Title
Chapter 25 Imaging and physically probing kinetochores in live dividing cells

Authors
Kuhn, Jonathan
Dumont, Sophie

Publication Date
2014

DOI
10.1016/b978-0-12-420138-5.00025-2

Peer reviewed

HHS Public Access

Author manuscript

Methods Cell Biol. Author manuscript; available in PMC 2015 May 22.

Published in final edited form as:

Methods Cell Biol. 2014 ; 123: 467–487. doi:10.1016/B978-0-12-420138-5.00025-2.

Imaging and physically probing kinetochores in live dividing cells

Jonathan Kuhn[1,2] and **Sophie Dumont**[1,2,3]

[1]Department of Cell & Tissue Biology, University of California, San Francisco, USA

[2]Tetrad Graduate Program, University of California, San Francisco, USA

[3]Department of Cellular & Molecular Pharmacology, University of California, San Francisco, USA

Abstract

The kinetochore mediates chromosome segregation at cell division. It is the macromolecular machine that links chromosomes to spindle microtubules, and is made of more than 100 protein species in mammalian cells. Molecular tools are presently revealing the biochemical interactions and regulatory mechanisms that ensure proper kinetochore function. Here, we discuss two approaches for imaging and physically probing kinetochores despite mitotic cell rounding and rapid kinetochore dynamics. First, we describe how mild spindle compression can improve kinetochore imaging, and how stronger compression can mechanically perturb the spindle and kinetochores. Second, we describe how simultaneously imaging two-colored kinetochore reporter probes at sub-pixel resolution can report on kinetochore structural dynamics under cellular forces. We hope that the experimental details we provide here will make these two approaches broadly accessible and help move forward our understanding of kinetochore function – and make these approaches adaptable to the study of other cellular structures.

Keywords

cell division; cell rounding; live mammalian cell; mechanical perturbation; spindle compression; sub-pixel imaging; kinetochore; structural dynamics

Introduction

The kinetochore

During cell division, the two daughter cells must inherit exactly one copy of each chromosome. Errors can lead to cell death or cancer in somatic cells or developmental disorders in the germline. Chromosome segregation is mediated by the kinetochore, a 100nm-sized macromolecular machine that anchors chromosomes to microtubules in the spindle. The kinetochore regulates chromosome segregation and generates forces for chromosome movement. We now know most of the proteins that make up the kinetochore – more than 100 of them in mammalian cells (Cheeseman and Desai, 2008) – and are currently uncovering the kinetochore's underlying biochemical interactions and regulatory

Correspondence: sophie.dumont@ucsf.edu.

mechanisms. In parallel, we have begun to elucidate how kinetochores generate and respond to mechanical force. Mechanical forces assemble the spindle (Karsenti and Vernos, 2001), move chromosomes within it (Nicklas, 1983), and stabilize and correct kinetochore-microtubule attachments (Nicklas and Koch, 1969). Force has also been proposed to regulate chromosome segregation (Li and Nicklas, 1995). To understand how kinetochores generate and respond to force, we need approaches for imaging kinetochore movement and structural dynamics under different forces, and externally perturbing mechanical forces. Here we focus on two such approaches that are conceptually simple and can be applied to live mammalian cells.

Mammalian cells: challenges

While there are many powerful genetic and biochemical techniques available in mammalian cells, the fact that mammalian cells round up at mitosis and that kinetochores are located deep within the cell make imaging and mechanical perturbations based on physical contact (such as with microneedles) difficult. In addition, kinetochores move rapidly, often in and out of a chosen focal plane over time, making it difficult to follow the movement of individual kinetochores over long periods. Kinetochores can also change their tilt angles with respect to the coverslip over time, confounding attempts to image molecular-scale rearrangements within kinetochores.

Chapter overview

In this chapter we first describe a simple approach utilizing an agarose pad and micromanipulator for compressing dividing mammalian cells and their spindles. Mild compression brings kinetochores closer to the coverslip to improve imaging, and brings the spindle and kinetochore-microtubule axis roughly parallel to the coverslip. In turn, medium compression directly flattens the spindle, confining all kinetochores to a smaller volume, bringing more kinetochores into the same focal plane, and limiting kinetochore movement in and out of focus. Even stronger compression can limit chromosome movements, externally controlling – and increasing – the cellular forces that kinetochores experience. Second, we describe the use of simultaneous two-color sub-pixel imaging of kinetochore reporter probes. In combination with mild compression to help confine and align kinetochores, this allows monitoring structural kinetochore dynamics in real time under different cellular forces.

Spindle compression to image and perturb kinetochores

Historical context

Several mechanical approaches have been used to probe chromosome segregation in live cells: some to improve imaging, and others to mechanically perturb mitotic cells and their macromolecular machines. Examples of mechanical approaches to improve live cell imaging include: coating coverslips (e.g. with Poly-L-Lysine) to help keep dividing cells flat; confining dividing cells in PDMS devices of different heights (Le Berre, Aubertin and Piel, 2012); and laying an agar pad on top of a cell to reduce mitotic rounding and movement (Fukui, Yumura and Yumura 1987; Pereira, Matos, Lince-Faria and Maiato, 2009). Examples of physical perturbation approaches include microneedles to exert and measure

tension on individual chromosomes and kinetochores in insect spermatocyte cells (Nicklas, 1983; Nicklas and Staehly, 1967); optical tweezers to move chromosomes inside mammalian cells (Liang, Wright, He and Berns, 1991); and laser ablation to probe kinetochore motility (Khodjakov and Rieder, 1996), kinetochore signaling (Rieder, Cole, Khodjakov and Sluder, 1995) and spindle mechanics (Snyder, Armstrong, Stonington, Spurck and Pickett-Heaps, 1991). We note that outside live cells, optical tweezers have given us unprecedented access to kinetochore-microtubule attachment mechanics (Akiyoshi, Sarangapani, Powers, Nelson, Reichow, Arellano-Santoyo et al., 2010).

Motivation

Here we describe how spindles can be compressed in live mammalian cells using a micromanipulator to controllably and reversibly press an agarose pad down on the cell (Figure 1). Unlike some methods of mechanical perturbation, spindle compression is compatible with high resolution live imaging and indeed improves image quality. Furthermore, spindle compression requires little equipment besides a micromanipulator, and is fully compatible with cell health. Mild compression has been used to improve conditions for sub-pixel kinetochore imaging (Dumont, Salmon and Mitchison, 2012), medium compression to study the response of spindle size to mechanical force (Dumont and Mitchison, 2009), and strong compression to exert extraordinary forces on kinetochores (Dumont et al., 2012). Below, we provide protocols for culturing cells in preparation for compression, preparing agarose pads, the experimental setup we use for compression, and details on executing and monitoring compression. We end this section with tips for troubleshooting common issues.

Methods

Choice of cell line—Protocols in this chapter will focus on one type of mammalian cells: rat kangaroo kidney epithelial (Ptk2) cells. These already remain relatively flat at mitosis, and have a small number of chromosomes – just 13 of them (Humphrey and Brinkley, 1969) – which are large and thus easy to image and distinguish from one another. They are amenable to RNAi, transfections and other molecular techniques (Guimaraes, Dong, McEwen and Deluca, 2008; Stout, Rizk, Kline and Walczak, 2006). Based on our experience, we anticipate that compression will work in a variety of cell types, and is easiest in flatter cells.

