Functional Assays to Identify and Characterize Regulators of Microtubule Behavior


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ABSTRACT
Previous experiments have clearly demonstrated that microtubule dynamic instability is regulated in living cells, but the molecular mechanisms that are responsible for this regulation are not well understood. We describe two rapid, functional assays that can be used to screen cell extracts for regulators of microtubule dynamic instability behavior. In both assays, highly purified tubulin is used to assemble microtubules from Tetrahymena axonemes. In the immunofluorescence assay, microtubules are visualized by fixation and staining with anti-tubulin antibodies. Alternatively, microtubule assembly has been visualized by the addition of rhodamine-labeled tubulin to axonemes, followed by low-light-level fluorescence microscopy. In either case, polymerization is quantified by measuring polymer length, total polymer and the number of microtubules per axoneme. In these assays, addition of brain microtubule-associated proteins (MAPs) results in a 2-fold–3-fold increase in average microtubule length, and addition of vinblastine results in a 50%–75% decrease in average microtubule length. The number of microtubules per axoneme was significantly increased by the addition of MAPs and significantly decreased by the addition of vinblastine. These functional assays can detect molecules that stimulate or suppress net microtubule assembly and provide a useful initial screen to isolate regulators of microtubule dynamic behavior.

INTRODUCTION
Microtubules are dynamic structures that alternate between periods of polymerization and rapid depolymerization, a process that has been termed dynamic instability (16). Although dynamic instability is a property of purified tubulin, it is likely that additional cellular factors modulate dynamic instability behavior in cells (3). For example, the rate of microtubule elongation is slower, and catastrophes occur less frequently for microtubules assembled in vitro than for microtubules in living cells (3). These observations indicate that molecules that promote elongation and stimulate transitions must be present in cells. Further, microtubule dynamics in cells can be suppressed, for example, following the establishment of polarity in epithelial cells and stimulated, for example, by growth factor treatment, demonstrating that microtubule behavior is subject to cellular regulation.

Microtubule-associated proteins (MAPs) were originally defined as molecules that co-purify with microtubules during cycles of polymerization and depolymerization and that stimulate microtubule assembly in vitro (20). Recent experiments performed both in vivo and in vitro have shown that the neuronal MAPs tau and MAP2 modulate microtubule dynamic instability behavior by decreasing the rate of rapid shortening, increasing the frequency of rescue and decreasing the frequency of catastrophes (6,7,14,18,22). Other methods have also been used to isolate molecules that interact with microtubules. For example, microtubule affinity columns have been used to isolate microtubule-binding molecules from soluble cytoplasmic extracts of early Drosophila embryos (13). Antibodies raised against sequences in tubulin that bind MAPs (10,24) and co-immunoprecipitation with tubulin dimers (21) have both been used to isolate novel molecules that interact with microtubules or tubulin, respectively. Finally, in vitro assembly assays have been used to isolate molecules that modulate microtubule assembly behavior. For example, XMAP, a microtubule-associated protein from Xenopus, was identified by its ability to stimulate microtubule growth, and Op18/stathmin was identified by its ability to destabilize microtubules (2,9).

We have characterized two rapid, functional assays to identify novel molecules that regulate microtubule assembly behavior. With these functional assays, we can not only screen cellular fractions for novel factors, but we can also test the effects of purified molecules on microtubule polymerization. These two assays are easy to perform and provide quantitative information regarding molecules that modulate microtubule behavior in vitro.

MATERIALS AND METHODS

Materials
Chemicals were purchased from either Fisher Scientific (Pittsburgh, PA, USA) or Sigma Chemical (St. Louis, MO, USA). DM1A anti-tubulin monoclonal antibody was purchased from Sigma Chemical, and Cy3™-labeled anti-mouse secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). 5,6-
Carboxy succinimidy]l ester of rhodamine for labeling tubulin was obtained from Molecular Probes (Eugene, OR, USA). Porcine brain tissue was obtained from a local slaughterhouse (Adams Farms, Athol, MA, USA).

**Tubulin Purification**

Tubulin from porcine brain tissue was isolated by two cycles of temperature-dependent assembly and disassembly in PM buffer containing 4 M glycerol to isolate assembly-competent tubulin. The final pelleted tubulin (PCC tubulin) was resuspended in PM buffer, centrifuged, quick-frozen in liquid nitrogen and stored at -80°C (17). Protein concentrations were quantified by modifications of the methods of Lowry et al. (15,19).

