In Vivo Protein Architecture of the Eukaryotic Kinetochore with Nanometer Scale Accuracy

Ajit P. Joglekar,1,* Kerry Bloom,3 and E.D. Salmon1
1Department of Biology
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599, USA

Summary

The kinetochore is a macromolecular protein machine [1] that links centromeric chromatin to the plus ends of one or more microtubules (MTs) and segregates chromosomes during cell division. Its core structure consists of eight multicomponent protein complexes, most of which are conserved in all eukaryotes. We use an in vivo two-color fluorescence microscopy technique to determine, for the first time, the location of these proteins along the budding yeast kinetochore axis at nanometer resolution. Together with kinetochore protein counts [2, 3], these localizations predict the 3D protein architecture of a metaphase kinetochore-microtubule attachment and provide new functional insights. We also find that the kinetochore becomes much shorter in anaphase as metaphase tension is lost. Shortening is due mainly to a decrease in the length of the Ndc80 complex, which may result either from intramolecular bending of the Ndc80 complex at the kink within the stalk region of the Ndc80-Nut2 dimer [4, 5] or from a change in its orientation relative to the microtubule axis. Conformational changes within the Ndc80 and Mtw1 complexes may serve as mechanical cues for tension-dependent regulation of MT attachment and the spindle-assembly checkpoint. The geometry of the core structure of the budding yeast kinetochore reported here is remarkably similar to that found in mammalian kinetochores, indicating that kinetochore structure is conserved in eukaryotes with either point or regional centromeres.

Results and Discussion

The budding yeast kinetochore is nucleated by one centromeric nucleosome containing the centromere-specific histone H3 variant Cse4 [6]. The centromere also binds the DNA-binding protein Mit2p and the CBF3 complex. Genetic, structural, and biochemical studies show that this assembly is stably linked to one microtubule (MT) plus end by a network of protein complexes comprising the Ctf19 complex [6], the Mtw1 complex [7, 8], the Spc105-Ydr532c complex [8], and the MT-binding Ndc80 complex [9, 10]. The MT-associated protein complex Dam1-DASH [11, 12] is also necessary for MT attachment. With the exception of the CBF3 and Dam1-DASH complex, these protein complexes are conserved in all eukaryotes [1, 13]. We have previously shown that the single MT attachment at the point centromere in budding yeast contains a specific number of each core structural protein complex [2]. Kinetochores at regional centromeres with 2–3 MT attachments in fission yeast also have nearly identical protein numbers per MT attachment (with the exception of the Dam1-DASH complex; see [3]), indicating that the protein architecture of individual MT attachment sites at these complex kinetochores is also conserved. The next critical task is determining the organization of these structural protein complexes within a kinetochore-MT attachment in living cells; this organization remains poorly understood because of poor visibility by electron microscopy methods [14].

We have used a two-color, in vivo fluorescence microscopy technique to determine the relative position of budding yeast kinetochore proteins along the kinetochore axis with ~10 nm resolution. Measurements are made pairwise, with one protein fused to EGFP (a green fluorescent protein) and the other fused to tdTomato (a red fluorescent protein [15]). Our technique is based largely on the in vitro method of Single-molecule High-Resolution Colocalization (SHREC [16]) and extends its scope to in vivo measurements. The ability to fuse fluorescent protein genes at the C terminus of budding yeast genes through homologous recombination—a technique not generally available in vertebrates—is critical for obtaining accurate localizations. The well-defined structure of the budding yeast mitotic spindle is also crucial. In a metaphase spindle, sister kinetochores on each chromosome are attached to MT plus ends from opposite poles and stretch their interconnecting chromatin apart by ~800 nm across the spindle equator [17]. The kinetochore from all 16 sister chromosomes pairs form two well-separated clusters, on opposite sides of the spindle equator, that appear as nearly diffraction-limited spots when imaged with wide-field fluorescence microscopy (Figure 1A). After spindle elongation in anaphase, the sister kinetochore clusters become separated by >4 μm (average spindle length in our mid- to late-anaphase measurements was 5–6 μm; see Figure S1, available online). In both metaphase and anaphase, kinetochores within the same cluster face the same pole (Figure 1A). At metaphase, opposing pulling forces produced by each pair of sister kinetochores stretch the chromatin between sisters and thus align the kinetochores and the axes of their attached MTs closely with the central spindle axis (Figure S1). In mid to late anaphase, the kinetochore axes can be expected to be roughly perpendicular to the face of the spindle pole body to which they are connected by very short (~60 nm) MTs [17].