Cell culture—We culture Ptk2 cells in MEM (Invitrogen 11095) supplemented with sodium pyruvate (Invitrogen 11360), nonessential amino acids (Invitrogen 11140), penicillin/streptomycin, and 10 % qualified and heat-inactivated fetal bovine serum (Invitrogen 10438). We plate cells on #1.5 25 mm round coverslips (HCl-cleaned, poly-L-lysine coated), and image them in Leibovitz's L-15 medium with L-glutamine without phenol red (Invitrogen 21083) with antibiotics and serum as above. When culturing Ptk2 cells, we recommend keeping cell confluency between 40 % and 90 %. When plating cells on coverslips for imaging, we typically dilute them to about 60 % confluency and grow them for two days prior to imaging. Hallmarks of healthy cells include a high fraction of mitotic cells (rapidly growing population), flat cells (including mitotic ones) that establish

strong junctions with neighbors and display a “football-shaped” spindle area free of mitochondria, reflecting high microtubule density.

Agarose pad preparation—Begin by mixing a solution of PBS and 2 % ultrapure agarose (Invitrogen 15510). The agarose concentration is chosen such that the pad is rigid enough to compress a large area of cells, and compliant enough for compression to be robust, easy and safe to perform. Boil this solution in the microwave until clear. Let cool for 5 min, and then use a plastic bulb pipet to fill a 60 mm plastic tissue culture dish with agarose solution around 2–3 mm deep (6–8 ml). Let the agarose solution cool until it appears “cloudy” and has solidified. Cut the agarose into 10–15 mm-wide squares, making sure to exclude any piece of agarose that touches the edge of the plate and curves up (such that the pad has uniform thickness). Store these squares in imaging media (described above) and let them sit overnight at 4 °C before using, so that they can equilibrate with the imaging media. At 4 °C, the pads will last at least one week. Before using any agarose pads on cells, warm them to the imaging temperature (we use 29–30 °C) immediately before imaging. If combining compression with pharmacological treatments (e.g. taxol), incubate the pads overnight in imaging media supplemented with the desired drug concentration. Adding a drug to, or washing a drug out from, a currently compressed cell may not be possible on a rapid timescale.

Experimental setup—We perform live imaging on a Nikon Eclipse Ti inverted microscope with 100X 1.45 Ph3 oil objective through a 1.5X lens yielding 105 nm/pixel. Before, during and after compression, we image cells by phase contrast and spinning disk confocal (Yokogawa CSU-X1) fluorescence imaging every few seconds on an Andor iXon3 camera, and keep cells in focus with the Nikon Perfect Focus System. We mount an oil hydraulic fine micromanipulator (Narashige MO-202) and coarse manipulator directly to our automated stage (ASI MS-2000 XYZ) (Figure 2). We attach a metal rod (2 mm wide) to the fine micromanipulator to directly contact the agarose pad. We image cells at 29–30 °C in a homemade heated aluminum coverslip holder accepting 25 mm round coverslips that allows access of the micromanipulator rod to the coverslip at shallow angles. To facilitate access of the rod to the coverslip, it is easier to use a low numerical aperture (NA) condenser (we use 0.52 NA).

Before spindle compression—Mount the coverslip in its temperature-controlled holder, fill the holder with 3–4 ml of 30 °C media, and mount the holder on the microscope. Use phase contrast imaging to locate a good cell for compression and imaging, and center it in the field of view. The ideal cell to compress (Figure 3) and image will be as flat as possible prior to compression so that additional flattening perturbs as little as possible, have significant contact with neighboring cells but still have cell-free space around it to flatten (an area about 80 % confluent is ideal for this) and have a clear spindle region. It is critical that the cell to be compressed is away from the coverslip edges so that the agarose pad can be centered over it, and the micromanipulator rod can access it (Figure 2). After finding a good cell, gently deposit the agarose pad on top of the coverslip by placing it in the media. Once the pad has dropped onto the coverslip, gently nudge the side with tweezers to put the center of the pad directly above the objective. Next, position the micromanipulator rod by

hand so that the rod end is directly above the objective and is touching the media but not the pad. Position the rod at as shallow an angle as possible (Figure 2). Take care to ensure that the micromanipulator Z-control knob is adjusted to the top of its range, so that the rod will have sufficient range to reach the pad. Then, finely adjust the position of the rod using fine micromanipulator X- and Y-control knobs to ensure it is centered above the cell (looking through the Bertrand lens is helpful).

Spindle compression—Once the rod is centered, slowly begin lowering it using the micromanipulator Z-control knob. If imaging is needed during compression, we typically send 80% of phase contrast imaging light to the camera and 20% to microscope eyepieces as these have a bigger field of view than the camera and thus allow more careful monitoring of compression progress. As the micromanipulator rod is lowered, the phase contrast image may begin to darken; if so, the condenser must be lowered as the rod is lowered to preserve Koehler illumination. Continue lowering the rod until particles in the media begin to rush (as seen through the eyepieces), reflecting first contact of the rod with the pad. From this point, only lower the rod a few microns at a time. The first sign that downward force is being exerted on the cell is that the spindle and chromosomes will spread outward. Adjustments to the focal plane are also typically necessary during compression as the position of the spindle with respect to the coverslip changes.

Choice of compression levels—We consider three compression levels, each with a different effect and purpose. Compression extent is fully under micromanipulator control, and is reversible. The extent of compression can simply be monitored by the surface area of the cell as viewed in phase contrast imaging, and can be further characterized by monitoring spindle thickness via 3D imaging (Z-stacks). While we do not know the precise amount of force that compression applies to the spindle, we estimate it to be on the order of hundreds of nanoNewtons (Dumont et al., 2009).

i. To achieve mild compression (Figure 1B), lower the rod but stop right when the cell begins to bleb. This level of compression (typically 100–150 μm of micromanipulator travel from the point of first contact of the rod and pad) flattens the cell mildly, brings the spindle and kinetochores closer to the coverslip without flattening the spindle, and aligns the spindle with the coverslip axis without affecting chromosome motion – making it easier to follow kinetochores over time. The response of the cells to this compression level is reversible when the compression force is removed.

ii. To achieve medium compression (Figure 1C), continue lowering the rod for a few microns after the cell begins to bleb (after which it may or may not continue to bleb). Medium compression flattens the spindle: it widens the spindle (passively, over seconds) and lengthens it (actively, over minutes) up to 40%, and does not affect kinetochore motility dynamics or inter-kinetochore distance, a proxy for kinetochore tension (Dumont et al., 2009). Medium compression improves imaging (Figure 4): it confines all kinetochores to a smaller volume to image, brings more kinetochores into the same focal plane and limits kinetochore movement in and out of the focal plane. For example, in one study we found that Ptk2 spindles were

about 5–7 μm in height (from top to bottom kinetochore-fibers) before and 3–4 μm in height after this level of compression (Dumont et al., 2009). As the pad is deformable, this can require 10 μm or more of micromanipulator travel beyond mild compression in our conditions. The response of the cell and spindle to this compression level is typically reversible.

iii. To achieve strong compression (Figure 1D), continue lowering the rod until some chromosomes can no longer move freely because cell height is not sufficient. This can require an additional 10 μm or more of micromanipulator travel beyond medium compression in our conditions. In addition to improving imaging as for milder compression, strong compression effectively pins down some of the chromosomes to the coverslip, while microtubules are still exerting pulling forces on kinetochores: strong compression can prevent one kinetochore from moving, while its sister kinetochore is free to move, yielding inter-kinetochore distances significantly above the normal range (Dumont et al., 2012). This results in extraordinary forces being exerted on kinetochores, and thus serves as a means to mechanically perturb kinetochores inside live cells. If such strong compression force is removed quickly by raising the rod rapidly, the spindle response is not reversible; however, if compression is removed more slowly (over ≫ 10 s), the spindle responds more reversibly. For strong compression, be particularly cautious to align the rod on top of the cell and objective to avoid cracking the coverslip.

