

# **Principles and Techniques of Electron Microscopy**

**BIOLOGICAL APPLICATIONS**

Volume 8

**M. A. HAYAT**

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**Volume 8**

**M. A. HAYAT**

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# PREFACE

This is the eighth volume of a multi-volume series on the principles and techniques employed for studying biological specimens with the aid of an electron microscope. Since its inception in 1970, the series has successfully reflected the growth of electron microscopy in instrumentation as well as in methodology. There was a pressing need to keep readers abreast of the remarkable expansion of the field in recent years and the ever growing importance of its contributions to the understanding of many problems in biological and medical sciences. This treatise serves as an international authoritative source in the field, and is designed to cover important new developments systematically. The treatise departs from the tradition that books on methodology present only the contemporary consensus of knowledge. It is written by scholars, and when they have anticipated the potential usefulness of a new method, they have so stated. The authors have not hesitated to include ideas in progress. The treatise should serve as a guide and survey, which can save a newcomer the tedious search for information scattered in biological journals.

This volume has developed over the years through the joint effort of ten distinguished author-scientists. As a result, a most comprehensive compilation of methods developed and used by a large number of competent scientists has been achieved. The book contains new viewpoints with particular regard to current problems. Areas of disagreement and potential research problems have been pointed out. It is hoped that the readers will become aware that correct interpretation of the information retrieved from electron micrographs is dependent

upon an understanding of the principles underlying the methodology and instrumentation.

The basic approach in this volume is similar to that in the previous seven volumes, in that the methods presented have been tested for their reliability, and are the best of those currently available. The instructions for the preparation and use of various solutions, media, stains, and apparatus are straightforward and complete, and should enable the worker to prepare his or her specimens without outside help. Before undertaking the processing, one should read the entire procedure and prepare necessary solutions and other media. Each chapter is provided with an exhaustive list of references with complete titles. Full author and subject indexes are included at the end of the book.

It is encouraging to know that the previous volumes have been received favorably. It is my impression that this volume will also fulfill its purpose: to provide an understanding of the usefulness, limitations, and potential applications of special methods employed for studying the structure, composition, size, number, and location of cellular components, and to provide details of current improvements in the instrumentation.

M. A. Hayat  
Berkeley Heights, N.J.

# Contents to

## Volume 1, M. A. Hayat

---

FIXATION

EMBEDDING

SECTIONING

STAINING

SUPPORT FILMS

## Volume 2

---

FREEZE-SUBSTITUTION AND FREEZE-DRYING, Lionel I. Rebhun

THE FREEZE-ETCHING TECHNIQUE, James K. Koehler

NEGATIVE STAINING, Rudy H. Haschemeyer and Robert J. Meyers

SHADOW CASTING AND REPLICATION, W. J. Henderson and K. Griffiths

HIGH RESOLUTION AND SHADOWING, R. Abermann, M. M. Salpeter and L. Bachmann

AUTORADIOGRAPHY, M. M. Salpeter and L. Bachmann

## Volume 3

---

THE ELECTRON MICROSCOPE, Saul Wischnitzer

ELECTRON MICROSCOPY OF SELECTIVELY STAINED MOLECULES,

T. Koller, M. Beer, M. Müller and K. Mühlethaler

HIGH RESOLUTION DARK-FIELD ELECTRON MICROSCOPY, Jacques Dubochet

IN-FOCUS PHASE CONTRAST ELECTRON MICROSCOPY, H. M. Johnson

ELECTRON MICROSCOPIC EVALUATION OF SUBCELLULAR FRAC-

TIONS OBTAINED BY ULTRACENTRIFUGATION, Russell L. Deter

STEREOLOGICAL TECHNIQUES FOR ELECTRON MICROSCOPIC MOR-

PHOMETRY, Ewald R. Weibel and Robert P. Bolender

CRITICAL POINT-DRYING METHOD, M. A. Hayat and B. R. Zirkin

**Volume 4**

---

OPTICAL SHADOWING, Glen B. Haydon  
RELATIVE MASS DETERMINATION IN DARKFIELD ELECTRON  
MICROSCOPY, G. J. Brakenhoff  
CORRELATIVE LIGHT AND ELECTRON MICROSCOPY OF SINGLE  
CULTURED CELLS, Zane H. Price  
DENATURATION MAPPING OF DNA, Ross B. Inman and Maria Schnöss  
EXAMINATION OF POLYSOME PROFILES FROM CARDIAC MUSCLES,  
Kenneth C. Hearn  
PARTICLE COUNTING OF VIRUSES, Mahlon F. Miller II  
ULTRAMICROINCINERATION OF THIN-SECTIONED TISSUE, Wayne R.  
Hohman  
PREPARATORY METHODS FOR ELECTRON PROBE ANALYSIS, James R.  
Coleman and A. Raymond Terepka