We simultaneously recorded red and green images of kinetochore clusters in cells expressing a selected pair of fluorescently labeled kinetochore proteins (Experimental Procedures; Figure 1B). After red-green image registration, the distance separating the centroids of each pair of EGFP and tdTomato spots reflects the average distance separating the labeled kinetochore proteins within a cluster, even if the kinetochores themselves were staggered as much as 150 nm along the spindle axis (Figure S2). The centroids of the EGFP and tdTomato spots were determined within the in-focus plane with accuracy better than 10 nm by fitting of the intensity distribution with a 2D Gaussian function (Figure S3 [16, 18]). Residual error after red-green image registration was 6 nm or less (Supplemental Experimental Procedures, Figure S4). Image registration and the random orientation of spindle axes within the image plane within each data set suppressed any bias due to chromatic aberrations to negligible levels.
Measured distances were also corrected for the tilt of the spindle axis along the optical axis, which projects actual distances in the image plane and thus underestimates the actual centroid separations (Supplemental Experimental Procedures). It has been previously established that the separation between the peaks of two normally distributed probability density functions is most accurately obtained with the use of maximum likelihood estimation (Supplemental Experimental Procedures; see also [19]). An additional source of error was incomplete maturation of the EGFP and tdTomato labels within each kinetochore cluster. However, this error only increases measurement variance and does not introduce any systematic error, leaving measurement accuracy unaffected. The minimum separation distance that could be directly measured by our technique was \( \approx 10 \) nm (Supplemental Experimental Procedures). As discussed below, the 68% confidence interval for measured separations >10 nm was less than \( \pm 3 \) nm. We have neglected the physical size of the fluorescent proteins (2 nm for EGFP and 2–4 nm for tdTomato; [20]), from our analysis because both EGFP and tdTomato are linked to a protein of interest via a flexible linker. The two monomers within tdTomato are also connected by a flexible linker [15]. These flexible linkers should allow the fluorescent proteins to rotate freely in space about the kinetochore protein end, thus significantly reducing their contribution to our distance measurements.

The NDC80 complex (Figure 2A) is a 56-nm-long, rod-shaped molecule with globular domains, separated by a long \( \alpha \)-helical coiled-coil rod domain [5, 21, 22]. Because of its well-characterized shape, we used the complex as an in vivo ruler to test the accuracy of our technique. The N-terminal heads of Ndc80-Nuf2 bind the microtubule lattice, and the C-terminal globular domains of Spc24-Spc25 link the Ndc80 complex to the inner kinetochore [21]. To characterize the in vivo structure and orientation of the Ndc80 complex within the kinetochore, we constructed four strains through combinations of N- and C-terminal tagging for Ndc80p, Nuf2p, and Spc24p. We found that the overall length of the complex was 55 nm, which is almost equal to its full length. In contrast, if the complex were bound to the MT lattice at a 40° angle, as observed in vitro [22], its end-to-end distance projected in the image plane would be 44 nm. Independent measurements of the lengths of the two sections of the complex yielded 38.5 nm for the Nuf2p-Ndc80p dimer and 17 nm for the Spc24p-Spc25p dimer (Figure 2B). The sum of these lengths is also close to the 55 nm measured for the total length. These results support the idea of an extended orientation for the Ndc80 complex along the MT axis in metaphase. They also demonstrate the accuracy of our measurement method.

We then measured the average location of other kinetochore protein complexes in metaphase cells relative to the C terminus of Ndc80p (Table S1). We display the results with respect to the C terminus of Spc24p, which is 17 nm inside (toward the centromere) the C terminus of Ndc80p, for ease of interpretation (Figure 3A). Relative to the C terminus of Spc24, three members of the Mtw1 complex were inside (toward the centromere). The C termini of Mtw1p and Nsl1p were localized 5 nm inside. For Dsn1p, the N terminus was 2 nm inside and the C terminus was 6 nm inside. Biochemical data link members of the Mtw1 complex with the centromere-proximal end of the NDC80 complex, as well as with the DNA-binding protein Mif2p [7, 23]. Structural studies measure a length of 30 nm for reconstituted Mtw1 complex molecules in vitro (Eva Nogales, personal communication). Our measurements are consistent
with both of these data. The Mtw1 complex is also linked to the centromere through the Ctf19 complex [23, 24]. We localized the C termini of three members of the Ctf19 complex [7]—Ctf19p, Ame1p, and Okp1p. Both Ame1p and Okp1p colocalized 13 nm inside the Spc24 C terminus, whereas Ctf19p was located 16 nm inside. These proteins were close to the N terminus of centromeric histone Cse4p, which was 17 nm inside the Spc24 C terminus.

In addition to depending on the Ndc80 complex, MT attachment within the kinetochore outer domain depends on Spc105p and the Dam1-DASH complex. The C terminus of Spc105p, a large, 100 kDa protein, colocalized with the Spc24p C terminus (1 nm inside; Figure 3A). The position of the N terminus of Spc105p could not be accurately determined with the use of the maximum likelihood method, but we estimate it to be 16 nm outside (toward the MT) the Spc24p C terminus (Table S1). This indicates that the protein probably extends outward along the microtubule axis. Surprisingly, we found that Ask1p, a key component of the Dam1-DASH complex, was 12 nm inside the microtubule-binding head domains of the Ndc80 complex (Figure 3A).

