Chapter 16

Quantification of Mitotic Chromosome Alignment

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Abstract

The alignment of chromosomes during metaphase is a hallmark of mitosis. For this reason, chromosome alignment has served as an informative functional assay for evaluating mitotic fidelity. The common approach of quantifying the number of mitotic cells with unaligned chromosomes within a population has led to the identification of many proteins required for this conserved process. However, more sensitive assays are now required to dissect the complex molecular control of chromosome alignment. In this chapter, we describe a microscopy-based method for objectively quantifying the distribution of fluorescently labeled chromosomes within the mitotic spindle that can be used to evaluate the extent of chromosome alignment within individual mitotic cells.

Key words Chromosome alignment, Mitosis, Mitotic spindle, Congression, Kinetochore

1 Introduction

The question of how chromosomes align at the center of the mitotic spindle has puzzled scientists since the process was first described over a century ago [1]. While many important contributions have been made towards understanding how this conspicuous event occurs, efforts to understand its molecular control are ongoing. Mitotic chromosome alignment depends both on stable attachments between chromosomes and mitotic spindle microtubules, via specialized protein structures called kinetochores, and the regulation of kinetochore microtubule lengths. Thus, chromosome alignment has frequently been used as a phenotypic reporter for abnormal kinetochore microtubule attachments or dynamics [2–9].

Mitotic chromosome alignment is typically quantified via visual inspection of chromosome organization within the mitotic spindle. This approach provides a binary data set reporting the percentage of mitotic cells with aligned and unaligned chromosomes, which limits determination of intermediate phenotypes. Furthermore, because of the subjective determination of what constitutes

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alignment, reproducibility can also be an issue. To address these issues, we have developed a quantitative assay for chromosome alignment that measures the distribution of fluorescently labeled kinetochores within the spindle [10–12]. This objective approach, which is described in detail below, yields reproducible results with the necessary sensitivity to detect partial alignment of mitotic chromosomes. This method can be used to measure the synergistic or antagonistic effects of multiple regulators on alignment, and can be utilized for structure–function analyses of individual mitotic proteins [11, 12].

2 Materials

2.1 Materials for Cell Culture and siRNA Treatment

1. Coverslips: Purchase high quality 12 mm diameter round glass coverslips from a reputable source.
2. Tissue culture plates: Sterile 10 cm and 24-well tissue culture plates are required.
3. Cultured cells: This assay is expected to work with any proliferating adherent cell line. We have specifically tested the method with human retinal pigment epithelial (RPE1) cells and HeLa cells.
4. Cell culture: Culture RPE1 and HeLa cells using standard sterile tissue culture techniques in Minimal Essential Medium Alpha (MEMα) supplemented with 10 % fetal bovine serum and grow in a 37 °C, 5 % CO_2 incubator.
5. Phosphate Buffered Saline (PBS): Prepare or purchase a 1× stock solution of 0.2 M monobasic sodium phosphate, 0.2 M dibasic sodium phosphate and 150 mM NaCl in ddH_2O at pH 7.4. Sterilize by autoclaving.
6. Trypsin: Use tissue culture grade trypsin.
7. Opti-MEM: Use a reduced serum medium such as Opti-MEM (Gibco) for transfection of siRNAs.
8. 1 M hydrochloric acid (HCl): Dilute concentrated HCl drop wise into ddH_2O to a final concentration of 1 M.
9. MG132: Prepare a 10 mM MG132 stock in DMSO and store at −20 °C.

2.2 Materials for Cell Fixation and Immunofluorescence

1. 1 % paraformaldehyde/methanol fix: Dilute high quality paraformaldehyde to a final concentration of 1 % in ice-cold methanol. Total volume should be 80 mL in a 250 mL beaker. The fixative should be made fresh just before use.
2. Tris Buffered Saline (TBS) 1×: Dissolve 8 g NaCl, 0.2 g KCl, 3 g Tris base in 800 mL ddH_2O and pH with HCl to 7.4. Bring volume up to 1 L and sterilize by autoclaving.