**Volume 5**

---

QUANTITATIVE MAPPING WITH THE ELECTRON MICROSCOPE, Peter  
Sterling  
PHOTOGRAPHIC ASPECTS OF ELECTRON MICROSCOPY, G. C. Farnell  
and R. B. Flint  
ENVIRONMENTAL DEVICES IN ELECTRON MICROSCOPY, David L.  
Allinson  
OPTICAL DIFFRACTOMETRY, Bjørn V. Johansen  
THE ANALYTICAL ELECTRON MICROSCOPY, EMMA-4, Barry A. Weavers

**Volume 6**

---

HIGH VOLTAGE ELECTRON MICROSCOPY, Colin Humphreys  
THE PRINCIPLES OF HIGH RESOLUTION ELECTRON MICROSCOPY,  
J. M. Cowley  
CONTRAST AND IMAGE FORMATION OF BIOLOGICAL SPECIMENS,  
R. E. Burge  
THE ANALYSIS OF BIOLOGICAL STRUCTURE WITH X-RAY DIFFRACTION  
TECHNIQUES, Alexander McPherson Jr.  
TILTING EXPERIMENTS IN THE ELECTRON MICROSCOPE, Rainer H.  
Lange  
ELECTRON AUTORADIOGRAPHY OF FREE SPECIMENS, Nadir M. Maraldi  
CRYOULTRAMICROTOMY, René Simard  
ELECTRON INTERFERENCE MICROSCOPE, T. Hibi and K. Yada

**Volume 7**


---

SPECIMEN DAMAGE IN THE ELECTRON MICROSCOPE, M. S. Isaacson  
 FREEZE-DRYING FOR ELECTRON MICROSCOPY, M. V. Nermur  
 IMAGE RECONSTRUCTION OF ELECTRON MICROGRAPHS BY USING  
 EQUIDENSITE INTEGRATION ANALYSIS, Klaus-Rüdiger Peters  
 G-BANDING OF CHROMOSOMES, F. Ruzicka  
 AUTORADIOGRAPHIC LOCALIZATION OF DNA IN NONMETABOLIC  
 CONDITIONS, M. Gueskens  
 OPTICAL ANALYSIS AND RECONSTRUCTION OF IMAGES, A. J. Gibbs  
 MIRROR ELECTRON MICROSCOPY, R. S. Gvosdover and B. Ya. Zel'dovich  
 ELECTON MICROSCOPY OF BANDED MAMMALIAN CHROMOSOMES,  
 Gary D. Burkholder  
 EQUIDENSITOMETRY: SOME NEUROBIOLOGICAL APPLICATIONS,  
 L. T. Ellison and D. G. Jones

**Volume 9**


---

ELECTRON MICROSCOPY OF ATOMS, John P. Langmore  
 ELECTRON MICROSCOPY OF DNA, John O. Thomas  
 TECHNIQUES FOR VISUALIZATION OF GENETIC MATERIAL, Aimée  
 H. Bakken and Barbara A. Hamkalo  
 LOCALIZATION OF ACETYLCHOLINE RECEPTORS, Mathew P. Daniels and  
 Zvi Vogel  
 ELECTRON MICROSCOPY OF ACTIN, Arthur Forer  
 ELECTRON MICROSCOPY OF GLYCOPROTEINS BY HIGH RESOLUTION  
 METAL REPLICATION, Henry S. Slayter  
 EXAMINATION OF THYROGLOBULIN MOLECULES IN THE ELECTRON  
 MICROSCOPE, Gertrud Berg  
 ELECTRON OPTICAL MEASUREMENT OF SURFACE CHARGES, Imre  
 Veres



5.19.10

# CONTENTS

id

## Preface

v

## 1 SPECIMEN SUPPORTS

W. Baumeister and M. Hahn

### Introduction

1

### Preparation and Properties of Specimen Supports

4

*Specimen Grids and Specimen Apertures*

4

*Microgrids and Perforated Films*

8

*Plastic Films*

21

*Supports for Unobstructed Mounting of Large Specimen Areas*

33

*Glass Specimen Supports*

36

*Carbon Films*

37

*Silicon and Silicon Oxide Films*

46

*Metal and Metal Oxide Films*

47

*Graphite Specimen Supports*

54

*Molybdenite Specimen Supports*

58

*Layer Silicate Specimen Supports*

59

*Self-Supporting Specimens*

64

<b>General Specimen Support Problems</b>	66
<i>Surface Conditioning</i>	66
<i>Sag and Radial Slope</i>	71
<i>Axial Oscillations</i>	73
<i>Radiation Damage</i>	75
<i>Temperature and Charging Effects</i>	82
<i>Methods for Thickness Measurement</i>	84
<i>Electron Optical Noise</i>	93
<i>Support Structure Suppression</i>	97
<b>References</b>	101