There were significant changes in the relative positions of kinetochore proteins from metaphase to anaphase. We found that the overall kinetochore length in late anaphase cells was reduced by 25 nm (Figure 3A, Supplemental Experimental Procedures, Table S2). The end-to-end length of the Ndc80 complex decreased from 55 nm in metaphase to 34 nm in anaphase. Within the Ndc80 complex, the separation between the two ends of the Ndc80p-Nuf2p dimer was reduced by 13 nm, whereas the Spc24-Spc25 dimer showed a smaller decrease of 5 nm. Also important was the movement of the Spc24-Spc25 end of the Ndc80 complex 5 nm closer to the centromeric nucleosome (Figure 3A). Components of the Mtw1 complex also showed a significant redistribution; notably, Nsl1p moved closer to the centromeric nucleosome. On the other hand, the position of the Ctf19 complex with respect to the N terminus of Cse4p did not change significantly, suggesting a rigid coupling between this complex and the centromeric nucleosome. It is known that the CBF3 complex binds to the CDE III region [25] of the centromeric DNA via Ndc10p and Cep3p in metaphase. We found that the C terminus of Ndc10p was 35 nm inside the N terminus of Cse4p in anaphase. This large distance is probably due to the anaphase dislocation of Ndc10p from the kinetochore [26]. Finally, the position of the Dam1-DASH complex does not change significantly with respect to the Ndc80 head domain. It should be noted that the average number of Dam1-DASH complex molecules per budding yeast kinetochore decreases from 16–20 in metaphase to 9 in anaphase, which is insufficient for formation of Dam1-DASH rings around the late anaphase MTs [2].

This study assembles the first in vivo, high-resolution map of kinetochore protein localization along the axis of a kinetochore-microtubule attachment (Figure 3A, [27]). It should be noted that these locations reflect average positions of kinetochore proteins. Furthermore, our technique can measure distances in the image plane, and it is insensitive to distance changes that may occur either along the optical axis or perpendicular to the spindle axis. Therefore, positional changes that take place within complexes of unknown shape (such as the Mtw1, Spc105-YDR532c, and Ctf19 complexes)
along these directions could not be detected. The position of the MT plus end within the kinetochore could not be determined with our technique. The location of the MT-associated Dam1-DASH complex suggests that the MT plus end extends at least 10 nm beyond the contact point between the MT and Nuf2p-Ndc80p head domains [28]. KNL-1, the C. elegans homolog of Spc105p [22], and the N-terminal domain of Spc7, the S. pombe homolog, show MT-binding activity [29]. These data suggest that the MT plus end may extend up to the Spc24-Spc25 end of the Ndc80 complex.

The localization data can be combined with protein numbers [2] and existing structural information to predict a 3D visualization of kinetochore-MT attachment assuming a symmetric distribution of proteins around the cylindrical MT lattice (Figure 3B). The end-to-end measurement of the metaphase length of the Ndc80 complex shows that it binds the MT lattice while making a small angle with the MT axis, in contrast to the 40° angle made by unbound Ndc80 complexes observed in vitro. This alignment of the Ndc80 complex and MT axes can be expected, given that the Ndc80 complex is one of the primary force generators at the kinetochore and that this force acts along the MT axis. Available biochemical data suggest that the contact between the Ndc80 complex and the inner kinetochore is achieved through interactions of Spc24-Spc25 globular domains with the Mtw1 and Ctf19 complexes [22]. Additional points of contact would be necessary for resisting the pulling forces tending to align the Ndc80 complex along the MT axis and maintaining its tilted orientation (with respect to the MT). The model displays a possible mechanism that relies on bending of the Ndc80-Nuf2p dimer at the kink as observed in vitro.

Figure 3. Protein Architecture of a Kinetochore-Microtubule Attachment
(A) The average location of kinetochore proteins along the axis of the kinetochore-microtubule attachment in metaphase and late anaphase. Each colored box represents a protein complex within the kinetochore. 68% confidence intervals on the mean position for all the measurements are <3 nm. The exception is Spc105p-C (indicated by stars), which could not be localized with the maximum likelihood estimation. The positions in this case reflect the average offset along the spindle axis, which is probably an underestimate of the actual distance. For the Mtw1 and Ctf19 complexes, we show only the spans as measured by the positions of the respective member proteins.

(B) 3D visualization of the metaphase budding yeast kinetochore-microtubule attachment, as predicted by the protein localization data, assuming a symmetric arrangement of kinetochore protein complexes around the cylindrical microtubule lattice. Black stars indicate the positions of fluorescent labels used in distance measurements. The configuration of the Dam1-DASH complex suggests two possibilities: a kinetochore that contains an oligomeric ring of the Dam1 complex (top) and a kinetochore that employs Dam1-DASH patches or incomplete rings (bottom). Dashed lines indicate established biochemical interactions between two protein complexes.

