Quantifying Mitotic Chromosome Dynamics and Positioning

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The proper organization and segregation of chromosomes during cell division is essential to the preservation of genomic integrity. To understand the mechanisms that spatially control the arrangement and dynamics of mitotic chromosomes requires imaging assays to quantitatively resolve their positions and movements. Here, we will discuss analytical approaches to investigate the position-dependent control of mitotic chromosomes in cultured cells. These methods can be used to dissect the specific contributions of mitotic proteins to the molecular control of chromosome dynamics.
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Chromosome segregation during cell division is highly regulated in order to preserve genomic integrity. To facilitate DNA segregation, cells assemble a protein-based machine, called the mitotic spindle, to capture, bi-orient and align chromosomes. These functions are highly dependent on microtubules, polar polymers that comprise the bipolar mitotic spindle (McIntosh et al., 2002). Spindle microtubules are the basis of the pushing and pulling forces necessary for chromosome movements. These forces can be generated by dynamic length changes of the microtubules themselves or molecular motors that use the microtubules as tracks (Inoue and Salmon, 1995). In order to orchestrate these forces to facilitate the proper alignment and segregation of mitotic chromosomes, accessory proteins are required to modulate the dynamics of microtubules and their attachments to chromosomes. Therefore, mutations that increase or decrease the function of proteins that regulate spindle microtubules often lead to mispositioning and altered movement of chromosomes in mitosis. As the cell division field continues to dissect the molecular mechanisms controlling chromosome segregation, there is an increased need for sensitive, quantitative assays to interrogate mitotic chromosome positioning and movement.

Chromosomes make stable attachments to the faster growing plus-ends of microtubules through specialized protein complexes called kinetochores. Proper attachment to the spindle is achieved when chromosome pairs become bi-oriented such that kinetochores on replicated sister chromosome pairs are attached to the plus-ends of microtubules emanating from opposite poles of the spindle. Classic live imaging studies of congressing chromosomes established that bi-oriented sister chromosomes are under tension, move at a relatively constant velocity and display abrupt changes in direction both before and after alignment (Skibbens et al., 1993; Waters et al., 1996). These oscillatory movements are surprisingly similar in unaligned and aligned chromosomes, with the exception that unaligned chromosomes tend to exhibit more directional persistence towards the center of the spindle between reversals (Skibbens et al., 1993). Thus, mechanisms that control congression regulate the persistent movement of chromosomes.

The persistent movement of chromosomes is at least in part controlled via modulation of kinetochore-microtubule dynamics. Previous work has established that assembly and disassembly of the 15–20 microtubules that bind each vertebrate kinetochore, and make up an individual kinetochore-fiber (K-fiber), are correlated with chromosome movement (Inoue and Salmon, 1995). Lengthening of K-fiber plus-ends is associated with away-from-pole (AP) movements and their shortening is associated with poleward (P) movements. Furthermore, reversals in the direction of chromosome movement occur when K-fibers undergo catastrophes (a switch from growth to shortening) or rescues (switch from shortening to growth). Because their movements are erratic and stochastic, each chromosome must find its own way to the spindle equator, independently of the others (Skibbens et al., 1993). This implies that local regulation of K-fiber dynamics provides a "spatial cue" required to align chromosomes. In the following sections, we will discuss quantitative approaches for investigating the molecular basis of these cues.

Quantification of chromosome alignment in fixed cells

Typical measurements of chromosome alignment yield a binary data set containing the percentages of cells with aligned versus unaligned chromosomes. While this type of analysis has been useful for identifying regulators of chromosome alignment in conjuction with siRNA or small molecule inhibition strategies, it lacks the sensitivity to detect intermediate phenotypes or determine how the level of expression of a particular protein or mutant correlates with chromosome alignment. Due to these limitations, we have developed a sensitive assay for chromosome alignment that measures the distribution of fluorescently labeled kinetochores within the spindle (Fig. 1) (Stumpff et al., 2012). This approach quantifies the degree of chromosome alignment in individual cells.

The distribution of kinetochores can be quantified in fixed cells expressing a fluorescently tagged kinetochore component

Fig. 1. Quantitative assay for chromosome alignment. A: HeLa and (B) RPE1 cells were fixed and stained for kinetochores (CREST serum) and centrosomes (anti-γ-tubulin antibodies) following treatment with the indicated siRNAs. C and D: Distributions of fluorescently labeled kinetochores were measured using the Plot Profile command in Image J and plotted using Igor Pro (black data points). Distributions were quantified using the full width at half max (FWHM) of a Gaussian fit (dashed red line) to the distribution (C) or calculation of the kinetochore distribution ratio (D).

