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Measuring mitotic forces

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Abstract

Productive chromosome movements require that a large multi-protein complex called the kinetochore assemble on sister centromeres. The kinetochore fulfills two critical functions as 1) the physical linkage between chromosomes and spindle microtubules, and 2) a mechano-molecular sensor that relays a spindle assembly checkpoint signal delaying anaphase onset until chromosomes are best-positioned to be equally segregated. Given its central roles in such a vital process, the kinetochore is one of the most important force-transducing structures in cells; yet it has been technically challenging to measure kinetochore forces. Barriers to measuring cellular forces have begun to be broken by the development of fluorescence-based tension sensors. In this chapter, two methods will be described for measuring kinetochore forces in living cells and strategies for applying these sensors to other force-transducing processes and molecules will be discussed.

I. Introduction

Cell division, at its core, is a physical process. Microtubule (MT)-based motors and the MTs themselves generate forces that move chromosomes through the cytosol, push spindle poles apart, and position the spindle; while actin and myosin drive the physical separation of the cell into two daughters during cytokinesis. This chapter will focus on the use of tension sensors to study kinetochores; but we hope that common principles highlighted here will inform the strategic engineering of force-transducing molecules to study other fundamental force-driven processes during cell division.

While knowledge of the kinetochore at the molecular and structural level has grown tremendously in the past decade, the question of how much force is applied to the kinetochore, especially in animals, has been difficult to measure. Force measurement techniques such as atomic force microscopy (AFM), magnetic traps, and optical tweezers, which are powerful methods *in vitro*, are not readily adapted to intracellular force measurements. To date, the most extensive measurements of mitotic forces come from a series of classic experiments using calibrated microneedles (Nicklas, 1988). However, the use of microneedles is limited to insect cell types because, unlike most animal cells, their plasma membranes can be deformed and chromosomes can be moved and “snagged”

without puncturing the cells, most likely due to the absence of conventional cytosolic intermediate filaments in insects. The emergence of single molecule-calibrated, fluorescence-based tension sensors (Freikamp, Mehlich, Klingner, & Grashoff, 2017) combined with live-cell imaging approaches has ushered in a new era in which the historical limitations to measuring forces in living cells are fading. This chapter is intended to be a launch-pad for the cell biologist's initial forays into probing cellular forces during cell division.

II. Selection and placement of tension sensors

This chapter will describe the use of FRET- and non-FRET-based tension sensors (Figure 1A, B). While different reporters have advantages and disadvantages, the use of multiple sensors as means of validation may be worthwhile. Also worth considering is that the calibrated FRET-based force sensor toolbox is rapidly expanding (Freikamp, Cost, & Grashoff, 2016; Freikamp, et al., 2017). New sensors have been designed with sensitivities that report over broader, narrower, higher, or lower force ranges than the TSMOD sensor described here (Grashoff et al., 2010).

Once the best reporter has been chosen, deciding upon the physical placement of that tension sensor into a force-transducing molecule is of central importance. While trial and error may be necessary, one should begin by carefully examining what is known structurally and organizationally about the protein and structure of interest. The ideal candidate will most likely be 1) a core component (not transient or highly dynamic) of the structure being studied and 2) positioned within the structure to be a force-transducing molecule. These two requirements are nicely highlighted by the kinetochore molecules that have been engineered to contain tension sensors to date: the kinetochore-microtubule attachment factor Ndc80 in budding yeast and a protein that links the inner and outer kinetochore called CENP-C in *Drosophila melanogaster* cells (Suzuki et al., 2016; Ye, Cane, & Maresca, 2016) since Ndc80 and CENP-C are core kinetochore components that are well-positioned to transduce force.

To measure tension, the reporter must be positioned internally into the core force-transducing molecule without disrupting the protein's localization or function. For budding yeast Ndc80, Suzuki et al. found that the position that supported insertion of the tension sensor was, surprisingly from a structural perspective, located within a region that typically forms an alpha helical coiled-coil with its binding partner Nuf2 (Suzuki, et al., 2016). However, this positioning made sense in light of prior work that had identified the sensor insertion region as insensitive to transposon insertion (Tien et al., 2013). Thus, genetic, structural, and biochemical studies should be fully harnessed to inform initial attempts at positioning a tension sensor. In the case of CENP-C, 1) the N- and C-termini had been characterized as the outer and inner kinetochore associating domains respectively, and 2) the middle of the protein is computationally predicted to be disordered (Figure 2 A – C). Disordered domains are by no means inert as they are enriched for protein modification sites (Holt et al., 2009) and deciphering the contribution of intrinsically disordered regions (IDR) to protein folding and phase transitions is an ascendant area of research (Shin & Brangwynne, 2017). However, we are of the belief that IDRs in force-transducing

components, will provide fertile ground for the insertion of tension sensors as long as the IDR is not a “stapled” or isolated loop within a tertiary/quaternary structure like the loop domain of Ndc80 (Ciferri et al., 2008; Maiolica et al., 2007). Interestingly, numerous kinetochore components contain IDRs suggesting this particular structure should be amenable to measuring tension at multiple locales. Useful protein disorder prediction tools are highlighted in Figure 2 B, C (Buchan, Minneci, Nugent, Bryson, & Jones, 2013; Oates et al., 2013). In addition to the internal tension sensor, all force-sensor experiments require accompanying control sensors that will not be subjected to force - typically by placing the sensor at either the N- or C-terminus of the force-transducing protein (Figure 2 D). Thus, one should be confident that tagging the ends of the protein of interest does not disrupt its localization or function before effort is put into developing these tools.

