The Microscope Series

Special Methods in Light Microscopy

Robert B. McLaughlin

SPECIAL METHODS IN LIGHT MICROSCOPY

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List of titles

Five volumes have now been published. Other titles will be published over the next few years. A complete listing follows:

1. **A Short History of the Electron Microscope**
2. **Modern Electron Microscopes** (SEM, TEM: design, applications, limitations)
3. **Accessories for the Transmission Electron Microscope** (stages, apertures, cameras, image enhancement SAD, SSE etc.)
4. **Preparation of Samples and Other Techniques for the Transmission Electron Microscope** (sectioning, staining, replication etc.)
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15. **The Optical Performance of the Light Microscope, Part II**
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51. **Dictionary for Microscopy**
52. **Teaching Microscopy**
53. **Image Processing**
54. **Microscopy for Art Conservators**
55. **Microscopy for Parents**
56. **Modern Optical Methods in Microscopy**

SPECIAL METHODS IN LIGHT MICROSCOPY

Dedicated to my wife Katharyn, daughter Susan, and son Scott, for their constancy of support and forbearance.

—about the author

R. B. McLAUGHLIN

—— received his bachelor's degree in electrical engineering at Tri-State College in Angola, Indiana in 1949. He was employed at the time of his recent retirement as Assistant Chief, Electronics Engineering Branch, Federal Aviation Administration, Anchorage, Alaska.

From 1949 to the present he has carried on the study and practice of microscopy. His major field of interest is in the diatomaceae. He is the author of Volume 16 of this series.

He is a member of the New York Microscopical Society, the Postal Microscopy Society, the Quekett Microscopical Club and a Fellow of the Royal Microscopical Society. In 1977 he was appointed a Research Associate of the McCrone Research Institute.

PREFACE—INTRODUCTION

The title of this book may imply to some that unusual or extraordinary methods of light microscopy are treated. That is not the case. The term "special methods" is used to indicate limited, particular, specific or selected methods of light microscopy.

Topics included herein are not new or unusual at all, most having been subject matter in numerous past publications, and will, no doubt, be included in many future publications.

The subject matter of the first two chapters, resolution and contrast, has been examined, discussed and treated both theoretically and practically from the early days of light microscopy. The remainder of the book also includes material that is not particularly new, although an attempt has been made to include current information.

Microscopy is not a "cookbook" process and those who would become more than uninformed or casual users of the instrument must acquire some technical background and familiarity with both theoretical and practical aspects of it.

Volumes 14 and 15 of this series provide a sound, though elemental, basis on the theory of the light microscope. It is intended that this volume provide the more practical follow-on aspects of adjustment and use, and, in addition, furnish an overview of some of the many techniques with which the light microscope is allied.

The practice of microscopy has its biases, personal viewpoints and prejudices in adjustment and technique. One book, such as this, cannot resolve all of the differences nor serve all of the purposes inherent in the practice of light microscopy. Only wide reading and assimilation of many viewpoints can approach this ideal.

I have attempted to include information that is both useful and interesting. Wherein certain possibly controversial topics are touched upon, I have tried not to be dogmatic. When it seemed necessary to provide some basic theory for a better understanding of an adjustment, procedure or technique, I have done so. On the other hand, many of the topics covered in this book are covered at book length in other volumes of this series. Therefore, many of them are presented "bare-bones", and it is assumed that the reader who wishes to obtain a detailed treatment of a specialized subject will avail himself of information contained in references listed in this book, or in companion volumes of the series. The choice of what to include and where the emphasis has been placed

has been mine. Any shortcomings, errors and/or oversights are entirely my responsibility.

Manufacturers of microscopes and associated equipment have been most generous in supplying photographs and other illustrations for my use. Many have provided technical papers, catalogs and other data and permitted me to include such information either verbatim or in abstracted or paraphrased form. Carl Zeiss Incorporated has supplied valuable information across the spectrum of light microscopy. Others that have contributed greatly to the content of this book are: Walter C. McCrone Associates, Inc., American Optical Corporation, Bausch & Lomb, Nippon Kogaku K.K. (Nikon), Unitron Instrument Company, Gaertner Scientific Corporation, R. P. Cargille Laboratories, Modulation Optics, Inc., M.E.L. Equipment Co. Ltd. (Watson, the Microscopy Division), Graticles Ltd., ATM Corporation, Universal Optics Ltd., Lemont Scientific, Olympus Corporation of America, and North American Philips. Where appropriate, credit is given for photographs, drawings or adapted drawings in the figure captions.

