Principles and Techniques of Electron Microscopy

BIOLOGICAL APPLICATIONS

Volume 8

M. A. HAYAT

Principles and Techniques of Electron Microscopy

BIOLOGICAL APPLICATIONS

Volume 8

M. A. HAYAT
Professor of Biology
Kean College of New Jersey
Union, New Jersey

VNR VAN NOSTRAND REINHOLD COMPANY
NEW YORK CINCINNATI ATLANTA DALLAS SAN FRANCISCO
LONDON TORONTO MELBOURNE

Van Nostrand Reinhold Company Regional Offices:
New York Cincinnati Atlanta Dallas San Francisco

Van Nostrand Reinhold Company International Offices:
London Toronto Melbourne

Library of Congress Catalog Card Number: 70-129544
ISBN 0-442-25693-0

Manufactured in the United States of America

Published by Van Nostrand Reinhold Company
135 West 50th Street, New York, N. Y. 10020

Published simultaneously in Canada by Van Nostrand Reinhold Ltd.

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Hayat, M A
Principles and techniques of electron microscopy.

Includes bibliographies.
Vols. 8 edited by M.A. Hayat.
1. Electron microscope—Collected works. I. Title.
[DNLM: 1. Microscopy, Electron. OH205 H413p H2VO]
QH212.E4H38 578'.4'5 70-129544
ISBN 0-442-25693-0 (v.8)

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 FRACTIONS OBTAINED BY ULTRACENTRIFUGATION, Russell L. Deter
STEREOLOGICAL TECHNIQUES FOR ELECTRON MICROSCOPIC MORPHOMETRY, Ewald R. Weibel and Robert P. Bolender
CRITICAL POINT-DRYING METHOD, M. A. Hayat and B. R. Zirkin

Volume 7

Volume 9

CONTENTS

2 PREPARATION AND ANALYSIS OF SERIAL SECTIONS IN ELECTRON MICROSCOPY

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

3 CALIBRATION OF MAGNIFICATION IN TRANSMISSION ELECTRON MICROSCOPY

Robert F. Dunn

4 CONTRAST ENHANCEMENT BY USING TWO ELECTRON MICROGRAPHS

D. L. Misell

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

Karl Hermann Andres and Monika von Düring

6 COMPUTER PROCESSING OF ELECTRON MICROGRAPHS
P. W. Hawkes

1. SPECIMEN SUPPORTS

W. Baumeister and M. Hahn

Institut für Biophysik und Elektronenmikroskopie der Universität Düsseldorf, Düsseldorf, Germany

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.

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

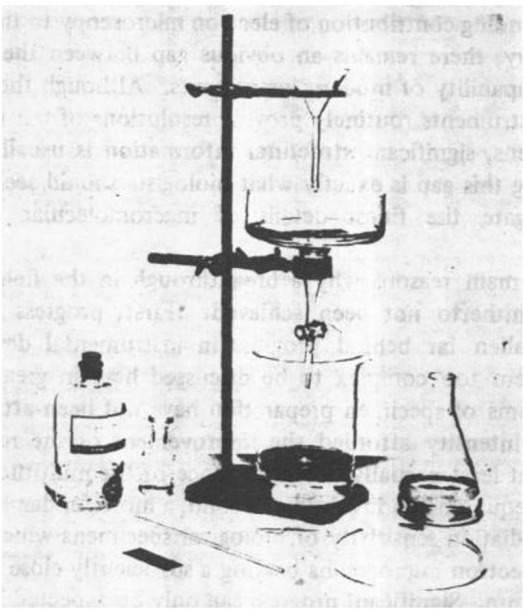

Fig. 1.1 Apparatus for spreading plastic films. (*From H. Ruska, 1939.*)

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 alumino-silicate 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 μm and 50 μm. 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.