(e.g., CENP-A) or immunostained with antibodies that recognize a kinetochore protein. Cells should also be co-stained with a centrosome component (e.g., γ-tubulin) to visualize spindle poles (Fig. 1). Single focal plane images of mitotic cells oriented parallel to the plane of focus are obtained using a standard widefield fluorescent microscope equipped with a 60× or 100× objective and CCD camera (Fig. 1A,B). The distribution of kinetochores between the two spindle poles within a rectangular region of interest can then be measured using image analysis software (e.g., ImageJ) (Stumpff et al., 2012). Note that the accuracy of this approach critically depends on choosing cells with bipolar spindles where both centrosomes are in the same plane of focus.

A single parameter that describes the kinetochore distribution can be used as a metric for chromosome alignment. For example, a normal distribution of kinetochores within the spindle can be fit with a Gaussian curve, and the full-width at half max (FWHM) of the Gaussian can be used as the metric for kinetochore alignment in that cell. This approach works well when the observed effect being quantified is a broadening of the metaphase plate, as is seen in cells depleted of the kinesin-like motor protein Kif18A (Fig. 1A,C) (Zhu et al., 2005). However, some molecular perturbations, such as depletion of the kinesin-like motor protein CENP-E, produce kinetochore distributions that are not well fit by a single Gaussian (Fig. 1B,D). In cells lacking CENP-E function, the majority of chromosomes congress to the spindle equator, but a few chromosomes remain positioned near the centrosomes (Schaar et al., 1997). In this case, a ratio of the kinetochore fluorescence near the poles and at the center of the spindle is a

more useful metric for evaluating the abnormal distribution. To produce this ratio one can measure the kinetochore fluorescence from one edge of the cell to the other within a rectangular region of interest along the normalized pole-to-pole axis. The ratio (r) is then calculated by summing the kinetochore fluorescence in the peripheral cell quarters (γ_1 and γ_2) divided by the fluorescence in the middle half of the cell (ε) using the following formula (Stumpff et al., 2012):

$$r = \frac{\gamma_1 + \gamma_2}{\varepsilon}$$

Quantification of kinetochore distributions can be used to compare the severity of depleting single or multiple suspected regulators of chromosome alignment (Stumpff et al., 2012). It is also an ideal approach for analyzing knockdown and replacement assays to determine the ability of engineered mutants to functionally substitute for an endogenous regulator of congression. If the mutant proteins are fluorescently tagged, chromosome alignment can also be analyzed as a function of mutant protein expression to evaluate concentration-dependent effects. Furthermore, by semi-automating the analysis process, one can quickly generate large data sets containing alignment measurements in hundreds of cells to facilitate robust statistical comparisons (Stumpff et al., 2012). Thus, kinetochore distribution measurements offer a number of advantages over the subjective approach of counting the percentage of cells with chromosome alignment defects.

Measurement of kinetochore movements in live cells

Abnormal chromosome alignment can result from the loss of stable attachments between kinetochores and microtubules or defects in the control of chromosome movements (Kops et al., 2010). Determining the effects of a particular perturbation on kinetochore-microtubule attachment is an important first step to understanding the molecular basis of altered chromosome alignment. Methods for evaluating kinetochore-microtubule attachment have been well described previously and will not be addressed here (Klebig et al., 2009; DeLuca, 2010). For perturbations that disrupt chromosome alignment without significantly altering kinetochore-microtubule attachments, live imaging analyses of kinetochore move-

ments can inform how the targeted protein functions to promote chromosome congression.

