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Microtubules, in vitro
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CONTENTS

Contributors xiii
Preface xix

SECTION I  Isolation and Biochemistry of Tubulin and Characterization of Antibodies and Isotypes

1. Preparation of Microtubule Protein and Purified Tubulin from Bovine Brain by Cycles of Assembly and Disassembly and Phosphocellulose Chromatography
   Herbert P. Miller and Leslie Wilson
   I. Introduction 3
   II. Protocols 4
   III. Buffer Compositions 13
   IV. Concluding Comments 14
   References 14

2. Isolating Tubulin from Nonneural Sources
   Dan L. Sackett, Karl A. Werbovetz, and Naomi S. Morrissette
   I. Why Tubulin from Nonneural Sources? 18
   II. General Principles of Tubulin Purification 18
   III. Specific Properties of Distinct Nonneural Sources 19
   IV. Genetic Manipulation of Tubulin Genes 22
   V. Isolation of Tubulin: Theme and Variation 24
   VI. Protocol Notes 28
   VII. Summary 29
   References 29

3. Characterization of Anti-β-tubulin Antibodies
   Anthony J. Spano and Anthony Frankfurter
   I. Introduction 33
   II. The Characterization of AA2, a Pan-specific Anti-β-tubulin Monoclonal Antibody That Reacts with All Vertebrate β-Tubulin Isotypes (Gene Products) 34
   III. The Characterization of Isotype-Specific β-Tubulin Monoclonal and Polyclonal Antibodies 36
   IV. The Characterization of Antibodies That Recognize the Glutamyl Side Chain of Glutamylated Proteins 37
   V. Summary 44
   References 44
4. Expression Profiling of Tubulin Isotypes and Microtubule-Interacting Proteins Using Real-Time Polymerase Chain Reaction

Sharon Lobert, Laree Hiser, and John J. Correia

I. Introduction and Rationale 48
II. Methods and Materials 48
III. Results and Discussion 54
IV. Summary 58
References 58

5. Nondenaturing Electrophoresis as a Tool to Investigate Tubulin Complexes

Mónica López Fanarraga, Gerardo Caranza, Raquel Castaño, Sofia Nolasco, J. Avila, and J. C. Zabala

I. Introduction 60
II. Rationale 60
III. Methods 61
IV. Materials 72
V. Discussion 73
VI. Summary 73
References 74

6. Mass Spectrometry Analysis of C-Terminal Posttranslational Modifications of Tubulins

Virginie Redeker

I. Introduction 78
II. Methods 82
III. Results and Discussion 87
IV. Conclusion 98
References 99

7. Methods in Tubulin Proteomics

Leah M. Miller, Hui Xiao, Berta Burd, Susan Band Horwitz, Ruth Hogue Angeletti, and Pascal Verdier-Pinard

I. Introduction 106
II. Methods 107
III. Summary 123
References 124

SECTION II  Microtubule Structure and Dynamics

8. Cryo-EM Studies of Microtubule Structural Intermediates and Kinetochore–Microtubule Interactions

Eva Nogales, Vincent H. Ramey, and Hong-Wei Wang

I. Introduction 130
II. Rationale 134
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. Methods</td>
<td>138</td>
</tr>
<tr>
<td>IV. Discussion</td>
<td>149</td>
</tr>
<tr>
<td>V. Summary</td>
<td>152</td>
</tr>
<tr>
<td>References</td>
<td>153</td>
</tr>
<tr>
<td>9. High-Resolution Imaging of Microtubules and Cytoskeleton Structures by Atomic Force Microscopy</td>
<td></td>
</tr>
<tr>
<td><em>Loic Hamon, Patrick A. Curmi, and David Pastré</em></td>
<td></td>
</tr>
<tr>
<td>I. Introduction to AFM Imaging of Biomolecules</td>
<td>158</td>
</tr>
<tr>
<td>II. Rationale</td>
<td>159</td>
</tr>
<tr>
<td>III. AFM Principle and Operation Mode</td>
<td>159</td>
</tr>
<tr>
<td>IV. Practical Aspects of Microtubules Adsorption on Surface and AFM Imaging</td>
<td>161</td>
</tr>
<tr>
<td>V. AFM Images in Air of Microtubule</td>
<td>167</td>
</tr>
<tr>
<td>VI. AFM Imaging in Air of Tubulin or Microtubule: Protein Interactions</td>
<td>169</td>
</tr>
<tr>
<td>VII. Conclusion</td>
<td>171</td>
</tr>
<tr>
<td>References</td>
<td>172</td>
</tr>
<tr>
<td>10. Using Computational Modeling to Understand Microtubule Dynamics: A Primer for Cell Biologists</td>
<td></td>
</tr>
<tr>
<td><em>Holly V. Goodson and Ivan V. Gregoretti</em></td>
<td></td>
</tr>
<tr>
<td>I. Introduction</td>
<td>176</td>
</tr>
<tr>
<td>II. Mathematical and Computational Modeling: A Primer</td>
<td>177</td>
</tr>
<tr>
<td>III. Using Modeling to Understand Microtubule Dynamics</td>
<td>181</td>
</tr>
<tr>
<td>IV. Conclusions</td>
<td>186</td>
</tr>
<tr>
<td>References</td>
<td>187</td>
</tr>
<tr>
<td>11. Analysis of Dynamic Instability of Steady-State Microtubules In Vitro by Video-Enhanced Differential Interference Contrast Microscopy with an Appendix by Emin Oroudjev</td>
<td></td>
</tr>
<tr>
<td><em>Mythili Yenjerla, Manu Lopus, and Leslie Wilson</em></td>
<td></td>
</tr>
<tr>
<td>I. Introduction</td>
<td>190</td>
</tr>
<tr>
<td>II. Method</td>
<td>192</td>
</tr>
<tr>
<td>III. Summary and Conclusion</td>
<td>200</td>
</tr>
<tr>
<td>References</td>
<td>201</td>
</tr>
<tr>
<td>Appendix</td>
<td>203</td>
</tr>
<tr>
<td>I. Introduction</td>
<td>203</td>
</tr>
<tr>
<td>II. MT-LHAP and Its Features</td>
<td>204</td>
</tr>
<tr>
<td>III. Instructions to Use MT-LHAP</td>
<td>204</td>
</tr>
<tr>
<td>IV. Conclusion</td>
<td>206</td>
</tr>
<tr>
<td>References</td>
<td>206</td>
</tr>
<tr>
<td>12. Nanometer-Resolution Microtubule Polymerization Assays Using Optical Tweezers and Microfabricated Barriers</td>
<td></td>
</tr>
<tr>
<td><em>Blake D. Charlebois, Henry T. Schek III, and Alan J. Hunt</em></td>
<td></td>
</tr>
<tr>
<td>I. Introduction</td>
<td>208</td>
</tr>
<tr>
<td>II. Rationale</td>
<td>209</td>
</tr>
</tbody>
</table>
13. Microtubule Dynamics Reconstituted In Vitro and Imaged by Single-Molecule Fluorescence Microscopy

Christopher Gell, Volker Bormuth, Gary J. Brouhard, Daniel N. Cohen, Stefan Diez, Claire T. Friel, Jonne Helenius, Bert Nitzsche, Heike Petzold, Jan Ribbe, Erik Schäffer, Jeffrey H. Stear, Anastasiya Trushko, Vladimir Varga, Per O. Widlund, Marija Zanic, and Jonathon Howard

I. Introduction 222
II. Single-Molecule TIRF Microscopy 224
III. List of Reagents 227
IV. Choice of Fluorophore/Protein Labeling 229
V. (Anti-)blinking/Photo-Toxicity/Photo-Bleaching Cocktails 230
VI. Preparation of GMPCPP-Stabilized Microtubules 233
VII. Glass Treatment and Sample Chamber Preparation 236
VIII. Binding of Microtubules and Passivation of Surfaces 239
IX. Dynamic Microtubule Assays 241
References 243

14. Studying Kinesin Motors by Optical 3D-Nanometry in Gliding Motility Assays

Bert Nitzsche, Volker Bormuth, Corina Bräuer, Jonathon Howard, Leonid Ionov, Jacob Kerssemakers, Till Korten, Cecile Leduc, Felix Ruhnow, and Stefan Diez

I. Introduction 248
II. Setup of Gliding Motility Assays 249
III. Analysis of Microtubule and Quantum Dot Movements 259
IV. Future Directions 266
Reagents 267
References 269

SECTION III  Drugs

15. Analysis of Tubulin Oligomers by Analytical Ultracentrifugation

John J. Correia

I. Introduction 276
II. Materials and Methods 276
III. Results and Discussion 282
IV. Summary 286
References 286
16. Determination of Drug Binding to Microtubules In Vitro
Jennifer A. Smith and Mary Ann Jordan

I. Introduction 290
II. Methods 292
III. Materials 298
References 298

17. Fluorescence Spectroscopic Methods to Analyze Drug–Tubulin Interactions
Bhabatarak Bhattacharyya, Sonia Kapoor, and Dulal Panda

I. Introduction 302
II. Colchicine Binding to Tubulin 303
III. Vinblastine Binding to Tubulin 310
IV. Taxol Binding to Microtubules 316
V. Determination of Binding Constants Using Extrinsic Fluorescent Probes 324
VI. Conclusion 325
References 326

18. A Tubulin Polymerization Microassay Used to Compare Ligand Efficacy
Ashley Davis, Sarah Martinez, Daniel Nelson, and Kim Middleton

I. Introduction 332
II. Rationale 333
III. Methods 342
IV. Summary 349
References 350

19. Fluorescent Taxoid Probes for Microtubule Research
Isabel Barasoain, J. Fernando Díaz, and José M. Andreu

I. Introduction 354
II. Materials 355
III. Methods 355
IV. Applications and Discussion 362
V. Summary 368
References 370

20. The Binding of Vinca Domain Agents to Tubulin: Structural and Biochemical Studies
Anthony Cormier, Marcel Knossow, Chunguang Wang, and Benoît Gigant

I. Introduction 374
II. Rationale 375
III. Structural Studies of the Tubulin–Vinca Domain Ligand Interactions 376
IV. Fluorescence Is Useful to Monitor Binding of Vinca Domain Ligands to Tubulin in Solution 382
V. Effects of Vinca Domain Ligands on Tubulin Biochemical Properties 383
VI. Discussion 387
References 388
21. Measurement of Ligand Binding to Tubulin by Sulphhydryl Reactivity

*Adrian Begaye and Dan L. Sackett*

I. Introduction and Rationale 392
II. Methods 393
III. Discussion 401
References 402

SECTION IV Interactions with Motors and MAPs

22. Probing Interactions of Tubulin with Small Molecules, Peptides, and Protein Fragments by Solution Nuclear Magnetic Resonance

*Marie-Jeanne Clément, Philippe Savarin, Elisabeth Adjadj, André Sobel, Flavio Toma, and Patrick A. Curmi*

I. Introduction 408
II. Rationale 409
III. Methods 414
IV. Application to Tubulin/Microtubules Interactions 422
V. Conclusion 441
VI. Appendix I. Materials 441
References 444

23. Microtubule and MAPs: Thermodynamics of Complex Formation by AUC, ITC, Fluorescence, and NMR

*François Devred, Pascale Barbier, Daniel Lafitte, Isabelle Landrieu, Guy Lippens, and Vincent Peyrot*

I. Introduction 450
II. Rationale 452
III. Materials and Methods 456
IV. Discussion 463
V. Concluding Remarks 474
References 475

24. Quantitative Analysis of MAP-Mediated Regulation of Microtubule Dynamic Instability In Vitro—Focus on Tau

*Erkan Kiris, Donovan Ventimiglia, and Stuart C. Feinstein*

I. Introduction and Rationale 482
II. Methods 490
III. Materials 495
IV. Summary 497
References 498

25. Structure and Dynamics of the Kinesin–Microtubule Interaction Revealed by Fluorescence Polarization Microscopy

*Hernando Sosa, Ana B. Aseujo, and Erwin J. G. Peterman*

I. Introduction 506
II. Rationale 507
26. Multiple Color Single Molecule TIRF Imaging and Tracking of MAPs and Motors

*Jennifer L. Ross and Ram Dixit*

I. Introduction 522
II. TIRF Optics 523
III. Labeling Molecules 528
IV. Examples and Protocols 528
V. Conclusions and Outlook 540
References 541

27. Studying Plus-End Tracking at Single Molecule Resolution Using TIRF Microscopy

*Ram Dixit and Jennifer L. Ross*

I. Introduction 544
II. Rationale 545
III. Methods 546
IV. Discussion 553
References 553

28. Fluorescence Microscopy Assays on Chemically Functionalized Surfaces for Quantitative Imaging of Microtubule, Motor, and +TIP Dynamics

*Peter Bieling, Ivo A. Telley, Christian Hentrich, Jacob Piehler, and Thomas Surrey*

I. Introduction 556
II. Rationale 557
III. Materials 558
IV. Methods 559
V. Surface Chemistry on Glass 561
VI. Fluorescence Microscopy Assays 565
VII. Discussion 576
VIII. Conclusion 577
References 578

SECTION V  Functional Extracts and Force Measurements

29. Quantitative Characterization of Filament Dynamics by Single-Molecule Lifetime Measurements

*Leonid A. Mirny and Daniel J. Needleman*

I. Introduction to Cytoskeletal Filament Dynamics 584
II. Single-Molecule Lifetime Measurements 585
III. Theoretical Foundations 587
IV. Results and Conclusion 597
   References 599

30. Extracting the Mechanical Properties of Microtubules from Thermal Fluctuation Measurements on an Attached Tracer Particle

Katja M. Taute, Francesco Pampaloni, and Ernst-Ludwig Florin

   I. Introduction 602
   II. Rationale 604
   III. Materials 606
   IV. Methods 607
   V. Discussion 613
   VI. Summary 614
   References 614

31. In Vitro Assays to Study Force Generation at Dynamic Microtubule Ends

Liedewij Laan and Marileen Dogterom

   I. Introduction 618
   II. Materials 620
   III. Methods 621
   IV. Results 633
   V. Conclusion/Discussion 634
   References 636

32. Reconstitution and Functional Analysis of Kinetochore Subcomplexes

Daniel R. Gestaut, Jeremy Cooper, Charles L. Ashby, Trisha N. Davis, and Linda Wordeman

