Isolation of Microtubules and Microtubule Proteins

J. Avila,[1] H. Soares,[2] M.L. Fanarraga,[3] and J.C. Zabala[3]

[1]Centro de Biología Molecular (CSIC). Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain
[2]Instituto Gulbenkian de Ciência, 2781-901, Oeiras, and Escola Superior de Tecnologia da Saúde de Lisboa, Lisboa, Portugal
[3]Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria-IFIMAV, Santander, Spain

ABSTRACT

This unit describes various protocols for the isolation and purification of the main constituents of microtubules, chiefly α- and β-tubulin, and the most significant microtubule associated proteins (MAPs), specifically MAP1A, MAP1B, MAP2, and tau. We include a classical isolation method for soluble tubulin heterodimer as the first basic purification protocol. In addition, we show how to analyze the tubulin and MAPs obtained after a phosphocellulose chromatography purification procedure. This unit also details a powerful and simple method to determine the native state of the purified tubulin based on one-dimensional electrophoresis under nondenaturing conditions (*UNIT 6.5*). The last protocol describes the application of a new technique that allows visualizing the quality of polymerized microtubules based on atomic force microscopy (AFM). *Curr. Protoc. Cell Biol.* 39:3.29.1-3.29.28. © 2008 by John Wiley & Sons, Inc.

Keywords: tubulin • microtubule • MAP1A • MAP1B • MAP2 • tau • native gel electrophoresis • atomic force microscopy

INTRODUCTION

The different morphologies displayed by cells from different tissues—for example the great diversity of neurons—is the result of the assembly of different cell scaffolds.

There are three different components in the cell scaffold known as the cytoskeleton. These are microtubules (Fig. 3.29.1), microfilaments, and intermediate filaments. Microtubules are particularly abundant in brain tissue and their components represent ∼20% of the total protein content from soluble brain extract, with α- and β-tubulin heterodimers the major components.

Microtubules are tubular cytoplasmic fibers with a diameter of 24 nm (Fig. 3.29.2). In addition to their role in the determination of cell shape they also play fundamental roles in other common cellular functions, such as intracellular transport, chromosome segregation (a specific form of transport), and flagella/cilia-mediated cell motility. In neurons, besides their function in determining neuron shape, microtubules seem to play essential roles in the formation and maturation of the axon and dendrites. The majority, if not all, of the microtubule functions are based on the capacity of these polymers to polymerize/depolymerize. The capacity of microtubules for assembly-disassembly was mimicked in vitro in the pioneer work of Weisenberg (1972), who reproduced conditions for the assembly of these polymers using a brain cell extract. In this work it was established that microtubule polymerization depends on the amount of tubulin and that, at least in vitro, a critical concentration of this protein is needed for polymerization into microtubules. Since there is a large amount of tubulin in a brain cell extract, it is possible

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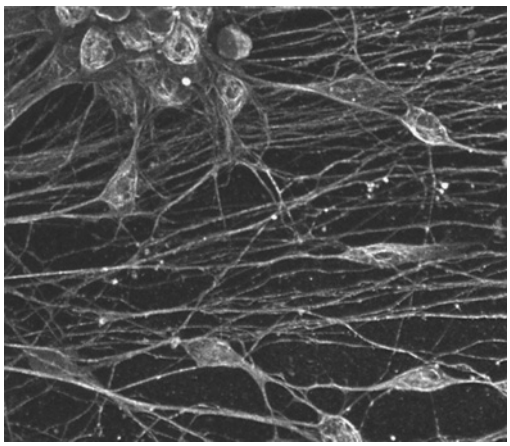

Figure 3.29.1 Microtubule networks of cerebellar cultured granule cells, as visualized by immunofluorescence microscopy using a specific antibody against the α-tubulin subunit (DM1A).

Figure 3.29.2 Electron micrograph of brain microtubules assembled after a single polymerization cycle. Scale bar represents 1 μm. For color version of this figure see *http://www.currentprotocols.com.*

to induce microtubule assembly in vitro. In addition, Weisenberg also found that the presence of GTP and the absence of calcium were both needed to properly carry out microtubule assembly. These observations served as the basis of various methods for microtubule protein purification (Borisy and Olmsted, 1972; Shelanski et al., 1973). At that time it was also found that microtubules are induced to assemble at warm temperatures. In contrast to tubulins from Antarctic fishes that polymerize at low temperatures, mammalian microtubules are not cold-stable and depolymerize very fast at 4°C. The observed temperature dependence seems to reside in differences in the polymerization energetics and kinetics of specific tubulin polypeptides. Thus, warming a cytosolic brain extract promotes microtubule polymerization, and the microtubules can be easily collected by centrifugation. But, because in vitro–polymerized mammalian microtubules depolymerize in the cold, after centrifugation, collected microtubules can be depolymerized using a cold buffer. After depolymerization, the solution can again be centrifuged to remove contaminants and those microtubule fragments that are not competent for depolymerization.

This sequence of warm-polymerization and cold-depolymerization can be repeated two or more times until the desired degree of purity of the microtubules is achieved.

The quality and quantity of tubulins and proteins associated with microtubules (MAPs) obtained can be followed by SDS-PAGE analysis (*UNIT 6.1*). From this analysis it is possible to observe the main microtubule components—two polypeptides or subunits, α- and β-tubulin (for reviews see: Vallee et al., 1986; Matus, 1988; Avila, 1990). MAPs are classified in three major families. The first protein family comprises the MAP1 polypeptides: MAP1A, MAP1B, and MAP1C (or dynein, a motor protein). The second family is composed of four structurally related proteins derived from a single gene: MAP2A, MAP2B, MAP2C, and MAP2D. The third family, present in a lower amount, is composed of several related polypeptides (tau proteins), or isoforms expressed from a unique gene. All these MAPs preferentially bind to the C-terminus of tubulins (Serrano et al., 1984a,b, 1986).

In this unit, we describe protocols for the purification of tubulins (Basic Protocols 1 and 3 and Alternate Protocol 2), MAP1 (Basic Protocol 4), MAP2 (Basic Protocol 5), and tau proteins (Basic Protocol 6), but not for other MAPs or motor proteins (such as dynein). We also describe how to assemble microtubules in vitro in the presence of paclitaxel (Taxol; Basic Protocol 2 and Alternate Protocol 1) and protocols for native gel electrophoresis (Support Protocol 1) and atomic force microscopy (AFM; Support Protocol 2).

ISOLATION OF MICROTUBULE PROTEIN BY CYCLES OF POLYMERIZATION AND DEPOLYMERIZATION

BASIC PROTOCOL 1

Many similar microtubule protein purification methods have been developed, published, and used for the purification of microtubule proteins (Shelanski et al., 1973; Williams and Lee, 1982; Vallee, 1986a). Most of these are based on the reversible assembly capacity of microtubule proteins in the presence or in the absence of assembly-promoting agents, such as glycerol or dimethyl sulfoxide (DMSO). Here we describe the isolation of brain microtubule proteins by temperature-dependent cycles of polymerization-depolymerization using glycerol and DMSO in the first and second polymerization cycles, respectively (Fig. 3.29.3). Figure 3.29.3 illustrates the results of the step-by-step protocol that we follow in our laboratories. This method can be applied to brain tissues from different sources, i.e., porcine, bovine, rat, etc. The protocol is appropriate for a medium-scale preparation (1 to 3 cow or pig brains).

Materials

One to three brains (100 to 500 g total start-up tissue; the weight of a pig brain is ~100 g)
Phosphate-buffered saline, pH 6.7 (PBS; *APPENDIX 2A*), 4°C
Isotonic buffer (see recipe), 4°C
Protease inhibitors (see recipe)
Buffer A (see recipe), 4°C
Glycerol
PMSF
GTP (see recipe)
Dimethyl sulfoxide (DMSO)

Plastic wrap
Ice-water bath
No.10 scalpel blade
500-ml beaker
Potter homogenizer (Teflon-in-glass homogenizer), as large as possible
Centrifuge (if possible two centrifuges, one at 4° to 6°C and the other at 25° to 30°C): Sorvall RC-5B or equivalent Beckman J2, J21 series, or Avanti J-25

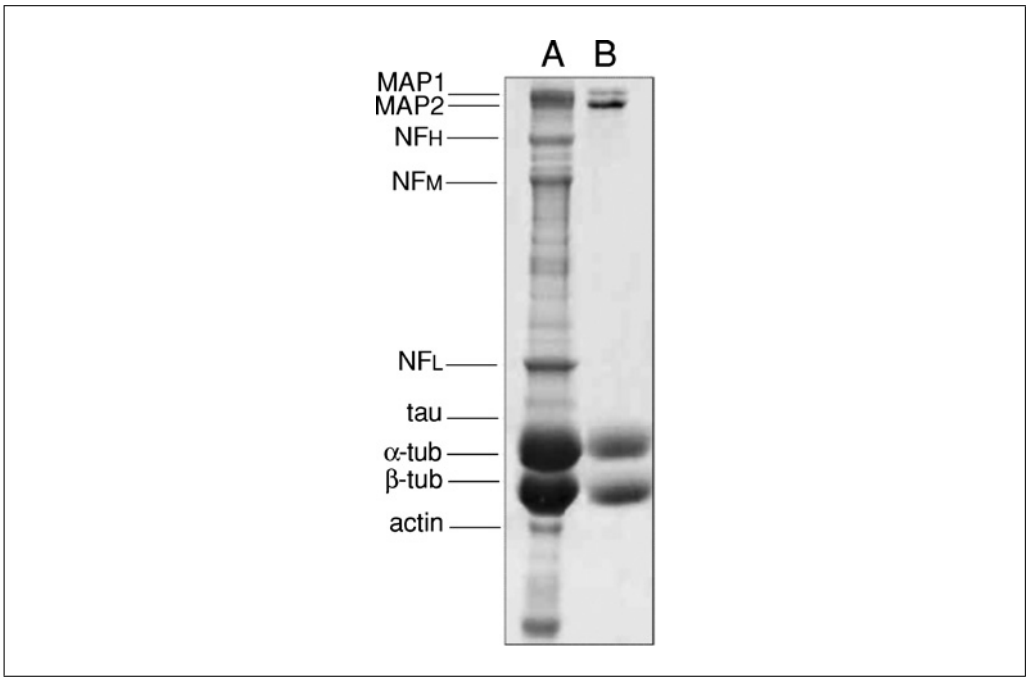

Figure 3.29.3 Characterization of pelleted protein after one (**A**), and three polymerization cycles. (**B**) In (A), the presence of MAP1, MAP2, neurofilament proteins (NF_H, NF_M and NF_L), tau protein, tubulin subunits, and actin are indicated. The gel was stained with Coomassie blue as explained in *UNIT 6.6.*

