans* female meiotic spindles is highly reminiscent of those found in *C. elegans* mitotic spindles: some microtubules have both their ends between the chromosomes, without touching the chromosomes (Figure 3.5A, center), while some microtubules make end-on contact with chromosomes, with their other end terminating between the chromosomes (Figure 3.5A, right). Our observation of numerous inter-chromosomal microtubules in anaphase of *C. elegans* female meiotic spindles is consistent with previous partial electron tomography reconstructions (Laband et al., 2017), and with expectations of a pushing body model (Figure 1.1).
Figure 3.5 (continued) Inter-chromosomal microtubules drive anaphase chromosome motion in *C. elegans* female meiotic spindles. (A) Electron tomographic reconstructions of microtubules in *C. elegans* meiotic spindles with inter-chromosomal distances (d) of 2 µm (upper row), 2.5 µm (middle row), and 4 µm (lower row), showing all microtubules (green) overlaid on tomograms. Some microtubules have both ends between chromosomes, with neither (red) or either one (orange) of their ends contacting chromosomes. (B) Time-lapse images of a GFP::tubulin (green) and mCherry::histone (magenta) *C. elegans* meiotic spindle, with the dotted line indicating the timing and location of laser ablation. (C) Inter-chromosomal distance versus time for spindles cut between chromosomes (orange), with uncut spindles (grey) for reference. (D) Images of a GFP::tubulin (green) and mCherry::γ-tubulin (magenta) spindle before and after photonbleaching (left), showing the position of the photobleached region (left). A kymograph of a spindle, with white dashed lines illustrating that the bleached regions and chromosomes move apart at the same speed; time zero is the onset of photobleaching (right). (E) Line-profiles of GFP-tubulin over the region corresponding to the kymograph in (D), with an arrow to indicate the shift of the bleached center.

To test the contribution of these inter-chromosomal microtubules to chromosome segregation, we used laser ablation to cut them during anaphase (Figure 3.5B), and found that, as in mitotic spindles, this damage caused an immediate cessation of chromosome segregation, with their speed reducing to 0.01 ± 0.01 µm/min (n=6; indistinguishable from zero, p=0.36), from a speed of 0.7 ± 0.1 µm/min (n=5) in controls (Figure 3.5C). Similar to mitotic spindles, chromosome motion resumed at a speed similar to controls after approximately 20 seconds, presumably due to the spontaneous repair of the damaged population of microtubules. Thus, this result demonstrates that inter-chromosomal microtubules are the primary driver of anaphase chromosome motion in *C. elegans* female meiotic spindles at the stages we studied. A previous laser ablation study also found that cutting inter-chromosomal microtubules halted chromosome motion in *C. elegans* meiotic spindles (Laband et al., 2017), though, in that study severed
spindles never recovered, likely due to severe collateral damage caused by using a UV laser for ablation.

We next further tested the applicability of a pushing body model for anaphase chromosome motion in *C. elegans* meiotic spindles by investigating the relative motion of inter-chromosomal microtubules and chromosomes (Figure 1.1C). We photobleached GFP-labeled tubulin to create a fiducial mark on inter-chromosomal microtubules adjacent to a set of sister chromosomes during anaphase, and found that they moved at a similar speed as chromosomes moved (Figure 3.5D). To better quantify this motion, we made line-profiles of the photobleached regions and tracked their motion, along with the motion of chromosomes (Figure 3.5E). Averaging measurements over multiple spindles gave a mean speed of microtubule sliding away from the spindle center of 0.5 ± 0.2 µm/min (n=6), indistinguishable from the mean speed of chromosome movement, 0.3 ± 0.1 µm/min (n=6, p=0.3). This result is consistent with a pushing body model, which predicts that anaphase chromosome motion should occur at the same speed as the sliding of inter-chromosomal microtubules (Figure 1.1C, right), as observed. In summary, in *C. elegans* female meiosis, the ultrastructure of the spindle, the microtubules driving chromosome segregation, and the relative motion of microtubules and chromosomes all support a pushing body model for anaphase chromosome motion (Figure 1.1). Therefore, we conclude that inter-chromosomal microtubules form a pushing body that drives anaphase chromosome motion in *C. elegans* female meiotic spindles.

