Theory and Practice of Histological Techniques

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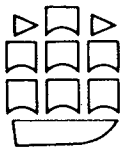

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Foreword

One of the more encouraging recent trends in medicine is the growing co-operation between clinician and histopathologist. Instead of the biopsy or resection done in the theatre, and the conventional histopathological report issuing from the laboratory, with only the hospital porter as a link between them, the clinician is tending more and more to tell the histopathologist beforehand what information he is seeking, and the histopathologist to keep the clinician informed as to how material can best be handled to give the most information.

This is a real advance, but all real advances have to be paid for. We are reaching the stage when each specimen has to be regarded as an entity in its own right, and fixed or preserved, stained or examined under phase contrast, and perhaps studied for its enzyme or mucosubstance content or for its immune competent celis, as the clinical diagnosis dictates. No longer is '10 per cent formalin' the only fixative, or paraffin embedding 'the only way to get a decent section'. It is here that this book comes into its own. Written by histopathologists and technical staff with a wide range of experience it outlines the ways in which material can be handled to yield the maximum information. It will be invaluable to all technical staff in training, and many histopathologists reading it may be pleasantly surprised at the amount of information which can be squeezed, and interest engendered, from a fuller study of what often appears at first sight to be rather dull routine material.

Nottingham 1977 **Ian Dawson**

Preface

In recent years histological techniques have become increasingly sophisticated, incorporating a whole variety of specialities and there has been a corresponding dramatic rise in the level and breadth of knowledge demanded by the examiner of trainees in histology and histopathology technology.

We believe that the time has arrived when no single author can produce a comprehensive book on histology technique sufficiently authoritative in the many differing fields of knowledge with which the technologist must be familiar. Many books exist which are solely devoted to one particular facet such as electron microscopy or autoradiography, and the dedicated technologist will, of course, read these in the process of self-education. Nevertheless the need has arisen for a book which covers the entire spectrum of histology technology, from the principles of tissue fixation and the production of paraffin sections, to the more esoteric level of the principles of scanning electron microscopy. It has been our aim then, to produce a book which the trainee technologist can purchase at the beginning of his career and which will remain valuable to him as he rises on the ladder of experience and seniority.

The book has been designed as a comprehensive reference work for those preparing for examinations in histopathology, both in Britain and elsewhere. Although the content is particularly suitable for students working towards the Special Examination in Histopathology of the Institute of Medical Laboratory Sciences, the level is such that more advanced students, along with research workers, histologists and pathologists, will find the book beneficial. To achieve this we have gathered a team of expert contributors, many of whom have written specialised books or articles on their own subject; most are intimately involved in the teaching of histology and some are examiners in the HNC and Special Examination in Histopathology. The medically qualified contributors are also involved in technician education.

All contributors have taken care to give, where applicable, the theoretical basis of the techniques, for we believe that the standard of their education has risen so remarkably in recent years that the time is surely coming when medical laboratory technicians will be renamed 'medical laboratory scientists'; we hope that the increase in 'scientific' content in parts of this book will assist in this essential transformation.

J. D. Bancroft
A. Stevens

Nottingham, 1977

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We must thank our colleagues in the Department of Pathology in the University of Nottingham for their tolerance, often in the face of extreme provocation, during the gestation period of this book, and particularly to Professor Ian Dawson who frequently turned a blind eye, but still had sufficient charity at the end to write our Foreword. We hope that everyone who has been associated with this book will think that it has been worthwhile. Lastly, we wish to thank the staff of our publishers, Churchill Livingstone, for their unfailing help and courtesy.

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Contents

1. Light microscopy

R. A. ROSE

LIGHT AND ITS PROPERTIES

Light in our daily lives we accept without further thought, but light is a form of energy that follows, like other forms of energy, certain rules, most of which are known to man. Knowing these rules we can design instruments to utilize this energy to our advantage.

Such an instrument is the microscope, one of the principal tools used in the laboratory. As with all tools, its operator should know its working to obtain the best results.

From its source, whether it be the sun, an oil lamp, an electric lamp, or whatever, light radiates in all directions. Each radiating ray, unless something interferes, travels in a straight line to infinity. Before we consider these interfering influences let us first note a point or two illustrating terms referring to the properties of light itself.