III. Equipment and Materials.

A. Live-cell imaging

Live-cell fluorescence microscopy resources are essential to begin probing your protein/structure of interest. An advantage of most of the protocols in this chapter, with the exception of acceptor photobleaching, is that the live-cell imaging can be done on standard wide-field microscopes. Microscopy systems with the required imaging capabilities are typically found in cell biology labs and, if not in your lab, one can access them through local core facilities. It is important to note that the imaging described here is of reporter ensembles consisting of many (typically 100s) fluorescently tagged proteins. Thus, the fluorescence readout of these assays will provide a population-scale view of force application to the structure of interest. In order to assess the behavior of individual proteins, such as the percentage of molecules that are under tension or whether variable tension is applied to different molecules within a structure requires more advanced imaging and data analysis that will not be discussed in detail here although consideration of more advanced approaches is discussed in the Conclusions section.

What follows are descriptions of two microscopes that were used in our published study on kinetochore forces (Ye, et al., 2016), followed by a summary of key features and other noteworthy points that we feel are relevant to getting a sense for the type of microscopes and accessories that are needed to get started.

1. Wide-field fluorescence microscopy equipment—Wide-field fluorescence imaging was conducted on a Ti-E inverted microscope (Nikon) equipped with an iXON EMCCD camera (Andor Technology) using a 100× 1.4 NA Plan Apo violet-corrected series DIC objective (Nikon) with a SOLA light engine (Lumencor) as a white light source. The microscope had a motorized turret containing the following filter cubes purchased from Chroma Technology Corp with set numbers in parentheses: 1) ET-DSRed (49005), 2) ET-EGFP (49002). The FRET imaging cubes were custom-built using the components from Chroma set number 89002 as follows: CFP cube: Excitation filter: ET430/24X, Dichroic mirror: 69008bs, Emission filter: ET470/24m; YFP cube: Excitation filter: ET500/20X, Dichroic mirror: 69008bs, Emission filter: ET535/30m; FRET cube: CFP excitation filter:

ET430/24X, Dichroic mirror: 69008bs, YFP emission filter: ET535/30m. MetaMorph (Molecular Devices) was used to control the microscope system and for data analysis.

2. Comments on wide-field microscopy equipment—An inverted microscope is ideal for imaging live cells in a petri dish containing media otherwise live-cell samples have to be sealed in a closed chamber. A sensitive camera is important, but it does not have to be an EMCCD camera, newer CMOS cameras produced and sold by companies like Andor and Hamamatsu have worked well in our hands, and if the fluorescence signal is bright enough then older digital CCD cameras may suffice. It is important to use high NA objectives (>1.2 NA) to collect as much sample fluorescence as possible and for higher resolution. The SOLA LED white light system is stable and bright but other white light sources such as mercury arc lamps are suitable if maintained properly. The area where there cannot be compromise is in the choice of filter sets. Significant effort should be put into matching your sensor fluorophores with the best possible filter sets. Furthermore, bleed-through for each fluorophore and filter cube should be measured on your microscope. To quantify bleed-through, cells expressing a single FP-tagged protein, typically either α -tubulin or a kinetochore component, are imaged with each of the relevant filter sets and cameras that will be used in the experiments (Ye, et al., 2016). One useful accessory for these protocols is a “beam splitter” such as the Dual-View (Photometrics) or Optosplit II (Andor), which allow for simultaneous imaging of multiple wavelengths such as mTurquoise2 and mVenus for the FRET-based sensor or TagRFP-T and EGFP for the talin-vinculin-based reporter. If beam splitters are used then bleed-through should also be measured for the accompanying filter sets. MetaMorph is not essential as any imaging software program that controls a user’s system will be sufficient and data analysis can be done with open source packages such as Fiji.

3. Scanning confocal microscopy equipment—The acceptor photobleaching was conducted on a Nikon Ti-E inverted microscope stand coupled to an A1 resonant (A1R) scanning confocal system (Nikon) using a 60 $\times$ 1.4 NA Plan Apo objective (Nikon). The A1R system was equipped with 6 solid-state laser lines (405 nm, 435 nm, 488 nm, 514 nm, 561 nm, 640 nm), 2 high-sensitivity photomultiplier tubes (PMTs), and 2 Gallium arsenide phosphide (GaAsP) detectors with greater sensitivity than the PMTs. NIS-Elements software (Nikon) was used to control the imaging system.

4. Comments on scanning confocal microscopy equipment—Image acquisition speed and the ability to rapidly bleach a user-defined region of interest (ROI) are key to the acceptor photobleaching protocol. The resonant scanning mode allows for rapid acquisition, but is not necessary if the scan region is small enough. The 435 nm, 514 nm, and 561 nm laser lines are necessary to visualize mTurquoise2, mVenus, and TagRFP-T respectively and the 514 nm laser power must be powerful enough (50 mW) to quickly bleach the mVenus. PMTs work well for bright samples, but the GaAsP detectors significantly improve detection and acquisition speed when the fluorescence signal is low. If using a Nikon A1R, the NIS-Elements software allows the user to easily define and quickly run the sequence of acquisition and bleaching parameters. If using a comparable microscope system from a

different manufacturer than the accompanying software package will suffice although upgrade modules may be necessary.

