CHAPTER

# Measuring Tau–microtubule affinity through cosedimentation assays

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**Emily O. Alberico[1], Aranda R. Duan[1], Holly V. Goodson[2]**

*University of Notre Dame, Notre Dame, IN, United States*

*[2]Corresponding author: e-mail address: hgoodson@nd.edu*

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[1]Cofirst authors.

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## Abstract

Microtubule cosedimentation assays have long been used to study the affinity of interactions between Tau protein and microtubules. While these assays are very useful for characterizing and comparing the effects of alterations to either Tau or the microtubule filaments, they can also be problematic. We provide a set of straightforward instructions for performing these assays and point out a number of challenges and pitfalls that can complicate their interpretation.

## 1 INTRODUCTION

Cosedimentation assays are the most commonly used approach for characterizing and quantifying the ability of a protein to bind microtubules (MTs), and Tau is no exception—these assays have used to study Tau since the 1980s (e.g., Ackmann, Wiech, & Mandelkow, 2000; Butner & Kirschner, 1991; Duan et al., 2017; Goode & Feinstein, 1994). However, while frequently employed, these assays are not without challenges. In performing and interpreting these assays, it is essential to be aware of the assumptions used in setting them up and in extracting quantitative information from them.

Briefly, in typical cosedimentation assays, a known concentration of microtubule-binding protein (MTBP) is mixed with different known concentrations of MTs, and the mixtures are allowed to incubate for a period of time (typically ~15–30 min). The mixtures are then centrifuged at high speed in an ultracentrifuge, where the heavy MT filaments sediment to the bottom of the tube, along with any MTBPs bound to them, leaving the unbound MTBPs in the supernatant. The resulting supernatants are then separated from the pellets, the pellets are resuspended, and equal proportions of the supernatants and pellets are analyzed by SDS-PAGE. Finally, the resulting gels are stained and quantified by densitometry, and the densitometry data are fit to a binding equation to extract information such as binding affinities and (depending on how the experiment is set up) binding ratios.

While such an overview of the basic experiment seems straightforward, "the devil" (as the saying goes) "is in the details." Any filament cosedimentation assay uses certain assumptions, which, if not met, interfere with or even prevent quantitative interpretation of the data. The central assumption of MT cosedimentation assays is that any protein found in the pellet after centrifugation ended up there by binding to the sedimenting MTs. If this assumption is not met (e.g., because some of the MTBP aggregates and sediments on its own), it is very difficult to obtain accurate $K_D$ measurements. In addition, validity of the assay assumes that the binding interaction has achieved equilibrium during the incubation time (generally a reasonable assumption), then stays at equilibrium through the postcentrifugation manipulations (perhaps questionable). Finally, as described more later, Tau has some characteristics and activities that further complicate interpretation of cosedimentation experiments.

Being aware of these complications is important for effective utilization of these assays in analyzing Tau function and mechanism.

Our text below first discusses some considerations to keep in mind when designing the experiments, then gives detailed protocols for both running the assays and analyzing the data. The protocols are described with respect to Tau and discuss some Tau-specific issues, but they can be applied to any MTBP. The text below assumes that readers are familiar with the basics of protein–ligand-binding equilibria such as can be found in typical biochemistry textbooks (e.g., chapter 5 of Nelson & Cox, 2012). For a good refresher on the topic, as well as a discussion of practical considerations, see Pollard (2010). Examples of Tau–MT-binding data produced by the methods described here can be found in Duan et al. (2017) and Duan and Goodson (2012).

# 2 INITIAL CONSIDERATIONS FOR EXPERIMENTAL DESIGN

## 2.1 WHICH COMPONENT SHOULD BE VARIED?

In theory, one might expect that identical answers would be obtained by holding the concentration of Tau constant and changing the concentration of MTs as described earlier, or by holding the concentration of MTs constant and varying the concentration of Tau (below we refer to these two approaches as TcMv and TvMc as abbreviations for "Tau constant, MTs varying" and "Tau varying, MTs constant," respectively). However, because free Tau binds to MT-bound Tau (Ackmann et al., 2000; Duan & Goodson, 2012), these two approaches produce different answers: apparent affinities as measured by TcMv are at least 10 × stronger than those measured by TvMc (Duan & Goodson, 2012; also compare Goode & Feinstein, 1994 to Ackmann et al., 2000). The reasons for this discrepancy are complex and are discussed more in Duan and Goodson (2012), but Tau—Tau interactions are minimized when Tau concentration is kept low and the incubation time is minimized. Therefore, from a practical standpoint, we suggest measuring Tau–MT affinity using the TcMv approach, keeping the concentration of Tau at or below 1 μM, and limiting the incubation time to ~15 min. However, because the TvMc approach is commonly used in the literature and may be useful for assessing Tau—Tau interactions, we provide protocols for both approaches later.

## 2.2 USEFUL CONCENTRATION RANGES

Concentration ranges used in these assays can vary depending on the goals of the assays and other experimental considerations. Briefly, it is important to have multiple datapoints above and below the $K_D$ if at all possible, which of course can be hard to do when you are trying to find the $K_D$. One limiting factor is that it is hard to work with microtubules at concentrations below 1 μM because they tend to depolymerize, even with stabilized by drugs such as Taxol (see also Section 2.5). Therefore, when performing TcMv assays with Tau or similar MTBPs that bind MTs strongly, total

MT concentrations typically range between 1 and 10 μM. Also, as noted earlier, our TcMv assays are generally performed with 1 μM Tau to minimize problems cause by Tau—Tau interactions. A typical TvMc assay might include 1 μM MTs and 1–30 μM Tau.