In diagrams showing the passages of light in the optics of the microscope, the rays and bundles of rays are drawn as straight lines, but in order to demonstrate the theory that light as an energy force is a series of pulses from that source it is frequently shown as a sine curve representing waves of energy, the peaks as it builds up, the troughs as the energy ebbs (see Fig. 1.1) *Amplitude* refers to the

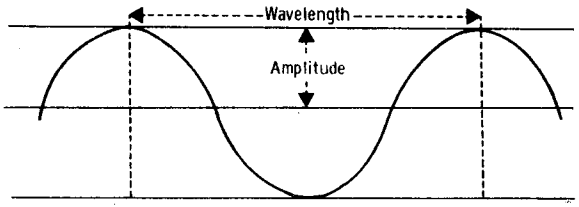

Fig. 1.1 Representation of light ray showing wavelength and amplitude.

strength of the energy, in the case of light, its brightness. The distance between the apex of one wave and the next is the *wavelength* and is measured in Angström or nm units. Wavelength determines colour. Most light sources emit energy in a wide range of wavelengths, some parts of the range at differing amplitudes. The number of waves per second is referred to as its *frequency*. The frequency of a light wave remains constant. Individual rays of identical frequency are able to combine or interfere with each other, and are from the same source. Such identical rays are said to be *coherent*. Rays from different sources or of different frequencies cannot interfere, and are said to be non-coherent.

When travelling through any medium, amplitude diminishes to a greater or lesser degree depending upon the medium, (see Fig. 1.2).

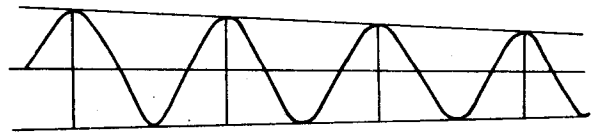

Fig. 1.2 Amplitude diminishes as it gets further from its source by absorption in the media through which it passes.

Media through which light is able to pass vary in their densities, and this variation affects the speed at which the light is able to pass through. Here is a simple analogy. If one walks across a closely mown grass field at a regular pace and then a patch of unmown longer grass is encountered, progress is slowed down until the denser medium is passed. To take the analogy a little further, a column of soldiers marching across our field encounter the long grass at right angles to its front, they are all slowed down together. If they encounter the long grass at any other angle one end of the column will be slowed down earlier. To keep the same step rhythm in the denser medium each man will tend to take shorter paces, with the result that the column deviates at an angle to its original track. The grass being a parallel strip, as they emerge, the column will revert to its original direction (see Fig. 1.3).

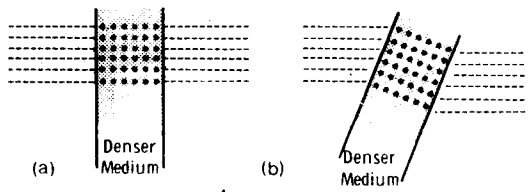

Fig. 1.3 (a) Rays passing from one medium to another, perpendicular to its interface, are slowed down at the same moment. (b) Rays passing at any other angle to the interface are slowed down in the order that they cross the interface and are deviated.

In a similar way, a bundle of light rays passing from, let us say, air to glass, cross the surface at right angles; they are all slowed down together, and they continue in the

same direction. When they cross at any other angle, a deviation will occur. This is known as *refraction*. The angle between the bundle of rays and the perpendicular to the surface at the point of contact, is known as the 'angle of incidence'. The angle to which the rays are bent is known as the 'angle of refraction'. A ratio of the sine values of these angles gives us the Refractive Index. The *Refractive Index* or R.I. of most transparent, or light passing, substances is known, and is of great value in the computation and design of lenses. Air has a refractive index of 1.00, water 1.30 and glass 1.50.

