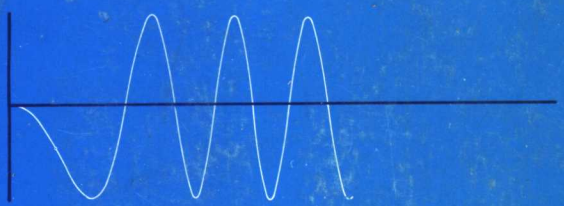
Image analysis, Enhancement and Interpretation

D.L. Misell



Practical Methods in Electron Microscopy

Editor: Audrey M. Glauert

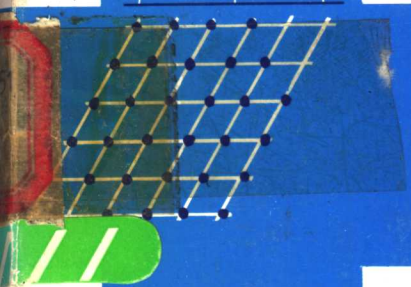


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IMAGE ANALYSIS, ENHANCEMENT AND INTERPRETATION

D.L. MISELL
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Editor's preface

Electron microscopy is now a standard technique with wide applications in all branches of Science and Technology, and every year a large number of students and research workers start to use the electron microscope and require to be introduced to the instrument and to the techniques for the preparation of specimens. Many books are available describing the techniques of electron microscopy in general terms, but the authors of Practical Methods in Electron Microscopy consider that there is an urgent need for a comprehensive series of laboratory handbooks in which all the techniques of electron microscopy are described in sufficient detail to enable the isolated worker to carry them out successfully. The series of books will eventually cover the whole range of techniques for electron microscopy, including the instrument itself, methods of specimen preparation in biology and the materials sciences, and the analysis of electron micrographs. Only well-established techniques which have been used successfully outside their laboratory of origin will be included.

Great care has been taken in the selection of the authors since it is well known that it is not possible to describe a technique with sufficient practical detail for it to be followed accurately unless one is familiar with the technique oneself. This fact is only too obvious in certain 'one author' texts in which the information provided quickly ceases to be of any practical value once the author moves outside the field of his own experience.

Each book of the series will start from first principles, assuming no specialist knowledge, and will be complete in itself. Following the successful innovation, made by the same publishers in the parallel series Laboratory

Techniques in Biochemistry and Molecular Biology (edited by T. S. Work and E. Work), each book will be included, together with one or two others of the series, in a hardback edition suitable for libraries and will also be available in an inexpensive edition for individual use in the laboratory. Each book will be revised, independently of the others, at such times as the authors and editor consider necessary, thus keeping the series of books continuously up-to-date.

Strangeways Research Laboratory
Cambridge, England

AUDREY M. GLAUERT, SC. D.
General editor

Author's preface

Beyond a visual examination of an electron micrograph, there are several methods of further analysing the image. This book aims to provide the necessary practical and mathematical background for the analysis of an electron microscope image in order to extract the maximum amount of structural information. Image analysis provides a quantitative way of assessing image defects (defocus, astigmatism) and image resolution. Also the state of preservation of the specimen can be assessed if it consists of an ordered array of subunits. On the basis of this analysis, structural and non-structural (noise) information may often be separated to give an enhanced image, which shows more clearly the structure of the specimen.

Instrumental methods of image enhancement are also described: these include the use of the energy-selecting electron microscope, and the scanning transmission electron microscope, which are specialised instruments or techniques. Non-standard imaging techniques such as the use of a long focal length objective lens, dark-field microscopy and single-sideband holography (optical shadowing) are easily achieved in the standard electron microscope.

The problems of image interpretation are considered with particular reference to the limitations imposed by radiation damage and specimen thickness. Only in favourable conditions can the normal two-dimensional image be interpreted in terms of a three-dimensional structure. A brief survey is given of the methods for producing a three-dimensional structure from a series of two-dimensional projections. However, in this book the emphasis is on the analysis, processing and interpretation of the two-dimensional projection of a structure.

This book is intended for the use of the biologist or materials scientist who wishes to improve the quality and interpretation of his electron micrographs, but who has little or no experience of image analysis or image processing. Although there is a substantial mathematical content in this book, it should be possible for non-mathematical scientists to understand the principles of image analysis and processing and be able to assess whether these techniques can be useful in their work.

Hamlet: Do you see yonder cloud that's almost in the shape of a camel?

Polonius: By th'mass and 'tis, like a camel indeed.

Hamlet: Methinks it is like a weasel.

Polonius: It is backed like a weasel.

Hamlet: Or like a whale?

