
**Basic Techniques for
Transmission
Electron
Microscopy**

M. A. HAYAT

Basic Techniques for Transmission Electron Microscopy

M. A. HAYAT

Department of Biology
Kean College of New Jersey
Union, New Jersey

1986



ACADEMIC PRESS, INC.

Harcourt Brace Jovanovich, Publishers

Orlando San Diego New York
Austin London Montreal Sydney
Tokyo Toronto

15286

Basic Techniques for Transmission Electron

COPYRIGHT © 1986 BY ACADEMIC PRESS, INC.
ALL RIGHTS RESERVED.
NO PART OF THIS PUBLICATION MAY BE REPRODUCED OR
TRANSMITTED IN ANY FORM OR BY ANY MEANS, ELECTRONIC
OR MECHANICAL, INCLUDING PHOTOCOPY, RECORDING, OR
ANY INFORMATION STORAGE AND RETRIEVAL SYSTEM, WITHOUT
PERMISSION IN WRITING FROM THE PUBLISHER

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD
24-28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

Hayat, M. A., Date

Basic techniques for transmission electron
microscopy.

Includes index.

1. Electron microscope, Transmission. 2. Electron
microscopy—Technique. I. Title.
QH212.T7H39 1985 578'.45 85-3957
ISBN 0-12-333925-1 (alk. paper)
ISBN 0-12-333926-X (paperback)

PRINTED IN THE UNITED STATES OF AMERICA

86 87 88 89

9 8 7 6 5 4 3 2 1

is to present only the best variation of a basic method, since for a particular study one variation may give a better result than another. For example, the variation for the preservation of yeast cells will differ from that ideal for preserving yeast antibiotic spores. Furthermore, the use of a superior variation may not be feasible in a certain laboratory because of the unavailability of a required piece of equipment, lack of funds, or lack of expertise. Variations on methods are therefore given so that readers can choose the most suitable approach to achieve their specific objectives.

Preface

Generally, no attempt was made to explain the theory or principles that govern preparatory procedures, because, because of the extensive of this volume is to present procedures in a step-by-step fashion. This book is a "Principles and Techniques of Electron Microscopy: Principles and Applications," 2nd ed., John Wiley & Sons, New York, 1971. By this author present the principles governing preparatory procedures. Original source references are provided throughout the text so that the reader can obtain additional information. It is almost impossible to personally test each of the methods included. I am hopeful that the methods presented in the text will be successful. However, preparatory methods are subject to modification depending on the objective of the study and available facilities. In order to achieve the best results, it is

Transmission electron microscopy has been making profound contributions to the solution of problems in biology and medicine in that the transmission electron microscope gives us the hope of direct examination of biological structures at the atomic level. Individual molecules and their components can be identified—thus the emergence of "molecular microscopy." Such information is of vital importance in understanding the correlation between structure and function at the cellular level. Since methodology is a major constraint in obtaining more detailed and accurate information on cell ultrastructure, the problems of biological specimen preparation need to be attacked with the same converging intensity as that afforded by the improvement of resolving power.

In the preface of my book, "Fixation for Electron Microscopy" (Academic Press, Inc., 1981), I expressed my wish to continue compiling information that might help to improve the quality of specimen preservation as well as the interpretation of the fine structure. This wish is partly fulfilled by publishing the present volume, which covers a vast variety of techniques and alternative approaches used for studying morphological and cytochemical aspects of specimens. Much of this volume is a compilation of methods scattered throughout a large number of scientific journals, and should therefore save readers valuable time in searching through journals for the desired method and/or in contacting various laboratories.

To minimize artifacts and to obtain the required information, each type of specimen requires special, optimal processing. The precise parameters of processing a wide variety of specimens are presented in Chapter 7. It is inappropriate

ate to present only the best variation of a basic method, since for a particular study one variation may give a better result than another; for example, the variation for the preservation of yeast cell wall differs from that ideal for preserving yeast mitotic spindle. Furthermore, the use of a superior variation may not be feasible in a certain laboratory because of the unavailability of a required piece of equipment, lack of funds, or lack of expertise. Variations on methods are therefore given so that readers can choose the most suitable approach to achieve their specific objectives.

