Manipulation and quantification of microtubule lattice integrity

Taylor A. Reid, Courtney Coombes and Melissa K. Gardner*

ABSTRACT

Microtubules are structural polymers that participate in a wide range of cellular functions. The addition and loss of tubulin subunits allows the microtubule to grow and shorten, as well as to develop and repair defects and gaps in its cylindrical lattice. These lattice defects act to modulate the interactions of microtubules with molecular motors and other microtubule-associated proteins. Therefore, tools to control and measure microtubule lattice structure will be invaluable for developing a quantitative understanding of how the structural state of the microtubule lattice may regulate its interactions with other proteins. In this work, we manipulated the lattice integrity of in vitro microtubules to create pools of microtubules with common nucleotide states, but with variations in structural states. We then developed a series of novel semi-automated analysis tools for both fluorescence and electron microscopy experiments to quantify the type and severity of alterations in microtubule lattice integrity. These techniques will enable new investigations that explore the role of microtubule lattice structure in interactions with microtubule-associated proteins.

KEY WORDS: Microtubule, Taxol, Structure, Lattice, GMPCPP

INTRODUCTION

Microtubules are long, hollow tubes that act as important structural and signaling components inside cells. Microtubules are typically closed tubes that are formed by 13 laterally associated individual protofilaments, each of which is composed of αβ-tubulin heterodimers that are stacked end-to-end (Zhang et al., 2015; Wang and Nogales, 2005). However, while this regular, stacked αβ heterodimer arrangement of microtubules is widely conserved, electron microscopy studies have revealed the presence of a wide range of microtubule lattice structures and irregularities. For example, cryo-electron microscopy studies have revealed that the lattice structures near to growing microtubule ends are frequently characterized by flattened, open sheets, rather than closed tubes (Chrétien et al., 1995; Guesdon et al., 2016). Further, variations in the number of individual protofilaments have been observed both within a microtubule (Vitre et al., 2008; Doodhi et al., 2016) and between microtubules that are nucleated under different conditions (Vitre et al., 2008; Moore et al., 2012; Wade and Chrétien, 1993; des Georges et al., 2008), leading to heterogeneity and defects in the microtubule lattice. It has been recently reported that hydrolysis of the β-tubulin subunit within the microtubule lattice leads to overall ‘compaction’ of the microtubule lattice (Alushin et al., 2014), likely leading to structural heterogeneity within the microtubule lattice. Finally, a range of microtubule-targeting drugs have been reported to alter the large-scale microtubule structure, introducing heterogeneity and defects into the microtubule lattice (Diaz et al., 1998; Doodhi et al., 2016; Kellogg et al., 2017).

Importantly, recent work has uncovered links between microtubule lattice integrity and the efficiency of kinesin-based transport (Liang et al., 2016), katanin-mediated microtubule severing (Davis et al., 2002), microtubule destabilization by Stathmin (Gupta et al., 2013) and tubulin acetylation in microtubules (Coombes et al., 2016). Similarly, disruption of the closed microtubule lattice structure near to the growing microtubule end hints that microtubule tip-tracking proteins could recognize this configuration to facilitate tip tracking (Guesdon et al., 2016; Bechstedt and Brouhard, 2012; Bechstedt et al., 2014). Thus, microtubule lattice integrity may significantly impact a variety of microtubule-associated cellular processes. For this reason, tools are required both to systematically manipulate microtubule lattice integrity in an in vitro setting, and also to quantitatively assess the associated microtubule lattice structure.

However, methods to systematically generate in vitro microtubule pools with common nucleotide states, but with differing states of lattice structural integrity, have not been described. These microtubule pools would be invaluable for assessing the contribution of microtubule lattice integrity to various microtubule-associated cellular processes. Similarly, while variations in lattice integrity have been observed using electron microscopy (Coombes et al., 2016), methods to quantify and describe these variations would be a useful contribution to this newly developing field of study.

In this work, we describe new methods for generating pools of in vitro microtubules with common nucleotide states, but with differing degrees and types of disruptions in lattice integrity. In addition, we have developed new analytical tools for quantifying these microtubule structural states through (1) a semi-automated image analysis platform for Electron Microscopy (EM) images, and (2) experiments and a semi-automated analysis method using Total Internal Reflection Fluorescence (TIRF) microscopy. Through our new quantitative tools, we found that the growth and storage conditions for in vitro microtubules had a strong impact on the lattice structural integrity of the microtubules. These results have implications that should be considered when investigating the interactions of microtubules with a range of microtubule-associated proteins, such as molecular motors, microtubule tip-tracking proteins, post-translational modification enzymes, and microtubule severing enzymes.

MATERIALS, METHODS AND RESULTS

Method: GDP-Tubulin microtubule pools with potential variations in lattice structural states

We first developed a method to generate stable pools of in vitro microtubules with a common GDP-tubulin nucleotide state, but with differing degrees and types of disruptions in lattice integrity. To prepare GDP-tubulin microtubules, a mixture composed of 33 μM tubulin (see Supplementary Materials and Methods) (25%
to ensure that the microtubules were fully hydrolyzed into GDP-tubulin, the microtubule solution was then separated into two tubes for overnight storage. One tube was stored overnight at 37°C, while the second tube was stored overnight at 25°C (Fig. 1A). Here, we predicted that the 37°C storage condition could potentially promote more efficient lattice repair of the microtubules, as compared to the solution stored at 25°C. This idea was supported by the observation that microtubules stored at 37°C were longer than those stored overnight at 25°C (Fig. 1B, left; quantitative length analysis Fig. S1A; P=2×10^{-9}, t-test), and preliminary EM images hinted that there were also differences in lattice structural integrity as a result of these alternative storage conditions (Fig. 1B, right).