5. Final comments on approaches to live-cell imaging—It is imperative that once cell lines expressing the various reporters have been made, sufficient time is spent at the microscope getting acquainted with the cells prior to doing serious experimentation. While the use of UV or far-red DNA dyes or live-cell cytoskeleton dyes such as SiR-tubulin is not recommended, if any vital dyes are used then carefully assess whether the visualization of the sensor fluorophores is impacted by the dyes. It is also worth noting that live-cell cytoskeleton dyes affect polymer dynamics and would; therefore, impact force measurements of structures linked to the dye-labeled polymers. If it is difficult to readily assess the extent of chromosome alignment by phase contrast or DIC microscopy, as is the case for *Drosophila* S2 cells, then “scanning” for cells will require the use of epifluorescence and; therefore, limiting photobleaching of the reporters is critical. For example, in our experiments, the FRET sensors were combined with an N-terminal TagRFP-T so that scanning could be done on the DSRred channel without bleaching the mTurquoise2 and mVenus fluorophores. If scanning cells with epifluorescence then aim for a level of proficiency in which the necessary cellular states (e.g. metaphase, interphase, mitotic cells following microtubule depolymerization, etc.) can be identified and centered in the camera’s field of view within ~3 – 5 s of moving into a new field.

IV. FRET-based tension sensor

In addition to building the internal and control TSMOD sensors (Figure 2D), experimental conditions for high force and low/no force must also be identified. In the case of the kinetochore TSMOD sensor, metaphase is the higher force condition and interphase (CENP-C constitutively localizes to the centromere) is the low/no force condition. If your protein of interest only localizes during mitosis then treatment with a MT (or actin) depolymerizing agent is a practical alternative for the low force condition, although confirm that the drug of choice does not exhibit fluorescence properties that could impact FRET measurements. For example, the MT depolymerizing agent colchicine, typically used in *Drosophila* cells since nocodazole doesn’t work, bound to tubulin possesses absorption and emission spectra (Bhattacharyya & Wolff, 1974) that overlap with the CFP filter set. If comparing interphase to metaphase cells then a single coverslip is required per experiment. If comparing drug versus DMSO treatments then 2 coverslips are necessary: one sensor cell line (internal or control) + 0.1% DMSO, and one sensor cell line (internal or control) + MT depolymerizing agent.

A. FRET emission ratio

1. Procedure

1. For semi-adherent *Drosophila* S2 cells, the cells are seeded on a concanavalin A coated glass bottom 35 mm dish (Cellvis, Part No. D35-20-1.5-N) at ~70% confluency. Once the cells have adhered (~15 minutes), bring the volume of the chamber up to 2 ml with fresh media and allow the cells to flatten for an additional 15 – 45 mins. For adherent cells, seed the glass bottom dishes ahead of

time such that the cells will be ~50–70% confluent on the day of imaging. If using drugs to create the low force condition then add either DMSO or MT (or actin) depolymerizing agent of choice to each dish for one hour prior to imaging. Note: Image each cell line for a 1–2 hour window after setting the dishes up on the microscope.

2. If using epifluorescence, scan on the DSRed channel to identify the metaphase/interphase cell of interest quickly (~3s), take 2–3 snapshots to identify the best focal plane.
3. Sequentially image the cell with the CFP, YFP, and FRET cubes using identical exposure times and camera settings.
4. Repeat steps 2 and 3 for 1–2 hours being sure to acquire both interphase (low force) and metaphase (higher force) cells. Aim to visualize 50–100 cells per coverslip.

2. Analysis

1. Apply region-in-region background correction to measure the fluoresce signal from the donor mTurquoise2 channel (CFP excitation, CFP emission) and the FRET channel (CFP excitation, YFP emission) (Figure 3A – C).
2. Ratio the background corrected FRET signal to the background corrected mTurquoise2 (donor) signal to determine the FRET emission ratio (Figure 3C, D).
3. Since the protocol described here captures the CFP, YFP and FRET channels, bleed-through corrected FRET (cFRET) can be determined. Knowledge of the bleed-through for each fluorophore and filter set combination is required for this method. On the wide-field microscope described here ~80% of the mTurquoise2 signal and ~8% of the mVenus signal bleeds-through into the FRET channel respectively. To calculate the corrected FRET signal the donor and acceptor bleed-through (bt) multiplied times the background-corrected donor and acceptor signals are subtracted from the background corrected FRET signal using the following equation:

$$cFRET = I_{FRET} - I_{donor} (0.8) - I_{acceptor} (0.08)$$

All intensities (*I*) above are background corrected measurements.

Software packages can apply the bleed-through correction (used defined) to FRET images on a pixel-by-pixel basis to generate corrected FRET images. Using this tool in MetaMorph, measuring the background-corrected intensity of the bleed-through corrected FRET signal yielded the same results as applying the correction to each channel manually. Thus, this feature can be time-saving for cFRET analysis. Since the total amount of FRET signal is concentration dependent, the cFRET values can be divided by the background corrected donor (mTurquoise2) signal to yield a cFRET ratio. Note 1: Knowledge of the bleed-through parameters is informative for understanding the FRET emission ratio values since the zero-

FRET emission ratio (all bleed-through and no FRET) can be determined. For the wide-field fluorescence microscope setup described here, zero-FRET yields a FRET emission ratio of ~1.0. By comparison, FRET emission ratios measured for the internal and C-terminal CENP-C TSMoD reporters typically ranged between 1.5 and 2.3 (Ye, et al., 2016).

B. Acceptor photobleaching

The *in vitro* calibration of the TSMoD measured FRET efficiencies over a range of applied forces (Grashoff, et al., 2010). Acceptor photobleaching is one approach to measure FRET efficiency in living cells. It requires measuring the donor (mTurquoise2) fluorescence before and after photo-bleaching the acceptor (mVenus). If a donor is engaged in FRET then the donor intensity will increase following photobleaching of the acceptor and the FRET efficiency can be calculated by measuring the extent to which the donor fluorescence changes. This procedure will describe running the experiment on the Nikon A1R system (described above) using Elements software.

