Supporting Information:

Tau binds to multiple tubulin dimers with helical structure

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Materials and Methods

Tau purification

The sequence of tau K16 was cloned into a pET-HT vector with a T7 promoter for expression and an N-terminal His-tag with a tobacco etch virus (TEV) cleavage site for removing the His-tag. Cysteine residues for site-specific fluorescent labeling (at the amino terminus for labeling with Alexa 488 for FCS and throughout the MTBR for labeling with acrylodan) were introduced at desired sites using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). Tau was expressed in e. coli and purified as previously reported.[1] Briefly, e. coli transformed with a desired plasmid were grown in 500 mL LB media incubated at 37°C with shaking until OD_{600}=0.4-0.6. Protein expression was induced by the addition of IPTG (1:2500 dilution of a 0.8M stock solution) and incubated for an additional 4 hours at 37°C. The cells were collected by centrifugation at 5000g at 4°C for 30min and the supernatant was discarded. The cell pellets were lysed in 15mL lysis buffer (50mM

Tris, pH 8, 500mM NaCl, 10mM imidazole with freshly added 1mg/mL lysozyme and 1mM PMSF) by ultrasonication followed by centrifugation at 20000g to remove cell debris. The supernatant was passed through a 0.22μm syringe filter and then incubated with Ni-NTA agarose for ~1h at 4°C with gentle mixing. The agarose was washed with 60mL Ni-NTA buffer A (50mM Tris, pH 8, 500mM NaCl, 10mM imidazole) before protein was eluted with Ni-NTA buffer B (50mM Tris, pH 8, 500mM NaCl, 400mM imidazole). The eluted protein solution was concentrated to ~1 mL. The His-tag was removed by overnight incubation at 4°C with TEV in the presence of 1mM DTT. The cleaved sample was buffer-exchanged back into Ni-NTA buffer A and purified again with the Ni-NTA agarose to remove TEV and cleaved tags. Final purification was by size-exclusion chromatography using a Superdex-200 column (GE HiLoad 16/600). The protein was concentrated and labeled immediately after elution for the Superdex-200 column, then flash-frozen for storage at -80°C until needed.

Darpin and RB3 purification

DARPin and RB3 were purified following previously published protocols[2] with slight modifications. Briefly, 1L cells expressing DARPin or RB3 were lysed by ultrasonication. After removal of the cell debris, the supernatant was incubated with fresh Ni-NTA resin at 4 °C for 1 hour. The resulting mixture was poured into a column and the protein was eluted by buffer containing 400mM imidazole, following extensive washing by buffer containing 10mM imidazole. For DARPin purification,

the protein solution was concentrated and purified by a Superdex 200 column (GE HiLoad 16/600). For RB3 purification, the eluted protein solution was concentrated to ~1mL, followed by TEV treatment to remove its N-terminal His-tag. The protein was then purified through a second Ni-NTA column to remove TEV and cleaved tags, before final purification by size exclusion chromatography using a Superdex 200 column (GE HiLoad 16/600). After SEC purification, the fractions containing the protein were combined and concentrated. Freshly purified protein was labeled immediately and flash-frozen for storage at -80°C until further use.

Tubulin purification and labeling

Tubulin was purified from porcine brain by the method of Castoldi and Popova[3], aliquoted, and stored at -80 °C. For FCS measurements requiring fluorescently labeled tubulin, freshly purified tubulin was labeled with rhoadamine green according to published protocols[4]. The labeled tubulin was aliquoted and stored at -80 °C.

At the beginning of each experiment, an appropriate number of tubulin aliquots were thawed quickly at 37 °C and then placed on ice. The tubulin was clarified by ultracentrifugation at 4 °C for 6 min at 98,000×g. Clarified tubulin was buffer exchanged into phosphate buffer (20mM phosphate, 20mM KCl, 1mM $MgCl_2$, 0.5mM EGTA, pH 7.2) using a Bio-rad spin column. The concentration of tubulin was determined by measuring the absorbance at 280nm using an extinction coefficient of 115,000 $M^{-1}cm^{-1}$.