We note that in order to generate stabilized GDP-tubulin microtubules, Taxol was added to the microtubule solution prior to overnight storage, replicating a widely used approach in microtubule research. Because Taxol itself has been reported to have an effect on microtubule structure (Kellogg et al., 2017; Díaz et al., 1998), the 25°C and 37°C storage condition microtubule mixture tubes were identically treated with Taxol prior to storage. Therefore, any observed changes in microtubule structure after overnight storage would be independent of the effect of the initial Taxol treatment itself.

**Method: GMPCPP-tubulin microtubule pools with potential variations in lattice structural states**

To potentially manipulate the microtubule structural states, and, especially to preserve the CaCl2-treated microtubules, we predicted that the Process #1 protocol would maximize the possibility of producing GTPtubulin microtubules with intact lattice structures, because (1) the relatively low tubulin concentration used in the initial microtubule assembly may promote a more ordered assembly process (Gardner et al., 2011), and (2) storage of Taxol-stabilized GTPtubulin microtubules at 37°C may promote more efficient lattice defect repair of the microtubules, as described above.

We then used an alternative process, Process #2, to produce GTPtubulin microtubules with potentially more disrupted lattice structures. This process was identical to Process #1, except that (1) the tubulin concentration used in the initial microtubule assembly was 25.5 μM rather than 12 μM, since we predicted that a higher free tubulin concentration may promote a more rapid, and thus less ordered, more defect-prone assembly process (Gardner et al., 2011), and (2) after 1 h of assembly, 10 μl of the microtubule mixture was diluted into 255 μl warm Brb80 with 50 mM KCl, 10 mM DTT, 0.1 mg/ml Casein, and 10 μl of Taxol (as compared to 24 μl as described above), and, (3) the mixture was stored overnight at 25°C (in contrast to 37°C as above) (Fig. 1E). Here, we predicted that by reducing the residual free tubulin concentration during storage, and by storing the microtubules at a lower temperature, this would discourage any scaffold defect repair of the GTPtubulin microtubules. Indeed, microtubules stored at 37°C, and with a higher residual free tubulin concentration (Process #1 above), were substantially longer than thosestored overnight at 25°C at a lower residual free tubulin concentration (Process #2) (Fig. 1F, left; quantitative length analysis Fig. S1B; P=2×10^{-16}, t-test), suggesting that polymerization and repair may have occurred during storage. Preliminary EM images hinted that both preparations had some degree of disruptions in large-scale microtubule lattice integrity, with Process #2 having more frequent disruptions (Fig. 1F, right).

Similar to the other microtubule preparations, we note that in order to generate stabilized GTPtubulin microtubules, Taxol was added to the microtubule solution prior to overnight storage, again replicating a widely used approach in microtubule research. Because Taxol itself has been reported to have an effect on microtubule structure (Kellogg et al., 2017; Díaz et al., 1998), the Process #1 and Process #2 microtubule mixture tubes were identical treated with Taxol prior to storage. Therefore, any observed changes in microtubule structure between Process #1 and Process #2 would be independent of the effect of Taxol treatment.

**Method: Quantitative lattice structural characterization tool for EM**

We then collected images of each microtubule preparation using Transmission Electron Microscopy (TEM), and analyzed the
images for potential structural disruptions using a newly developed semi-automated analysis tool. It should be noted that the negative stain TEM method used here provides a simple method for comparative analysis of our microtubule pools, especially since both pools of microtubules in each nucleotide case were identically prepared and imaged with TEM over multiple trials. However, our new automated tool for quantitative structural characterization of microtubules as is described below would be equally applicable to cryo-electron microscopy, a method that may allow for improved preservation of microtubule structure.

For TEM imaging, microtubules were prepared identically to those as described above. A drop of the mixture was then placed on a 300-mesh carbon-coated copper grid for 1 min. After 1 min, the grid was stained with 1% uranyl acetate for 1 min. The stain was wicked away with filter paper and the grid was left to dry and then stored. Specimens were imaged using a Technai Spirit BioTWIN transmission electron microscope (FEI, Thermo Fisher Scientific). All images were acquired at 18.5k× magnification (pixel size, 1 nm), 2048×2048 image size, and saved to a lossless image format.

Analysis of the EM images was performed using a novel custom MATLAB (MathWorks) script (see Supplementary Materials and Methods). First, microtubules in the EM images were traced manually using connected line segments (Fig. 2A,B). We note that the segment size resulting from manual tracing was dependent on the degree of curvature, with higher curvature leading to shorter segments. The segments were refined using an automated algorithm to reduce human error or bias (Fig. 2C-E). This automated refinement involved first smoothing the image to improve edge detection (Fig. 2C), followed by the use of an edge filter and nonmaximal suppression, which is an intensity based thinning technique to identify the center of the edges (Fig. 2D). Finally, the 'strong', high intensity, microtubule edges were identified using a multi-level implementation of the Otsu thresholding algorithm with which the manual edge traces could be refined (Fig. 2E). From the refined microtubule traces, microtubule width ($W$) and curvature ($C$) were measured automatically (Fig. 2F) (for details of trace segmentation and midline calculation see Supplementary Materials and Methods).

