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Measuring kinetochore-microtubule interaction in vitro

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Abstract

Many proteins and protein complexes perform sophisticated, regulated functions in vivo. Many of these functions can be recapitulated using in vitro reconstitution, which serves as a means to establish unambiguous cause-effect relationships, for example between a protein and its phosphorylating kinase. Here, we describe a protocol to purify kinetochores, the protein complexes that attach chromosomes to microtubules during mitosis, and quantitatively assay their microtubule binding characteristics. Our assays, based on DIC imaging and laser trapping microscopy, are used to measure the attachment of microtubules to kinetochores and the load-bearing capabilities of those attachments. These assays provide a platform for studying kinase disruption of kinetochore-microtubule attachments, which is believed to be critical for correcting erroneous kinetochore-spindle attachments and thereby avoiding chromosome mis-segregation. The principles of our approach should be extensible to studies of a wide range of force-bearing interactions in biology.

1. Introduction

Kinetochores are protein complexes that attach chromosomes to microtubules of the mitotic spindle (Fig. 1). These remarkable microtubule binding machines are capable of tracking the dynamic tips of growing and shortening microtubules for many minutes without detaching (Nicklas & Koch, 1969). They are also regulatory hubs that can break improper kinetochore-microtubule attachments using kinase reactions to prevent chromosomal mis-segregation (Biggins, Severin, Bhalla, Sassoon, Hyman & Murray, 1999; Hauf, Cole, LaTerra, Zimmer, Schnapp, Walter et al, 2003; Tanaka, Rachidi, Janke, Perieira, Galova, Schiebel et al, 2002; Lampson, Renduchitala, Khodjakov & Kapoor 2004; Pinsky, Kung, Shokat & Biggins, 2006; Cheeseman, Anderson, Jwa, Green, Kang, Yates et al, 2002). On the other hand, kinetochore-microtubule attachments are paradoxically *stabilized* when tension is applied to them (Nicklas et al, 1969; Nicklas & Ward, 1994; Akiyoshi, Sarangapani, Powers, Nelson, Reichow, Arrellano-Santoyo et al, 2010). *In vitro* biophysical studies provide powerful tools for dissecting these regulatory mechanisms.

Our *in vitro* assays have already revealed that tension directly stabilizes kinetochore-microtubule attachments through effects on microtubule dynamics (Akiyoshi et al, 2010). Tension reduces the frequency of ‘catastrophes’, or switches from polymerization-driven microtubule growth to de-polymerization/shortening, during which a kinetochore-

microtubule attachment is particularly susceptible to spontaneous rupture. This stabilization mechanism is completely independent of the aforementioned kinase activity and would have been nearly impossible to detect *in vivo* using other existing technologies since tension cannot be measured inside a cell. The *in vitro* approach also makes quantitative comparison of mutant or modified kinetochores straightforward, sidestepping the problems presented by subtle phenotypes (where small but measureable attachment strength changes do not lead to observable changes in spindle structure) and pleiotropic effects (where multiple changes occur at once, producing a confusing result). We have shown that the Ipl1/Aurora B kinase very likely uses a small set of phosphorylation sites on the Ndc80 and Dam1 kinetochore subcomplexes to disrupt attachments, and that phosphorylation on these two subcomplexes contributes additively to attachment weakening (Sarangapani, Akiyoshi, Duggan, Biggins & Asbury, 2013).

In this article, we present methods for studying kinase disruption of kinetochore-microtubule attachments *in vitro*, which involve modifications to the assays used in our previous work (Akiyoshi et al, 2010; Sarangapani et al, 2013). These experiments directly test how kinases affect the strength of microtubule-kinetochore attachment strength, which is important for understanding the error correction process of improper chromosome attachment (Maure, Kitamura & Tanaka, 2007). These experiments also enable more advanced tests of mechanoregulation of the kinases, which is believed (but not proven) to help the kinases identify improper attachments (Maresca & Salmon, 2010). A deeper understanding of the error correction process is of wide interest because chromosomal mis-segregation is associated with birth defects and the development of cancers (Ganem, Godinho & Pellman, 2009; Rajagopalan & Lengauer, 2004).

2. The Leap to *in vitro*: Purification of Intact, Functional Kinetochores

2.1 An antibody-based approach

Purifying native protein complexes can be challenging. Antibody-based strategies are an attractive option since dissociation of the complex from the antibody can be achieved by introducing an excess amount of the protein antigen, as opposed to altering solution pH or ionic strength or introducing reactive small molecules which might disrupt the complex. We use antibodies raised against the 3xFlag peptide, a 24 (3x`DYKDDDDK`) amino acid sequence that we fused to the Dsn1 protein, which is part of the Mtw1 complex and a central structural subunit of the kinetochore. Dsn1 is the kinetochore subunit bound by the monopolin complex to promote sister kinetochore co-orientation during meiosis I, so it can be thought of as a handle for the kinetochore (Corbett, Yip, Ee, Walz, Amon & Harrison, 2010; Corbett & Harrison, 2012). By replacing the endogenous Dsn1 gene with a gene coding for Dsn1-6xHis-3xFlag, we can effectively immunoprecipitate whole kinetochores (Akiyoshi et al, 2010) using a protocol similar to that previously used to purify minichromosomes (Akiyoshi, Nelson, Ranish & Biggins, 2009). The histidine tag is used in the assay protocols and is not involved in the purification. Yeast with this new, tagged Dsn1 gene have no phenotype relative to wild-type strains. We find magnetic beads to be a superior purification substrate, because they produce very clean kinetochore preparations with few contaminants.