Generally, no attempt was made to explain the theory or principles that govern preparatory procedures, because the primary objective of this volume is to present procedures in a step-by-step fashion. Other books (e.g., "Principles and Techniques of Electron Microscopy: Biological Applications," 2nd ed., International Pub., Berkeley Heights, New Jersey) by this author present the principles governing preparatory procedures. Original source references are provided throughout the text so that the reader can obtain additional information.

It is almost impossible to personally test each of the methods included. I am hopeful that the methods presented in the text will be successful. However, preparatory methods are subject to modification depending on the objective of the study and available facilities. In order to achieve the best results, it is imperative that an attempt be made to optimize even a basic method.

Preparatory procedures for both eukaryotic and prokaryotic groups are presented. Optimal preparatory requirements for these two groups differ from each other. Since plant specimens frequently present special problems (cell wall and large vacuoles), optimal protocols for these specimens differ from those used for animal specimens. Special preparatory methods for plant specimens as well as for viruses are also presented. The processing of uncommon specimens and the solution of unusual, individual problems are included.

Electron microscopy is playing an increasingly crucial role in the confirmation of human diseases; important diagnostic information is provided by electron microscopy. Accordingly, I have attempted to include techniques useful for clinical medicine. Presently, diagnostic information can be obtained by electron microscopy within three hours after a biopsy or collection of a surgical specimen. I have not attempted to present every preparatory procedure used in diagnostic electron microscopy, but have focused instead on well-established, routinely used techniques (for example, negative staining for viral diagnosis).

This book departs from tradition in that books on methodology present only the contemporary consensus of knowledge. Here relatively new methods are also presented, provided that they show potential usefulness. Some of these methods are in the developmental stage, and will probably be refined and standardized; all are restricted in their scope to the preparation of specimens for transmission electron microscopy. Because of the availability of an enormous number and variety of techniques for transmission electron microscopy, limited available

space did not allow the inclusion of techniques for scanning electron microscopy. Esoteric techniques that require very expensive instruments are also excluded because of space limitations.

This book is intended for students, technicians, teachers, and research scientists in biology and medicine, and is essentially a laboratory handbook that can be used in formal courses and by individuals. I hope that it will yield practical advice to beginners who are learning the preparatory techniques as well as to experienced and busy scientists who cannot afford to spend time searching for procedures in literature. It is suggested that the entire procedure be read and necessary solutions prepared prior to undertaking the processing. An exhaustive list of references with complete titles is provided, as is a detailed subject index.

Because this is a book on methodology, many techniques are included that were extracted and synthesized from scientific journals, personal communications, and laboratory testing. A vast majority of the methods presented were checked for accuracy and updated by their originator. Numerous scientists were very kind to allow me the use of their illustrations, for which I am most grateful. I am also thankful to the publishers of various scientific journals who were more than prompt in granting me permission for reproducing the illustrations.

This volume could not have been completed without the help, encouragement, and inspiration that were graciously extended to me by a large number of very able scientists.