For all three compression levels, we generally lower the compression rod over 10–20 s, and keep it down in position for as long as we want to image or perturb the cell. For compression levels (i) and (ii), anaphase entry is not significantly delayed during or after compression. Monitoring the time to anaphase entry is excellent practice.

After spindle compression—After compression is no longer needed, raise the rod upward with the micromanipulator. If other cells are to be used in the area of the compressed cell, raise the micromanipulator rod slowly to encourage reversible responses to compression removal. Importantly, make sure to never move the rod or the pad in X-Y while a cell is under compression as this could shear cells off the coverslip. First raise the micromanipulator rod before moving it in the X-Y plane.

Troubleshooting tips

i) Poor phase contrast image in compressed cells: Both the agarose pad and micromanipulator rod are in the transmitted light path and can thus affect phase contrast imaging. Koehler illumination should be maintained for good phase contrast generation, which in our experience may require bringing the condenser down during compression. Having about a 1.5 cm high pool of imaging media in the holder (several milliliters of media in our holder) is also helpful, and makes imaging more robust to media evaporation.

ii) Difficulty maintaining compression: If compression is not maintained and the cell rounds up during compression, this suggests that the area being compressed is not directly over the cell. Either the rod or pad is not directly above the objective. Slowly release

compression and correctly center the rod and pad in the X-Y plane. Cells at the edge of the coverslip may be hard to compress, either because the rod or pad center cannot reach them.

iii) Cell death upon compression: When compressing, cell bleb expansion and retraction are often observed and are expected and normal. However, excessive blebbing and lack of bleb retraction indicate compression is too strong for cell health maintenance. In particular, if the cytoplasm slowly leaks or suddenly bursts – leading to cell death – this indicates that the compression level was too strong.

iv) Coverslip cracking upon compression: If this occurs, it is most likely because the rod or pad is miscentered and not above the cell and objective. If the cell does not begin to compress shortly after the media rushes when the pad is brought down, raise the rod, re-center the rod and pad, and try again.

Imaging kinetochore dynamics at sub-pixel resolution via two-color reporter probes

Historical context

In the previous section we described spindle compression as a method to improve kinetochore imaging under cellular forces and to externally perturb these forces. Here, we describe a method for monitoring the structural dynamics of kinetochores under force in live cells. Most of what we know about the global organization of mammalian kinetochores has come from electron microscopy, which reveals a multilayered structure (Dong, Vanden Beldt, Meng, Khodjakov and McEwen, 2007): an inner plate at centromeric chromatin and outer plate near microtubule plus-ends. In turn, biochemical analysis has revealed information on relative positions of different kinetochore proteins between chromatin and microtubules (Cheeseman et al., 2008). Until a few years ago, obtaining high resolution positional data with molecular specificity presented a major challenge: while kinetochore proteins could be tagged with fluorescent markers, the distance between chromatin-binding and microtubule-binding kinetochore proteins (about 100 nm) is below the diffraction limit of light. FRET (Förster resonance energy transfer) only informs on distances smaller than 10 nm. The application of Gaussian-fitting to find the center of diffraction-limited objects (Yildiz, Forkey, McKinney, Ha, Goldman and Selvin, 2003) and the distance between them (Churchman, Okten, Rock, Dawson and Spudich, 2005) now provides us with a means to use light imaging to position kinetochore proteins with respect to each other, and to measure linkage deformations under different conditions.

Motivation

Here we describe an adaptation of SHREC, single molecule high-resolution colocalization (Churchman et al., 2005), to measure the distance between two groups of differently colored reporter probes within a single kinetochore in live mammalian cells, which we refer to as “intra-kinetochore distance” (Wan, O’Quinn, Pierce, Joglekar, Gall, DeLuca et al., 2009). SHREC in fixed cells has revealed the architecture of the mammalian kinetochore-microtubule attachment site with 5 nm accuracy (Wan et al., 2009). Three key features make kinetochore live cell SHREC possible: i) the high copy number of most protein species

within one kinetochore means that enough photons can be collected with genetically-encoded fluorescent proteins; ii) the natural orientation of the kinetochore-microtubule axis roughly parallel to the coverslip axis makes it possible to measure intra-kinetochore distances along this axis; iii) the cyclical nature of kinetochore movements in mammalian metaphase chromosome oscillations makes data averaging possible by providing clear synchronization points. However, challenges to live kinetochore SHREC measurements are significant: kinetochores are found deep inside round mitotic cells, move up and down – and can tilt – with respect to the imaging plane, and move fast; in addition, not all copies of the same protein may localize in a similar manner. Yet, using live kinetochore SHREC, it has been possible to relate structural dynamics and different microtubule forces (Dumont et al., 2012; Joglekar, Bloom and Salmon, 2009). Below we describe cell preparation, the experimental setup, imaging, and basic data analysis guidelines for live kinetochore SHREC, and mention key questions to consider in data interpretation.

Methods

Gaussian-fitting for sub-pixel resolution—If a single fluorescent molecule is imaged, it forms a diffraction limited image of width $\lambda/(2NA)$, with λ the wavelength of light and NA the collection objective numerical aperture. If we fit the image to a 2D Gaussian, the mean corresponds to the position of the single molecule. The standard error of the mean, which reflects our ability to estimate the mean, will depend on photon noise, the effect of the detector's finite pixel size, and background noise (Thompson, Larson and Webb, 2002; Yildiz et al., 2003). Thus, for improved accuracy we must collect as many photons as possible and choose a camera carefully. Although Gaussian-fitting approaches were developed for single molecules, they have been applied to groups of kinetochore proteins: there are several copies of each kinetochore protein per microtubule (Johnston, Joglekar, Hori, Suzuki, Fukagawa and Salmon, 2010), and multiple microtubules per mammalian kinetochore (20–25 in Ptk cells, (McEwen, Heagle, Cassels, Buttle and Rieder, 1997)), and thus there are a couple to a few hundred copies of some proteins at each mammalian kinetochore. As discussed below, care must be taken to understand the assumptions behind the use of a single-molecule technique for groups of molecules.

Choice of cell line and reporter probes—We focus on Ptk2 rat kangaroo kidney epithelial cells for the same reasons as for spindle compression. To measure intra-kinetochore distances via live SHREC, two reporter probes (Figure 5A) should be chosen such that i) the labeled kinetochore proteins are expected to be sufficiently distant from each other (50 nm and above are good starting points) – based on protein distances in fixed cells (Wan et al., 2009) and related biochemical data – for live SHREC to robustly resolve this distance; ii) they are fused to fluorescent proteins with non-overlapping spectra; iii) they can be expressed at high enough levels in cells without adversely affecting function, to get high photon counts and a good estimate of the Gaussian center; iv) they are not known to bind anywhere else near kinetochores, as this could affect Gaussian-fitting (e.g. to microtubules in a kinetochore-independent manner). In addition, to aid in data interpretation on the relationship between intra-kinetochore distances and microtubule forces we recommend choosing reporter probes which take on structural (as opposed to regulatory) roles at kinetochores when possible given above constraints.