## 2 PREPARATION AND ANALYSIS OF SERIAL SECTIONS IN ELECTRON MICROSCOPY

Robert L. Knobler, Jerome G. Stempack, and Mary Laurencin

<b>Introduction</b>	113
<b>Tissue Fixation</b>	115
<b>Staining</b>	119
<b>Dehydration and Embedding</b>	121
<b>Grid Preparation</b>	123
<b>Sectioning</b>	127
<b>Section Collection</b>	134
<b>Microscopy</b>	141
<b>Analysis and Reconstruction</b>	144
<b>Concluding Remarks</b>	148
<b>References</b>	148

### 3 CALIBRATION OF MAGNIFICATION IN TRANSMISSION ELECTRON MICROSCOPY

Robert F. Dunn

Introduction	156
Factors Affecting Magnification	156
Calibration Specimens	159
<i>Diffraction Grating Replicas</i>	160
<i>Glass Spheres</i>	161
<i>Latex Spheres</i>	162
<i>Crystal Lattices</i>	163
<i>Viruses</i>	166
Lens Distortions	166
Methods of Calibration	169
Appendix: Derivation of Equation (3.9)	176
References	178

### 4 CONTRAST ENHANCEMENT BY USING TWO ELECTRON MICROGRAPHS

D. L. Misell

Introduction	181
<i>Detrimental Effects of Inelastic Scattering</i>	181
Image Formation by Inelastically Scattered Electrons	188
<i>Inelastic Images: Experimental Results</i>	190
<i>Inelastic Images: Theoretical Results</i>	195
<i>Inelastic Images: Effect of Chromatic Aberration</i>	197
<i>Comparison of Elastic and Inelastic Images</i>	199
Image Subtraction	204
<i>Theoretical Results</i>	204

**x CONTENTS**

<b>Practical Problems in Image Subtraction</b>	<b>209</b>
<i>Determination of Defocus in Bright-field Microscopy</i>	<i>210</i>
<i>Determination of Defocus in Dark-field Microscopy</i>	<i>217</i>
<i>Alignment of Two Images</i>	<i>219</i>
<i>Image Subtraction in Practice</i>	<i>222</i>
<b>Dark-Field Versus Bright-Field Microscopy</b>	<b>224</b>
<b>Image Subtraction For Low Resolution Images</b>	<b>233</b>
<b>Concluding Remarks</b>	<b>239</b>
<b>References</b>	<b>243</b>

**5 INTERFERENCE PHENOMENON ON OSMIUM  
TETROXIDE-FIXED SPECIMENS FOR SYSTEMATIC  
ELECTRON MICROSCOPY**

**Karl Hermann Andres and Monika von Düring**

<b>Introduction</b>	<b>246</b>
<b>Apparatus</b>	<b>247</b>
<b>Fixation</b>	<b>248</b>
<b>Production of Interference Reflecting Light (IRL) Phenomena</b>	<b>248</b>
<b>Natural Surface</b>	<b>249</b>
<b>Section Surface</b>	<b>249</b>
<b>Precise Excision of Specimen for TEM</b>	<b>257</b>
<b>TEM Analysis of CNS with IRL Method</b>	<b>257</b>
<b>References</b>	<b>261</b>

**6 COMPUTER PROCESSING OF ELECTRON MICROGRAPHS****P. W. Hawkes**

<b>Introduction</b>	262
<i>Electron Image Formation and Digital Processing</i>	264
<i>Preparation Artifacts and Radiation Damage</i>	266
<i>Electron Microscope Parameters</i>	267
<i>Scanning Electron Microscope Images</i>	268
<b>Equipment</b>	269
<i>Microdensitometry</i>	269
<i>Computing</i>	272
<i>Filmwriting</i>	275
<b>Types of Image Processing</b>	278
<i>Improvement of Image Appearance: "Aesthetic" Processing</i>	279
<i>Removal of Electron Optical Artifacts</i>	280
<i>The Phase Problem</i>	283
<i>Three-dimensional Reconstruction</i>	285
<i>Very Fragile Periodic Specimens</i>	288
<i>Pattern Recognition; Particle Counting; Automation</i>	289
<b>Picture-Handling Languages</b>	292
<b>Concluding Remarks</b>	297
<b>References</b>	298
<b>Author Index</b>	307
<b>Subject Index</b>	315