   I. Introduction 642
   II. Methods 642
   III. Conclusion 656
   References 656

33. In Vitro Assays to Study the Tracking of Shortening Microtubule Ends and to Measure Associated Forces

Ekaterina L. Grishchuk and Fazly I. Ataullakhanov

   I. Introduction 658
   II. Rationale 659
   III. Materials and Methods 660
   IV. Summary and Discussion 673
   References 674

Index 677

Volume in Series 693
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PREFACE

It has been almost 30 years since the first Methods in Cell Biology volumes that contained methods for analysis of tubulin and microtubules were published. These were two volumes on The Cytoskeleton, edited by Les Wilson, covering the entire cytoskeleton field, which at the time was a relatively new emerging area of cell biology. Volume 24, Part A of the two-part series, focused on the isolation and characterization of cytoskeletal proteins. Of the 24 chapters, 18 were on microtubules; 5 on purification methods, 6 more on length distributions, polarity, assembly kinetics, and tubulin flux measurements, 3 on tyrosination, tubulin tyrosine ligase reactions, and only 2 on dynein. The nontubulin methods chapters covered actin, myosin, and intermediate filament purification and characterization, with 2 chapters on actin assembly measurements. The second volume, Part B, was Volume 25 and focused on biological systems and in vitro models. Of the 18 chapters, 1 discussed axonal transport, 1 discussed microtubule mediated motility of sperm flagella, 2 were on the role of microtubules in secretion and intracellular transport, 3 discussed mitotic spindles and mitosis in either PtK1 cells, sea urchin eggs, or Aspergillus nidulans, and 1 described electron microscopic methods for visualizing microtubules in spindles. The remaining methods chapters focused on actin-based systems with only one chapter devoted to intermediate filaments in muscle. While most of the chapters in the earlier volumes are classics and remain extremely useful today, so much has happened since then. At the time these volumes were assembled and published, dynamic instability had not yet been described, kinesin had not been isolated, video-enhanced differential interference contrast microscopy had not yet been developed, laser trap methods were just being discovered, and the three-dimensional (3D) structure of tubulin and microtubules was a distant dream. As advanced as the state of knowledge on microtubules was at that time, a continuous wave of new discoveries, dominated by the advancement of single-molecule methods and high-resolution techniques and their application to new families of remarkable motor proteins and microtubule-associated proteins (MAPs), has emerged that has sent the microtubule field into unimagined directions.

Here, with a focus on analysis of purified (in vitro) systems, 33 groups of experts have written state-of-the-art methods chapters that reflect many of the advances that have taken place in the analysis of tubulin and microtubules and their functions that have occurred in the subsequent 28 years. Volume 95, Microtubules, In Vitro, is organized into five sections. Section 1, on Isolation, Biochemistry, and Characterization of Antibodies and Isotypes, consists of seven chapters on tubulin isolation, β-tubulin antibody production and characterization methods, PCR methods for tubulin isotypes and MAPs, and native electrophoresis methods together with two chapters on mass spectroscopy, C-terminal posttranslational modifications, and tubulin proteomics. Section 2, Microtubule Structure and Dynamics, consists of seven chapters on cryo-electron microscopic- and atomic
force microscopic imaging of microtubules, analysis of microtubule dynamic instability at steady state, and at nanometer resolution, single-molecule imaging of microtubule dynamics and kinesin motors, and mathematical modeling of microtubule dynamics. Section 3 (Drugs) includes seven chapters on methods for measuring drug binding to tubulin and to microtubules. Section 4 (Interactions with Motors and MAPs) includes seven methods chapters useful for studying MAP tubulin interactions. These include analysis by nuclear magnetic resonance spectroscopy, isothermal titration calorimetry (ITC), fluorescence, total internal reflection fluorescence (TIRF), fluorescence polarization microscopy (FPM), and the methods for functionalizing the surfaces for these measurements. Finally, Section 5 (Functional Extracts and Force Measurements) includes five chapters describing methods for performing dynamics measurements in functional extracts, for extracting mechanical properties from thermal fluctuations of microtubules, for measuring force at dynamic microtubule ends, and for reconstructing kinetochore complexes and measuring the associated forces.

This volume in part reflects the continuing importance of tubulin and microtubule biochemistry and the emergence of modern physical methods for studying molecular interactions including immunological and PCR techniques. It also reflects the critical importance of antimitotic drugs as drugs for treatment of cancer and as tools in cell and molecular biology, and the development of assays to study them, especially at high resolution. While the antimitotic field is currently dominated by the drug discovery biotechnology and questions about biomarkers and clinical outcomes (see the recent volume edited by Tito Fojo, The Role of Microtubules in Cell Biology, Neurobiology and Oncology), the assays described here play a major role in drug development and in our understanding of the microtubule cytoskeleton. Recent advances in cryo-electron microscopy, nuclear magnetic resonance spectroscopy, atomic force microscopy, and proteomic methodology are driving detailed studies into the structure and function of microtubule-based systems. The single-molecule imaging and the techniques that allow nanometer and 3D-nanometer resolution, microtubule plus end tip tracking, and detailed structural pictures of kinesin motility clearly play a major role in the new experimental approaches described in this volume. The merging of single-molecule nanotechnology with high-resolution imaging has significantly changed the questions we can ask and the way microtubule structure, function, and regulation can be analyzed. While this volume is not meant to be all inclusive, it does bring together the breath of biochemical, physical, structural, dynamic, microscopic, and nanomolecular techniques that are now available for study of the microtubule cytoskeleton with purified microtubule systems. An accompanying Methods in Cell Biology volume that will be published later this year, Microtubules, In Vivo, edited by Lynne Cassimeris and Phong Tran, will focus on analysis of microtubule function in living cells.

We wish to thank all the authors for their contributions and the immense effort they put into making this volume an overwhelming success. Their willingness to contribute to this project has been essential. (To those authors who were unable to meet the deadline for this volume, we anticipate assembling a follow-up volume on microtubules in vitro to this volume). We especially want to thank Tara Hoey, Zoe Kruze, and Narmada Thangavelu at Elsevier for their support and organizational skills
throughout this long and seemingly endless process. Finally, we thank our colleagues, collaborators, and families, who only politely harassed us when we seemed to be distracted from our other more noble pursuits.

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SECTION I

Isolation and Biochemistry of Tubulin and Characterization of Antibodies and Isotypes
Preparation of Microtubule Protein and Purified Tubulin from Bovine Brain by Cycles of Assembly and Disassembly and Phosphocellulose Chromatography

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I. Introduction

Tubulin can be purified from a number of nonmammalian and mammalian nonneural sources (e.g., Farrell, 1982; Detrich and Wilson, 1983; Newton et al., 2002; Bellocq et al., 2005), but for most applications mammalian brain is the most commonly used starting material (Borisy et al., 1975; Lee and Timasheff, 1975; Asnes and Wilson, 1979; Murphy, 1982; Williams and Lee, 1982; Sloboda and Belfi, 1998; Andreu, 2007). There are several important reasons that mammalian brain is so highly favored. First, the tubulin concentration in brain is very high, especially in
brains from young animals (Bamburg et al., 1973). Second, microtubules from mammalian brain readily assemble at 30–37°C and quickly depolymerize at cold temperature (0–4°C), facilitating purification by straightforward alternating cycles of warm temperature polymerization and cold temperature depolymerization. Third, mammalian brain contains a high content of assembly-promoting/stabilizing microtubule-associated proteins (MAPs) such as MAP2 and tau. These MAPs, which are mainly found in large amounts in the axonal and dendritic processes of neurons, strongly promote microtubule nucleation and elongation and reduce the critical tubulin concentration required for self-assembly. Beginning with fresh brains from cows, pigs, chickens, goats, mice, rats (or other mammals), and especially from young animals, one can obtain tens of milligrams of assembly-competent microtubule protein (MTP) and purified tubulin in 6–10 h.

Solvents such as glycerol or dimethylsulfoxide (DMSO), and drugs like taxol, strongly promote microtubule polymerization, and a number of purification protocols based upon alternating cycles of warm assembly and cold disassembly have been developed over the years in which such solvents or taxol are added to promote polymerization. While the use of these agents increases the yields of tubulin, because of the high MAP content, purification of tubulin from brain tissue by cycles of assembly and disassembly is very efficient in the absence of such agents as long as the concentration of tubulin present during the assembly reaction is well above the critical tubulin concentration required for polymerization in the presence of the MAPs (~0.3–0.7 mg/ml). This is easy to accomplish simply by careful adjustment of the buffer volume when resuspending microtubules assembled during the preceding cycle into cold depolymerizing buffer (see below).

Also depending upon the application, the use of assembly-promoting solvents or assembly-promoting agents could present important disadvantages. The most important is that they modify the polymerization, treadmilling, and dynamic instability behaviors of microtubules and can mask the activities of drug molecules or specific MAPs that one would like to study (e.g., Schlistra et al., 1991; Panda et al., 1999). Thus, if protocols that involve assembly in assembly-promoting agents are used, it is critical to ensure that all of the agents are completely removed in order to study the effects of modulating proteins or drugs—a procedure that takes additional time and could result in some degradation of the tubulin. Here we present an efficient high yield and relatively easy protocol for purification of MTP (tubulin plus stabilizing MAPs, consisting of ~70–75% tubulin and 25–30% MAPs) and, subsequently, for purifying tubulin from the MTP, in the absence of assembly-promoting solvents.

### II. Protocols

#### A. Optimal Starting Material and Initial Processing

1. **Freshness of Brains: Age and Sex of the Animals**

   The yield of polymerization-competent tubulin is highly dependent upon the freshness of the brain tissue, which is difficult to control. We obtain bovine brains at a commercial slaughter house from Mr. Ramero Carlos of Manning Beef LLC, in Pico Rivera, CA, which is about a 3-h drive from our laboratory. There is a complex and somewhat time-consuming process defined by law that slaughter houses must
follow, which affects brain freshness. As a rule, when cattle arrive at the slaughter house they are first inspected to ensure good health. Animals are then filed onto a killing floor where they are rendered unconscious with a captive bolt pistol fired against the forehead centered just above the eyes. The jugular vein and carotid artery are then cut to drain the blood. Although head removal from the carcass is one of the initial steps in the slaughtering process, the brain cannot be removed from the head until the rest of the carcass has been inspected. First the internal organs are excised and inspected for parasites or disease. Then the tongue is taken and the glands around the head are examined. Only after the carcass passes inspection can the brain be removed from the head. We must arrange to obtain the brains in advance. Murphy (1982) has reported the half-time for loss of tubulin polymerization activity from death of the animal until brain removal to be approximately 19 min. So this process should be carried out quickly. We always request that removal of the brain from the skull be carried out as rapidly as possible—a process that under good conditions takes 20–30 min from the time of kill. The brain is immediately separated into two approximately 125 g hemispheres that are individually placed in plastic sealable bags and buried in ice. This reduces the time to chill the brain to ~0°C and greatly increases the half-time for loss of assembly competence. We have obtained good yields of assembly-competent tubulin from brains that were quickly buried in ice and kept on ice for as long as 5 h after slaughter.

We have found that cow brains yield only one-third the amount of assembly-competent MTP as steer brains. Thus we try to obtain steer brains. Perhaps most important is the age of the animals. Dairy cows at the slaughter house we use are usually 5–7 years old. On the other hand steers, which are raised primarily for beef, are usually no more than 2 years old when slaughtered. Since we have found that young heifers yield similar amounts of assembly-competent protein as steers, it seems that the age of the animal is much more important than the gender, with younger being better. Bulls, like cows, are usually older animals when sent to slaughter and also yield less MTP per gram of fresh brain tissue than steers. Thus, it is best to obtain brains from young steers.

2. Condition of the Brains

An important factor that influences the yield of MTP is the condition of the brains. Because the animals are initially stunned with a pistol fired against the forehead, the skull is usually shattered at the point of impact, sending fragments of bone deep into portions of the brain. This results in localized hemorrhage and damage to the surrounding brain tissue (perhaps due to protease activity), which in our experience reduces yields. Thus, we remove all damaged brain tissue as soon as possible.

3. Removal of the Meninges

We also have found that the yield of assembly-competent tubulin is greatly increased by removing the meninges and free-flowing and coagulated blood. We do this as quickly as possible at the slaughter house. The presence of blood and blood clots seems to reduce yields. Removal of the meninges also facilitates homogenization of the brain tissue. We remove the meninges with blunt tipped forceps. It is important to handle the brain tissue as gingerly as possible during the process to
minimize tissue damage, to minimize the amount of blood forced into the interstitial spaces of the tissue, and to minimize the presence of free-flowing and coagulated blood prior to homogenization.

4. Processing Speed and Optimal Cycling Temperatures

Once the brain is homogenized (see below) it is important to isolate the tubulin and associated proteins as quickly as possible. The tubulin degrades rapidly once the brain is homogenized even at 0°C and each successive polymerization cycle removes proteases and other degradative activities. It is important to keep in mind that all steps (except for warm temperature polymerization) should be carried out at 0°C with 1 mM GTP and 1 mM Mg\(^{++}\) present. It is also important that soluble free calcium is removed with EGTA and that the pH is maintained in a narrow range. We use 30°C to polymerize MTP rather than 37°C. Polymerization is robust at 30°C and experience has shown that recovery of assembly-competent tubulin drops off dramatically as the temperature rises above 37°C. There can be as much as a 5°C variation in temperature above the set point on centrifuges that use a friction-based heating system. In general superspeed centrifuges use a refrigeration unit to cool the chamber and thus the rotor, but rely on friction from the movement of air to heat the chamber and rotor. Temperature is maintained by balancing refrigerated cooling and friction-generated heating. By contrast, most ultracentrifuges are equipped with both heating and cooling units. It is important to confirm the temperature of samples and adjust the centrifuge controls accordingly.