(C) Loss of centromeric tension and changes induced the cell-cycle regulation result in a shorter kinetochore in late anaphase. A striking change occurs in the Ndc80 complex: the Nuf2p-Ndc80p dimer shows a length reduction that is 40% larger than the reduction predicted by an overall change in the orientation of the molecule with respect to the MT. The model displays a possible mechanism that relies on bending of the Ndc80-Nuf2p dimer at the kink as observed in vitro.
complex molecules to attach the MT lattice at angles of 50°–60° to accommodate the Dam1-DASH ring underneath. These large angles are inconsistent with the measured end-to-end length of 55 nm for the Ndc80 complex. Therefore, the Dam1-DASH ring will have to encircle both the MT lattice and the rod domains of Ndc80 complex molecules (Figure 3B). Individual Dam1-DASH monomers can still interact with the MT lattice via the projections spanning the gap between the inner surface of the ring and the MT lattice. This configuration may also promote rapid re-binding of Ndc80 heads to the MT lattice by limiting their diffusion. Alternatively, the Dam1-DASH complex may not form a single ring structure at the kinetochore. Instead, spiral oligomers that incompletely surround the microtubule lattice at several locations along the microtubule axis may assemble (Figure 3B). In this configuration, direct binding between the Dam1-DASH monomers and the Ndc80 complex becomes necessary for their stable association with the kinetochore. Although a direct biochemical link between the Dam1-DASH complex and other kinetochore complexes has not been established, such a linkage is necessary for transmitting the force generated through interactions between the Dam1-DASH complex and the MT lattice to the rest of the kinetochore for the participation of either configuration in force generation [31, 34].

The anaphase measurements reveal tension and/or cell-cycle-dependent changes within the kinetochore (Figure 3C). The reduction in the end-to-end length of the Ndc80 complex in late anaphase indicates that the Ndc80 complex directly participates in force generation and transmits this force to the inner kinetochore components through the Mtw1 complex. The observed decrease may be explained through either intramolecular bending at the kink domain within the complex, observed in vitro (depicted in Figure 3C), or a reorientation of the entire complex so that it makes an angle of 45°–50° with the axis of the MT. The latter configuration requires a large extension (~40 nm) of the inner kinetochore complexes perpendicular to the MT axis to stably link the Ndc80 complex back to the inner kinetochore and the centromere. The elongated shapes of the Mtw1 complex and the Ctf19 complex may facilitate such an alignment of the Ndc80 complex in anaphase. The total length of such a linkage in anaphase would predict a much longer distance between the centromere and the Spc24-Spc25 end of the Ndc80 complex under the metaphase pulling forces acting along the axis of the MT. We therefore show the simpler anaphase kinetochore configuration that relies on intramolecular bending of the Ndc80 complex.

Many of the structural proteins and protein complexes are conserved in all eukaryotes [1], although the complex architecture of the regional centromeres probably necessitates significant modifications, especially to the centromere-proximal proteins [35–37]. Architecture of the kinetochore-microtubule attachment site built on either the point or the regional centromere foundation, however, is probably conserved in all eukaryotes, as evidenced by the conserved stoichiometry of kinetochore proteins between point and regional centromeres [2, 3]. Indeed, kinetochore protein localizations obtained by antibody labeling in fixed HeLa cells show a strikingly similar pattern (E.D.S., unpublished data). This conservation of kinetochore protein structure and the protein architecture of the kinetochore-MT attachment demonstrate that the core structure of the kinetochore, along with its basic functional mechanisms in force generation and spindle assembly checkpoint signaling, are conserved throughout eukaryotic phylogeny.

Experimental Procedures

Strains and Growing Conditions

Strains (Table S3) were grown in complete media, with either glucose or galactose as the carbon source, at 32°C. Proteins were tagged with either EGFP or tdTomato through homologous recombination, mostly at the C terminus, with the use of PCR-amplified cassettes. Cells from mid-log phase cultures were resuspended in synthetic media and immobilized on concanavaline A (cat. no. 7279, Sigma, St. Louis, MO)-coated coverslips for imaging.

Imaging

Cells were imaged at room temperature on a Nikon TE-2000E (Nikon Instruments, Melville, NY) inverted microscope equipped with a 1.4 NA, 100× DIC objective and 1.5× optovar lens (1 pixel = 107 nm). A dual-excitation filter set (FITC/TRITC ET set no. 59004, Chroma Technology, Rockingham, VT) was used for simultaneous excitation of both EGFP and tdTomato. Images were acquired with a DV-887B iXon camera (Andor Technology, South Windsor, CT), with the use of the conventional acquisition mode mounted on the bottom port of the microscope. The Dual-View attachment (MAG-Bio-systems, Pleasanton, CA) was used for simultaneous acquisition of images at both wavelengths with preselected dichroic and emission filters for EGFP and tdTomato. Before each experiment, 100 nm TetraSpek (cat. no. T-7279, Invitrogen, Carlsbad, CA) bead images were acquired for image registration (Figure S3). For each cell, 10 image slices were obtained through moving the piezoelectric Z-stage (MadCity Labs, Madison, WI) through 200 nm steps, and a 300×300-pixel-wide, centrally located region was recorded in each image. The exposure time was set at 800 ms per image, for maintenance of a high signal-to-noise ratio with minimal bleaching during image acquisition. The imaging and image acquisition hardware was run by Metamorph 7 (Molecular Devices, Sunnyvale, CA).