From the diagrams of rays passing from air to glass (Fig. 1.4a), and into air again, first as a parallel plate (1.4b), then as a wedge (1.4c), it can be seen how by calculation of the Angle of Incidence and the Angle of Refraction, a curved surface can be created that directs

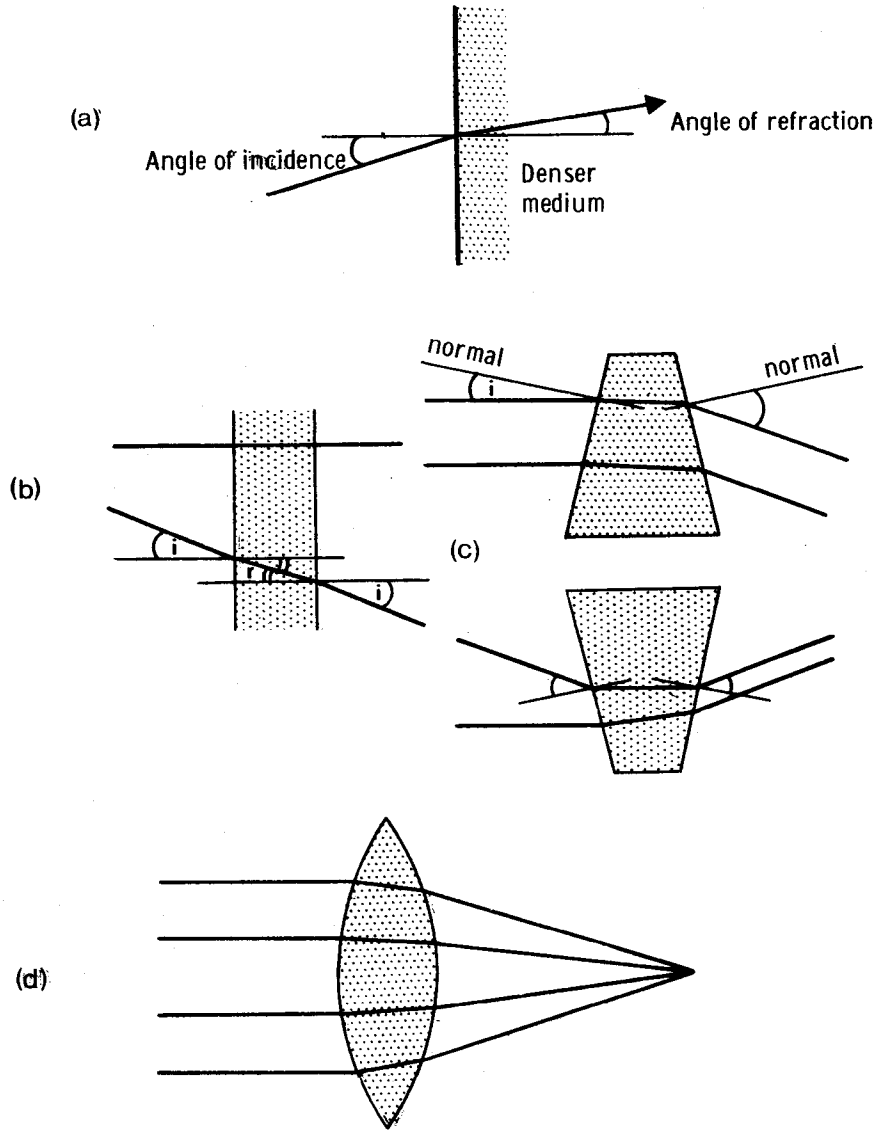

Fig. 1.4 Behaviour of light rays: (a) Angles of incidence and refraction. (b) Light rays passing through glass resume their original direction on emergence, if the surfaces are parallel. (c) By shaping the glass, rays can be directed in predictable directions. (d) A lens can be computed by plotting the paths of rays from all directions at every infinite point off its surface, given the Refractive Index and therefore knowing how the rays will behave.

every light ray impinging upon it to meet at the same point of focus (1.4d). Using glass, or other materials of known Refractive Index, surfaces of lenses can be computed to give a desired result. At one time these calculations were a long and tedious task, but with the aid of computers this is now rapid.

A lens placed between an object and a screen can form an image of the object from every ray from that object that is intercepted by the lens. The lens is able to collect each ray from each infinite point of the object and bring them together to the same plane of focus. If the screen is placed at that focal plane then the image will be sharp. This is known as a 'Real Image' and can be demonstrated, (see Fig. 1.5).