M. A. Hayat

Contents

Preface

xxv

1 Chemical Fixation

Introduction

1

Hazards, Precautions, and Safe Handling of Reagents

5

Fixatives

8

Purification of Glutaraldehyde

8

The Charcoal Method

8

The Distillation Method

9

Storage of Glutaraldehyde

9

Preparation of Formaldehyde Solution from Paraformaldehyde Powder

10

Preparation of and Precautions in Handling Osmium Tetroxide Solution

10

Regeneration of Used Osmium Tetroxide

11

Procedure for Preparing OsO_2

12

Preparation of 50 ml of 2% OsO_4 Solution

12

Preparation of Fixatives

13

Acrolein

13

Acrolein-Glutaraldehyde

13

Acrolein-Glutaraldehyde-Formaldehyde	13
Acrolein-Glutaraldehyde-Dimethyl Sulfoxide	13
Acrolein-Glutaraldehyde-Formaldehyde-Dimethyl Sulfoxide	13
Carbodiimide-Glutaraldehyde	14
Dalton's Chrome-Osmium	14
Dimethyl Suberimide (DMS)	14
Formaldehyde-Chromic Acid	15
Formaldehyde-Picric Acid	15
Glutaraldehyde-Phosphate Buffer	15
Glutaraldehyde-Cacodylate Buffer	15
Glutaraldehyde-Alcian Blue	15
Glutaraldehyde-Caffeine	15
Glutaraldehyde Containing High Concentration of Potassium	16
Glutaraldehyde-Digitonin	16
Glutaraldehyde-Formaldehyde	16
Glutaraldehyde-Formaldehyde-Nitrogen Mustard <i>N</i> -Oxide	16
Glutaraldehyde-Hydrogen Peroxide	17
Glutaraldehyde-Lead Acetate	17
Glutaraldehyde-Formaldehyde-Terpenoids	17
Glutaraldehyde-Malachite Green	18
Glutaraldehyde-Phosphotungstic Acid	18
Glutaraldehyde-Potassium Dichromate	18
Glutaraldehyde-Potassium Permanganate	18
Glutaraldehyde-Potassium Permanganate-Phosphotungstic Acid-Hematoxylin	19
Glutaraldehyde-Tannic Acid-Saponin	19
Glutaraldehyde-Trinitro Compounds	19
Glutaraldehyde-Uranyl Acetate	20
Lanthanum Permanganate	20
Osmium Tetroxide Quick-Fix Method	20
Potassium Permanganate	21
Acid Permanganate	21
Neutral Permanganate	21
Sodium Permanganate	21
Ruthenium Red	21
Ruthenium Tetroxide	22
Preparation of Buffers	22
2-Amino-2-methyl-1,3-propanediol (Ammediol) Buffer	22
Acetate Buffer	22
Barbital Buffer	23
Cacodylate Buffer	23
Carbonate-Bicarbonate Buffer	24

Citrate Buffer	24
Collidine Buffer	24
Glycine-HCl Buffer	25
Maleate Buffer	25
Phosphate Buffer (Sörensen)	25
Phosphate Buffer (Karlsson and Schultz, 1965)	26
Phosphate Buffer (Maunsbach, 1966)	26
Phosphate Buffer (Millonig, 1961)	26
Phosphate Buffer (Millonig, 1964)	26
Piperazine (PIPES) Buffer	27
PM Buffer	27
Ryter-Kellenberger Buffer	27
Succinate Buffer	28
Tris(hydroxymethyl)aminomethane Buffer	28
Tris(hydroxymethyl)aminomethane Maleate Buffer	29
Veronal Acetate Buffer	29
Preparation of Tissue Blocks	30
Fixation	31
Vascular Perfusion	31
Recommended Osmolality of Perfusate for Selected Tissues	31
Recommended Perfusion Pressure for Selected Tissues	32
Anesthesia	32
Aorta	33
Arteries	34
Central Nervous System	34
Embryo	36
Fish	37
Heart	38
Method 1 (Fetus)	38
Method 2 (Adult)	38
Kidney	40
Method 1 (Animal)	40
Method 2 (Human)	40
Liver	41
Method 1 (Adult)	41
Method 2 (Embryos and Very Small Animals)	42
Method 3 (Biopsy)	43
Lung	43
Muscle (Skeletal Muscle of Rat Hind Limb)	45
Ovary	46
Region at Transition between Peripheral and Central Nervous System	47