Finally, I wish to express my appreciation to Dr. McCrone for his assistance and continuing confidence in me, and to his staff for their unflagging support.

Robert B. McLaughlin

VOLUME 17

SPECIAL METHODS IN LIGHT MICROSCOPY

by Robert B. McLaughlin

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SPECIAL METHODS IN LIGHT MICROSCOPY

CHAPTER 1
METHODS IN ACHIEVING IMPROVED RESOLUTION

A. RESOLUTION - A DEFINITION: Resolution may be defined as the minimum separation of parallel lines or adjacent points in a given subject that can be made visible as separate lines or points in the image under actual conditions.

A key word in this definition is "visible". The human eye, its characteristics and the conditions under which it functions are very important to the ability to perceive detail. In critical light microscopy the microscope, the eye and the brain function as a system.

To achieve the ultimate use of the light microscope, the microscopist must not adhere blindly to some of the more established "rules" of microscopy as immutable doctrine. On the other hand, he must accept the fact that to obtain best results from the instrument, certain fundamental considerations must be attended to.

The effect of cumulative errors with improper adjustment will prevent the best results from being obtained. There are literally dozens of factors which affect the ultimate interpretation of what is seen by the observer. While, in many cases, each has a very small effect, the cumulative result of neglecting them results in a comparatively large degree of image degradation. It has been estimated that at least a 50 percent improvement in results can be obtained by adhering to the more fundamental adjustment procedures quite often neglected completely by the practicing microscopist.

In the following paragraphs of this chapter are some of the more important considerations which, if taken into account in operation of the light microscope, will provide the best resolution of detail. Enhancement of the image by methods to improve contrast are treated in the next chapter.

B. RESOLVING POWER FORMULA - A GUIDE: The resolving power of a lens (the ability to separate detail), used under a given set of conditions, is essentially a physical limit which generally is only approached, seldom reached, and never surpassed.

There have been many equations developed for expressing the resolving power of a lens. The one that has the greatest practical value is:

$$d = \frac{1.22C\lambda}{NA} \qquad \text{Equation (1)}$$

where: d = separation distance of two self-luminous points in the object plane.
C = a factor varying between a minimum of 0.4 and 1.0 depending on such factors as the correction of the objective, and the individual capacity of observers to detect minute differences in intensity.
(lambda) = wavelength of the illuminating light.
NA = numerical aperture of the objective.
1.22 = a constant.

The equation above is developed for conditions of self-luminosity, and is valid for equivalence to self-luminosity.

If we use objectives and condensers of the highest perfection, the factor C can be assumed to be slightly more than 0.4. If this becomes the case then the equation reduces to approximately:

$$d = \frac{0.5\lambda}{NA}$$

or

$$d = \frac{\lambda}{2NA} \qquad \text{Equation (2)}$$

and the minimum separation distance to be resolved with white light of approximately 0.55 μm wavelength (λ), and an objective with a 1.25 NA is:

$$d = \frac{0.5 \times 0.55}{1.25} = 0.22\ \mu m$$

Objectives with a slightly higher NA (1.40) and light of somewhat shorter wavelength can provide a resolution figure of somewhat less than 0.2 μm which is the generally accepted limit for the visual light microscope (Figure 1).