3. Antibody dilution buffer (Abdil): Prepare a solution containing 1 % BSA (IgG-free, protease-free), 0.1 % Triton X-100, and 0.02 % sodium azide in 1× TBS.
4. Primary antibody working solutions: Dilute Human anti-centromere antibodies (ACA, Antibodies Inc.) to 2 μg/mL and mouse anti-γ-tubulin antibodies (Sigma) to 1 μg/mL in Abdil. Store working stocks at 4 °C or prepare fresh just before use.
5. Secondary antibody mix: Dilute Alexa Fluor 647 goat anti-mouse IgG (H + L) and Alexa Fluor 594 goat anti-human IgG (Life Technologies) to 4 μg/mL each in Abdil.
6. Cell mounting media: Use an anti-fade cell mounting media containing a DNA stain such as DAPI (e.g., Prolong Gold with DAPI, Life Technologies).

2.3 Materials for Image Acquisition and Analysis

1. Fluorescence microscopy: A standard wide field fluorescence microscope with a CCD camera, high quality 60× or 100× objective lens and filter sets for imaging at least two fluorophores is required.
2. Image analysis software: An image analysis software package that allows quantification of fluorescence intensity in user-defined regions of interest will be needed. We routinely use ImageJ, which is freely available at http://rsb.info.nih.gov/ij. The specific commands given for quantifying fluorescence in Subheading 3.3.2 are for the Macintosh version of ImageJ 1.48d.

3 Methods

3.1 Cell Culture and siRNA Treatment

3.1.1 Prepare Acid Washed Coverslips

Acid washing coverslips increases cell adherence to the glass.

1. In a glass beaker, incubate glass coverslips in 1 M HCl at room temperature for 12–18 h. Cover beaker with Parafilm during the incubation.
2. Carefully remove the HCl by decanting and wash coverslips five times with ddH_2O.
3. In the same glass beaker, wash coverslips five times with 95 % ethanol.
4. Dry coverslips on Kimwipes making sure they are spread out to prevent them from sticking to one another.
5. Place coverslips in a sterile petri dish and keep sterile for future use.

3.1.2 Seed Cells onto Coverslips

When performing cell culture, clean all equipment with 70 % ethanol and work inside a biosafety cabinet using sterile technique to prevent contamination.

1. Grow adherent cells to 70 % confluency in a 10 cm plate containing 10 mL of culture media with antibiotics.
2. Gently suction off media and wash cells with 10 mL of 1× PBS. This is necessary to remove residual serum, which will inactivate trypsin and prevent cell dissociation from the culture dish.
3. Gently remove PBS and add 1 mL of 37 °C trypsin to the 10 cm plate. Gently rock side-to-side making sure the entire culture is bathed in trypsin. Incubate cells in trypsin for 5–10 min until all cells have dissociated from the plate.
4. Once cells have dissociated, add 4 mL of antibiotic-free, 37 °C cell culture medium to the cells. Very slightly tip the culture dish at an angle and mix the cells by pipetting the entire 5 mL 10–15 times with a 10 mL serological pipette. This will pool all the cells in one area of the dish and break up cell clusters.
5. Immediately after pipetting the cells, count them using a hemocytometer or similar method (*see* **Note 1**).
6. Using forceps, place one 12 mm acid washed coverslip into the well of a 24-well culture plate. Double check to make sure the coverslip is sitting flat on the bottom of the dish. Repeat for each condition being tested in the experiment (*see* **Note 2**).
7. Dilute cells to an appropriate concentration to seed at 50 % confluency in a 24-well plate. For HeLa and RPE1 cells, dilute cells to 1.0×10^5 per mL and pipette 0.5 mL (0.5×10^4 cells) directly onto the center of the 12 mm coverslip (*see* **Note 3**).
8. Place the cells in an incubator until they have adhered to the coverslip and started to spread. Incubation time can vary from 1 h to overnight depending on the cell type.

3.1.3 Treat Cells with siRNAs

Cells should be in antibiotic free media when siRNAs are added, and siRNA reagents should be warmed up to room temperature.

1. For each coverslip of cells being treated, dilute and gently mix the following reagents separately in microfuge tubes containing reduced serum media (*see* **Note 4**).
   - 50 µL Opti-MEM + 1.5 µL RNAiMax.
   - 50 µL Opti-MEM + 30 pmol siRNA.
2. Combine siRNA and RNAiMax solutions and allow to complex at room temperature for 15 min.
3. Add each complexed siRNA solution to the appropriate coverslip in the 24-well plate, and gently shake the plate to mix.
4. Incubate the culture dish for 24–72 h in a 37 °C incubator with 5 % CO_2 (*see* **Note 5**).

