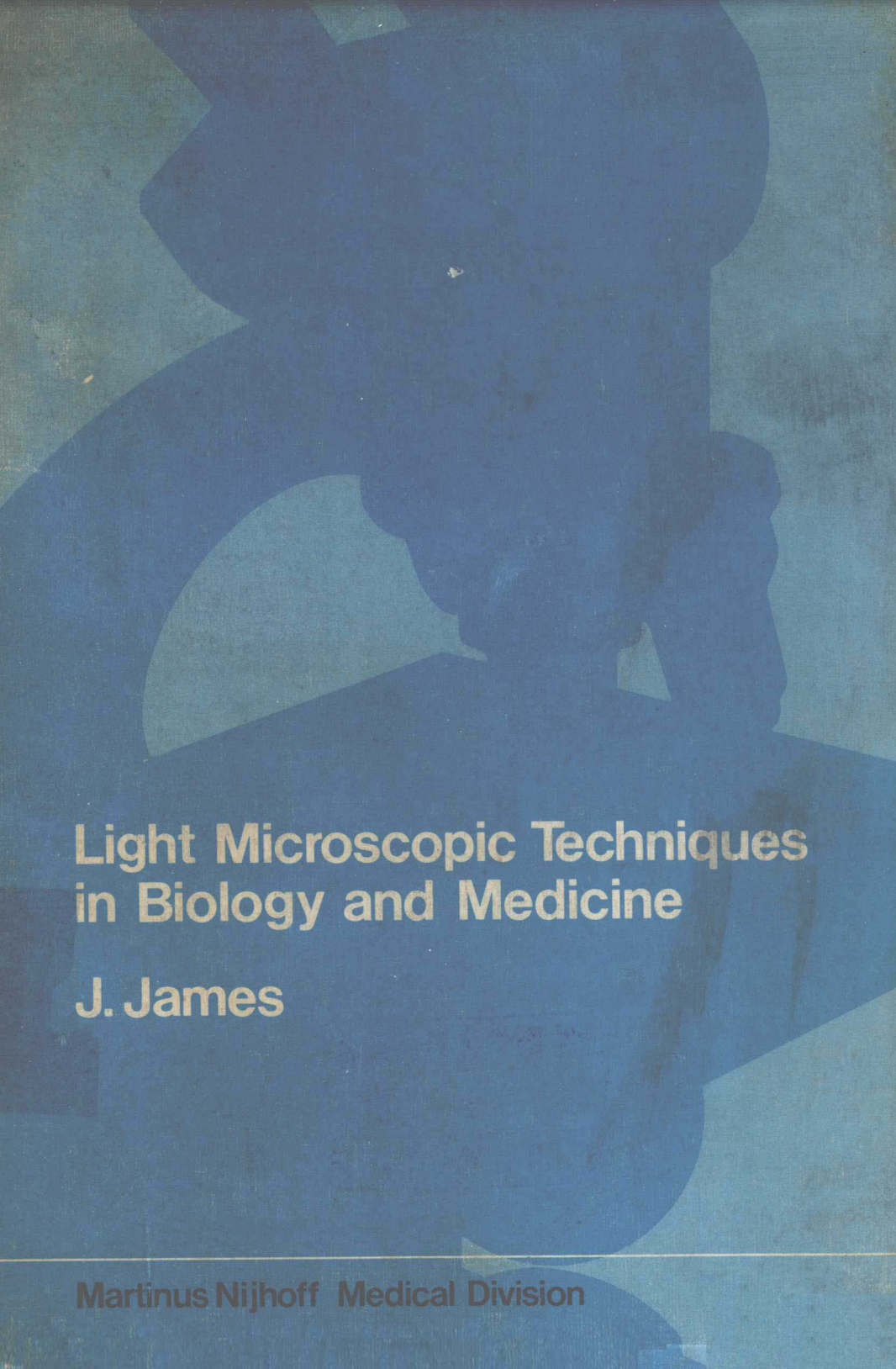

Light Microscopic Techniques in Biology and Medicine

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LIGHT MICROSCOPIC TECHNIQUES IN BIOLOGY AND MEDICINE

PREFACE

Up to about twenty-five years ago, virtually the entire field of microscopy could be overseen and even practized by any active research worker. The rapid evolution which microscopy in its broadest sense has since undergone and which has contributed greatly to our insight in many fields of biological science and medicine has, however, lead to a progressive specialisation. Both experienced investigators in clinical and biological laboratories and post-graduate students, confronted with a limited number of microscopic techniques in their daily research work, have increasing difficulty in keeping (or obtaining) a general idea of the many time-honoured and new possibilities which microscopy has to offer.

This book has been written with the aim of presenting general information on light microscopic techniques, at a level somewhere in between booklets like those provided by microscope manufacturers (which are often too much focussed on the production program of a particular make) and very advanced treatises with a thorough mathematical treatment of all phenomena concerned. The physically oriented texts moreover often do not sufficiently take into account the practical situation in a medical or biological laboratory; on the other hand, the value of really understanding what one is doing in using a microscopic technique is often underestimated. Attempt has been made, therefore, to present sufficient background information necessary for a rational application of the different microscopical techniques in their mutual relationship. The text has thus deliberately been given a twofold character, that of a practical guide and of a scientific introduction with references for further reading. Both aspects have a somewhat different emphasis in the two main sections of the book, the first seven chapters dealing with conventional techniques and the second part devoted to advanced techniques of microscopy. In this second part, much attention has been paid to new image-forming principles and quantitative aspects of micro-

scopy. Unlike an earlier version of this book in Dutch published in 1969, electron microscopy has only been dealt with in passing to show certain similarities and dissimilarities between light and electron rays as image-forming agents; several recently published books deal adequately with this rapidly expanding group of techniques. Moreover, the author's experience lies more in the domain of light microscopy and it is a sufficient challenge to deal with this field alone.