The oscillatory movements of bi-oriented chromosomes are characterized by periods of poleward (P) and away-from-pole (AP) motion interrupted by abrupt directional changes (Skibbens et al., 1993). To determine if a protein regulates chromosome movements, one can measure the velocity, switch rate and oscillation amplitude of fluorescently labeled kinetochores in live cells, as well as the distance between paired sister kinetochores (Fig. 2). To obtain these data, 4D fluorescent microscopy is used to image cells transiently or stably expressing fluorescent kinetochore markers with high temporal resolution (i.e., frame rates of 2–5 sec) (Fig. 2A). To facilitate the optical
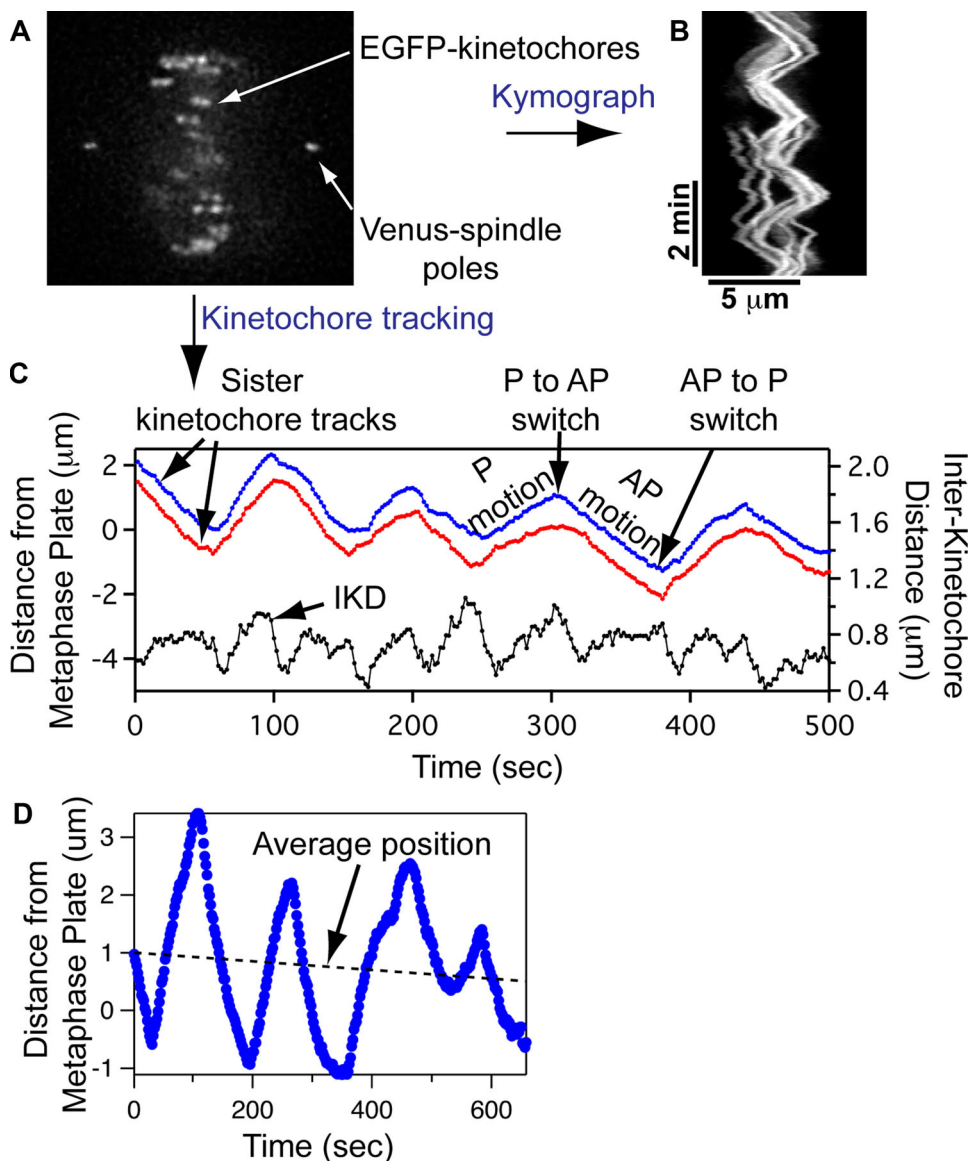
Fig. 2. Quantitative analysis of kinetochore movements in live cells. A: Cells were tranfected with fluorescent kinetochore (EGFP-CENP-B) and spindle pole markers (Venus-centrin) and imaged at 2 sec intervals. The movements of kinetochores were then analyzed by kymograph (B) and by tracking positions over time relative to the metaphase plate (C). Specific movement parameters such as the velocity of poleward (P) and away-from-pole (AP) motion, switches between these two states and the distance between paired sister kinetochores (IKD) were measured from track data. D: The deviation of a kinetochore from its average position (DAP) can also be used as a metric for oscillation amplitude. DAP is defined as the standard deviation of the distances between a kinetochore (blue points) and its average position (dashed black line) at every time point.

