Handbook of Microscopy

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Preface

The late Maurice Langeron and I shared two common passions, microscopy and mycology. They soon brought us together for, having corresponded with him just before the Second World War, I met him on my arrival in Paris and we soon discovered that our personalities were very similar. In 1946, we started a series of weekly discussions on the then spectacular developments of microscopy and its biological applications, particularly in relation to parasitology and mycology. Just before the publication of the seventh edition of his standard work *Précis of Microscopy*, which proved to be his last publication, we had agreed on the necessity to publish a completely revised eighth edition, recast in a new form on which we had jointly decided. However, the death of Mr Langeron prevented us from completing this new project.

In 1957, we had undertaken to write and publish a *Treatise of Microscopy* in collaboration with Albert Policard and Marcel Bessis. This was not intended either to replace or to follow Langeron's *Précis*, because it remains irreplaceable as far as the information contained in it is concerned.

Both of these books, published by Masson and Co., are now out of print and since light microscopy has made great strides during the past twenty years, I started again on the project we had once drawn up in the quiet isolation of his laboratory in the rue de l'Ecole de Médecine, and I decided to complete it, with the valuable collaboration of our joint editor, in the form of this new *Handbook of Microscopy*.

When a microscopist finds a new object, he is often in an awkward situation. If his systematic and anatomical knowledge is sufficient, he will probably find another similar object, starting from which he might conceive of a new efficient method of study. His literature search may lead him on to difficult questions: how to choose the best of several hundred fixatives, how to choose the best stain among thousands of which many are known under several names, and how to remember the few applicable techniques from among the hundreds of thousands which have been published, many with variations. The encyclopaedic collections, like those of McClung or the Bolles Lee, will not help him unless he has already acquired a good grounding of histology and cytology. Specialist books may confuse him because of the multiplicity of overlapping techniques, or dishearten him by the omission of essential details. Any logical thread may be scattered in different books, more physicochemical than biological, and he will have to integrate the different disciplines, with which he may not be acquainted, to be able to extract the main ideas.

I undertook the task of writing this book, despite the justified pessimism shown by Mr Gabe in 1968 concerning the possibility of writing an up-to-date manual of microscopy. Optimally, it should comprise, within a manageable length, both a logical choice of staining systems mentioned earlier, and a concise and detailed account of old and new methods assessed critically. Unfortunately, I had to do it on my own, since Maurice Langeron is no longer with me to help with this *Handbook of Microscopy*. It is

published in English by Butterworths and in France by Masson, and is intended to complement the classic book by Mr Gabe, *Histological Techniques*, which goes more deeply into histochemistry. It deals mainly with biological applications of light microscopy, leaving quantitative analysis to more specialized books such as the one mentioned above.

The selection is critical, since repetitive uses have been eliminated; the best reagents have been chosen for routine histology irrespective of current practice, such as the unjustifiable preference of ethanol over acetone, of toluene over benzene and of the fixatives of Hollande or Zenker over those of Bouin and Kopsch-Regaud for routine use. The selections have been progressive, starting with the optimal reagent or technique, discussing the variants in general use and following this by more specific adaptations. Finally, it is intended to be broadly based, since the general charts, indispensable concepts and brief systematic survey of most of the basic methods used in modern light microscopy are intended to enable any specialist to find information about the application of known techniques to a particular research project and to the development of entirely new techniques.

Since the French edition of this book was published by Masson in 1978, about 60 new techniques have been added. In particular, a large section has been devoted to the study of the organic matter in microfossils preserved in rocks, in view of its stratigraphic importance in mining and oil prospecting.