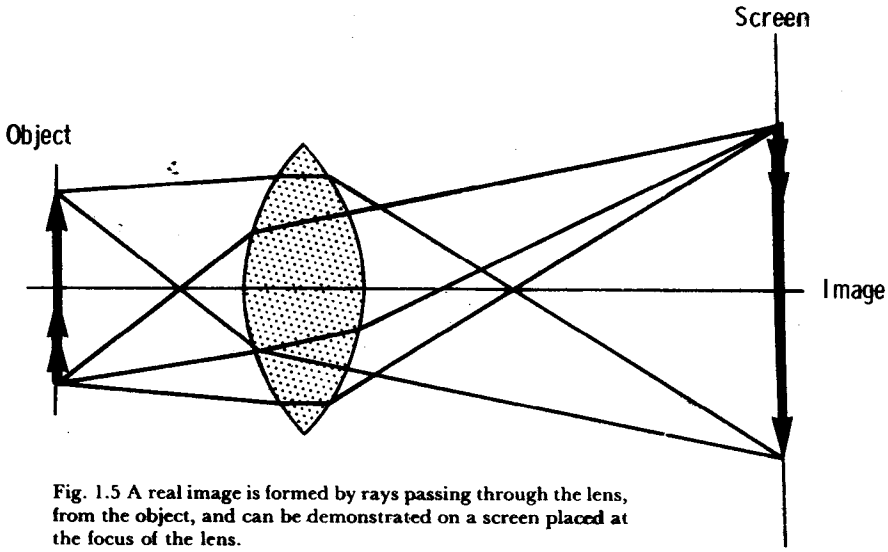

Fig. 1.5 A real image is formed by rays passing through the lens, from the object, and can be demonstrated on a screen placed at the focus of the lens.

When one looks at an object through a lens, the rays diverge through the focal point and appear to the eye as an enlarged image on the object side of the lens. This is known as a 'Virtual Image' (see Fig. 1.6). The compound microscope uses both kinds of image, Real and Virtual.

So-called 'white light' is composed of light of many wavelengths. Unfortunately each wavelength will have its own Angle of Refraction, and will therefore have a different focal point, so that images from a single lens will show fringes of the longer wavelength colours, are unsharp, and would be disturbing and of little use. The 'dispersions', or wavelength-splitting properties, of the various types of glass used for optical instruments also vary in the extent to which they disperse light. It is possible to combine elements of different glass to form composite lenses that correct this fault, which is known as 'chromatic aberration'. Other distortions in the image are known as 'spherical aberration' and are due to lens shape and the distance the light actually travels through the lens, often much shorter at the edge than at the centre. These faults are also corrected by making combinations of lens elements of differing glass and differing shape.

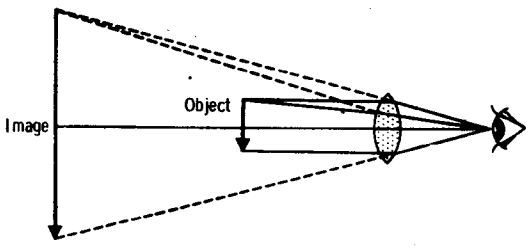

Fig. 1.6 A virtual-image is viewed through the lens. It appears to be on the object side of the lens. If the lens is convex the image is enlarged.

THE COMPONENTS OF A MICROSCOPE

Light source

Light of course is an essential part of the system; at one time daylight was the usual source. In many countries daylight is unreliable, both in its intensity and duration. A progression of light sources have developed, from oil lamps to the low voltage electric lamps of today. These operate via a transformer and can be adjusted to the intensity required. The larger instruments have their light sources built into them. Dispersal of heat, collection of the greatest amount of light, direction and distance, are all carefully calculated by the designer for greatest efficiency. In simpler models the lamp is often an attachment to the

stand, its efficiency possibly running in parallel relationship with the cost of the instrument.

Condensers

Light from the lamp is directed into the first major optical component, the condenser. In many microscopes the lamp is on the opposite side of the instrument to the operator. This reduces the possibilities of stray light disturbing images, and keeping hot lamps well out of the way, of the optics as well as the user. This arrangement means that the light beam starts in a horizontal path and must be directed into the condenser by a mirror.

The main purpose of the condenser is to focus or concentrate the available light into the plane of the object (see Fig. 1.7). Within comfortable limits, the more light at the specimen, the better is the resolution of the image, resolution being the ability to separate minute adjacent details.