Expression of reporter probes—Two reporter probes must be expressed either using transient transfection or infection, or in stable cell lines. For example, we have recently used transient transfection to express Hec1-EGFP or EYFP-Cdc20 (outer kinetochore proteins) and CenpC-mCherry (inner kinetochore protein) in Ptk2 cells (Figure 5B–C): we transfect cells on a 25mm coverslip in a 6-well plate using 0.5 μg of each plasmid with 3 μl Fugene6 (Promega E2693) with 2.5 ml of media without antibiotics. Cells are either plated on coverslips 24 h before transfection, or plated and transfected together. Cells are imaged 36–48h post-transfection in imaging media as described above. Cells expressing one probe will typically also express the other, and using the above protocol we find that 30–50% of cells express both probes.

Experimental setup—We use the setup described for spindle compression, with a Yokogawa CSU-X1 spinning disk confocal with 488 nm and 561 nm diode lasers (100–150 mW lasers, used at a fraction of their power) and add a DualView (Photometrics, Chroma 565dcxr dichroic and ET525/50M and ET630/75M emission filters) module for simultaneous EGFP or EYFP and mCherry camera acquisition (Figure 5B–C). We have confirmed the lack of channel crosstalk with EGFP or EYFP and mCherry (Semrock Yokogawa dichroic Di01-T405/488/561). We use an Andor iXon3 camera with 5X pre-amp gain and no EM gain: EGFP or EYFP and mCherry are simultaneously excited, and emissions collected simultaneously on each camera half. Simultaneous imaging is critical because of fast kinetochore movements at mammalian mitosis (if kinetochores move at 1 μm/min, this means 17 nm each second!).

Before live cell imaging: two-color bead registration—Before the distance between two different kinetochore protein populations can be measured with EGFP/EYFP and mCherry probes, one must map chromatic aberrations (how the same object appears in two different colors) over the field of view (Figure 6A). To do this, we mount scattered TetraSpeck 100 nm beads (Invitrogen T-7279) to a coverslip surface and simultaneously image the same beads in both green and red channels. We then use the 2D-Gaussian-fitted centers (*lsqcurvefit*, Matlab) of these beads in both channels to create a position-dependent 2D transform (we find that Matlab's *cp2tform* with polynomial degree 2 works well) that accounts for chromatic aberrations (Churchman et al., 2005). This transform can then be applied to other bead slides to probe its error. If performance is satisfactory, it can then be used to register (i.e. correctly align and relatively position) EGFP/EYFP and mCherry kinetochore images together and ultimately measure intra-kinetochore distances. In our experience, it is helpful to perform this bead registration every day before beginning imaging.

Sub-pixel resolution kinetochore imaging via two-color reporter probes—We use phase contrast to find metaphase cells without bleaching fluorophores, and then confocal imaging to assess whether both probes are expressed, and whether their expression level (i.e. collected photon count) is high enough for needed localization accuracy. For CenpC-mCherry and Hec1-EGFP or EYFP-Cdc20, we typically collect 4000–7000 photons/kinetochore (which we can estimate using the electron-to-photon conversion factor obtained after camera calibration), and the signal-to-noise ratio (SNR) is typically 15–20 (SNR=I_{max}/

$\sqrt{(I_{max}+b^2)}$, with I_{max} the maximum pixel photon count and b the background photon standard deviation). Once a proper cell has been identified, we perform medium compression (as described above) to i) bring more kinetochores in the same plane, which means faster data collection; ii) limit out of plane movement, which allows us to follow a single kinetochore pair over long times as it experiences different forces; iii) help align the kinetochore-microtubule axis to the coverslip, since this is the axis along which we measure distance. We typically wait a few minutes between compression start and imaging start. At every time point, we acquire a phase contrast image to monitor cell health and associate kinetochores in pairs (a proxy for tension) by identifying chromosomes, and a simultaneous two-color confocal image to monitor the distance between the two kinetochore probes (Figure 6B). Images are acquired at 105 nm/pixel (bin=1), and exposure times are kept as short as possible to avoid blurring the distributions due to movement. Because we attempt to follow the same kinetochore over long times as microtubule forces change, we do not typically collect Z-stacks to avoid photobleaching, and thus only perform Gaussian fitting in 2D. If Z-stacks can be acquired, Gaussian fitting in 3D has the advantage of reporting on kinetochore tilt.

Data analysis for sub-pixel resolution kinetochore imaging—After data collection, we begin by tracking each kinetochore's position over time (SpeckleTracker, Matlab program written by Xiaohu Wan), and then determine the centroids of the Hec1-EGFP or EYFP-Cdc20 and CenpC-mCherry probes at each time point by fitting a 2D Gaussian (*lsqcurvefit*, Matlab) in a 10×10 pixel box (Figure 6C–D). Applying the two-color bead registration map to the EGFP/EYFP and mCherry images, we then find the inter-probe distance at each time (Figure 6E): this distance fluctuates broadly over time, and thus we pool together inter-probe distances from different times, kinetochores and cells in conditions we believe to be similar (Figure 6F). Metaphase chromosome oscillations can be used as a system where averaging can be performed over well-defined periodically recurring events: for example, in recent work we found that the inter-probe distance was different by an average of 8 nm in kinetochores moving toward and away from the spindle pole (Figure 6E–F) (Dumont et al., 2012). To validate such conclusions, it is essential to check whether individual kinetochores behave – on average – like the means do. We calculate inter-probe distance as the mean of the Gaussian fit of the distance distribution, and note that because the inter-probe distance cannot be negative, this can overestimate the inter-probe distance – particularly for small distances (Churchman, Flyvbjerg and Spudich, 2006). In our conditions, we measure the standard deviation of the inter-probe distance distribution to be about 20 nm, and this includes contributions from centroid determination with limited photon counts (4–6 nm accuracy for our conditions (Mortensen, Churchman, Spudich and Flyvbjerg, 2010)), two-color registration map estimation errors (target registration error as high as 7 nm in our tests (Churchman et al., 2005)), map application to inhomogeneous environments deep inside the cell, and biological variation (Dumont et al., 2012).