Centrifuge rotors: GSA or JA-10; type 50.2Ti and 70.1 Ti for the Beckman
Graduated flask
26.3-ml ultracentrifuge tubes
35°C rocking incubator
Ultracentrifuge Beckman L8-70, Optima XL-100 or 120

Additional reagents and equipment for measuring protein concentration (*APPENDIX 3B*)

Obtain the brains

1. Make an appointment with veterinarians at the slaughterhouse and request fresh brains to be picked up immediately after animal slaughter.
2. Wrap brains carefully with plastic before introducing in the ice-water bath. Place brains on ice during transport to the laboratory (<1 hr in our case). After arriving at the laboratory start the preparation of the cytosolic extracts (immediately).

Prepare brains

3. Rinse brains well, three times, each time with 100 ml cold PBS buffer and keep them cold throughout the following steps until the first polymerization cycle.
4. Remove the meninges, the blood clots, and most of the peripheral blood vessels from the brain.
5. Chop the brains into smaller pieces (1- to 2-cm) using a no. 10 scalpel blade.
6. Weigh the tissue in a tared 500-ml cold beaker.

Homogenize the brain tissue

7. Add 0.75 to 1 ml of isotonic buffer per gram of tissue.

   The use of the isotonic buffer for tissue homogenization facilitates the removal of neurofilaments in the following microtubule isolation process.

8. Add PMSF to a final concentration of 1 mM. Add aprotinin, leupeptin, and pepstatin to a final concentration of 1 μM each (see recipe for protease inhibitors in Reagents and Solutions).

9. Disrupt brain tissues at 4°C by using a Potter homogenizer, which allows a fast and reproducible homogenization.

   Use the largest Potter you have and homogenize as many times as required depending on the total volume (see Critical Parameters).

10. Clear the crude brain homogenate by centrifuging 30 min at 25,000 × *g*, 4°C.

    If necessary, since the volume is too high, a rotor type GSA (Dupont-Sorvall) or equivalent JA-10 in a Beckman J2-21, or Avanti J-25 centrifuge can be used.

11. Decant the supernatant in a cold 500-ml beaker and, if necessary for further clarification, centrifuge again for 30 to 45 min at 25,000 × *g*, 4°C.

12. Discard the pellet and take the supernatant. Take a small aliquot for analysis in SDS-PAGE (*UNIT 6.1*), native electrophoresis (Support Protocol 1), and for protein determination (*APPENDIX 3B*).

    From an initial 100 g we usually get 150 ml at 40 mg/ml at this point, and, of this amount, ~20% represents microtubule proteins.

13. Measure the total volume of the cytosolic extract in a graduated flask and make it 1× buffer A by adding for each 90 ml of extract, 10 ml of 10× buffer A. Add glycerol to a final concentration of 25%(v/v) to 30%(v/v) (Krauhs et al., 1981).

    Be aware that some commercial glycerol preparations have a concentration of 87%.

14. Add PMSF to a final concentration of 1 mM to prevent protein degradation by nonspecific proteolysis. Finally, add 1 M GTP (1000×) to a final concentration of 1 mM.

Perform first polymerization

15. Transfer the solution to 26.3-ml ultracentrifuge tubes and balance them to ±0.01 g. Incubate solution for 30 min at 35°C with gentle agitation.

16. Ultracentrifuge the mixture 45 min at 100,000 × *g*, 25°C, using a prewarmed (30°C) rotor.

    We use the 50.2 Ti rotor in a Beckman centrifuge.

17. Discard the supernatant and take the pellet, which should be translucent.

    If necessary, pellets can be frozen at −80°C at this point without much loss of function of the polymerized tubulin. We usually get 300 to 400 mg protein at this point, determined by Lowry's method.

Perform first depolymerization

18. Resuspend the pellet in cold buffer A at 10 to 20 mg/ml. Let the mixture to stand in an ice-water bath for 30 min.

    Intermittent homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate microtubule depolymerization.

19. Centrifuge 15 to 20 min at 25,000 × *g*, 4°C using a precooled rotor Beckman 50.2 Ti. Discard the pellet and keep the supernatant.

    The pellet at this point is enriched in the so-called cold-resistant microtubule fraction and denatured protein. It is not advisable to centrifuge at very high speed, to avoid loss of "competent" microtubule proteins.

20. Add PMSF and GTP to the supernatant to a final concentration of 1 mM (add 1/1000 volume of 1 M GTP). Add DMSO to a final 10% concentration.

CAUTION: DMSO is hazardous: see APPENDIX 2A for guidelines on handling, storage, and disposal.

Perform second polymerization

21. Transfer the solution to 26.3-ml ultracentrifuge tubes and balance them to within ±0.01 g. Incubate the tubes 30 min at 35°C with gentle agitation.

22. Collect the microtubules polymerized in the second cycle by ultracentrifuging 40 min at 180,000 × *g* (Beckman 70.1 Ti at 50,000 rpm), 25° to 30°C.

If at this point there is only one rotor available, wash the rotor with hot water to rapidly reach the appropriate temperature.

23. Discard the supernatant. To finish the second cycle resuspend the pellet in 6 ml of cold 0.5× buffer A. Let the mixture stand in an ice-water bath for 30 min.

The pelleted protein can be frozen in liquid nitrogen or at −80°C for further use. We usually get 60 to 80 mg protein at this point.

Occasional homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate depolymerization of microtubules.

24. Centrifuge 20 min at 25,000 × *g*, 4°C, using a precooled rotor. Discard the pellet. Measure protein concentration in the supernatant by using a spectrophotometer absorbance at 280 nm and 260 nm. (*APPENDIX 3B*)

The extinction coefficient of tubulin at 280 nm is 115,000 $M^{-1}cm^{-1}$ [1.15 $(mg/ml)^{-1}$ cm^{-1}]. This extinction coefficient is calculated from tubulin sequences and includes the contribution of the two bound guanine nucleotides to the absorbance at 280 nm. For this reason, we use the formula $A_{280}/1.15$ = mg/ml of the sample. Also, this formula might be improved if we take into account the contribution of nucleotides to the absorbance at 260 nm: $1.45 \times A_{280} - 0.74 \times A_{260}$ = mg/ml of tubulin. The isolated tubulin competent to polymerize can be stored at −80°C for several months or until further steps.

BASIC PROTOCOL 2

ISOLATION OF MICROTUBULE PROTEIN USING PACLITAXEL (TAXOL)

An alternative single-step method to isolate polymerized microtubule proteins is based on the fact that microtubules assemble and remain stable in the presence of paclitaxel (Shiff et al., 1979). This allows one to sediment them and to resuspend them in a cold buffer without depolymerization (Borisy and Olmsted, 1972). Moreover, an advantage of this procedure is the fact that MAPs can be easily dissociated from these stable microtubules by exposing them to an elevated ionic strength. Additionally, the use of paclitaxel permits the purification of tubulin from sources where the amount of this protein is low.

Materials

Bovine or porcine brain tissue (10 g)
Phosphate-buffered saline (*APPENDIX 2A*)
Buffer A (see recipe)
Protease inhibitors (see recipe)
Paclitaxel (Taxol; see recipe)
GTP (see recipe)
PMSF
Sucrose underlayer solution (1 ml per centrifuge tube containing 10% sucrose, 10 μM paclitaxel, and 0.5 mM GTP)
Scalpel blade, No.10
500-ml beaker

Potter homogenizer (Teflon-in-glass homogenizer)
Centrifuge rotors: GSA or JA-10. Type 50.2Ti and 70.1 Ti for the Beckman Ultracentrifuge Beckman L8-70, Optima XL-100 or 120
13.5-ml ultracentrifuge tubes
30°C rocking incubator
Pasteur pipet

Prepare cytosolic extract

1. Obtain fresh brains from the slaughterhouse as described in Basic Protocol 1.
2. Place and keep brains on ice until arrival at the laboratory, as described in Basic Protocol 1.

   Although we use fragments of any region of porcine brain, whole brains from other sources can also be used.

3. Rinse brains well, three times, each time with 100 ml cold PBS buffer and keep them cold throughout the following steps until the first polymerization cycle.
4. Remove the meninges, the blood clots, and most of the peripheral blood vessels from the brain.
5. Chop the brains into smaller pieces (1 to 2 cm) using a scalpel blade, no. 10. Weigh the tissue in a tared 500-ml cold beaker.
6. Add 15 ml buffer A (1.5 ml per g of tissue).
7. Disrupt brain tissues at 4°C by using a Potter homogenizer.

   Follow recommendations in Basic Protocol 1.