B. Protocol for Purification of Microtubule Protein (Tubulin Plus Microtubule-Associated Proteins) in the Absence of Glycerol

While the protocol has been considerably fine-tuned and standardized, the basic protocol for isolating microtubules by temperature-dependent cycles of assembly and disassembly in the absence of glycerol remains similar to the protocols that were first introduced (Borisy et al., 1975; Asnes and Wilson, 1979; Murphy, 1982). The procedure we describe here is a modification of the Asnes and Wilson procedure (1979). It should work well with some modifications for purification of MTP and tubulin from any brain source. Purification of tubulin from cultured cells or other sources that contain much less tubulin than brain presents special problems that will not be described here (see Farrell, 1982; Detrich and Wilson, 1983; Newton et al., 2002; Bellocq et al., 2005). A flowchart documenting the purification protocol is shown in Fig. 1.

1. Preparation of the Crude Brain Extract (CBE)

We usually begin with two steer brains, weighing a total of between 400 and 600 g. Processing this quantity of tissue requires the equivalent of six Sorvall refrigerated superspeed centrifuges. For example Sorvall RC5B Plus centrifuges equipped with SS-34 rotors work well for the initial purification steps. It is convenient the day before carrying out the purification to prepare two buffers. The first is one l of homogenizing buffer, L-GNPEM buffer, consisting of 100 mM sodium glutamate, 20 mM sodium phosphate, 1 mM EGTA, and 0.5 mM MgCl\(_2\), pH 6.85,
and the second is 250 ml of the same buffer at pH 6.75. Both buffers should be refrigerated. Beginning at the slaughter house, the meninges, superficial blood vessels, and blood clots are removed and each brain hemisphere is placed in a separate plastic bag and buried in ice for transport back to the laboratory (see above). Once back at the laboratory, add 1 mM dithiothreitol (DTT) to one l of L-GNPEM buffer, pH 6.85. This buffer will be used to blend the brains. Brains are initially blended in a Waring Commercial Blender at a ratio of 1.5 ml of buffer per gram of wet brain weight at low speed for 30 s. Next, the blended brains are homogenized by using one pass in a motor-driven Teflon pestle/glass homogenizer (Kimble-Chase, Vinland, NJ) operated at the maximum speed (we use a Tri R Stir R motor). Next, the brain homogenate is centrifuged at 32,500 \( \times g \) (average RCF) for 40 min at 4°C.

Fig. 1 Flowchart illustrating the steps in the purification of microtubule protein and phosphocellulose-purified tubulin.
(we use 50-ml centrifuge tubes). The supernatant, now called the crude brain extract or CBE (Fig. 1), is collected in a liter graduated cylinder to measure the volume and serve as the polymerization vessel.

2. First Polymerization and Depolymerization Cycle

The appropriate amount of GTP is first added in dry form to the CBE at a final concentration of 2.5 mM. Next, the CBE is warmed to 30°C and incubated in a water bath for 30 min to polymerize the MTP into microtubules. We use a 20-gal aquarium with a Fisher Isotemp immersion circulator for all warm incubations. This is followed by centrifugation at 45,000 × g for 30 min at 30°C again using 50-ml centrifuge tubes to obtain microtubule pellets (the H₁P). It is important to note the total volume of the warm supernatant, now called the H₁S, which is then discarded.

Next the H₁P microtubule pellets are resuspended in 10% of the volume of the H₁S into cold L-GNPEM buffer, pH 6.75. The suspended microtubules are then homogenized with three passes in a 15-ml glass pestle “A”/glass tissue homogenizer (we prefer the Kontes K885300–0015) and incubated on ice for 40 min to fully depolymerize the microtubules. The solubilized H₁P fraction is then centrifuged at 45,000 × g for 45 min at 4°C to clarify it and remove any particulate debris. Next the clarified supernatant, the C₁S, is collected and retained and the C₁P pellet is discarded (Fig. 1).

3. Second Polymerization Cycle

First, 2.5 mM GTP from a stock solution of 100 mM GTP is added to the C₁S, which is then incubated for 30 min at 30°C. The C₁S, now a suspension of microtubules, is centrifuged at 45,000 × g for 60 min at 30°C. The H₂S is decanted and the volume is noted before it is discarded. At this point, the H₂P can be quick-frozen (see below) in liquid nitrogen and stored as pellets in a −70°C ultracold freezer until further purification or processing is convenient. As a rule, this will be ~8–9 h after beginning to prepare the CBE. The H₂P can either be taken through another cycle of assembly/disassembly to obtain a clean MAP-rich tubulin preparation or be subjected to phosphocellulose chromatography (see below) to prepare MAP-free purified tubulin, often called PC-Tubulin (Pc-Tu), for use in experiments.

4. Third Purification Cycle

We often use MTP that has been processed through three cycles of warm assembly and cold disassembly. This third cycle involves centrifuging through 50% sucrose cushions to remove any proteins that do not adhere to the microtubules. The third cycling process is similar to the two previous cycles with the following modifications. The frozen H₂P pellets are quickly thawed by initially placing the tubes containing the pellets in a beaker of room temperature water, followed by cooling the tubes on ice. The thawed H₂P pellets are then suspended in L-GNPEM buffer, pH 6.75, using one-third the volume of the former H₂S, homogenized with three passes in a 15-ml glass pestle “A”/glass tissue homogenizer, and incubated on ice for 20 min to fully depolymerize the microtubules. The solubilized H₂P solution is then centrifuged to clarify it at 105,000 × g (average RCF) for 1 h at
4°C. This centrifugation step and the subsequent centrifugation step require a refrigerated ultracentrifuge. We use a Beckman/Coulter Optima L-90 ultracentrifuge with a type 70.1 Ti rotor.

The C\textsubscript{2}S supernatant is removed and 2.5 mM GTP is added. The solution is then incubated for 30 min at 30°C to polymerize the microtubules. A 50% sucrose solution (w/v) in L-GNPEM buffer, pH 6.75, is warmed to 30°C. Five milliliters of this solution is dispensed into 10-ml Oakridge centrifuge tubes, and 1 ml of the polymerized microtubule suspension is layered on top of the 50% sucrose cushion. Microtubules are centrifuged through the sucrose cushion at 150,000 \( \times \) g for 2 h at 30°C to collect microtubule pellets. The supernatant, now called the H\textsubscript{3}S, is aspirated, as is the subnatant of 50% sucrose. This will leave a translucent MTP pellet, called the H\textsubscript{3}P (Fig. 1), at the bottom of the tube.

The H\textsubscript{3}P is resuspended at 0°C in a small volume of PEM buffer, consisting of 100 mM PIPES, 1 mM EGTA, and 1 mM MgSO\textsubscript{4}, pH 6.8, such that the protein concentration is between 5 and 10 mg/ml. The suspension is then Dounce homogenized with three passes in a 7-ml glass pestle “A”/glass tissue homogenizer (Kontes K885300-0007) and incubated on ice for 20 min to depolymerize the microtubules. The solubilized MTP from the H\textsubscript{3}P is then centrifuged at 45,000 \( \times \) g for 20 min at 4°C. The supernatant containing the solubilized MTP is removed (now called the C\textsubscript{3}S), and 100 µM GTP is added. The MAP-rich MTP concentration is measured and the solution is drop-frozen in liquid nitrogen (see below). In our experience, the C\textsubscript{3}S MTP solution can be stored as frozen beads in an ultracold freezer at \(-70°C\) for at least 1 year without noticeable loss of assembly competence.

5. Preparation of Frozen Beads

We prepare frozen beads as follows. Four tri-corner polypropylene beakers (250 ml), which are easily held for decanting the liquid nitrogen, are each completely filled with liquid nitrogen. Using a Pasteur pipette, one drop of MTP solution is added per beaker, alternating the beakers, allowing each bead to freeze before another drop is added. The liquid nitrogen is decanted and discarded and the frozen beads are placed into 50-ml plastic tubes for storage. The average bead volume with this procedure is \( \sim 32 \mu l \).

C. Protocol for Purification of Tubulin from Microtubule Protein by Phosphocellulose Column Chromatography

An alternative to the third cycle for purification of MTP (tubulin plus MAPs) is to purify tubulin devoid of MAPs beginning with the C\textsubscript{2}S using phosphocellulose column chromatography. A similar procedure is also described by Ross and Dixit in this volume. Prepare the C\textsubscript{2}S as described earlier. However, instead of dissolving the H\textsubscript{3}P in L-GNPEM buffer with one-third the volume of the H\textsubscript{3}S, resuspend the microtubule pellet in one-tenth the volume of the previous H\textsubscript{3}S using PEM\textsubscript{50} buffer, which is composed of 50 mM PIPES, 1 mM EGTA, and 1 mM MgSO\textsubscript{4}, pH 6.8. The supernatant (C\textsubscript{3}S) from the ultracentrifugation run is retained and supplemented with 100 µM GTP.
1. Preparation of the Phosphocellulose

The required column volume for the procedure can be calculated in terms of ml by multiplying the total number of mg of protein in the C₂S by 0.67. The amount of dry phosphocellulose (actually cellulose phosphate, Whatman P-11) required for the column is 0.17 g dry phosphocellulose per ml settled column volume, which is stirred into 25 volumes of 0.5 M NaOH. The phosphocellulose resin is allowed to settle and the liquor containing the fines is decanted. The phosphocellulose resin is now resuspended in a large excess volume of glass-distilled water. After the resin has settled, the water is decanted. The water wash is repeated three more times. Next, the phosphocellulose resin is resuspended in 25 volumes of 0.5 M HCl. The resin is allowed to settle and the HCl solution is decanted. The resin is then washed four times with a large excess of glass-distilled water. The phosphocellulose resin is equilibrated in the buffer of choice (we use PEM₅₀ buffer). A 10× solution of PEM₅₀ buffer (consisting of 0.5 M PIPES, 1 mM EGTA, and 1 mM MgSO₄, pH 6.8), enough to cover the phosphocellulose resin to double the volume, is stirred in. After settling, the liquid is decanted and the resin is washed four times with 1× PEM₅₀ buffer. The pH of the liquor is measured after the resin has settled and compared to the pH of the buffer to ensure that the resin is equilibrated and at the correct pH.

2. Building and Developing the Column

To retain maximal assembly competence of the tubulin, the column should be run in a cold room or cold cabinet, buffers should be stored and used at 4°C, and the proteins kept on ice. For 150 mg of C₂S, we use a 2.5 × 30 cm glass Flex Column (Kontes K420401-2550) with a bed volume of 100 ml. The column is filled with the phosphocellulose resin to the 100 ml mark, and 250 ml of PEM₅₀ buffer + 100 μM GTP (PEM₅₀G buffer) is passed through the column as the final step in the equilibration process. The pH of the filtrate is checked after it has passed through the column and compared to the pH of the buffer. Equilibration is continued until the pH of the emerging filtrate is the same as the starting buffer. The buffer on top of the column is drained to the top of the resin bed, being careful not to let the column dry out. The C₂S is loaded onto the top of the phosphocellulose resin bed and allowed to flow completely into the resin bed. Six to ten milliliters of PEM₅₀G buffer is layered on top of the resin bed, a reservoir with 175 ml of PEM₅₀G buffer is attached to the column, and the column is developed, collecting 2–3 ml fractions. The phosphocellulose-purified tubulin (PC-Tu) will begin to elute from the column around 40 ml and should be completely eluted from the column by 100 ml (Fig. 2). The protein-containing fractions consisting of pure PC-Tu are pooled, placed on ice, and concentrated from approximately 1.5–2 mg/ml to 7–10 mg/ml. We use an Amicon Ultra 15 Centrifugal Filter device (Millipore Corp, Billerica, MA) with a 30,000 molecular weight cut-off and a Sorvall super-speed centrifuge with an SS-34 rotor equipped with a lidless run device to concentrate the PC-Tu. The concentrated PC-Tu is collected and 100 μM GTP and 1 mM MgSO₄ are added. The PC-Tu concentration is determined and the solution is drop-frozen in liquid nitrogen (previously described). The PC-Tu can be stored as frozen beads in an ultracold freezer at −70°C for at least 1 year without loss of assembly capability. A typical elution profile is shown in Fig. 2.
PAGE gels showing the CBE, the MTP, and PC-Tu (α- and β-tubulin are labeled) are shown in Fig. 3. Typical yields at various steps are shown in Table I.

D. Characteristics and Polymerization Properties of Purified Proteins

The MTP, C3S, after being stored in a −70°C freezer for 13 months, self-assembles into microtubules robustly, displaying quick self-nucleation as the temperature is raised from 0 to 30°C, followed by rapid elongation and eventual attainment of a stable polymer mass plateau (Fig. 4, solid line). As a rule, the composition is ∼70–75% tubulin and 20–25% MAPs.

For efficient polymerization of PC-Tu (Fig. 4, dashed line), assembly should be initiated by addition of nucleating seeds. The seeds we use consist of short glycerol-/DMSO-stabilized microtubules. For the assembly curve shown in Fig. 4, we used PC-Tu that had previously been stored for 15 months in a −70°C freezer. The seeds were prepared as follows. Ten percent of the PC-Tu solution to be assembled was removed and to it was added a total of 20% glycerol and 10% DMSO. The solution was warmed to 30°C and incubated for 20–30 min to assemble microtubules. The microtubules were then sheared by passing the suspension three times through a 25-gauge needle to reduce the microtubule mean length to ∼5 µm and to increase the microtubule number concentration. After 10 min of additional incubation to allow the microtubules to reattain a stable polymer mass steady state, the seeds were ready to nucleate microtubule polymerization. The original 10% volume of the microtubule seed suspension was then added back to the remaining 90% of the PC-Tu sample (prewarmed to 30°C) to nucleate microtubule polymerization in the remaining

Fig. 2 Typical elution profile of tubulin from a 100-ml phosphocellulose column equilibrated in PEM50G buffer and loaded with 150 mg of microtubule protein, C2S. Ninety-nine percent of the protein in the entire peak is tubulin.
sample. The final glycerol concentration was 2% and the final DMSO concentration was 1%. The nucleated microtubules elongated efficiently and achieved a stable polymer mass (Fig. 4, dotted line). The difference in overall absorbance between the MTP microtubule sample and the PC-Tu microtubule sample is indicative of the polymer mass and reflective of the difference in the tubulin critical concentration for assembly of the two samples.

Fig. 3  SDS PAGE of crude brain extra (CBE) and purified proteins. Lane A is the CBE. Lane B is purified microtubule protein after three cycles of assembly and disassembly and passage through a sucrose cushion. Lane C is phosphocellulose-purified tubulin. Ten micrograms of protein was loaded onto each lane and the developed gel was stained with Coomassie Blue. Note the separation between α- and β-tubulin, which is accomplished by using Sigma L-5750 as the source of SDS.