Axoneme fragments were isolated from *Tetrahymena* cilia as described previously (4). Axonemes were stored at -20°C in 50% glycerol and washed in column buffer (CB) (50 mM PIPES, pH 6.9, 1 mM EGTA, 0.5 mM MgSO₄, 1 mM GTP) or JPM (80 mM PIPES, pH 6.9, 1 mM EGTA, 5 mM MgCl₂, 2 mM GTP) before use (23,27).

**Preparation of Rhodamine-Labeled Tubulin**

Rhodamine-labeled tubulin was prepared with slight modifications to previously published methods (12,25). PC tubulin was assembled in glycerol-containing buffer (50 mM PIPES, pH 6.9, 1 mM EGTA, 4 M glycerol, 10 mM MgSO₄, 1 mM GTP) at 37°C for 30 min and labeled with a 40-fold molar excess of 5,6-carboxy succinimidyl ester of rhodamine. The labeled microtubules were sedimented at 200,000×g for 2 h at 25°C through a sucrose cushion, and the pellet was subjected to three cycles of assembly and disassembly in glutamate buffer (1 M sodium glutamate, 0.5 mM MgSO₄, 1 mM EGTA, 1 mM GTP, pH 6.9) (11) to remove unreacted dye and inactive tubulin. The final pellet of microtubules was then resuspended in injection buffer (20 mM glutamate, 0.5 mM MgSO₄, 1 mM EGTA, pH 7.2) and centrifuged, and the supernatant was separated into aliquots, quick-frozen in liquid nitrogen and stored at -80°C (6).

**Microtubule Assembly Assays**

**Immunofluorescence assay.** The assembly of individual axoneme-nucleated microtubules was adapted from methods described by Vasquez et al. (23) and Walker et al. (27). A 50-µL mixture containing 12 µM unlabeled PC tubulin, 1 mM GTP and CB (with or without vinblastine or MAPs) was placed on a coverslip with axonemes in a humid chamber. No glycerol or dimethyl sulfoxide (DMSO) was present in the reaction mixture. The humid chamber was constructed by placing a piece of moist filter paper in a petri dish. Coverslips, previously cleaned with 70% ethanol, were placed on broken cotton-tipped applicators above the moist filter paper. The extent of microtubule assembly was visualized by fixation and immunofluorescence staining for tubulin as described previously (26). The slides were stable at 4°C for at least one week.

**Rhodamine tubulin assay.** A mixture containing 2 µM rhodamine-labeled and 4 µM unlabeled PCC tubulin in JPM buffer, 2 mM GTP and axonemes in JPM buffer was placed in a 1.5-µL microcentrifuge tube and put in a 37°C water bath for 20 min to induce microtubule assembly as described by Belmont and Mitchison (2). No glycerol or DMSO was present in the reaction mixture. After 20 min, a 2.0-µL sample from the microtubule assembly mixture was gently pipetted into 35 µL of JPM containing 60% glycerol to stabilize microtubules. A 2.5-µL sample from the glycerol solution was placed on a slide and visualized with a Silicon Intensified Target (SIT) 66 Camera (DAGE MTI, Michigan City, IN, USA) connected to a fluorescence microscope. The concentration of axonemes was adjusted so that there were approximately 1–2 axonemes per 25 µm².

**Microscope and Image Acquisition**

Axoneme-nucleated microtubules were observed using a Model IM-35 Inverted Microscope (Carl Zeiss, Thornwood, NY, USA) with a 40×1.3 NA objective lens (Nikon, Melville, NY, USA) or a 63×1.4 NA objective lens (Carl Zeiss). Images were acquired using a SIT camera connected to Macintosh® Quadra® Computer (Apple Computer, Cupertino, CA, USA) equipped with a PixelTools NuBus video board (Percepts, Knoxville, TN, USA) and running Oncor Image™ Imaging Software (Oncor, Gaithersburg, MD, USA). Fields were selected at random, and the unprocessed image was integrated (100 frames) and saved for analysis. Images were stored using an optical drive.

**Quantification and Analysis of Individual Microtubule Lengths**

Individual axoneme-nucleated microtubules were measured from the saved images using Oncor Image software, and pixel values were converted to microns for analysis. The number of microtubules per axoneme was also determined from the images. In the experiments reported here, microtubule growth from both ends of the axoneme was pooled, so information about the differential effects of molecules on the two ends of the microtubule was not obtained. However, data from the two ends could be analyzed separately using axonemes in which the plus and minus ends can be morphologically distinguished. Axonemes from *Chlamydomonas* are frayed at the distal (plus) ends, and these could be used in the assays. *Tetrahymena* axonemes also fray at the plus end, but not every plus can be identified when examined using fluorescence microscopy. The difference in the rates of microtubule growth at the two ends of axonemes has also been used to distinguish the plus from the minus end, but this method has the disadvantage that an unknown factor might alter the elongation rate and result in errors in assigning the end as plus or minus (23). The data were imported into Microsoft® Excel®.
Immunofluorescence Assay