Labeling: acrylodan

To label tau with acrylodan (Life Technologies), 200µL of approximately 500µM protein in purification buffer (25mM Tris pH 7.4, 100mM NaCl, 1mM EDTA) was treated with 1mM DTT at room temperature for 30 min. The solution was passed through two coupled desalting columns (GE, HiTrap) to remove DTT and buffer exchanged into labeling buffer (20mM Tris pH 7.4, 50mM NaCl). Acrylodan was freshly dissolved in DMSO and titrated into the protein solution stepwise with constant stirring, taking care that the final DMSO concentration did not exceed 10%[5], to reach a final dye:protein ratio 4:1. The reaction mixture was incubated in the dark at room temperature for 4 hours with stirring. Excess acrylodan was removed by passing the solution over two coupled desalting columns (GE, HiTrap). The fluorophore concentration was calculated using ε_{372nm}=16400 cm^{-1} M^{-1}.[6] Protein concentration was determined by protein DC assay (Biorad) and the labeling efficiency was calculated accordingly.

Due to the low solubility of acrylodan in water, the final labeling efficiency ranged from 57% to 84%. To test if the extent of labeling had an impact on our measurements, unlabeled tau was mixed with labeled protein to create a solution with ~12% labeled tau (to mimic a poor labeling reaction). The mixture was tirated with tubulin with a final concentration of 300nM tau and 10µM tubulin. The emission spectrum of the mixture was compared to a measurement made with 61% labeled tau under same concentrations of tau and tubulin. The emission spectra of both samples overlapped with the emission peaks at ~497 nm for both samples (Figure S5A). In order to further

ensure that there were no other systematic trends in the data related to labeling efficiency, we plotted the emission peak positions of tau when bound to tubulin for each construct against its labeling efficiency. No correlation was observed between these two values (Figure S5B).

Labeling: Alexa 488

Labeling of tau, DARPin, and RB3 with Alexa 488 proceeded as above through the DTT removal step. After removal, 4X Alexa 488 maleimide (Life Technologies) dissolved in DMSO was titrated into the protein solution stepwise with stirring, taking care that the final DMSO concentration did not exceed 1%. The reaction mixture was incubated in dark at room temperature for 30min and then 4°C overnight before excess dyes were removed by passing the solution over two coupled desalting columns (GE, HiTrap). The fluorophore concentration was calculated using ε_{494nm}=71000 cm^{-1} M^{-1}. Protein concentration was determined by protein DC assay (Biorad) and the labeling efficiency was calculated accordingly. All reactions had a labeling efficiency greater than 98%,

Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed on a lab-built instrument based on an inverted Olympus microscope as described previously.[1] The laser power was adjusted to 5-6 μW prior to measurements. Each sample was placed in a well of an 8-well NUNC chamber treated with PEG-PLL to prevent any non-specific adhesion. FCS

measurements were performed in phosphate buffer (20mM phosphate, 20mM KCl, 1mM $MgCl_2$, 0.5mM EGTA, pH 7.2). This buffer was chosen because it resulted in tight binding of tau, DARPin, and RB3. For each measurement, 30 traces of 10 seconds were recorded and averaged together to obtain statistical variations. The averaged curve was analyzed by a single component fit to the following equation, using the inverse of the variance as a weighting factor (1):

$$G(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\frac{s^2\tau}{\tau_D}\right)^{-\frac{1}{2}} \quad (1)$$

to obtain the average diffusion time for each system, where $G(\tau)$ is the autocorrelation as a function of time τ, N is the average number of fluorescent molecules, τ_D is the mean diffusion time of labeled protein, s is the ratio of the radial to axial dimensions of the focal volume which was determined to be 0.17 for the system. At least three independent measurements were performed for each protein combination. The average diffusion times and standard deviations were reported.

For each measurement with tubulin, 15 μM tubulin was used in order to ensure that essentially all of the tau, DARPin, or RB3 was bound. This was verified by measuring autocorrelation curves at 5μM, 10μM and 15μM tubulin. No significant difference between these curves was observed indicating that each of the fluorescently labeled proteins was close to 100% bound to tubulin under the conditions of our measurements (Figure S6).

FCS measurements of 20nM fluorescently labeled tubulin, DARPin, RB3, and tau were used to determine the diffusion times of each of these proteins, respectively (Table S1). Measurements of tau-tubulin, DARPin-tubulin, and RB3-tubulin

complexes were made using 20nM of fluorescently lableled tau, DARPin, or RB3 and 15μM of unlabeled tubulin (Figure 2 and Table S1). Control measurements were made of 20nM fluorescently labeled tau and 30μM unlabeled DARPin or RB3 in the absence of tubulin to ensure that tau was not binding to these proteins (Table S1). For measurements of tau binding to DARPin-tubulin or RB3-tubulin complexes, we used 20nM fluorescently labeled tau in the presence of 15μM unlabeled tubulin and 30μM unlabeled DARPin or RB3.