*Note:* it is very difficult to measure Tau concentration by standard methods because Tau binds poorly to both Coomassie and Bradford reagent (Barghorn, Biernat, & Mandelkow, 2005), and it also has a low extinction coefficient ($7700\,M^{-1}\,cm^{-1}$) (Devred et al., 2004). Therefore, it can be a good idea to use amino acid analysis (e.g., as performed by Sigma-Aldrich, see http://www.sigmaaldrich.com/analytical-chromatography/analytical-reagents/amino-acid-analysis.html) to determine the concentration of protein in your Tau preps and use this information as the foundation for interpreting any Coomassie-stained gels. It is helpful to save sufficient samples of this initial characterized prep for comparison to future preps. An alternative is to measure the concentration of Tau by using absorption of the peptide bond at A214 (Barghorn et al., 2005).

## 2.3 NECESSARY CONTROLS

A fundamental assumption of all microtubule cosedimentation assays is that any test protein that ends up in the pellet is there because it bound to the MTs. Therefore, in designing any cosedimentation experiment, it is essential to include a control (or controls) in which the test protein is centrifuged by itself, without MTs. When characterizing a new protein, it is also a good idea to include a sample that has all components except the test protein to determine whether the test protein or a component of its buffer might change the amount of tubulin that sediments.

*Note:* Tau itself does not generally alter the amount of Taxol MTs that sediment, but it is still good to verify this because evidence to the contrary could indicate that something is wrong either with the prep (e.g., the protein is stored in an inappropriate buffer) or with the way the assay is set up (e.g., the amount of active Taxol is insufficient, so Tau-promoted MT stabilization causes more tubulin to sediment in samples with Tau than without).

## 2.4 TAU PROTEIN PURIFICATION

The assays described here should be useful regardless of the identity of Tau protein being studied or the details of its preparation. Having said this, the values for parameters measured will likely depend on both the sequence used (e.g., three-repeat vs four-repeat, human vs mouse, tagged or untagged) and details of the preparation (e.g., native vs bacterially expressed). We have generally used four-repeat Tau expressed in bacteria and purified by boiling (Duan & Goodson, 2012). For other examples of approaches to Tau purification, see manuscripts including Barghorn et al. (2005), Karikari et al. (2017), and Lindwall and Cole (1984).

## 2.5 MT POLYMERS

Because microtubules are unstable structures, MTs used in MTBPcosedimentation assays are almost always stabilized by the drug Taxol (or a compound with similar activity).[a] Taxol MTs (e.g., prepared using the protocols of the Mitchison Lab; https://mitchison.hms.harvard.edu/files/mitchisonlab/files/tubulin_polymerization_with_gtp.pdf) can be prepared ahead of time and frozen in convenient aliquots, then thawed warm (e.g., in a water bath). Technically, it is possible to use glycerol or DMSO to shift dynamic MTs into the polymer form in MTBP-binding assays—indeed, cosedimentation of Tau with glycerol-stabilized MTs is part of a classic approach to purifying Tau (see Lindwall & Cole, 1984 and references therein). In practice, however, this approach can be difficult to use because of assay-to-assay variation in the fraction of tubulin dimers that spin down. Another possibility would be to use MTs stabilized with the slowly hydrolyzable analog GMPCPP (Hyman, Salser, Drechsel, Unwin, & Mitchison, 1992). However, even GMPCPP and Taxol have caveats as stabilizing agents because both have been reported to alter Tau–MT binding (Duan et al., 2017; Kar, Fan, Smith, Goedert, & Amos, 2003). In the absence of other considerations, we suggest using Taxol to stabilize MTs because Tau is a lattice-binding protein (Taxol-GDP MTs are models for the GDP-lattice) and because of its familiarity and relative ease of use.

## 2.6 TEMPERATURE

MT-binding assays are generally conducted (from incubation through centrifugation) at room temperature or above, since unstabilized MTs depolymerize at colder temperatures. Because Taxol makes MTs cold-stable (see, for example, Miller, Field, Alberts, & Kellogg, 1991), it is likely possible to conduct experiments at lower temperatures as long as care is taken to ensure that the MT polymers are remaining stable under the conditions used. However, we do not have experience with this approach.

## 2.7 USE OF GLYCEROL CUSHIONS AND/OR PELLET WASHING?

Historically, MTBP cosedimentation assays were typically conducted by layering the binding reaction on top of a "cushion" made of glycerol or similar dense material, and then determining whether the putative MTBP binds tightly enough to sediment through the cushion (see, for example, Gache, Waridel, Luche, Shevchenko, & Popov, 2007). Such assays have been very useful for identifying MTBPs. However, we do not recommend the use of cushions for quantitative assays because significant fractions of both tubulin dimers and MTBPs are often found in the cushion, and it is

[a]When using Taxol, experimental design should include approaches to making sure that the concentration of Taxol is held approximately constant (e.g., ~10 μM) across all experimental conditions. Also, because Taxol is poorly soluble in water, intermediate dilutions of concentrated frozen stocks should be made in DMSO, not water.

not clear how to account for these "lost" proteins in the affinity calculations. Utilization of such cushions can be useful under some circumstance, e.g., it can provide qualitative insight into the dynamics of how a protein binds to MTs (rapidly exchanging proteins are more likely to dissociate in the cushion). For similar reasons, we do not recommend that pellets be washed after centrifugation is complete. If you are concerned about nonspecific trapping of your protein by the sedimenting MT filaments, you can perform controls with a protein that does not bind to MTs to convince yourself that this effect is minimal unless MT concentrations are very high.