# 1. SPECIMEN SUPPORTS

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## INTRODUCTION

Despite the achievement of great insights into the structural organization of matter and the outstanding contribution of electron microscopy to the development of modern biology, there remains an obvious gap between the actual performance and the capability of modern instruments. Although the best commercially available instruments routinely provide resolutions of 0.2 to 0.3 nm with biological specimens, significant structural information is usually restricted to 2-3 nm. Bridging this gap is exactly what biologists should seek to achieve in order to investigate the finest details of macromolecular and molecular organization.

There are two main reasons why a breakthrough in the field of molecular microscopy has hitherto not been achieved. First, progress in preparatory techniques has fallen far behind progress in instrumental development, for reasons which seem too complex to be discussed here in greater detail. No doubt, the problems of specimen preparation have not been attacked with the same converging intensity afforded the improvement of the resolving power. This problem is, at least partially, a consequence of the multitude and diversity of experimental requirements involved. Second, a more fundamental reason for this gap is the radiation sensitivity of biological specimens which makes it difficult to obtain electron micrographs bearing a sufficiently close resemblance to the original structure. Significant progress can only be expected if the damaging effects of the electron irradiation can be circumvented or drastically reduced.

## 2 PRINCIPLES AND TECHNIQUES OF ELECTRON MICROSCOPY

This improvement, in turn, needs to be preceded by a thorough reconsideration of conventional preparatory procedures with regard to achieving optimal preservation. The problem of radiation damage has attracted wide attention over the past years, but we are still far from completely understanding the interactions involved among the specimen, stain, and support film during electron bombardment.

Since the early days of electron microscopy, the problem of obtaining adequate specimen supports has been one of the major obstacles to achieving a maximum utilization of instrumental capabilities. Ideally, specimen supports should meet three basic criteria: (1) high electron transparency, (2) minimum intrinsic structure, and (3) high mechanical stability under the electron beam. Of only slightly less importance are the ease and reliability needed in preparing a given specimen support. The first electron micrographs taken at higher than light-microscopical magnifications were obtained from "self-supporting" specimens, such as metal foils or cotton fibers spanning over the apertures of specimen diaphragms (Ruska, 1934). However, as early as 1935, Marton used "thin" aluminum foils as supports for microtome sections of biological specimens. Shortly thereafter, plastic (nitrocellulose) films, previously developed for experiments with ultrasoft X-rays (Trenktrog, 1923) and cathode rays (Kirchner, 1930), were introduced for electron microscopy (Marton, 1936, 1937).

In 1939, H. Ruska published the first review on the preparatory techniques. In the same paper he described an improved method and an apparatus (Fig. 1.1) for the routine production of thin nitrocellulose films. This progress in support

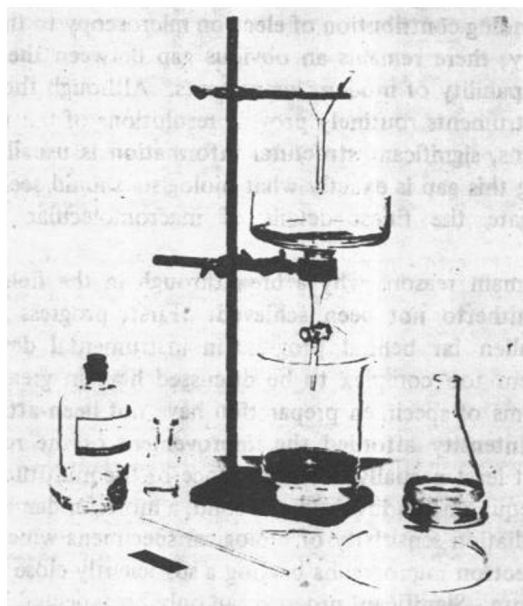


Fig. 1.1 Apparatus for spreading plastic films. (From H. Ruska, 1939.)

film techniques was immediately followed by a great success in biological electron microscopy, the visualization of virus particles (Kausche *et al.*, 1939).

In 1942, Schaefer and Harker introduced a new polymer, polyvinylformal, which, because of its improved mechanical stability in the electron beam, became the most commonly used material for support films. At moderate resolution levels criteria (1) and (2) are satisfactorily met by these conventional plastic films; nevertheless, the radiation sensitivity remains unsatisfactory. It seems astonishing that the potential of extremely radiation-resistant polymers developed in the last two decades has only scarcely been explored.