The relative 'brightness' per molecule of tau and tau-tubulin were calculated to exclude the presence of multiple tau in the tau-tubulin complexes. The resulting values, 1.20 kHz μW^{-1} per molecule for tau and 1.28 kHz μW^{-1} per molecule for tau-tubulin, are comparable, indicating that only a single fluorescent tau is present in the complex.

Acrylodan fluorescence

The acrylodan measurements were made on a Horiba Fluorolog 3 Fluorometer using a quartz cuvette (Starna Cells, Inc.). The excitation and emission slit widths were set to 5nm. Tau (300nM) and tubulin (15μM) were mixed in phosphate buffer (20mM phosphate, 20mM KCl, 1mM $MgCl_2$, 0.5mM EGTA, pH 7.2). The mixture was transferred to a quartz cuvette and equilibrated at 20°C. The excitation wavelength was set to 390nm, and the emission was scanned from 400 to 600nm. The position of the emission maximum of a buffer-subtracted emission trace was recorded as the peak position. For each constructs, at least 3 independent measurements were

performed; the reported values are the average and standard deviation of these measurements.

Models of tau-tubulin complexes

In order to understand to what sort of tau-tubulin complexes the measured diffusion times may correspond, we created models of tau bound to tubulin, DARPin-tubulin, and RB3-tubulin and calculated their diffusion times. To do this, a model of tau was first generated by random sampling dihedral angles from Ramachandran plot. Residues defined as 'potentially helical' (P251 to K267 in R1, L284 to K298 in R2, L315 to H329 in R3, F346 to T361) based on previous reports[7] and acrylodan fluorescence results in this work only sampled dihedral angles in the helical region, while the rest sampled the whole plot. The model was then adjusted to bring it into agreement with our previously reported smFRET measurements[1]. Four different tau models with different conformations were used as input for docking (Figure S7). The models were docked to tubulin (chain C and chain D from PDB: 4DRX), DARPin-tubulin (chain C, chain D and chain E from PDB: 4DRX), or RB3-tubulin (PDB: 3N2G) using ZDOCK[8] with default parameters. The 10 top-scored hits for each of the four models were used to calculate diffusion coefficients and diffusion times.

Calculation of diffusion coefficients and diffusion times

The crystal structures of DARPin-tubulin (PDB: 4DRX) and RB3-tubulin (PDB:

3N2G), as well as the predicted docking structures of tau to tubulin, DARPin-tubulin and RB3-tubulin, were used as input to WinHYDROPRO [9] to calculate the diffusion coefficient for each complex. The diffusion coefficient for the tubulin dimer was calculated using chain C and chain D in DARPin-tubulin structure (PDB: 4DRX). The pdb files used for input were prepared using PyMOL[10].

After noting that the diffusion coefficients calculated by WinHYDROPRO were consistently different from values quoted in the literature, we determined a calibration curve to allow us to convert the WinHYDROPRO values. We carried out WinHYDROPRO calculations on a number of globular proteins with reported diffusion coefficients[11] and performed linear regression between the calculated ($D_{HYDROPRO}$) and reported (D_{report}) values (Figure S8, red line).

WinHYDROPRO was then used to calculate diffusion coefficients for tubulin (chain C and chain D in PDB: 4DRX), DARPin-tubulin (chain C, chain D and chain E from PDB: 4DRX), and RB3-tubulin (PDB: 3N2G) and these values were plotted against the experimentally determined diffusion coefficients (D_{exp}) of the same molecules. D_{exp} values were calculated by taking the diffusion times measured by FCS, converted using equation (2):

$$D_P = D_{Alexa488} \frac{\tau_{Alexa488}}{\tau_P} \quad (2)$$

where D_P and τ_P are the diffusion coefficient and diffusion time of the protein complex of interest, respectively, $\tau_{Alexa488}$ is the diffusion time of Alexa 488 measured on the same instrument, and $D_{Alexa488}$ is the diffusion coefficient of Alexa 488 ($D_{Alexa488}$= 435 μm^2/s) reported in the literature[12]. Both tubulin and DARPin-tubulin

fall on the linear calibration curve described above, while RB3-tubulin deviates from this line (Figure S8, blue). This is most likely because the structure of RB3-tubulin is elongated[13] and thus diverges from the canonical globular structures used in the calibration. Thus, a separate calibration equation was determined by fitting the calculated diffusion coefficients $D_{HYDROPRO}$ against experimentally determined diffusion coefficients D_{exp} using a linear model (Figure S8, blue line). The calibrated diffusion coefficients ($D'_{HYDROPRO}$) from were calculated as:

$$D'_{HYDROPRO} = \frac{D_{HYDROPRO} + 0.582}{1.08} \quad (3)$$

(units of 10^{-7} cm^2/s). Subsequently, the predicted diffusion time ($\tau_{predict}$) was calculated for each model as:

$$\tau_{predict} = \tau_{Alexa488} \frac{D_{Alexa488}}{D'_{HYDROPRO}} \quad (4)$$

A total of 40 different predicted diffusion times (10 top-scored hits from docking for each of the 4 tau models) were used to generate statistical variance for each of the tubulin complexes with one tau molecule bound.