Key considerations for interpretation of inter-probe distances

i) Meaning of the Gaussian center and of inter-probe distances: It is important to keep in mind that the Gaussian-fit center of many copies of the same kinetochore protein does not necessarily correspond to the location of all protein copies (Figure 7A). If protein copies are

tightly clustered, mean localization will not be affected; however, if protein copies are too scattered, then assumptions behind Gaussian fitting break down (Joglekar et al., 2009; Wan et al., 2009). In an extreme example, if the protein copies were distributed in a bimodal distribution, the centroid could be in a region where few protein copies actually reside. The position of the centroid reflects the position of the center of mass of a group of proteins.

ii) Contributions of non-structural factors to changes in inter-probe distances: While it is tempting to interpret changes in inter-probe distance as structural changes within the kinetochore, other events may lead to inter-probe distance changes. Most notably, both kinetochore tilt with respect to the coverslip and changes in protein binding sites at the kinetochore could lead to apparent inter-probe changes. First, given that most microtubules terminate at the kinetochore within a 30 nm band along the microtubule axis (McEwen and Heagle, 1997) and that microtubule plus-ends are located in a 400 nm diameter circle in Ptk kinetochores (McDonald, O'Toole, Mastronarde and McIntosh, 1992), one can estimate the amount by which tilt would be expected to widen the kinetochore probe image standard deviation (Dumont et al., 2012). While such tilt has thus far been insufficient to explain observed changes in distance between Gaussian centers, its possible effects must always be considered (Figure 7B). Second, dynamic changes in protein binding may always contribute to apparent inter-probe distance changes (e.g. if reporter proteins bound different sites on the kinetochore over time; Figure 7C), and such possibilities can be carefully evaluated by measuring changes in parameters such as protein intensities, recovery kinetics after photobleaching, and Gaussian standard deviations over time.

Conclusion and outlook

Mammalian cells round up when they divide, and many key structures that mediate division are highly dynamic. Together, these make imaging and physically probing cell division structures difficult. In this chapter, we have presented two conceptually simple methods to image and physically probe kinetochores in live dividing mammalian cells: i) spindle compression improves imaging inside round cells, and stronger compression can be used as a tool to mechanically perturb the spindle and kinetochores; ii) in turn, sub-pixel imaging of kinetochore linkages can probe kinetochore structural dynamics under cellular forces. We hope that the experimental details we provide, as well as our open discussion of common technical and interpretation pitfalls, will make these two approaches broadly accessible – and together move forward our understanding of kinetochore function. Lastly, we note that these methods – once adapted – may help us image and physically probe other cellular structures.

Acknowledgments

We thank Tim Mitchison and Ted Salmon for conceptual and technical suggestions, Stirling Churchman and Jennifer Waters for quantitative microscopy advice, Xiaohu Wan for sharing the SpeckleTracker Matlab program, Jagesh Shah for the stable Ptk2 EYFP-Cdc20 line, Jennifer DeLuca for constructs and help culturing and transfecting Ptk cells, Christopher Carroll for constructs, and the Dumont Lab for discussions and critical reading of the manuscript. Work was supported by NIH T32GM007810 (J.K.), NIH R00GM09433 and UCSF PBBR funds. S.D. is a Searle, Kimmel and Rita Allen Foundation Scholar and a Sloan Research Fellow.

References

Akiyoshi B, Sarangapani KK, Powers AF, Nelson CR, Reichow SL, Arellano-Santoyo H, et al. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. Nature. 2010; 468:576–579. [PubMed: 21107429]

Cheeseman IM, Desai A. Molecular architecture of the kinetochore-microtubule interface. Nature Reviews Molecular and Cell Biology. 2008; 9:33–46.

Churchman LS, Flyvbjerg H, Spudich JA. A non-Gaussian distribution quantifies distances measured with fluorescence localization techniques. Biophysical Journal. 2006; 90:668–671. [PubMed: 16258038]

Churchman LS, Okten Z, Rock RS, Dawson JF, Spudich JA. Single molecule high-resolution colocalization of Cy3 and Cy5 attached to macromolecules measures intramolecular distances through time. Proceedings of the National Academy of Sciences. 2005; 102:1419–1423.

Dong Y, Vanden Beldt KJ, Meng X, Khodjakov A, McEwen BF. The outer plate in vertebrate kinetochores is a flexible network with multiple microtubule interactions. Nature Cell Biology. 2007; 9:516–522.

Dumont S, Mitchison TJ. Compression regulates spindle length by a mechanochemical switch at the poles. Current Biology. 2009; 19:1086–1095. [PubMed: 19540117]

Dumont S, Salmon ED, Mitchison T. Deformations within moving kinetochores reveal different sites of active and passive force generation. Science. 2012; 337:355–358. [PubMed: 22722252]

Fukui Y, Yumura S, Yumura TK. Agar-overlay immunofluorescence: high-resolution studies of cytoskeletal components and their changes during chemotaxis. Methods in Cell Biology. 1987; 28:347–356. [PubMed: 3298995]

Guimaraes GJ, Dong Y, McEwen BF, Deluca JG. Kinetochore-microtubule attachment relies on the disordered N-terminal tail domain of Hec1. Current Biology. 2008; 18:1778–1784. [PubMed: 19026543]

Howell BJ, Moree B, Farrar EM, Stewart S, Fang G, Salmon ED. Spindle checkpoint protein dynamics at kinetochores in living cells. Current Biology. 2004; 14:953–964. [PubMed: 15182668]

Humphrey RM, Brinkley BR. Ultrastructural studies of radiation-induced chromosome damage. Journal of Cell Biology. 1969; 42:745–753. [PubMed: 4895598]

Joglekar AP, Bloom K, Salmon ED. In vivo protein architecture of the eukaryotic kinetochore with nanometer scale accuracy. Current Biology. 2009; 19:694–699. [PubMed: 19345105]

Johnston K, Joglekar A, Hori T, Suzuki A, Fukagawa T, Salmon ED. Vertebrate kinetochore protein architecture: protein copy number. Journal of Cell Biology. 2010; 189:937–943. [PubMed: 20548100]

Karsenti E, Vernos I. The mitotic spindle: a self-made machine. Science. 2001; 294:543–547. [PubMed: 11641489]

Khodjakov A, Rieder CL. Kinetochores moving away from their associated pole do not exert a significant pushing force on the chromosome. Journal of Cell Biology. 1996; 135:315–327. [PubMed: 8896591]

Le, Berre M.; Aubertin, J.; Piel, M. Fine control of nuclear confinement identifies a threshold deformation leading to lamina rupture and induction of specific genes. Integrative biology: quantitative biosciences from nano to macro. 2012; 4:1406–1414. [PubMed: 23038068]

Li X, Nicklas RB. Mitotic forces control a cell-cycle checkpoint. Nature. 1995; 373:630–632. [PubMed: 7854422]

Liang H, Wright WH, He W, Berns MW. Micromanipulation of mitotic chromosomes in PTK2 cells using laser-induced optical forces (“optical tweezers”). Experimental Cell Research. 1991; 197:21–35. [PubMed: 1915660]

McDonald KL, O’Toole ET, Mastronarde DN, McIntosh JR. Kinetochore microtubules in PTK cells. Journal of Cell Biology. 1992; 118:369–383. [PubMed: 1629239]

McEwen BF, Heagle AB. Electron microscopic tomography: A tool for probing the structure and function of subcellular components. International Journal of Imaging Systems and Technology. 1997; 8:175–187.

McEwen BF, Heagle AB, Cassels GO, Buttle KF, Rieder CL. Kinetochore-fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. Journal of Cell Biology. 1997; 137:1567–1580. [PubMed: 9199171]

Mortensen KI, Churchman LS, Spudich J, Flyvbjerg H. Optimized localization analysis for single-molecule tracking and super-resolution microscopy. Nature Methods. 2010; 7:377–381. [PubMed: 20364147]

Nicklas RB. Measurements of the force produced by the mitotic spindle in anaphase. Journal of Cell Biology. 1983; 97:532–548.