I am indebted to some colleagues from Holland and abroad for help in reading some parts of the manuscript and/or providing material, tips or references; in particular I want to thank Dr. Goldstein (Sheffield), Dr. Ploem and Dr. de Bruin (Leiden). The photographic work has again been performed by Mr. J. Peeterse. New drawings and graphs have been made by Mr. J. van Dusschoten (those kept from the older Dutch edition were executed by Mr. E. J. J. Eerkens).

Dr. R. D. R. Birtwhistle has been the invaluable adviser in the preparation of the English text, reviewing the entire manuscript, which was read with the proofs by Prof. D. B. Kroon; Dr. P. Mestres (Bochum) helped in translating technical terms into Spanish for the four-lingual technical vocabulary. Miss M. E. Tollenaar performed the laborious task of typing the entire manuscript and bibliography from start to finish. The publisher has met with a great number of reasonable and unreasonable wishes and good co-operation has been maintained during the production of the printed text.

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January 1976

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PART I

CONVENTIONAL TECHNIQUES OF MICROSCOPIC OBSERVATION

CHAPTER 1

SOME ESSENTIALS OF GEOMETRICAL OPTICS

GEOMETRICAL OPTICS AND WAVE OPTICS

Light is a form of electromagnetic radiation and as such a wave phenomenon. The wavelength is so small, however, that light rays can be considered often as rays propagating linearly. What happens when e.g. a band of light passes a lens, can be illustrated quite adequately with geometric constructions. When phenomena are studied near the level of the wavelength of light, however, geometrical optics fail to explain what happens and light should be treated purely as a wave phenomenon. This is the case when light is used in microscopy; objects with a diameter of about 0.0005 mm – such as bacteria and parts of animal cells, e.g. mitochondria – are in the same size range as the wavelength of the light with which they are to be studied.

In view of these small – and even much smaller – dimensions of object size and wavelength, which would enforce the use of increasingly smaller fractions of millimeters, the following measures have been introduced in microscopy:

micrometer,	abbreviation μm (formerly micron or μ) = 0.001 mm $= 10^{-6}$ m
nanometer,	abbreviation nm = 0.001 μm = 10^{-9} m.