Sequence alignment and pairwise distance calculation

Sequence alignment of R1 through R4 was performed using MEGA version 6[14]. Alignment was performed using ClustalW.

Chemical cross-linking

Cross-linking experiments were performed according to a previously published protocol[15] with slight modifications. DARPin, RB3, or tau (300 nM) labeled with Alexa 488 and unlabeled tubulin (15 μM) were used for these reactions. Tubulin was mixed with one of the fluorescent proteins in phosphate buffer (20 mM phosphate, 20 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.2) to a final volume of 30 μL. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was dissolved in water to make a 100mM stock solution immediately before cross-linking reaction and was then diluted to a final concentration of 2mM in each sample. The samples were incubated at room temperature for 30 minutes. The reactions were then quenched with 1μL solution of a 1:1 mixture of 0.5M EDTA and 1M pH 7.4 Tris buffer. The samples were then subjected to SDS-PAGE analysis. The gels were imaged by a Typhoon gel scanner both in fluorescence mode for Alexa 488 signal (tau, DARPin or RB3) and in digitizing mode for SimplyBlue (Life Technologies) stain (tubulin). For controls, each of the proteins was incubated individually with the cross-linker at the same concentrations used in the experiments.

Table S1 Measured diffusion times

construct	- tubulin (ms)	+tubulin (ms)
tubulin (20nM labeled)	-	0.65±0.02
DARPin (20nM labeled)	0.40±0.01	0.72±0.01
RB3 (20nM labeled)	0.47±0.02	0.91±0.02
tau (20nM labeled)	0.60±0.01	1.24±0.11
tau (20nM labeled) + DARPin (30μM unlabeled)	0.62±0.01	1.06±0.01
tau (20nM labeled) + RB3 (30μM unlabeled)	0.62±0.01	1.23±0.05

Table S2 Predicted diffusion times for tau-tubulin complexes using ZDOCK models

	tubulin	DARPin-tubulin	RB3-tubulin
$\tau_{predict}$ (ms)	0.77	0.82	0.99
Range of $\tau_{predict}$ (ms)	0.12	0.14	0.11

Table S3 Summary of acrylodan data

labeling position	labeling efficiency	peak position (nm) -tubulin	peak position (nm) +tubulin
I260C	61%	526	500
C291	84%	526	495
L315C	67%	525	489
S316C	70%	525	481
V318C	66%	524	490
T319C	68%	524	484
S320C	78%	525	487
C322	78%	525	491
G323C	75%	525	488
S324C	66%	525	489
L325C	69%	526	494
G326C	78%	525	488
N327C	68%	525	487
I328C	69%	526	498
I354C	57%	525	497