2.1.1 Antibody conjugation to magnetic beads—Immobilization of anti-Flag antibodies (M2 anti-Flag, Sigma F3165) to magnetic beads (protein G Dynabeads, Life Technologies 10003D) is achieved through protein G. To begin, rinse 300 μ L Dynabeads twice with 1 mL sodium phosphate buffer (0.1 M Na-PO₄, pH 7.0) using a magnetic particle concentrator (MPC, Life Technologies). After removing the second mL of buffer, resuspend the beads in 60 μ L of 2.5 μ g/ μ L M2 antibodies (in sodium phosphate buffer) with gentle rotation for 30 min. Rinse the beads twice with 1 mL PBST (0.1 M Na-PO₄, 0.01% Tween 20, pH 7.0) and twice with triethanol amine buffer (0.2 M triethanol amine, 20 mM dimethyl pimelimidate, pH 8.2) using the MPC. Initiate covalent coupling through DMP chemistry by resuspending the beads in 1 mL of 20 mM DMP in triethanol amine buffer. Quench the reaction after 30 min (do not over-couple) by resuspending in 1 mL Tris buffer (50 mM Tris-HCl, pH 7.5), and then rinse three times with 1 mL PBST. Resuspend in 300 μ L PBST. M2 antibody-coated Dynabeads can be stored at 4°C for use within one month.

2.1.2 Yeast lysate preparation—Grow a 2 L culture of a Dsn1-6xHis-3xFlag epitope tagged yeast strain (SBY8253) to late log phase ($OD_{600} = 1.5$ -2.0) and harvest the yeast by centrifugation. All subsequent steps should be performed at 4°C by keeping buffers and yeast on ice. Rinse (resuspend with 25 mL pipette + re-pellet) the pellet with cold H₂O + 0.2 mM PMSF, and then with BH0.15 (25 mM HEPES, 2 mM MgCl₂, 0.1 mM EDTA, 0.5 mM EGTA, 15% glycerol, 0.1% NP-40, 150 mM KCl) + protease inhibitors (0.2 mM PMSF, 10 μ g/mL Leupeptin, 10 μ g/mL Pepstatin, 10 μ g/mL Chymostatin) + phosphatase inhibitors (100 ng/mL Microcystin-LR, 1 mM sodium pyrophosphate, 2 mM sodium-beta-glycerophosphate, 100 nM sodium orthovanadate, 5 mM sodium fluoride). Freeze the pellet in liquid nitrogen and weigh (usually ~9 g) in preparation for mechanical disruption. The pellet can be stored at -80°C if necessary. We use a small blender (Oster brand) to lyse the yeast, but other methods may be effective as well (Aitchison, Blobel & Rout 1996; Schultz, M. C., Hockman, D. J., Harkness, T. A. A., Garinther, W. I. & Altheim, B. A., 1997). Regardless of the method used, care must be taken to keep the pellet frozen during the grinding process, which invariably creates heat. Shake the pellet into the blender cup and add dry ice to the blender at around five times the volume of the pellet, grind for 10-12 min, replace the evaporated dry ice, and repeat once more. Allow the dry ice to evaporate at room temperature, occasionally stirring the powder. Once the dry ice evaporates and the ground cell mass begins to melt, lyse the pellet in 16.6 mL of BH0.15 + protease inhibitors + phosphatase inhibitors (same as pellet rinse buffer except double the amount of leupeptin, pepstatin, chymostatin, and microcystin-LR). Use a 25 mL pipette to completely resuspend the cell mass and then incubate on ice for an additional 15 min with occasional mixing. Clarify by ultracentrifugation (24,000 rpm in a Beckman-Coulter SW-41 Ti or 71,124xg for 90 min at 4°C). Three layers are visible in the centrifuge tube after clarification: (1) an insoluble cell debris mass at the bottom, (2) an aqueous layer containing the protein, (3) a thin film of lipid at the top. Extract only the aqueous layer using a needle and syringe to puncture the wall of the plastic centrifuge tube, being very cautious not to disturb the lipid layer which would contaminate the lysate. Use a Bradford assay to estimate protein concentration and total protein yield. If needed, the lysate can be flash frozen in liquid nitrogen and stored at -80°C for future use.