Image Analysis

Image analysis was carried out with custom software written in MatLAB 7 (MatWorks, Natick, MA). The tdTomato image stack was registered with the EGFP image stack (described in detail in Figure S3). For centroid determination, the area of interest for centroid localization was determined through placing an 8×8 pixel region (for metaphase measurements) on an EGFP image such that the cumulative intensity within the centrally located 2×2 pixel square was maximized. The corresponding region from the registered tdTomato image was then extracted for centroid localization. A similarly selected 10×10 pixel region was used for analyzing anaphase cells.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00809-4.

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References

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Supplemental Data

**In Vivo Protein Architecture**

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**Supplemental Experimental Procedures**

**Effects of Tilting of the Kinetochore Axis along the Optical Axis**

The separation between the EGFP and tdTomato centroid determined in the “in focus” plane is projection of the true distance on the image plane. Since the long axes of all sixteen kinetochores are closely aligned with the spindle axis, the orientation of the spindle axis can be used to deduce the average tilt in the kinetochore axes. In this study, we analyzed spindles in which, the centroids of the two kinetochore clusters were separated by less than 600 nm along the optical axis. We compensate for the spindle axis tilt along the optical axis by dividing the measured separation with the cosine of the angle that the spindle axis (as determined by the centroids of sister kinetochore clusters) makes with the image plane.

\[ \theta_{\text{tilt}} = a \tan \left( \frac{L_{K-K}}{z} \right) \]

where,

- \( L_{K-K} \) = Separation between two EGFP centroids in the image plane (nm)
- \( z \) = 100 + separation between the two in-focus planes along the optical axis (nm)

and,

\[ D_{\text{true}} = \frac{D_{\text{measured}}}{\cos(\theta_{\text{tilt}})} \]

where,
$D_{true} = \text{the true separation between the EGFP and tdTomato centroids}$

$D_{measured} = \text{the measured separation between the EGFP and tdTomato centroids}$

The kinetochore axes have yet another degree of freedom, which is the curling of the kinetochore axis along the optical axis, and it can’t be detected. If we assume that the curling of the kinetochore axis within the image plane and along the optical axis are the same, we can estimate its effect from the measured curling of the kinetochore axis within the image plane. This angle is given by the angle of the kinetochore axis with the spindle axis in the image plane. We chose the full-length measurements for the NDC80 complex for two reasons – the complex exists as a single macro-molecule, and the distance is larger than the uncertainty in centroid determination. The average value of this angle for the entire dataset was $23 \pm 16^\circ$ ($\cos$ of this angle is 0.92). If the kinetochores have a similar tilt along the optical axis, all of our measurements would have to be increased by $\sim 8\%$. E.g. the full length of the NDC80 complex with the correction would be $\sim 57$ nm instead of the measured length of 55 nm. However, because of the absence of direct measurements, and because of the small magnitude of the underestimation involved, we did not apply this correction to our measurements.

**Maximum Likelihood Estimation of the Mean Centroid Separation**

Measurements of the distance separating two centroids that are defined by Gaussian probability distributions have been shown to be distributed according to a 2-D non-Gaussian probability density function given by [2]:

$$P_{2D}(r) = \left(\frac{r}{\sigma^2}\right) * \exp\left(\frac{\mu^2 + r^2}{2\sigma^2}\right) * I_0\left(\frac{r\mu}{\sigma^2}\right)$$

(1)

Where,

$r = \text{measured magnitude of distance separating the two centroids}$

$\sigma = \text{expected standard deviation for each centroid location measurement (experimental errors)}$
\( \mu = \) true distance separating the two centroids
\( I_0 = \) modified Bessel function of order zero

This is an asymmetric probability distribution skewed towards larger values of \( r \). A maximum likelihood method was devised for estimating the true distance that is based on fitting the observed probability distribution of distance measurements with the theoretical probability distribution given by equation (1). The ability to accurately measure the distance separating the two centroids depends on the relative magnitudes of the true distance being measured (\( \mu \)) and the expected uncertainty in centroid determination (\( \sigma \)). It follows that for correct determination of \( \mu \) using this method, the measurements of the two centroids must be normally distributed. We have implemented this method for our measurements of the centroids that reflect the centroid of a cluster of fluorophores rather than single molecules. The implicit assumption is that the centroid localization errors will be normally distributed as in the single molecule case.