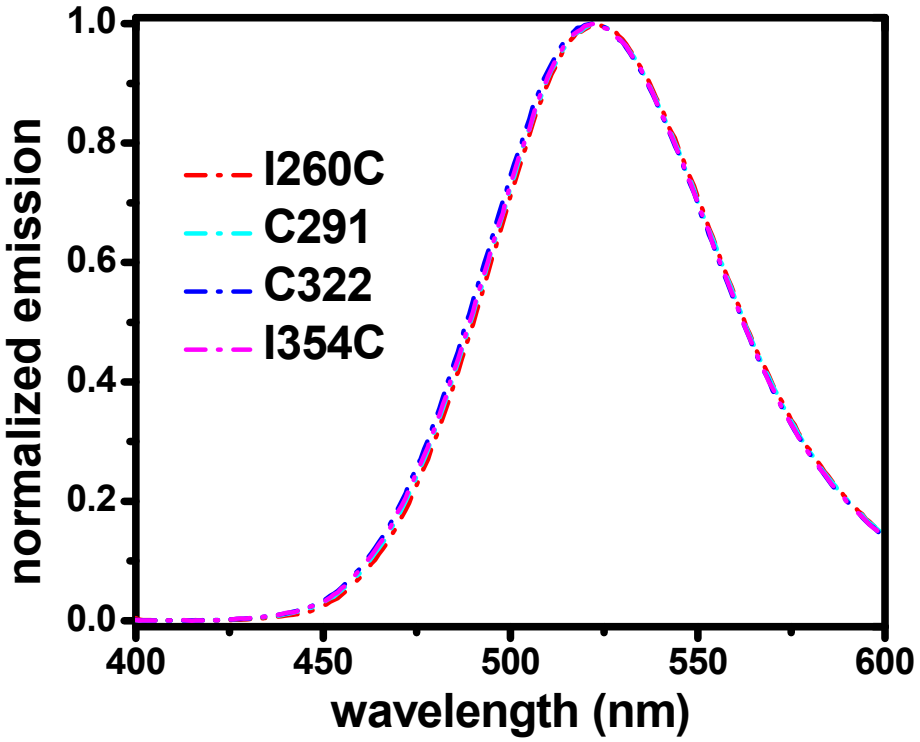


Figure S1 Normalized fluorescence emission spectra of tau labeled with acrylodan at equivalent residues in each of the four repeats in the absence of tubulin.

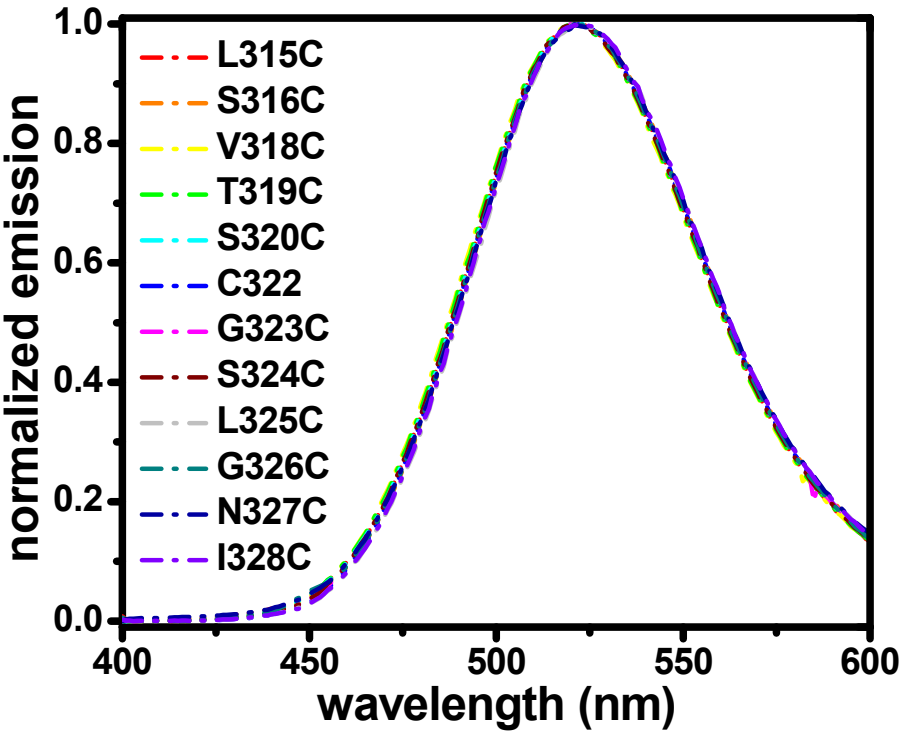
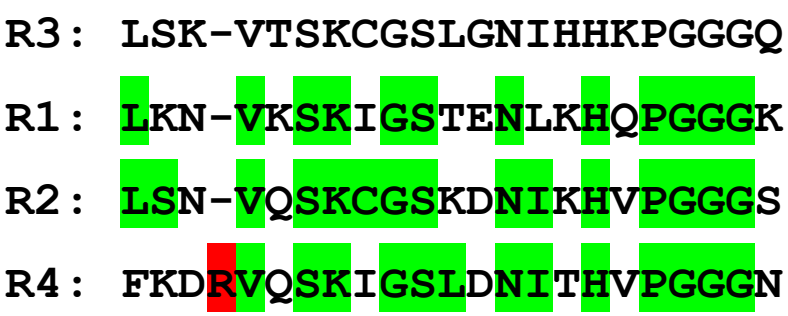


Figure S2 Normalized fluorescence emission spectra of tau labeled with acrylodan scanning repeat 3 in the absence of tubulin.



repeat	identity%	gaps
R1	54	0
R2	68	0
R4	59	1

Figure S3 Sequence alignment comparing R3 with R1, R2, and R4. Identity=green, gap=red. R2 has ~14% and ~9% greater identity to R3 than R1 or R4, respectively.