Spinal Cord	47
Method 1 (Goldfish)	47
Method 2 (Rat)	48
Spleen	48
Testes	49
Uterus	50
Vapor Fixation and Staining	52
Method 1	52
Method 2	53
Microwave Fixation	54
Artifactual Electron-Dense Granules Occurring during Double Fixation	54
2 Rinsing, Dehydration, and Embedding	
Introduction	56
Standard Procedure for Fixation, Rinsing, Dehydration, and Embedding	57
Gradual, Progressive Dehydration and Embedding	61
Procedure for Minimizing Chemical Hazards during Specimen Preparation	64
Low-Temperature Infiltration and Embedding	66
Glycol Methacrylate	66
Lowicryl K4M (Polar)	67
Lowicryl HM20 (Nonpolar)	67
Incomplete Dehydration for Lipid Preservation	68
Water-Immiscible Embedding Media	68
Epoxy Resins	68
Epon 812 (LX-112)	68
Araldite	70
Maraglas	71
LR White	72
Quetol 651	72
Dow Epoxy Resins	73
DER 334	73
DER 332	73

Vinylcyclohexene Dioxide (VCD, ERL 4206, Spurr Mixture)	73
Vinylcyclohexene Dioxide- <i>n</i> -Hexenyl Succinic Dioxide (HXSA)	74
Polyester Resins	74
Polymaster 1209AC	74
Poly- <i>N</i> -vinylcarbazole	75
Rigolac	76
Vestopal W	76
Methacrylates	76
Styrenes	76
Water-Miscible Embedding Media	76
Acrylamide-Gelatin-Jung Resin	76
Durcupan	77
Gelatin	78
Glutaraldehyde-Carbohydrazide (GACH)	78
Glycol Methacrylate (GMA)	78
Method 1	78
Method 2	79
Hydroxypropyl Methacrylate (HPMA)	80
Method 1	80
Method 2	80
LEMIX	81
Lowicryl K4M	81
Melamine Mixture	82
Method 1	82
Method 2	82
Polyacrylamide	83
Polyampholyte (Polyamph 10)	84
Polyethylene Glycol (PEG; Carbowax)	84
Diethylene Glycol Distearate	86
Polyvinyl Alcohol	86
Protein Aldehydes	87
Mixed-Resin Embedding Media	87
DER 332-DER 732	87
Epon 812 (LX-112)-Araldite 502	88
Epon 812-DER 736	89
Epon 812-Thiokol LP-8	89
Epon 812 (LX-112)-Maraglas	90
ERL 4206-Quetol 653	90
Methacrylate-Styrene	90
Rigolac-Styrene	90

Silicone (Rhodorsil 6349)—Araldite CY 212	90
Vinylcyclohexene Dioxide- <i>n</i> -Hexenylsuccinic Anhydride—Araldite RD-2	91
Embedding	91
Viscosity of Embedding Media	91
Agar Preembedding (Encapsulation)	92
Bovine Serum Albumin Preembedding	92
Fibrin Clot Preembedding	93
Direct Pelleting of Cells	93
Pelleting of Cells in Agar	94
Pelleting of Cells in Agarose	95
General Embedding Methods	95
Capsule Embedding	95
Embedding of Buoyant Specimens	96
Flat Embedding	97
Labeling	97
Rapid Embedding	97
Method 1	97
Method 2	98
Specimen Orientation	99
Embedding of Hard Plant Tissues	101
Orientation and Embedding of Small Specimens	102
Orientation and Embedding of Single-Cell Organisms	103
Embedding of Individual Cells	104
Embedding of Ascomycetes (Cell Walls)	106
Embedding of Bacteria and Tissue Culture Cells	106
<i>In Situ</i> Embedding of Cells Grown on Millipore Filters	107
<i>In Situ</i> Embedding of Cell Monolayers on Untreated Glass Surfaces	108
<i>In Situ</i> Embedding of Virus for Immunoelectron Microscopy	109
Open-Face Embedding for Correlative Microscopy	110
Resin Slide Embedding for Correlative Microscopy	112
Method 1	112
Method 2	113
Epoxy Resin Slides for Handling Unfixed Cryostat Sections	113
Wafer Embedding	115
Identification of Areas of Interest in Human Breast Tissue before Embedding	115
Reembedding of Paraffin-Embedded Tissue in Resin	117
One-Step Methods	117
Reembedding of Paraffin-Embedded Tissue Sections in Resin	118
Method 1	118