5. *Optional*: To increase the number of metaphase cells, treat cells with the proteasome inhibitor MG132 for 1–2 h prior to fixation. Add MG132 to a final concentration of 20 μM in each well (*see* **Note 6**).

3.2 Cell Fixation and Immunofluorescence

3.2.1 Fix Cells

1. Using two pairs of forceps, move coverslips with cells into a coverslip staining rack and submerge the rack into a beaker of 1 % paraformaldehyde/methanol fixative (*see* **Note 7**).
2. Fix for 10 min on ice.
3. Wash coverslips three times in 100 mL of 1× TBS for 5 min each (*see* **Note 8**). Perform washes in 250 mL beakers and use caution when lowering the coverslip rack to avoid displacing coverslips.

3.2.2 Immunofluorescently Label Centrosomes and Kinetochores

1. Prepare a staining chamber from a 25 mm Petri dish. Cover the bottom of the dish with Parafilm and place wet Kimwipes around the sides of dish. Replace the lid for all incubation steps to create a humidified chamber (*see* **Note 9**).
2. Place coverslips onto the Parafilm in the chamber with the cell side facing up.
3. To reduce nonspecific binding of antibodies, block cells by adding 30 μL of 20 % goat serum in AbDil to each coverslip. Incubate at room temperature for 1 h on a horizontal shaker.
4. Move coverslips back to staining rack, and wash three times in 1× TBS for 5 min each.
5. Place coverslips cell side up in staining chamber, and add 30 μL of one of the diluted primary antibody solutions to each coverslip. For mouse anti-γ-tubulin antibodies, which label centrosomes, incubate at room temperature for 1–4 h. For human ACA antibodies, which label kinetochores, incubate overnight at 4 °C.
6. Remove excess liquid by dabbing the edge of the coverslip on an absorbant wipe and move coverslips back to the staining rack. Wash three times in 1× TBS for 5 min each.
7. Repeat **steps 5** and **6** for the remaining primary antibody.
8. Move coverslips back to staining rack, and wash three times in 1× TBS for 5 min each.
9. Add 30 μL of diluted secondary antibody mix to each coverslip in the staining chamber and incubate for 1 h at room temperature with shaking. Cover staining chamber with foil to reduce light exposure during incubation.
10. Remove excess liquid by dabbing the edge of the coverslip on an absorbant wipe and wash three times in 1× TBS for 5 min each.

11. Remove excess liquid from coverslips by touching the edge of the coverslip to an absorbant wipe and place onto a glass slide cell side down in a drop of cell mounting media with DAPI.
12. Gently remove the excess mounting media by blotting the top of the coverslip/slide with an absorbant wipe.
13. Allow the mounting media to cure in the dark at room temperature overnight. Slides can be stored at 4 °C or –20 °C.

3.3 Image Acquisition and Analysis

3.3.1 Acquire Images of Mitotic Cells

1. Acquire single focal plane images of fluorescently labeled centrosomes, kinetochores and DNA using a wide field fluorescent microscope (Fig. 1). Image acquisition settings should be optimized and kept consistent when imaging each experimental condition. The criteria for choosing which cells to image should include (a) late prometaphase and metaphase cells that have both poles in the same plane of focus and (b) cells with kinetochores that are attached to microtubules and under tension (i.e., the distance between paired sister kinetochores is higher than is observed at resting length during prophase) (*see* **Note 10**).

3.3.2 Image Analysis to Measure Kinetochore Distribution

The analysis methods described here can be performed within a number of software programs. We describe specific steps for performing the analysis with ImageJ, which is freely available. We have also written a custom macro to partially automate this process in ImageJ, which we will distribute upon request.

1. Export all images as TIFF files from the microscope acquisition software.
2. Open the centrosome and kinetochore images from a cell in ImageJ.