From Equation (1) it is obvious that an increase in resolving power (decrease in the value of d) can be obtained by a decrease in the value of C, a decrease in the wavelength of the light used and an increase in the numerical aperture (NA) of the objective. The converse, of course, is true. These, alone, comprise the significant factors on which the microscopist can base his actions to achieve the best resolution. There are other equations expressing the relationships of factors affecting the resolution obtainable, but Equation (1) has been chosen because it recognizes,

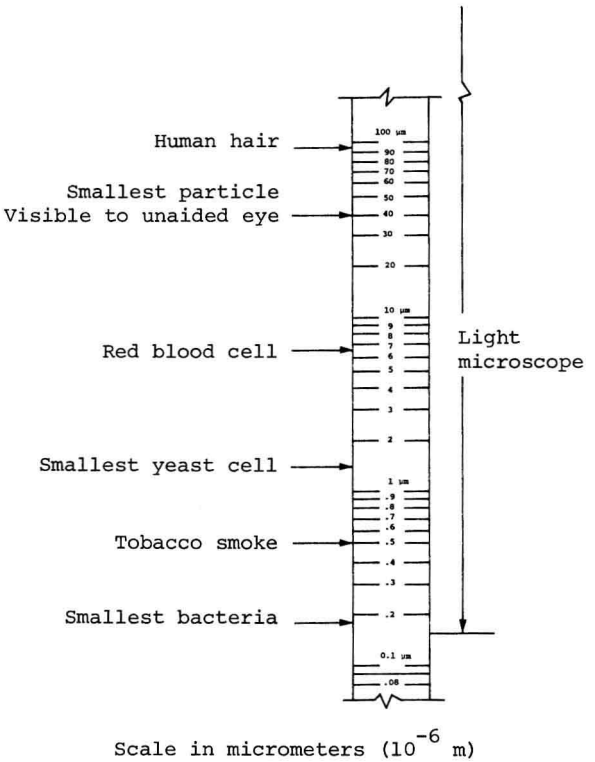

Figure 1. The range of the light microscope.

through the inclusion of the factor C, that lens aberrations and the human eye are a part of the relationship. This simple expression, then, will provide much on which to base a choice of optical elements, lighting requirements and adjustment of the system.

C. CHOOSING OPTICS: A most important factor in optimizing the resolution of the light microscope is the intelligent selection of the optical components to be used. Those components are the objective, the substage condenser and the ocular(s).

1. Objectives: Choice of objectives for optimum resolution capability is based on numerical aperture (NA) and on aberration

corrections applied (factor C in Equation [1]) to the particular objective. Refer to Figure 2 for some typical lens arrangements.

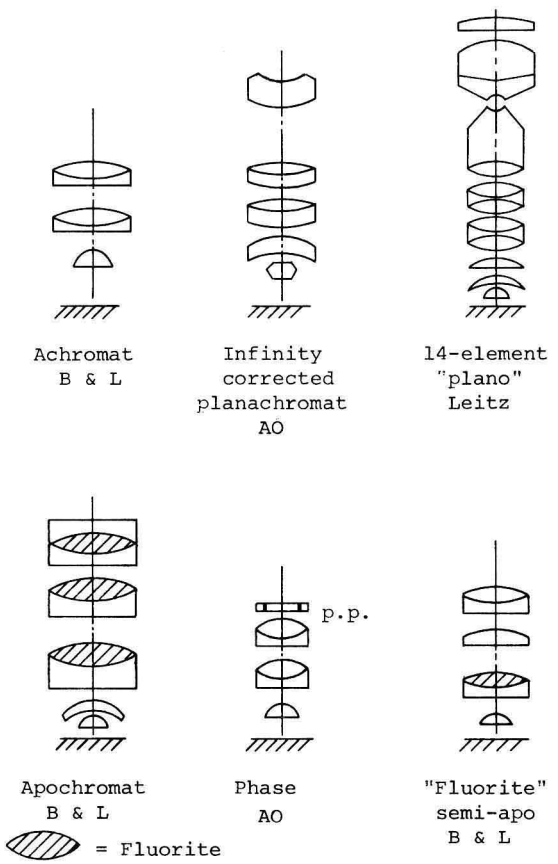

Figure 2. Lens arrangement. Some objective types.

a. Achromat: The achromatic objective is corrected for chromatic aberrations at two wavelengths, one in the red and one in the blue, and is fully corrected for spherical aberration at only one wavelength in the yellow-green (D line). At other wavelengths in the visible spectrum the correction for spherical abberation is good but not complete. Field curvature is present.