The width and curvature metrics were then combined to calculate an overall ‘Structure Metric’ ($S$), which provides a quantitative measure of the morphology of microtubule lattice. To do this, the total absolute curvature was calculated by summing the absolute value of curvature for each segment of an individual microtubule midline [Fig. 3A, left; ($C_{Total}$)]. Then, a microtubule ‘width deviation’ metric was automatically calculated by measuring the width of the microtubule for each segment [Fig. 3A, right, ($W$)], and then by subtracting the width of a typical intact microtubule width ($W_{Expected}$) in pixels as measured based on typical intact microtubules in the images. The absolute value of this width deviation was summed across the whole microtubule, normalized to microtubule length, and used as the width deviation metric ($W - W_{Expected}$). The final Structure Metric ($S$) was then calculated by summing the width deviation and curvature metrics, each respectively normalized by the parameters $N_W$ and $N_C$ to provide...
approximately equal weight of curvature and width to the final score, as follows:

\[ S = \frac{|W - W_{\text{Expected}}|}{N_W} + \frac{C_{\text{Total}}}{N_C} \]  

(1)

The values of \( N_W \) and \( N_C \) were set to 2 and 0.1 respectively, such that a deviation in width of 2 pixels was weighted equally with a total absolute curvature of 0.1 radians. These values were chosen according to the scale of variation observed in width and curvature in order that each contributed approximately 50% to the final overall

FIG. 2. Automated quantification of large-scale microtubule lattice integrity. Description (left) and example visualization (right) of the automated EM quantification method. The boxed region in B (right) is shown enlarged in C to E (right).
Fig. 3. Large-scale lattice integrity is shifted within microtubule pools. (A) Visualization of EM quantification method. Left: width measurement in the Structure Metric. Right: curvature measurement in the Structure Metric. (B) Sample EM images of 37°C storage GDP microtubules (top) and 25°C storage GDP microtubules (bottom; arrows indicate structural disruptions). (C) Structure Metric is increased at 25°C storage, suggesting that large-scale microtubule structure is disrupted. (D,E) Width measurements and curvature measurements contribute to the Structure Metric. (F) Sample EM images of untreated GMPCPP microtubules (top) and CaCl₂ treated microtubules (bottom; arrows indicate structural disruptions, inset is enlargement). Scale bars: 100 nm. Structure Metric is increased with CaCl₂ treatment (bottom), suggesting that large-scale microtubule structure is disrupted. (H,I) Width and curvature measurements contribute to structure metric. (J) Sample EM images of GTPγS microtubules prepared and stored using Process #1 (top) and Process #2 (bottom; arrows indicate disrupted structure). Scale bars: 100 nm. Structure Metric is increased with Process #2, suggesting that large-scale microtubule structure is further disrupted with Process #2 as compared to Process #1. (L,M) Width and curvature measurements contribute to the overall Structure Metric. The bar graphs in C, G and K show mean±s.e.m.; P-values were calculated from t-test of independent means.
Structure Metric (S). We note that a larger value of the EM Structure Metric (S) would reflect more frequent or more drastic morphological structural disruptions in microtubule lattice integrity, such as bends, partial tubes, and open sheet-like regions, which would tend to increase both microtubule width and curvature. Smaller disruptions in microtubule lattice integrity, such as defects or holes in the lattice, would be less efficiently detected by this measure.

Results: Microtubule pools have alterations in lattice structural integrity as measured by EM

The automated MATLAB code was then applied to analyze EM images for each pooled batch of microtubules. By using a common Structure Metric based on microtubule width and curvature (S, Eqn 1), the large-scale structural integrity of microtubule preparations could be compared between pools of microtubules with different preparations. Significance was assessed using two-tailed Student’s t-test of independent means. All imaged microtubules were included in the analysis regardless of apparent structural condition. Images in Fig. 3 were chosen to match the average structure metric of each condition.

First, the lattice integrity of the GDP microtubules was evaluated. Qualitatively, the pool of 37°C storage-condition GDP microtubules was characterized in the EM images by straight edges and uniform widths (Fig. 3B, top). In contrast, the 25°C storage-condition microtubule pool appeared to have more frequent bends along the length of the microtubule, and higher variability in width along the microtubule length, frequently coincident with regions of reduced intensity (Fig. 3B, bottom). These disruptions may be associated with an open, sheet-like, or incomplete tubes. We then used our automated analysis tool to measure the Structure Metric (S) of numerous microtubules in each pool. We found that there was a significant increase in the Structure Metric for the 25°C storage-condition pool of GDP microtubules as compared to the 37°C storage-condition (Fig. 3C; \( P = 2 \times 10^{-6}, t\)-test), and that this increase was due to shifts towards larger widths and higher curvature for the 25°C microtubules (Fig. 3D,E).

Importantly, we have described methods that allowed us to shift the lattice structural integrity within a given nucleotide pool of stable microtubules. Quantification of microtubule EM images suggested that the large-scale lattice structural integrity of a microtubule, as assessed by its width and curvature, is tunable for Taxol-stabilized GDP, GMPCPP and GTPγS microtubules. The ability to shift the large-scale structural integrity of microtubules within a common nucleotide state will allow for new studies that directly examine the effect of microtubule structural state on steady-state binding, mobility, and on/off kinetics of microtubule-associated proteins.

Method: Lattice structural characterization by TIRF reporter assay

The automated EM quantification tool described above provided a method to characterize lattice structural changes in in-vitro stabilized microtubules. However, this EM quantification method was not efficient in characterizing smaller, submicrotubule-scale disruptions in microtubule lattice integrity, such as gaps or holes. For such an analysis, we developed an alternative automated method.

Recent work by Schaedel et al. (2015) demonstrated that new tubulin could be incorporated into defects or gaps in the microtubule lattice. Based on this result, we developed a TIRF ‘reporter’ assay, which allowed us to quantitatively probe the structural integrity of our microtubule pools using fluorescence microscopy.

The automated MATLAB code was then applied to analyze EM images for each pooled batch of microtubules. By using a common Structure Metric based on microtubule width and curvature (S, Eqn 1), the large-scale structural integrity of microtubule preparations could be compared between pools of microtubules with different preparations. Significance was assessed using two-tailed Student’s t-test of independent means. All imaged microtubules were included in the analysis regardless of apparent structural condition. Images in Fig. 3 were chosen to match the average structure metric of each condition.