Marcel V. Locquin

Acknowledgements

The collection and compilation of this manual has been long and exacting and had to be constantly updated. The late Maurice Langeron devoted at least one hour every day to this work. At present, systematic abstracting of the leading analytic journals in all relevant branches of science makes it possible to obtain a fairly complete collection of the literature. The most fruitful sources are the comprehensive textbooks, colloquia or congresses and the major microscopic journals. There is no recent work in French on light microscopy, unlike electron microscopy. Periodic and systematic enquiries made at a representative sample of laboratories doing microscopy made it possible to keep abreast with current techniques. Such enquiries must obviously be supranational. Far too many people have helped us in this way to enable us to thank them individually. Indeed, even before the fifth edition of his *Précis*, Maurice Langeron had given up trying to check himself the value of all the techniques he mentioned.

Marcel Locquin insisted on being present when procedures were performed in a specialist laboratory whenever he was unable to carry them out himself. He personally performed experiments on the following subjects at the places indicated: the chemistry of stains and fixatives at the Institute of Chemistry in Lyon and at Rhône Poulenc; light microscopy at the Institute of Optics under the guidance of Françon and Nomarski; colorimetry and electron microscopy at the Physics Laboratory of the National Museum of Natural History, Paris (Prof. Y. Le Grand); techniques of light microscopy at Wild Co.; photomicrography at the Société Lumière; cinematography at the Collège de France with J. Dragesco; telemicroscopy at the Thomson Society and later with Philips; phase contrast and interference contrast microscopy at Wild Co.; polarized light microscopy with Messrs. Cotton and Manigault at Bellevue; spodography at the Histology Laboratory of the Faculty of Medicine in Lyon (A. Policard); spark spectrography at the Forensic Laboratory with Prof. Sannie; different methods of lighting with Messrs. Claude Paz and Silva; surface treatments and inteference filters with Messrs GAB; flashlights and lasers with the Orthotron Co.; botany and mycology at the Laboratory of Cryptogamy of the Paris Museum (Prof. R. Heim); palynology and micropalaeontology at the Paris Museum laboratory (G. Deflandre), at the Micropalaeontology Laboratory of the University of Paris VI with Mrs Taugourdeau and Mr Lashkar, and at the Laboratory of the Ecole Pratique des Hautes Etudes with Mr Taugourdeau; haematology and cytology at the Laboratory of the Blood Transfusion Centre with Mr Bessis; documentation with Phototechnique and with Mr Cordonnier; microphotometry with Europelec Co.; parasitology and mycology at the Institute of Parasitology with Mr Langeron; protistology at the Collège de France with E. Faure Fremiet; automation in the analysis of images at CENFAR with Dr Le Gô. I wish to express my very sincere thanks to all of them and to all their collaborators who made my task much easier.

Abbreviations

Acidity or alkalinity	pH	Millimetre	mm
Centimetre	cm	Minute	min
Contrast	γ	Molar concentration	M
Equal quantities	aa	Nanometre	nm (= mμm
Gram	g	Normality	N
Hour	h	Optical density	δ
Joule	J	Oxidation–reduction potential	r_H
Litre	l	Second	s
Micrometre	μm	Temperature	°C or K
Milligram	mg	Wavelength	λ
Millilitre	ml (= cm^3)		

Contents

1 Instruments and Techniques

1.1 Basic principles

Light and colours

The luminous flux emitted by an object can be analysed by different methods. For this purpose, the units used are defined as follows:

c = the speed of light in a vacuum = 299 776 km s^{-1}
λ = the wavelength = c/υ, where υ is the frequency
$\mu m = \mu$ = micron = 10^{-4} cm
nm = mμm = millimicron = 10^{-7} cm
0.1 nm = Å = angström = 10^{-8} cm
n = the refractive index; n_{D20} = the refractive index of the yellow D line of sodium at 20 °C

Figure 1.1 shows the range of radiations which encompasses the octave of radiations visible to the eye, comprising about one-seventieth of the total known range. In microscopy, exploration of the ultraviolet and infrared regions extends the known regions to barely three octaves (*Figure 1.2*).