8. Add freshly prepared PMSF to a final concentration of 1 mM in DMSO. Also add the following protease inhibitors to a final concentration of 1 μM: aprotinin, leupeptin and pepstatin (see recipe for protease inhibitors in Reagents and Solutions).
9. Clear the crude brain homogenate by ultracentrifuging 40 min at 100,000 × *g*, 4°C. Use a precooled rotor (Beckman 50.2 Ti or similar).

Polymerize microtubules

10. Discard the pellet and add paclitaxel to the supernatant to a final concentration of 20 μM, GTP to a final concentration of 1 mM, and PMSF to a final concentration of 1 mM.
11. Transfer the solution to 13.5-ml ultracentrifuge tubes and balance them to within ±0.01 g. Incubate at 30 min at 30°C with gentle agitation.
12. With a Pasteur pipet introduce 1 ml of a 30°C prewarmed sucrose solution very gently at the bottom of the tube.
13. Collect the assembled microtubules in the polymerization cycle by ultracentrifuging 30 min at 180,000 × *g* (prewarmed rotor Beckman 70.1 Ti), 25° to 30°C.
14. Discard the supernatant and keep the translucent pellet. Resuspend pellet in 3 ml buffer A containing 20 μM paclitaxel and 1 mM GTP.

    These microtubule preparations, containing assembled microtubules, can be used as a source for isolating MAPs, such as MAP1B (see below) or they can be stored in the refrigerator (4°C). You must be aware that storage for >1 week will enrich the preparation in unpolymerized tubulin. To store samples for longer periods keep the preparation at −80°C.

ALTERNATE PROTOCOL 1

MICROTUBULE POLYMERIZATION IN THE PRESENCE OF PACLITAXEL

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Microtubule proteins (tubulin and MAPs) are prepared by repeated cycles of assembly/disassembly using a temperature-dependent procedure as described in Basic Protocol 1. Purified microtubule heterodimers are incubated 30 to 40 min with 20 to 25 μM paclitaxel (taxol) at 35°C in BRB80 buffer (see recipe) containing 1 mM GTP and 30% glycerol. The pellet corresponding to the polymerized microtubules is obtained by centrifuging 30 min at 45,000 × *g*, 30°C through a 50% sucrose cushion containing 1 mM GTP.

BASIC PROTOCOL 3

ISOLATION OF TUBULIN DIMERS FROM MICROTUBULE PROTEINS

Tubulin contains very acidic regions, mainly in its C-terminal domain (Krauhs et al., 1981), which allows its fractionation from many other proteins by ion-exchange chromatography (anionic or cationic). Two types of resins have been successfully used to purify tubulin: cationic resins such as DEAE-cellulose (or DEAE-Sephadex) and anionic resins like phosphocellulose. However, it is possible to use any other type of ion-exchange resins such as Mono-Q or Mono-S from Pharmacia, or HiTrap columns. Nevertheless, all these chromatographic columns show advantages and disadvantages. For example, while the use of DEAE-cellulose is a slow procedure, the use of phosphocellulose columns might result in a partial inactivation of tubulin due to magnesium ion chelation (Williams and Detrich, 1979). Likewise, highly purified tubulin can be obtained from cationic columns, but the fact that tubulin binds to these resins requires the use of a salt gradient to elute the protein, which might result in tubulin denaturation. Therefore, tubulin elution from these columns requires a further step of salt removal by dialysis or ultrafiltration. In this protocol we describe the classical procedure to purify tubulin using phosphocellulose chromatography. This method has been chosen because it is the fastest and does not need the additional dialysis step that could also result in a partial denaturation of tubulin.

Materials

Resin: Whatman P11 Cellulose Phosphate
0.5 M NaOH
0.5 M HCl
Buffer A (see recipe)
50 mg of porcine brain microtubules stored at −80°C or in liquid nitrogen (Basic Protocol 1)

Buchner funnel
XK50/20 (Length 20 cm, i.d. 50 mm; Pharmacia) column including:
  Thermostat jacket
  Flow adaptor
  Flanged tubing at both ends for direct connection to valves
  Pumps
  UV monitors if using an FPLC or an AKTA system
Teflon-in-glass homogenizer
Ice-water bath
Ultracentrifuge

Additional reagents and equipment for isolating microtubule proteins (Basic Protocol 1), determining protein concentration (*APPENDIX 3B*), SDS-PAGE (*UNIT 6.1*), and nondenaturing gel electrophoresis (Support Protocol 1)

Precycle the ion exchanger

1. Take 100 g of resin and stir into 1.5 liter of 0.5 M NaOH for 30 min at room temperature. Filter the resin in a Buchner funnel and wash with water several times.

   Alternatively, stir the resin in a beaker and let it settle by gravity for a few minutes and decant the supernatant.

2. After the wash, treat the resin with 1.5 liter of 0.5 M HCl as described above. Filter or decant and wash several times with water.
3. Repeat this procedure until a neutral pH is attained.
4. Next, filter and stir the resin in 0.5× buffer A.
5. Add the resin to an XK 50 column (~150 ml) and equilibrate with 0.5× buffer A until the absorbance at 280 nm returns to baseline.

Prepare sample

6. Collect microtubule proteins (10 mg/ml), isolated following Basic Protocol 1, by depolymerization of microtubules preparing a homogenate in cold 0.5× buffer A with the help of a Teflon-in-glass homogenizer.
7. Leave homogenized sample in ice-water for 30 min.

   Intermittent homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate microtubule depolymerization.

8. Ultracentrifuge the homogenate 15 min at 25,000 × *g*, 4°C, using a precooled rotor. Discard the pellet and keep the supernatant.

   We currently use 50.2 Ti or 70.1 Ti rotors in a Beckman centrifuge in our laboratory.

9. Adjust the final protein concentration of the supernatant to ~10 mg/ml and load it onto a prepacked XK 50 phosphocellulose column (1 ml column/mg protein) equilibrated in 0.5× buffer A.

   The first time you use the resin you must take into account that you will probably lose some tubulin. This is due to the fact that some will irreversibly bind to the phosphocellulose. This could be overcome or minimized by previously loading in the column with 1 g of BSA diluted in the same buffer, followed by several washes with the salt-containing buffer before re-equilibration.

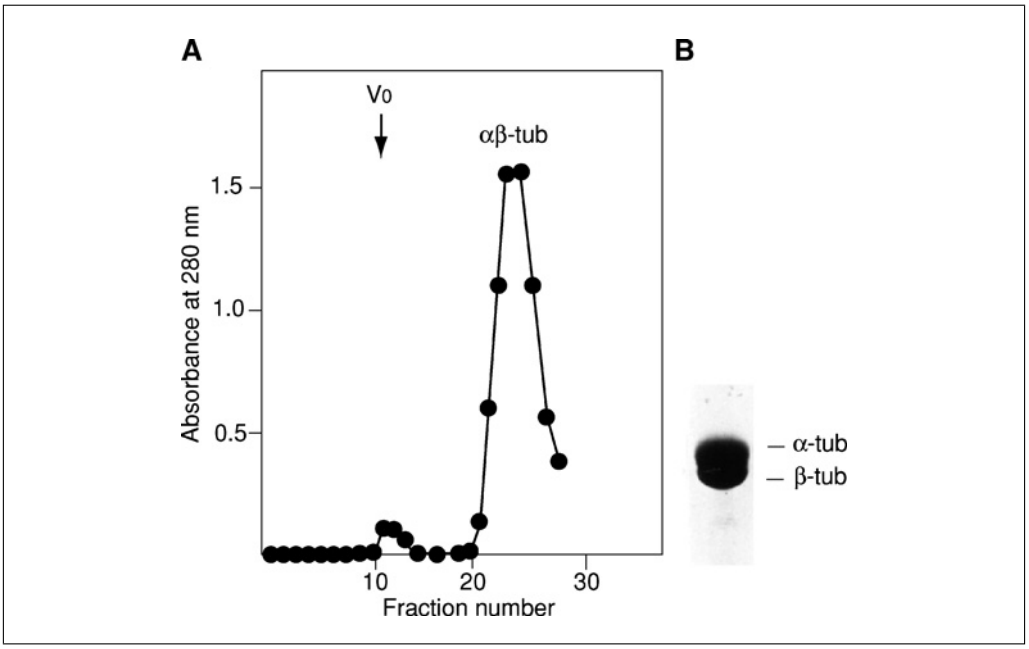

Figure 3.29.4 (**A**) Chromatographic elution profile obtained at 280 nm of absorbance from a gel filtration analysis of phosphocellulose-purified tubulin chromatographed through a Sepharose 4B column. (**B**) Denaturing SDS gel (8.5% polyacrylamide) analysis of the peak corresponding to a molecular mass of 110 kDa eluted from the column. The gel was stained with Coomassie blue as explained in *UNIT 6.6.* Abbreviations: V_o, void volume.

10. Collect 0.5-ml fractions from the column.

    Tubulin, which will be present in the first fractions after the void volume of the column, can be detected by measuring absorbance at 280 nm (Fig. 3.29.4).

11. Determine protein concentration by Bradford, Lowry, or BCA, and measure the absorbance at 280/260 as described in *APPENDIX 3B*.

12. To finish, analyze the quality of purified tubulin by SDS-PAGE (*UNIT 6.1*) and by nondenaturing gel electrophoresis (Support Protocol 1).

13. Add to the tubulin-containing fractions the amount of 10× buffer A to obtain 1× buffer A, and GTP to a final concentration of 1 mM. Freeze fractions immediately in liquid nitrogen or at −80°C.

    Other microtubule associated proteins (MAPs) can be eluted from the column using buffer A containing 2 M NaCl (Basic Protocol 2 and Alternate Protocols 4 and 5).