## 3 EXPERIMENTAL METHODS

Notes:

**I.** Here and in most publications using cosedimentation assays, the phrase "concentration of MTs" refers to the concentration of polymerized alpha–beta tubulin dimers.

**II.** The protocol below assumes that the experimenter has access to a Beckman tabletop ultracentrifuge and a TLA-100 rotor. This combination allows the use of small (100 μL) samples. Floor model ultracentrifuges with larger rotors can be used if appropriate adaptors exist for small tubes, or if the assay is scaled up. Airfuges can also be used, assuming that air pressure is sufficient to achieve the necessary G-force and that temperature regulation is not a problem. Regardless of which centrifugation set-up is used, it is helpful to use narrow tubes where it is easy to separate the top supernatant fraction from the pellet at the bottom of the tube.

**III.** As discussed more later, the type of plastic can influence whether your protein sticks to the sides of the tubes. We have performed most of our Tau work with Beckman thickwall polyallomer tubes (Beckman Part # 343621). Unfortunately, these polyallomer tubes have been discontinued, but it is still possible (as of Summer 2017) to order them by talking to Beckman customer service. The other options presently available for the TLA-100 rotor are polycarbonate (Beckman # 343775), polypropylene (Beckman # 343621), and cellulose propionate (Beckman # 342303). The polycarbonate tubes can work if they are relatively new, but become sticky as they get older. We have not yet tested the polypropylene or cellulose propionate tubes with Tau.

**IV.** The protocol below assumes that the assay will be run at 37°C, but it is also possible to run the assay at 30°C or room temperature. If the assay is run at room temperature, it is suggested that efforts be made to ensure that this temperature does not change significantly from day to day to avoid any problems caused by temperature variation.

## 3.1 EQUIPMENT, INSTRUMENT, AND SAMPLE PREPARATION

1. At least 1 day before the assay, prepare a sufficient number of thickwall ultracentrifuge tubes (see note above about plastic type): one tube per concentration to be tested, plus "Tau only" and (if desired) "MTs only" controls. New tubes can be used directly from the box; after use, tubes should be cleaned by washing with vigorous shaking/vortexing in soapy water. Tubes should be rinsed well in water, rinsed briefly in ethanol, rinsed again in distilled water, then air dried.
2. Assemble the necessary buffers and materials:
   a. PEM buffer: 100 mM PIPES, pH 6.8, 2 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA) (PEM stores well at RT as long as it is kept in the dark)
   b. 1 mM Taxol stock dissolved in DMSO (can be stored at −20°C)
   c. Sufficient Taxol-stabilized MTs and Tau to prepare the assays. (Both can be frozen and stored long term at −80°C; thawed MTs should be left at RT or kept warm; Tau should be kept on ice until it is ready to be used.)
   d. 6× SDS sample buffer for the gel electrophoresis (stock can be stored at −20°C)
3. In addition, make sure that you have access to sufficient supplies to run the experiments below, including MTs, Tau, and materials for running and staining SDS gels.
4. The morning before the assay, determine which type of assay you will run (TvMc or TcMv, see Sections 3.2 and 3.3), which concentration ranges of proteins you will use, and how many different experimental conditions you will test. We typically run six samples plus one "Tau-only" control because this number of trials allows one to put all supernatant and pellet fractions and a molecular weight marker on a single 15-well gel.
5. ∼One hour before the assay (more if necessary), place the TLA-100 rotor in the warm room (37°C) and turn on the centrifuge, setting the temperature to the desired temperature (37°C).
6. While the rotor is warming, calculate the necessary volumes of Taxol, MTs, and Tau for each reaction, with a total solution volume of 100 μL. Label the centrifuge tubes as well as the Eppendorf tubes used after centrifugation.

## 3.2 PROTOCOL FOR THE TcMv APPROACH

This approach is the traditional MTBP-MT-binding assay using ultracentrifugation to cosediment Tau bound to MTs. In this TcMv approach, a set of samples is prepared in which the Tau concentration is held constant at 1 μM, and the MT concentration is increased across the samples, typically from 1 to 10 μM. As noted earlier, it is important to include a Tau-only control; a MT-only control may also be useful. As also discussed earlier, the Tau concentration is typically set at 1 μM to minimize problems

caused by Tau—Tau interactions, while still being high enough to be straightforwardly measurable by gel band intensity analysis. This approach gives what might be considered to be "normal" looking binding curves that can be fit to the standard-binding curve equation (e.g., Duan & Goodson, 2012; Goode & Feinstein, 1994; see also Section 4). The protocol below assumes that the MTs have been stabilized by Taxol; the procedure will need to be altered for MTs stabilized by other methods.

1. Pipette the required amount of PEM buffer (calculated in step 6 in Section 3.1) into each labeled centrifuge tube at room temperature.
2. Pipette 1 μL of 1 mM stock Taxol into the PEM buffer to obtain a final concentration of 10 μM. Vigorously pipette to mix the Taxol into the PEM buffer to create a clear suspension.
3. Deliver to each tube the volumes of concentrated MTs necessary to achieve the required final concentrations for each tube. Sometimes we use wide-bore tips or cut tips for this work to avoid shearing the MTs, but with Taxol-stabilized MTs, we have generally found that special tips are not necessary.
4. Pipette the calculated volumes of Tau to reach the final desired concentration of 1 μM (constant in all the tubes except the MT-only control).
5. Mix the whole solution gently but thoroughly with a pipette tip; avoid bubbles.
6. Incubate for 15 min at the desired temperature (37°C). Place a protective covering over the mouths of the tubes (the ultracentrifuge tubes are typically capless) to prevent evaporation and catching dust.
7. Move to the steps outlined in Section 3.4.