### 3.3 Discussion

The canonical model of chromosome segregation was originally developed by Hans Ris (Ris, 1949), in opposition to the pushing body model championed by Karl Bělař (Belar, 1929). These
models are distinct conceptions of the mechanism of chromosome segregation that make distinct predictions (Figure 1.1). Most fundamentally, the canonical model postulates that chromosome motion results from the combination of two mechanistically distinct processes: chromosome-to-pole motion (i.e. anaphase A), driven by microtubules between chromosomes and poles, and chromosomes dragged apart by pole separation (i.e. anaphase B), driven by interpolar and/or astral microtubules. In contrast, in a pushing body model, all anaphase chromosome motion is primarily driven by elongation of the inter-chromosomal microtubule array (irrespective of if the motion results in chromosomes moving closer to poles or not). Here we have investigated the mechanism of chromosome segregation in *C. elegans* mitotic spindles, human mitotic spindles, and *C. elegans* female meiotic spindles using genetic perturbations, laser ablation, quantitative optical microscopy, and electron tomography. Structurally, we found that all of these spindles contain inter-chromosomal microtubules in anaphase. Functionally, damaging these inter-chromosomal microtubules with laser ablation caused immediate cessation of chromosome segregation. In contrast, interfering with microtubules between chromosomes and poles had no or only a minimal influence on chromosome motion in *C. elegans* and human mitotic spindles, and such microtubules are almost entirely absent in *C. elegans* female meiotic spindles at the stages we studied. Dynamically, inter-chromosomal microtubules slide apart at the same speed as chromosomes separate. All of these structural, functional, and dynamical characterizations of the spindle’s behaviors are inconsistent with the canonical model of chromosomes segregations (Figure 1.1, left). Rather, these results all conform to the predictions of the pushing body model (Figure 1.1, right). Therefore, this evidence collectively suggests that, even though these three spindles have different morphologies and dynamics, in all of them, inter-chromosomal
microtubules act as a pushing body that is the primary driver of anaphase chromosome motion (Figure 3.6).
Figure 3.6 Model of chromosome segregation. (A) Despite their different structures, inter-chromosomal microtubules serve as the primary driver of chromosome segregation in *C. elegans* mitotic and human mitotic spindles, and *C. elegans* female meiotic spindles, suggesting that this mechanism is widely conserved.

Past work has demonstrated that chromosome segregation can proceed without the direct attachment of chromosomes to spindle poles (Elting et al., 2014, Forer et al., 2015, Khodjakov et al., 2004, Laband et al., 2017, Nahaboo et al., 2015, Nicklas, 1989, Sikirzytski et al., 2014). It has recently been shown that a single chromosome oriented away from the spindle can segregate without any connection, direct or indirect, to one pole (Vukusic et al., 2017). With the exception of a recent study on *C. elegans* meiotic spindles in oocytes (Laband et al., 2017), this work has still been interpreted in a frame work in which chromosome motion is the sum of two mechanistically distinct sets of processes: one driving chromosome-to-pole motion, the other resulting from chromosomes being dragged apart by pole-to-pole separation. Our work expands on these previous studies by providing new information on structure, function, and dynamics of the spindle behaviors during chromosome segregation. Our results indicate that pushing from inter-chromosomal microtubules is the primary driver of chromosome motion in anaphase, even when chromosomes move closer to spine pole. This is inconsistent with the canonical model,
that postulates that chromosome segregation is the sum of two mechanistically distinct processes associated with anaphase A and anaphase B, and instead supports a model in which inter-chromosomal microtubules form a pushing body which is the single dominant mechanism of chromosome segregation (Figure 1.1).