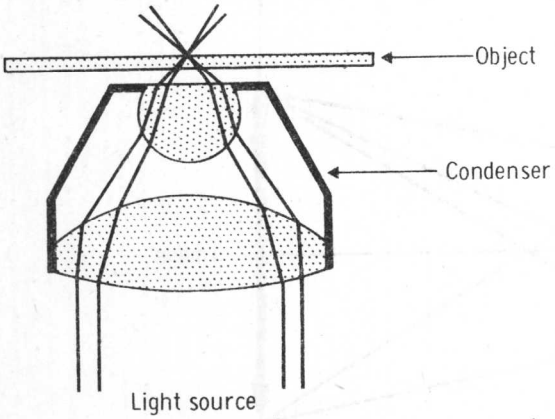

Fig. 1.7 The function of the condenser is to concentrate, or focus, the light rays at the plane of the object.

Many microscopes have condensers capable of vertical adjustment, in order to allow for varying heights or thickness of slides. Once the correct position of the condenser has been established, there is no reason to move it, unless there is a variation in slide thickness or some major adjustment required for photographic purposes. It is not a satisfactory method of adjusting light intensity, though there are some microscopists who persist in lowering the condenser when light intensity is too high.

In most cases condensers are provided with adjustment screws for centring the light path. Checking and, if necessary, adjusting the centration before using the instrument should be a routine procedure for every microscopist. All condensers have an aperture diaphragm with which the diameter of the light beam can be controlled. Reducing the aperture reduces the amount of oblique light reaching the subject and has the effect of increasing the apparent contrast of the image. No hard and fast rule can be made as to the amount of closure, though many people advise reducing to two-thirds of the field, when viewed through the empty eyepiece tube. There must always be a compromise with the intensity of light and resolution. Many condensers are fitted with a swing-out top lens. This is turned into the light path when the higher power objectives are in use. It focuses the light into a field more suited to the smaller diameter of the objective front lens. Swing it out of the path with the lower power objectives, or the field of view will only be illuminated at the centre.

Object stage

Above the condenser is the object stage, which is a rigid platform with an aperture through which the light may pass. The stage supports the glass slide bearing the specimen, and should therefore be sturdy and perpendicular to the optical path. In order to hold the slide firmly, and to allow the operator to move it easily and smoothly, a mechanical stage is either attached or built in. This allows controlled movement in two directions, and in most cases Vernier scales are incorporated to enable the operator to return to an exact location in the specimen at a later occasion, providing a note has been made of the readings.

Objectives

The next and most important piece of the microscope's equipment is the objective, the type and quality of the objective having the greatest influence on the performance of the microscope as a whole.

Within the objective may be lenses and elements from five to fifteen in number, depending on image ratio, type and quality (see Fig. 1.8). It is not always realised that the

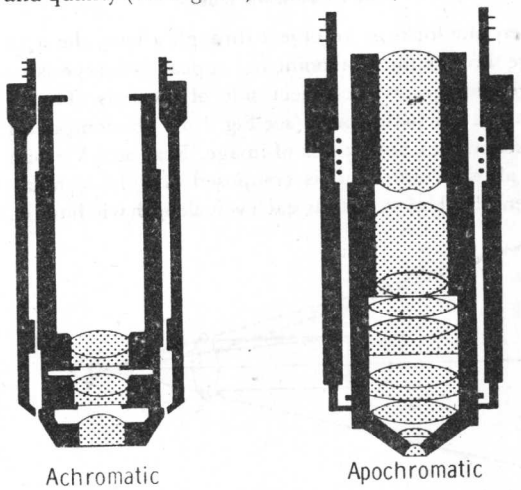

Fig. 1.8 Diagram of achromatic and apochromatic objectives. Some examples of the latter may have as many as fourteen separate lens elements.

tiny lenses still have to be ground by hand, taking time, patience and a great deal of skill.

The objective's task is to collect light rays from the object and with them form a magnified Real Image some distance above. This distance varies with the microscope, and with the type of work being performed. For instance, for biological transmitted light examinations, objectives are computed for an optical tube length of 160 mm or 170 mm whereas for metallurgy (reflected light microscopy) the objectives are computed for infinity.

Magnifying power, or more correctly, object-to-image ratios of objectives are from 1:1 to 100:1 in normal biological instruments.

Several types of objective are available, differing in performance, construction and cost. The simplest and most widely used are *Achromats* which can be used for most routine purposes. They are corrected as far as colour is concerned for two colours, red and blue. That is to say the dispersion in the lenses has been corrected so that both the red and blue wavelengths are equalized in their intercept length and will be focused at the same point. Between red and blue lie the majority of wavelengths acceptable to the human eye, and therefore the image is comfortable to observe.