Young and Fresnel, using interference and diffraction, at the beginning of the nineteenth century, showed that light varies periodically in both time and space. Thus, at a given point the intensity of light reaches its maximum value many times per second; this is its frequency υ (cycles per second—Hz). In space, the distance separating two wave surfaces having the same intensity is the wavelength λ. These two quantities are connected by the relationship $\lambda = c/\upsilon$.

The periodic nature of the wave does not lead to an easy interpretation of light emission and absorption, although it readily accounts for propagation. A discrete aspect is assumed—a quantization of the energy transported by light—to interpret these phenomena. Max Planck (1905) showed that this quantization involves multiples of an elementary quantity

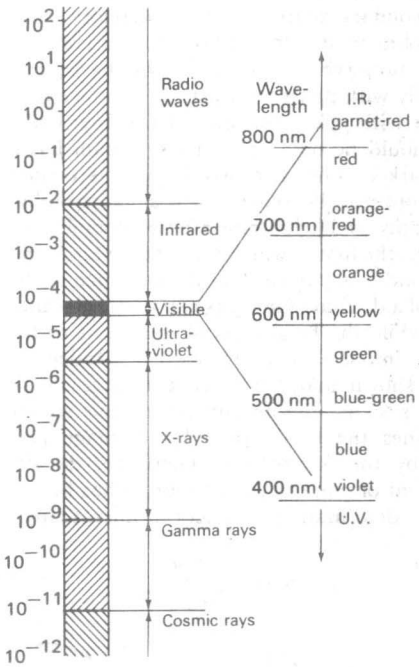

Figure 1.1. Diagram of radiations.
Left—wavelengths (m); middle—conventional regions; right—enlarged diagram of the visible range and conventional regions of colour sensations

called a quantum of energy: $\epsilon = h\upsilon$. Each quantum is present in the light wave in the form of a photon which constitutes its elementary particle but which cannot exist in a state of rest. According to Yves le Grand, light can be said to consist of a 'wave of disembodied particles'.

Point sources are seen by the observer or by the receiving aperture of the instrument used as a small angle, not exceeding a few arc minutes. The smaller the point source, the more the light is said to be 'coherent'; the importance of this concept will be seen later.

Extended sources may be of low coherence, partially coherent or incoherent. They are called primary sources if they are self-luminous and secondary sources if they modify a portion of the radiation they receive. If a diffusing surface is placed in front of a coherent source, an incoherent secondary source results. Therefore, such an attachment should not be used if it is necessary to preserve the coherence of the source, especially with phase contrast, interference contrast, or with polarized light microscopy. Briefly, it should be noted that a laser beam has a very marked coherence, which permits certain special optical phenomena such as holography.

Radiance and brightness are two homologous concepts, the first of which relates to volume and the second to surface. Only the radiance of the sky or of a discharge in a gas can be of relevance to us, while the brightness of sources is of the greatest importance in microscopy. Indeed it can be shown that, for a given aperture of an optical system, the brightness of the source determines the luminosity of the image produced by the microscope. Therefore, the development of more powerful sources has been a factor in determining advances in microscopy.

The spectrum of a radiation source may be continuous or discontinuous. In the latter case we speak of a line spectrum; some sources give rise to a mixture of both. Excitation of a gas such as sodium or mercury vapour produces a line spectrum, while heating a tungsten filament results in a continuous spectrum. A continuous spectrum is characterized by its spectral energy curve, and a line spectrum by the wavelength and intensity of each line.

The eye constitutes the basic radiation receptor, since no optical instrument is capable of using an image by analysing photographed, displayed or recorded results directly or indirectly. Photocells or photosensors belong to different categories, depending on their mode of functioning. They are dealt with in specialized electronic and optical textbooks.

In accordance with their chemical structure, photosensitive surfaces record densities in black and white or in colour. These receptors have one characteristic in common: they record only amplitudes or degrees of grey in the image but never the phases. In optics, a black body is a concept of considerable theoretical interest. In microscopy, it is useful only as a standard of reference. Generally speaking, all bodies observed under the microscope are non-black bodies.