In a pushing body model, the speed of chromosome motion in anaphase is primarily set by the speed of extension of the inter-chromosomal array of microtubules. The validity of a pushing body model is thus further supported by a classic experiment demonstrating that the speed of chromosome separation is only changed upon locally heating the inter-chromosomal region, not the region between chromosomes and poles (Nicklas, 1979). The inter-chromosomal microtubules that drive chromosome segregation are also expected to resist forces that attempt to move chromosomes faster than their rate of extension, acting as a break. The simultaneous function of inter-chromosomal microtubules in driving chromosome segregation and resisting cortical pulling forces naturally resolves the apparent paradox that chromosomes move apart in anaphase of *C. elegans* mitotic spindles if either the central spindle (Grill et al., 2001) or the spindle pole (Nahaboo et al., 2015) is destroyed. If the central spindle is destroyed in anaphase, then unbalanced cortical pulling forces can lead to rapid separation of the two half spindles, even though such cortical pulling forces only minimally contribute to normal chromosome segregation (Figure 3.1). If the spindle poles are destroyed, chromosome segregation can continue almost unaffected as it is driven primarily by the inter-chromosomal microtubules. It will be interesting for future work to investigate if a pushing body model can also explain anaphase chromosome motion in other spindles in which microtubules between chromosomes act as a brake which resist cortical pulling forces (Aist and Berns, 1981, Aist and Bayles, 1991, Kronebusch and Borisy, 1981, Aist et al., 1993).
We speculate that the inter-chromosomal microtubules that form the pushing body that drives chromosome segregation may be the so-called “overlap”, “interzone”, or “midzone” microtubules which were previously proposed to drive pole separation (McIntosh et al., 2012, Scholey et al., 2016), and that they may become the “central spindle” at later stages of anaphase, and the “midbody” during cytokinesis (Glotzer, 2009, Uehara and Goshima, 2010, Maton et al., 2015). The validity of this hypothesis, and the applicability of the pushing body model for chromosome segregation in other spindles, is an exciting topic for future work. The same experimental approaches we apply in this study should be readily applicable to other spindles. It will also be important to further investigate whether inter-chromosomal microtubules move chromosomes by directly pushing on them, by indirectly pushing on kinetochore microtubules (Vukusic et al., 2017), or by a combination of both. Whether similar mechanisms drive the initial separation of chromosomes at the onset of anaphase is an interesting open question.

3.4 Materials and methods

C. elegans strains

Strain SA250 (tjIs54 [pie-1p::GFP::tbb-2 + pie-1p::2xmCherry::tbg-1 + unc-119(+)]; tjIs57 [pie-1p::mCherry::his-48 + unc-119(+)]), and a strain expressing GFP::tubulin and mCherry::histone (a gift from Marie Delattre lab) were used for experiments of fluorescence imaging, laser ablation, and fluorescence recovery after photobleaching. Strain AZ244 (unc-119(ed3) III; ru1s57[unc-119(+)] pie-1::GFP::tubulin]) was used for second-harmonic-generation microscopy and two-photon fluorescence microscopy imaging. Strain MAS37 (unc-119(ed3) III; abcIs3 [pie-1p::ebp-2::GFP + unc-119(+)]) was used for measuring the polymerization profile of microtubules. Wildtype (N2) C. elegans embryos and oocytes were used for the preparation of
electron tomography. All strains were cultured at 24°C and fed on OP50 bacteria on nematode growth medium plates.

**Human cell lines and culture**

Human Bone Osteosarcoma Epithelial (U2OS) cells were engineered to stably express fluorescence-labeled proteins by retroviral transfections. Three stable fluorescence U2OS lines were used: one expresses GFP::CENP-A, and mCherry::tubulin; another expresses GFP::centrin, GFP::CENP-A, and mCherry::tubulin; the other expresses GFP::centrin, GFP::CENP-A, and mEOS3.2::tubulin. U2OS cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), and 50 IU/ml penicillin and 50 μg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere with 5% CO2.

**C. elegans imaging preparation and RNA interference**

For imaging of mitotic spindles, gravid *C. elegans* hermaphrodites were cut in half, and the released embryos were transferred onto a 4% agarose pad between a slide and a coverslip (Walston and Hardin, 2010). Meiotic spindles in oocytes were observed in uterus, as adult hermaphrodites were mounted between a coverslip and a thin 4% agarose pad on a slide. Polystyrene microspheres (Microspheres 0.10 μm, Polysciences, Inc.) in solution were added to help immobilize worms.

RNA interference (RNAi) was carried out following the RNAi feeding protocol from the Ahringer lab (Kamath et al., 2001). Plasmid pMD082 (a gift from Marie Delattre lab) containing the sequence of gpr-1/2 was cloned into the L4440 plasmid, which was transformed into HT115 bacteria. L2 hermaphrodites were transferred to gpr-1/2 (RNAi) plates and fed on the RNAi bacterial lawn for 36–48 h at 24°C.