Finally, the lattice structural state of the GTPγS microtubules was evaluated. The pools of GTPγS microtubules generated using Process #2 appeared qualitatively more curved, and of less uniform width, than the GTPγS microtubules from Process #1 (Fig. 3J). This observation was quantitatively confirmed by evaluating the Structure Metric score: there was a significant increase in the Structure Metric value for the GTPγS microtubules produced and stored via Process #2 as compared to Process #1 (Fig. 3K, \( P = 1 \times 10^{-15}, t\)-test). This increase came about by concurrent shifts towards larger widths and higher curvature for the Process #2 GTPγS microtubules as compared to Process #1 (Fig. 3L,M). These results suggest that the GTPγS microtubules produced by Process #2 tended to have more frequent regions with open sheets and partial tubes relative to those produced by Process #1.

While the preparation protocols for microtubules using the three different nucleotides were distinct, each of these protocols reflect commonly used methods for producing stable in vitro microtubules. As such, our new analysis method highlights structural differences in the microtubule lattice that are produced when these protocols are used in a typical laboratory setting. In particular, we note that even the intact microtubules from Process #1 for the GTPγS microtubules had a much higher structure metric (~13), and thus substantially lower structural integrity, than the microtubules produced from either of the common base protocols for the GDP and GMPCPP microtubules (~4 and ~7, respectively).

The automated EM quantification tool described above provided a method to characterize lattice structural changes in in-vitro stabilized microtubules. However, this EM quantification method was not efficient in characterizing smaller, submicrotubule-scale disruptions in microtubule lattice integrity, such as gaps or holes. For such an analysis, we developed an alternative automated method.

Recent work by Schaedel et al. (2015) demonstrated that new tubulin could be incorporated into defects or gaps in the microtubule lattice. Based on this result, we developed a TIRF ‘reporter’ assay, which allowed us to quantitatively probe the structural integrity of our microtubule pools using fluorescence microscopy. The experimental portion of our reporter assay was completed as follows. First, each red-labelled microtubule pool (as described above) was incubated with green-labelled ‘reporter’ tubulin. To do this, the final microtubule preparations as described above were spun down in an air-driven ultracentrifuge at 20 psi for 5 min, resuspended in 50 μl of ‘reporter’ solution [1.5 μM 66% Alexa Fluor 488 (Thermo Fisher Scientific)-labelled tubulin, 1 mM MgCl2, 250 μM GTP, and 10 μM taxol in Brb80], and then incubated for 3 h at 37°C (Fig. 4A, left). This microtubule solution was then introduced into an imaging chamber, after which between 30 s and 5 min were allowed for the microtubules to adhere to the imaging coverslip, and the solution was subsequently replaced with warm imaging buffer (see Materials and Methods & Techniques).
The microtubules were then imaged at 488 nm and 561 nm wavelengths (Fig. 4B).

Over the course of the incubation period, the green reporter tubulin incorporated as normal microtubule growth at the plus ends of microtubules (Fig. 4C, right), but was also occasionally incorporated along the length of the microtubule (Fig. 4C, left, white arrow). Here, we expected that microtubules with more gaps, holes, or other lattice defects would lead to an increased occurrence of green reporter patches along the length of the microtubule due to new reporter tubulin incorporation into the lattice (Fig. 4A, right, bottom). In contrast, a perfectly intact microtubule lattice would only have green reporter tubulin incorporation extending beyond the red-labelled lattice at its plus-end, due to normal microtubule end assembly (Fig. 4A, right, top).

Method: Lattice structural characterization by TIRF reporter assay—quantitative analysis

We then developed a new MATLAB (Mathworks) analysis tool (see Supplementary Materials and Methods) to provide a quantitative measure of the degree of disruption in submicrotubule-scale lattice integrity, as evidenced by the fraction of green reporter tubulin that was incorporated along the length of the red microtubule lattice. This was accomplished first by automatic processing of the red microtubule channel to determine the microtubule-positive regions, which then allowed conversion of the red channel into a binary image with white microtubules and a black background (Fig. 5; extended details in Fig. S2). The green reporter tubulin channel was then also pre-processed to smooth high-frequency noise and to correct for TIRF illumination inhomogeneity (Fig. 5B). The green channel threshold was then manually increased to just above background level (Fig. 5C). The choice of threshold at just above background maintains consistent analysis across multiple experiments while also reliably detecting dim reporter tubulin incorporations into the microtubule (Fig. 5C, right-bottom image represents final thresholded image). Measurements of the reporter tubulin length were then automatically collected from the identified microtubule regions, as indicated by the red outline in Fig. 5D.

To analyze the degree of lattice disruption for each microtubule, the extent of green tubulin incorporation was quantified by the Reporter Fraction (RF). This metric was automatically calculated as the total length of green reporter tubulin signal (G; Fig. 6A) divided by the total length of red microtubule signal (R; Fig. 6A):

\[ RF = \frac{G}{R} \]  

This length-based metric has two key advantages to an intensity-based readout, as (1) it is not sensitive to the variation in image intensity between experiments, and (2) it is not biased by overlap with the green extensions at the growing plus end, which are typically much brighter than most gap-filled sites, and are present on most microtubules regardless of the structural condition. We note that while the repair assay is theoretically sensitive enough to pick up the addition of a single labeled tubulin dimer, our TIRF microscope diffraction limit leads to reporting of the repair length of gaps that are actually significantly smaller than \( \sim 250 \) nm (even a single reporter tubulin dimer) as repair lengths between 160 nm (our pixel size) and \( \sim 250 \) nm [diffraction limit, \( \sim 31 \) dimers in length (250/8)]. Here, the thresholding of the green reporter signal results in some of the dimmer repair regions registering only the brightest pixels in their diffraction pattern, which is why we might detect lengths as low as the pixel size.