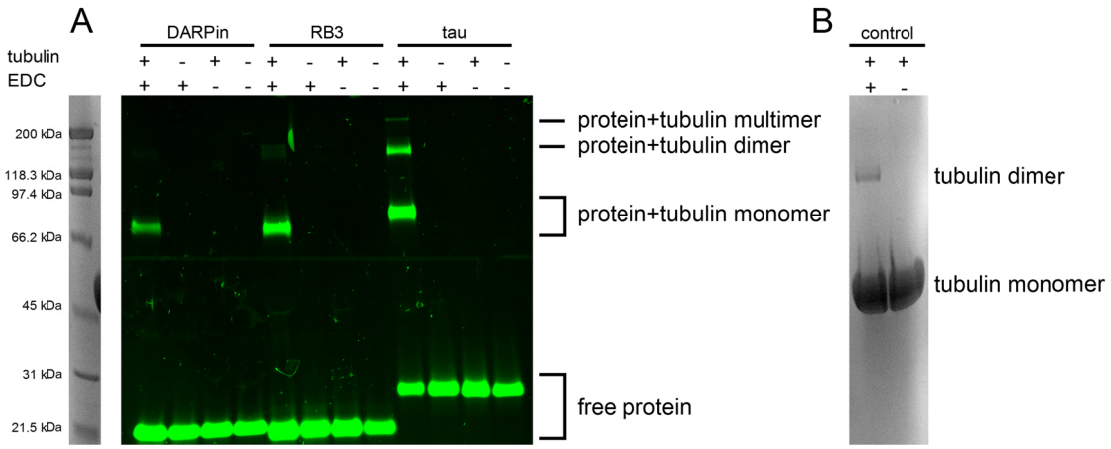


Figure S4 Cross-linking of DARPin, RB3 and tau to tubulin. Both tubulin and the cross-linking reagent must be present for cross-linking to occur (Panel A). While labeled protein cross-linked to tubulin monomer served as the major cross-linking product for all three proteins, an increase of the amount of protein cross-linked to tubulin dimer was observed for RB3 (1:2 binding to tubulin),with a larger increase in this species for tau (Panel A). Moreover, tau shows a clear band for protein cross-linked to tubulin multimer which is not observed with the other two proteins (Panel A), indicating that tau is able to bind to multiple tubulin dimers. As RB3 serves as a standard for a 1:2 protein:tubulin binding stoichiometry, the comparison of the cross-linking results between tau and RB3 strongly support our hypothesis that tau binds to multiple tubulin dimers. Tubulin alone shows a very small fraction of cross-linked dimer (Panel B).