1. Procedure

1. Seed cells as described in step A.1.1.
2. Scan the coverslip by eye using the wide-field red fluorescence channel. This ensures that the FRET fluorophores will not be excited prior to the acceptor photobleaching protocol.
3. Once ideal cell is found, “scan” with only the 561 nm laser selected in the A1plus Compact GUI, which will show live scans of the RFP channel, to quickly define the best focal plane with the most kinetochores/centromeres in focus. Note 1: Define the area to be scanned to encompass the cell keeping in mind that the smaller the area the faster the scan. Aim for ~1 second per channel. Faster scans can also be achieved through other means including changing the dwell time per pixel or selecting the resonant scan mode. Note 2: Prior to conducting the photobleaching experiments, scan the CFP and YFP channels as well as RFP by selecting the 445 nm, and 514 nm, and 561 nm laser lines in the “A1plus Compact GUI” to define the ideal imaging parameters (scan speed, laser power, field size, etc.) for each wavelength. While each system will differ, for comparison, imaging on the A1R system described here was done at 10%, 5%, and 10% laser power for CFP (445 nm), YFP (514 nm), and RFP (561 nm) lines respectively.
4. Once the imaging parameters for each channel have been determined, scan on the RFP channel for a cell in metaphase for the high force condition or interphase for the low-force condition, define the best focal plane, and take a scan with the 561 nm laser.
5. Using the simple ROI editor menu select “Rectangle” and manually draw a rectangular region in the RFP scan around the group of kinetochores or centromeres you want to bleach. Designate the region “S1” by right clicking on the rectangle and selecting “Use as Stimulation ROI: S1” in the drop-down menu.

6. In the A1plus Compact GUI select the 456 nm and 514 nm lasers and set the imaging parameters defined in Step 3.
7. In the A1plus Stimulation GUI select the 514 nm laser (YFP) as the photobleaching laser and define the laser power to achieve maximal bleaching in the shortest period of time (55% in our experiments).
8. Use the ND Stimulation GUI to configure the time phases of the experiment. In the acceptor photobleaching experiments there will be three phases. The first pre-bleach phase will be set as an “acquisition” scan (parameters from step 6 above for the CFP and YFP channels will be applied) with “no delay” for one loop. The second phase is the YFP bleach and requires selection of “stimulation” with S1 selected in the drop-down of the “ROI” column. The stimulation step was typically looped 6 times but this can be adjusted accordingly to achieve maximum bleaching in the shortest period of time. The final phase is a post-bleach scan in which the same “acquisition” settings as the pre-bleach scan must be applied. Note: In the CENP-C TSMOD experiments, our defined imaging parameters for this protocol (pre-bleach CFP and YFP scans, 6 bleach loops of the S1 ROI with the YFP laser, post-bleach CFP and YFP scans) took ~ 7 seconds.
9. Repeat the acceptor photobleaching protocol for 1–2 hours being sure to acquire both interphase (low force) and metaphase (higher force) cells. Aim to visualize 50–100 cells per coverslip.

2. Analysis

1. Measure the fluorescence intensity of the mTurquoise2 signal before and after photobleaching using the region-in-region or duplicate region approach (Figure 3). Note 1: It is also worthwhile to measure the YFP intensity to confirm the extent of acceptor photobleaching. We limited analysis to kinetochores/centromeres in which >95% of the YFP signal was bleached. Note 2: If movement of kinetochores/centromeres out of the focal plane is evident over the course of the ~7 second imaging/bleaching protocol then exclude the cell from analysis.
2. The FRET efficiency can be calculated using either of the following equations:

$$FRET_{eff} = 1 - \frac{I_{donor} (pre-bleach)}{I_{donor} (post-bleach)}$$

$$FRET_{eff} = \frac{I_{donor} (post-bleach) - I_{donor} (pre-bleach)}{I_{donor} (post-bleach)}$$

All intensities (*I*) above are background corrected measurements.

3. In our experimental conditions, there was variation in the FRET efficiency measurements (typically ranging from 0 - ~50% with a standard deviation of +/-

~10%). Depending on the variation and FRET efficiency changes (if any) between experimental conditions large sample sizes may be required for your analyses. For example, we attained FRET efficiency measurements for ~200–500 kinetochores/centromeres per condition.

V. Talin-vinculin-based force sensor

Talin is a large integral component of the focal adhesion complex that links integrins to the actin cytoskeleton. Talin has an N-terminal head domain that binds integrins and a C-terminal talin rod (TR) domain containing numerous vinculin binding sites (Critchley & Gingras, 2008). Unlike the self-contained TSMOD reporter, the talin-vinculin reporter is a two component system. The first component, which is inserted into the force-transducing protein of interest, is a portion of the chicken TR domain (aa 482–889) containing five potential vinculin binding sites (Figure 1B). The second component is the minimal domain of vinculin required to bind TR – the vinculin head (VH). Using a magnetic trap with purified components *in vitro*, the TR domain has been shown to increase the number of VH molecules it binds upon the application of 2–12 pN of force with a baseline level of one VH-bound per TR under zero force (del Rio et al., 2009).

This line of experimentation requires the generation of cell lines co-expressing 1) the FP-tagged force-transducing protein of interest containing the TR, and 2) soluble VH tagged with a different FP. The rationale underpinning this reporter is that the application of sufficiently high cellular force to the TR-containing protein will expose VH binding sites and, distinct from the FRET reporter that exhibits reduced FRET under tension, the talin-vinculin-based reporter becomes brighter (aka more VH bound to the TR) when force is applied (Figure 1B). Since the number of VH molecules associated with the TR domain has been counted *in vitro* at three different forces (0, 2, 12 pN), measuring the number of VH bound per TR in cells yields a force estimate. To count the VH per TR requires the generation of cells expressing a reference protein (Figure 2E). Thus, three cell lines (1 reference, 1 experimental (internal) sensor, and 1 control (N- or C-terminal) sensor) will be required for this protocol.