At present, incandescent bulbs equipped with a filament heated by the Joule effect are still the most widely used light sources. Their spectral energy curve varies markedly with the current passed (*see Figure 1.21*). Their colour temperature provides a convenient means of marking the dominant wavelength of their energy spectrum (*Figure 1.3*).

Retinal heterogeneity limits the resolving

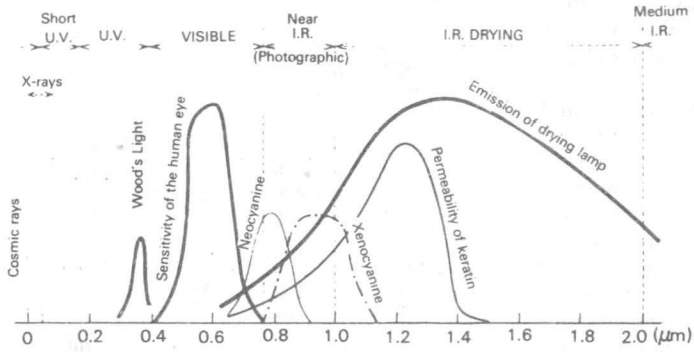

Figure 1.2. Relationship between the infrared, visible and ultraviolet regions (According to M. Déribéré, 1944)

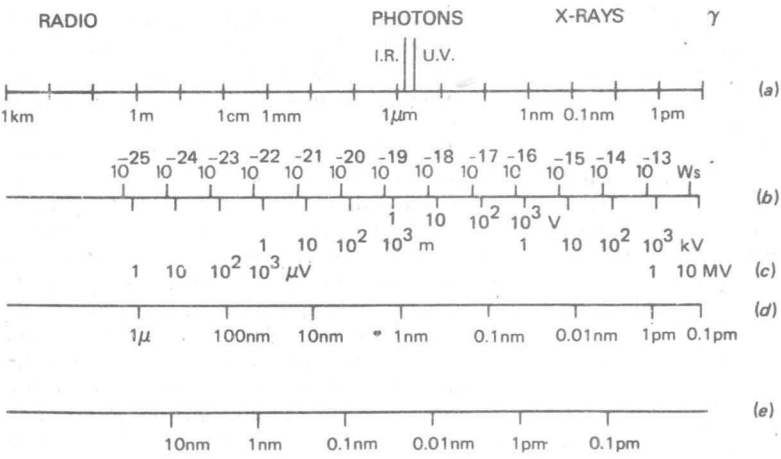

Figure 1.3. Physical data relating to waves and particles utilized in microscopy.
(a) wavelengths; (b) energy quanta; (c) acceleration of electrons; (d) wavelength associated with electrons; (e) wavelength associated with protons

power of the eye. It is now known that cones and rods are connected by lateral fibres. This makes obsolete the previously held hypotheses put forward to deduce the theoretical resolving power of the eye from their dimensions. Furthermore, the eye oscillates continuously about a mean position and explores more than it fixes.

The eye has a complex spatio-temporal memory. It establishes an apparent continuity when discontinuous images follow one another as they do in cinematography. An apparent continuity of our consciousness masks the image of discontinuities in our conscious thought—a process which takes place in the fully awake state.

The visual field of the eye is always greater than that of the instrument into which the eye is looking. The operator must be trained to use peripheral vision for a rapid exploration of what he sees. He must be able to avoid central vision consciously, in favour of peripheral vision, without moving his eyes. This is indispensable for acquiring the technique of rapid exploration zone by zone, of which high speed reading is a particular case.

The limit of visibility varies between observers and depends upon the intensity of illumination. The maximum visibility shifts from yellow to green when the illumination is diminished. The image at the eyepiece of a microscope is not analogous to a 'hole of light' at the bottom of a black tunnel. In order to obtain greater sharpness associated with greater comfort, it would be necessary to have a weak auxiliary illumination at the periphery of the field. This has not yet been tested in microscopy.