## 3.3 PROTOCOL FOR THE TvMc APPROACH

In the TvMc approach, a set of samples is prepared simultaneously in which the MT concentration is held constant, and the Tau concentration is increased across the samples. As noted earlier, it is essential to include at least one Tau-only control, and because Tau concentration varies across the experiment, it would be better to have controls from both low and high Tau concentrations. While the TvMc approach has been used frequently (e.g., Ackmann et al., 2000), it is problematic for measuring Tau–MT affinity because of complications caused by Tau—Tau interactions (discussed in detail in Duan & Goodson, 2012). We include it here because it is found frequently in the literature, and because it may be useful for studying Tau—Tau interactions. Steps 1–2 and 5–7 are identical to the TcMv approach; steps 3–4 have some minor alterations.

1. Pipette the required amount of PEM buffer into each labeled centrifuge tube. For this you will need to have calculated in advance the volumes of the other components (Taxol, MTs, and Tau) and dispense the remaining volume as PEM to reach 100 μL total solution volume.
2. Pipette 1 μL of 1 mM stock Taxol into the PEM buffer to obtain a final concentration of 10 μM. Vigorously pipette to mix the Taxol into the PEM buffer to create a clear suspension.

3. Deliver to each tube the volumes of concentrated MTs necessary to achieve the required final concentrations. A typical concentration would be 1 or 4 μM; this concentration is constant across all tubes except the Tau-only control(s). Sometimes we use wide-bore tips or cut tips for this work to avoid shearing the MTs, but with Taxol-stabilized MTs, we have generally found that special tips are not necessary.
4. Pipette into each tube the volume of Tau necessary to reach the final desired concentrations for that tube. A typical range might be 1–30 μM.
5. Mix the whole solution gently but thoroughly with a pipette tip.
6. Incubate for 15 min at the desired temperature (37°C). Place a protective covering over the mouths of the tubes (the ultracentrifuge tubes are typically capless) to prevent evaporation and catching dust.
7. Move to the steps outlined in Section 3.4.

## 3.4 ULTRACENTRIFUGATION FOR THE SEDIMENTATION OF PROTEIN POLYMERS

Tubulin polymer (or microtubules) has a high enough weight to sediment or pellet out of solution using ultracentrifugation under conditions where most MTBPs stay in solution. The name "cosedimentation assay" reflects the idea that any protein bound to the MTs will sediment out of solution along with the MTs, while unbound protein will remain in the supernatant. In the case of Tau binding to MTs, the pellet will contain free MTs and MTs with bound Tau. However, it is important to remember that Tau fibrils (which can form in this assay; see Duan & Goodson, 2012) will also sediment out of solution under these conditions, as will many nonspecific protein aggregates.

1. Place tubes in prewarmed rotor.
2. Centrifuge at 37°C for 20 min at 65,000 rpm in a TLA-100 or similar rotor.
3. Remove rotor promptly; open the rotor and place the tubes in a rack at room temperature (warmer is OK, but probably not necessary as long as Taxol is present).
4. Quickly pipette off the top 50 μL of the supernatants into prelabeled Eppendorf tubes. This is the primary supernatant fraction.
5. Quickly pipette off the remaining supernatant volume into a separate set of prelabeled Eppendorf tubes. When removing this liquid, be very careful to not touch or disrupt the pellet. In some cases the pellet is not visible, so be aware of the position of the tube in the rotor to know approximately where the pellet would be inside the tube (to make it easier to keep track of the position of the pellet, it is helpful put a mark on each tube and position the tubes in the rotor so that the pellets will line up with the marks). *Note*: This second supernatant fraction is normally treated as waste but is temporarily maintained in case the pellet is accidentally removed with this liquid. In addition, it can be useful to

come back to this fraction and analyze it if the sum of the supernatant and pellet fractions does not correspond to the expected amount of each protein.

6. After the supernatant and pellet fractions have been fully separated, move all tubes to ice to reduce protein degradation.
7. Resuspend the pellets in 100 μL of PEM. Use the pipette tip to scrape along the inner tube wall and pipette thoroughly to resuspend the pellet. *Note*: It is also possible to resuspend directly in 1 × SDS protein sample buffer; just be careful to choose a volume that will correspond properly to however you prepare your supernatant sample. For example, if you add 10 μL of 6 × SDS sample buffer to your 50 μL supernatant fraction, you should resuspend your whole pellet in 120 μL of 1 × SDS sample buffer.

## 3.5 SDS-PAGE GEL ELECTROPHORESIS

1. Mix standard stock SDS loading buffer into the supernatant and pellet samples for a final 1 × SDS loading buffer (we typically use 6 × sample buffer to maximize the amount of protein we can load on the gel).
2. Heat the samples at 95°C for 5 min. At this point, it is OK to freeze the samples at −20°C or proceed with the SDS-PAGE.
3. Prepare a 10% SDS-PAGE gel for the samples.