Table 1  Protein Yields in Typical Preparations Starting with Two Brains

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg/ml)</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet brain weight</td>
<td></td>
<td></td>
<td>562,000</td>
</tr>
<tr>
<td>CBE</td>
<td>15.8</td>
<td>930</td>
<td>14,700</td>
</tr>
<tr>
<td>H1P</td>
<td></td>
<td></td>
<td>980</td>
</tr>
<tr>
<td>C1S</td>
<td>9.6</td>
<td>64</td>
<td>600</td>
</tr>
<tr>
<td>H2P</td>
<td></td>
<td></td>
<td>360</td>
</tr>
<tr>
<td>C2S</td>
<td>23</td>
<td>12</td>
<td>280</td>
</tr>
<tr>
<td>H3P</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C3S</td>
<td>11</td>
<td>18</td>
<td>200</td>
</tr>
<tr>
<td>PC-Tu</td>
<td>11</td>
<td>14</td>
<td>150</td>
</tr>
</tbody>
</table>

Herbert P. Miller and Leslie Wilson
III. Buffer Compositions

L-GNPEM buffer plus DTT, pH 6.85
- 100 mM sodium glutamate
- 20 mM sodium phosphate
- 1 mM EGTA
- 0.5 mM MgCl$_2$
- 1 mM DTT (added at the time of use)

L-GNEPEM buffer, pH 6.75
- 100 mM sodium glutamate
- 20 mM sodium phosphate
- 1 mM EGTA
- 0.5 mM MgCl$_2$

PEM buffer, pH 6.8
- 100 mM PIPES
- 1 mM EGTA
- 1 mM MgSO$_4$

PEM50 buffer, pH 6.8
- 50 mM PIPES
- 1 mM EGTA
- 1 mM MgSO$_4$

10× PEM50 buffer, pH 6.8
- 500 mM PIPES
- 1 mM EGTA
- 1 mM MgSO$_4$

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Fig. 4  Polymerization characteristics of purified proteins as determined turbidimetrically (350 nm). MTP, 19 µM tubulin, solid line, and 18 µM PC-Tu, dashed line. Proteins were polymerized at 30°C in PEM buffer. The MTP was self-nucleated, whereas assembly of PC-Tu was initiated by addition of microtubule nucleating seeds.
IV. Concluding Comments

The methods we have described here for isolation of MTP and purified tubulin from bovine brain in the absence of assembly-promoting solvents have been used for a great many years in our laboratory. Mammalian brain has been an extremely valuable source of polymerization-competent tubulin for many experimental applications in cell and molecular biology. While the basic strategy for warm polymerization and cold depolymerization cycling has changed little, the specific steps and buffer conditions (e.g., GTP concentration, pH values to use) have been fine-tuned and refined extensively to optimize yields and purity. We have tried to be as thorough as possible in indicating critical steps and ensuring that the protocols are as simple as possible to follow. Finally, we want to emphasize that there is a considerable variation in the tubulin and MAP contents of mammalian tissues and, especially, among tissues from different eukaryotic organisms. Thus, the procedures described here may need to be substantially modified, or may not work at all, for many tissues or species.

Acknowledgments

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References

CHAPTER 2

Isolating Tubulin from Nonneural Sources

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Abstract

I. Why Tubulin from Nonneural Sources?
II. General Principles of Tubulin Purification
III. Specific Properties of Distinct Nonneural Sources
   A. Metazoan Sources (e.g., Tissue Culture Cells, Chicken Red Blood Cells)
   B. Tetrahymena thermophila
   C. Kinetoplastid Parasites (Leishmania, Trypanosoma)
   D. Saccharomyces cerevisiae
   E. Land Plants
IV. Genetic Manipulation of Tubulin Genes
   A. Genetic Manipulation of Tubulin Genes in S. cerevisiae
   B. Genetic Manipulation of Tubulin Genes in T. thermophila
V. Isolation of Tubulin: Theme and Variation
VI. Protocol Notes
VII. Summary
Acknowledgments
References

Abstract

Tubulin is a highly conserved, negatively charged protein that is found in essentially all eukaryotic cells. These properties ensure that isolation protocols successful in one system will likely work, with a few modifications, in most systems. Tubulin has been isolated most frequently from mammalian brain, and the main difference encountered in other systems versus brain is that tubulin is much less abundant in nearly all other sources than it is in brain. This means that attempting to purify
tubulin by direct polymerization from a homogenate will often fail or be quite inefficient. However, the conservation of negative charge on tubulin means that an initial ion exchange step can be used to both purify and concentrate the protein from most systems. Polymerization-competent tubulin can usually be obtained by inducing polymerization in the salt eluate from the ion exchange step. We describe protocols for this procedure and describe its application to a number of vertebrate, fungal, protozoal, and plant sources.

I. Why Tubulin from Nonneural Sources?

Although researchers have isolated tubulin from a large variety of organisms ranging from yeast to sea urchin to higher land plants, the source of pure tubulin for most biochemical assays is clearly vertebrate brain (Borisy et al., 1974; Farrell and Wilson, 1978; Morejohn and Fosket, 1982). Neurons contain abundant microtubules, and cow or pig brains obtained from slaughterhouses represent an economical and straightforward source for tubulin. However, researchers may have a variety of critically important reasons to exploit tubulin purified from alternative sources. These include characterization of tubulin isoforms that are distinctly expressed in nonneural cells or tissues such as flagellar-specific isoforms or altered tubulin expression in cancer cells (Aoki et al., 2009; Banerjee, 2002; Cucchiarelli et al., 2008; Galmarini et al., 2008; Kemphues et al., 1979; Kimble et al., 1990; Ohishi et al., 2007; Rao et al., 2001; Rawlings et al., 1992; Shalli et al., 2005; Verdier-Pinard et al., 2003). Another reason is a need for a tubulin sample containing fewer isotypes and fewer posttranslational modifications (PTMs) than the notoriously complex brain tubulin (Williams et al., 1999). Tubulin from cultured cell lines selected for resistance to particular drugs might be sought in order to study the binding site for the drugs (Sackett et al., 1997). Moreover, there may be a need to work with tubulin from specific species to characterize small molecule activity that is specifically directed to phylogenetically restricted tubulin subsets, such as the activity of the benzimidazoles (benomyl, albendazole, and mebendazole) on helminth and fungal tubulins or the action of dinitroanilines on plant and protozoan tubulins (Gill and Lacey, 1992; Hugdahl and Morejohn, 1993; Morejohn et al., 1987). Lastly, genetically tractable unicellular eukaryotes such as Saccharomyces cerevisiae or Tetrahymena thermophila can be used to express tubulins with directed mutations for characterization in biochemical assays (Callahan et al., 1984; Gaertig et al., 1995; Gupta et al., 2003, 2002). In this chapter, we discuss alternative sources of tubulin and how to purify tubulin dimers from these other sources.

II. General Principles of Tubulin Purification

The principal consideration in developing a method for purifying tubulin from most nonneural sources is the relatively low concentration of tubulin in the cell or tissue extract, compared to that found in brain homogenate. While tubulin can be assembled directly from clarified brain homogenate, it is inefficient or impossible to use induced polymerization as a first step to purify tubulin from these alternative sources. Instead, the first step following preparation of a clarified homogenate is to
concentrate tubulin while also effecting purification without microtubule assembly. Anion exchange chromatography is well suited to both of these roles. A benefit of using chromatography to isolate and concentrate tubulin is that cells can be lysed in a much larger volume, since there is no need to maintain a critical concentration for tubulin. While the details of each step in the protocol that follows may need to be adjusted somewhat for each source material, the general methods are the same. Rather than attempting to describe one exact protocol applicable to all source materials, we will outline a general series of steps and discuss the parameters to consider when applying this general protocol to a particular situation. The essential tubulin purification protocol entails these steps:

1. **Homogenize** cells/tissues to release soluble tubulin into an appropriate buffer
2. **Clarify** the tubulin-containing extract by centrifugation
3. **Isolate** tubulin from the extract by anion exchange chromatography
4. **Concentrate** tubulin eluted from the anion exchange material
5. **Polymerize** the tubulin and pellet microtubules
6. Adjust the **tubulin concentration**, aliquot, and **store** at −80°C or lower

We will discuss each of these procedures in greater detail in Section VI.

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### III. Specific Properties of Distinct Nonneural Sources

#### A. Metazoan Sources (e.g., Tissue Culture Cells, Chicken Red Blood Cells)

Nonneural sources of metazoan tubulin have included tissue culture cells, nucleated erythrocytes, and various tissues or organs. This list includes tissues such as liver (Jennett *et al.*, 1987) and testis (Lessman, 1993); specialized cells such as human lymphocytes (Liebes *et al.*, 1980), mammalian platelets (Castle and Crawford, 1978; Ikeda and Steiner, 1978), avian erythrocytes (Murphy, 1991), surf clam oocytes (Suprenant and Rebhun, 1984), sea urchin sperm (Shelanski and Taylor, 1968); and tumor cells cultured in monolayer (Sackett *et al.*, 1997) and ascites (Doenges *et al.*, 1977). These materials can be obtained in a variety of ways, but will eventually feed into the same protocol following homogenization. In all cases, the steps needed are to obtain and wash sufficient material for the prep and efficiently disrupt the washed cells to release soluble tubulin.

Tissue culture cells are in principle a good source for tubulin since they may express isotypes or bear PTMs of specific interest in the particular cells under study (Sackett, 1995; Sackett *et al.*, 1991, 1997). The main problem with tissue culture cells as a source of tubulin is obtaining sufficient starting material. A small-scale preparation with 1 g of cells to start can yield about 0.5 mg of tubulin, which is ~0.5% of the total protein. To obtain the starting material, cells are grown under appropriate culturing conditions, harvested from flasks, pelleted in a centrifuge, washed once with PBS, resuspended in an equal volume of PME buffer (see below), snap frozen on dry ice, and stored at −80°C or in liquid nitrogen. A typical T75 flask with a confluent (adherent) mammalian cell line can yield about 10^7 cells, and 1 g = ~2 × 10^8 cells, so many flasks will be required for a tubulin preparation of any size. Frozen cell pellets can be accumulated until sufficient material is obtained; we found no problem keeping the pellets frozen for extended periods of time, as we
earlier found for brain tissue which had been frozen quickly and stored appropriately (Sackett et al., 1991).

Red blood cells (RBCs) have been used as a source of tubulin due to the nearly single isotype nature of the protein obtained (Murphy, 1991). The original description of this isolation used chicken blood obtained at a slaughterhouse, but we have found that washed, packed RBCs from PelFreez (Rogers, AR) are a suitable alternative. These preparations are shipped so that they arrive on ice the morning after initial collection, and this time delay seems not to cause a problem.

B. *Tetrahymena thermophila*

*T. thermophila* is easily grown in large volumes of a simple inexpensive media (Sequestrene proteose peptone, SPP) at either room temperature or 30°C. We typically grow 6 l of culture at a time to isolate tubulin dimers; each 2-l flask contains 1 l of media and is inoculated with 30 ml of a starter culture (~2 × 10^4 cells/ml). Healthy growth of cultures requires good aeration (150 rpm on a rotary shaker). For optimal tubulin recovery, it is important to collect the cultures before they reach late log phase (no more than ~4 × 10^5 cells/ml) since log phase cultures secrete tetrain, a cysteine protease that effectively degrades tubulin (Suzuki et al., 1998). Pelleted *Tetrahymena* can be frozen overnight or for up to 2 months before tubulin purification (~80°C).

C. Kinetoplastid Parasites (*Leishmania, Trypanosoma*)

The purification of assembly-competent tubulin from *Leishmania* is simplified by the fact that tubulin constitutes an estimated 11% of the total cellular protein in the promastigote stage of this parasite (Fong and Chang, 1981). However, the large-scale growth of *Leishmania* species that are pathogenic to humans is undesirable due to biosafety concerns and because the media required for culturing these parasites to high cell densities is expensive. We have found that tubulin purified from *Leishmania tarentolae*, a species of *Leishmania* that infects reptiles rather than humans, is an attractive alternative. Higher cell densities can be achieved with *L. tarentolae* (up to 2 × 10^8 parasites/ml) (Morgan et al., 2008; Yakovich et al., 2006) compared to pathogenic species such as *Leishmania amazonensis* (up to 5 × 10^7 parasites/ml) (Werbovetz et al., 1999). Also, *L. tarentolae* is grown in relatively inexpensive brain heart infusion medium supplemented with hemin, while culturing pathogenic *Leishmania* species to high cell densities typically requires Schneider’s Drosophila medium containing up to 20% fetal bovine serum. The *L. tarentolae* α- and β-tubulin amino acid sequences are >98 and >96% identical to those found in pathogenic *Leishmania* species, respectively, and the susceptibility of *L. tarentolae* tubulin to antimitotic dinitroanilines is indistinguishable from that of the *L. amazonensis* protein (Yakovich et al., 2006).

Cultures of *L. tarentolae* can be passed in standard T_25_ flasks at 25°C and transferred to 21 flasks for large-scale growth on a shaker incubator set to 125 rpm and 25°C (Yakovich et al., 2006). We have not experienced decreased tubulin yields from cultures grown to late log phase and typically allow the parasites to reach this growth stage prior to harvesting the organisms by centrifugation at 1200 × g and 4°C. After washing the organisms with PBS, cell pellets can be stored at −80°C until use. The purification of assembly-competent tubulin from *Trypanosoma brucei* has
been reported by MacRae and Gull (MacRae and Gull, 1990), and our isolation of tubulin from *Leishmania* species (Morgan *et al.*, 2008; Werbovetz *et al.*, 1999; Yakovich *et al.*, 2006) was based on their protocol. Since MacRae and Gull reported that *T. brucei* procyclic parasites were cultured to a density of $2 - 4 \times 10^7$ cells/ml in medium containing 10% serum, we speculate that it would be more challenging to conduct large-scale tubulin purification from trypanosomes.