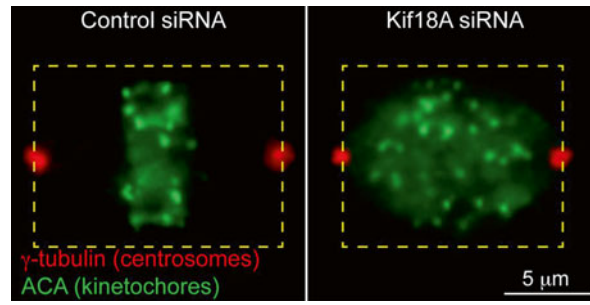

Fig. 1 Example centrosome and kinetochore images for analysis of kinetochore distribution. After a 24 h incubation with control siRNAs or siRNAs targeting the kinesin-like motor Kif18A, RPE1 cells were fixed and immunofluorescently labeled with anti-centromere (ACA, *green*) and anti-γ-tubulin antibodies (*red*). The *dashed yellow boxes* illustrate the region of each cell that was analyzed for ACA fluorescence distribution

3. Use the line tool to draw a single line from the centroid of one γ-tubulin focus to the centroid of the other.
4. Run the measure command under the Analyze menu, which will report the angle and length of the pole-to-pole line.
5. Rotate the kinetochore and the centrosome images based on the angle of the line drawn above so that the pole-to-pole axis is horizontal (Image menu, Transform, Rotate).
6. Using the rectangular selection tool, draw a box that has two sides centered on the centroids of the γ-tubulin foci (*see* yellow boxes in Fig. 1). Adjust the height of the box so it will surround all of the labeled foci in the kinetochore image (*see* **Note 11**). Make note of this box height and use it for all subsequent image analyses.
7. Add the box to the Region of Interest (ROI) Manager (Analyze menu, Tools, ROI Manager), then place the region of interest onto the kinetochore image.
8. Using the Plot Profile command (Analyze menu), measure the average fluorescence intensity in each pixel column within the ROI in the kinetochore image. Save the values from this measurement as a text file and export or copy them into a graphing software program (e.g., Excel).
9. Normalize the average kinetochore fluorescence intensity values (y values) to a zero to one scale, and convert the distances along the pole-to-pole axis (x values) from pixels to microns (Fig.2a, b).
10. Plot the normalized fluorescence values as a function of the distance along the pole-to-pole axis. Fit a Gaussian function to this distribution and use the full width at half maximum (FWHM) as a metric to quantify chromosome alignment in that cell. $\mathrm{FWHM} = 2 \times \mathrm{sqrt}(2 \times \ln 2) \times \sigma$, or $\sim 2.354 \times \sigma$, where σ is the standard deviation of the Gaussian fit function.
11. Alternatively, a ratio of the kinetochore fluorescence near the poles to the kinetochore fluorescence at the center of the spindle, which we call the kinetochore distribution ratio, can be used as a metric. For this analysis, divide the spindle into quarters and sum the average kinetochore fluorescence values in the two quarters near the poles (γ_1 and γ_2) and divide by the sum of the fluorescence in the middle two quarters (ε) ($r = \gamma_1 + \gamma_2 / \varepsilon$). Normalizing the spindle length to one is useful for this analysis (Fig. 2d–e).
12. Repeat **steps 2–11** for each cell imaged and evaluate the distributions of FWHM and kinetochore distribution ratios (Fig. 2c, f).