To calculate the Reporter Fraction for each microtubule, the red microtubules were automatically detected using the MATLAB script, as described above. False-positives (nonmicrotubules) were deselected manually. For each (red) microtubule, the microtubule length was automatically recorded as the red signal (R; Fig. 6A). For each microtubule, the green signal (G) was defined as the...
cumulative length of green reporter tubulin signal that overlapped with the red microtubule, which allowed for exclusion of plus-end extensions (i.e. the green signal that did not overlap with a red microtubule) from the analysis (Fig. 6A). The Reporter Fraction (RF) was then calculated as the cumulative length of reporter signal divided by the length of the microtubule (Eqn. 2).

**Results: Microtubule pools have alterations in lattice structural integrity as measured by TIRF Reporter Assay**

The TIRF reporter assay was then used to characterize each of our microtubule pools (Fig. 6B-J). First, the lattice integrity of the GDP microtubule pools was evaluated by calculating the Reporter Fraction for each pool. Qualitatively, segments of green tubulin reporter incorporation into the microtubule lattice were more commonly observed in the 25°C storage pool of GDP microtubules than in the 37°C storage pool (Fig. 6B, white arrows). After quantification using the Reporter Fraction (RF), we found that there was a 55% higher Reporter Fraction for the 25°C storage pool GDP microtubules as compared to the 37°C storage pool microtubules (Fig. 6C; \(P<10^{-9}\)), suggesting that the higher overnight storage temperature led to a decrease in defects, gaps, and holes in the microtubules. The mean repair length was similar between the two microtubule pools (Fig. 6D; \(P=0.124\), \(t\)-test), suggesting that the higher Reporter Fraction for the 25°C pool resulted from an increase in the number of repair regions, rather than an increase in repair region length.

Then, the lattice integrity of the GMPCPP microtubules was evaluated by calculating the Reporter Fraction for each pool (Fig. 6E). GMPCPP microtubules treated with CaCl\(_2\) exhibited ~80% more incorporation of green reporter tubulin (higher Reporter Fraction) than untreated GMPCPP microtubules (Fig. 6F; \(P<10^{-9}\)). Further, the distribution of repair lengths was shifted upon CaCl\(_2\) treatment, such that a larger mean repair length was observed for the CaCl\(_2\)-treated microtubules relative to the untreated microtubules (Fig. 6G; \(P<10^{-13}\), \(t\)-test). Thus, CaCl\(_2\) treatment caused the introduction of holes and gaps in the lattice, in addition to the disruptions as were reported by the EM Structure Metric (Fig. 3C). Finally, the lattice integrity of the GTP\(_{\gamma}\)S microtubules was evaluated by calculating the Reporter Fraction for each pool (Fig. 6H). We observed ~85% more incorporation of green reporter tubulin (higher Reporter Fraction) in the GTP\(_{\gamma}\)S microtubules produced by Process #2 as compared to Process #1 (Fig. 6I; \(P<10^{-9}\)). The mean repair length was similar between the two microtubule pools (Fig. 6J; \(P=0.054\), \(t\)-test), suggesting that the increased reporter fraction was due primarily to more reporter repair regions per micron of microtubule. Therefore, by initially growing the GTP\(_{\gamma}\)S microtubules at a lower free tubulin concentration, and then by storing them in Taxol under conditions that promoted repair of defects (37°C, higher residual free tubulin concentration in storage solution), this allowed defects in the lattice, such as missing subunits, holes, and gaps, to repair.
Results: Insight into the microtubule repair process

Finally, we used our new TIRF reporter assay and automated quantification method to dissect a potential mechanism for how the overnight storage temperature, and the associated residual tubulin concentration during storage, may alter the lattice integrity of Taxol-stabilized GDP microtubules.

To test whether the Taxol-stabilized microtubules were indeed self-repairing their lattice during overnight storage, we compared the Reporter Fraction for newly prepared microtubules as compared to those stored overnight. Here, at Day 0, immediately after the microtubules were prepared and Taxol-stabilized, the microtubule solution was split into two tubes. One tube was stored overnight at 25°C, and the other was stored overnight at 37°C. We observed that the Reporter Fraction was reduced after overnight storage at 37°C (Day 1) as compared to newly prepared, Taxol-stabilized microtubules (Day 0) (Fig. 7A; \(P = 0.0014\)). In contrast, there was a
slight but nonsignificant decrease in Reporter Fraction after overnight storage at 25°C (Fig. 7A; \(P=0.160\)). Thus, newly prepared, Taxol-stabilized GDP microtubules exhibited lattice defects, which were repaired upon overnight storage at 37°C. However, storage at room temperature (25°C) did not facilitate a similar level of repair, suggesting that the storage temperature of Taxol-stabilized in vitro microtubules has a significant effect on their structure due to an innate self-repair process.

Because microtubules stored in Taxol solution may repair themselves, even at very low residual tubulin concentrations, we then asked whether changes in the residual tubulin concentration of the storage solution could alter this repair process. In our original GDP-tubulin microtubule preparation, 10 μl of the original polymerized microtubule mixture was diluted into 990 μl warm, 10 μM Taxol Brb80 solution (Fig. 1A, left), which resulted in a measured residual free tubulin concentration in solution of 90 nM. To test whether residual free tubulin could contribute to a repair process during storage, 2, 2.5 and 5 μl of the freshly prepared, polymerized GTP-microtubule solution as described above was diluted into 1000 μl, 250 μl and 100 μl warm Taxol solution (in Brb80), respectively, resulting in residual free tubulin concentrations of 18 nM, 90 nM and 450 nM, respectively. We found that after overnight storage at 25°C, there was a decrease in the Reporter Fraction value with increasing residual free tubulin concentration (Fig. 7B; \(P=0.0053\) from 90 nM to 450 nM)). For example, when there was a fivefold increase in residual free tubulin concentration (90 nM to 450 nM), there was a 15% decrease in Reporter Fraction, and, in contrast, when there was a fivefold decrease in residual free tubulin concentration (90 nM to 18 nM), there was a 15% increase in Reporter Fraction, although this change was not statistically significant due to variability in the Reporter Fraction results (\(P=0.249\)). Additionally, we observed, in a separate experiment, that when there was a 10-fold decrease in residual free tubulin concentration (90 nM to 9 nM), overnight storage at 25°C caused the microtubules to depolymerize completely (data not shown).