The progress in instrumental resolution, the growing insight into the mechanism of contrast and image formation, and the need for more radiation-resistant supports stimulated the development of various techniques for the production of low mass thickness metal films. Hass and Kehler (1941) described a method for the preparation of thin aluminum oxide films by anodizing an aluminum foil, a method that was later refined by several workers. Hast (1947), Cosslett (1948), and Kaye (1949) described techniques to produce thin aluminum, beryllium, or aluminum-beryllium alloy films by evaporation onto solid surfaces. Hast (1948) presented an elegant method to prepare extremely thin and smooth aluminum films by evaporation onto a glycerol surface. More than 20 years later, Müller and Koller (1972) revived this method of producing aluminum oxide films, which proved suitable for electron microscopy at atomic resolution.

Further progress was initiated by the development of a method to evaporate carbon to form thin, highly electron-transparent, and exceptionally stable amorphous films (Bradley, 1954). The deposition of thin evaporated carbon layers on top of plastic films became the most successful way of stabilizing these supports against drift and shrinkage under electron bombardment. Presently, thin, pure carbon films are by far the most common specimen supports used for high resolution electron microscopy. However, when the goal of visualizing single atoms was envisaged during the sixties, it became obvious that their expected faint contrast would be obscured by the pronounced phase contrast structure of carbon films.

As early as 1960, Fernández-Moran had put forward the attractive suggestion to use exfoliated graphite or mica single crystals as specimen supports, thus avoiding random variations of mass thickness which lead to the undesired granular appearance. Subsequently, Beer and coworkers explored various techniques for the production of suitable graphite crystals. The superiority of graphite over carbon films in the field of molecular microscopy has now convincingly been demonstrated (Wiggins and Beer, 1972; Hashimoto *et al.*, 1974; Johansen, 1975). Nevertheless, difficulties in achieving large scale production of sufficiently thin graphite films, the relatively small size of crystallites obtained, and entirely new adsorption properties necessitating new surface conditioning and specimen mounting procedures have hitherto prevented widespread application.

Some progress in the large scale production of very thin crystalline layers has



been achieved recently with vermiculite, a native hydrated aluminosilicate with a layered structure similar to that of the micas. It has been shown that the specimen information can further be enhanced if the periodic background displayed by these crystalline supports is separated from the specimen structure by optical filtering (Baumeister and Hahn, 1974a). One might suspect now that electron irradiation randomizes atom positions in the crystal and thus creates new random pictorial noise, which, because of its "white" spatial spectrum, cannot be discriminated any more from the usually extended object spectrum. Loss of crystallinity can, in fact, be observed, but it is nevertheless of minor importance, because the doses tolerated by crystalline supports without significant changes of crystalline order are greater by several orders of magnitude than those leading to the "steady state" of destruction in biomolecules.

More serious at present are the problems of surface conditioning of "low noise" supports for proper adsorption of biomolecules and avoidance of surface contamination during mounting and observing biogenic materials. Contaminated areas show a noise pattern quite similar to that of amorphous carbon films, which, if superimposed onto the specimen structure under investigation, obliterate the finest details.

## PREPARATION AND PROPERTIES OF SPECIMEN SUPPORTS

### Specimen Grids and Specimen Apertures

A wide variety of grid types for mounting very thin specimen supporting films or, whenever possible, for directly supporting the specimen is commercially available. The majority of the grids are supplied in two standard sizes, 2.3 mm and 3.05 mm in diameter. Usually grids are made of copper; for special fields of application (e.g., where reactive reagents are involved, for high-temperature investigations, for X-ray and microprobe analysis) titanium, stainless steel, nickel, molybdenum, rhodium, palladium, silver, tungsten, platinum, gold, and carbon-coated nylon grids are available. For some applications it may be sufficient to use the cheaper gilded, silvered, or platinized copper grids, instead of those manufactured completely of the respective noble metal.

Figure 1.2 shows a selection of commonly used specimen grid patterns. The standard square mesh grids are offered in various mesh sizes, ranging from 50-mesh/inch to 500-mesh/inch. The open area decreases with the increasing number of mesh/inch. The 50-mesh grids have an open area of approximately 80%, while the open area of the 500-mesh grids is usually as low as 35%. The exact values depend upon the specific bar thickness, which may vary between 15  $\mu\text{m}$  and 50  $\mu\text{m}$ . It is clearly economical to keep the ratio between the open area and the total area as high as possible. On the other hand, one has to consider that very large open areas with thin bars unduly weaken the grid and enhance the effects of specimen heating and charging.