**Human cell imaging preparation**
In preparation for imaging, cells were grown on a 25-mm diameter, #1.5-thickness, round coverglass coated with poly-D-lysine (GG-25-1.5-pdl, neuVitro) to 80~90% confluency. The cells were incubated in imaging media, which is FluoroBrite™ DMEM (Gibco) supplemented with 4mM L-glutamine (Gibco) and 10mM HEPES, for 15~30 minutes before imaging. The coverglass was mounted on a custom-built temperature controlled microscope chamber at 37°C, while covered with 1.5 ml of imaging media and 2 ml of white mineral oil (VWR). An objective heater (Bioptech) was used to maintain the objective at 37°C.

**Sample preparation for electron tomography**

Wild-type (N2) *C. elegans* embryos and oocytes collected in cellulose capillary tubes (Pelletier et al., 2006) and HeLa Kyoto cells grown on sapphire discs (Guizetti et al., 2011) were high-pressure frozen as described using an EM PACT2+RTS high-pressure freezer (Leica Microsystems, Vienna, Austria)(Muller-Reichert et al., 2007). Freeze substitution was performed over 2-3 d at -90 °C in anhydrous acetone containing 1 % OsO4 and 0.1 % uranyl acetate. Samples were embedded in Epon/Araldite and polymerized for 2-3 d at 60 °C. Serial semi-thick sections (300 nm) were cut using an Ultracut UCT Microtome (Leica Microsystems, Vienna, Austria), collected on Formvar-coated copper slot grids and post-stained with 2 % uranyl acetate in 70 % methanol followed by Reynold’s lead citrate. For dual-axis electron tomography (Mastronarde, 1997) series of tilted views were recorded using a TECNAI F30 transmission electron microscope (FEI Company, Eindhoven, The Netherlands) operated at 300 kV and equipped with a Gatan US1000 CCD camera (2k x 2k). Images were captured every 1.0° over a ±60° range. The IMOD software package (http://bio3d.colourado.edu/imod) was used for the calculation of electron tomograms (Kremer et al., 1996). The Amira software package with an
extension to the filament editor was used for the segmentation, automatic tracing and 3D visualization of microtubules, and for data analysis (Weber et al., 2012, Stalling et al., 2005).

**Spinning disk confocal fluorescence imaging**

Live imaging was performed using a spinning disk confocal microscope (Nikon Ti2000, Yokugawa CSU-X1), equipped with 488-nm and 561-nm diode lasers, an EMCCD camera (Hamamatsu), and a 60X water-immersion objective (CFI Plan Apo VC 60X WI, NA 1.2, Nikon). Acquisition parameters were controlled by a home-developed LabVIEW program (LabVIEW, National Instruments). For *C. elegans* mitotic spindles and *C. elegans* female meiotic spindles, images were acquired every 2 second or 4 second with a single z-plane. For Human mitotic spindles, images were acquired every 2 second or 4 second with 3 z-sections every 1 µm, and the middle planes were presented.

**Laser ablation**

The laser ablation system was constructed on the above-mentioned spinning disk confocal microscope. Femtosecond pulsed near-infrared lasers with either 80-MHz or 16-kHz repetition rate were adapted to perform laser ablation. 80-MHz femtosecond pulses with 0.3-nJ pulse energy and 800-nm center wavelength came directly from a Ti:sapphire pulsed laser (Mai-Tai, Spectra-Physics, Mountain View, CA). A 16-kHz femtosecond pulse train with ~6-nJ pulse energy was produced by selecting pulses from the above Ti:sapphire pulsed laser using a pulse picker (Eclipse Pulse Picker, KMLabs). The ablation laser was focused through the same objective for imaging, and laser ablation was performed by moving the sample on a piezo-stage (P-545 Plnano XYZ, Physik Instrumente) in three dimensions controlled by a home-developed LabVIEW program (LabVIEW, National Instruments). Scanning line-cuts with z-steps were created by moving the stage perpendicular to the spindle long axis back and forth on the focal
plane while lowering the stage in the z direction. The parameter for ablation in length by depth was 8 x 6 \( \mu m \) for \textit{C. elegans} mitotic spindles; 12 x 6 \( \mu m \) for human mitotic spindles; 6 x 2 \( \mu m \) for \textit{C. elegans} meiotic spindles. The moving speed of the stage was 50 \( \mu m/sec \).