### D. *Saccharomyces cerevisiae*

Purification of tubulins from budding yeast was first accomplished in 1981 by John Kilmarten who purified assembly-competent dimers from 50 g of pelleted cells using diethylaminoethyl (DEAE)-Sephadex to isolate tubulin from other cytosolic proteins and ammonium sulfate to concentrate the column eluate (Kilmartin, 1981). These samples were assembled into microtubules for electron microscopy and used in assembly assays to assess the effects of colchicine and benomyl on tubulin polymerization. The technique was further optimized by Barnes, Louie, and Botstein, who purified *S. cerevisiae* tubulin with copurifying microtubule-associated proteins (Barnes *et al.*, 1992) and by Davis *et al.*, who purified tubulin for biochemical analysis (Davis *et al.*, 1993). Himes and colleagues have purified tubulins with directed mutations for characterization in vitro (Gupta *et al.*, 2002, 2003) and recently, tubulin has been purified from fission yeast (*Schizosaccharomyces pombe*) for biochemical assays (des Georges *et al.*, 2008).

There are many advantages to working with yeast (genetic tractability and ease of growth); however, the yeast cell wall and the relatively low concentration of tubulin dimers in budding yeast require starting with a large amount of packed cells and working to ensure that as many cells are disrupted as is possible. Growth in rich media (yeast extract peptone dextrose, YPD) is best for tubulin purification; bulk cultures that have reached late log or stationary phase after overnight growth can be supplemented with additional 2% glucose and allowed to grow for several more hours (Gupta *et al.*, 2002). This strategy achieves the dual purpose of providing additional starting material and getting the yeast out of late log/stationary phase, where their cell walls are harder to disrupt (Mohan Gupta, personal communication). Pelleted and washed yeast is stored at 4°C overnight to destabilize the microtubules prior to tubulin purification. Researchers have purified yeast tubulin from 500 g to >2 kg of packed wet cells. This can yield ~5 mg pure tubulin from 500 g of packed wet cells (Gupta *et al.*, 2002, 2003). Yeast cell wall disruption can be carried out enzymatically or, for larger amounts, mechanically, using a bead beater, coffee grinder, or microfluidics device with on-going monitoring to ensure that >95% of the cells are disrupted.

### E. Land Plants

Assembly-competent plant tubulin dimers were first isolated from cultured cells of Paul’s Scarlet rose by Morejohn and Foskett (Morejohn and Fosket, 1982). Tubulin has also been isolated from plant tissues, but isolation of material from tissues is more problematic than isolation of tubulin from cultured cells due to polyphenol secondary metabolites which can cause protein crosslinking and compromise the quality of recovered tubulin. One strategy to reduce secondary metabolites is to use
plants grown in the dark; etiolation is associated with decreased polyphenolic production (Davies, 1972). However, the best solution is to use cultured plant cells, which are low in secondary metabolites. Cultured cells in early exponential growth are preferable since cellular proteins are being synthesized rapidly and polyphenolic compounds are at their lowest concentrations (Davies, 1972; Morejohn and Fosket, 1982). Assembly-competent plant tubulin dimers can be isolated from a variety of cultured plant cells including carrot (Daucus carota) (Dawson and Lloyd, 1985; Morejohn et al., 1984), hibiscus (Hibiscus rosa-sinensis) (Morejohn et al., 1984), and tobacco (Nicotiana tabacum) (Yadav and Filner, 1983). To maximize success, choose a nonpigmented cell culture (e.g., cells cultured in the dark or cell types that lack pigment production) and be sure the culture has a high percentage of actively dividing cells. Before starting tubulin isolation check the health of the plant culture under the microscope. Living cells have centrally located nuclei with distinct cytoplasmic strands and the cytoplasm appears smooth in texture. In contrast, dead and dying cell nuclei are appressed to the side of the cell; they lack cytoplasmic strands and the cytoplasm appears rough in texture (Richard Cyr, personal communication).

Different plant cells have diverse properties and grow at distinct rates. For example, BY2 cells (tobacco) grow quickly, requiring subculturing each week, whereas most Arabidopsis cultures grow more slowly and only require subculturing every other week. Cells such as carrot that have a dense cytoplasm resembling the apical meristem are superior to cells such as BY2 that have a parenchymal character (less cytoplasm and more vacuole) for protein purification since greater cytoplasm per cell provides more total protein for purification. Cells that grow faster also tend to have more recoverable tubulin. A good beginning strategy is to grow 100 ml cultures in ten 500 ml Erlenmeyer flasks to mid-log phase (e.g., 3–4 days for BY2 cells). It is possible to scale up to as many as 120 flasks for a single isolation. Plant cell collection requires porous cloth with a biased weave such as the backing material used for furniture upholstery or Miracloth (sold by Calbiochem, La Jolla, CA). Three sheets of cloth are arranged at 90° to each other to construct a collection filter which is seated into a large Erlenmeyer flask. Cells are retained by the cloth and excess culture media is removed by wringing the cloth. Isolated cells should be rinsed in ice-cold PM buffer to remove secreted factors that adversely affect protein purification. Cells from the wrung-out cloth should be weighed so that PME can be added at a ratio of 2 ml per 3 g of cells. After the suspended cells are >95% disrupted (see later), centrifuge the lysate at 4°C for 20 min at 7000 × g. Remove the floating lipid layer, collect the supernatant, and centrifuge a second time at 48,000 × g for 30 min. Skim additional floating lipids and pool the supernatant for application to a DEAE column (Richard Cyr, personal communication).

IV. Genetic Manipulation of Tubulin Genes

A. Genetic Manipulation of Tubulin Genes in S. cerevisiae

S. cerevisiae has been used to extensively dissect tubulin function by generation of either spontaneous or directed mutations to α-, β-, or γ-tubulin. Budding yeast has two α-tubulin genes, TUB1 and TUB3, and a single β-tubulin gene, TUB2 (Neff
et al., 1983; Schatz et al., 1986a). TUB1 and TUB2 are essential genes, but TUB3 can be deleted without loss of viability, although the tub3 null has some meiotic/sporulation defects (Schatz et al., 1986b). Well-established methods allow researchers to delete essential genes in S. cerevisiae by supplying a copy of the gene in trans on a nonintegrating plasmid that segregates as a minichromosome. These strains can be used to test the effects of mutant forms of the gene in the absence of the wild-type gene by performing a “plasmid shuffle” which uses positive and negative selectable markers to simultaneously select for transformation of a second plasmid that carries an altered allele and selects against the original complementing plasmid containing the wild-type allele (Boeke et al., 1987; Caron et al., 2001; Schatz et al., 1988). If altered α- or β-tubulin alleles can be shuffled into yeast with chromosomally deleted copies of the gene, the resulting strain can be used to purify a homogeneous population of tubulin with an altered amino acid sequence.

An alternative strategy to express altered tubulins in yeast is to generate a tubulin gene replacement in diploid cells. This is accomplished by linking an altered tubulin gene to a marker which permits selection for homologous integration into one of the two copies of the tubulin gene. In the case that sporulation yields four viable haploids, the cells that bear the gene replacement as the sole source of tubulin can be isolated by tetrad dissection. These cells express a homogeneous population of tubulin bearing the directed mutation. In the case that the diploid is viable but the sporulated haploid mutant is not, mutant tubulins coexpressed with wild-type tubulin can be purified to homogeneity using an affinity tag such as a carboxy-terminal His6 tag. Himes and colleagues used a gene replacement strategy to examine the effect of mutations to C354 of β-tubulin on microtubule dynamics (Gupta et al., 2002) and to probe the binding site amino acid requirements for tubulin–taxol interactions (Gupta et al., 2003). In both studies, the haploid mutant lines were viable, permitting isolation of homogeneous tubulin populations without purification away from wild-type tubulin dimers.

B. Genetic Manipulation of Tubulin Genes in T. thermophila

One of the original biochemical sources for tubulin is the free-living protozoan T. thermophila; tubulin was first purified from Tetrahymena in 1976 (Kuriyama, 1976). Tetrahymena is a ciliate, with an abundant microtubule cytoskeleton. In addition to its ease of growth and abundant tubulin, Tetrahymena is genetically tractable, containing a single, essential α-tubulin gene (ATU1) and two β-tubulin genes (BTU1 and BTU2) which encode identical proteins (only one gene is essential) (Barahona et al., 1988; Callahan et al., 1984; Gaertig et al., 1993). Due to its unusual nuclear organization and life cycle, it is possible to knock out essential genes in Tetrahymena and to later select for genes to complement this loss. Tetrahymena contains two nuclei: a “germ line” micronucleus which is a transcriptionally silent “backup” copy of the genome and a “somatic” macronucleus which contains the actively transcribed genome. When an essential gene is knocked out in the micronucleus, asexually growing cells are unaffected because they retain the expressed macronucleus copy of the gene. However, when Tetrahymena undergo sexual recombination, macronuclei are destroyed and new ones are reconstituted from the micronucleus after meiosis. Therefore, when Tetrahymena bearing an essential gene deletion in the micronucleus are forced to undergo sexual recombination, they only
survive if the missing gene is supplied in trans for integration into the new macronucleus. We have used existing α-tubulin knockout strains (Gaertig et al., 1995; Hai and Gorovsky, 1997) to substitute modified α-tubulin genes in Tetrahymena. Modified α-tubulin genes are introduced into mating pairs using a biolistic gene gun and only Tetrahymena that successfully integrate this gene copy into the macronucleus survive. We have introduced directed point mutations into the Tetrahymena α-tubulin gene that corresponds to amino acid substitutions that are associated with dinitroaniline resistance in the protozoan parasite Toxoplasma gondii. The α-tubulin genes bearing these mutations both complement the α-tubulin knockout and confer dinitroaniline resistance in the transformed Tetrahymena. We have purified tubulin with single amino acid substitutions that confer dinitroaniline resistance for drug binding and microtubule assembly assays. Tubulin isolated from these engineered strains assembles in the presence of the dinitroaniline oryzalin, while wild-type Tetrahymena microtubule assembly is inhibited (N. Morrissette and S. Lyons-Abbott, in preparation).

V. Isolation of Tubulin: Theme and Variation

You may anticipate using the following equipment:

- Centrifuge
- Water bath (37°C)
- DEAE-Sepharose column
- Peristaltic pump
- Disruption device (e.g., sonicator, microfluidics device, French Press)
- Glass wool
- Tubes, pipettes
- Ultracentrifuge tubes

Buffers for cell lysis, column chromatography, and tubulin assembly are typically simple variations on a physiological buffer (phosphate buffers, MES buffers, etc.) with additional components such as protease inhibitors, potassium chloride, and glutamate for column elution and guanosine triphosphate (GTP) and dimethylsulfoxide (DMSO) for microtubule assembly.

You may anticipate using the following solutions:

- **PME + P buffer (PME plus protease inhibitors)**
  0.1 M Pipes pH 6.9
  1 mM MgCl₂
  1 mM EGTA (ethylene glycol tetraacetic acid)
- **PME + P column wash buffer**
  0.1 M Pipes pH 6.9
  1 mM MgCl₂
  1 mM EGTA
  0.1 M KCl
  0.25 M glutamate

If the wash buffer causes tubulin loss of the column, the KCl can be omitted and the pH adjusted to 6.5 as an alternative method of increasing wash stringency.
**PME + P column elution buffer**
- 0.1 M Pipes pH 6.9
- 1 mM MgCl\(_2\)
- 1 mM EGTA
- 0.3 M KCl
- 0.75–1.0 M glutamate

**Microtubule assembly buffer**
- 2 mM GTP
- 8% (v/v) DMSO
- 10 mM MgCl\(_2\)

Suspend in 1× PME

***Protease inhibitors***: Different cell types vary in protease content; therefore, investigators may need to optimize protease inhibitor additions for the lysis and elution buffers. We list a few considerations for specific cell types later.

- *T. thermophila* has a secreted cysteine protease (tetrain) that influences tubulin purification. Effective inhibitors include 1 mM benzamidine, 1 mM 4-(2-aminoethy)benzenesulfonyl fluoride hydrochloride, 800 nM aprotinin, 50 µM bestatin, 15 µM E64, 10 µM pepstatin, and 25 µg/ml leupeptin.
- *S. cerevisiae* proteolysis does not appear to be a significant issue during tubulin purification. Himes and colleagues supplement lysis buffer with 2 mM phenylmethylsulfonyl fluoride (PMSF) (Gupta *et al.*, 2003).
- *Leishmania major* is known to contain genes encoding 65 cysteine proteases (Ivens *et al.*, 2005; Mottram *et al.*, 2004). In the absence of cysteine protease inhibitors, immunoblotting of *L. amazonensis* lysates prepared by sonication using an anti-α-tubulin antibody indicates the appearance of an approximately 35-kDa band in addition to the expected 50-kDa band. We therefore include 25 µg/ml leupeptin during the lysis, clarification, and chromatography phases of our purification protocol. Serine peptidases are also present in the *Leishmania* genome (Mottram *et al.*, 2004), so benzamidine (1 mM) and PMSF (0.5 mM) are used in these buffers as well.
- Plant cells have proteases and polyphenol oxidase activity which can degrade and crosslink proteins during the purification process. Reducing agents inhibit polyphenol oxidase activity: 15 mM dithiothreitol (DTT) is an effective inhibitor (Moore *et al.*, 1997). If the buffer solution turns brown during extraction (indicating polyphenol oxidase activity) add more DTT to the sample. Abundant protease inhibitors will improve the quality of the isolated tubulin: use antipain, aprotinin, chymostatin, leupeptin, and pepstatin at 10 µg/ml, BAME and TAME at 50 µg/ml, and PMSF at 1 mM. Make stocks of each using the appropriate solvents rather than the commercially available combination tablets. Keep the extraction buffer pH neutral and use Mg\(^{2+}\) and EGTA to further inhibit protease activity (Richard Cyr, personal communication).