   *Note:* running a duplicate gel in parallel can be helpful to improve confidence in the numbers by providing a technical replicate and provide insurance against standard problems that can occur in running gels (tearing, aberrant behavior in particular lanes, etc.). In any case, be sure to save the samples (store at −20°C) in case you need to run another gel.
4. Load the protein ladder and 10 μL of each sample into the wells. It is important to have the supernatants and pellets from a given experiment on the same gel to avoid ambiguity that might result from gel-to-gel staining variations. We generally load in an alternating pattern "S, P #1; S, P #2; S, P #3," etc. Don't be tempted to leave out the molecular weight markers: aside from providing reassurance that your proteins have the appropriate size (i.e., they are not degraded), they provide a useful rule for judging consistency of staining across experiments.
5. Attach electrodes and run at 25 mA for 1.5 h.
6. Carefully remove the gel and stain the gel using fresh Coomassie Blue buffer in a covered container overnight on a tilting or shaking platform. Ensure the gel is free floating in the solution and moving with the tilting/shaking (if it is not or if it gets stuck at any point, the gel will stain very unevenly). For consistency of staining, it is a good idea to use the same volume (e.g., 200 mL) of stain and to utilize the same type of staining box from experiment to experiment. The use of fresh (never used) stain is also important for reduction of variability; the used stain can then be used again for other experiments where consistency is less of an issue.

7. Destain the gel in approximately the same volume (~200 mL) of standard destain for 1–3 h in a covered container on a tilting or shaking platform. A rolled up paper towel should be placed in the destain solution to soak up the stain. It is important to ensure the gel is free floating during this process and does not stick to the walls or paper towel, which could cause uneven destaining.
8. Scan the gel to acquire a digital image for band intensity analysis. Although it is possible to use more sophisticated equipment, we typically use a standard office scanner/printer (e.g., HP Photosmart C4150), which can work well. Because these devices can have a dizzying array of options, some of which could alter the resulting data, be sure to use a set of standard settings from experiment to experiment and turn off any automatic image adjustment options.

*Note:* Acquisition of high quality, reproducible data requires that all steps of the SDS-PAGE process above are performed in a consistent (preferably identical) manner each time. For example, while the protocol above says that the gel should be stained "overnight" and then destained "1–3 h," variation from one experiment to the next will cause alterations to band intensity, which can then impact the reproducibility of the data. In theory, it is possible to account for this variation, but in practice, it is best to rigorously adhere to a particular protocol (e.g., 12 h of stain followed by 1.5 h of destain, with each run performed in a similar container with a similar volume of liquid and a similar amount of paper towel). In addition, while the times above work for us, it is important to empirically determine what works best for you in your lab.

# 4 DATA ANALYSIS

## 4.1 OVERVIEW OF DATA ANALYSIS

In most cases, the purpose of experiments such as those described earlier is to estimate the binding affinity ($K_D$) of a particular Tau–microtubule interaction, e.g., to determine the effect of alterations to Tau or the tubulin polymer. If this is the goal, we recommend that researchers use the TcMv approach as discussed more earlier, measure the band intensities as discussed in Section 4.2, and extract the $K_D$ value from the band intensity data as described in Section 4.3. Interpretation of TvMc data is much less straightforward and is discussed briefly in Section 4.4. To assist in following the discussion later, it might be useful to first review the mathematics used to model equilibrium-binding reactions. For a straightforward overview, see Pollard (2010).

To estimate a $K_D$ from TcMv-binding data, the first step is to determine what fraction of Tau is bound to (cosediments with) MTs at each MT concentration. To proceed beyond this point and estimate a $K_D$ value, it is first necessary to make an assumption of the stoichiometery of the Tau–MT interaction, i.e., the binding ratio. As discussed more later, it is reasonable to start with assuming a 1:1 Tau–tubulin ratio (Duan & Goodson, 2012). Once you have calculated the concentration of bound

Tau and have assumed a particular Tau–tubulin-binding ratio, you can calculate the concentration of bound and free MTs (i.e., bound and free polymerized tubulin dimers) in each sample. *Once you have this information, you are close to having your answer!*

It is possible to make a rough estimate of the $K_D$ value by making simple plot (e.g., in Excel) relating bound Tau (on the *y*-axis) to [free MTs] (on the *x*-axis) and eyeballing the resulting curve to determine the concentration of free MTs at which 50% of the Tau is bound. To make a quantitative estimate of the $K_D$, one should use a curve fitting program such as Prism (Graphpad) or Kaleidagraph (Synergy) to fit the standard bimolecular-binding equation ($\theta = [L_{Free}]/([L_{Free}] + K_D)$) to the data. In this equation, $\theta$ is the fraction of Tau that is found in the pellet (assumed to be bound to MTs), and $L_{Free}$ is the concentration of free (unbound) polymerized tubulin dimers. Using this approach, the $K_D$ is the only free parameter, and the fitting program should provide to you an estimate of the apparent $K_D$, with fitting error. However, it is important to remember that in performing this calculation, you have assumed a particular Tau–tubulin-binding ratio (typically 1:1). The issue of potential ambiguity in binding ratio is discussed more later.

Notes:

**I.** In interpreting the binding data, it is essential to calculate the concentration of *free MTs* (i.e., the concentration of polymerized tubulin dimers that are not bound to Tau) that are present at each concentration and use this value for further analysis. Failure to do this and instead trying to estimate the $K_D$ using the *total* MT concentration (i.e., simply performing calculations using the concentration of MT polymer placed in each tube) will result in significant (perhaps egregious) errors. This is because the "$[L_{Free}]$" in the binding equation above is specifically the concentration of *free* ligand (i.e., unbound tubulin dimers). In cases where the test protein is present at concentrations far below the concentration of ligand, one can assume that [free ligand] $\asymp$ [total ligand], but that is not the case in the assays discussed here. See Section 4.3 for more discussion.

**II.** In the discussion below, it is assumed that the controls and other aspects of the assay worked as expected. In particular, it is assumed that no Tau sedimented in the absence of MTs, that little if any tubulin was found in the supernatant, and that all of the tubulin and Tau is accounted for, i.e., that the sum of supernatant + pellet for each of these proteins is approximately equal to what was put into the tubes at the beginning of the experiment. If any of these assumptions is not met (e.g., because part of the pellet got stuck in a pipette tip), then the ability of the experiments to yield quantitative data may be severely compromised.