Polonius: Very like a whale.

from *Hamlet* by William Shakespeare

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I am grateful to the following copyright holders for permission to reproduce the illustrations indicated: Academic Press Inc. (*Journal of Molecular Biology*; Figs. 2.1, 3.28, 3.39, 3.41, 4.20, 4.24, 4.25, 4.26, 7.1, 7.2, 7.3, 7.11 and 7.12) (*Journal of Ultrastructure Research*; Figs. 4.9, 4.10, 4.11, 4.12, 4.14, 4.15, 4.16, 5.9, 5.14, 5.18 and 5.19); Alan R. Liss Inc. (*Journal of Supramolecular Structure*; Figs. 3.24, 4.18, 4.19 and 4.20); North-Holland Publishing Company (*Ultramicroscopy*; Fig. 4.7); The Rockefeller University Press (*Journal of Cell Biology*; Figs. 4.12 and 4.13); Nature (*Nature*; Figs. 4.22, 7.9 and 7.10); The Royal Society (*Philosophical Transactions of the Royal Society*; Figs. 4.27, 4.28 and 5.15); The American Association for the Advancement of Science (*Science*; Fig. 4.30); The Royal Microscopical Society (*Journal of Microscopy*; Figs. 4.34, 5.1, 5.2, 5.3, 5.4, 5.5, 5.7 and 5.16); Pergamon Press (*Micron*; Figs. 5.23, 5.24 and 5.25); The Institute of Physics (*Journal of Physics*; Figs. 6.3 and 7.5); and the Company of Biologists (*Journal of Cell Science*; Fig. 7.13).

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London, April 1978 D.L. MISELL

Contents

Chapter 1

Introduction

Electron microscopy is a visual science, but observation of the image or the electron micrograph does not necessarily lead to the extraction of the maximum amount of available information. By using the techniques detailed in this book, it should be possible to extract all the structural information that is present in the electron micrograph and to quantify it.

Biological materials are prepared in several different ways in the hope of finding a method which most nearly preserves the original structure. For example, in the examination of a system such as the head of the bacteriophage T4 (Aebi et al. 1976, 1977), one of the objectives is to determine the geometrical arrangement and shape of the protein molecules in the capsid. Negatively-stained, freeze-dried, freeze-fractured or metal-shadowed preparations may be used, together with various imaging techniques that minimise radiation damage to the specimen. It is virtually impossible, however, to assess quantitatively the differences between the images obtained without using additional techniques such as optical diffractometry (Horne and Markham 1972) and image reconstruction (Aebi et al. 1976, 1977). For T4 phage heads the fact that the capsid is an assembly of regularly arranged protein subunits enables a determination of their geometrical arrangement to be made, whilst the highest order diffraction spot observed in the *optical transform* (§ 3.3), or *diffractogram*, gives some indication of the order preserved in the specimen. In addition, in such preparations, the images of the top and bottom surfaces are superimposed. This leads to an uninformative moiré pattern, but careful analysis of the optical transform of the image allows the contributions of the two surfaces to be separated.

The next step is to use the optical transform to produce an *enhanced image*, based on the regular (periodic) arrangement of protein subunits. More

accurately this procedure should be referred to as *image reconstruction*, image enhancement being the rebuilding of the original micrograph from only that information which is relevant to the specimen structure. Image enhancement can also be produced using computer based analysis of a numerical *Fourier transform* (§ 3.4). In this procedure the optical density variations in the micrograph are converted to numbers for *digital processing*. This has some distinct advantages: for example, the image can be corrected for small distortions in the positions of subunits nominally in a regular array (Crowther and Sleytr 1977). Both optical and digital processing of electron images provide quantitative information on how well the biological structure has been preserved, the artefacts introduced by the techniques used to prepare the specimen, and, in high resolution applications (0.5–2.0 nm), the effects of radiation damage.

Image reconstruction may be practical in only a few laboratories, but the analysis of images by optical diffractometry should be a routine procedure for all microscopists who wish to make the best use of their micrographs, irrespective of specimen resolution. The basic principles of image analysis by both optical and computer methods are discussed in Chapter 3, and some typical applications in materials science and biology are described.

It will be evident that image analysis is most useful when the specimen has some type of symmetry, such as translational (as in a two-dimensional array of units), helical or rotational. This is not at all uncommon in biology. A number of membrane systems show a regular arrangement of protein subunits, for example gap junctions and bacterial outer membranes (Thornley et al. 1974). Also, some viruses, like adenovirus and T-even phages, show a periodic arrangement of subunits. Helical symmetry is found in microtubules, tobacco mosaic virus (TMV), T4 phage tails and in some nucleoprotein complexes from viruses, such as Sendai virus. Rotational symmetry, where a repetition of the structure occurs on rotating the particle about its centre through $360°/n$ for n-fold symmetry, is exhibited by T4 phage base plates ($n = 6$), TMV stacked disc protein ($n = 17$), and assemblies of proteins from virus surfaces such as the 'groups of nine' hexons of adenovirus ($n = 3$).