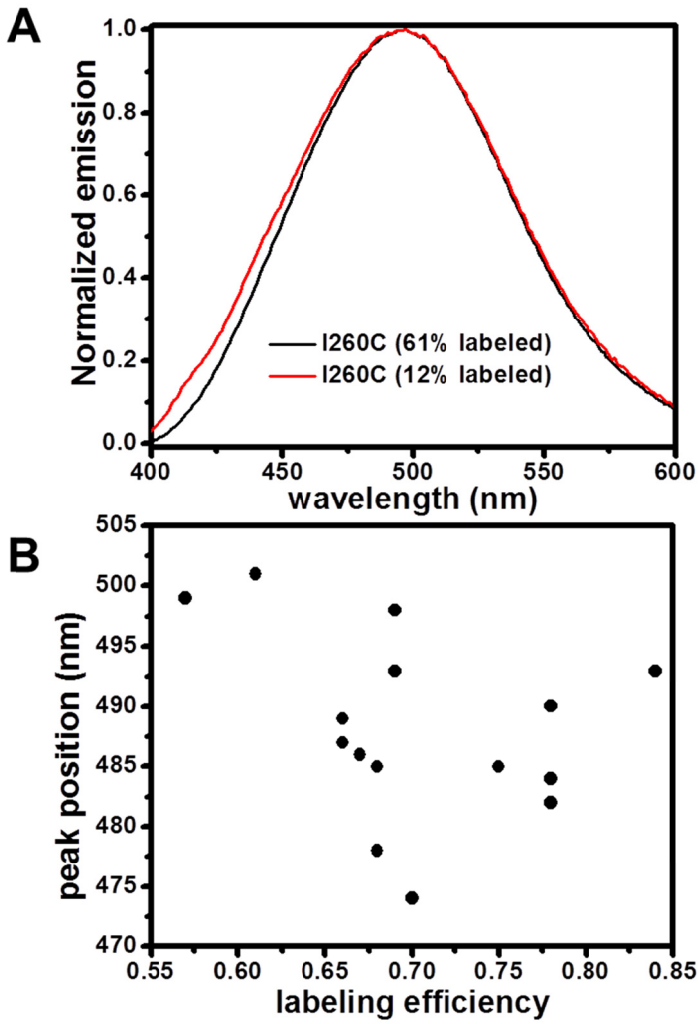


Figure S5 A) Spectra of tau I260C labeled with acrylodan in the presence of 10μM tubulin, with labeling efficiency noted. The slight discrepancy between the spectra at shorter wavelengths likely results from scattering of the excitation beam (390 nm); when the total labeling efficiency is low, the scatter will contribute a relatively greater amount to the emission at wavelengths closer to the excitation beam wavelength. B) A plot of acrylodan emission peak wavelength of tau in the presence of tubulin versus labeling efficiency shows no evidence of correlation. These data suggest that the differential shifts in the peak positions are not due to differences in the labeling efficiency.

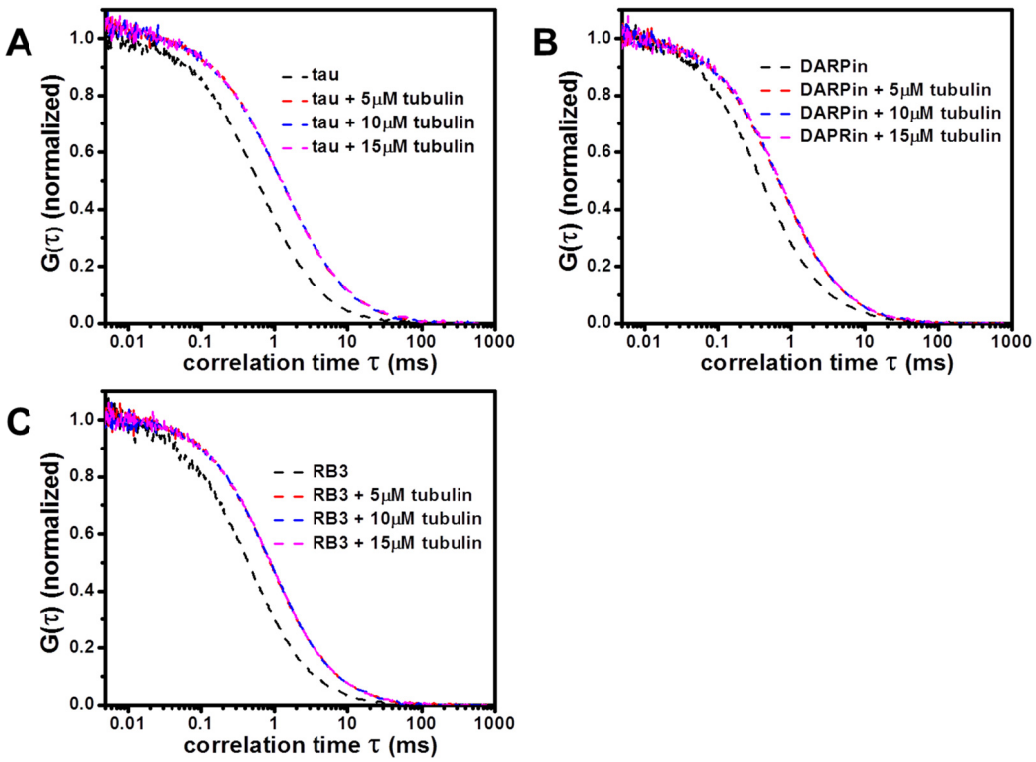


Figure S6 Autocorrelation curves of fluorescently labeled tau (A), DARPin (B) and RB3 (C) in the presence of increasing concentrations of tubulin