A. Reference and sensor proteins

The key features of the reference protein are 1) it localizes to a discrete cellular structure, preferably the structure that is being studied, and 2) it is tagged with both the FP-tags that will be used in the sensor components (Figure 2E). For example, we used TagRFP-T and EGFP because both are bright and photostable; however, an alternative pairing (reasons discussed in comments below) could be mTurquoise2 (or mTFP1) and a bright photostable red fluorophore. For the remainder of this protocol, TagRFP-T (RFP) and EGFP (GFP) will be the example fluorophores. In our kinetochore study the reference protein was CENP-C tagged with RFP and GFP at the N- and C-termini respectively (Figure 2E). However, an alternative reference design could be an N- or C-terminal RFP-tag with the GFP placed at the internal location that the TR domain will eventually be inserted. In this case, generating the sensor protein would simply require swapping out the internally positioned GFP for the TR domain. As with the TSMOD reporter, a control sensor must also be made in which the

TR domain is positioned at the N- or C-terminus (Figure 2D). A stable “parental” cell line must be generated that expresses soluble VH-EGFP. VH-EGFP expression should not be under the control of a strong promoter such as CMV since the VH-EGFP cannot be highly over-expressed. Once the VH-EGFP parental cell line is built, the following cell lines must be generated: 1) “reference” cells expressing RFP-GFP double tagged reference protein, 2) “experimental” cells expressing RFP-tagged internal TR + VH-EGFP, and 3) “control” cells expressing RFP-tagged, N- or C-terminal TR + VH-EGFP.

B. Counting vinculin heads per talin rod sensor

1. Procedure

1. For the internal sensor one should identify experimental conditions for high force and low/no force. In the case of the kinetochore talin-vinculin-based sensor, metaphase is the higher force condition and unattached kinetochores are the low/no force condition. Unattached kinetochores were generated using the MT depolymerizing agent colchicine. Thus, one experimental day requires setting up 3 coverslips: one reference cell line + 0.1% DMSO, one internal sensor cell line + 0.1% DMSO, and one internal sensor cell line + 25 μ m colchicine.
2. Seed cells as described in step A.1.1.
3. Image the reference cell line to define the imaging parameters that will be used in your experiments. If using epifluorescence, scan for cells (for the reference protein the cell cycle state is not critical although mitotic cells were typically imaged) on the DSRed channel with the Neutral Density (ND) 4 filter inserted in the light path to limit bleaching. Center cell in field of view quickly (~3s) and take a 2–3 quick snapshots with the ND4 filter in place to identify the best focal plane.
4. Remove the ND4 filter from the light path and sequentially image the GFP and RFP signals.
5. Repeat steps 3 and 4 as many times as necessary to best define the camera settings and exposure times that yield an ideal signal aiming for enough signal to noise (at least one hundred counts above local background pixel intensities) without saturating any pixels. Note: Defining the imaging parameters is a critical step as these settings will be used for all subsequent imaging.
6. Once the imaging parameters have been determined, scan the reference cells on the DSRed channel with the ND4 filter inserted into the light path. Center cell in field of view quickly (~3s), take 2–3 quick snapshots with the ND4 filter in place to identify the best focal plane, remove the ND4 filter from the light path and sequentially image the GFP and RFP. Visualize ~30–50 reference cells in this manner not exceeding 1–2 hours of total imaging time.
7. After imaging the reference cells, immediately place the internal sensor cells + 0.1% DMSO on the microscope and image them exactly as the reference cells were imaged (Step 6) capturing as many metaphase cells as possible in the 1–2 hour window.

8. After imaging internal sensor cells + 0.1% DMSO, immediately place the internal sensor cells + MT depolymerizing agent (treated for 1 hour) on the microscope and image them exactly as the reference cells were imaged (Step 6) capturing as many mitotic cells (condensed chromosomes with paired but unattached kinetochores) as possible in the 1–2 hour window.
9. Once sufficient data (from a minimum of 3 separate experiments) has been acquired for the internal sensor cells, image the control sensor cells exactly as described in steps 6 – 8. In this case an experimental day requires setting up the following 3 coverslips: one reference cell line + 0.1% DMSO, one control sensor cell line + 0.1% DMSO, and one control sensor cell line + 25 μ m colchicine.

2. Analysis

1. To define a “correction ratio”, the background corrected fluorescence intensities of TagRFP-T and EGFP from 50–100 structures in the reference cells is quantified using the region-in-a-region method (Figure 3). The ratio of background corrected EGFP to TagRFP-T intensities for the reference protein represents the correction ratio that will be applied to the experimental (sensor cell) imaging done on that same day. While labor intensive, we recommend calculating the correction ratio for each day of experiments because there was variability in the correction ratio on a day-to-day basis.
2. Images from the sensor cells are quantified in the same manner as the reference cells to determine the background corrected ratio of VH-EGFP to TagRFP-T-sensor.
3. Apply the correction ratio from step 1 to each of the VH-EGFP:TagRFP-T-sensor ratios determined in step 2 to attain the number of VH-EGFP bound per TagRFP-T-sensor. For example, if the correction ratio for a given day was measured to be 3.1 and the background corrected ratio of VH-EGFP to TagRFP-sensor at a kinetochore is 7.2 then this value divided by the correction ratio yields 2.3 VH molecules per TR. This number represents the mean value of an ensemble of TR-containing proteins within the structure.