Thus, we found that by increasing the residual free tubulin concentration in the overnight storage solution, we could tune the microtubule Reporter Fraction for GDP microtubules, suggestive of a change in microtubule lattice integrity. Consistent with the change in Reporter Fraction from Day 0 to Day 1 (Fig. 7A), this suggests that freshly prepared microtubules, stabilized by Taxol in a single-step process, had disruptions in lattice integrity (Fig. 7C, top), as previously reported (Diaz et al., 1998; Matesanz et al., 2011). A mechanism by which in vitro microtubule lattice integrity may be altered during storage is by direct lattice incorporation and repair by free tubulin dimers from solution, especially when stored at warm temperatures (Fig. 7C, bottom). Alternatively, the more disrupted subpopulation of microtubules could also selectively depolymerize during storage. However, since Taxol-stabilized microtubules tended to increase in length as a function of storage time (Fig. S4), it seems likely that damaged microtubules may undergo repair as well.

**DISCUSSION**

Through quantification of fluorescence and electron microscopy experiments, we demonstrated that the structural state of microtubules could be manipulated by changes in the growth and storage conditions of those microtubules. These results suggest that the protocols used to prepare, stabilize, and store in vitro microtubules can impact the microtubule lattice integrity. This could in turn affect experimental results in studies of motor proteins (Liang et al., 2016).
or other microtubule associated proteins (Bechstedt et al., 2014; Davis et al., 2002; Gupta et al., 2013).

It is important to note that our two quantification methods, EM and TIRF microscopy, provide information on different elements of microtubule structural states. The EM Structure Metric (S) reports on the width and curvature of the microtubules, both of which are characteristics of larger-scale changes in the microtubule structural state. The curvature is indicative of more flexibility in the microtubule lattice, which could result from any or all of gaps (Schaedel et al., 2015), unclosed regions of the microtubule (Guesdon et al., 2016), inherent lattice flexibility due to the nucleotide state (Lopez and Valentine, 2014; Valdman et al., 2014; Alushin et al., 2012), Taxol treatment (Mickey and Howard, 1995; Lopez and Valentine, 2014; Hawkins et al., 2013), or temperature (Kawaguchi and Yamaguchi, 2010). Similarly, increased width is suggestive of an open lattice structure, which may originate from the loss of individual protofilaments, while decreased width is suggestive of small gaps or quantification based on intensity.

The TIRF reporter assay is ideal for identifying gaps in the microtubule structure. While gaps and defects can be observed in EM images, the higher throughput nature of TIRF imaging allows for rapid quantification of many hundreds of microtubules for changes in lattice integrity. Additionally, TIRF imaging is sensitive enough to detect repair by a single fluorescent tubulin dimer. Since the Reporter Fraction (RF) depends on the relative lengths of red and green fluorescence, it is therefore more sensitive to small gaps than a quantification method based on intensity.

It is important to note that the manipulation of lattice structure for GMPCPP microtubules differed in its implementation and results from that of GDP or GTP/S microtubules. GDP and GTP/S microtubules were initially of low structural integrity as a result of their growth processes and/or the addition of Taxol, and then they were subsequently placed in favorable or unfavorable conditions for repair. In contrast, GMPCPP microtubules were initially characterized by high structural integrity, and were subsequently damaged by the addition of calcium. This resulted in a characteristic difference in the lower structural integrity pool of GMPCPP microtubules as compared to the other two nucleotides in both the EM and TIRF measurements. In the EM measurements, calcium treatment had the distinct phenotype of reducing the median width while increasing the number of curvature outliers. In the TIRF reporter repair assay, GMPCPP calcium treatment was the only condition to shift the repair region length distribution, despite the fact that all three low structural integrity conditions had similar increases in reporter fraction. This is indicative that the method of structural integrity manipulation plays an important role in the resultant characteristics of the microtubule.

Previous work has identified methods for the alteration of microtubule structure through protofilament number control (Bechstedt and Broughard, 2013). However, the methods and tools described in our new work manipulate a separate element in microtubule structure, namely the lattice integrity, and thus expands the available options for investigations concerned on the influence of microtubule structure in microtubule-based cellular processes. The manipulation and quantification of microtubule structure will be useful for future studies focused on the role of microtubule interactions with microtubule-associated proteins.

Acknowledgements

Parts of this work were carried out in the Characterization Facility, University of Minnesota, a member of the NSF-funded Materials Research Facilities Network (www.mrfm.org) via the MRSEC program. The steerable line filter implementation for the automated microtubule detection code was provided by the Computational Image Analysis in Cellular and Developmental Biology course at Marine Biological Laboratories. We thank Mark McClellan for technical assistance and helpful discussions.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

This work was supported by the National Institutes of Health (GM-103833).

Supplementary information

Supplementary information available online at http://bio.biologists.org/lookup doi:10.1242/bio.025320.supplemental

References


Modulation of microtubule interprotofilament interactions by modified taxanes. 