To identify novel factors that modulate microtubule dynamic instability, we used two in vitro assembly assays. In the immunofluorescence assay, axonemes were allowed to adhere to a cleaned coverslip for 10 min in a humid chamber. The coverslips were then rinsed twice in phosphate-buffered saline (PBS) containing 0.1% Tween® 20 and 0.02% azide. Tubulin in PM buffer containing GTP was then added to the coverslip, and the humid chamber was placed on a slide warmer at 30°C to initiate microtubule polymerization from the axoneme seeds. The tubulin concentration was adjusted to 12 μM; at this concentration, spontaneous nucleation was not observed. The polymerization reaction was stopped at various time points by fixation in ice-cold methanol. The microtubules were visualized by immunofluorescence using a tubulin antibody (DM1A) and Cy3-conjugated secondary antibodies. Images of the axoneme-nucleated microtubules were acquired using a SIT camera; it should be noted, however, that the microtubules are sufficiently bright to be recorded using conventional photography. The tubulin antibody (DM1A) stained both the individual microtubules and axonemal nucleating structures (Figure 1A).

At each time point, length measurements were made for each individual microtubule nucleated from an axoneme, and average microtubule lengths for each time point (and treatment, see below) were calculated (Figure 1B). When 130 μg/mL of brain MAPs (20) were added to the reaction, a 3-fold increase in average length was observed at 30 min (Figure 1). Addition of 500 nM vinblastine to the reaction induced a 75% decrease in average length at 60 min, while 50 nM vinblastine induced a nearly 50% decrease in average length at 30 min. Furthermore, the number of microtubules nucleated per axoneme was decreased in the presence of vinblastine and increased in the presence of MAPs (Figure 1C). The decrease and increase in the number of microtubules nucleated per axoneme in the presence of vinblastine or MAPs, respectively, were statistically different ($P = 0.001$) from the number of microtubules nucleated per axoneme in controls. Thus, this nucleated assembly assay can detect factors that increase or decrease average microtubule length and number of microtubules per axoneme.

Figure 1. Immunofluorescence assay. (A) Integrated SIT images of methanol-fixed microtubule polymerization reactions visualized by immunocytochemistry. A control polymerization reaction and reactions containing 13 μg/mL of brain MAPs or 50 nM vinblastine are shown. (B) Microtubule polymerization reactions were incubated at 30°C for various times, and average microtubule length was determined for control (diamond; $n = 160$), 50 nM vinblastine (X; $n = 85$), 500 nM vinblastine (square; $n = 62$) and 130 μg/mL brain MAPs (triangle; $n = 154$). Error bars are similar for all these reactions but are shown for only one for clarity in presentation. (C) Average number of microtubules per axoneme was measured for control, 50 nM vinblastine and 130 μg/mL brain MAPs. Bar in Panel A = 5 μm.
Rhodamine Assay

As an alternative to immunofluorescence visualization of nucleated microtubules, we added rhodamine-labeled tubulin to assembly reactions and visualized the resulting microtubules by low-light-level fluorescence microscopy as previously described by Belmont and Mitchison (2). This approach was used to screen fractions of a calf thymus extract for microtubule modulating factors and resulted in the identification of the catastrophe promoter Op18. Reaction mixtures were incubated for 20 min, at which point steady-state microtubule growth was achieved. In these reactions, the rhodamine tubulin also labeled the nucleating structures (Figure 2A), presumably by non-specifically adhering to the axoneme surface. Length measurements were made for each individual microtubule nucleated from an axoneme as in the immunofluorescence assay. Histograms of the distribution of microtubule lengths in the various reactions are shown in Figure 2 (B–D). The data show that the distribution of microtubule length is dramatically altered by vinblastine and brain MAPs. Average microtubule lengths (Figure 3A), total polymer per axoneme (Figure 3B) and microtubule nucleation per axoneme (Figure 3C) were calculated and plotted. In the vinblastine-treated reactions, the average microtubule length was less than half that of the controls, while addition of brain MAPs resulted in an approximately 2-fold increase in average length. Total polymer calculations showed that treatment with brain MAPs resulted in a 3-fold increase in total polymer, while vinblastine treatment showed a dramatic 87.5% decrease in total polymer. Moreover, brain MAP-treated reactions had twice as many microtubules per axoneme as the controls, while vinblastine-treated reactions had less than half the number of microtubules per axoneme. The results of these experiments demonstrate that both assays can be used to examine the effects of known modulators on microtubule assembly; quantitatively, the effects of both vinblastine and MAPs were very similar in the two assays.