**Photobleaching experiments**

Photobleaching experiments used the same set-up and software control as the laser ablation, except that a 80MHz Ti:sapphire pulsed laser was used (800nm wavelength, \(~70\) fs pulse-width, \(~0.1\)-nJ pulse energy, Mai-Tai, Spectra-Physics, Mountain View, CA). The parameter for photobleaching in length by depth was 7 x 6 \( \mu m \) in \textit{C. elegans} mitotic spindles, and 6 x 2 \( \mu m \) in \textit{C. elegans} meiotic spindles. The moving speed of the stage was 50 \( \mu m/sec \).

**Photoconversion experiments**

Photoconversion experiments used the same set-up and software control as the laser ablation, except that a 405nm continuous-wave diode laser was used (Thorlabs, Inc.). The parameter for photoconversion in length was 12 \( \mu m \) in human mitotic spindles. The moving speed of the stage was 50 \( \mu m/sec \).

**Nonlinear optical imaging and polarity analysis**

Our nonlinear optical imaging system was constructed around an inverted microscope (Eclipse Ti, Nikon, Tokyo, Japan), with a Ti:sapphire pulsed laser (Mai-Tai, Spectra-Physics, Mountain View, CA) for excitation (900 nm wavelength, 80 MHz repetition rate, \(~70\) fs pulse-width), a commercial scanning system (DCS-120, Becker & Hickl, Berlin, Germany), and hybrid detectors (HPM-100-40, Becker & Hickl). The maximum scan rate of the DCS-120 is \(~2\) frames/s for a 512 X 512 image. The excitation laser was focused on the cells with a 40X water-immersion objective (CFI Apo 40X WI, NA 1.25, Nikon). A half-wave plate (AHWP05M-980) and a quarter-wave plate (AQWP05M-980) were used in combination to achieve circular polarization.
at the focal plane, resulting in equal second-harmonic-generation (SHG) signals of all orientations of microtubules in the plane. Two-photon (TP) fluorescence was imaged with a non-descanned detection scheme with a 510/42 emission filter (FF01-510/42-25, Semrock) for GFP-labeled tubulin. Forward-propagating SHG signals were collected through an oil-immersion condenser (1.4, Nikon) with a 448/20 nm filter (FF01-448/20-25, Semrock). Both pathways contained short-pass filters (FF01-650/SP-25, Semrock) to block the fundamental laser wavelength. Different anaphase stages of wildtype spindles and gpr-1/2 (RNAi) spindles were exposed to excitation laser for 2 seconds to acquire simultaneous SHG and TP images. Detailed polarity analysis using SHG and TP signals was described in the reference (Yu et al., 2014).

**Quantitative analysis in C. elegans mitotic spindles**

Quantitative analysis of the chromosome distance with a sub-pixel resolution was achieved using a home-written Matlab (The MathWorks, Natick MA) program. The center positions of two centrosomes in each frame of the time-lapsed images were either manually selected in spindles expressing GFP-labeled tubulin or automatically located in spindles expressing mCherry-labeled γ-tubulin based on a public Matlab program for particle tracking (https://site.physics.georgetown.edu/matlab/index.html). The center positions of the two centrosomes in each frame determines the spindle long axis as well as the spindle length, and were used to generate line-scans of overlaid images of mCherry-labeled histones (corresponding to chromosomes). Averaged mCherry fluorescence intensities from histones along these line-scans were extracted, and the fluorescence profile around histone-enriched regions were Gaussian-like shapes. A double-peak Gaussian function was used for fitting the line-scans to locate the centers of two groups of histones, presumably reflecting the central positions of sister chromosomes. The distance of these two fitted center positions was defined as the chromosome-
to-chromosome distance. A straight line was fitted to the change of chromosome distance versus time to extract the velocity of chromosome separation before and after laser ablation. In controls, the velocity was computed before and after the chromosome distance was 2.4 µm, the chromosome distance around which most laser-ablation experiments were performed.

Movement and recovery of photobleaching makers were also tracked using a program written in Matlab. Line-scans of GFP-labeled tubulin between chromosomes along the spindle axis were extracted over the course of anaphase. Each line-scan at each time point can be divided into two halves by the middle plane of the spindle. The half of the line profile with the bleaching mark was normalized to the other half of the profile by a reflection of symmetry around the middle plane of the spindle. This profile normalization was used to remove spatial variations in the background fluorescence, a valid procedure assuming mirror symmetry of the spindle around its middle plane. A Gaussian function was used to fit the normalized profile to locate the center of the bleached mark, and thus the position of the bleached mark versus time was extracted. A straight line was fitted to the position of the bleached mark versus time to retrieve the velocity of the bleached mark.