sectioning and signal to noise ratios required for accurate kinetochore tracking, the microscopy system should be equipped with a sensitive CCD camera and a fast z-stage, as well as temperature control. Time-lapse images can then be analyzed to determine the movements of kinetochores relative to the metaphase plate or spindle poles (Fig. 2B,C). The former can be objectively determined by a linear fit to the fluorescent kinetochores, while the latter requires co-expression of a centrosome marker in the cells (Stumpff and Asbury, 2008; Stumpff et al., 2012). Note that using a spindle pole marker, such as centrin, that is labeled with a fluorescent tag that has similar excitation and emission spectra as the kinetochore label being imaged improves imaging speed and reduces light damage to the cells (Fig. 2A).

Once high quality time-lapse images of kinetochore movements are obtained, kinetochore trajectories can be visualized via kymograph and quantitatively tracked using image analysis software (Fig. 2B,C). Kinetochore movements can be manually tracked in two or three dimensions using freely available software programs, such as ImageJ (Stumpff and Asbury, 2008; Magidson et al., 2011; Stumpff et al., 2012). Automated tracking of kinetochore movements has been a challenge due to the oscillatory nature of the movements, which lead to frequent trajectory crossover. However, recent advances in particle tracking algorithms has made automated analysis of kinetochore movements a reality, greatly enhancing the ability of researchers to produce and process large data sets (Jaqaman et al., 2010). Each of these general approaches can be used to identify significant quantitative changes in particular movement parameters following inhibition of a mitotic regulator, which informs protein function. For example, increases or decreases in the velocity of kinetochore movement suggest a change in the rate of K-fiber growth or shortening, while altered rates of kinetochore directional changes suggest that K-fiber rescue or catastrophe rates are modified. Changes in either velocity of switch rate will alter the amplitude of kinetochore oscillations. Similarly, increases or decreases in interkinetochore distance suggest that either the amount of tensile force on kinetochores or the stiffness of the connection between them has changed.

Quantification of irregular kinetochore movements

Analyses of switch rates and oscillation amplitudes requires unambigous determination of directional changes in kinetochore movements, which is not always possible. For example, in cases where kinetochore oscillations are significantly reduced, such as when cells overexpress Kif18A or are depleted of the chromokinesin Kid, directional changes in the resulting irregular kinetochore trajectories are not readily apparent (Stumpff and Asbury, 2008; Stumpff et al., 2012). In these cases, the deviation of a kinetochore away from its average position (DAP) can be used as an alternative method to quantify the extent of altered movement (Stumpff and Asbury, 2008) (Fig. 2D). The average position of a kinetochore is defined as a regression fit to the track of its position as a function of time. The distance of the kinetochore from this line at each time point is measured and DAP is calculated as the standard deviation of these distances. DAP is a linear measurement of oscillation amplitude that is sensitive to changes in either kinetochore velocity or switch rate. Note that this measurement is also sensitive to changes in the basic shape of kinetochore oscillations, such as those that might occur if kinetochores displayed excessive pauses in their movements. While standard measurements of velocity, switch rate and oscillation amplitude provide more information about chromosome movements, DAP serves as a useful alternative for analyzing irregular or dampened trajectories.