BASIC PROTOCOL 4

ISOLATION OF MAP1A/1B

Microtubule-associated proteins comprise a heterogeneous group of polypeptides which display different structural characteristics, are differentially expressed during development, and are localized at different sites in neurons. A good example of these differences can be found between proteins termed MAP1A and MAP1B (Bloom et al., 1984; 1985). MAP1B is localized in the developing rat brain (Tucker et al., 1989), decreasing its expression dramatically at the end of the neuronal differentiation. On the other hand, MAP1A is expressed at later developmental stages and its level of expression remains constant through adulthood. MAP1B and the protein tau are mostly axonal proteins, whereas the high-molecular-weight forms of MAP2 are mostly found in dendrites (Matus, 1988).

Different MAPs display different characteristics which have served in developing diverse strategies and schemes to purify them. For instance, MAP2 and tau proteins are heat-resistant proteins, whereas MAP1A/1B denature at high temperature. Yet, since MAP1A/1B preferentially bind to a cluster of acidic amino acids present at the carboxy-terminus of tubulin (Tucker et al., 1989; Cross et al., 1991), molecules like poly-L-aspartic acid can be employed to compete with the interaction of these MAPs with tubulin (Fujii et al., 1990). Moreover, MAP1B can be isolated from the brain of newborn rats, whereas the most appropriate source for other MAPs is the adult brain. Similarly, the differential proportion of axons/somatodendritic tissues between white and gray matters can also be used to differentially purify MAP1 (white matter) and MAP2 (gray matter), minimizing contaminations (Vallee, 1986b), while either of the two matters can be used to purify MAP1A. In the case of tau, the expression pattern of the different isoforms is clearly different between white and gray matter, and there is a dramatic decrease in the amount of two tau isoforms in gray matter (de Ancos and Avila, 1993).

MAP1B/1A are both thermolabile proteins and cannot be isolated through a heat-treatment step. Instead, the addition of poly-L-aspartic to taxol-stabilized microtubules detaches MAP1A/1B proteins from these polymers. This step is performed in a PIPES buffer where the interaction of MAP1 proteins with tubulin is weakened (Pedrotti et al., 1993). MAP1 proteins are next fractionated from taxol-polymerized microtubules by centrifugation, being further purified using an FPLC (fast protein liquid chromatography) or AKTA system (Pazzagli and Avila, 1994). This particular protocol uses adult rat or porcine brains as the source of MAP1A. MAP1B is isolated from whole newborn-rat brains. These sources were chosen taking into consideration the information described above.

Materials

White matter from a cow or ten brains from adults rats (MAP1A)
Twenty brains from newborns rats (MAP1B)

Buffer B (see recipe)
GTP
Paclitaxel (Taxol; see recipe)
Poly-L-aspartic
Buffer C (see recipe)
Buffer D (see recipe)
Buffer A (see recipe)

10.4-ml centrifuge tubes
Ice-water bath
Centrifuge
Centrifuge rotors: GSA or JA-10. Type 50.2Ti and 70.1 Ti for the Beckman
100-ml Erlenmeyer flask
37°C incubator
Ultracentrifuge Beckman L8-70, Optima XL-100 or 120
1-ml Mono-Q column (or another with similar characteristics) connected to a FPLC or AKTA system

Additional reagents and equipment for preparing and homogenizing the brains (Basic Protocol 1), FPLC (Pazzagli and Avila, 1994), and SDS-PAGE (*UNIT 6.1*)

1. Follow steps 1 to 17 of Basic Protocol 1.
2. Resuspend the pelleted protein in buffer B to a final concentration of 4 mg/ml. Transfer to 10.4-ml centrifuge tubes. Let stand 30 min in an ice-water bath.
3. Centrifuge 15 min at 20,000 × *g*, 4°C.
4. Collect the supernatant in a 100-ml Erlenmeyer flask and add GTP, paclitaxel, and poly-L-aspartic acid to final concentrations of 1 mM, 10 μM, and 50 μM, respectively. Incubate for 30 min at 37°C.
5. Ultracentrifuge at 30 min at 100,000 × *g*, 25° to 30°C.

   Prewarm the rotor.

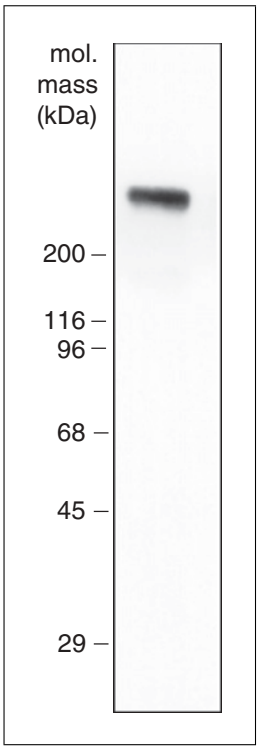

Figure 3.29.5 Characterization of the isolated MAP1A protein (Basic Protocol 4) by denaturing SDS-PAGE (7% polyacrylamide). The gel was stained with Coomassie blue as explained in *UNIT 6.6*.

6. Load the MAP1-containing supernatant (at a concentration of 0.5 mg/ml) onto a 1-ml mono-Q column and fractionate by fast protein liquid chromatography (FPLC; Pazzagli and Avila, 1994). Wash and equilibrate the column with buffer C until the A_{280} of the eluent returns to the baseline.
7. Next, elute using a linear gradient from buffer C to buffer D.
8. Characterize the protein content of the different collected fractions by SDS-PAGE (*UNIT 6.1*).
9. Select only MAP1-containing fractions (Fig. 3.29.5) and dialyze against buffer A. Store samples at −20°C or −70°C.

Be aware that usually MAP1 proteins are eluted in more than one peak.

BASIC PROTOCOL 5

ISOLATION OF HIGH-MOLECULAR-WEIGHT MAP2

The MAP2 gene gives rise to different isoforms that are developmentally regulated. For instance, those showing a higher molecular weight are the major isoforms in adult brain (Matus, 1988). As already mentioned, MAP2 is heat resistant and remains stable and fully functional after a high temperature treatment. Consequently, the inclusion of a heat-treatment step in the purification protocol facilitates the separation of MAP2 from other microtubule-associated proteins that should denature and precipitate (Herzog and Weber, 1978; Sloboda and Rosembaum, 1982). An alternative protocol for MAP2 purification has also been published (Williams and Lee, 1982).

Materials

Porcine or bovine brains or alternatively microtubule protein stored at −80°C or liquid nitrogen (Basic Protocol 1)
Buffer A (see recipe)
NaCl
2-mercaptoethanol
Ammonium sulfate
Sepharose 4B resin (GE Healthcare-Pharmacia)

Teflon-glass homogenizer
Ice-water bath
Centrifuge
Centrifuge rotors: GSA or JA-10, Type 50.2 Ti and 70.1 Ti for the Beckman
Boiling water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*)

Isolate MAP2

1. Follow steps 1 to 17 of Basic Protocol 1.
2. Depolymerize microtubules in cold buffer A using a Teflon-glass homogenizer to obtain microtubule proteins (10 to 20 mg/ml).

Intermittent homogenization of the mixture with a Teflon-in-glass homogenizer (10 strokes) will facilitate microtubule depolymerization.

3. Incubate in an ice-water bath for 30 min.
4. Centrifuge the mixture 15 min at 25,000 × *g*, 4°C.
5. Collect the supernatant and dilute to a protein concentration of 1 mg/ml with buffer A. Then, add NaCl and 2-mercaptoethanol to a final concentration of 1 M and 10 mM, respectively.
6. Heat the resulting protein solution for 5 min at 100°C in a boiling water bath.

7. Centrifuge 10 min at 10,000 × *g*, 25°C.

   A white precipitate containing denatured protein should be obtained.

8. Then, collect the supernatant in an Erlenmeyer flask and add ammonium sulfate salt to 35% (w/v) saturation. Incubate 10 min at room temperature.

   A total of 20.9 g of ammonium sulfate should be added to 100 ml of solution to obtain a 35% saturated solution.

9. Centrifuge 10 min at 10,000 × *g*, 4°C, then discard the supernatant and resuspend the pellet in 2 to 5 ml of buffer A containing 1 M NaCl.