Figure S1: Microtubules are longer when stored at higher temperature. (A) Cumulative distribution plot as a function of microtubule length for 25 °C storage (dark blue) and 37 °C storage (light blue) GDP microtubules. Microtubules stored in Taxol at the higher temperature are characteristically longer. (B) Cumulative distribution plot as a function of microtubule length for 25 °C storage (dark orange) and 37 °C storage (light orange) GTPγS microtubules. Microtubules stored at the higher temperature are characteristically longer. (C) Cumulative distribution plot as a function of microtubule length for calcium treated (dark purple) and non-calcium treated (light purple) GMPCPP microtubules. A net shift in lengths from calcium treatment was not observed.
Figure S2: Detailed TIRF image processing example. (A-L) Example images for the entire red (microtubule) channel processing pipeline as summarized in the main figures (Fig. 5A). (M-O) Example images for the green (reporter tubulin) channel pre-processing step from in Fig. 5B. (A-O) See supplemental methods text for details of each step.
Figure S3: Shorter microtubules have highly variable structural integrity. (A) A plot of Reporter Fraction (RF) as a function of microtubule length for 37 °C storage GDP microtubules. Each point represents one microtubule (N = 199). Short microtubules show high variability in reporter tubulin incorporation, as measured by Report Fraction(RF). Microtubules are observed spanning the entire possible scope of reporter fraction; from 0 to 1. The longer microtubules approach the mean Reporter Fraction (represented by the grey dashed line). This behavior is to be expected if the regions of low structural integrity occur stochastically across the microtubule, as opposed to individual microtubules having an inherent tendency towards low or high structural integrity. (B) Alternate representation of the Reporter Fraction (RF) variability changes at different lengths for 37 °C storage GDP microtubules, shown as a box and whisker plot. Bins show microtubules of less than 75px, 75-149px and 150px or greater. Reporter Fraction variance decreases with increasing length. (C) A plot of Reporter Fraction (RF) as a function of microtubule length for 25 °C storage GDP microtubules. Each point represents one microtubule (N = 653). The same trend of highly variable short microtubules, and low variability long microtubules, holds for the pool of “ragged” 25 °C storage microtubules. As shown in Fig S1, there is a lower proportion of long microtubules in this pool as compared to 37 °C storage GDP microtubules. (D) Alternate representation of the Reporter Fraction (RF) variability changes at different lengths for 25 °C storage GDP microtubules, shown as a box and whisker plot. Bins show microtubules of less than 75px, 75-149px and 150px or greater. Reporter Fraction variance decreases with increasing length.
Figure S4: Taxol-stabilized microtubules increase in length during storage. (A) Cumulative distribution plots of microtubule length for increasing storage times for 37 °C storage conditions of Taxol-stabilized microtubules. Overall, microtubules increase in length over time, shifting to a longer length distribution after 2-3 days of storage, relative to newly prepared Taxol-stabilized microtubules (Day 0). (B) Cumulative distribution plots of microtubule length for increasing storage times for 25 °C storage conditions of Taxol-stabilized microtubules. Overall, microtubules increase in length over time, shifting to a longer length distribution after 3 days of storage, relative to newly prepared Taxol-stabilized microtubules (Day 0).
Supplemental Materials and Methods

Tubulin Purification and Labelling

Tubulin was purified and labelled as per (Gell et al., 2010).

Analysis of TEM Images

Analysis of TEM images was conducted using a custom MATLAB (mathworks) script. Microtubules in the TEM images were traced by hand using connected line segments to mark both edges of the microtubules. This edge data was then fed into an automatic processing script. The script first attempts to refine the manual edge segments. The images are Gaussian smoothed and subsequently filtered using a steerable edge detection filter (function provided by the “Computational Image Analysis in Cellular and Developmental Biology” course at Marine Biological Laboratories) to identify all the edges present in each image. In order to distinguish microtubule edges (high contrast) from background edges (low contrast), the edge-filtered image is thresholded using a 2-level Otsu threshold algorithm, with only the strongest edges being preserved.

For each end point of the manual edge segments, the script searches for the nearest “strong” edge point within a 30x30px area centered around the given edge point. If on the first pass no strong edges are found, the image is locally re-thresholded in a 100x100px area, and the script again searches for the nearest strong edge within a 30x30px area. Any adjustment made in the second pass must be verified and/or corrected by the user before the script will continue, as it is not considered a high confidence refinement.

After all edge points have been refined, the curvature is calculated for each edge by calculating the angle deviation between each consecutive edge segment. The absolute value of curvatures for each end segment are summed, and the total absolute curvature for the entire microtubule is considered to be the average absolute total curvature of its two edges.
Next the script must calculate the microtubule width deviations. As not all segments are of equal width nor are they necessarily lined up with segments from other edge of the corresponding microtubule, accurate measurements of the width require the edge traces be sub-segmented and microtubule midlines be calculated. Sub-segmentation ensures that each end-point of an edge segment has a unique end-point on the microtubule’s opposite edge, which is the closest point on the corresponding edge segment. The end result is that the edge trace segments are sub-divided such that they have an equal number of segments and the Nth end point on one edge is the closest neighbor to the same Nth end point on the opposite edge. For details on this sub-segmentation algorithm, please see the code itself.

After sub-segmentation, the midline is calculated as the halfway point between each pair of segment end-point coordinates. Due to the sub-segmentation, the mid-line accurately tracks the microtubule midline even around curves. Segment widths are also easily calculated by taking the distance between each pair of segment end points. The microtubule average width is determined by taking the average of all segment widths, weighted on segment length (as determined by the distance between points on the midline).