Correction for a wider range of colours is possible by including lenses made from fluorite and other minerals. These objectives, known as *Fluorites*, are important where subtle differences in specimen colours are to be observed, and are therefore much in demand in pathology and by photographers.

The most highly corrected objectives are known as *Apochromats*; they are also the most expensive. By using many more elements made from a variety of types of glass and minerals, chromatic and spherical aberrations are eliminated. In some, the *Plan Apochromats*, the field of view is perfectly flat from edge to edge in contrast to others that suffer from a curvature of field and have a different focal plane in the centre from that at the edge. Plan Apochromats are therefore very suitable for photomicrography.

In addition to its type of construction, an important indication of the performance of an objective is its Numerical Aperture. The ability of the microscope to resolve depends entirely upon the Numerical Aperture of the objective and not upon the magnifying or reproduction ratio. The Numerical Aperture or N.A. is expressed as a figure, and will be found engraved on the body of the objective. The figure expresses the product of two factors and can be calculated from the formula:-

$$N.A. = n \times \sin u$$

where n is the Refractive Index of the medium between the coverglass over the object and the front lens of the objective e.g. air, water or immersion oil; and
u is the angle included between the optical axis of the lens and the outermost ray which can enter the front lens.

In Figure 1.9 we regard the point where the axis meets the specimen as a light source; rays radiate from this point

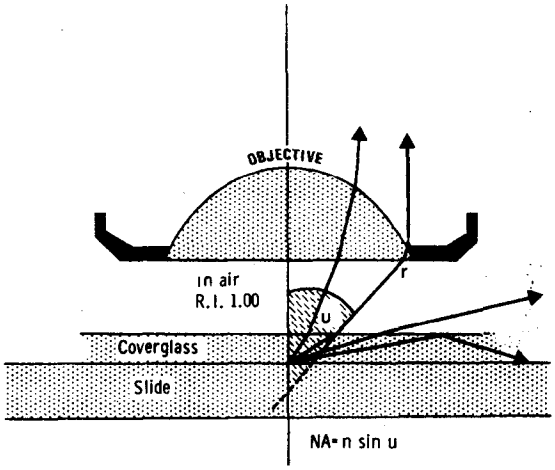

Fig. 1.9 The Refractive Index of the medium between the coverglass and the surface of the objective's front lens (in this case air, R.I. = 1.00), and the sine of the angle (u) between the optical axis and the widest accepted ray (r), gives the Numerical Aperture. (See text below)

in all directions. Some will escape to the outside, and some will be reflected back from the surface of the coverglass. We are interested in the ray r; the angle u between ray r and the axis gives us the *sin* value we require. In theory the greatest possible angle would be if the surface of the front lens coincided with the specimen, giving a value for u of 90°. In the above formula, with air (RI=1.00) as the medium, and a value for u of 90° ($\sin u = 1$), we get the result N.A.=1.00. Of course this is impossible as there must always be some space between the surfaces and a value of 90° for u is unobtainable. In practice the maximum N.A. attainable with a dry objective is 0.95. Similar limitations apply to water and oil immersion objectives; theoretical maximum values for N.A. are 1.30 and 1.50 respectively. In practice values of 1.20 and 1.40 are the highest obtainable.

In short, the higher the numerical aperture the better is the light gathering power and therefore the better the resolving power of the objective.

Most objectives are designed for use with a cover glass protecting the object. If so, a figure giving the correct cover glass thickness should be found engraved on the objective. Usually this is 0.17 mm. Some objectives, notably Apochromats between 40:1 and 63:1, require coverslip thickness to be precise. Some are mounted in a correction mount and can be adjusted to suit the actual thickness of the coverglass used.

Nosepiece

On many modern microscopes the objectives are screwed into a revolving nosepiece that can accommodate four, five or six objectives. Some are interchangeable, facilitating changes of sets of objectives of one type to another, and avoiding the need to unscrew the objectives from their places. This is often the moment when they are in danger of being dropped or damaged. It should also be noted that an objective can never be replaced in exactly the same position in its threaded mount. Parfocality and parcentricity with other objectives on the same nosepiece will therefore suffer.