Nicklas RB, Koch CA. Chromosome micromanipulation III: Spindle fiber tension and the reorientation of mal-oriented chromosomes. Journal of Cell Biology. 1969; 43:40–50. [PubMed: 5824068]

Nicklas RB, Staehly CA. Chromosome micromanipulation. I. The mechanics of chromosome attachment to the spindle. Chromosoma. 1967; 21:1–16. [PubMed: 5339860]

Pereira J, Matos I, Lince-Faria M, Maiato H. Dissecting mitosis with laser microsurgery and RNAi in Drosophila cells. Methods in Molecular Biology. 2009; 545:145–164. [PubMed: 19475387]

Rieder CL, Cole RW, Khodjakov A, Sluder G. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. Journal of Cell Biology. 1995; 130:941–948. [PubMed: 7642709]

Snyder JA, Armstrong L, Stonington OG, Spurck TP, Pickett-Heaps JD. UV-microbeam irradiations of the mitotic spindle: spindle forces and structural analysis of lesions. European Journal of Cell Biology. 1991; 55:122–132. [PubMed: 1915410]

Stout JR, Rizk RS, Kline SL, Walczak CE. Deciphering protein function during mitosis in PtK cells using RNAi. BMC Cell Biology. 2006; 7:26. [PubMed: 16796742]

Thompson RE, Larson DR, Webb WW. Precise nanometer localization analysis for individual fluorescent probes. Biophysical Journal. 2002; 82:2775–2783. [PubMed: 11964263]

Wan X, O'Quinn RP, Pierce HL, Joglekar AP, Gall WE, DeLuca JG, et al. Protein Architecture of the Human Kinetochore Microtubule Attachment Site. Cell. 2009; 137:672684.

Yildiz A, Forkey JN, McKinney SA, Ha T, Goldman YE, Selvin PR. Myosin V walks hand-over-hand: single fluorophore imaging with 1.5-nm localization. Science. 2003; 300:2061. [PubMed: 12791999]

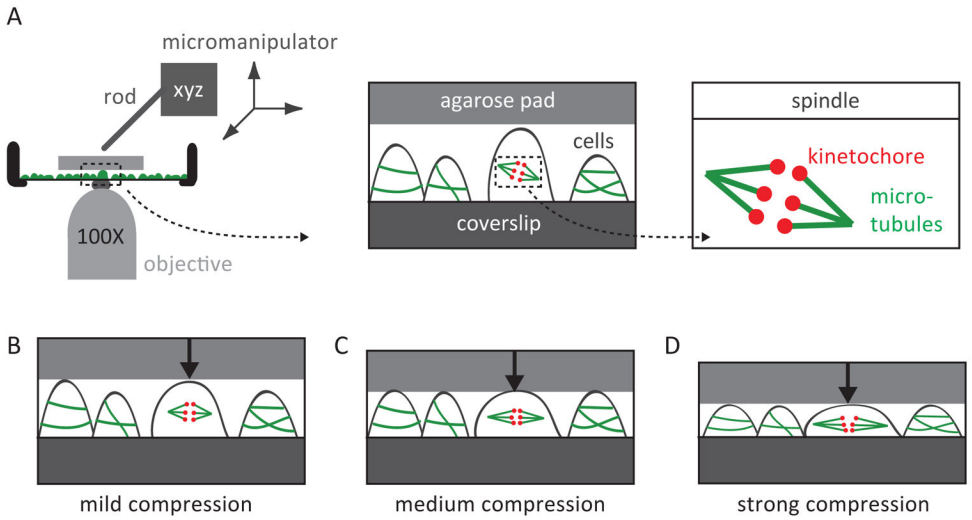

Figure 1.

Spindle compression. **(A)** Experimental sketch for spindle compression. A micromanipulator presses a rod down on an agarose pad to compress the cells below it. **(B)** Mild compression flattens the cell, brings the spindle and kinetochores closer to the coverslip, and aligns the spindle with the coverslip axis – making it easier to follow kinetochores over time (Dumont et al., 2012). **(C)** Medium compression (Dumont et al., 2009) widens and lengthens the spindle, does not affect kinetochore motion and tension, and improves imaging by bringing more kinetochores into focus and limiting their movement in and out of the focal plane. **(D)** Strong compression improves imaging and pins down some of the chromosomes to the coverslip, preventing them from moving and resulting in inter-kinetochore distances significantly above the normal range (Dumont et al., 2012). Part (A) adapted from (Dumont et al., 2009).

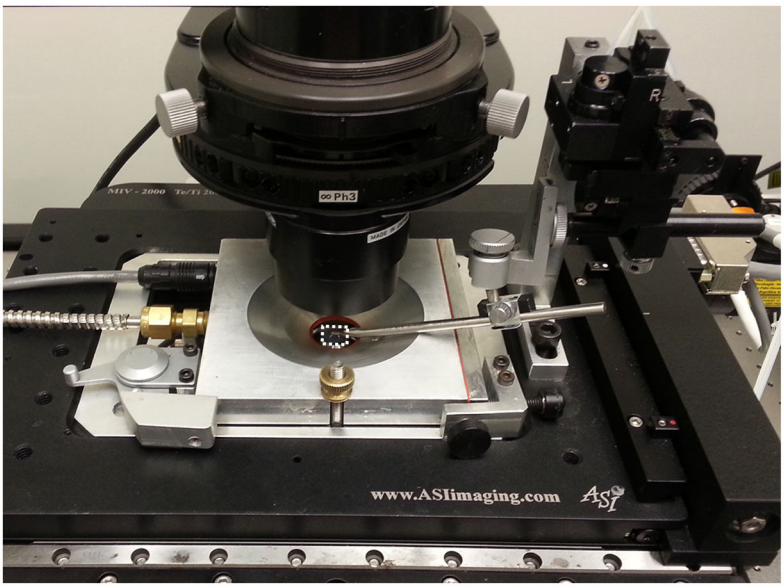

Figure 2.

Experimental setup for spindle compression. The shallow angle of the micromanipulator rod and coverslip holder walls allow the rod access to the agarose pad (highlighted with a white dotted rectangle). The micromanipulator is attached directly to the stage, and positioned in the center of the objective and pad.

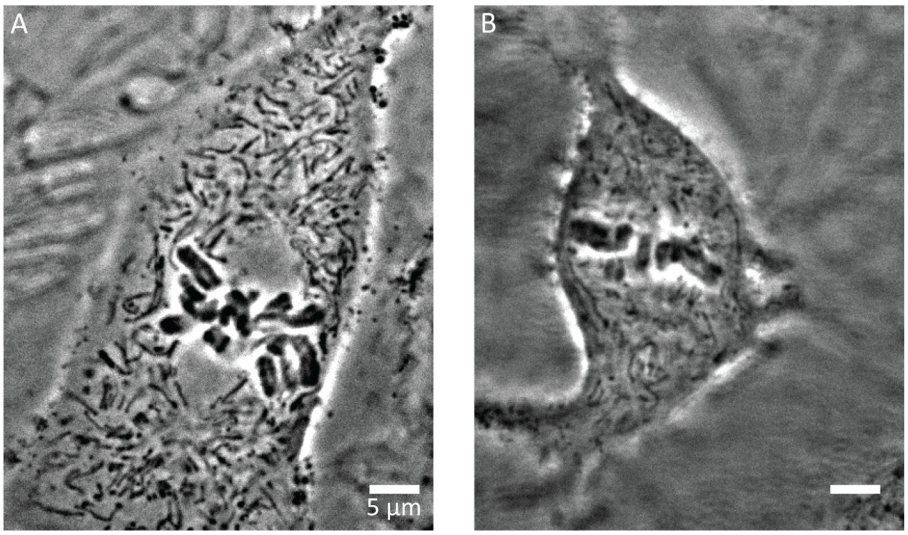

Figure 3.