3. Comments—Unlike the FRET-based sensor, which is entirely self-contained within the TSMoD, the vinculin-talin-based sensor has two components. Consequently, the low force condition for the kinetochore force measurements was unattached mitotic kinetochores rather than interphase centromeres because the VH-EGFP was mostly cytosolic in interphase. It is essential to confirm that the VH-EGFP does not bind your structure of interest in the parental cells prior to building the internal and control sensor lines. Also, TagRFP-T and EGFP will engage in FRET if the fluorophores are close enough in your experimental conditions, which was not an issue for the CENP-C reporters (Ye, et al., 2016). Nonetheless, it is worth considering the use of mTurquoise2 (or comparable CFP-variant) in place of EGFP for your TR-VH reporter system.

While calibrated at three forces (0, 2, and 12 pN), the *in vitro* calibration of the TR-VH interactions is limited in comparison to the single-molecule force spectroscopy methods

used to measure, not only the FRET efficiency over a dynamically applied force range, but also response times and hysteresis. However, one notable caveat for the FRET calibrations is that they are done using fluorescent dyes rather than the FPs that will be used when the reporter is expressed in cells. Finally, since the rationale of the TR reporter is that tension exposes additional VH binding sites, individual reporter molecules must exhibit switch-like responses (as opposed to the graded response of the TSMOD) in which fluorescence incrementally increases with each VH-EGFP that binds when force is applied to the TR (Figure 1). Careful evaluation of VH-EGFP turnover dynamics may, therefore, be informative as to the fraction of TR-containing reporters that are under sufficiently high force to expose VH binding sites.

VI. Conclusions

As mentioned in section III, the protocols outlined here will yield the mean fluorescent properties of an ensemble of sensor molecules. There is a giant leap between the single molecule resolution by which the sensors have been calibrated and an ensemble of proteins within a cell. Thus, interpretation of these data should be viewed as a jumping off point (or not) for a deeper dive into understanding the forces that act upon and regulate a structure. For example, as a starting point for the kinetochore, our force estimates were based on experimentally measured values for the number of CENP-C linkages at the kinetochore and assumptions about the structural organization of these linkages as well as how poleward-directed forces may be distributed across them. While these assumptions are within the boundaries of accepted conceptualizations of kinetochore organization, they are also inherently simplified so as to propose a framework in which to think about physiologically relevant kinetochore force ranges. Undoubtedly, each molecule within an ensemble is not behaving identically. At any given moment some sensor molecules will be actively engaged with the force-producing machinery and under tension while others will be in a relaxed state (Figure 4).

To better dissect the complex behavior of molecules within an ensemble requires an advanced fluorescence imaging modality called fluorescence-lifetime imaging microscopy (FLIM). FLIM measures the lifetime (time between absorption and emission of photons by a fluorophore) of the FRET donor. A commonly applied (and intuitive) method for imaging FRET-based reporters is time-correlated single photon counting (TCSPC) in which the time between fluorophore excitation with a pulsed laser source and detection of emitted photons at a PMT is measured over many cycles. Since the lifetime of the donor decreases when it is engaging in FRET with an acceptor, FLIM can be used to accurately calculate not only FRET efficiencies, but the fraction of molecules engaged in FRET, which can be useful for defining the percentage of sensors that are under tension. Other advantages of FLIM are that, unlike FRET intensity measurements, 1) it is insensitive to sensor concentration and, 2) FLIM is less sensitive to photobleaching, although significant levels of photobleaching will affect lifetime measurements (Sun, Day, & Periasamy, 2011).

Unlike the protocols described in this chapter, FLIM requires advanced (often custom-built) systems and computational analyses. Commercially available FLIM systems are available from specialty companies (e.g. Becker & Hickl, PicoQuant Photonics) and as upgrade

packages from major microscope companies (e.g. Zeiss, Leica); however, these systems may not be available in your lab or local imaging facility. Nonetheless, to maintain the option of doing FLIM at a future point it is important that the tension sensor of choice contains a donor that has a mono-exponential fluorescence decay. These include mTFP1, mTurquoise, mTurquoise2, and mCitrine (paired with RFP variants). FLIM based applications of newer FPs (Murakoshi, Shibata, Nakahata, & Nabekura, 2015; Shcherbakova, Hink, Joosen, Gadella, & Verkhusha, 2012) have led to the development of a powerful new dual-color FLIM method that could have significant implications for studying mitotic forces (Demeautis et al., 2017). In this method, a single wavelength (~440 nm) is used to excite two separate sensors with the donors mTFP1 and long stoke shifted mOrange (LSSmOrange), both of which efficiently absorb photons from CFP laser lines and have mono-exponential decays, paired with the acceptors ShadowG (“dark” GFP variant) and mKate2 respectively. Since there is no overlap in the emission spectra of mTFP1 and LSSmOrange the lifetimes of each donor can be simultaneously measured following their excitation with a single CFP laser line. This approach was recently applied to multiplex (two different tension sensors) force-sensing in focal adhesions (Ringer et al., 2017) and will likely be informative for studying the distribution of forces through force-transducing structures such as the kinetochore during cell division. The arrival of calibrated tension sensors combined with widely available and more advanced live-cell imaging technologies has produced an opportune moment for researchers interested in fundamental processes that are regulated by cellular forces. We hope this chapter will allow force-inclined cell biologists to feel confident that there are tension sensors that suit their needs and microscopes to begin studying them.