2.1.3 Purification step—The final step is immunoprecipitation. Incubate M2 anti-Flag Dynabeads (15 μ L per 12.6 mg protein in lysate) with lysate for 3 hr at 4°C with gentle rotation. Rinse the beads four times with 1 mL BH0.15 + protease inhibitors + phosphatase inhibitors + 2 mM DTT, then three times with 1 mL BH0.15 + protease inhibitors. Incubate with elution buffer (BH0.15 + protease inhibitors + 0.5 mg/mL 3xFlag peptide) in the original volume of Dynabeads for 30 min with constant gentle vortexing to elute the kinetochores from the beads. We use a custom-synthesized 3xFlag peptide, but it is also commercially available (Sigma F4799). Freeze the product in liquid nitrogen in small (~2-5 μ L) aliquots.

2.1.4 Analysis of kinetochore particles—To analyze the kinetochore purification, run an aliquot on a 4-12% NuPage Bis-Tris gel (Life Technologies) and analyze the stoichiometry of kinetochore components after silver staining the gel. After a successful purification, the stoichiometry of the Ndc80, Spc105 and Dsn1 components are similar. On the same gel, run BSA protein standards to estimate the Dsn1 concentration of the preparation, which is usually between 1.5-6.0 ng/ μ L. Particles can also be analyzed by immunoblotting for specific candidate proteins that are present at substoichiometric amounts (Akiyoshi et al, 2010).

2.2 Microbeads for visualization and manipulation of single kinetochores

Polystyrene microbeads provide a handle for manipulating protein complexes such as microtubule motors, DNA enzymes, and kinetochore particles (Vale, Reese & Sheetz, 1985; Yin, Wang, Svoboda, Landick, Block & Gelles, 1995; Akiyoshi et al, 2010). The complex of interest can be targeted to the microbead surface through an antibody recognizing a tag on the complex. We cannot use anti-Flag antibodies for this since our kinetochore preparations contain excess 3xFlag peptide; instead, we use antibodies that bind the His epitope on Dsn1-6xHis-3xFlag. We purchase streptavidin-conjugated microbeads and biotin-conjugated anti-His antibodies that self-associate. Alternatively, antibodies can be covalently linked directly to microbeads using established protocols (Hermanson, 1996; Bangs Laboratories, 2002).

To prepare microbeads coated in anti-His antibody, combine 112.5 μ L H₂O, 45 μ L 5xBRB80 (400 mM PIPES, 62.5 mM NaOH, 5 mM MgCl₂, 5 mM EGTA, pH 6.9), and 22.5 μ L PSS4 (SpheroTech, 0.44 μ m) beads in a 500 μ L eppendorf tube. Sonicate with a tip sonicator in an ice bath for 1 min. Add 45 μ L biotin-conjugated anti-His antibody (Qiagen #34440) to the mixture, and incubate at 4°C for 1 hr. Add 275 μ L bead storage buffer (1xBRB80 (80 mM PIPES, 12.5 mM NaOH, 1 mM MgCl₂, 1 mM EGTA, pH 6.9) + 8 mg/mL BSA + 1 mM DTT) and mix well, sonicate again for 1 min and centrifuge at 16.1xg for 9 min at 4°C. Aspirate the supernatant gently. Repeat the resuspension and centrifugation steps 5 times, resuspending in 500 μ L bead storage buffer each time. After the last centrifuge spin and aspiration step, resuspend the bead pellet in 225 μ L bead storage buffer, sonicate for 1 min, and store at 4°C with inversion mixing.

Microbeads prepared this way (which we refer to as PSS4/10) can be stored and used for 2-3 months. Over time, the beads will clump/deposit on the sides of the tube. Sonication should

correct this. Clumping is more pronounced in samples that have been insufficiently washed (too few resuspensions/centrifugations).

3. The Assays

3.1 Bead binding assay

Sometimes a simple measure of a complex's ability to bind its target (microtubule, DNA, etc.) is useful. Polystyrene microbeads are plainly visible in DIC, so they can be used as a marker instead of a fluorescent tag on the complex. The principle behind the assay is to densely coat a coverslip with microtubules so that all microbeads with active kinetochores have an opportunity to bind (Sassoon, Severin, Andrews, Taba, Kaplan, Ashford et al, 1999; Biggins et al, 1999). Measure the dimensions of the field only if you wish to compare measurements between different instruments. Of course, bead binding is a function of the amount of kinetochore material used to coat the beads, so this is a design parameter that may need to be optimized for different experiments to maximize sensitivity of the assay to differences between the intrinsic binding capabilities of the different kinetochores in the comparison.

3.1.1 Materials—All coverslips should be cleaned with KOH solution (80 g KOH in ethanol, 300 mL total volume in a 1 L beaker). This solution is corrosive and flammable and should be handled with care in a fume hood. Load coverslips into a Teflon rack (this rack needs hooks so that a metal positioning rod can be used to pull it in and out of the KOH), and immerse them in the KOH solution. Sonicate the beaker with the coverslips for 10 min using a bath sonicator. Prepare two beakers with 1.5 L and 0.8 L deionized water. Using a positioning rod, remove the coverslip rack from the KOH and dunk it twenty times in the beaker with 1.5 L of water, then remove and place in the smaller water beaker. Sonicate the small beaker for 10 min. Take the coverslip rack out and spray the coverslips forcefully with deionized water from a spray bottle. Use about 250 mL of water. Last, spray the coverslips with around 250 mL of ethanol. Place the rack in an oven at around 50°C for a few hours to dry, and then transfer the rack to a covered container for storage.