- If Tau is sedimenting on its own (i.e., without MTs), it is probably best to abandon that particular set of experiments and try another prep. One might be tempted to just subtract off any Tau that sediments by itself, and indeed, this can be a reasonable approach if the amount is small (<10% of the total). However, the resulting ambiguities in how to handle the

rest of the calculations (e.g., how much active Tau is actually present?) make it better to avoid working with self-sedimenting preps if at all possible. If self-sedimentation is unavoidable, both this issue and the approach used to account for this in the calculations should be made clear in the methods.

- If more than ~5% of tubulin dimers are found in the supernatant, it is best to try a different tubulin prep or perhaps try new Taxol (Taxol can go bad).
- If Tau seems to be disappearing, but the amount of tubulin is as expected, try new centrifuge tubes (Tau does tend to stick to old tubes; certain types of plastic are worse than others; see note III in Section 3 about tubes).
- If the amounts of both Tau and tubulin are varying, it is usually a sign that the supernatant and pellet are not being appropriately separated; in this case, it is useful to go back to the "waste" supernatant fraction—the missing protein can usually be found there. More careful pipetting might ameliorate this problem in future runs.
- If the reaction volumes do not appear consistent across the reactions (e.g., some tubes have less volume before removal of supernatant fractions), it is best to throw out those samples. The loss of volume may be due to an improper seal on the rotor, and the O-rings may need be to be replaced if cracked.

**III.** In principle, you should arrive at the same $K_D$ value if you determine the concentration of bound Tau by measuring the amount that sediments with MTs or the amount that is left in the supernatant (i.e., that is unbound). In practice, this expectation can fail because the fraction of Tau that sticks to the tubes often depends on the concentration of MT. In other words, Tau sticks to the MTs if sufficient MTs are present, but can stick to the tubes otherwise. For this reason, we base our measurements on the amount of Tau in the pellet, but it is still a good idea to follow the amount that is in the supernatant, observe whether any Tau is unaccounted for, and think carefully about what this might mean for how you interpret your results.

## 4.2 MEASURING BAND INTENSITY

1. The first step is to choose a reasonable image analysis program. While there are many possibilities, we recommend the FIJI version of ImageJ (https://fiji.sc/) because it is free, powerful, and relatively easy to use.
2. Using your chosen software package, measure the intensity of the Tau bands in the supernatant and pellet fractions. Briefly, the basic version of this process consists of drawing boxes around the supernatant and pellet fractions, measuring the integrated intensity of each band, subtracting off the background (typically determined by measuring the intensity immediately above or below the bands), and recording the values in an Excel spreadsheet. In performing the measurements in this way, it is important to maintain a constant box size when measuring both the background and band intensities.

This allows a consistent number of pixels to be measured in each lane and assists in subtraction of the background intensity. *See step 2 in Section 3.2 for an alternative approach.*

3. Although only the Tau band intensities are used in the standard analysis, it can be a good idea to perform a parallel analysis of the tubulin band intensities in both the supernatant and pellet fractions to make sure that the experiment worked as planned. For example, it is useful to determine whether the tubulin bands as observed on the gel are consistent with the amounts added to the samples. If the tubulin bands appear abnormal, it can be evidence of a problem with that sample (e.g., loss of part of the pellet), which would justify throwing out that particular data point.
4. Once the intensities have been measured, proceed with analysis as described in Section 4.3.

Notes:

I. For the TcMv method, it is important to make sure that the sum of the $Tau_{bound}$ and $Tau_{free}$ intensities adds up to approximately the same value in each sample and is similar to the amount in your Tau-only control.
   - If the value $Tau_{bound}$ and $Tau_{free}$ is observed to vary in a noisy way, there may be problems with pipetting or loss of the pellet during removal of the supernatant. In this case, examination of tubulin fractions can be informative, and problems can often be rectified by being more careful during separation of supernatant and pellet fractions and/or gel loading.
   - If the sum $Tau_{bound} + Tau_{free}$ is varying in a systematic way (e.g., more Tau is lost at lower tubulin concentrations), Tau may be sticking to the tubes. In this case, it is a good idea to try new centrifuge tubes or consider changing type of centrifuge tubes.
   - It is important to remember that systematic variation in the amount of a MT-binding protein in the supernatant or pellet fraction can cause major problems in these types of assays. For example, disappearance of protein from the supernatant (e.g., by binding to the tubes) without change in the amount that pellets could be mistakenly interpreted as increased fractional binding. Thus, it is essential to be alert to unexpected behaviors.

II. The procedure as outlined earlier sounds straightforward but can be somewhat challenging to accomplish in a reproducible way. One potential problem is uneven gel staining, which can make it very difficult to appropriately subtract the background. For a more sophisticated protocol for measuring band intensity, one that handles backgrounds in a different way, consult resources such as http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/ and http://www.navbo.info/DensitometricAnalysys-NIHimage.pdf. We have obtained similar results by both approaches. It is a good idea to use both approaches to make sure that similar results are obtained in your hands, but it is ultimately up to the user to decide which is preferred.