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REFERENCES

- Bhattacharyya B, & Wolff J (1974). Promotion of fluorescence upon binding of colchicine to tubulin. *Proc Natl Acad Sci U S A*, 71(7), 2627–2631. [PubMed: 4527949]
- Buchan DW, Minneci F, Nugent TC, Bryson K, & Jones DT (2013). Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res*, 41(Web Server issue), W349–357. doi: 10.1093/nar/gkt381 [PubMed: 23748958]
- Ciferri C, Pasqualato S, Screpanti E, Varetti G, Santaguida S, Dos Reis G, ... Musacchio A (2008). Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell*, 133(3), 427–439. doi: 10.1016/j.cell.2008.03.020 [PubMed: 18455984]
- Critchley DR, & Gingras AR (2008). Talin at a glance. *J Cell Sci*, 121(Pt 9), 1345–1347. doi: 10.1242/jcs.018085 [PubMed: 18434644]
- del Rio A, Perez-Jimenez R, Liu R, Roca-Cusachs P, Fernandez JM, & Sheetz MP (2009). Stretching single talin rod molecules activates vinculin binding. *Science*, 323(5914), 638–641. doi: 10.1126/science.1162912 [PubMed: 19179532]
- Demeautis C, Sipietter F, Roul J, Chapuis C, Padilla-Parra S, Riquet FB, & Tramier M (2017). Multiplexing PKA and ERK1&2 kinases FRET biosensors in living cells using single excitation wavelength dual colour FLIM. *Sci Rep*, 7, 41026. doi: 10.1038/srep41026 [PubMed: 28106114]
- Freikamp A, Cost AL, & Grashoff C (2016). The Piconewton Force Awakens: Quantifying Mechanics in Cells. *Trends Cell Biol*, 26(11), 838–847. doi: 10.1016/j.tcb.2016.07.005 [PubMed: 27544876]

- Freikamp A, Mehlich A, Klingner C, & Grashoff C (2017). Investigating piconewton forces in cells by FRET-based molecular force microscopy. *J Struct Biol*, 197(1), 37–42. doi: 10.1016/j.jsb.2016.03.011 [PubMed: 26980477]
- Grashoff C, Hoffman BD, Brenner MD, Zhou R, Parsons M, Yang MT, ... Schwartz MA (2010). Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*, 466(7303), 263–266. doi: 10.1038/nature09198 [PubMed: 20613844]
- Hoffman DB, Pearson CG, Yen TJ, Howell BJ, & Salmon ED (2001). Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at Ptk1 kinetochores. *Mol Biol Cell*, 12(7), 1995–2009. [PubMed: 11451998]
- Holt LJ, Tuch BB, Villen J, Johnson AD, Gygi SP, & Morgan DO (2009). Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science*, 325(5948), 1682–1686. doi: 10.1126/science.1172867 [PubMed: 19779198]
- Maiato H, Hergert PJ, Moutinho-Pereira S, Dong Y, Vandenbeldt KJ, Rieder CL, & McEwen BF (2006). The ultrastructure of the kinetochore and kinetochore fiber in *Drosophila* somatic cells. *Chromosoma*, 115(6), 469–480. doi: 10.1007/s00412-006-0076-2 [PubMed: 16909258]
- Maiolica A, Cittaro D, Borsotti D, Sennels L, Ciferri C, Tarricone C, ... Rappsilber J (2007). Structural analysis of multiprotein complexes by cross-linking, mass spectrometry, and database searching. *Mol Cell Proteomics*, 6(12), 2200–2211. doi: 10.1074/mcp.M700274-MCP200 [PubMed: 17921176]
- Murakoshi H, Shibata AC, Nakahata Y, & Nabekura J (2015). A dark green fluorescent protein as an acceptor for measurement of Forster resonance energy transfer. *Sci Rep*, 5, 15334. doi: 10.1038/srep15334 [PubMed: 26469148]
- Nicklas RB (1988). The forces that move chromosomes in mitosis. *Annu Rev Biophys Chem*, 17, 431–449. doi: 10.1146/annurev.bb.17.060188.002243 [PubMed: 3293594]
- Oates ME, Romero P, Ishida T, Ghalwash M, Mizianty MJ, Xue B, ... Gough J (2013). D(2)P(2): database of disordered protein predictions. *Nucleic Acids Res*, 41(Database issue), D508–516. doi: 10.1093/nar/gks1226 [PubMed: 23203878]
- Ringer P, Weissl A, Cost AL, Freikamp A, Sabass B, Mehlich A, ... Grashoff C (2017). Multiplexing molecular tension sensors reveals piconewton force gradient across talin-1. *Nat Methods*. doi: 10.1038/nmeth.4431
- Shcherbakova DM, Hink MA, Joosen L, Gadella TW, & Verkhusha VV (2012). An orange fluorescent protein with a large Stokes shift for single-excitation multicolor FCCS and FRET imaging. *J Am Chem Soc*, 134(18), 7913–7923. doi: 10.1021/ja3018972 [PubMed: 22486524]
- Shin Y, & Brangwynne CP (2017). Liquid phase condensation in cell physiology and disease. *Science*, 357(6357). doi: 10.1126/science.aaf4382
- Sun Y, Day RN, & Periasamy A (2011). Investigating protein-protein interactions in living cells using fluorescence lifetime imaging microscopy. *Nat Protoc*, 6(9), 1324–1340. doi: 10.1038/nprot.2011.364 [PubMed: 21886099]
- Suzuki A, Badger BL, Haase J, Ohashi T, Erickson HP, Salmon ED, & Bloom K (2016). How the kinetochore couples microtubule force and centromere stretch to move chromosomes. *Nat Cell Biol*, 18(4), 382–392. doi: 10.1038/ncb3323 [PubMed: 26974660]
- Tien JF, Fong KK, Umbreit NT, Payen C, Zelter A, Asbury CL, ... Davis TN (2013). Coupling unbiased mutagenesis to high-throughput DNA sequencing uncovers functional domains in the Ndc80 kinetochore protein of *Saccharomyces cerevisiae*. *Genetics*, 195(1), 159–170. doi: 10.1534/genetics.113.152728 [PubMed: 23833183]
- Ye AA, Cane S, & Maresca TJ (2016). Chromosome biorientation produces hundreds of piconewtons at a metazoan kinetochore. *Nat Commun*, 7, 13221. doi: 10.1038/ncomms13221 [PubMed: 27762268]

Figure 1. Schematic of the two FRET reporters described in this chapter.