Next, the width deviations are calculated. Because an individual microtubule sometimes has different regions that are wider and thinner than the “standard width,” it is important to cumulatively track the deviation over all segments. Due to the sub-segmentation described above, all segments have either constant width or linearly changing width from start to end of each segment. This allows the continuous width deviation to more easily be calculated. If both ends of the segment are above standard width, or both ends of the segment are below standard width, the width deviation of that segment is equivalent to the average of the width deviation at the two segment endpoints. If, however, the beginning and end of the segment have widths on either side of “standard width”, simply taking the average would result in partial cancelling of the width deviation and is no longer cumulative and continuous. Therefore, the width deviation for that segment is calculated by effectively taking the separate absolute width deviations from the segment beginning to where it reaches “standard width” and from where it reaches “standard width” to the end point of the segment, then combining
them via weighted average. The cumulative width deviation of the whole microtubule (as used in the Structure Metric) is the average width deviation of all segments, weighted by segment length.

Construction and Preparation of Flow Chambers for TIRF Microscopy Imaging

Imaging flow chambers were constructed as in Section VII of (Gell et al., 2010), with the following modifications: two narrow strips of parafilm replaced double-sided scotch tape as chamber dividers; following placement of the smaller coverslip onto the parafilm strips, the chamber was heated to melt the parafilm and create a seal between the coverslips; typically only three strips of parafilm are used, resulting in two chambers per holder. Chambers were prepared with anti-rhodamine antibody followed by blocking with Pluronic F127, as described in Section VIII of (Gell et al., 2010).

TIRF Imaging

A flow chamber was prepared as described above. Microtubules were adhered to the chamber coverslip, and the chamber was flushed gently with warm BRB80. The flow chamber was heated to 35°C using an objective heater on the microtubule stage, and then 3-4 channel volumes of imaging buffer containing 110 μg/ml Glucose Oxidase, 20 μg/ml Catalase, 20 nM D-Glucose, 10 mM DTT, 0.1 mg/ml Casein, 1% Tween-20, and 10 μM Taxol were flushed through the chamber.

Microtubules were imaged on a Nikon TiE microscope using 488 nm and 561 nm lasers sent through a Ti-TIRF-PAU for Total Internal Reflectance Flourescence (TIRF) illumination. An Andor iXon3 EM-CCD camera fitted with a 2.5X projection lens was used to capture images with high signal to noise and small pixel size (64 nm). Images were collected using TIRF with a Nikon CFI Apochromat 100X 1.49 NA oil objective.

Analysis of Reporter Assay

Analysis of the repair assay was performed using a custom MATLAB (Mathworks) script. Unless indicated otherwise, all steps detailed below are performed automatically
by the analysis script. Each image (Fig S2A) was corrected for uneven TIRF illumination using an multi-image median filter (Fig S2B). The median image (illumination map) is created by taking the median across multiple images from the same experiment to determine the typical illumination intensity at each pixel location (this is intended for use when each image is of a different field of view, not for time-course images of one field of view). We found that a minimum of 25 images from the same experiment were needed to generate an appropriately accurate illumination map at the density of microtubules shown in the example image (if density of microtubules is lower, fewer images are needed, if density is higher, more are needed). Any fewer images and there were artifacts in the illumination map created by frequent overlap of microtubules from different images. For best results, 100+ images from the same experiment were used in creating the illumination map. The illumination map was then normalized to its maximum value (Fig S2C) such that all values are less than or equal to 1. The original image was then divided by the normalized illumination map (Fig S2D). This caused the dim outer areas of the image to become equally as bright as the center of the image. This was necessary for consistent processing and thresholding of the image.

In preparation for microtubule detection, the image was denoised using a-trou wavelet denoising (Fig S2E, now showing zoomed region for clarity). Microtubules were detected using a steerable line filter (Fig S2G) (function provided by the “Computational Image Analysis in Cellular and Developmental Biology” course at Marine Biological Laboratories). The line-filter response was thinned to identify the center-line of the microtubules using non-maximal suppression (Fig S2H). The thinned response image was thresholded (threshold adjusted manually for the first image of a given experiment, typically valid for all further images) to identify strong candidates for selection as microtubules (Fig S2I). The identified “microtubule lines” were then thickened to match the point spread function width in our imaging setup (Fig S2K). False-positives were deselected by the user prior to further analysis (Fig S2L). The green reporter-tubulin channel (Fig S2M) was also corrected using an illumination map following the same process as used for the red channel (Fig S2N), and subsequently smoothed slightly using a 0.5-pixel sigma Gaussian filter (Fig S2O). The user then set the green reporter channel threshold just above background level. Any green channel pixel
with intensity over the designated threshold was considered a signal positive pixel. Any signal positive pixel overlapping the identified microtubules was added to that microtubule’s total signal count.

The Reporter Fraction was calculated by dividing the total number of green positive pixels by the number of pixels composing the microtubule. The uniform width of the microtubules arising from the fixed thickening step of the processing makes this roughly equivalent to taking the cumulative green signal length divided by the microtubule length, but is simpler in terms of computation.

To account for the possibility that a microtubule overlaps a coverslip bound, non-specific, green reporter tubulin, we calculated the expected contribution of the non-specific signal and subtracted it from our measurements. The non-specific contribution was calculated by summing the total number of signal positive pixels, determined by the same threshold as previously, that did not contact a microtubule. This value was divided by the total non-microtubule area, in pixels, of the image, providing a signal fraction resultant from non-specific background binding. The non-specific signal fraction was typically very small in comparison to the microtubule-associated Reporter Fraction. The non-specific signal fraction was subtracted from each microtubule’s Reporter Fraction.

To calculate the Reporter Fraction for each pool of microtubules, an average Reporter Fraction was calculated for all microtubules in that pool, weighted based on the length each given microtubule. This is functionally equivalent to measuring the individual Reporter Fractions for fixed length segments of microtubules and taking the average over all segments. The length-weighted average is also functionally equivalent to a hypothetical stacking of each microtubule end to end and treating it as one long microtubule, with the pool’s reporter fraction being equivalent to reporter fraction of said stacked microtubule.