To prepare taxol-stabilized microtubules, mix 48.8 μ L of polymerization buffer (1xBRB80 + 5.7% DMSO + 4.1 mM $MgCl_2$ + 9.8 mM GTP) with 12.8 μ L tubulin (100 μ M). Incubate 45 min at 37°C. Add 200 μ L warm BTAX (1xBRB80 + 10 μ M paclitaxel) to the polymerization mix to stabilize the microtubules. Transfer to a TLA 100 centrifuge tube and spin at 58,000 rpm (129,797xg) for 10 min at 37°C. Discard the supernatant and resuspend the pellet in 200 μ L BTAX with a cut P200 tip to avoid excessive shearing of the microtubules.

Prepare a flow chamber using a glass slide, coverslip, and double-sided tape. Place two pieces of tape on the slide parallel with its short dimension with a 1 mm gap between them centered on the slide. The width of the gap is important; it influences the speed of the flow through the channel as well as the total channel area over which the bead binding is distributed. The best practice is to measure the width with calipers and to only use channels that fall within ± 0.1 mm of the target width. Place the coverslip over the channel so that it

covers it and overhangs each edge, standardizing the length of the channel to exactly the width of the slide.

For the assay, you will need rigor kinesin, which serves as a linker between the microtubules and the surface (we use the G234A mutant: Rice, Lin, Safer, Hart, Naber, Carragher et al, 1999). Typically, a five-fold dilution of BL-21 expressed, Ni^{2+} -NTA purified motor will do well, but optimization may be needed to achieve high microtubule density. In this protocol, a 6xHis-tagged GFP (0.5 mg/mL working concentration) is used to saturate the antibodies on the microbeads that remain unoccupied after the kinetochore incubation so that they do not bind directly to the 6xHis-tagged kinesin on the surface (Fig 2a).

3.1.2 Basic binding assay—To test the ability of the kinetochores to bind microtubules, mix 20 μL of BTAX with 5 μL of PSS4/10 to make PSS4/50. Sonicate the PSS4/50 under a tip sonicator in an ice bath for 1 min. Dilute a kinetochore aliquot with BTAX so that the concentration of Dsn1 is 5 nM (0.33 ng/ μL), then mix 5 μL of PSS4/50 with 5 μL of diluted kinetochores and incubate at 4°C for 45 min, with rotational mixing. Add 10 μL of GFP and incubate 15 min more at 4°C. Five min into the incubation, fill the chamber with rigor kinesin and incubate 5 min. Using an aspirator or filter paper to draw the liquid through the channel, rinse the channel with 30 μL blocking buffer (1xBRB80 + 2 mg/mL κ -casein + 10 μM paclitaxel) and then add 20 μL of the taxol-stabilized microtubules and incubate for 5 min. Rinse the channel once more with 30 μL blocking buffer. When the GFP incubation completes, add 20 μL blocking buffer to the mixture and sonicate it for 45 sec using the tip sonicator. Flow 20 μL of this final mixture through the slide, and begin counting the number of surface associated beads in each field 5 min later. Count as many fields as you can in 10 min. For a control measurement, substitute pure BTAX for diluted kinetochores.

While you are counting, qualitatively assess the microtubule coverage of the coverslip. The microtubules should densely cover the coverslip in a “mat” (Fig. 2b). If the microtubules become sparse, non-specific binding will increase and specific binding will decrease. High concentrations of casein and 6xHis-GFP are necessary to prevent non-specific binding of the beads to the surface (binding not mediated by kinetochore-microtubule attachments). Do not attempt to differentiate beads that are associated with microtubules on the coverslip vs. beads associated with the coverslip directly. Be sure to count beads attached to microtubules protruding from the surface; they are valid attachments. Develop a system for counting beads that are on the edge of a field and be consistent (e.g., all beads visible on the right edge count, all beads on the left edge do not). Make sure that you traverse the channel in such a way as to sample across its width while avoiding the very edges. This will average out spatial variations in bead deposition. Stick to the time limits. Beads continue to accumulate slowly throughout the experiment.

3.1.3 Timecourse experiment for assaying kinase activity—A protein kinase called Mps1 co-purifies with the kinetochores from section 2.1 (London, Ceto, Ranish & Biggins, 2012). It phosphorylates the kinetochores *in vitro* in the presence of ATP and this phosphorylation may interfere with the formation of attachments to microtubules (Maure et al, 2007). The progress of this reaction can be monitored in the binding assay, with minor modifications. Instead of diluting the PSS4/10 bead stock in BTAX, dilute it in bead buffer

that contains the phosphatase inhibitors described in section 2.1.2 for rinsing the cell pellet. The phosphatase inhibitors stop any phosphatases in the prep from undoing the phosphorylation by Mps1 so that its effects can be measured. One may also deactivate the Mps1 kinase using a temperature-(Winey, Goetsch, Baum & Byers, 1991; Schutz & Winey, 1998) or nucleotide analogue-sensitive mutant (Jones, Huneycutt, Pearson, Zhang, Morgan, Shokat, et al, 2005; Tighe, Staples & Taylor, 2008) and then add recombinant Ipl1/Sli15 to the reaction mixture below prior to adding ATP.