### Step 1. Homogenize cells/tissues

Methods for disrupting cells vary extensively. Plant cells and budding yeast have cell walls that need to be efficiently broken in order to effectively obtain a cytoplasmic lysate. Protozoans such as *Tetrahymena* and the kinetoplastid parasites have much of their tubulin in stable structures such as flagella/cilia and subpellicular/corset microtubules.
Devices and methods to disrupt specific cell types are listed below:

- **Vertebrate tissues**: Tissues are homogenized in 2 ml/g of PME with protease inhibitors appropriate to the particular tissue. Sonication on ice can also be quite effective. Check for the degree of cell lysis by microscopy.
- **Avian erythrocytes**: Sonication is the usual method of lysis. Packed erythrocytes are resuspended in 2 ml/g of PME and sonicated on ice in 30-s bursts. For volumes of greater than 100 ml, it is important to use the stub horn on the sonicator, rather than the microtip attachment. Lysis can be easily monitored by microscopy.
- **Tetrahymena disruption**: *T. thermophila (~2 × 10⁹ cells, 6 l of mid-log culture)* are collected by centrifugation at 2500 × g for 10 min at room temperature and frozen overnight at −80°C. Pellets thawed on ice are resuspended in 40 ml PME + P buffer on ice and sonicated (BioRad, Hercules, CA) with ten 30-s bursts at 25 W and a 2-min cooling interval between each burst.
- **S. cerevisiae** disruption has been carried out using coffee grinders, bead beaters, or by a microfluidics device. The cost-effective coffee grinder method disrupts a 50% slurry of yeast in buffer flash frozen into liquid nitrogen using commercially available grinders. By far the most efficient method of disruption employs a microfluidizer device to treat yeast cells 5–10 times. Disruption of >95% of the cells is essential to obtain efficient tubulin purification.
- **Leishmania** disruption: extensive sonication is required to liberate tubulin heterodimers from the stable subpellicular and flagellar microtubules present in *Leishmania*. Lysis is carried out on ice employing a probe sonicator set at full power (25 W). We use ten 30-s bursts with 2-min cooling intervals between bursts; additional rounds of sonication do not increase the yield of *Leishmania* tubulin in our experience.
- **Plant cell disruption**: plant cell walls from different species vary considerably in thickness and therefore may require different methods for disruption. Plant cells can be disrupted by homogenization or by using a basic blender or bead beater. Flash-frozen material can be disrupted using the coffee grinder method described for yeast. Pressure disruption using a French Press at 5000 psi consistently disrupts plant cells and is the best choice, if available. The degree of cell disruption should be monitored by microscopy; it is essential to identify conditions that result in >90% cell disruption yet leave cell nuclei intact (the presence of nucleoli separated from nuclei indicates that the disruption conditions are unnecessarily harsh). Once suitable disruption conditions are established, it is critical to work quickly and keep the sample ice cold.

**Step 2. Clarify** the tubulin-containing extract by centrifugation

Once cells are disrupted, the cell suspension is cooled on ice for 30 min, then centrifuged at 40,000 × g for 40 min at 4–80°C. The decanted supernatant can also be filtered through glass wool to remove particulates prior to loading onto a column for purification.

**Step 3. Isolate** tubulin from the extract by anion exchange chromatography

Both α- and β-tubulin subunits are slightly acidic, with isoelectric points of approximately 4.8–5.2. They are effectively bound to positively charged resins
such as DEAE-Sepharose until eluted with buffers containing a high salt concentration. See Section VI, Note 1, for more details about this step. The clarified homogenate is poured over a preequilibrated column of DEAE-Sepharose or equivalent and the flowthrough collected in fractions. The column is washed with 1 column volume of PME + P wash buffer and then eluted with 2 column volumes of PME+P column elution buffer, again collecting fractions. Most of the tubulin will be in the fractions corresponding to the first column volume of elution, but this should be checked by sodium dodecyl sulfate (SDS) gel. See Section VI, Notes 2 and 3, for discussion of optimization.

**Step 4. Concentrate** tubulin eluted from the anion exchange material

In order for tubulin to assemble after elution, it must be above the critical concentration. Methods to concentrate tubulin include concentration by ammonium sulfate precipitation and concentration in devices that use dialysis filter and centrifugation. For small volumes (<~50 ml) we have found that centrifugal concentrators, such as the conical devices from Pierce (Thermo Scientific, Rockford, IL), are very convenient. For larger volumes, ammonium sulfate precipitation is useful (Andreu, 2007). For this purpose, ammonium sulfate is added to 43% saturation, which is done by adding, with constant stirring on ice, 25.3 g ammonium sulfate per 100 ml solution. If the volume is not too large, the solution may be combined with a saturated solution of ammonium sulfate kept at 4–80°C, in the ratio of 2 volumes of saturated ammonium sulfate to 3 volumes of column eluant. In either case, the solution is allowed to stand on ice for at least 10 min after all the solid is dissolved or all of the saturated solution is added. The solution is then centrifuged for 10 min at ~20,000 × g, the supernatant removed, and the pellet redissolved in a small volume (~2 × the pellet volume) of 1 M sodium glutamate buffer.

**Step 5. Polymerize** the tubulin and pellet microtubules

Microtubule assembly from eluted tubulin is induced with GTP and DMSO. Tubulin polymerization is promoted by the sodium glutamate present in the elution buffer or resolubilization buffer. The tubulin solution is incubated at 37°C for 60 min for assembly and then spun at 50,000 × g at 30°C for 30 min. The pelleted microtubules are resuspended in cold PME buffer (to a concentration of ~10 mg/ml or higher) and further solubilized via probe sonication (ten to thirty ~5-s bursts at 10 W). This tubulin-rich solution is incubated on ice for 30 min and then spun at 50,000 × g at 4°C for 30 min to remove insoluble contaminants.

**Step 6. Adjust the tubulin concentration**, aliquot, and store at −80°C or lower

The supernatant containing heterodimeric tubulin is stored at −80°C in 50 µl aliquots. Alternatively, the solution may conveniently be drop-frozen in liquid nitrogen and stored at −80°C or in liquid nitrogen. Tubulin concentration may be determined by UV absorbance, using extinction for native tubulin of $E_{276} = 1.16 \text{ g/(l/cm)}$, or by dilution in neutral SDS, with extinction $E_{276} = 1.07 \text{ g/(l/cm)}$ (Andreu, 2007). See Section VI, Note 4, for information on quantifying tubulin in whole cells to assess purification efficacy.
VI. Protocol Notes

1. For the ion exchange step, it is important that the DEAE be equilibrated and that there be sufficient column material for the preparation that is being loaded onto it. For equilibration, consult the manufacturer for specific details, but a useful general protocol is to wash the resin with 1 volume of 10x loading buffer, followed by 5 volumes of 1x loading buffer. To be certain of equilibration, one should be certain that the pH and conductivity of the final wash buffer are not changed following exposure to the resin, but we have found that this is usually not necessary. The capacity of the column should be considered. These exchangers have a rated capacity of ~50–100 mg of albumin per milliliter of settled bed. We have found that using a volume of exchanger with capacity equal to the total amount of soluble protein in the clarified homogenate is usually a good guideline. In this regard, the yield of protein differs considerably between different starting materials, but a guide is as follows: tissues yield soluble protein ~1–2% of the wet weight of tissue, while cells yield more, ~8–10% of wet weight, although for some cells such as erythrocytes, the yield is even higher, ~20% of wet weight. Finally it is worth noting that the column geometry should be short and squat, since for a given volume of exchanger, added length does not improve separation, but does significantly slow flow.

2. To optimize the prep, it is important to know that all tubulin is being absorbed from the loading solution and to know what volume of elution is sufficient to recover the bulk of tubulin. For both of these, running an SDS gel is a good first step. If the loading solution contains so many other proteins that the tubulin band is unclear, then it may be necessary to do an immunoblot. In either case, the fractions that were collected while loading allow a determination of what volume of solution could be loaded before tubulin began to “break through” in the loading phase. For the elution, it may be sufficient to take all fractions that have OD_{280} ≥ 0.1 times the highest tube. However, it may be desirable to keep more than this in some cases. For example, the highly glutamylated species will probably be eluted later, so if these are of particular interest it may be necessary to continue to collect fractions beyond the arbitrary cutoff.

3. After the presence of tubulin in column fractions can be demonstrated by gel electrophoresis or immunoblotting, microplate assembly assays can be performed as a fast and routine method to verify the presence of assembly-competent tubulin. These assays, which involve the addition of column fractions, DMSO (to 10% v/v), and GTP (to 1 mM) to PME buffer, provide a more rapid means of identifying tubulin-rich fractions than gel electrophoresis or immunoblotting.

4. It may be useful to quantify the concentration of tubulin per cell in order to have a basic sense of its abundance in order to assess the efficiency of extraction and purification. Prepare cell lysates for your system of choice using a defined number of cells per volume SDS polyacrylamide gel electrophoresis (PAGE) loading buffer in order to be able to load total protein from a set cell number onto a protein gel. Run a dilution series of this lysate along with standards consisting of known amounts of purified tubulin. Purified tubulin at a defined concentration is available from Cytoskeleton, Inc. (Denver, CO). After resolving samples and standards by SDS PAGE blotted to a suitable membrane for immunoblot analysis,
blots should be probed with pan-specific tubulin antibody such as the 1-5-2 mouse anti-α-tubulin monoclonal antibody (available from Sigma, St. Louis MO). The relative intensity of individual samples can be quantified relative to the tubulin standards.

### VII. Summary

Tubulins are highly conserved proteins found in all eukaryotes, and tubulin dimers from diverse organisms have similar biochemical properties such as GTPase activity, assembly and disassembly properties, and polymer structure. Historically, the bulk of the work characterizing tubulin structure and biochemistry has focused on tubulin from neural sources. Although the high degree of amino acid conservation among α- and β-tubulins allows researchers to draw parallels between vertebrate brain tubulin properties and tubulins from other sources, there are examples of differences in amino acid sequences between tubulin types that impart distinct drug sensitivities or permit specific functions such as construction of the flagellar apparatus. Moreover, in order to understand how tubulin mutations, changes in isoform levels, or PTMs affect the properties of a tubulin population, it is essential to purify tubulin from specific sources to compare its behavior to that observed with the omnipresent brain tubulin samples. The protocol and variations presented here are intended to assist researchers to develop protocols that can be optimized to purify tubulin from diverse nonneural sources for such studies.

### Acknowledgments

NM would like to thank Mohan Gupta (University of Chicago), Richard Cyr (Pennsylvania State University), and Sally Lyons-Abbott (UCI) for information, comments, and corrections. This work was supported in part by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH (DLS), and NIH grant AI067981 (NSM).

### References


CHAPTER 3

Characterization of Anti-β-tubulin Antibodies

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Abstract

Tubulin antibodies are among the most extensively used immunological reagents in basic and applied cell and molecular biology. In this chapter, we provide a brief overview of the practices and reagents developed in our laboratory during the past 25 years for characterizing anti-β-tubulin antibodies.

I. Introduction

Many of the commercially available anti-β-tubulin antibodies are marketed with minimal information regarding their specificity and the epitopes to which they bind. These shortcomings limit their utility. Here, we describe the characterization of pan-specific and isotype-specific monoclonal and polyclonal anti-β-tubulin antibodies. These antibodies have been used extensively for Western blotting, affinity purification, and immunohistochemistry. We also discuss our more recent efforts to generate and characterize monoclonal and polyclonal antibodies that specifically recognize...
glutamylated tubulin. Hopefully, this overview will be useful to investigators interested in producing their own antibodies, as well as to those who depend on commercially available reagents for their research.

II. The Characterization of AA2, a Pan-specific Anti-β-tubulin Monoclonal Antibody That Reacts with All Vertebrate β-Tubulin Isotypes (Gene Products)

Pan-specific α- or β-tubulin antibodies are frequently used in Western blot experiments to demonstrate protein abundance relative to tubulin. They are also used extensively to illuminate changes in cell morphology and cytoskeletal organization during development, or as a result of disease, drug treatment, or genetic manipulation. These antibodies are presumed to recognize equally all of the expressed α- or β-tubulin isotypes. A recent report demonstrates that at least one of the widely used commercially available monoclonal antibodies, Tub 2.1, fails to meet this requirement (Yang et al., 2009). Presently, we know of two commercially available pan-specific anti-β-tubulin mouse monoclonal antibodies whose epitopes have been partially mapped, DM1B and AA2. Both antibodies react with epitopes located within the C-terminal domain spanned by amino acids 412–430. This region is the most highly conserved in β-tubulin, in both animal and plant species (Arevalo et al., 1990; Blose et al., 1984; de la Vina et al., 1988; Sullivan, 1988). The sequence of these 31 amino acids is entirely conserved in seven of the eight human β-tubulin isotypes, and with one exception, a conservative substitution, in seven of the eight mouse β-tubulin isotypes (Fig. 1). Only the megakaryocyte-specific class VI β-tubulin isotype (βVI) contains nonconservative substitutions in this region that appear likely to interfere with the binding of either DM1B or AA2.

DM1B was generated with native chick brain tubulin as the immunogen, whereas we generated AA2 with phosphocellulose-purified bovine brain tubulin (Blose et al., 1984). When it became apparent that AA2 recognizes a highly conserved epitope present in vertebrate and nonvertebrate tubulins, we used a bovine serum albumin-conjugated synthetic peptide β(412–430) and a bacterially expressed fusion protein consisting of the maltose-binding protein (MBP) and the extended C-terminal domain of β-tubulin (400–429) to establish that the epitopes for AA2 and DM1B were located in the same region. A Western blot demonstrating the specificity of AA2 is presented in Fig. 2.

The characterization of AA2 exemplifies the procedures that we have employed to characterize isotype-specific tubulin antibodies as well as other proteins.

We use standard procedures to generate mouse monoclonal antibodies, and commercial suppliers to provide rabbit polyclonal antisera (Binder et al., 1985; Caceres et al., 1984). Mouse IgGs are purified from tissue culture supernatants by conventional Protein-G chromatography. Rabbit IgGs are purified first from serum proteins by Protein-A chromatography, and specific IgGs are then isolated by peptide affinity chromatography.

The mouse monoclonal antibodies and the affinity purified rabbit polyclonal antibodies are screened first by enzyme-linked immunosorbent assay (ELISA) to determine their reaction with nondenatured purified bovine or rat brain tubulin. This
3. Characterization of Anti-β-tubulin Antibodies

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### Mouse

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**Fig. 1** An alignment of the amino acid sequences comprising the extended C-terminal domains of the eight human and eight mouse β-tubulin isotypes. Highlighted residues (gray) indicate the common domain containing the epitopes for the monoclonal antibodies AA2 and DM1B. Residues highlighted in yellow represent non-identical positions within the common domain. (See Plate no. 1 in the Color Plate Section.)