Choosing a cell for spindle compression. Phase contrast images of an **(A)** ideal and **(B)** non-ideal Ptk2 cell for spindle compression. An ideal cell remains flat at mitosis prior to compression, and has space available around it to increase its surface area upon compression. Note that in phase contrast imaging a healthy spindle appears as a clear "football-shaped" region around chromosomes.

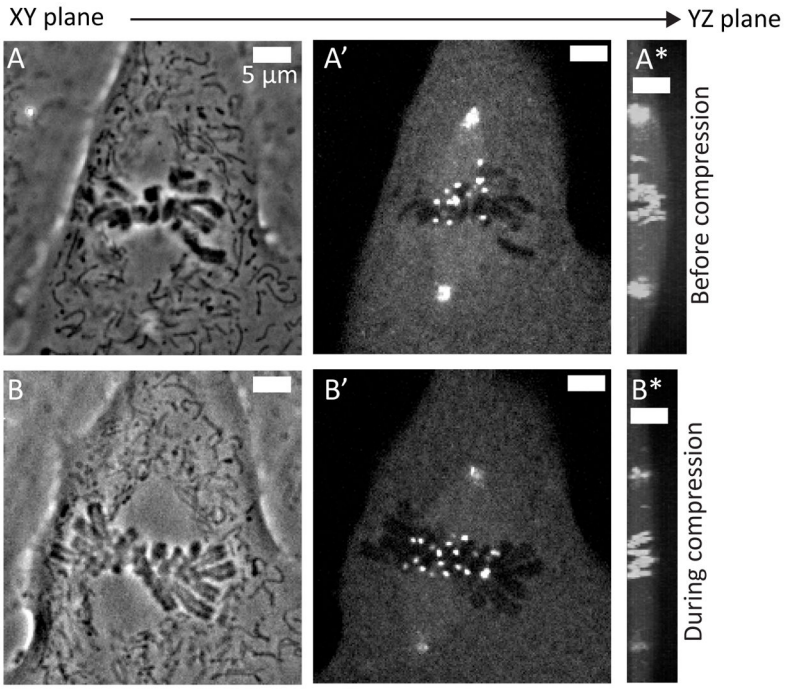

Figure 4.

Images of a Ptk2 cell **(A)** before and **(B)** during medium spindle compression: phase contrast (A, B) and spinning disk confocal EYFP-Cdc20 (A′, B′) images. Compression increases cell area, spindle width and length, number of in-focus chromosomes and kinetochores (from about 10 to about 22). A Z-stack of confocal images was acquired (350nm between planes) and a maximum intensity projection along the Y-Z plane (A* and B*) confirms that kinetochores are confined to a narrower volume slice during compression. Intensities should be compared with care between images in (A) and (B) as 5 min of imaging separates them; during this time, photobleaching takes place and Cdc20 localization changes as mitosis progresses (Howell, Moree, Farrar, Stewart, Fang and Salmon, 2004). Intensity display scaling was adjusted for YZ planes (to show cell shape changes as revealed by cytoplasmic EYFP-Cdc20). All XY images in this figure were acquired with 2×2 binning, yielding 210 nm/pixel.

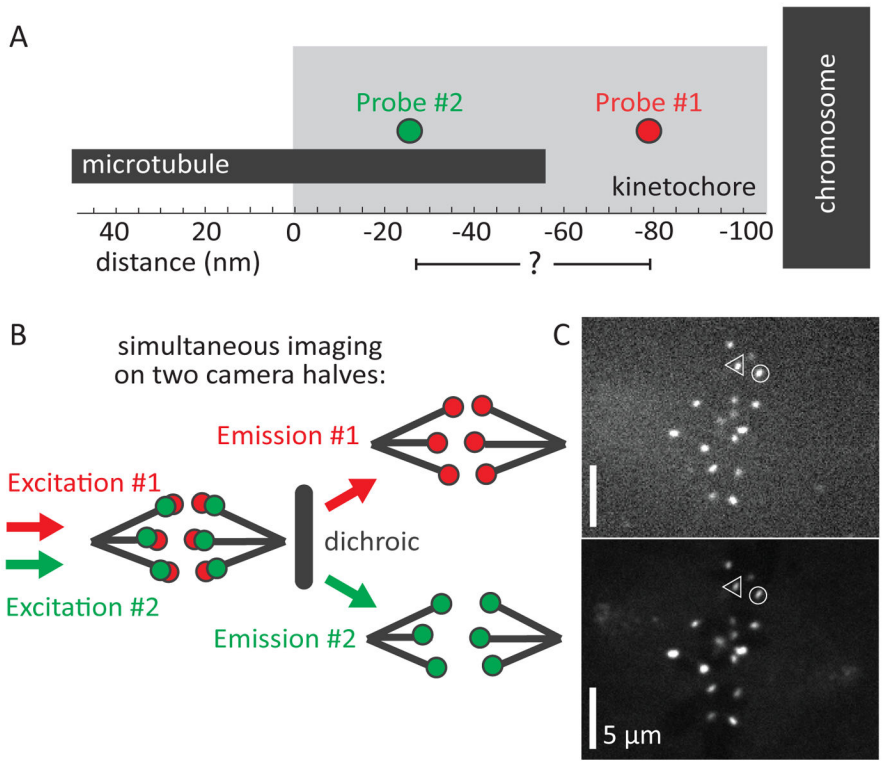

Figure 5.

Simultaneously imaging two kinetochore reporter probes in live cells. **(A)** Reporter probes must be chosen with care and here we depict the expected position (Wan et al., 2009) of two probes we have used: CenpC-mcherry (probe #1) and EYFP-Cdc20 (probe #2). **(B)** To monitor kinetochore structural dynamics live, both probes are simultaneously imaged on each half of the camera by using a dichroic to separate emission photons from each probe. **(C)** Simultaneous imaging of CenpC-mcherry (top, probe #1) and EYFP-Cdc20 (bottom, probe #2) on two camera halves. The identified kinetochore pair will be analyzed in Figure 6. Part (C) adapted from (Dumont et al., 2012).