Before starting, prepare four slides as described before. Follow the previous instructions, but use 10 μ L of PSS4/50 in bead buffer (1xBRB80 + phosphatase inhibitors), 10 μ L of diluted kinetochores, and 20 μ L of GFP. After the 15 min GFP incubation, add 4 μ L of 100 mM ATP and move the reaction to a 37°C incubator. This creates a one-pot reaction that will be sampled at four different time points. Prep for each slide requires ~15 min, and they should be prepared immediately before use. Two people must be involved: one to prepare the samples, another to count the beads per field in each sample. For time points of 5, 25, 45, and 65 min after ATP introduction, start the preparation of the first slide at the same time as in the original protocol. Extract 10 μ L of material from the pot at each time point, dilute with 10 μ L blocking buffer, and sonicate for 45 sec. Formally, the person preparing the slides has 5 min to begin preparing the next sample, but practically the 5 min of extra time will be taken by the various aspiration and sonication operations required at each step. Use a master timer that monitors the overall time in the 65 min experiment rather than counting off 5 min for each step. This will help with comparisons between runs. If 3 people and 2 microscopes are available, the kinetochore-bead-GFP mixture volume can be doubled and then split in half into experimental (ATP) and control (H_2O or ADP) reactions to be counted simultaneously.

3.2 Rupture Force

With an optical trap, it is possible to measure the force that a single complex attachment can withstand (Fig. 3a). In essence, optical traps are simply microscopes with powerful, focused laser beams in the sample plane, but the optics and instrumentation required to focus, image, and detect the laser require expertise to set up (Block, 1990). Like most, our traps are feedback-controlled such that the separation between the laser focus and a trapped polystyrene bead can be maintained or changed in a pre-programmed manner (Visscher & Block, 1998). We translate a piezoelectric sample stage (to which the polystyrene bead is attached) relative to the trap laser, which is fixed (Frank, Powers, Gestaut, Davis & Asbury, 2010), but one may alternatively steer the trap beam using an acousto- or electro-optic deflector. Our custom instrument control software is written in LabView and is freely available upon request. In these experiments, the control software is used to steadily increase or “ramp” the separation between the bead and the trap laser, consequently increasing the restoring force applied by the trap on the bead (Fig. 3b). Trap control software can be easily written to facilitate other measurements, such as attachment lifetime under oscillating loads (as occurs during metaphase (Dumont, Salmon & Mitchison, 2012)) to test whether attachment strength is affected by the pattern or structure of the applied load in addition to its magnitude. Since single complexes detach from their targets stochastically under any load (or none) the result of the force ramp experiment is a rupture force distribution that is

dependent on both the intrinsic properties of the attachment and the rate at which load on the attachment is increased (Evans, 2001). With this measurement, one can cope with some amount of heterogeneity in a sample and distinguish relatively small differences between species.

3.2.1 Microtubules for optical trapping—The microtubules are anchored to the coverslip surface so that they remain fixed while force is applied to the kinetochores through the microbeads. Through a serial polymerization strategy, microtubules with two compositionally distinct ends are synthesized. One end binds the surface through biotin/streptavidin interactions while the other remains unbound and projects into solution so that it can interact with beads held away from the surface with the optical trap. This second end can either be dynamic (spontaneously polymerizing and depolymerizing throughout the experiment) or static (stabilized using paclitaxel). Kinetochores couple to the ends of dynamic microtubules *in vivo*, so reconstituting this behavior *in vitro* is desirable. However, for consistent polymerization behavior, buffer conditions must be strictly controlled.

Before starting, prepare biotinylated microtubule seeds. These can be stored in small (~5 μ L) aliquots at -80°C . Mix 10 μ L biotin tubulin (2 $\mu\text{g}/\mu\text{L}$ in 1xBRB80, Cytoskeleton #T333P) with 33 μ L unlabeled tubulin (100 μM in 1xBRB80). Withdraw 40 μ L of this mixture and add to it 250 μ L of seeds buffer (2xBRB80 + 20.8% glycerol + 2 mM GMPCPP + 1.6 mM DTT). Incubate 1 hr at 37°C , then aliquot and flash freeze in liquid nitrogen.