10. Load resuspended protein onto a Sepharose 4B column equilibrated with buffer A containing 1 M NaCl.

11. Characterize the proteins present in the different collected fractions by SDS-PAGE (*UNIT 6.1;* Fig. 3.29.6).

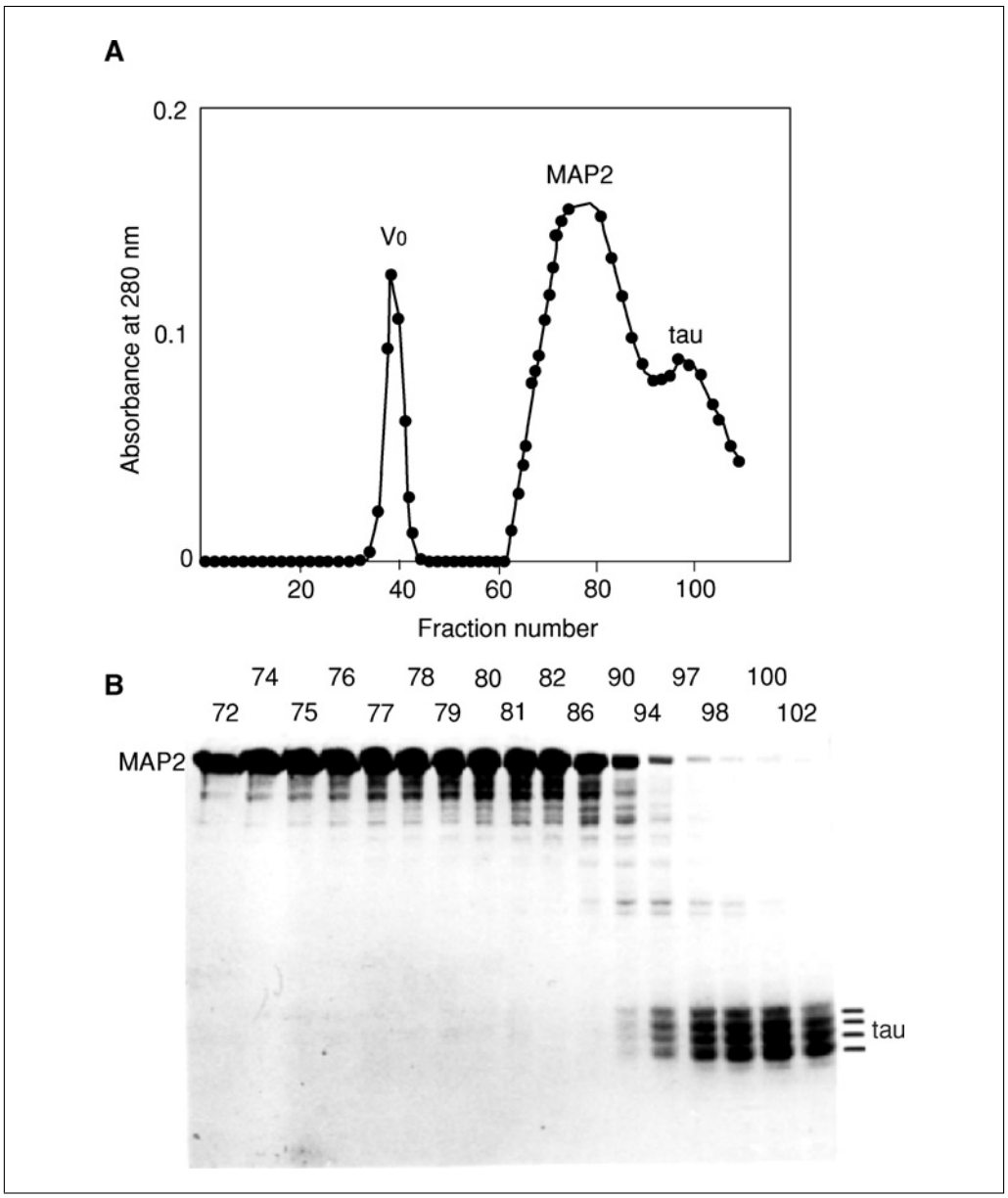

Figure 3.29.6 (**A**) Fractionation of heat-resistant MAPs by chromatography on Sepharose 4B (Basic Protocol 5). (**B**) SDS-PAGE analysis of the corresponding eluted fractions. The gel was stained with Coomassie blue as explained in *UNIT 6.6.* Abbreviations: V_o, void volume.

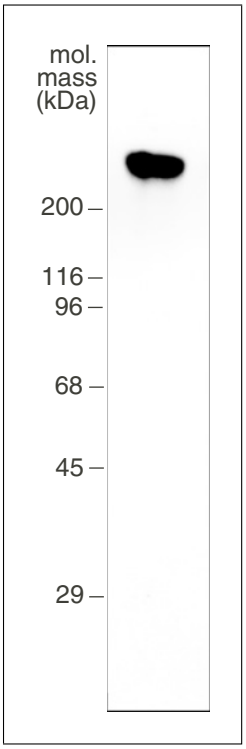

Figure 3.29.7 Characterization of the isolated high-molecular-weight MAP2 protein (Basic Protocol 5) by denaturing SDS-PAGE (7% acrylamide). The gel was stained with Coomassie blue as explained in *UNIT 6.6.*

12. Pool fractions containing MAP2 and precipitate protein by the addition of ammonium sulfate to 50% saturation. Collect the precipitated protein by centrifuging 10 min at 15,000 × *g*, room temperature.

    A total of 31.3 g of ammonium sulfate should be added to 100 ml to obtain a 50% saturated solution.

13. Add 1 ml of buffer A to resuspend the pellet and dialyze against 1 liter of the same buffer.

14. Store protein samples in a freezer at −20°C (Fig. 3.29.7).

BASIC PROTOCOL 6

ISOLATION OF TAU PROTEIN

The primary gene transcript of tau is a well-known example of alternative splicing in the mammalian brain (Himmler et al., 1989). Furthermore, tau possesses multiple phosphorylation sites that are selectively used to create polypeptides with different functions. The post-translational modifications of the various tau isoforms result in multiple protein behaviors (Lindway and Cole, 1984). For example, not all the phosphorylated tau isoforms bind to microtubules. For this reason there are two alternate procedures depending on whether we want to isolate all the isoforms, or just those that bind to microtubules (Garcia de Ancos et al., 1993).

To isolate total tau isoforms (Garcia de Ancos et al., 1993), adjust the concentration of NaCl and DTT to 1 M and 1 mM, respectively in the homogenate obtained in step 12 of Basic Protocol 1, and boil for 5 min and chill on ice. Further steps of the purification are indicated below. In this protocol we describe the isolation of microtubule binding tau isoforms. In addition to this protocol, there are other tau purification procedures that have also been described (Herzog and Weber, 1978; Sobue et al., 1981; Drubin and Kirschner, 1986; Garcia de Ancos et al., 1993).

Materials

Porcine brain
Buffer A (see recipe)
Perchloric acid
Ammonium sulfate
Glycerol

Ice-water bath
Boling water bath
Centrifuge
Centrifuge rotors: GSA or JA-10. Type 50.2Ti and 70.1 Ti for the Beckman

Isolate tau

1. Follow steps 1 to 17 of Basic Protocol 1.
2. Resuspend microtubule proteins in 25 ml of buffer A. Leave 30 min in an ice-water bath.
3. Centrifuge 15 min at 20,000 × *g*, 4°C.
4. Collect the supernatant in a 100-ml Erlenmeyer flask and correct the concentrations of NaCl and DTT to 1 M and 1 mM, respectively. Then boil samples for 5 min and immediately chill on ice for 5 min.
5. Centrifuge samples 30 min at 35,000 × *g*, 4°C.
6. Add perchloric acid to the supernatant to a 2.5% (v/v) final concentration. Keep on ice for 10 min.
7. Centrifuge 15 min at 35,000 × *g*, 4°C.
8. Recover the supernatant, collecting it in a 100-ml Erlenmeyer flask, and add ammonium sulfate up to 50% saturation. Incubate 30 min at 4°C.

   A total of 313 g of ammonium sulfate should be added to 1 liter to obtain a 50% saturated solution.

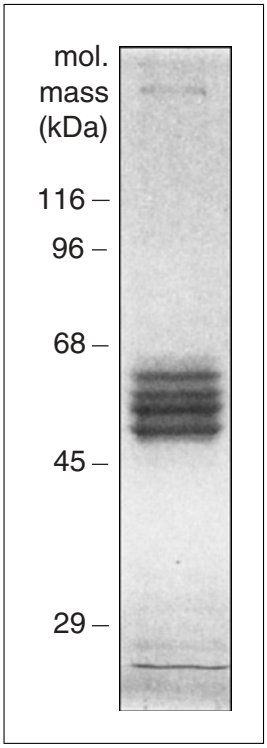

Figure 3.29.8 Characterization of different isoforms of the isolated tau protein by denaturing SDS-PAGE (10% acrylamide). The gel has been stained with Coomassie blue as explained in *UNIT 6.6.*

9. Centrifuge 15 min at 35,000 × *g*, 4°C.

10. Dissolve the pellet in buffer A (to a final concentration of 1 to 5 mg/ml), bring up to 2.5% perchloric acid, and centrifuge 15 min at 35,000 × *g*, 4°C.

11. Decant supernatant and add glycerol to a final concentration of 25% (v/v). Centrifuge 15 min at 35,000 × *g*, 4°C.

12. Resuspend the pellet—which should appear as a translucent film stuck to the centrifuge tube wall—in buffer A to a final concentration of 0.5 to 2 mg/ml. Store samples in a freezer at −20°C (Fig. 3.29.8).

SUPPORT PROTOCOL 1

NONDENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS AS A SIMPLE METHOD TO QUANTIFY/CHECK POLYMERIZATION-COMPETENT TUBULIN

The determination of the purity of the different protein fractions from the various purification steps can be monitorized using SDS-denaturing gels (*UNIT 6.1*). Purified tubulin is usually analyzed by SDS-PAGE. Under denaturing conditions, α- and β-tubulin polypeptides have different mobilities, both polypeptides in the molecular mass range of 55 kDa. However, the analysis and quantification of the amount of tubulin by most of the known biochemical methods does not provide information about the native state of the tubulin in the preparation. To distinguish between folded and unfolded tubulin (noncompetent for microtubule assembly) we can take advantage of an old, but still very important, nondenaturing gel electrophoresis technique first applied by Ornstein (1964) for the analysis of water-soluble serum proteins (see *UNIT 6.5*). This method can be used only under conditions that preserve the native state of soluble individual proteins or complexes. In contrast to classic SDS-denaturing electrophoresis, the migration of proteins in native gels is essentially dependent on their isoelectric point and the pH of the buffer (Safer, 1994). Consequently, in native electrophoresis it is crucial to select the appropriate buffer for each protein under analysis. The behavior of native tubulin under nondenaturing conditions was established by Zabala and Cowan (1992). Although many times it is advantageous to use a discontinuous system to maximize the resolution of the protein bands, we have found that this is not required for tubulin, and it is easier and faster to prepare a continuous system. To prepare and to run a native electrophoresis, carefully follow the safety indications explained in Chapter 6.