Ultraviolet light is harmful to the eye; furthermore, it excites fluorescence and forms a bluish 'halo' around the images.

The intensity of the light at the retina regulates the diameter of the pupil reflexly. In the microscope this reflex has no effect on the flux entering the eye, since the field visible at the eyepiece has generally a much smaller diameter at high magnification. This explains why the eye is easily dazzled by intense light. Furthermore, the sensitivity of the retina changes very rapidly with increasing distance of the incident beam of light from the axis of the eye; this is the Purkinje effect. The eye behaves as if it consists of two receptors functioning differently with regards to sensitivity to wavelength in full daylight and in dim light.

Most of the objects examined under the microscope are either naturally coloured or dyed artificially. Therefore, it is essential to bear in mind the following principles of colour measurement, to make the best use of an instrument. The colour white is only white with reference to a standard. This is a set standard: it is daylight at 5200 K; it is known that 'colour temperature' varies considerably during the course of a day and from month to month. A monochromatic ray is determined completely by its wavelength and its flux. A coloured ray is defined completely by its flux and by two additional variables: a white background and a monochromatic colour. In practice, preference is given to the synthesis of colours by the addition of three variable colours; this is the trichrome synthesis currently used in photography and printing. However, in

this case, the coloured lights must cover broad bands and not be monochromatic.

Every colour has its complementary colour which, when added to it, produces white; for example, yellow is complementary to blue–violet; these are all colours of the spectrum, except for the complement of green which is purple, the only pure colour not represented in the spectrum. If the pure colours are arranged in a circle, the complementary colours are diametrically opposite each other. The two triads possible for trichrome synthesis are found at the apices of an equilateral triangle.

In microscopy, five categories of colours can be distinguished:

(a) the colours formed by selective absorption of a pigment;
(b) the colours formed by absorption–reflection;
(c) the colours formed by interference in natural light;
(d) the colours formed by diffusion–dispersal;
(e) the colours formed by interference in polarized light.

The eye is incapable of differentiating between these various methods of formation in the same way that it is incapable of recognizing the composition of two different mixtures if they give the same visual colour impression.

In colorimetry a so-called XYZ system has been adopted internationally, in which the trichromatic coordinates of all the visible colours have been transferred to the inside of a triangle. Thus, the coloured filters currently available are all represented within such a triangle by means of a point and its three coordinates. The colour temperatures of the sources lie on a parabola referred to as the black body locus.

When a coloured filter is interposed between a source and the eye, the transmitted light is modified. If this filter is neutral, that is if it absorbs all wavelengths equally, then the optical densities add together when the filters are superimposed; if two filters are complementary their superimposition stops all light; with other filters, results may vary considerably, depending on whether the source has a line spectrum or a continuous spectrum. In practice, a graphic superimposition of the filter curves with the spectral distribution curve of the source makes it possible to predict the result. When the details of the object are of one colour, they can be assimilated on a filter. If they are multicoloured, an understanding of what is happening can be gained by having previously separated out the trichromatic factors, if necessary, by spectroscopy.

Most coloured solutions follow Beer's law, i.e. their absorption is proportional to the concentration and the thickness traversed. The constituents of a mixture of dyes in general also follow this law if they do not react with one another. In the case of microscopy using an eyepiece, as opposed to photographic microscopy, the overall amount of light which reaches the eye from the source after passage through filters and the optical observation system must be taken into consideration. In addition, one has to take into account the passage of light through the media of the eye. It must not be forgotten that some filters change colour as a result of ageing. In particular, the aqueous and vitreous fluids yellow with age, which increases the perception of the white colour used as reference.