3.2.2 Dynamic microtubule rupture force assay—To perform our dynamic microtubule rupture force assay, use chambers that are identical to those described in section 3.1.1, though the channel width does not need to be stringently controlled. The solutions required for this assay are: assay buffer (1xBRB80 + 1 mg/mL κ -casein), GB2x (1xBRB80 + 2 mg/mL κ -casein + 2 mM GTP), GB1x (50% GB2x in H_2O), and GBTB (50% GB2x in H_2O + 12% KTB + 16 μM tubulin + 0.8 mM DTT + 50 mM glucose + 0.5 mg/mL glucose oxidase + 60 $\mu\text{g}/\text{mL}$ catalase). The glucose oxidase, catalase, and glucose consume oxygen in the GBTB solution so that kinetochores are protected from oxidative damage when they are in the trapping laser. One must wait to add the glucose oxidase and catalase until just before the GBTB is used so that the reaction does not run its course prior to the measurement. Also wait until the end to add the tubulin to avoid unnecessary polymerization. Prepare PSS4/50 as before using assay buffer to dilute. Dilute the kinetochores to 1 ng/ μL Dsn1 with assay buffer. Combine 5 μL of each to make KTB and incubate at 4°C . Fifteen minutes into the KTB incubation, add biotin BSA (Vector Labs #B-2007, 10 mg/mL) to the flow chamber and incubate 15 min. Rinse the chamber with 100 μL warm 1xBRB80, then introduce avidin (Vector Labs #A-3100, 0.33 mg/mL) and incubate 3 min. Rinse again with 100 μL 1xBRB80, then dilute a 5 μL aliquot of microtubule seeds with 15-35 μL of warm 1xBRB80 and flow the entire dilution through the chamber and incubate 3 min. The appropriate seeds concentration can vary based on properties of the coverslip. After the seeds incubation, rinse the channel with 100 μL GB1x. Finish preparation of the GBTB by adding KTB, glucose oxidase and catalase, sonicating under the tip sonicator for 45 sec, and then adding the tubulin. Warm up the GBTB to approximately room temperature just before introduction to the chamber to avoid

depolymerization of the seeds without risking polymerization of the free tubulin in the GBTB. Seal the ends of the chamber with nail polish and take the sample to the optical trap.

To test the attachment strength of the kinetochores, you may capture beads floating in solution and attach them to microtubules (only a fraction will bind), or find pre-attached beads. With each bead, apply a load in the direction of the microtubule's free end. Start with a very low load (e.g., 1 pN) and increase it only as necessary to pull the bead to the microtubule tip. Kinetochores can slide along the lattice of a microtubule without detaching, but some amount of force is needed to overcome protein friction. Once at the tip, the bead will advance at a rate of ~10 nm/s during microtubule assembly (which is far more common than disassembly). Increase the load to a pre-determined level (e.g., 4 pN) and hold for 30-60 sec before initiating a force ramp to increase the force at a constant rate (e.g., 0.25 pN/s) until the attachment ruptures. Repeat this for as many attachments as you wish within a 2 hr time window.

3.2.3 Taxol microtubule rupture force assay for kinase activity—In the cell, kinases must recognize erroneous attachments that have already formed and disrupt them; we have modified the previous assay to match this order of events. In this last protocol, beads that do not spontaneously bind are rinsed away with a reaction buffer so that subsequent enzyme reactions are only carried out on attached kinetochores (which may be acted upon differently than unattached kinetochores). This rinsing operation is sensitive because the flow driven through the channel can easily be forceful enough to remove attached kinetochore-beads. The most ideal solution to the problem is to use a syringe pump to drive a slow, smooth, consistent rinse (Fig. 4, diagram). If this is not possible, wicking with wet filter paper may be a viable alternative. Note, use of a syringe pump may require custom-machined adaptors to interface the tubing to the slide/chamber (Gestaut, Cooper, Asbury, Davis & Wordeman, 2010). Channel width must again be closely controlled.

Be sure to rinse the channel with H₂O thoroughly before you begin. Refer to the dynamic microtubule assay protocol and figure 4. New solutions are: 2xBTAX+PIs (2xBRB80 + 2xphosphatase inhibitors + 20 μM paclitaxel), BTAX+PIs (50% 2xBTAX+PIs in H₂O), GB1x+tubulin (50% GB2x + 18 μM tubulin in H₂O; reserve tubulin until just before use), KTB mix (50% 2xBTAX+PIs + 20% KTB in H₂O; KTB will be added after incubation), and reaction buffer (50% 2xBTAX+PIs in H₂O + 0.8 mM DTT + 50 mM glucose + 0.5 mg/mL glucose oxidase + 60 μg/mL catalase + 2 mM ATP; reserve glucose oxidase, catalase, and ATP until just before use). During the KTB incubation, construct the slide (Fig. 4, table). The GB1x+tubulin polymerizes non-biotinylated extensions from the seeds, and the BTAX+PIs stops the polymerization and stabilizes the extensions. Kinetochore-beads (KTB mix) are then introduced at low velocity so that they can bind the extensions, and finally the free kinetochore-beads are rinsed away with reaction buffer containing ATP, which activates the kinase. Use a razor blade to cut the tubing away from the tubing adaptor to avoid disturbing the chamber. Take the sample to the optical trap. There should be some beads attached to microtubules 'wagging' just off the coverslip surface. The strength of those attachments can be tested in a manner similar to that in the previous assay, but if kinase activity has a strong disruptive effect on the attachments, a lower initial load may be required.