**Imbalanced Fluorophore Maturation and the Limit of Resolution**

Centroid separation measurements for kinetochore clusters contain an additional source of variance besides the uncertainty in centroid determination, residual errors from image registration, and biological variation in the kinetochore structure. Each diffraction-limited spot represents a cluster of 16 kinetochores that are spread over an area, and kinetochore each carrying multiple copies of EGFP and tdTomato. Furthermore, the maturation efficiency for fluorescent proteins is less than 100%. Maturation efficiency has been estimated [3] at 90% for EGFP. Since the maturation half-life for tdTomato is \(~3\)-fold higher than that for EGFP, its maturation efficiency can be expected to be lower [4]. These two factors combine to introduce a significant error in individual distance measurements. The effective error in each measurement is:

\[
\sigma_{\text{Total}}^2 = \sigma_{\text{centroid}}^2 + \sigma_{\text{registration}}^2 + \sigma_{\text{maturation}}^2
\]  
(2)
\( \sigma_{\text{centroid}} \) = uncertainty in centroid determination (< 10 nm, Supplemental Data 3)

\( \sigma_{\text{registration}} \) = average residual error after image registration (~ 6 nm, Supplemental Data 4)

\( \sigma_{\text{maturation}} \) = error due to differential fluorophore maturation within a kinetochore cluster

Centroid determination error and error due to registration account for only 50% of the measurement variance. Exact values for fluorophore maturation are unknown. The distribution of kinetochores within the cluster is less than 150 nm.

The magnitude of the effective measurement error is also the smallest distance that can be measured using the maximum likelihood method described in the previous note. The maximum likelihood method only considers the probability distribution of EGFP-tdTomato centroid vector magnitudes for a given pair of labeled kinetochore proteins. In cases where the actual separation between two protein domains is smaller than the measurement error, the maximum likelihood method will therefore estimate the mean value of the measurement error rather than the expected distance. E.g. the C-termini of Ndc80p and Nuf2p are separated by an 80 amino acid tail on Ndc80p (ref. [5]). We localized the C-termini of Ndc80p and Nuf2p with respect to the C-terminus of Ctf19p that is far removed from these protein domains, and hence can be accurately determined by the maximum likelihood estimation method. We found that in anaphase, the distance separating the C-termini of both Ndc80p and Nuf2p from the Ctf19p C-terminus is 24 nm and 25 nm respectively, whereas in metaphase, these distances were 37 nm and 33 nm respectively. The difference between these separations provides the separation between the C-termini of Ndc80p and Nuf2p: -1 nm in anaphase and 4 nm in metaphase. These values compare well with the separation of Ndc80p and Nuf2p C-termini projected along the spindle axis (1 ± 5 nm; N=160 in anaphase and 6 ± 11 nm; N=98 in metaphase). In contrast, the maximum likelihood estimation method predicts an anaphase separation of 10 nm between these two protein domains (metaphase data does not
generate a satisfactory fit), which can be used as an estimate of the measurement error for our technique. We thus estimate the resolution limit for our technique to be approximately 10 nm.

**Estimation of the Systematic Underestimation in Anaphase Measurements**

The release of tension in anaphase can be expected to provide more freedom to the orientation of the two SPBs that nucleate spindle microtubules. Kinetochore-microtubules in anaphase cells are only 60 nm in length on average [1], and can therefore be assumed to be perpendicular to the face of the SPBs. The increased rotational freedom in SPB orientation along the Z-axis will introduce a systematic underestimation in distance measurements for the kinetochore. To estimate this distance, we carried out distance measurements in a strain that had two SPB proteins: Spc72p and Spc110p labeled at their C-termini with EGFP and tdTomato respectively. We found that the metaphase distance separating these two ends was 75 nm, whereas the anaphase distance was found to 70 nm. If we ascribe this decrease in SPB dimension to the greater orientations freedom for the SPB face along the optical axis, we can expect an underestimation of no more than 7% in anaphase measurements.

**Residual Chromatic Aberrations**

Chromatic aberrations can introduce a systematic bias in the distance measurements. Although the image registration procedure described in the previous section will also correct for chromatic aberrations. Generally, however, the bead chosen for image registration procedure is not at the same depth as the average depth at which kinetochore clusters are imaged. A small bias due to residual chromatic aberrations may still be expected. We offer two data that demonstrate that the bias due to chromatic aberrations is negligibly small in our data. The first evidence is the measurement of the distance between two SPB proteins carried out in two strains that have the color of the fluorescent label switched. The random orientation of the spindle axis within the image plane in any data set will also average out the directional offset between the red and green PSFs resulting from chromatic aberrations. The average value of the angle subtended by the spindle axes in the set for a given distance measurement is $0 \pm 40^\circ$. 
A Case for Centromeric Nucleosome Versus Hemisome in Budding Yeast