Method 2	118
Method 3	119
Pop-Off Method for Reembedding	119
Reembedding of Tissue Culture Cells	121
Reembedding of Thick Resin Sections	121
Method 1	121
Method 2	122
Osmication and Flat Embedding of Large Tissue Sections	124
Reembedding of Autoradiograms of Semithin Sections for Correlative Microscopy	124
Retrieval of Poorly Resin-Embedded Tissue	125

3 Sectioning

Introduction	126
Examination of Glass Knives	127
Diamond Knives	30
Grids	133
Carbon-Polymer Support Grids for X-Ray Microanalysis	136
Specimen Block Trimming	137
Preparation of Troughs	142
Mounting the Specimen Block and Knife	144
Sectioning Procedure	147
Defects Appearing during Sectioning	150
Serial Sectioning	156
Specific Methods	157
En Face Sectioning	157
Sectioning Hard Mineral Fibers in Tissues	158
Sectioning Hard Laminated Fibrous Tissues	159
Vertical Sections of Cultivated Anchorage-Dependent Cells	160
In Situ Thin-Section Microscopy of Cell Cultures	160
Resectioning of Semithin Sections	162
Semithin Sectioning	164
Introduction	164
Ralph Knife	165

Sectioning	168
Serial Sectioning	169
Cryofixation and Cryoultramicrotomy	174
Specimen Collection	175
Encapsulation	175
Fixation and Cryoprotection	175
Coolants	176
Freezing	176
Freezing Instrument	178
Operation	179
Sectioning	179
4 Positive Staining	
Introduction	182
Stains	183
Acridine Orange	183
Alcian Blue	184
Bismuth	184
Specific <i>en Bloc</i> Staining	184
Enhancing Contrast in General	184
Chromosome Staining	185
Staining of Mucosubstances and Polysaccharides	185
Cobalt	185
Cobalt Sulfide-Silver Intensification	186
Concanavalin A	186
Two-Step Lectin-Peroxidase	186
One-Step Lectin-Peroxidase	187
Diaminobenzidine-Osmium Tetroxide	187
Gold	187
Preparation of Colloidal Gold	188
Gold-Protein Complex	188
Injection and Fixation	188
Indium	188
Iodide-Osmium Tetroxide	189
Sodium Iodide-Osmium Tetroxide	189
Zinc Iodide-Osmium Tetroxide	189
Iodine	189
Tetraiodophthalic Acid	190

Iron	190
Colloidal Ammonium Ferric Glycerate	190
Negative Colloidal Ferric Hydroxide	190
Positive Ferric Oxide	191
Negative Ferric Oxide	191
Staining Procedures	191
<i>En Bloc</i> Staining	191
Staining of Thin Sections	191
Lanthanum	192
Lanthanum Hydroxide	192
Lanthanum Nitrate	192
Lead	192
Lead Acetate	192
Lead Aspartate	193
Lead Citrate	193
Lead Hydroxide	194
Lead Tartrate	195
Molybdenum Blues	195
Osmium Amine	196
Osmium Tetroxide	196
Osmium Tetroxide-Acrylic Acid	196
Osmium Tetroxide-Dimethylenediamine (Os-DMEDA)	196
Osmium Tetroxide- <i>p</i> -Phenylenediamine	197
Osmium Tetroxide-Potassium Ferricyanide or Ferrocyanide	197
Osmium Tetroxide Buffered with Imidazole	197
Oxalate-Glutaraldehyde	198
Phosphotungstic Acid (PTA)	198
Ethanollic Phosphotungstic Acid (EPTA)	198
Phosphotungstic Acid-Acridine	199
Phosphotungstic Acid-Chromic Acid	199
Phosphotungstic Acid-Hematoxylin	199
Platinum	199
Potassium Permanganate	200
Potassium Permanganate and Lead Citrate	200
Potassium Pyroantimonate-Osmium Tetroxide	201
Ruthenium Red	201
Silicotungstic Acid	202
Silver	202
Silver Methenamine	202
Periodic Acid-Silver Methenamine	203
Periodic Acid-Chromic Acid-Silver Methenamine	203
Golgi Impregnation Method	204