Spatial resolution of kinetochore movement parameters

Mechanisms that control chromosome congression must regulate the persistent movement of individual chromosomes relative to their spindle position by modulating one or more movement parameters in a position-dependent manner. Therefore, in order to investigate the mechanisms controlling chromosome alignment, it is useful not only to measure kinetochore movement parameters, but also to determine how they change as a function of a kinetochore's position within the spindle (Fig. 3). For example, we previously determined that the rate of kinetochore directional switching is dependent on position within the spindle in control HeLa cells (Stumpff et al., 2012) (Fig. 3B). This indicates that switch rates are spatially regulated. Furthermore, depletion of proteins that disrupt chromosome alignment, such as Kif18A and Kid, reduce the correlation between switch rate and spindle position (Stumpff et al., 2012). To facilitate spatially resolved analyses, a coordinate system that measures a kinetochore's K-fiber length relative to the half-spindle can be used to define the absolute position of each individual kinetochore (Fig. 3A). Changes in the dependence of kinetochore velocities, switch
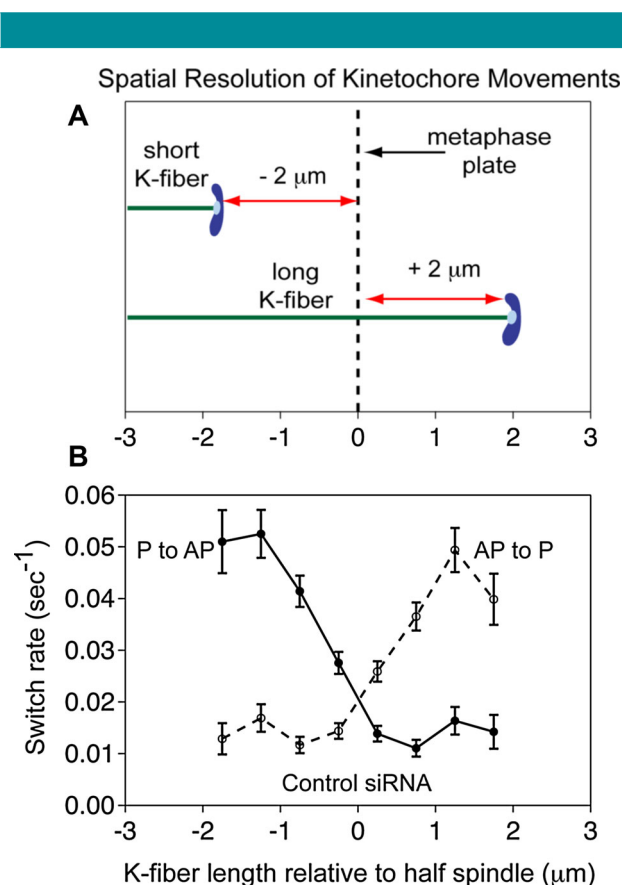
Fig. 3. Spatial resolution of kinetochore movements. A: Schematic of a coordinate system used to determine the absolute positions of kinetochores relative to the metaphase plate. K-fiber length relative to the half spindle is determined by measuring the distance between a kinetochore and the metaphase plate. The distance is positive if the kinetochore is on the opposite side of the metaphase plate relative to the pole it is attached to and negative if it is on the same side of the metaphase plate as the pole it is attached to. B: This coordinate system was used to analyze the rate at which kinetochores switch directions as a function of position. Data for poleward to away from pole (P to AP) switches and away from pole to poleward (AP to P) switches are shown for measurements made in control siRNA treated HeLa cells.

rates and interkinetochore distance on position can then be measured. Spatially resolved analyses of chromosome movements will likely be necessary to fully illuminate the molecular basis of the spatial cues facilitating chromosome congression.

Future Directions

The quantitative approaches for analyzing the positioning and movements of kinetochores described here are currently being used to advance our understanding of the mechanisms that control cell division. By permitting determination of the details and severity of changes in chromosome movement and alignment, these approaches will reveal the relative contributions of different regulators and pathways to the alignment and segregation of chromosomes. While it is important to keep in mind that there are many direct and indirect ways that an individual protein could influence a specific kinetochore movement parameter at the molecular level, determining the precise effects of reducing the regulator's function on chromosome movements will permit the formation of specific testable hypotheses regarding its molecular activity.

As our knowledge of mitotic spindle function and the list of its regulators continues to grow, increasingly sensitive assays will be needed to advance our knowledge of the complex mechanisms controlling chromosome movements. For example, mechanical regulation of chromosome arms and kinetochores are thought to provide important contributions to this control. However, the molecular basis of these mechanical mechanisms remain unclear. Thus, development of methods for resolving and tracking the movements of chromosome arms relative to kinetochores, as well as new approaches for measuring changes in kinetochore tension will be essential. Furthermore, the 3D organization of chromosomes within the spindle is likely more complex than the 2D alignment between the spindle poles that is typically analyzed (Magidson et al., 2011). Thus, expanding the abilities of the kinetochore distribution and spatially resolved tracking methods described here to three dimensions would better facilitate investigations into the position-dependent control of chromosome organization and movement.

In summary, advances in imaging technology are continually improving what we can observe during cell division. To make sense of this information and ultimately understand how this process is controlled will require paralleled advances in our quantitative evaluation of imaging data. The methods discussed here represent an ideal starting point for quantitatively determining how a mitotic protein contributes to the control of chromosome movements and positioning.

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