**Fig. 2** The anti-β-tubulin pan-specific monoclonal antibody AA2 reacts with an epitope located within the extended C-terminal domain of β-tubulin. MBP-tubulin fusion protein (β-tubulin, amino acids 400–429) (lane 1); purified rat brain β-tubulin (lane 2). Lanes 3 and 4 are the corresponding Coomassie-stained gel bands of protein samples loaded in lanes 1 and 2, respectively.
step is obligatory. We have produced numerous antibodies that react well with the synthetic peptide used as an immunogen, but react weakly or not at all with tubulin. Following this initial screening, we probe a set of bacterially expressed MBP fusion proteins by Western blotting to more precisely determine antibody specificity. The construction of these MBP fusion proteins has been described previously (Hiser et al., 2006).

III. The Characterization of Isotype-Specific $\beta$-Tubulin Monoclonal and Polyclonal Antibodies

Our laboratory has produced a number of isotype-specific $\beta$-tubulin antibodies that can be used for both biochemical analyses and immunocytochemistry (Bhattacharya et al., 2008; Katsetos et al., 2001; Lee et al., 1990a,b; Moody et al., 1996). Isotype-specific $\beta$-tubulin antibodies are in great demand. They are used to determine tubulin isotype abundance, cellular distribution, and subcellular localization during early brain development as well as to identify changes in isotype expression associated with oncogenesis and in response to drug therapy (e.g., Akasaka et al., 2009; Anthony et al., 2004; Arai et al., 2002; Dumontet et al., 2009; Gan and Kavallaris, 2008; Hayashi et al., 2009; Jaglin et al., 2009; Keays et al., 2007; Lee et al., 1990b; Miller et al., 2008; Nakamura et al., 2003; Saussedee-Aim et al., 2009; Sugita et al., 2005; Terada et al., 2005; Terry et al., 2009; Wu et al., 2009). For example, there is steadily increasing evidence that the aberrant expression of the neuronal-specific $\beta$-tubulin isotype ($\beta$III) is correlated with tumorigenesis and increased resistance to chemotherapeutic agents (e.g., Dumontet et al., 2009).

With one exception, all our isotype-specific $\beta$-tubulin mouse monoclonal and rabbit polyclonal antibodies were produced with synthetic peptides conjugated to keyhole limpet hemocyanin. Each peptide was identical in sequence to all or a portion of the extreme C-terminal isotype-defining domain of one of the eight human and eight mouse $\beta$-tubulin isotypes. The amino acid sequences comprising the entire isotype-defining domains of each $\beta$-tubulin isotype (431 to the C-terminus) are shown in Fig. 1. In order to determine antibody specificity by Western blotting, dot blotting, and ELISA, we constructed a set of MBP fusion proteins in which each one of the human and the mouse $\beta$-tubulin C-terminal isotype-defining domains is represented (Hiser et al., 2006). These MBP fusion proteins, approximately 45 kDa, offer several important advantages compared to synthetic peptides. They are extremely easy to purify, are highly soluble in aqueous solutions, and adhere well to a solid support. They can also be used as standards for estimating $\beta$-tubulin isotype abundance.

A complete list of the isotype-specific anti-$\beta$-tubulin monoclonal and polyclonal antibodies that we have produced is shown in Table I. On dot blots and Western blots, each of these antibodies recognizes only purified tubulin and the MBP fusion protein related to the peptide immunogen.

The anti-$\beta$II and $\beta$III antibodies were generated without any difficulty. In contrast, we have been unsuccessful in producing antibodies that are specific for either $\beta$I or $\beta$IV. Our antibodies react with both isotypes. This failure may be due to the fact that the sequences of the synthetic peptides used as immunogens are identical to the last
seven C-terminal amino acids for βI and βIV. These sequences are highly similar, different by a single residue. However, other investigators have been successful. Monoclonal antibodies specific for these two isotypes are commercially available (Banerjee et al., 1992; Roach et al., 1998).

Our first attempt to isolate an anti-human βV also resulted in failure. The immunogen was a synthetic peptide identical to the last C-terminal 20 amino acids of human βV (Fig. 1). All of the selected monoclonal antibodies not only reacted strongly with the βV MBP fusion protein, but also reacted, albeit less strongly, with the βIII MBP fusion protein. An alignment of the βIII and βV C-terminal isotype-defining sequences indicated that an internal sequence of seven amino acids was the most probable source of the cross-reaction (YEDDEEE/FEDEEEE).

To overcome such potential sources of cross-reaction, we devised a strategy of immunizing with truncated synthetic peptides that minimize the influence of flanking sequences on the immune response to the targeted epitope. For example, to generate specific anti-human βV and anti-mouse βV polyclonal antibodies, we used as immunogens synthetic peptides that are identical to human βV and mouse βV only at the last four C-terminal amino acids, CGGGEIDG and CGGGEINE (Bhattacharya et al., 2008).

More recently, we have used this strategy of immunizing with truncated peptides that minimize the influence of flanking sequences to generate antibodies that recognize the γ-glutamyl link in glutamylated tubulin.

### IV. The Characterization of Antibodies That Recognize the Glutamyl Side Chain of Glutamylated Proteins

Tubulin is a substrate for several types of evolutionarily conserved posttranslational modifications: detyrosination and retyrosination, acetylation, phosphorylation, palmitoylation, glutamylation, and glycylation (Alexander et al., 1991; Argarana et al., 1977; 1978; Caron, 1997; Edde et al., 1990; Eipper, 1972; Fukushima et al., 1984; Table I).
2009; Janke et al., 2005; L’Hernault and Rosenbaum, 1985; Ozols and Caron, 1997; Redeker et al., 1994; Rosenbaum, 2000; Westermann and Weber, 2003). Of these posttranslational modifications, three, the detyrosination and retyrosination of α-tubulin and the glycylation and glutamylation of α- and β-tubulin, are catalyzed by amino acid ligases belonging to the ATP-grasp superfamily of enzymes (Galperin and Koonin, 1997; Janke et al., 2005).

Our laboratory has a longstanding interest in the glutamylation of brain tubulin (Alexander et al., 1991; Redeker et al., 1998, 2004). Glutamylation is a covalent, reversible modification that results in the formation of a string of glutamate residues linked through the side chain of a glutamate residue in the polypeptide backbone. The number of glutamate residues in the glutamylation side chain can vary considerably. Whereas the initial glutamate residue is added through an obligatory γ-carboxyl link, the remaining residues are added through α-carboxyl links (Redeker et al., 1996). This modification was initially considered unique to tubulin; however, recent evidence indicates that a large, heterogeneous population of proteins is a substrate for glutamylation (Janke et al., 2008; Regnard et al., 2000). In tubulin, the target residues are located in the C-terminal isotype-defining domains. The position of the glutamylation sites in βI, II, III, and IV has been identified by mass spectrometry (Alexander et al., 1991; Mary et al., 1994; Redeker et al., 1992; Rudiger et al., 1992). BV, a minor β-tubulin isotype that is not present in neural tissue, appears to be glutamylated, but the site has not been identified (Verdier-Pinard et al., 2005). The megakaryocyte-specific β-tubulin, βVI, is not a substrate for glutamylation (Rudiger and Weber, 1993). Examples of the structures of monoglutamylated and diglutamylated peptides identical in their primary sequences to the βIII and βII C-terminal isotype-defining domains, respectively, are shown in Fig. 3.

**The structures of mono- and di-glutamyl branched peptides**

(A) Met-Tyr-Glu-Asp-Asp-Glu-Glu-Ser-Glu-Ala-Gly-Pro-Lys

(B) Asp-Glu-Gln-Gly-Glu-Phe-Glu-Glu-Glu-Gly-Asp-Glu-Ala

**Fig. 3** The structures of a monoglutamyl and a diglutamyl branch are shown. The first glutamate in the branch is added through an obligatory γ-carboxyl peptide bond and the additional glutamate residues are added only through α-carboxyl peptide bonds. There is no evidence indicating that polyglutamylation occurs on any other amino acid. The sequence of the monoglutamylated peptide shown in (A) is identical to the C-terminal isotype-defining domain of mouse class III β-tubulin. The sequence of the diglutamylated peptide shown in (B) is identical to the C-terminal isotype-defining domain of mouse class II β-tubulin.
Of the different tubulins characterized by mass spectrometry, mammalian brain tubulin ranks as perhaps the most extensively glutamylated. More than 96% of the tubulin purified from mature rodent and bovine brain is glutamylated (D. Hunt and A. Frankfurter, unpublished observations). Compared to both adult and neonatal brain tubulin, the proportion of glutamylated tubulin subunits to nonglutamylated tubulin subunits is considerably lower in tubulin from other organs (V. Redeker and A. Frankfurter, unpublished observations). Further, during early postnatal brain development, the proportion of glutamylated α- and β-tubulin subunits increases dramatically, as does the average number of residues in the γ-glutamyl side branch (Redeker et al., 2004). Since, the α- and β-tubulin isotypes expressed in brain are not glutamylated to the same extent, this developmentally regulated posttranslational modification produces in the adult brain an extremely heterogeneous population of glutamylated tubulin subunits. Because there is also evidence that tubulin subunits undergo deglutamylation, by as yet unidentified hydrolases, the process of glutamylation and deglutamylation produces a microtubule surface on which the distribution of charge and the charge density is continuously changing.

In order to facilitate the study of this unusual posttranslational modification, we have generated several antibodies that recognize the γ-glutamyl link in the glutamyl side chain. We have focused on generating antibodies that recognize the glutamylated isoforms of βII and βIII, the two most abundant β-tubulin isotypes expressed in brain.

The first step in generating such antibodies is to locate the services of a competent, willing synthetic chemist, commercial or academic. The cost of this custom synthesis is considerable. Unfortunately, we have been unable to depend on a reliable source for the manufacture of glutamylated peptides. Consequently, when we locate a source, we order a greater quantity of peptide(s) than we estimate is required for a single round of antibody production and characterization. We require a quantity of peptide sufficient to immunize one to five mice (0.3–1.5 mg) or one to two rabbits (2–4 mg). Next, we require a sufficient quantity of peptides, the glutamylated peptide and a sister nonglutamylated peptide (1–2 mg per peptide), for dot blotting and ELISAs. And finally, for polyclonals, we require glutamylated peptide (5–10 mg) sufficient for purification by affinity chromatography. Upon receipt, the structures of the peptides are verified by mass spectrometry. We view this as essential because we have received a number of peptides that were incorrectly synthesized.

We have produced six specific γ-Glu antibodies, two mouse monoclonal antibodies, and four rabbit polyclonal antibodies. The sequences of the six immunogens and the six parent peptides from which the immunogens were derived are shown in Table II.

The following discussion focuses primarily on one reagent, a mouse monoclonal antibody, 7D2. A synthetic, monoglutamylated peptide whose primary amino acid sequence was identical to the βIII C-terminal isotype-defining domain was used to generate 7D2 (Table II).

The antibody is an ideal research reagent. An IgG2a, it purifies by Protein-G and Protein-A affinity chromatography and is not labile at the acidic pH required for purification. 7D2 concentrates well without aggregating and can be coupled to cyanogen bromide (CNBr)-activated Sepharose without difficulty. It can be used for Western blotting, in competitive ELISAs, and for immunoaffinity purification of glutamylated proteins.
The initial characterization of 7D2 was accomplished with dot blots. A dot blot demonstrating the specificity of 7D2 for the \( \gamma \)-glutamyl linkage is shown in Fig. 4. In this experiment, the anti-\( \beta \)III antibody TuJ1 was used as a control (Lee et al., 1990a,b). The blot shows that TuJ1 reacts with purified brain tubulin, with the unmodified fusion protein containing approximately the last third of the \( \beta \)III polypeptide and with all of the \( \beta \)III peptides containing the last six amino acids. By contrast, 7D2 reacts only with the purified brain tubulin and with the monoglутamylated peptide. It does not react with the unmodified \( \beta \)III sequence in the MBP fusion protein or the other peptides.

The specificity of the antibody was established further by performing competitive ELISAs. The binding of the antibody to purified brain tubulin was determined in the presence of nonglutamylated, monoglутamylated, diglutamylated, and triglutamylated \( \beta \)III peptides. It does not react with the unmodified \( \beta \)III sequence in the MBP fusion protein or the other peptides.

The binding of the antibody to purified brain tubulin was determined in the presence of nonglutamylated, monoglутamylated, diglutamylated, and triglutamylated \( \beta \)III peptides. The results from one experiment are shown in Fig. 5.

### Table II

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<sup>a</sup> S, species; m, mouse monoclonal; r, rabbit polyclonal.
<sup>b</sup> (C), cysteine added for maleimide coupling.
<sup>c</sup> {E} or {EE}, one or two glutamate residues in side chain. All polyclonal antibodies were affinity purified using peptide-cyanogen bromide Sepharose.

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**Demonstration of the specificity of antibodies 7D2 and TuJ1**

![Diagram](image)

**Fig. 4** The dot blot demonstrates the specificity of antibodies 7D2 and TuJ1. Underlined residues indicate posttranslational modifications. \( Y \) and \( S \) are phosphorylated, and \( E \) is monoglутamylated. The (C) represents a cysteine added for conjugation.
The assay clearly demonstrates that 7D2 preferentially reacts with the monoglutamylated peptide. It binds approximately one to two orders of magnitude less efficiently to the diglutamylated peptide and does not react at all with the triglutamylated or nonglutamylated peptides.

Lastly, purified adult rat brain tubulin was passed through a column loaded with 7D2 coupled to CNBr and the retentate analyzed by mass spectrometry. The \( \beta \)-tubulin subunits identified were primarily monoglutamylated \( \beta \)III, and to a much lesser extent diglutamylated \( \beta \)III, validating the competitive ELISA (V. Redeker and A. Frankfurter, unpublished observations).

To determine whether 7D2 recognizes proteins other than tubulin, Western blots of whole human organ extracts were probed with the antibody. The Western blot presented in Fig. 6 demonstrates that 7D2 recognizes multiple polypeptides with masses ranging from ~25 to 200 kDa.