This means that in practice we cannot do without reference colours. These are supplied by the 'colour codes'. Only *Chromotaxia* by M. Locquin, Paris (1965) allows comparison by transmission. In 1975, the same author published a *Guide to Natural Colours* followed by a table showing the correspondence between the colours given in this guide with the basic works of Saccardo and Ridgway, and with the Munsell notation. The latter work only enables comparison with colours examined by reflection.

Tonality, saturation and value are the psychological variables which correspond to the physical quantities of dominant wavelength, clarity and luminosity. Only the former variables are taken into account in practice, and they serve as the basis of the most universally adopted system of colour notation.

Tonality is related to the dominant pure colour, saturation to its degree of purity or to the reciprocal value of white in the colour, and the value to the percentage of grey in the colour.

However, it must be admitted that so far there is no satisfactory theory of colour vision capable of integrating all the known phenomena, but this will not be discussed further here.

Image formation

Optical treatises usually begin with an explanation of a simple magnifying lens, then a compound apparatus, simulating the objective–

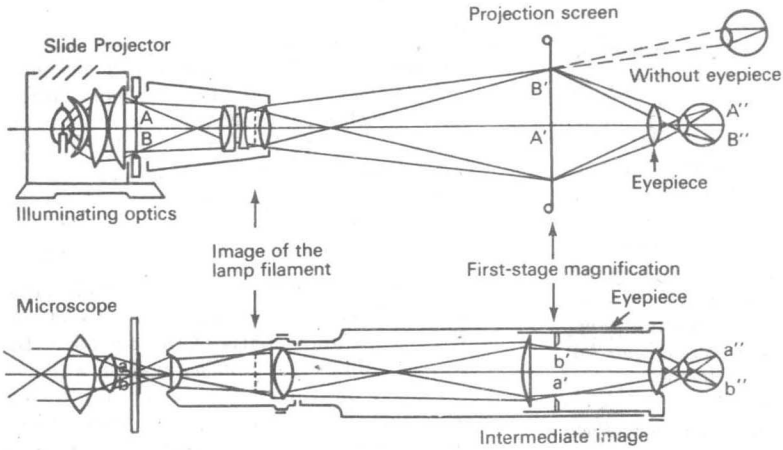

Figure 1.4. Path of light rays in a slide projector and in a microscope (Leitz-Wetzlar)

eyepiece relationship of the microscope by means of two single lenses. This arrangement, which is not very convincing to those who have not studied physics, has been described so many times that it will not be repeated here. However, it is widely used at present. In our opinion it is closer to reality than the model of the microscope lens, whether we are dealing with the common photographic slide projector or with the enlarger used for photographic negatives. In *Figure 1.4*, the direction of the rays of light is horizontal—at the top in a projector and at the bottom in a microscope. These two diagrams do not require long explanations. Finally, the reader is referred to treatises dealing with the optics and mechanics of the microscope for more precise details.

Mechanical conventions

Some mechanical parameters relating to microscopes have been standardized for some time. The tube length of monocular instruments is usually 160 mm, but in Leitz instruments it is 170 mm and in inverted microscopes it is generally 250 mm. This length is measured between the objective at rest and the eyepiece at rest. When an accessory is introduced which alters this length, an optical system must be incorporated to compensate for this change. This is nearly always the case for binocular tubes, draw tubes and almost all accessories between the stand and the observation device (*Figure 1.5*).

The standardization of objectives was developed from standards established by the Royal Microscopical Society in Great Britain, especially in respect of the screw thread to mount the objective on the revolving nosepiece. The length of the objectives varies about a mean of 35 mm from one designer to another.

When the designer incorporates corrected objectives in the microscope to produce an image at infinity, this image is brought to the observation end point by a lens, and intermediary devices can be interposed directly in front of this lens. This obviously represents the best solution. In particular, it allows the introduction of an optical device specially designed to realign the focal plane of the objective, thereby making various operations more convenient, e.g. the introduction of phase plates and various compensators.