DISCUSSION

We describe two functional assays that can be used to screen cellular extracts for modulators of nucleated microtubule assembly in vitro. With both assays, the total polymer, average length and number of microtubules nucleated per axoneme can be easily determined. Using brain MAPs and vinblastine, we show that both assays can detect factors that increase or decrease net microtubule assembly in
vitro. Furthermore, these assays have the ability to distinguish between factors that might differentially affect total polymer, average microtubule length and microtubules per axoneme.

Comparison of the two assays shows that each has unique advantages and disadvantages. The immunofluorescence assay provides better image quality, and the preparations are stable for at least one week after preparation. Moreover, image visualization does not require a low-light-level video camera; a standard fluorescence microscope and a 35-mm camera can be used. In the rhodamine tubulin assay, however, smaller volumes are used, an important factor when fractionating small quantities of cytoplasm. It might be possible to modify the immunofluorescence assay, for example, by using fragments of coverslips or 96-well plates to reduce the amount of sample needed. The rhodamine tubulin assay is performed in plastic tubes, so nonspecific sticking of proteins to the coverslip does not occur (18); in the immunofluorescence assay, the coverslips can be coated with casein to prevent nonspecific adsorption of protein to the slide (23). Finally, the rhodamine assay is very rapid because the immunofluorescence staining steps are eliminated. A hybrid method in which the microtubules are polymerized in solution and then placed on coverslips and stained with antibodies to tubulin is also possible.

The changes in net microtubule assembly following addition of MAPs and vinblastine were very similar in both assays. For example, in the presence of MAPs, microtubule assembly was stimulated 3-fold in the immunofluorescence assay and 2-fold in the rhodamine tubulin assay. It should be noted, however, that the absolute values for the microtubule lengths varied between the two assays. In the rhodamine tubulin assay, average microtubule length for controls was 5.4 µM, while in the immunofluorescence assay, average microtubule length was 9.7 µM. One possible explanation for this difference is that some breakage of microtubules might occur during pipetting in the rhodamine tubulin assay, reducing the average microtubule length. Moreover, the addition of rhodamine tubulin has been shown to perturb microtubule dynamic behavior, specifically by increasing the frequency of catastrophe transitions (1), which would further reduce microtubule average length. However, the changes in microtubule behavior in the presence of MAPs and vinblastine were qualitatively similar with both assays.

The strength of these two assays lies in their ability to rapidly identify factors that modulate microtubule dynamic behavior. While techniques such as microtubule co-sedimentation and microtubule affinity columns can identify factors that bind to microtubules, they cannot detect factors that might alter dynamics without binding to microtubule polymer. For example, recent work suggests that molecules that modulate microtubule dynamics can bind to microtubule ends or to tubulin dimers. For example, low concentrations of vinblastine markedly suppress microtubule dynamic turnover by interaction with microtubule ends, and Op18/stathmin increases the frequency of catastrophe transitions through an interaction with tubulin dimers (2,5).

Figure 2. Rhodamine assay. (A) Integrated SIT images of control polymerization reactions and reactions treated with 130 µg/mL of brain MAPs and 50 nM vinblastine. (B–D) Histograms of the distribution of microtubule lengths for control reactions (B) and reactions containing 130 µg/mL brain MAPs (C) and 50 nM vinblastine (D). Bar in Panel A = 5 µm.
assays described here can detect these molecules by their effect on microtubule assembly; such molecules might be difficult or impossible to detect in microtubule binding assays. It is important to note that when crude cell fractions are tested, it is necessary to account for the tubulin present in the lysate that is added to the assay. This can be done using quantitative immunoblotting (8,9). Finally, a disadvantage of these assays is that the effects of a given molecule on the parameters of individual microtubule dynamic behavior cannot be determined directly. For example, suppression of microtubule assembly in the assay could result from a decrease in the rate of elongation, an increase in the frequency of catastrophe transitions, a decrease in the frequency of rescue transitions, or some combination of these. However, once an effect on net microtubule assembly is detected, then tracking of individual microtubules in real time can be used to determine which parameters of dynamic instability are modulated. In conclusion, we describe methods to rapidly and quantitatively monitor microtubule behavior in vitro. These assays are useful screens for novel molecules that modulate microtubule dynamic behavior.

REFERENCES


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