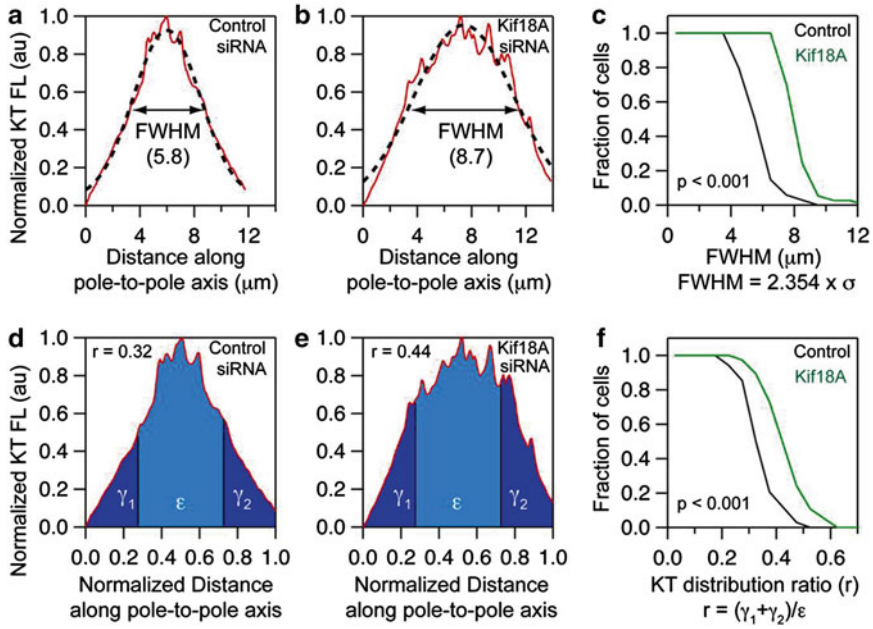

Fig. 2 Two methods for quantifying kinetochore distribution within the spindle. (**a**, **b**) Plots of normalized ACA fluorescence (*red* line and data points) from single RPE1 cells treated with control (**a**) or Kif18A (**b**) siRNAs. Plots were fit with Gaussian functions (*dashed black lines*). The full width at half maximum (FWHM) values calculated from each Gaussian fit are reported in parentheses. (**c**) Plot of the FWHM values measured in a population of RPE1 cells treated with control (*black*) or Kif18A (*green*) siRNAs. (**d**, **e**) The same fluorescence distribution data shown in A and B color-coded to indicate the portion of each plot used to calculate a kinetochore (KT) distribution ratio (*r*). The ratios (*r*) calculated for each cell are reported. (**f**) Plot of KT distribution ratios measured from the same population of cells analyzed in (**c**)

4 Notes

1. The solution of cells should be homogenous before counting and seeding onto coverslips. The cells settle out of solution quickly, so make sure to mix cells well by pipetting before counting and again before plating.
2. The 24-well culture plate can be prepared while cells are trypsinizing to save on time.
3. This suggested density is based on our experience with HeLa and RPE1 cells incubated with siRNAs for 24–48 h. The number of cells plated may need to be adjusted for other cell types or incubation times to prevent overgrowth.
4. This step can be performed with any transfection reagent optimized for delivery of siRNAs. The optimal ratio of RNAiMax and siRNA may need to be determined empirically for each siRNA target.

5. The optimal incubation time for each siRNA must be determined empirically by quantitative evaluation of the target protein via Western blot or immunofluorescence.
6. MG132 is a potent inhibitor of the proteasome that arrests mitotic cells in metaphase [13, 14]. For this assay, it increases the number of cells at the correct stage for analysis and facilitates comparisons between cells at the same point in mitosis. This is especially useful when it is difficult to distinguish prometaphase from metaphase cells by morphology after siRNA depletion of a target. Arresting cells in metaphase also increases the sensitivity of the chromosome alignment assay when analyzing cells that spend more time in prometaphase than metaphase (e.g., RPE1 cells), as asynchronously dividing populations of these cell types will have a low percentage of mitotic cells with aligned chromosomes. However, one should be aware that a prolonged mitotic delay has the potential to reduce the severity of some chromosome alignment phenotypes by providing extra time for the process to occur.
7. Any staining rack that will hold 12 mm round coverslips can be used for this step. We typically use cover glass staining outfits from Thomas Scientific. Carefully keep track of which side of the cover glass the cells are adhered to. This can be facilitated by placing all coverslips in the same orientation with the cell side facing towards a piece of tape on one side of the handled coverslip holder. It is also important to prevent the cells from drying during the entire fixing and staining process.
8. For more stringent washing, include 0.01 % Triton X-100 in the TBS.
9. We use gridded 25 mm dishes and number the squares of the grid with a permanent marker. Placing each coverslip over a number assists with keeping track of individual coverslips through the staining process.
10. Accurate measurement of kinetochore distribution within the spindle depends greatly on the signal to noise ratio of the kinetochore image. A good goal is to optimize imaging conditions to obtain an ACA fluorescence signal at kinetochores that is 2-3 fold higher than the fluorescence signal in the cytoplasm of the cell.
11. If a box drawn from pole-to-pole does not contain all of the kinetochores in the cells being analyzed (e.g., if there are kinetochores on both sides of one pole), the width of the box can be increased to include a region that runs from the cell membrane near one centrosome to the cell membrane near the other centrosome. However, it is critical to define the size of the box consistently for each cell analyzed within one data set (e.g., either by the length of the spindle or the diameter of the cell).

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