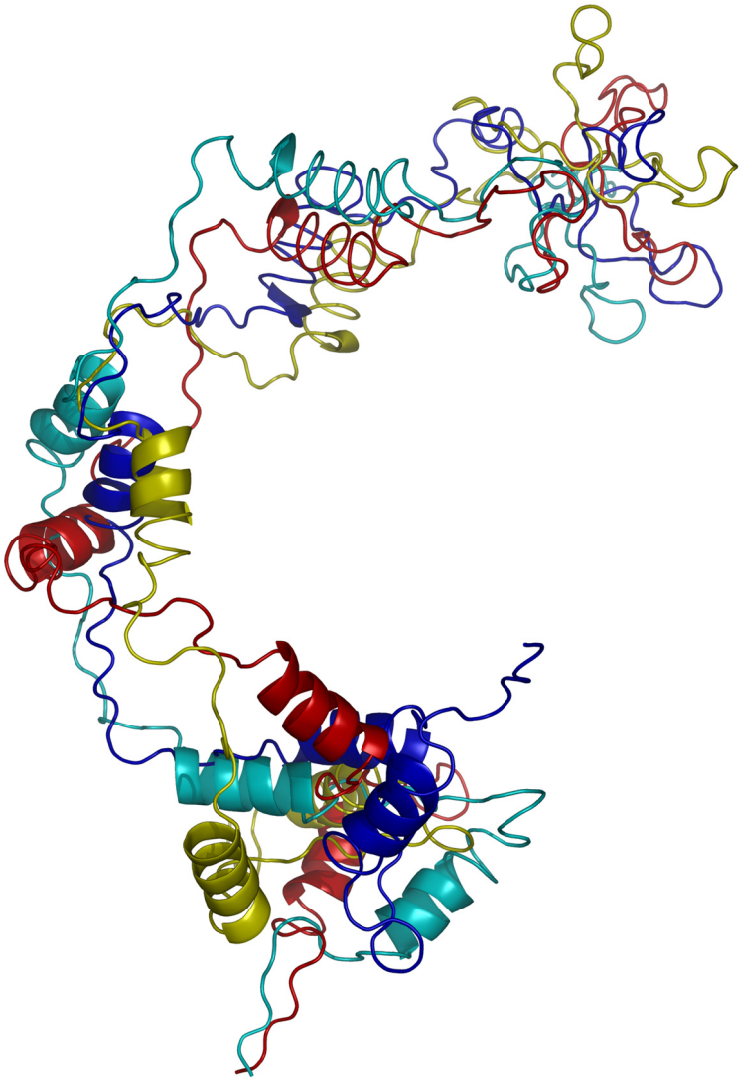


Figure S7 Models of tau used for docking.

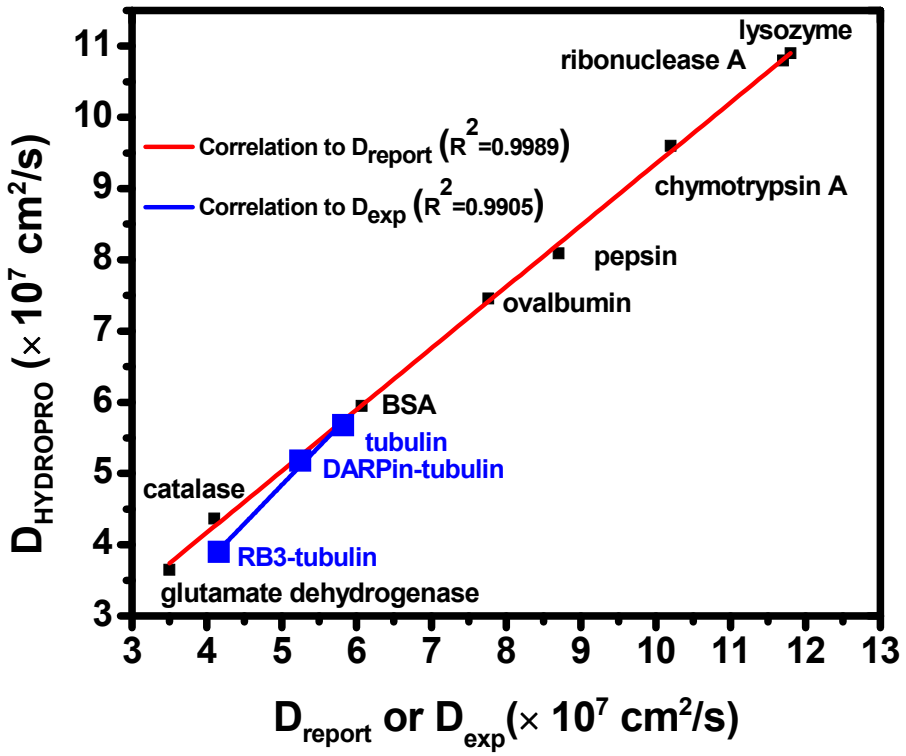


Figure S8 Relationship between diffusion coefficients calculated from crystal structures ($D_{HYDROPRO}$) and experimentally determined diffusion coefficients reported previously in the literature[11] (D_{report}, black square) or measured in this study by FCS (D_{exp}, blue square). The linear fits for $D_{HYDROPRO}$ against D_{report} or D_{exp} are colored red or blue, respectively.

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