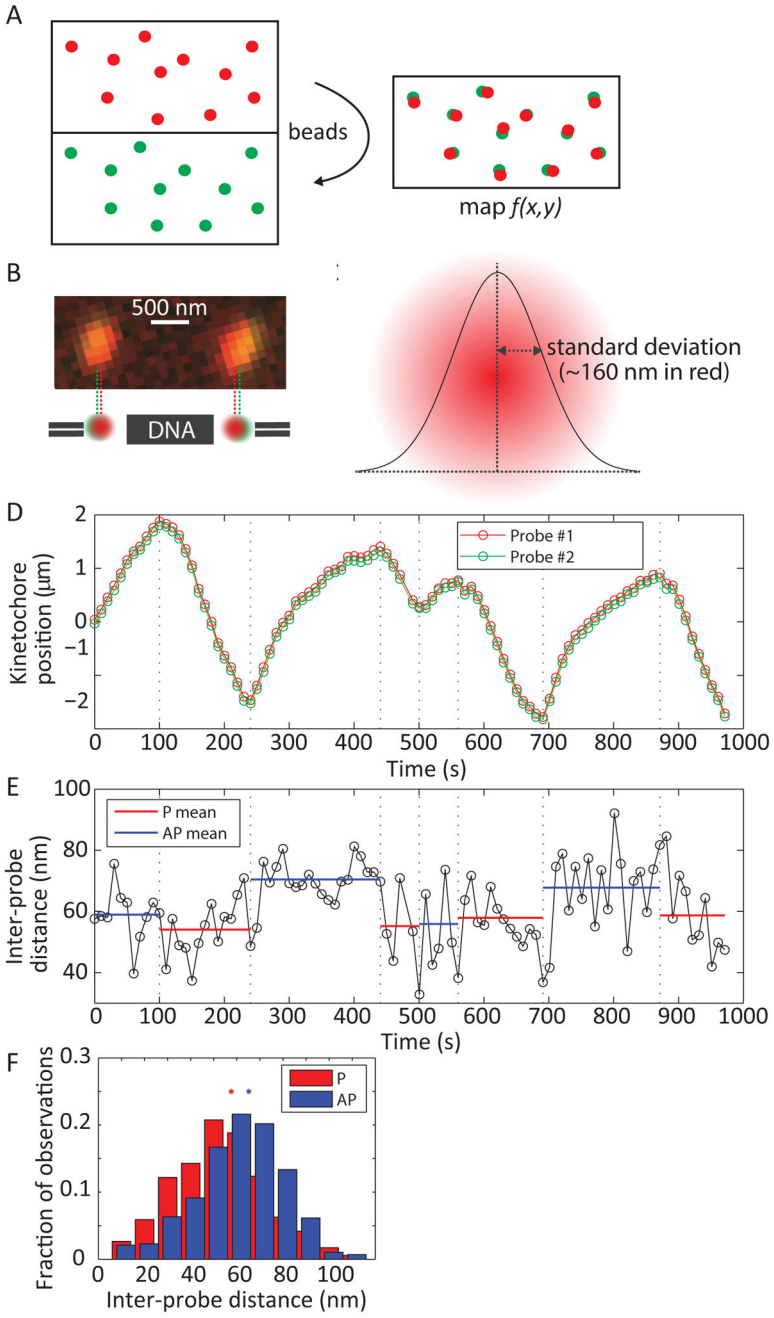

Figure 6.

Measuring kinetochore inter-probe distances. **(A)** We image two-color beads in both green and red channels, and find the transform *f(x,y)* that maps Gaussian-fitted position differences in both channels. **(B)** Enlarged two-color image of the kinetochore pair identified in Figure 5C (left = triangle, right = circle). **(C)** Each kinetochore probe leads to an image that is fit to a 2D Gaussian (which we find has a standard deviation of about 160 nm along the microtubule axis, slightly larger than 100 nm beads (Dumont et al., 2012)). **(D)** Tracks of one kinetochore's (the right one in (B)) two probes (EYFP-Cdc20 and CenpC-mCherry, as

for (E) and (F)), moving during chromosome oscillations (dashed lines = reversals). **(E)** Inter-probe distance versus time from the tracks in (D), highlighting poleward (P, red) and away-from-pole (AP, blue) movement. **(F)** As an example measurement, we show data suggesting that kinetochores are in different structural states during poleward and away-from-pole movement. Histograms of inter-probe distances over different times, kinetochores and cells for poleward (red) and away-from-pole (blue) movement: 47±20 nm poleward (n=525) and 55±19 nm away-from-pole (n=569). Parts (B), (D–F) adapted from (Dumont et al., 2012).

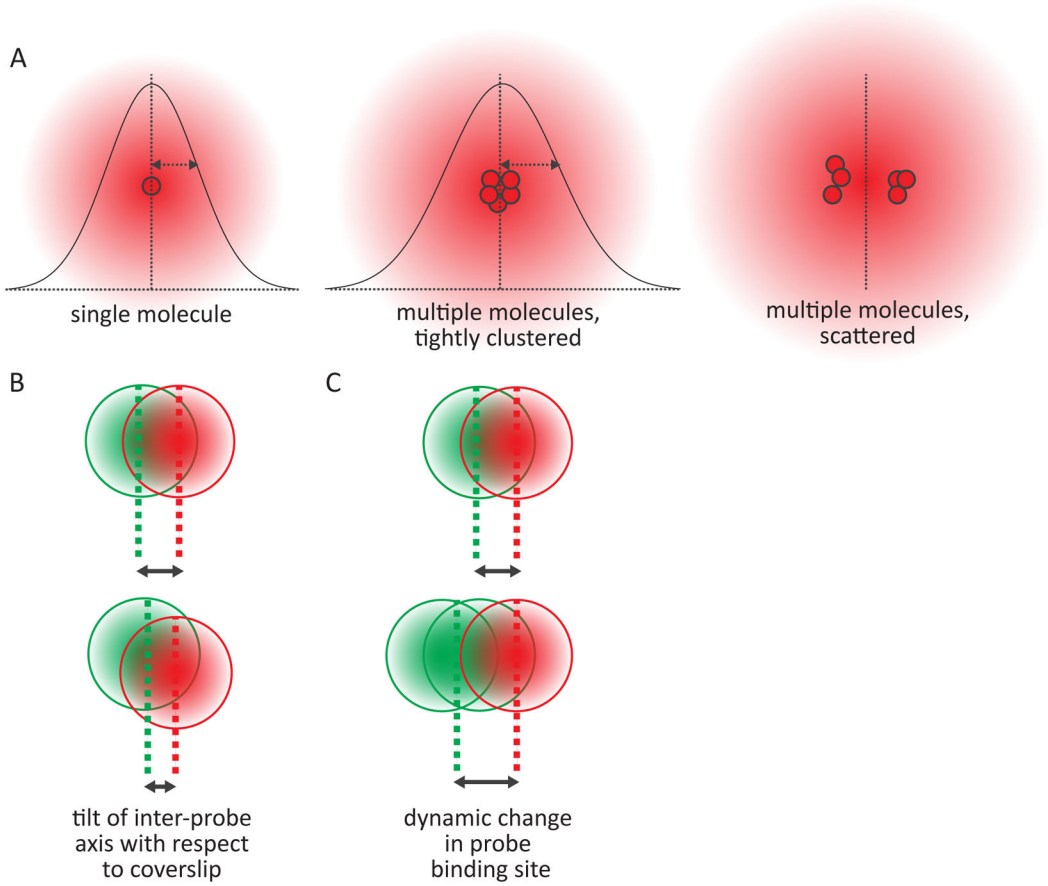

Figure 7.

Considerations for interpreting inter-probe distances. **(A)** The Gaussian-fit center of many copies of the same kinetochore protein does not necessarily correspond to the location of all protein copies. Protein copies that are tightly clustered will not affect mean localization, but if protein copies are too scattered then assumptions behind Gaussian fitting break down (Joglekar et al., 2009; Wan et al., 2009). Also, non-structural factors can lead to apparent changes in inter-probe distances, such as **(B)** tilt of the inter-probe axis with respect to the coverslip and **(C)** dynamic changes in probe binding sites (such as recruitment to a new binding site, as depicted here). In the text we provide pointers for evaluating the effects of (B–C).