These measures form a part of the internationally adopted SI (Système International) system in which the meter has become the basic unit of length (with this system, which also holds for weight, the prefix milli- means 10^{-3}, micro- 10^{-6} and nano- 10^{-9}). The term millimicron (mμ) thus has become obsolete, although it is still occasionally used. Another unit of length which does not fit into the SI system is the Ångström-unit, abbreviation ÅU or Å (called after a Swedish physicist from the nineteenth century) which is 0.1 nm or 10^{-10} m. It is still often employed in electron microscopy and for the expression of wavelengths, although a tendency now exists to replace this unit which dates from before the international metric system by an universal expression in μm and nm.

The term microscopy is associated in first instance with the use of *visible light*, with a wavelength from about 380 nm to 760 nm viewed by the eye as violet or red, respectively. In microscopy other types of radiation from the electromagnetic spectrum than light are also used, such as *infrared light* with a longer wavelength than that of visible light and *ultraviolet light* with a shorter one. Infrared and ultraviolet light do not differ essentially in their physical properties from visible light. This is no longer the case with *X-rays* or *röntgen-rays* with wavelengths of a few Ångström-units, or fractions thereof (chapter 12). With bundles of accelerated electrons (comparable in their physical behaviour with electromagnetic waves) the wavelength is likewise a small fraction of an Å. In contrast to the situation with X-rays, electron waves can be influenced in their course by electromagnetic fields, which has made possible the development of electron microscopy. Both in X-ray microscopy and in electron microscopy, mainly geometric phenomena have to be taken into account in explaining the formation of the image. Objects which come even approximately within the size range of these short wavelengths cannot be observed with X-rays or electron microscopy, but wave optics can be applied here for submicroscopic analysis (X-ray diffraction, electron diffraction).

All image forming agents used in microscopy have in common physically that they are electromagnetic radiations, or behave as such. They have been shown to propagate with equal velocity in the same matter. Because in a homogeneous medium the propagation velocity of an electromagnetic wave equals the product of wavelength and vibration frequency, the latter quantity is inversely proportional to the wavelength.

RESOLVING POWER AND THE EYE

With a normally built, so-called emmetropic eye an object at infinity (in practice: far removed from the eye) forms a sharp image on the retina (fig. 1.1A). When the object is approximated to the eye, the image remains sharp up to a distance of about 250 mm. This is brought about by the fact that the refractive power of the eye lens is continuously enhanced by what is called *accommodation*. The closest distance to the eye at which a sharp image can be formed is called the *near point* (punctum proximum); although large individual variations exist, it has been standardized at 250 mm for optical calculations. The observation of objects at a distance of much less than 250 mm is as a rule possible with younger persons; the accommodation (brought about by an increase of the curvature of the eye lens when the