4. Summary

Kinetochores form load-bearing attachments with microtubules to link chromosomes to microtubules during cell division. Mechanoregulation of these attachments is very difficult to study in living cells since load on the attachments cannot be precisely controlled or measured. As a solution, we have recently shown that kinetochores can be purified from yeast by immunoprecipitation using an epitope tag on the Dsn1 protein. This one-step strategy gives high yield sufficient for quantitative biophysical assays. The bead binding assay measures the propensity of kinetochores to form attachments, while the rupture force assay measures their strength. Kinase disruption of these attachments can be studied using (1) genetic manipulation to introduce phosphorylation-mimicking aspartic acid mutations at kinase target sites and (2) by phosphorylation *in vitro* by active kinase. These strategies provide the most direct test of the effects of phosphorylation at specific sites by specific kinases, and do so with instrumentation and expertise that is becoming increasingly accessible to biochemists. It should be possible to adapt these assays to study a wide range of protein binding interactions regulated by enzyme activity.

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References

- Aitchison JD, Blobel G, Rout MP. Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science*. 1996; 25:624–627. [PubMed: 8849456]
- Akiyoshi B, Nelson CR, Ranish JA, Biggins S. Quantitative proteomic analysis of purified yeast kinetochores identifies a PP1 regulatory subunit. *Genes and Development*. 2009; 23:2778–2899. [PubMed: 19952112]
- Akiyoshi B, Sarangapani KK, Powers AF, Nelson CR, Reichow SL, Arellano-Santoyo H, Gonen T, Ranish JA, Asbury CL, Biggins S. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature*. 2010; 468(7323):576–579. [PubMed: 21107429]
- Bangs Laboratories, Inc. Tech note 205: Covalent Coupling. 2002. <http://www.bangslabs.com/support/technotes>
- Biggins S, Severin F, Bhalla N, Sassoon I, Hyman AA, Murray AW. The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes and Development*. 1999; 13(5):532–544. [PubMed: 10072382]
- Block, SM. Optical tweezers: a new tool for biophysics. In: Grinstein, S.; Foskett, K., editors. *Modern Biology Series: Volume 9. Noninvasive Techniques in Cell Biology*. Wiley-Liss; New York: 1990. p. 375–401.
- Cheeseman IM, Anderson S, Jwa M, Green EM, Kang J, Yates JR, et al. Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell*. 2002; 111(2):163–172. [PubMed: 12408861]
- Corbett KD, Harrison SC. Molecular architecture of the yeast monopolin complex. *Cell Reports*. 2012; 1(6):583–589. [PubMed: 22813733]
- Corbett KD, Yip CK, Ee LS, Walz T, Amon A, Harrison SC. The monopolin complex crosslinks kinetochore components to regulate chromosome-microtubule attachments. *Cell*. 2010; 142(4):556–567. [PubMed: 20723757]