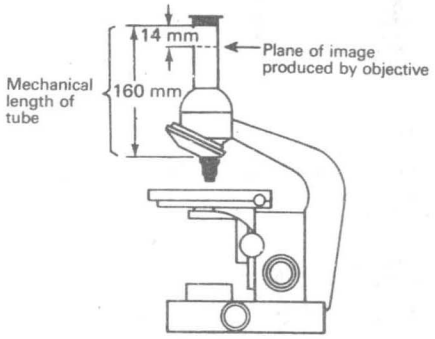

Figure 1.5. Mechanical standardization of the microscope (Wild-Heerbrugg)

Aberrations

Aberrations are called the correction defects of an optical system. In microscopy there are three important types of aberration:

(1) Spherical aberration, which must not be confused with field curvature. Spherical aberration is caused by the prism effect of a single lens which forms a band of rays from a point source rather than a point image as expected. This can be corrected by appropriate design or modifications, bonding together two or three refracting lenses with different dispersive powers (*Figure 1.6*).

(2) Chromatic aberration, due to the variation of focal distance with wavelength. This can be corrected in the same manner (*Figure 1.7*).

(3) Field curvature, due to the fact that generally the image given by the objective, formed from a plane object, is in the form of a spherical cap. The centre and the edge of the image cannot simultaneously be brought into focus. This defect becomes more awkward as the field becomes larger. It is much more obvious in photomicrography than in observation by eye, because the eye frequently refocuses, and also during observation one frequently manipulates the fine focus, which results in a continuous combination of several planes of the object.

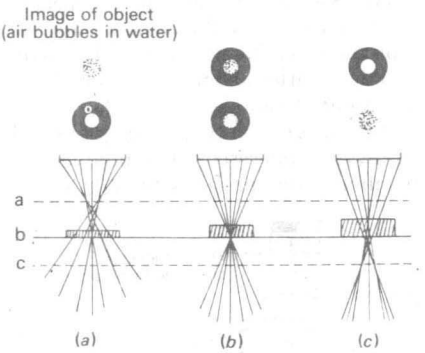

Figure 1.6. Checking the correction of the spherical aberration of a microscope. Top—two defocalized images

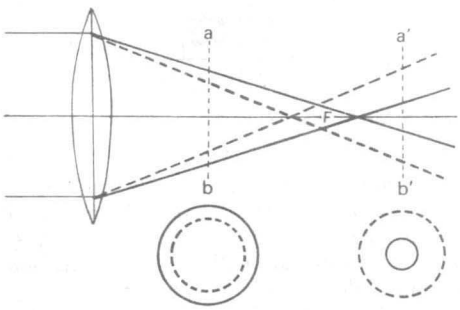

Figure 1.7. Chromatic aberrations of a simple lens. Continuous line—red; dotted line—blue. Going out of focus on both sides of F, we obtain a blue image on the far side and a red one on the near side

A fourth aberration—the coma—which is less apparent, is caused by centring defects of the different constituent parts of the objective. By examining the small details against a black background, one can check whether the centring is perfectly symmetrical; the image from a luminous point on a black background shows a comet-like lateral tail if it is not.

Depth of field

The term depth of field refers to the distance along the optical axis which separates the extreme parts of the object which are observed sharply without mechanically changing the focus. During observation with the eye, the operator compensates for a shallow field depth by moving the fine focus back and forth on either side of the mean plane of observation. Photography achieves the same effect by using a successive superimpression technique, as described later.

To increase the field depth, the lowest magnification by the objective compatible with the desired resolution and the highest magnification by the eyepiece are chosen. In photography the print may be enlarged, the negative being taken with both objective and eyepiece of the lowest possible power.

Flatness of field

A field is flat if the image is distinct, thin and flat up to the edge. The objective–eyepiece combination gives this result. Therefore, it should not be altered subsequently.

The microscopic image in wave optics

So far only the geometrical aspect of the path of light rays through the microscope has been considered. This explains image formation at very low power magnification, but at high magnification the diffraction caused by the structure