Golgi Impregnation Method with Gold	204
Silver Lactate–Osmium Tetroxide	205
Sodium Tungstate	205
Tannic Acid–Ferric Chloride	206
Tannic Acid–Uranyl Acetate	206
Tetraphenylporphine Sulfonate	206
Thallium Ethylate	207
Thiosemicarbazide and Thiocarbohydrazine	207
Periodic Acid–Thiocarbohydrazine or Thiosemicarbazide– Silver Proteinat	207
Uranyl Acetate	208
Preparation of Staining Solutions	208
<i>En Bloc</i> Staining	208
Section Staining	209
Specific Staining of Neuroendocrine Granules	209
Semiselective Staining of RNA	209
Vanadium	209
Lipid Preservation and Staining	209
Multiple Staining	213
Double Staining of Thin Sections with Uranyl Acetate and Lead Citrate	214
Multiple-Grid Staining	217
Removal of Bound Osmium from Thin Sections	222
Staining for High-Voltage Electron Microscopy	222
Staining Methods	223
Precautions to Minimize Artifactual Staining Precipitates	224
Removal of Artifactual Staining Precipitates from Thin Sections	225
Removal of Epoxy Resins from Semithin Sections before Staining	225
Staining Methods for Semithin Sections	226
Azure B for Plant Tissues	226
Basic Fuchsin and Methylene Blue	226
Methylene Blue–Azure II–Basic Fuchsin	227
Methylene Blue–Azure II	227
Giemsa	228

Hematoxylin-Phloxine B	228
Hematoxylin-Malachite Green-Basic Fuchsin	229
Sudan III	230
Chromotrope 2R-Methylene Blue	230

5 Negative Staining

Introduction	232
--------------	-----

Negative Stains	233
-----------------	-----

Wettability of Support Films	234
------------------------------	-----

Choice of Support Film	236
------------------------	-----

General Methods	236
-----------------	-----

Basic Considerations	236
----------------------	-----

One-Step (Simultaneous) Method	237
--------------------------------	-----

Spray Method	238
--------------	-----

Negative Stain-Carbon Method	239
------------------------------	-----

Two-Step (Sequential) Method	242
------------------------------	-----

Staining after Fixation	244
-------------------------	-----

Single- or Double-Carbon-Layer Method	245
---------------------------------------	-----

One-Side Negative Staining Method	247
-----------------------------------	-----

Paper Filtration Method	247
-------------------------	-----

Pseudoreplica Method	248
----------------------	-----

Agar Filtration Method	248
------------------------	-----

Freeze-Dry Negative Staining	250
------------------------------	-----

Viruses	252
---------	-----

General Methods for Animal Viruses	252
------------------------------------	-----

Viruses from Skin Lesions	253
---------------------------	-----

Viral Detection in Fecal Specimens	254
------------------------------------	-----

Direct Staining Method	254
------------------------	-----

Microsolute Concentration Method	254
----------------------------------	-----

Pseudoreplica Method	254
----------------------	-----

Ultracentrifugation Method	255
----------------------------	-----

Other Methods	255
---------------	-----

Direct Detection of Viruses with the Beckman Airfuge	256
--	-----

Virus Particle Counting	256
-------------------------	-----

Virions in Bacteriophage Plaques	259
----------------------------------	-----

General Methods for Plant Viruses	259
-----------------------------------	-----

Rapid Procedures	259
------------------	-----

Viruses in Crude Extract	260
--------------------------	-----