Bodytube

Above the nosepiece is the body tube. Three main forms are available, monocular, binocular, and the combined photo-binocular. This latter sometimes has a prism system allowing 100 per cent of the light to go either to the observation eye-pieces, or to the camera located on the vertical part, sometimes with a beam-splitting prism dividing the light 20 per cent to the eyes, 80 per cent to the camera. This facilitates continuous observation during photography. Provision is made in binocular tubes for the adjustment of the interpupillary distance, enabling each observer to adjust for the individual facial proportions. Alteration of this interpupillary distance may alter the mechanical tube length, and thus the length of the optical path. This can be corrected either by adjusting the individual eyepiece tubes, or by a compensating mechanism built into the body tube. It is especially important in the combined photo-binocular tubes, as, in earlier designs this alteration of tube length occurred in the observer side but not in the photo side and so the camera attachment had to be fitted with a focusing device to ensure that the camera was in correct focus. The modern systems avoid this; what the observer sees, the camera sees, and he does not have to keep changing his viewing position.

Modern design tends towards shortening the physical lengths of the components and in consequence intermediate optics are sometimes included in the optical path to compensate. These in some cases add a magnifying factor to the total magnification of the instrument.

Eyepiece

Eyepieces are the final stage in the optical path of the microscope: their function is to magnify the image formed by the objective within the body tube, and present the eye with a virtual image, apparently in the plane of the object being observed; usually this is an optical distance of 250 mm from the eye.

Early types of eyepieces, like objectives, were subject to aberrations, especially of colour. Compensating eyepieces were designed to overcome these problems. Today eyepieces similar to those designed by Huygens, together with compensating or periplanatic eyepieces, are still available. In addition, there are eyepieces giving much wider fields of view, eyepieces holding graticules for measuring and for photography with adjustable focusing eyelenses, and eyepieces with a higher focal point for spectacle wearers.

These are the principal components of a microscope constructed for brightfield transmitted light; they are summarised together with the light path in Figure 1.10a, and shown in the body of the microscope in 1.10b.

MAGNIFICATION VALUES

Magnification values are important when considering which optics to bring into use for studying microscopic objects, and more especially when comparisons are to be made between known accepted objects and those to be examined.

Total magnification is the product of the reproduction ratio of the objective (the relationship in length of the object to the length of its real image, reproducible on a

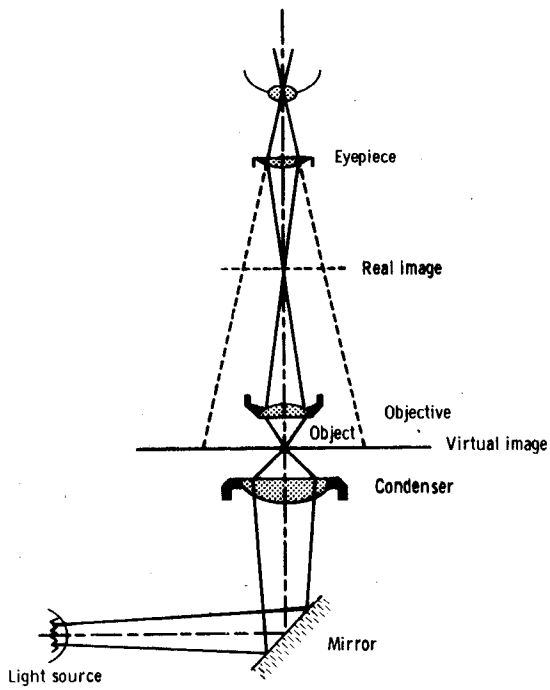

Fig. 1.10a Ray path through the microscope. The eye sees the magnified virtual image of the real image, produced by the objective.