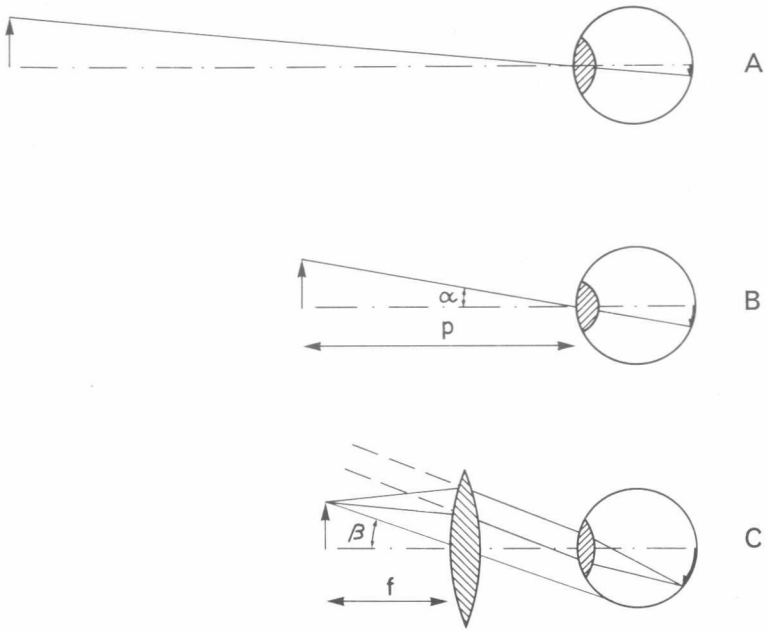

Fig. 1.1. A Observation of a distant object. *B* Observation of the same object, moved towards the eye up to the near point. The accommodation is maximal, the angle of vision α as large as possible. *C* Observation of the same object with a simple microscope (loupe). The angle of observation has been enlarged to β, the image is far removed ('in the infinite'), due to the positioning of the object in or near the focal plane of the lens; accommodation is slight or absent, the eye lens has a flattened form.

circular tension exerted on it is relieved by muscular contraction) then puts a sensible strain on the eye.

In moving an object towards the eye, the image on the retina will grow: one takes a 'closer look' (fig. 1.1A and B). This enlargement of the image is obviously limited by the nearest point to the eye at which distinct vision is still possible as mentioned before (P in fig. 1.1B). This distance increases with age, from about 7 cm at ten to about 20 cm as forty is approached, after which it increases slowly. Apart from age, variations in this distance can also be due to other causes, e.g. myopy in which it may be considerably shorter.

Except by the mutual distance of the photoreceptors in the retina, which can be considered as fairly constant, the ability of the eye to recognize two closely related points as discrete entities is determined by the angle at which the light rays from both points enter the eye. This angle will be the greater when the near point is closer to the eye. When the smallest distance which can be separated is 8 mm, the *resolving power* is defined as 1/8.

Normal visual acuity is sufficient to distinguish points which subtend an angle of one minute of arc, which corresponds with a distance between retinal images of 4-5 μm. As the diameter of the retinal receptors is just less than 3 μm, it can be concluded that two adjacent points of light can be distinguished if their images fall on two separate photoreceptors with an intermediate, unstimulated retinal receptor. If it is assumed that the near point at which the largest sharp image is obtained is at the standard 250 mm, this would correspond with a minimal distance of about 0.07 mm. This can be reached under the most ideal circumstances, however; this minimal distance is usually reckoned at 0.1-0.2 mm.

It is obvious that if the near point is closer to the eye than the standardized 250 mm, the image in the retina will increase correspondingly, enhancing the resolving power. As explained before, this is possible with younger persons and in the case of myopy. These two factors are independent from one another so that they can have a cumulative effect; a myopic teen-ager sometimes can accommodate until 50 mm, enabling the separation of distances of 0.020 to 0.015 mm[1]. On the other hand, the difference in refractive power of the eye in the two states of complete relaxation and maximal accommodation decreases with age; hence the near point recedes, so that between 40 and 50 years it comes to exceed 250 mm. This involves a corresponding decrease in resolving power of the eye, without the quality of the retina being involved. As a rule, this phenomenon can be adequately compensated by reading glasses.

Apart from the formation of the geometric image of the object discussed so far, *contrast* and *brightness* of the image play a role. It can be easily demonstrated that two closely applied dots of ink on brown paper will fuse more easily for the eye than two similar dots on white paper; illumination with a candle or a strong light bulb will also make a great difference. These are influences on the resolving power which affect the quality and not the size of the retinal image. In contrast to the effects related to the accommodation, these phenomena occur also in observing objects at larger distances.

THE SIMPLE MICROSCOPE

Assuming that brightness and contrast are optimal, it is obvious that the resolving power of the eye can be increased only when the angle of vision,

1. Probably this forms an explanation for the extremely fine detail discernible only with a loupe in some miniatures from the early middle ages. It is beyond question that these small paintings were made in a period when magnifying glasses were not yet in use.