A recent study of the conformation of chromatin using chromatin immunoprecipitation against CENP-A in Drosophila cells demonstrated that the CENP-A bound DNA is packed as hemisomes containing one molecule each of CENP-A, H2A, H2B and H4 (ref. [7]). Several lines of evidence suggest that the budding yeast centromere may not follow this pattern. (1) Histone depletion experiments [8] show that the depletion of histone H3 leaves the nucleosomal compaction within centromeric chromatin unaltered, whereas depletion of the histone H4 or Cse4p disrupt the wild-type pattern of nucleosomal compaction. (2) Fitzgerald-Hays et al. created two mutant alleles of Cse4p – one with a mutation within the histone-fold-domain, and the other with a mutation in the N-terminal tail of the protein [9]. Both these alleles were non-functional, and hence lethal to the cells. The authors found that when co-expressed in the same strain, the two mutant alleles could function together to restore wild-type Cse4p function. This synthetic restoration of protein function is possible only if the two mutant alleles dimerize, so that one could provide the essential function of the HFD, whereas the other monomer could provide the function of the N-terminal tail. (3) The length of nuclease resistant centromeric DNA (220 base pairs long) and the recent finding that Cse4p localization is limited only to the centromeric nucleosome indicate that the kinetochore is likely supported by a single centromeric nucleosome containing Cse4p (or two hemisomes, ref. [10],[11]). Based on these findings, we use a representation of a nucleosome in the model 3-D drawing of the yeast kinetochore-microtubule attachment.
Figure S1: Estimation of the Effect of the Relative Angle between MT and Spindle Axes

The average tilt in the axis of a kMT with respect to the spindle axis was estimated from a published serial section EM reconstruction of a metaphase budding yeast spindle (Fig. S1a). The average of angles for all the kMTs from this reconstruction was found to be \(6.3 \pm 5.0^\circ\) (Fig. S1b). This angle would result in a negligible underestimation (0.001) in distances measured along the spindle axis. We found that the average separation between the two kinetochore clusters in a strain expressing Nuf2p-GFP & Ndc80p-tdTomato from our metaphase dataset is \(1.05 \pm 0.26 \, \mu m\) (\(N = 75\)) and \(5.01 \pm 1.07 \, \mu m\) (\(N = 80\)) for the anaphase dataset. These values are typical for all of our data, and they correctly predict the expected spindle length (assuming a mean kMT length of ~ 300 nm) from previous experiment. We can thus very reliably distinguish metaphase cells from cells in early/late anaphase based on the position of the kinetochore spots and the distance separating the two spots within a cell.
Figure S2: Centroid Localization Accuracy Is Insensitive to the Spatial Staggering of Kinetochores

Nanometer-scale distances can be accurately measured along the axis of the kinetochore, even if the kinetochores themselves are staggered along the spindle axis as shown in the schematic at the top (variation in kMT lengths in metaphase is less than 150 nm; ref. [1]). The accuracy of such measurements directly depends only on the accuracy with which the location of the centroid of the image of a cluster of kinetochores can be determined, and not directly on the staggering of the kinetochores. The extent of kinetochore staggering can have an indirect effect, since for large spatial staggering, the image of a
kinetochores do not appear as one spot as shown in the bottom figure. To illustrate this point, we simulated images of kinetochores by randomly dispersing only six kinetochores over a square area of the indicated dimension. Each kinetochore is represented by a pair of green and red fluorophores separated by a distance of 56 nm – the length of the Ndc80 complex molecule. An overlay of the images made by such a cluster of six kinetochores is shown in the middle panel. The bottom panel displays an overlay of the 2-D Gaussian fits obtained for the simulated images. These representative images clearly show that as the staggering of the kinetochore cluster exceeds 200 nm, the corresponding microscope image acquires a distinct structure with multiple peaks, instead of the single peak seen for the first two cases. The least squares fitting routine for determining the centroid location with nanometer accuracy described in the previous section makes the assumption that the observed intensity distribution can be described by a 2-D Gaussian function. The last two images show that this key assumption breaks down for kinetochore staggers over a 200x200 nm region, which is much larger than the expected spatial staggering of the sixteen kinetochores in a metaphase as well as anaphase spindle is much smaller. Therefore, the accuracy with which distances can be determined is unaffected by the staggering of the kinetochore along the axis of the budding yeast spindle.
The accuracy in centroid localization for an imaging and image acquisition setup is determined by the signal-to-noise ratio of the image. To estimate the centroid localization accuracy for the budding yeast kinetochore clusters in metaphase, we first determined the average GFP and tdTomato signal and background values for strains containing Ctf19p-GFP, Ndc80p-tdTomato, and Ndc80p-GFP. Ctf19p-GFP represents kinetochore proteins with low copy numbers, and also a higher background signal, whereas Ndc80p represents proteins with high copy number per kinetochore. Ndc80p-tdTomato (or Nuf2p-tdTomato) was used as the reference point on the kinetochore for all the measurements. For each strain, the maximum signal intensity as well as the standard deviation of the background (from intra-nuclear regions) was determined. The spread of the intensity distribution in the image plane was determined through 1-D Gaussian curve fitting. Images of kinetochore clusters were then simulated by randomly distributing fluorophores in an area of appropriate size, and then convolving this object space with a 2-D Gaussian point spread function. For each signal and background condition, 100 image pairs were simulated such that one image had the centroid of kinetochore distribution in the object space displaced by a known, fixed amount with respect to its partner image. The images included background signal containing noise with Gaussian characteristics (modeled after the camera dark noise) and noise due to
Poisson statistics (shot noise dependent on signal magnitude). Such image pairs were then analyzed by least squares fitting the intensity distribution with a 2-D Gaussian surface. The standard deviation in the measured separation between the centroids of each image pair was designated as the expected precision and plotted as a function of the brightest pixel value within the spot (the dotted lines mark the approximate range for our measurements). The results show that for the lowest signal-to-noise ratio conditions (Ctf19p-GFP), the expected precision in determining the distance is ~ 10 nm. Due to a more compact distribution of kinetochores in anaphase cells, the signal-to-noise ratio improves in anaphase.
Registration between the EGFP and tdTomato images was carried out prior to each experiment as follows [2]. Tetraspek microspheres (diameter = 100 nm, cat. # T-7279, Invitrogen) were suspended in 5% polyacryl amide gel (refractive index ~ 1.37) and sandwiched between a slide and a coverslip. Single, isolated beads were selected, and scanned over a 150x150 pixel image area in the center of the field of view with a spacing of 1 μm or 1.5 μm using an XY translation stage (MS-2000, ASI, Eugene, OR). An image of the bead was recorded at each position. Irregular movements of the XY translation stage resulted in a slightly irregular displacements of the bead. Stage drift along the optical axis over the time of acquisition was minimized through the use of Perfect Focus system (Nikon) to maintain the bead in focus. The image
acquisition time for each image ranged from 600-1800 ms. Acquisition time was selected so as to maintain a signal to noise ratio > 30 in both imaging channels. The top panel in this figure shows the maximum intensity superposition of 240 image planes obtained in this manner. The EGFP and tdTomato images are high-lighted with green and red squares respectively. The high signal to noise ratio ensures centroid localization accuracy approaching 1 nm for each bead image along both X and Y axes using 2-D Gaussian curve fitting. The image transform for registration between the EGFP and tdTomato fields was computed using the local weighted mean algorithm implemented in MatLAB (MathWorks, Natick, Md). The use of Dual-View attachment to acquire the EGFP and tdTomato channels simultaneously introduces spatially varying offsets between the centroids of the EGFP image of a bead and the corresponding tdTomato image (displayed as vectors), which are apparent in the left middle panel. A histogram of the absolute offset between the centroids of an EGFP spot and the corresponding tdTomato spot prior to registration is displayed in the bottom left panel. This variation along with chromatic aberrations is corrected by the image registration procedure (vector map in the right middle panel, and a histogram of corresponding offset magnitude). The average offset magnitude after image registration was found to range from 3-6 nm (mean offset magnitude of 3.7 ± 1.9 nm in this particular example). This procedure was carried prior to or just after each experiment. Each experiment lasted for 30-40 minutes to minimize effects of drift during the experiment.