## 4.3 ESTIMATION OF APPARENT $K_D$ VALUES FROM TcMv DATA

As a reminder, to estimate an apparent $K_D$ from a TcMv dataset, you need to plot [bound Tau] vs [free MTs]. The apparent $K_D$ can be roughly estimated by "eyeballing" the resulting graph to see the concentration of free MTs at which 50% of the Tau is bound. A more quantitative approach is to fit the data to the bimolecular-binding equation ($\theta = [L_{Free}]/([L_{Free}]+K_D)$), where $\theta$ is the fraction of input Tau that is found in the pellet, and $L_{Free}$ is the concentration of free (unbound) polymerized tubulin dimers. However, in interpreting these analyses, it is essential to remember the assumptions that were used in setting up the analysis, namely: (a) that any Tau that cosedimented with MTs did so because it was bound to MTs; (b) that Tau binds to tubulin dimers in a 1:1 ratio. Assumption (a) is essential to the analysis; if it is not met (and sometimes with Tau it is not; see Duan & Goodson, 2012), then the analysis may be significantly flawed. On the other hand, it is easy for the user to explore the impact of changes to the assumption of a 1:1 binding ratio as discussed more later.

1. Determine the fraction of Tau that is bound to MTs in each sample. This value (given the symbol "$\theta$" in the binding equation) is assumed to be the fraction of Tau that cosediments with MTs in each sample. Perform this calculation by dividing the intensity of bound Tau observed at a given concentration (i.e., the pellet intensity $P$) by the intensity of total Tau that was observed at that concentration (determined by summing together the supernatant intensity $S$+pellet intensity $P$)

$$\text{Fraction Tau}_{\text{Bound}} = (\text{Intensity of Tau}_{\text{Bound}})/(\text{Intensity of Tau}_{\text{Total}}) = P/(S+P) = 0.$$

2. Next determine the concentrations of bound Tau ($\text{Tau}_{\text{Bound}}$) for each Sample. To make these calculations, multiply the fractions obtained immediately above by the concentration of total Tau used in your experiments (obtained from the original experimental design). Concentrations here are assumed to be in micromolar

$$[\text{Tau}_{\text{Bound}}] = \text{Fraction Tau}_{\text{Bound}} * [\text{Tau}_{\text{Bound}}] = \theta * [\text{Tau}_{\text{Bound}}].$$

3. For each sample, determine the concentration of bound MTs (i.e., the concentration of polymerized tubulin dimers) that are bound to Tau ($[\text{MT}_{\text{Bound}}]$). This step typically assumes a 1:1 Tau to tubulin dimer-binding stoichiometry, which may be inaccurate, but is a reasonable place to start (see discussion below and Duan & Goodson, 2012). Under this assumption, if 1 μM of Tau is bound at a given MT concentration, then 1 μM of polymerized tubulin dimers must also be bound

$$[\text{MT}_{\text{Bound}}] = [\text{Tau}_{\text{Bound}}].$$

4. For each sample, determine the concentration of free MTs

$$[\mathrm{MT_{Free}}] = [\mathrm{MT_{Total}}] - [\mathrm{MT_{Bound}}].$$

5. To generate the fractional saturation plot, plot $\theta$ (i.e., the fraction of Tau bound) on the $y$-axis and $[\mathrm{MT_{Free}}]$ on the $x$-axis.
6. Inspect the data. Ideally, the fractional saturation will approach 1.0 at the highest of the MT concentrations used (i.e., 100% of your protein will be observed to cosediment with MTs). If the fractional saturation is still increasing at the highest MT concentrations used, then you should repeat the experiment with higher tubulin concentrations, if possible. If the data do level off, but at a value lower than 1.0 (e.g., 80%), then some of your protein may be inactive. See further discussion of these issues in the note later.
7. The experiment should be repeated in at least triplicate, and the average values $\pm$ standard deviation plotted on a graph as described in step 5.
8. Using a program such as Graphad Prism, Origin, or Kaleidegraph, you can fit the data from step 7 to the equation $\theta = B_{max}{}^{*}\ [\mathrm{MT_{Free}}]/([\mathrm{MT_{Free}}]+K_D)$, where for the $Y$ values you enter your data for $\theta$ (i.e., your fraction of $\mathrm{Tau_{Bound}}$ values) and for the $X$ values you enter your calculated $[\mathrm{MT_{Free}}]$ concentrations (*not* the total input MT concentrations). $B_{max}$ represents the asymptote that the binding curve approaches when saturation is achieved. You should generally set this to 1.0 (i.e., 100%), unless it is clear that your data saturate at a lower fractional saturation (e.g., if it appears to saturate at 80%, input 0.8 for the $B_{max}$). The program should then be able to use this information to fit for the apparent $K_D$.
9. To test the effect of altering the assumed stoichiometry of the Tau–MT interaction, explore the effect of changing this ratio in step 3. Note that changing the ratio so that one Tau occupies more tubulin dimers (e.g., using a 1:2 Tau: tubulin ratio instead of a 1:1 ratio) will result in a decrease in the apparent $K_D$ (i.e., it will result in a stronger apparent affinity). You can use this approach to set an upper limit on the binding ratio because if you choose a ratio that is too extreme, you will obtain impossible negative values for $[\mathrm{MT_{free}}]$. In contrast, changing the ratio so that more than one Tau can bind to a given tubulin dimer will increase the apparent $K_D$ (i.e., result in a weaker apparent affinity). In theory, one could determine the Tau: tubulin-binding ratio by a TvMc experiment as discussed later, but interpreting these data is difficult because of Tau—Tau interactions (Ackmann et al., 2000; Duan & Goodson, 2012). Our approach has been to assume a 1 Tau:1 tubulin dimer-binding ratio for purposes of comparing the affinity of different Tau constructs and/or types of tubulin polymer, but recognize that the ratio could be different.