Materials

Gel solutions (Table 3.29.1)
4× loading buffer [50 mM MES, pH 6.7, containing 30%(v/v) glycerol or 25%(w/v) sucrose
Electrophoresis (running) buffer (see recipe)
GTP
Protein sample to be analyzed
10× transfer buffer (see recipe), optional
Electrophoresis equipment (Chapter 6; e.g., Miniprotean from Bio-Rad and 0.75-mm spacers)

Additional reagents and equipment for gel staining (*UNIT 6.2*)

1. Assemble the gel glass-plate sandwich and fit it to the gel electrophoresis unit casting stand according to manufacturer's instructions.

2. Prepare the gel solution as indicated in Table 3.29.1.

3. Pour the gel mix to the top of the gel mold and insert the comb (0.75-mm spacers and 10 wells per comb). Avoid trapping air bubbles under the comb teeth. Allow gel to polymerize for 30 to 60 min.

Table 3.29.1 Recipe for Gel Preparation Using a Native Continuous Buffer System[a]

Stock solution (ml)[b]	Final acrylamide concentration in gel (%)[c]			
	4	5	6	7
Distilled water	6.48	6.15	5.81	5.48
30% acrylamide/0.8% bisacrylamide[d]	1.33	1.66	2	2.33
0.5 M MES, pH 6.7	2	2	2	2
1 M $MgCl_2$	0.01	0.01	0.01	0.01
0.5 M EGTA	0.02	0.02	0.02	0.02
1 M GTP	0.01	0.01	0.01	0.01
10% (w/v) APS (ammonium persulfate)[e]	0.14	0.14	0.14	0.14
TEMED[f]	0.01	0.01	0.01	0.01

[a]Preparation of the gel: Use a 75 or 100-ml Erlenmeyer flask or in order to speed polymerization, degas the mix under vacuum several minutes in a side-arm flask. Mix the ProtoGel[c] solution with 0.5M MES pH 6.7, 1M $MgCl_2$, 1M GTP and 0.5M EGTA. Add 10% (w/v) ammonium persulfate and TEMED.
[b]All reagents and solutions used must be prepared with Milli-Q purified water or equivalent.
[c]The recipe is for 10 ml solution, which is adequate for 2 minigels (7 cm × 8 cm) 0.75 mm thickness.
[d]The polyacrylamide can be prepared as described in Chapter 6, but it is more convenient to use a ready-to-use commercial solution, we usually use ProtoGel 30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide from National Diagnostics, England.
[e]It can be prepared and stored frozen at −20°C in aliquots but we prefer to use freshly made.
[f]Add just before polymerization.

4. Mix the protein sample to be analyzed with 4× native loading buffer [50 mM MES, pH 6.7, containing 30% (v/v) glycerol or 25% (w/v) sucrose] for a final concentration of 1×.

   For better sample visualization small amounts [0.025% (w/v)] of bromphenol blue can be added in the loading buffer.

5. Assemble the gel unit and fill the upper tank with enough electrophoresis buffer with GTP added to 0.01 M. Use the rest of the 800 ml of electrophoresis buffer, without GTP, to fill the rest of the cell. Remove the comb and rinse wells with buffer.

6. Carefully load a maximum of 25 μl of the samples starting up from the bottom of the wells.

   Use a micropipet or alternatively a Hamilton syringe to load the gel.

7. Connect the unit to the power supply. Set at constant voltage of 80 V for 2 hr.

   We usually run one gel per cell unit, for no longer than 2 hr at room temperature.

   Gels can run longer than 2 hr if the electrophoresis buffer is replaced with freshly prepared electrophoresis buffer in order to maintain the pH. The apparatus needs to be turned off before exchanging the buffer.

8. Turn off the power supply. Disassemble the unit and remove gel from glass sandwich. Stain the gel according to *UNIT 6.6*.

   Coomassie blue or silver staining is the usual method to detect tubulin depending on the amount of tubulin loaded into the gel (Fig. 3.29.9). Alternatively, gels can be transferred onto nitrocellulose or PVDF membranes prior to antibody detection in immunoblots (UNIT 6.2) by using a typical transfer buffer where methanol is omitted.

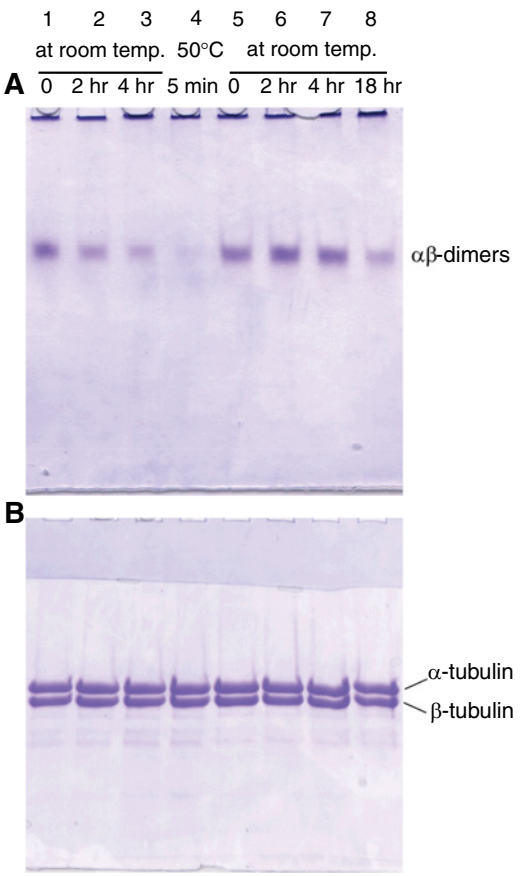

Figure 3.29.9 Assessment of the quality of native tubulin heterodimers by non-denaturing and denaturing gel electrophoresis. (**A**) Nondenaturing PAGE (8.5% acrylamide) characterization of 1-μl aliquots (Support Protocol 1) of purified tubulin (at 6 μg/μl) diluted to 10 μl with water Milli-Q (lanes 1 to 4) or 50 mM MES, pH 6.7 (lanes 5 to 8), and incubated for the indicated times at room temperature with the exception of the aliquot analyzed in lane 5, which was incubated 5 min at 50°C. (**B**) The same tubulin samples were loaded on a gel prepared and run as described in Support Protocol 1. Both gels were stained with Coomassie blue and then scanned before drying. For color version of this figure see *http://www.currentprotocols.com*.

SUPPORT PROTOCOL 2

VISUALIZATION OF POLYMERIZED MICROTUBULES BY ATOMIC FORCE MICROSCOPY (AFM)

Atomic Force Microscopy (AFM) is a powerful technique with a wide range of applications in biology, allowing not only imaging, but also different types of probing and manipulation at nanometric scale. AFM systems are becoming widely available, and new developments are reported everyday, many of them in biological research. In AFM a sharp tip (local probe) is moved over a surface at constant (van der Waals) interaction force between the tip and the surface, allowing the reconstruction of the surface topography. This measurement process can be performed in different ambient conditions, e.g., in vacuum, air, or liquid, over a broad temperature range. These characteristics and the possibility to observe features at nanometric scale in a nondestructive manner are a strong motivation for the use of AFM on biological systems. As compared to existing microscopy techniques in the same range (as electron microscopy), sample preparation is also much easier and less time-consuming, allowing one to perform measurements even on live systems (Fotiadis et al., 2002). The list of examples is now almost endless. For example, AFM

techniques have been extensively used to image the surface of cells (Dufrêne et al., 1999; Charras and Horton, 2002), nucleic acids (Pande et al., 1998; Scheuring et al., 1999), proteins (Round et al., 2002), chromosomes (Radmacher et al., 1994; Fritzsche et al., 1997), viruses (Dubrovin et al., 2007), and biomaterials (Wallwork et al., 2001). Besides being used as a powerful imaging technique, AFM shows a strong potential as a unique tool for nanometric probing of physical (e.g., elasticity or viscosity of surfaces, binding forces, strength of protein folding) and chemical properties (by using a chemically activated tip) and for precise local manipulation (Rief, et al., 1997; Sritharan et al., 1998; Raab et al., 1999; Dufrêne, 2000). It is nowadays a fundamental tool for nanomedicine (medicine approached at a molecular level) and its relevance can no longer be overlooked

Microtubules may be characterized by AFM (Ramalho et al., 2007), which permits nanometer-scale resolutions and topographic characterization of the sample, allowing one to visualize the assembled microtubules.

Here we introduce a protocol adapted from Martins et al. (2005) and Moreno-Herrero et al., (2002, 2005) describing how to adsorb microtubules on a substrate to visualize them by AFM. In this protocol, microtubules are adsorbed to mica functionalized with APTES, a silane with an amine group that is positively charged at around pH 7. Since mica is composed of layers, which can be cleaved ("peeled" off) to expose a new clean surface and, unlike more traditional glass, no preliminary washing procedures are necessary.

Materials

Mica (muscovite; SPI Supplies)
Nail polish or water-resistant glue (e.g., epoxy resin)
Silanization solution: 0.1% (v/v) 3-aminopropyl-triethoxysilane (APTES; Fluka, 96%) freshly prepared with Milli-Q water
Milli-Q water; if unavailable, bidistilled water filtered through a 0.22-μm pore filter
GTP
Paclitaxel (taxol)
Sample of purified tubulin (Basic Protocol 3)
0.1 M PIPES, pH 6.9
1 mM EGTA
1 mM $MgCl_2$
1 mM PMSF

Sharp scissors
Glass coverslips or slides
Duct tape
Micropipet
30°C thermostatic bath
AFM Si tapping mode tips (Olympus, OTESP)

Functionalize the mica

1. Use sharp scissors to cut mica squares of appropriate dimensions (2 to 5 mm) and mount them on glass coverslips or slides (for easier handling) with nail polish or water-resistant glue (e.g., epoxy resin). Remove the upper layers of the mica squares by pulling off the top layer with a piece of duct tape.