(A) The TSMOD tension sensor reports on a force range between 1–6 pN. The reporter is FRET-based and works via force-dependent extension of a central linker positioning the FRET pairs such that the reporter exhibits a graded reduction in FRET efficiency from ~24% at zero force to 0% at 6 pN. (B) The Talin Rod (TR)-Vinculin Head (VH)-based reporter incrementally increases in fluorescence intensity over a force range of 2–12 pN as the application of force exposes additional binding sites in the TR domain to which fluorescent protein (FP)-tagged VH can associate.

Figure 2. Protein disorder and sensor design strategies.

(A) Schematic of the linker protein CENP-C in which the two sensors were inserted to study kinetochore forces in *D. melanogaster* cells. (B) The PSIPRED protein sequence analysis workbench (Buchan, et al., 2013) predicts the central ~1200 amino acids of CENP-C are highly disordered. (C) The disorder prediction aggregator at the database of disordered protein predictions (D²P²) (Oates, et al., 2013) allows the user to type in a search term and lists results of IDR-containing proteins sorted by species. In this example, one of the hits for the search “CENP” in *D. melanogaster* is the protein CENP-C. The results of the search show the predictions of nine separate disorder predictors and a color-coded disorder agreement bar to highlight consensus amongst the predictors across all the amino acid positions. The example shows the D²P² results for the span of CENP-C highlighted in the boxed region of the trace in B. (D) Outline of tension sensor (TS) design using TagRFP-T-tagged CENP-C as an example. The internal tension sensor is inserted into the intrinsically disordered region (IDR) of the force-transducing component CENP-C. The control requires placement of the tension sensor at the C-terminus although the N-terminus could also be used. (E) Two reference protein design strategies for the TR-VH-based reporter.

Figure 3. Background correction techniques.

(A) Equations for the region-in-region adapted from (Hoffman, Pearson, Yen, Howell, & Salmon, 2001) and the duplicate region background correction methods. (B) Examples of a metaphase and interphase cell expressing the CENP-C internal TSMoD reporter. The asterisks denote the position of the spindle poles in the metaphase cell and the nucleus is outlined in the interphase cell. The mTurquoise2 (donor) and FRET (CFP excitation, YFP emission) channels are shown because the background corrected intensities from these two channels will be used to calculate the FRET emission ratio. Dashed boxes highlight the regions shown in the zoomed images in C and D. (C) Sample region-in-region background corrections and calculation of FRET emission ratios. The inner region (R1) should capture the entirety of the signal from the kinetochore/centromere of interest while the larger region (R2) must encompass the smaller region, but not signal from neighboring structures. While boxes are shown in these examples, we have found that freely drawn concentric regions of any shape sufficient to capture the structure (smaller region) and representative surrounding pixels (larger region) yields fine results. In this example the FRET emission ratios of metaphase (1.69) versus interphase (2.08) are indicative of the TSMoD reporter experiencing lower tension in interphase and higher tension in metaphase. (D) Sample duplicate region background corrections and calculation of FRET emission ratios of the same structures analyzed in C. In this method an identical background region (R1) as the region drawn around the kinetochore/centromere of interest (R2) is placed in the cytosol/nucleoplasm in close proximity to R2 avoiding nearby structures. In these examples, the two methods yield nearly identical FRET emission ratios. Nonetheless, we favor the use of the region-in-region method for data analyses in which multiple structures are measured in a single image as this technique yields an accurate measurement of local pixel background intensities around each individual structure. If using the duplicate method a single background region should not be

used to correct for structures that are microns apart as local background pixel intensities may vary throughout the cell. Thus, we typically use the duplicate region method when a small number of closely situated structures will be analyzed in an image.

Figure 4. Interpreting results from reporter ensembles.

Model ensembles are envisioned for a single microtubule (MT) with twelve CENP-C linkers containing the (A) internal TSMOD or (B) internal TR-VH reporter. (A) The approximate FRET efficiencies for a TSMOD under 0 pN, 1.5 pN and 6 pN are 24%, 20% and 0% respectively. A mean FRET efficiency of ~20% was measured for metaphase cells expressing the internal TSMOD CENP-C. This result could reflect even distribution of force through all linkers such that each sensor experiences 1.5 pN. Alternatively, a comparable result could be attained from a highly asymmetric distribution of forces in which 10 linkers are under zero tension and 2 linkers experience forces 6 pN. While there are many combinations of force distributions between these two extremes, we favor scenarios closer to the even distribution end of the spectrum. (B) The number of FP-tagged VHs bound to the TR reporter increases, above a baseline of 1 VH-FP, incrementally over a force range of 2–12 pN. A mean VH per TR of 1.3 was measured for metaphase cells expressing the internal TR CENP-C. This value could be a consequence of one of three different scenarios: 1) four linkers under moderate force (2–7 pN), 2) two linkers under moderate force and one linker under high force (7–12 pN), or 3) two linkers under high force. Determining the true physiological condition is further complicated by the fact that each kinetochore binds an average of 11 MTs in *Drosophila* S2 cells (Maiato et al., 2006) meaning an ensemble of ensembles is being measured at each kinetochore. Thus, it is possible that in the same kinetochore one MT could be generating higher forces than the overall mean estimate while a neighboring MT generates comparatively low/no forces.