Note:

I. It is possible to skip the calculation of $\mathrm{MT_{Free}}$ and directly fit the Tau fractional saturation data to a quadratic version of the binding equation in which the the Tau

fractional saturation is a function of $MT_{Total}$ (see equation 8 in Pollard, 2010). We prefer the process outlined earlier because it is more intuitive, but the end result should be similar.

## 4.4 ESTIMATION OF APPARENT $K_D$ VALUES AND BINDING RATIOS FROM TvMc DATA

In TvMc analyses, the concentration of MTs is held constant, and the concentration of Tau is varied. Then plots of $[Tau_{Cosed}]$ vs $[Tau_{Free}]$ are generated, and the data are fit to a variation of the binding equation used earlier. Because this approach utilizes only data on the behavior of Tau, it avoids making any assumptions about the Tau:MT-binding ratio. As a result, this approach can in theory be used to determine both the Tau–MT affinity and the Tau–MT-binding ratio. However, in practice this approach is problematic because MTs promote Tau—Tau interactions that become common at the high Tau concentrations used in these assays; these Tau—Tau interactions include both Tau binding to MT-bound Tau- and microtubule-promoted formation of Tau-only filaments that sediment in response to centrifugation (Ackmann et al., 2000; Duan & Goodson, 2012). The existence of these interactions violates the central assumption of cosedimentation assays, i.e., that all protein cosedimenting with MTs does so by binding to MTs. The failure of this assumption means that it is impossible to interpret these assays in a straightforward way. Regardless, these assays can still be useful for characterizing and comparing aspects of Tau behavior as long as this significant caveat is remembered, so we will briefly discuss the calculations here.

The first 2 steps are the same as in TcMv approach. After this point, the calculations diverge.

1. Determine the fraction of Tau that cosediments with MTs in each sample. In the following calculations, this is assumed to be the fraction of Tau that is binding to MTs in each sample, though, as noted earlier, this is generally a bad assumption in TvMc assays. Perform this calculation by dividing the intensity of cosedimenting Tau observed at a given concentration (i.e., the pellet intensity $P$) by the intensity of total Tau that was observed at that concentration (determined by summing together the supernatant intensity $S$+pellet intensity $P$)

$$\text{Fraction } Tau_{Cosed} = Tau_{Cosed}/Tau_{Total} = P/(S+P).$$

2. Next, determine the concentration of cosedimenting Tau ($Tau_{Cosed}$) for each sample. To make these calculations, multiply the fractions obtained immediately above by the concentration of total Tau used in your experiments (obtained from the original experimental design). Concentrations here are assumed to be in micromolar

$$[Tau_{Cosed}] = \text{Fraction } Tau_{Cosed}{}^{*}[Tau_{Total}].$$

3. Next, determine the concentrations of free Tau ($Tau_{Free}$) for each sample. To make these calculations, utilize the conservation of mass equation shown below

$$[Tau_{Free}] = [Tau_{Total}] - [Tau_{Cosed}].$$

4. To generate the binding plot, plot the $[Tau_{Cosed}]$ on the $y$-axis and $[Tau_{Free}]$ on the $x$-axis.
5. The experiment should be repeated in at least triplicate, and the average values ± standard deviation plotted on the graph.
6. The plot generated in steps 4 and 5 can be used to compare the behavior of different Tau constructs without making any potentially problematic assumptions about whether cosedimenting Tau = Tau bound to MTs. However, if you have reason to think that Tau—Tau interactions will not cause problems in your experiments (i.e., that $[Tau_{Cosed}] = [Tau_{Bound}]$), you can use a program such Graphad Prism, Origin, or Kaleidegraph to attempt to fit the data to the equation:

$$[Tau_{Bound}] = N^*[MT_{Total}]^*[Tau_{Free}]/([Tau_{Free}] + K_D).$$

In the absence of Tau—Tau interactions, the resulting fit should provide both $N$ (the binding ratio) and the apparent $K_D$ value. However, under conditions where Tau—Tau interactions are occurring (i.e., many TvMc experiments), the binding will not saturate as would normally be expected for a standard binding reaction, and the resulting fit will be very poor (Ackmann et al., 2000; Duan & Goodson, 2012). The combination of the poor fit and ambiguities created by violation of the assumption that sedimentation occurs though binding to MTs means that any $N$ and $K_D$ values obtained from this type of analysis should be treated with extreme caution. For more discussion of these issues, see Duan and Goodson (2012).

## 5 SUMMARY OF USEFUL RESOURCES

As mentioned earlier, good protocols for preparing different types of tubulin polymers can be found at the website of the Tim Mitchison lab (https://mitchison.hms.harvard.edu/files/mitchisonlab/files/tubulin_polymerization_with_gtp.pdf). As also noted, the following websites provide useful information on how to quantify bands on SDS gels: (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/; http://www.navbo.info/DensitometricAnalysys-NIHimage.pdf). You can download the FIJI image processing package (a batteries included version of imageJ) at https://fiji.sc/. In addition, the following publication is an excellent resource for discussions of how to set up and interpret protein-binding assays more generally (Pollard, 2010).

## 6 CONCLUSIONS

Cosedimentation assays are far from ideal approaches for studying interactions between Tau and MTs, but in the absence of better methods, they can yield useful data as long as researchers take pains to remember the assumptions used in performing the assays, pay attention to detail, and are careful to perform the assays in a consistent manner. The TcMv approach is strongly preferred for measuring Tau–MT affinity because of complications caused by Tau—Tau interactions in TvMc assays. However, TvMc experiments are likely to be used more frequently as Tau—Tau interactions are studied in more depth.

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