The recovery time of GFP::tubulin fluorescence after photobleaching in the center of the inter-chromosomal region was determined by using an exponential fit. The fluorescence intensities of photobleached marks were calculated by summing intensities over 5 pixels (~0.9µm) around the center of the bleach mark.

**Quantitative analysis in C. elegans meiotic spindles**

Chromosome distance in *C. elegans* meiotic spindles was computed with a combination of Fiji (Schindelin et al., 2012) and a Matlab program. Time-lapse images of spindle expressing mCherry::histone (corresponding to chromosomes) were realigned in a routine for matching,
rotation and translation using Rigid Body of Fiji’s StackReg plug-in, so that the random displacement of the spindle due to the spontaneous motion of the worm was corrected. On the realigned time-lapse stack, a straight line passing through sister chromosomes was manually drawn in line with the spindle axis, and the kymograph of mCherry:histone intensities along this line was generated. Upon a Matlab program, each line-scan of mCherry:histone was fitted to a double-peak Gaussian function for computing the center positions of sister chromosomes, and thus the chromosome distance. A straight line was fitted to the data of chromosome distance versus time to extract the velocity of chromosome separation before and after laser ablation. In controls, separation velocity was computed before and after the chromosome distance was 3.3 \( \mu \text{m} \), the chromosome distance around which most laser-ablation experiments performed.

Movement of photobleaching makers were also tracked using a program written in Matlab. Line-scans of GFP-labeled tubulin between chromosomes along the spindle axis were extracted over the course of anaphase. A Gaussian function was used to fit the line-scanning profile to locate the center of the bleached mark, and thus the position of the bleached mark versus time was extracted. A straight line was fitted to the position of the bleached mark versus time to retrieve the velocity of the bleached mark.

**Quantitative analysis in Human mitotic spindles**

Distance information of interest was extracted from spindles expressing GFP::CENP-A (corresponding to kinetochores) and GFP::centrins (corresponding to spindle poles) in time-lapse z-stacks based on the following steps. First, using approaches from a particle tracking algorithm (Pelletier et al., 2009), a GUI Matlab program was developed to retrieve the three-dimensional coordinates of the center positions of each kinetochores and the two poles. Next, a support vector machine (SVM) algorithm was adapted to find the best plane to separate kinetochores of one
half-spindle from those of the other half-spindle. This best plan is the one with the largest margin between two sides of kinetochores, and corresponds to the middle plane of the spindle. A unit vector perpendicular to this plane was thus in line with the direction of spindle long axis. All positions of kinetochores and spindle poles in space were projected onto this unit vector, and thus converted to one-dimensional information of projection lengths. Finally, chromosome-to-chromosome distance was computed as the length difference between the averaged projection lengths of kinetochores at one side of the half-spindle and those at the other side. Similarly, the chromosome-to-pole distance was computed as the length difference between averaged projection lengths of kinetochores and the projection length of the pole at the same side of the half-spindle. The above procedure was performed for each time point of a z-stack, so that the rotation and translation of spindles in space over the course of anaphase was corrected. A straight line was fitted to the data of chromosome-to-chromosome distance, and chromosome-to-pole distance versus time to compute the separation velocity of the chromosomes, and the velocity between chromosomes and poles before and after laser ablation, individually. In controls, the separation velocity of chromosomes was computed before and after the chromosome distance was at 6.1 µm.

Movement of photoconverted mEOS3.2-labeled microtubules were also tracked using a program written in Matlab. Line-scans of photoconverted tubulin between chromosomes along the spindle axis were extracted over the course of anaphase. A Gaussian function was used to fit the profile to locate the center of the photoconverted microtubules, and thus the position of the photoconverted microtubules versus time was extracted. A straight line was fitted to the position of the photoconverted microtubules versus time to retrieve the velocity of the photoconverted microtubules.
**Statistical analysis**

Statistics are presented as mean ± SEM, and p-values were calculated by “ttest2” function in Matlab.
Table 1. Key resources.

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<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
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References