2. Immediately apply 10 μl silanization solution on the exposed surface, taking care not to spill on the sides. To complete silanization let it rest for 10 min.

   While the nail polish will secure the mica even if wet, silanization solution may still get between the mica layers and distort them.

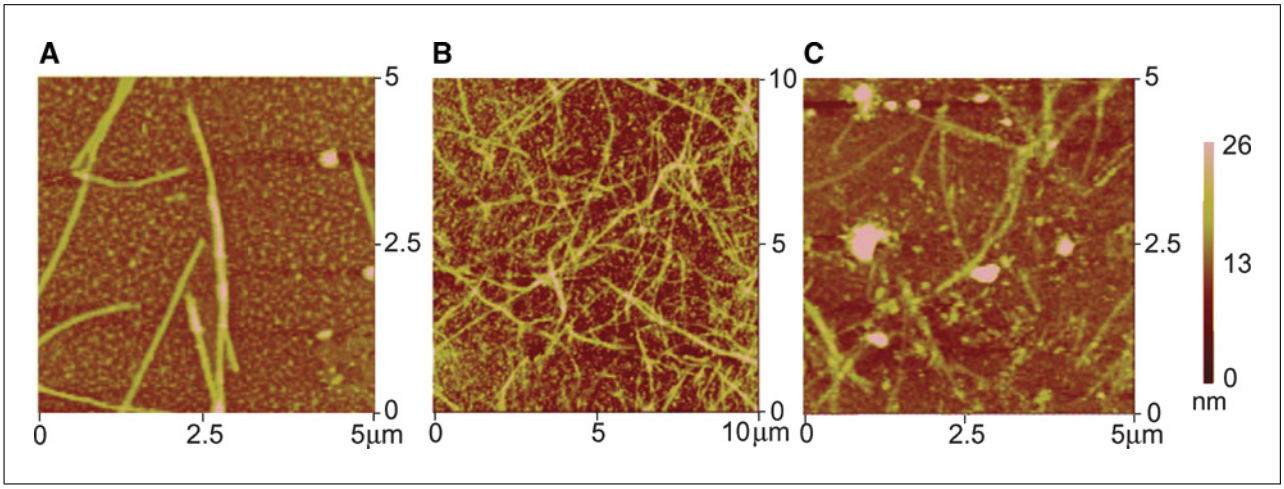

Figure 3.29.10 (**A**) AFM topography images of polymerized microtubules adsorbed to silanized mica where a few individual microtubules with a well preserved structure are easily observed. (**B**) A microtubule-rich region in the sample showing contaminant material. (**C**) Distortion of measurements due to the contaminants in a dirty sample. For color version of this figure see *http://www.currentprotocols.com*.

3. Remove the silanization solution as much as possible with a micropipet and avoid scratching the mica surface. Wash off the mica three times, each time with 10 µl of Milli-Q water and allow the mica to air dry.

   It is quite important that mica surface is maintained clean during this process in order to guarantee that environmental dust does not stick to it during the process of silanization.

Polymerize and adsorb microtubules

4. Add 2 µl of 100 mM GTP (to a final concentration of 1 mM) to 200 µl of a purified tubulin solution in 0.1 M PIPES pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, at 1 mg/ml on ice.

5. Transfer to a 30°C thermostatic bath and incubate for 45 min.

6. Add 4 µl of 1 mM paclitaxel (taxol) to give a final concentration of 20 µM. Incubate for another 15 min at the same temperature.

   Optionally, microtubules may be concentrated by sedimenting them at 15,000 × g for 120 min. This will usually require more than one aliquot to give a visible pellet.

   If you are not going to immediately use the assembled microtubule suspension then aliquot it in suitable volumes, promptly freeze in liquid nitrogen, and store at −80°C.

7. When appropriate, thaw the microtubule suspension by placing on ice. Apply 5 µl of the microtubule suspension on the surface of the functionalized mica. Incubate for 5 to 10 sec. Immediately carefully remove the liquid with a micropipettor.

8. Gently wash the preparation twice with 10 µl Milli-Q water. Allow remaining water to air dry and observe with AFM as soon as possible.

   Avoid touching the mica surface with the tip of the micropipet in steps 1 and 2.

   For AFM measurements we use a Dimension 31 00 (Veeco/Digital Instruments) microscope with nanoscope III a controller and silicon nitride tips (SiN_2). All samples are observed in tapping mode at atmosphere in order to minimize de-adsorption (Fig. 3.29.10).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BRB80 buffer

80 mM PIPES, pH 6.8
1 mM EGTA
1 mM $MgCl_2$
Store up to 2 months at 4°C

Buffer A, 10×

1 M MES, pH 6.7
20 mM EGTA
10 mM $MgCl_2$
Store up to 2 months at 4°C

Buffer B

0.15 M PIPES, pH 7.0 (see recipe)
2 mM EGTA
0.1 mM $MgCl_2$
Store up to 2 months at 4°C

Buffer C

0.1 M PIPES, pH 7.0 (see recipe)
2 mM EGTA
Store up to 2 months at 4°C

Buffer D

0.1 M PIPES, pH 7.0 (see recipe)
2 mM EGTA
1 M NaCl
Store up to 2 months at 4°C

EGTA, 0.5 M

Dissolve 19 g EGTA [ethylene glycol-bis-(2-aminoethyl)-N,N,N′,N′-tetraacetic acid] in 80 ml water. Titrate to pH 8 with KOH or NaOH. Adjust to a final volume of 100 ml. Store up to 2 years at room temperature.

Electrophoresis (running) buffer

0.1 M MES, pH 6.7
1 mM $MgCl_2$
1 mM EGTA

Dilute and adjust with distilled water 160 ml 0.5 M MES, pH 6.7 (see recipe), 800 µl 1 M $MgCl_2$, and 1.6 ml 0.5 M EGTA (see recipe) to 800 ml. Prepare fresh just prior to use.

GTP, 1 M

1.5 ml distilled H_2O
1 g Na_2GTP
Bring to final volume of 1.91 ml with H_2O.

Do not let stand at room temperature or at 4°C. Aliquot in small volumes and store at −80°C.

Isotonic buffer

0.32 M sucrose
1 mM EGTA
1 mM $MgCl_2$
10 mM phosphate buffer, pH 7 (*APPENDIX 2A*)
Store up to 2 months at 4°C

MES, 0.5 M, pH 6.7

Dissolve 53.3 g MES monohydrate in 400 ml water. Titrate to pH 6.7 with KOH or NaOH. Adjust to a final volume of 500 ml with water. Store up to 6 months at 4°C.

Paclitaxel, 10 mM

10 mM paclitaxel (taxol; Sigma). Prepare in DMSO. Store 50-µl aliquots at −80°C and use at final concentration of 20 µM.

PIPES, 1 M (pH 7.0)

151.2 g PIPES (free acid)
H_2O to 400 ml
Add concentrated KOH dropwise to achieve pH 7.0.
Bring to final volume of 500 ml with H_2O
Store up to 2 months at 4°C

Protease inhibitors

100 mM phenylmethylsulfonyl fluoride, (PMSF; *APPENDIX 2A*) prepare fresh, use at 1 mM, dissolve in DMSO
1000× leupeptin, prepare 1 µg/µl in Milli-Q water
1000× pepstatin A, prepare 1 µg/µl in DMSO
1000× aprotinin, 10 µg/ml in Milli-Q water (sodium azide or 0.2% Triton X-100 can be added to avoid contamination)

CAUTION: *Phenylmethylsulfonyl fluoride is toxic.*

Transfer buffer, 10×

Dissolve the following in water:
15.13 g Tris base (250 mM)
72.067 g glycine (1.92 M)
Adjust to a final volume of 500 ml with H_2O

COMMENTARY

Background Information

The generation of complex neuronal morphologies during vertebrate neurodevelopment is characterized by the production of long cytoplasmic processes known as neurites. These processes, referred to as axons and dendrites, will eventually generate a complex network of neuronal synaptic contacts. Although microtubules are universal polymers in all eukaryotic cell types, in neurons they are highly specialized and more abundant, and play crucial roles in the determination of the cell shape, being organized in long bundles within the axons and dendrites of mature neurons. Microtubules are dynamic structures in vitro, where possible regulatory factors of the process have been thoroughly studied. These investigations led to the discovery of a group of proteins that bind to tubulin "in vitro" during microtubule polymerization assays, promoting the polymerization and further stabilizing the polymer structure. These proteins are referred to as microtubule-associated proteins or MAPs. Three major families of MAPs have been identified and characterized: MAP1 (Vallee, 1990), MAP2 (Murphy et al., 1977), and tau proteins (Cleveland et al., 1977).

MAP1 proteins

The MAP1 protein family consists of two distinct, but related, polypeptides, MAP1A and MAP1B (Schoenfeld et al., 1989). MAP1A has a molecular mass of 299 kDa, whereas MAP1B has a molecular mass of 255 kDa as predicted from their respective amino acid sequences (Noble et al., 1989; Langkopf et al., 1992).

There are also three low-molecular-weight proteins associated with the microtubule-binding domains of both MAP1A and MAP1B; these are referred to as light chains: LC1 (34 kDa), LC2 (30 kDa), and LC3 (19 kDa; Schoenfeld et al., 1989).