- Dumont S, Salmon ED, Mitchison TJ. Deformations within moving kinetochores reveal different sites of active and passive force generation. *Science*. 2012; 337:355–358. [PubMed: 22722252]
- Evans E. Probing the relation between force, lifetime, and chemistry in single molecular bonds. *Annual Reviews of Biophysics and Biomolecular Structure*. 2001; 30:105–128.
- Frank AD, Powers AF, Gestaut DR, Davis TN, Asbury CL. Direct physical study of kinetochore-microtubule interactions by reconstitution and interrogation with an optical force clamp. *Methods*. 2010; 51(2):242–250. [PubMed: 20096784]
- Ganem NJ, Godinho SA, Pellman D. A mechanism linking extra centrosomes to chromosomal instability. *Nature*. 2009; 460:278–282. [PubMed: 19506557]
- Gestaut DR, Cooper J, Asbury CL, Davis TN, Wordeman L. Reconstitution and analysis of kinetochore subcomplexes. *Methods in Cell Biology*. 2010; 95:641–656. [PubMed: 20466157]
- Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, et al. The small molecule Heperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *The Journal of Cell Biology*. 2003; 161(2):281–294. [PubMed: 12707311]
- Hermanson, GT. *Bioconjugate Techniques*. Academic Press; San Diego: 1996.
- Jones M, Huneycutt B, Pearson C, Zhang C, Morgan G, Shokat K, et al. Chemical genetics reveals a role for Mps1 kinase in kinetochore attachment during mitosis. *Current Biology*. 2005; 15(2):160–165. [PubMed: 15668173]
- Lampson MA, Renduchitala K, Khodjakov A, Kapoor TM. Correcting improper chromosome-spindle attachments during cell division. *Nature Cell Biology*. 2004; 6(3):232–237.
- London N, Ceto S, Ranish JA, Biggins S. Phosphoregulation of Spc105 and Mps1 and PP1 regulates Bub1 localization to kinetochores. *Current Biology*. 2012; 22(10):900–906. [PubMed: 22521787]
- Maresca TJ, Salmon ED. Welcome to a new kind of tension: translating kinetochore mechanics into a wait-anaphase signal. *Journal of Cell Science*. 2010; 123(6):825–835. [PubMed: 20200228]
- Maure J-F, Kitamura E, Tanaka TU. Mps1 kinase promotes sister-kinetochore bi-orientation by a tension-dependent mechanism. *Current Biology*. 2007; 17(24):2175–2182. [PubMed: 18060784]
- Nicklas RB, Koch CA. Chromosome micromanipulation III. Spindle fiber tension and the reorientation of mal-oriented chromosomes. *The Journal of Cell Biology*. 1969; 43(1):40–50. [PubMed: 5824068]
- Nicklas RB, Ward SC. Elements of error correction in mitosis: microtubule capture, release, and tension. *The Journal of Cell Biology*. 1994; 126(5):1241–1253. [PubMed: 8063861]
- Pinsky BA, Kung C, Shokat KM, Biggins S. The Ipl1-aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores. *Nature Cell Biology*. 2006; 8(1):78–83.
- Rajagopalan H, Lengauer C. Aneuploidy and cancer. *Nature*. 2004; 432:338–341. [PubMed: 15549096]
- Rice S, Lin AW, Safer D, Hart CL, Naber N, Caragher BO, et al. A structural change in the kinesin motor protein that drives motility. *Nature*. 1999; 402(6763):778–784. [PubMed: 10617199]
- Sarangapani KK, Akiyoshi B, Duggan NM, Biggins S, Asbury CL. Phosphoregulation promotes release of kinetochores from dynamic microtubules via multiple mechanisms. *Proceedings of the National Academy of Sciences*. 2013; 100(18):7282–7287.
- Sassoon I, Severin FF, Andrews PD, Taba M-R, Kaplan KB, Ashford AJ, et al. Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. *Genes and Development*. 1999; 13(5):545–555. [PubMed: 10072383]
- Schutz AR, Winey M. New alleles of the yeast Mps1 gene reveal multiple requirements in spindle pole body duplication. *Molecular Biology of the Cell*. 1998; 9(4):759–774. [PubMed: 9529376]
- Schultz MC, Hockman DJ, Harkness TAA, Garinther WI, Altheim BA. Chromatin assembly in a yeast whole-cell extract. *Proceedings of the National Academy of Sciences*. 1997; 94(17):9034–9039.
- Tanaka TU, Rachidi N, Janke C, Pereira G, Galova M, Schiebel E, et al. Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell*. 2002; 108(3):317–329. [PubMed: 11853667]

- Tighe A, Staples O, Taylor S. Mps1 kinase activity restrains anaphase during an unperturbed mitosis and target Mad2 to kinetochores. *The Journal of Cell Biology*. 2008; 181(6):893–901. [PubMed: 18541701]
- Vale RD, Reese TS, Sheetz MP. Identification of a novel force generating protein, kinesin, involved in microtubule-based motility. *Cell*. 1985; 42:39–50. [PubMed: 3926325]
- Visscher K, Block SM. Versatile optical traps with feedback control. *Methods in Enzymology*. 1998; 298:460–489. [PubMed: 9751903]
- Winey M, Goetsch L, Baum P, Byers B. Mps1 and Mps2: novel yeast genes defining distinct steps of spindle pole body duplication. *The Journal of Cell Biology*. 1991; 114:745–754. [PubMed: 1869587]
- Yin H, Wang MD, Svoboda K, Landick R, Block SM, Gelles J. Transcription against an applied force. *Science*. 1995; 270(5242):1653–1657. [PubMed: 7502073]

Figure 1.

A metaphase chromosome is coupled to microtubules from opposite sides of the mitotic spindle through its kinetochores. The kinetochores remain attached as the microtubules undergo assembly and disassembly.

Figure 2.

(a) Diagram of a kinetochore-coated bead binding to a microtubule immobilized to a coverslip through rigor kinesins. The blocking protein described in the text is 6xHis-GFP, but any protein that has a 6xHis tag and that does not stick to the surface will work. (b) DIC image of kinetochore beads bound to a microtubule mat.

Figure 3.

(a) Diagram of a kinetochore-microtubule attachment under tension in an optical trap. (b) Example rupture force measurement. The microbead returns to the center of the trap (zero force) after the attachment ruptures. The peak force reached during the force ramp is the “rupture force” that is recorded for that attachment.

Solution	Volume (μL)	Rate (μL/min)	Wait (min)
Biotin BSA	25	50	15
1xBRB80	100	50	0
Avidin	75	50	3
1xBRB80	100	50	0
Seeds	25	50	3
GB1x	100	50	3
GB1x+tub.	50	50	10
BTAX+PIs	200	50	0
KTB mix	50	10	5
Rxn buff	50	25	0

Figure 4.

(Diagram) Illustration of a flow chamber for syringe-pump driven buffer exchange. **(Table)** Flow protocol for creating a chamber with kinetochore-beads bound to surface-immobilized microtubules.