Frequently the order within the specimen is only preserved over a small number of subunits, but this is sufficient for at least a preliminary structural analysis by electron microscopy. Certainly, if large (≥ 200 μm) three-dimensional crystalline arrays are available, electron microscopy cannot compete with X-ray crystallography, but often such crystals cannot be made. For example, the purple membrane of *Halobacterium halobium* occurs in

two-dimensional, highly ordered sheets; only limited information is available using X-ray diffraction techniques (Blaurock 1975; Henderson 1975), but careful (low radiation dose) electron microscopy of unstained purple membrane sheets and image reconstruction techniques have led to a three-dimensional structure of the membrane protein at 0.7 nm resolution (Henderson and Unwin 1975; Unwin and Henderson 1975).

In addition to naturally occuring ordered systems, there are several examples where, by biochemical methods, normally irregular (aperiodic) structures can be encouraged to form quite well ordered crystalline arrays; examples include neuraminidase and haemagglutinin, proteins from the envelope of influenza virus. Whole viruses may also be encouraged to form large two-dimensional ordered arrays (Horne and Ronchetti 1974).

Even if the specimen shows no order, image analysis provides a rapid way of assessing image quality; that is, how good it is electron-optically, with respect to focus, astigmatism, specimen drift and other image defects (§ 3.3). Of course, the microscopist tries to minimise these defects by correct use of the microscope, but image analysis (particularly optical diffractometry) will provide a rapid post-diagnostic measurement of the magnitude of image defocus and other defects.

Most image reconstruction techniques are designed for specimens exhibiting symmetry (see Chapter 4), but there are several methods of image enhancement for aperiodic or amorphous specimens. These are more subjective than the methods for ordered specimens, but they have sometimes led to a gain in structural information. For example, in attempting to resolve single heavy atoms in labelled biological macromolecules, such as deoxyribonucleic acid (DNA), and organo-metallic compounds, *spatial* (*Fourier*) *filtering* (§ 4.7) is used to reduce the high frequency noise in the image and facilitate the determination of atom positions (Ottensmeyer et al. 1972). At a much lower resolution the enhancement of boundary structures in sectioned material can be achieved by, for example, differentiating the image (§ 6.3).

Image processing is the next step to follow image analysis. Its objective is to produce a structurally enhanced version of the original image. It is not a cosmetic for making poor images into good ones, but it does enable the extraction of the maximum amount of structural information from the original image. It is no substitute for the use of the best specimen preparation photographed under minimum irradiation conditions, but is a complementary technique. There are published examples where the reconstructed image gives no more information about the specimen structure than was available

by a careful examination of the original image. So methods for obtaining the 'best' images (as assessed by optical diffractometry) are emphasized, before proceeding with the long operation of image processing.

The major part of this book is concerned with the analysis and processing of images after they have been photographed by conventional transmission electron microscopy (CTEM), but instrumental methods of improving image contrast are also discussed (Chapter 5). Clearly 'non-conventional' electron microscopy which can produce clearer images is to be preferred to post-processing. Some of these methods require the use of specialist instruments, such as the scanning transmission electron microscope (Crewe and Wall 1970; Crewe 1971) and the energy-selecting electron microscope (Henkelman and Ottensmeyer 1974), while other techniques, such as dark-field electron microscopy, where only a small modification to the conventional electron microscope is required (Kleinschmidt 1971), may be unfamiliar to the majority of biologists.

Assuming that image analysis and image processing have been fully exploited, the final step is to interpret the image in terms of structure, with a consequent inference concerning the biological function of a system which has been examined under the harsh conditions existing in the electron microscope (Chapter 7). However detailed a picture emerges as a result of image processing, the material examined in the electron microscope is in the dried state. Only where other physical techniques confirm the dimensions or arrangement of known structural features can there be said to be a fair understanding of the organisation of the original (and probably hydrated) structure.

The scope of this book excludes a detailed account of one major aspect of image processing, namely, three-dimensional reconstruction techniques (DeRosier and Klug 1968; DeRosier 1971). Only the analysis of two-dimensional projections of a three-dimensional object is included; here the superposition arising from observing a three-dimensional structure in an electron microscope limits the information available (§ 7.3).

The purpose of this book is to convince the biologist that image analysis and image processing or reconstruction offer an additional dimension to electron microscopy, that may lead to structural information over and above that seen directly in the image. So far these techniques have failed to become well established in biological electron microscopy, probably because of the complexity of the mathematics associated with them. Thus in the main part of this book I hope to convince the biologist that image analysis is a valuable tool by showing many practical examples, and providing the mathematical basis as simply as possible.

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