In the mammalian brain there is a strong developmental control of the expression patterns of these MAPs. The first MAP expressed in neurons is MAP1B (Tucker et al., 1988). Immunohistochemical analyses in developing brain tissue have shown that this protein is highly concentrated in the growing axons of the developing neurons, exhibiting a more moderate expression in both axons and dendrites of mature neurons (Schoenfeld et al., 1989). Indeed, MAP1B expression is down-regulated during brain development (Binder et al., 1984; Bloom et al., 1985; Riederer et al., 1986; Tucker et al., 1989; Garner et al., 1990). On the contrary, MAP1A is more abundant in mature neurons (Bloom et al., 1984).

MAP2

There are several developmentally regulated isoforms of MAP2 arising from alternative mRNA splicing. MAP2B is a high-molecular-weight isoform which contains 1828 amino acids and has an apparent molecular mass of 270 kDa as determined by SDS-PAGE (Lewis et al., 1988). Also, MAP2A shows a slower electrophoretic mobility (Matus, 1988). There is a smaller form, referred to as MAP2C that contains 467 amino acids and has an apparent molecular mass of 70 kDa as determined by SDS-PAGE. MAP2B is a neuron-specific phosphoprotein which is selectively localized in the cell bodies and dendrites (Caceres et al., 1984; Huber and Matus, 1984). MAP2C is expressed perinatally in rats, coincident with the period of maximal dendritic outgrowth and synaptogenesis (Riederer and Matus, 1985). There is an additional MAP2 isoform which is more abundant in glial cells named MAP2D (Doll et al., 1993).

Tau

There are several tau proteins with apparent molecular masses ranging from 55 kDa to 68 kDa (as determined by SDS-PAGE); they are found in the central nervous system (Weingarten et al., 1975). This large number of tau isoforms is generated by alternative splicing from a primary transcript (Himmler et al., 1989; Goedert et al., 1992). Based on their domain characteristics, two classes of tau isoforms have been described. One class contains three tubulin-binding motifs that are predominantly expressed in the developing brain; a second class contains four motifs and is mostly expressed in the adult brain (Kosik et al., 1989; Goedert et al., 1991).

An additional tau isoform, with an apparent molecular mass of 110 kDa, has also been identified in the peripheral nervous system (Georgieff et al., 1991). This high-molecular-weight tau contains four repeated motifs in its microtubule-binding domain; it is similar to the adult brain tau isoform but has an additional insertion of 254 amino acids in its amino-terminal region (Goedert et al., 1991; Couchie et al., 1992).

Critical Parameters and Troubleshooting

Our experience suggests that, although a higher yield of microtubule proteins is usually obtained from porcine brain tissue, cow brains do also give good tubulin yields. Tubulin has traditionally been considered an extremely labile protein that becomes noncompetent for polymerization if left on ice for long periods of time. However, as Figure 3.29.9 shows, tubulin is a stable dimer for >4 hr at room temperature in MES buffer at pH 6.7 (at a concentration as low as 0.6 μg/μl, with no magnesium ions or GTP added). In fact, the tubulin dimer becomes unstable only after 18 hr or longer incubation periods. Our experience also shows that tubulin can be stored at temperatures between −70 and −80°C. Regarding the size of the preparation, we usually prepare medium-scale tubulin preparations. This guarantees a faster protein extract preparation, which is key to preserving a good-quality tubulin, as explained in the Time Considerations section. The intermediate-step tubulin aliquots obtained can be fast-frozen and stored at −80°C instead of in liquid nitrogen. For each experiment, appropriate-size aliquots can be thawed, mixed well by flicking, and centrifuged 20 min at 20,000 × *g*, 4°C, before use, to discard insoluble material. Although repeated freezing and thawing of aliquots is not recommended, we find that tubulin will still work fine in freeze-thawed aliquots. Another

crucial point to obtaining a good homogenate when isolating tubulin from brain extracts is not to destroy the tissue excessively. The use of a classical blender, even at low speed, is not recommended since vigorous homogenization leads to the formation of aggregates that persist during the purification. Moreover, this procedure also provokes the formation of tubulin aggregates. We recommend the use of a Polytron set at low speed, or just a Teflon-in-glass homogenizer at 2,000 rpm for 5 to 10 sec, with the pestle moving up and down the homogenizer. A commercial drill adapted as a homogenizer is a cheaper alternative.

Preparation of microtubule samples for AFM entails adsorption onto an adequate substrate (positively charged) and washing off the buffer as much as possible in order to avoid artifacts resulting from salt crystallization. Otherwise, salt crystals will rapidly grow over the mica surface after the microtubule suspension has dried, completely impairing the AFM measurements. Washes must be carefully performed to avoid microtubule damage or removal from the surface. So, drop and remove water from the surfaces containing microtubules during washes.

Since microtubules are adsorbed to mica functionalized with APTES, the time of incubation of microtubules on the functionalized mica is one of the most important parameters in this method. Longer incubation times tend to yield "dirty" samples due to the adsorption onto the surface of nonpolymerized tubulin and other contaminants (even if they are scarce in the sample). On the other hand, shorter incubation times will result in sparser distributions of microtubules on the surface, allowing a better visualization. The more microtubules are adsorbed onto the surface, the more difficult it will be to observe detailed features. Incubation times of 5 to 10 sec will usually give good results with microtubules polymerized from ~1 mg/ml brain tubulin solution. However, longer times may be necessary for lower concentrations. Attention should also be paid to the concentration of microtubules in suspension, since an increase in polymer concentration would lead to aggregation and microtubule bundle formation.

Anticipated Results

The cytosolic extract obtained after the first centrifugation of the isolation procedure of microtubule proteins usually contains a protein concentration in the range of 20 to 40 mg/ml of protein and ~4 to 8 mg/ml of tubulin. Phosphocellulose-FPLC chromatography purified bovine brain tubulin should give a single band upon electrophoresis under native conditions—in the presence of GTP—which serves as a reference marker for native tubulin heterodimers. The amount of tubulin in this unique band is extremely useful to quantify the amount of tubulin dimers capable of polymerization. Proteins that have denatured or aggregated during these purification steps will remain at, or close to, the origin of the native gel (Fig 3.29.9). Therefore, this type of electrophoresis will serve to estimate the amount of tubulin that should be used in any other experiment requiring native tubulin. The use of GTP during native gel electrophoresis is critical for this type of analysis. In the absence of GTP we have found that the intensity of the native tubulin heterodimer band decreases, indicating that tubulin dimers have became unstable in the absence of GTP. This could be due to denaturation of the β-tubulin that loses GTP during the running of native gel electrophoresis in the absence of this nucleotide (Zabala and Cowan, 1992). Be aware that ATP (used instead of GTP for actin) cannot replace GTP in this type of electrophoresis.

There are a number of different gel staining protocols that can be used (*UNIT 6.6*). Coomassie blue or silver staining are the usual ways to detect tubulin depending on the amount of sample loaded on the gel. Protein in these gels can also be transferred to nitrocellulose or PVDF membranes prior to antibody detection by immunoblots (*UNIT 6.2*). In this case, a typical transfer buffer without methanol should be used.

In vitro polymerized microtubule samples observed by AFM are typically heterogeneous. These samples will characteristically present areas with fewer microtubules, but also fewer interfering contaminants (Fig. 3.29.10A), along with areas denser in microtubules but presenting more contaminants (Fig. 3.29.10B) or areas with many amorphous structures (precipitates from the microtubule solution) that will make the analysis more difficult (Fig.3.29.10C). Denser areas, as well as bundles of microtubules, and generally more contaminants, are often observed in samples prepared from more concentrated microtubule solutions. On the other hand, concentrations lower than those recommended will result in very scattered samples where microtubules may be difficult to find.

Time Considerations

The most critical step in the tubulin protein purification protocol is the time spent from the

time of slaughter to the end of the first microtubule polymerization. It is crucial to obtain brains of freshly sacrificed animals and to remove the skulls as soon as possible. These must be immediately stored at 4°C wrapped in plastic bags or similar wrapping material, to avoid direct contact with ice. The brain cytosolic protein extract should be prepared as soon as possible. Everything needed for the isolation/purification procedure should be already prepared in the laboratory. In the same way, extreme care should be taken to avoid any delay in washing and cleaning the brains.

For AFM, waiting periods for sample drying should typically be of 0.5 to 1 hr, depending on room temperature and humidity. For reproducibility, microtubule incubation times must be carefully observed, even if chosen times differ from those detailed in this protocol. Nonetheless, this procedure should take a maximum of 4 hr. Similarly, samples must be observed with AFM as soon as microtubules are adsorbed to functionalized mica, thus avoiding artifacts caused by dust or degradation. Samples can be kept for at least a week at 4°C (in a sealed container) although some degradation and contamination can occur.

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Internet Resources

Web sites for protocols and advice on microtubule preparation, tubulin purification, and handling:

http://mitchison.med.harvard.edu/protocols/tubprep.html

Web site for large scale tubulin preparation protocol.

http://mitchison.med.harvard.edu/protocols/recycle.html

http://www.bio.com/protocolstools/protocol.jhtml?id = p1426

http://www.ciwemb.edu/labs/koshland/Protocols/MICROTUBULE/cyclingmicrotub.html

Web sites for handling recycled tubulin.

http://www.bio.com/protocolstools/protocol.jhtml?id = p1434

Protocol for tubulin/MAP co-sedimentation under high ATP conditions and MAP/motor/tubulin co-sedimentation.

http://www.bio.com/protocolstools/protocol.jhtml?id = p1600

In this protocol purified tubulin is stabilized with Taxol.

http://www.bio.unc.edu/faculty/salmon/lab/protocolsporcinetubulin.html

Web site for tubulin purification from 3 pig brains similar to Basic Protocol 1.

http://www.borisylab.northwestern.edu/pages/protocols.html

Web page for microtubule protein preparation.