The distance measurements were conducted with a custom interactive graphical user interface written in MatLab. In each experiment, the tdTomato image stack was first registered with respect to the corresponding EGFP stack based on the image transform obtained as discussed above. Two kinetochore clusters (distinguished as metaphase and anaphase cells) in each cell were manually selected using the EGFP image. The region of interest was then automatically defined for the selected kinetochore cluster as described in the methods section. The same region was then transferred to the corresponding tdTomato image. The centroids of these regions were then determined by 2-D Gaussian fitting, and the data was appropriately stored.
Table S1. Comparison of Raw Means and Maximum Likelihood Estimates

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<th>Protein</th>
<th>Raw data</th>
<th>Max. likelihood fit</th>
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<tbody>
<tr>
<td></td>
<td>Mean (nm)</td>
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<tr>
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<tr>
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<td>19</td>
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<tr>
<td>Nuf2p-C:Ctf19p-C</td>
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<td>15</td>
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<tr>
<td>Ndc80p-C:Ctf19p-C</td>
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* - Metaphase localizations for these proteins could not be determined reliably using the non-Gaussian probability distribution fits due to high variation in the data relative to the distance being measured. The value cited in these cases is the separation *along the spindle axis*.

Negative signs for distances were assigned to indicate that the distance inside (towards the centromere) of the C-terminus of Ndc80p or Nuf2p. Positive sign implies that the protein localizes on the outside (away from the centromere) of this reference point.

Confidence limits obtained from maximum likelihood estimation are typically asymmetric [6]. The 68% confidence interval cited above is the larger limit of the two obtained from maximum likelihood estimation.
Table S2. Protein Localizations in Metaphase and Anaphase Cells

<table>
<thead>
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<td>-12</td>
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* - Metaphase localizations for these proteins could not be determined reliably using the maximum likelihood method due to high variation in the data relative to the distance being measured.

68% confidence limit cited is the larger limit obtained from maximum likelihood estimation.

Negative signs for distances were assigned to indicate that the distance inside (towards the centromere) of the C-terminus of Ndc80p or Nuf2p. Positive sign implies that the protein localizes on the outside (away from the centromere) of this reference point.

† - Although the distances were measured with respect to Ndc80p-C, Fig. 3 was constructed by using Spc24p-C as the point of reference. These columns display each distance from Spc24p-C. The metaphase values was carried out by subtracting 17 nm from the original measurement, while the anaphase values were obtained by subtracting 12 nm.
### Table S3. List of Strains and Plasmids Used in This Study

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Supplemental References