Since the \( \beta \)III gene is not expressed in nonneural tissues, it is unlikely that the immunoreactive polypeptides at ~50 kDa represent tubulin. The Western blot experiments confirm previous reports in which the monoclonal antibody GT335 was used to identify a large number of glutamylated polypeptides (Regnard et al., 2000; van Dijk et al., 2007).
Recently, we devised a purification scheme involving both ion exchange and immunoaffinity chromatography to isolate glutamylated polypeptides. We have had considerable success in using arginine-Sepharose chromatography to purify tubulin and tubulin C-terminal peptides for analysis by mass spectrometry (Redeker et al., 1998, 2004). We extended this procedure by combining it with 7D2 immunoaffinity chromatography and have purified rat brain and rat testes nucleosome assembly proteins (NAPs) as well as other glutamylated polypeptides. The procedure involves the following steps:

1. Tubulin is removed from rat brain and rat testes soluble extracts by taxol-driven microtubule assembly followed by ultracentrifugation.
2. The tubulin-depleted supernatant is fractionated by arginine-Sepharose chromatography. Bound polypeptides are eluted with 400 mM MES pH 6.5 or 1.5 M Na glutamate, dialyzed against PBS pH 7.4, and then applied to a 7D2-Sepharose column.
3. Polypeptides are eluted from the 7D2 column with 1.2 M NaCl and then analyzed by gel electrophoresis and/or mass spectrometry.

A silver-stained gel and a companion Western blot from one experiment are shown in Fig. 7. The silver-stained gel shows the eluted polypeptides contained within three separate fractions. Lane 1 shows 7D2-binding rat brain polypeptides previously eluted from an arginine-Sepharose column with 400 mM MES, pH 6.5. Lane 2 shows 7D2-binding rat brain polypeptides previously eluted from an arginine-Sepharose column with 1.5 M Na glutamate, and lane 3 shows 7D2-binding rat testes polypeptides previously eluted from an arginine-Sepharose column with 1.5 M Na glutamate.

These three experiments demonstrate that in addition to tubulin, an impressive number of putative glutamylated polypeptides are present in soluble extracts of rat...
Glutamylated NAPS recovered by 7D2 immunoaffinity chromatography

Fig. 7 The silver-stained gel shows the profiles of eluted polypeptides following three rounds of 7D2 immunoaffinity chromatography. Lane 1 shows 7D2-binding rat brain polypeptides eluted from an arginine-Sepharose column with 400 mM MES, pH 6.5. Lane 2 shows the 7D2-binding rat brain polypeptides eluted from an arginine-Sepharose column with 1.5 M Na glutamate, and lane 3 shows 7D2-binding rat testes polypeptides eluted from an arginine-Sepharose column with 1.5 M Na glutamate. These three experiments demonstrate that in addition to tubulin, an impressive number of putative glutamylated polypeptides are present in soluble extracts of rat brain and testes, and these can be captured by arginine-Sepharose and further enriched for by 7D2 immunoaffinity chromatography. The Western blot in this figure (lanes 4, 5, and 6) demonstrates that the three fractions shown in the companion silver stain contain glutamylated NAPs.

brain and testes and can be captured by arginine-Sepharose chromatography and further enriched for by 7D2 immunoaffinity chromatography. The Western blot shown in Fig. 7 demonstrates that each of the three silver-stained fractions contain glutamylated NAPs. The anti-NAPs monoclonal antibody used in this experiment was a gift from Ishimi et al. (1985).

The identity of the other silver-stained polypeptides has not yet been established. Overall, the results from these experiments confirm an earlier study in which the monoclonal GT335 was used to capture glutamylated NAP1 and NAP2 as well as other putative glutamylated polypeptides (Regnard et al., 2000). It would be worthwhile to determine the extent to which GT335 and 7D2 capture the same polypeptides, since the primary amino acid sequences of their immunogens were different.

We are most interested in studying the three polyclonal antibodies: TTβIIglu, TTSG1, and TTSG2. Their immunogens were designed so that their primary amino acid sequences possess minimal similarity with the primary amino acid sequences of βII and βIII. This was done to minimize the antigenic influence of the amino acids flanking the glutamylation site in the expectation that these antibodies would recognize a large number of glutamylated proteins. Conversely, it seems unlikely that any anti-γ-Glu antibody devoid of polypeptide context could be generated that recognizes a single glutamylated protein.
V. Summary

We have provided a brief overview of the methods and reagents developed in our laboratory for characterizing anti-β-tubulin antibodies. We have purposely not included a discussion of anti-α-tubulin antibodies. The difficulties inherent in producing and characterizing antibodies that react with individual α-tubulin isotypes and their posttranslational modifications are considerably greater than those associated with generating and characterizing β-tubulin antibodies. We also described the progress we have made in generating a small library of monoclonal and polyclonal antibodies that recognize the γ-glutamyl link in glutamylated proteins.

Without minimizing their potential utility, anti-γ-Glu antibodies should be used with considerable forethought. In view of the fact that numerous proteins appear to be targets of glutamylation, these antibodies will have to be used very carefully for immunocytochemistry. Further, caution should be used in identifying immunoreactive bands on a Western blot following one-dimensional sodium dodecyl sulfate gel electrophoresis. For example, glutamylated tubulin and NAP1 comigrate in the first dimension. Lastly, antibodies like 7D2 will be valuable in identifying glutamylated polypeptides, but they cannot be used to determine the extent of glutamylation, since the polypeptides with glutamyl side chains longer than two residues will remain undetected.

References


3. Characterization of Anti-β-tubulin Antibodies


CHAPTER 4

Expression Profiling of Tubulin Isotypes and Microtubule-Interacting Proteins Using Real-Time Polymerase Chain Reaction

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Abstract

Real-time polymerase chain reaction (PCR) has been used for quantification of intracellular mRNA levels in cell culture and tissue samples. It is an important tool for studying antimitotic drug effects on tubulin isotype and microtubule-interacting protein levels and for measuring differences in normal and tumor tissue samples that
could have predictive or prognostic applications. Both quantitative and comparative methods are valuable approaches; however, the selection of either approach requires an understanding of their benefits and challenges. In this chapter, we provide detailed protocols for real-time PCR experiments, discuss issues to consider in selecting real-time PCR methodologies, and give examples utilizing either quantitative or comparative approaches.

I. Introduction and Rationale

Real-time analysis of reverse transcriptase polymerase chain reactions (RT-PCRs) is a useful tool for studying tubulin isotype and microtubule-interacting protein (MIP) mRNA levels under varying experimental conditions. We are able to measure drug effects on tubulin isotype and MIP transcription in cell culture. Quantitative or comparative RT-PCR experiments, using templates from cells grown in culture, provide insight into drug effects on transcription of specific messages over time. We can also study patterns of tubulin isotype and MIP expression in tissues, allowing us to assess potential biomarkers for disease pathology and evaluate the utility of expression profiles for predicting therapeutic outcomes (Cucchiarelli et al., 2008; Dozier et al., 2003; Hiser et al., 2006). The methods (quantitative or comparative) are also employed to compare normal and tumor tissue samples from patients and can be used to examine the impact of chemotherapy on tubulin isotype and MIP expression profiles.

Inherent in the use of quantitative or comparative RT-PCR are different models for setting up experiments and analyzing data. There are several excellent technical guides describing RT-PCR protocols on the Stratagene (http://www.Stratagene.com/tradeshows/feature.aspx?fpId=1) and the Invitrogen webpages (http://tools.Invitrogen.com/content.cfm?pageid=12257). While there are pros and cons for the utilization of either method, some types of experiments are more readily handled by one method as opposed to the other. Issues to consider when selecting quantitative or comparative RT-PCR as the method for data collection are discussed in this chapter and data examples are provided.

II. Methods and Materials

A. RNA Extraction from Cells or Tissues

Total RNA that is suitable for use in expression studies may be obtained using a combination of TRIZOL Reagent (Invitrogen, Carlsbad, CA) and spin columns (e.g., RNeasy kit; Qiagen, Valencia, CA). The result is a high yield of good-quality RNA.

1. Prepare 100 µl of tissue or whole cell lysate in an RNase-free microcentrifuge tube. For tissues, start with 0.25 G or less and homogenize with mortar and pestle in liquid nitrogen. For cell culture studies, we start with about $10 \times 10^6$ cells. Cells are washed with phosphate-buffered saline (PBS), centrifuged into a pellet, and resuspended in 100 µl PBS.
2. Add 1 ml TRIzol prewarmed to room temperature. Avoid contact with skin and breathing the vapor. Let the mixture stand at room temperature about 5 min to complete cell lysis and dissociation of nucleoprotein complexes.

3. Add 0.2 ml chloroform to the homogenate. Shake vigorously for 15 s. Do not vortex because of the possibility of RNA breakage. Allow the sample to stand at room temperature for 2–3 min.

4. Separate the aqueous and organic phases in a 1.5 ml microcentrifuge tube by centrifugation at 12,000 × g for 15 min at 4°C.

5. Carefully remove aqueous phase (top) and transfer to another RNase-free tube. It is extremely important not to get any of the material from the aqueous/organic interface; it is suggested to sacrifice aqueous material rather than risk taking this precipitate. From this step on, work in an area that is RNase free.

6. Measure the volume of the aqueous sample. If it is more than 750 µl, divide it equally into two tubes.

7. Slowly add an equal volume of 70% ethanol (EtOH) prepared in RNase-free water, mixing as it is added. Do this by pipeting up and down. Measure the full amount of EtOH, but expel only a little from the pipet tip before drawing up more solution. Continue this process as a way of mixing the contents of the tube at the same time you are adding the EtOH. Slow introduction of the EtOH is important to avoid localized precipitation of RNA.

8. Load the sample (up to 700 µl) into an RNeasy column seated in a collection tube. Centrifuge for 30 s at ≥ 8,000 × g. Discard the flow-through. If the sample volume is larger than the loading capacity of the column, repeat this step until the entire sample has been passed over the column.

9. Add 350 µl Buffer RW1 (from the RNeasy kit) onto the column and centrifuge for 15 s at ≥ 8,000 × g. Discard flow-through.

10. Transfer the spin column into a new collection tube (from the kit) and discard the old collection tube.

11. Add 10 µl of DNase I stock solution to 70 µl Buffer RDD. Mix by gentle inversion and centrifuge briefly.

12. Add the DNase I incubation mix (80 µl) directly to RNeasy spin column membrane and place on the benchtop to incubate RT for 15 min.

13. Add 350 µl Buffer RW1 and centrifuge for 15 s at ≥ 8,000 × g. Discard the flow-through.

14. Transfer the spin column into a new collection tube (from the kit) and discard the old collection tube.

15. Add 500 µl Buffer RPE and centrifuge for 30 s at ≥ 8,000 × g. Discard the flow-through.

16. Add another 500 µl Buffer RPE and centrifuge for 2 min at ≥ 8,000 × g.

17. Centrifuge the empty tube for 2 min more at ≥ 8,000 × g to dry the column.

18. Transfer column into a 1.5-ml collection tube (with cap) and pipet 30 µl of RNase-free water directly onto the center of the column membrane. Allow the sample to sit at room temperature for 1–2 min, and then centrifuge for 1 min at ≥ 8,000 × g to elute the RNA. Yields can be improved by using warm (preheated to 98°C) RNase-free water.

19. For greater recovery of RNA, repeat the previous step using 20 µl prewarmed RNase-free water for a total of 50 µl RNA.
20. Measure RNA concentration spectrophotometrically (NanoDrop 1000 UV-Vis spectrophotometer; NanoDrop Technologies, Inc., Wilmington, DE): 
$$\lambda_{260} = 15,100 \text{ M}^{-1} \text{ cm}^{-1}$$.

21. Store the purified RNA at $-80^\circ\text{C}$.

The RNA quality is determined using three assessments: (1) $A_{260/280}$, (2) 28S/18S ribosomal RNA ratio, and (3) the RIN (RNA integrity number). Good-quality RNA typically has a ratio of absorbance at 260/280 nm between 1.8 and 2.0 (this may be influenced by the quantity of RNA and thus the size of the peaks, with larger peaks having higher ratios than shorter peaks). We do this using the Agilent 2100 Bioanalyzer RNA chip and Agilent software (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The maximum 28S/18S ribosomal RNA ratio is near 2.0 as determined using an Agilent 2100 Bioanalyzer. The RIN, ranging from 1 to 10, is determined by a proprietary formula in the Agilent 2100 Bioanalyzer software. This quantification includes factors for all RNA, including the degraded material. The optimum RIN for tracking good-quality RNA should be determined experimentally (Imbeaud et al., 2005; Schroeder et al., 2006). We generally consider samples as acceptable when the RIN is greater than 8.5.

### B. cDNA Preparation

We use a two-step RT-PCR procedure in which cDNA is first produced and can be stored at $-20^\circ\text{C}$ for use in many PCR experiments. This allows us to study multiple targets from a single cDNA sample and to replicate experiments with each target. In the first step, the total RNA is treated with DNase I in solution and then, after DNase treatment, a poly (dT) primer is used in reverse transcription reactions to generate cDNA from all mRNA transcripts. We use 2 µg of total RNA, as determined by absorbance at 260 nm using a NanoDrop 1000 UV-Vis spectrophotometer (NanoDrop Technologies, Inc.) in our RT reactions. An avian myeloblastosis virus (AMV) RT (Promega Corporation, Madison, WI) is used because of its temperature stability and RNaseH activity.

1. Start with 2 µg of RNA and add RNase-free water to give a total volume of 7.5 µl in a 0.2- or 0.5-ml microcentrifuge tube.
2. Add the following in this order:
   a. 0.5 µl of RNase OUT.
   b. 1.0 µl 10× DNase I for a total of 10 µl.
   c. 1.0 µl of DNase I for a total of 10 µl.
3. Incubate for 15 min at 25°C in thermal cycler.
4. Add 1 µl of Stop solution to end the DNase I digestion.
5. Incubate for 10 min at 70°C to disrupt secondary structure and then immediately place on ice.
6. Add 0.5 µg of poly (dT)$_{15}$ (2 µl of 500 µg/ml).
7. Heat to 70°C for 5 min.
8. Chill on ice for 5 min and centrifuge briefly.
   a. While tubes are on ice, warm sodium pyrophosphate to 42°C.
9. Add the following in the order shown (Promega Corporation):
   a. 5 µl AMV RT 5× reaction buffer.
   b. 2.5 µl of 40 mM dNTP mix.