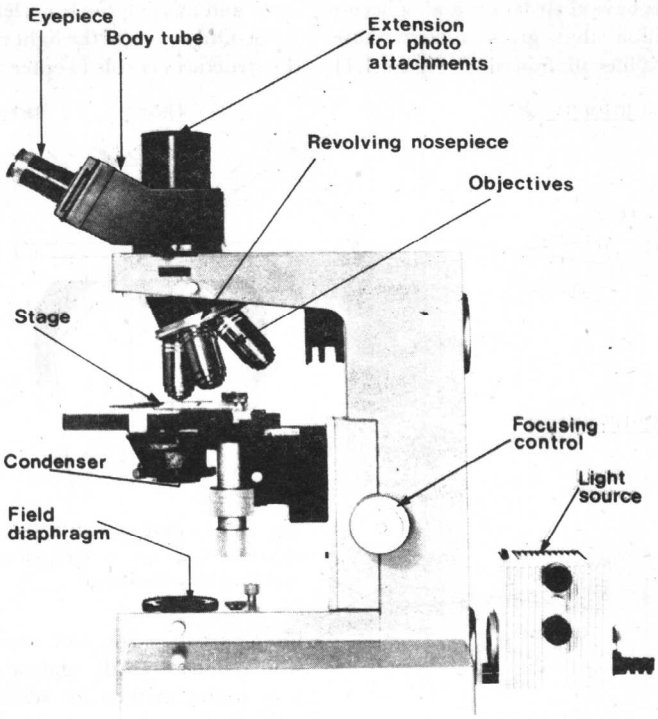

Fig. 1.10b A modern microscope.

ground glass screen) multiplied by the magnification factor of the eyepiece. If, as in many cases, an additional tube lens system is built into the instrument, this additional factor must also be included. For example, in a microscope composed of an objective 40:1, eyepiece 10 ×, and an intermediate factor of 1.25, the total magnification would be 40 × 10 × 1.25 = 500. The factors involved are usually to be found on the appropriate parts. A table of magnification values is often supplied with the instrument, also such a table may be published in catalogues and handbooks relating to the instrument. It should be remembered that these tables can only be generalisations and approximate values since every objective is an individual, no two exactly alike, and when accurate measurements are to be made the instrument must be calibrated with an accurate micrometer.

Changing the combination of objective and eyepiece values will give different total magnifications, and here the N.A. of the objective will be of value in deciding how high the total magnification may be taken, for a stage is reached when increased magnification shows only enlargement with no further resolution; this condition, 'empty magnification', gives no further information about the object. Total magnification should never exceed 1000 times the N.A. of the objective. For example, an objective 100/1.30 could be used in a combination giving a total of 1300, so an eyepiece higher than 12.5 x would be of no advantage. In general it is better to increase total magnification by an increase in objective ratio, rather than by raising eyepiece magnification.

KÖHLER ILLUMINATION

Most microscope descriptions mention Köhler or Köhler-type illumination. *Critical illumination*, when the best observation conditions are obtained, is when the light source is focused by the condenser in the same plane as the object. When this condition is fulfilled using modern filament lamps as a source, the image of the filament itself with 'hot spots' and uneven illumination would be disturbing. Köhler illumination includes an adjustable collector lens in front of the lamp which focuses the source in the plane of the iris diaphragm of the condenser; the illumination at the object plane is then even and homogenous, much more suitable for photographic pur-

poses. Present day stands have their lamps and collector lenses fixed in a position that gives a very close approximation to true Köhler illumination. Figure 1.11

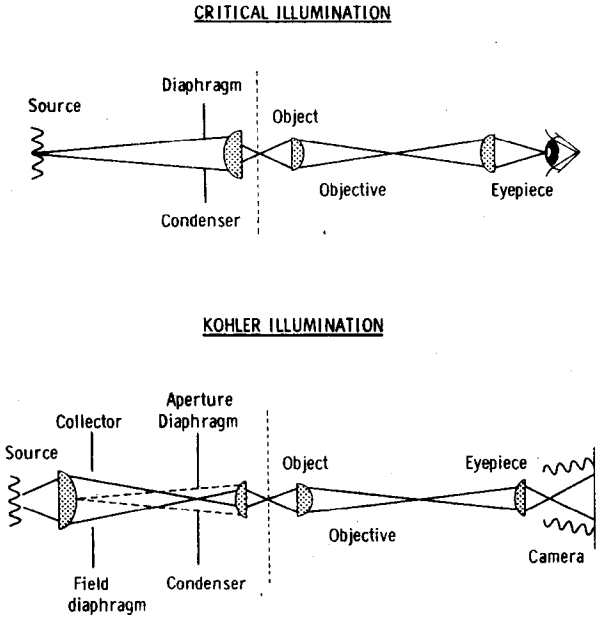

Fig. 1.11 Critical, and Köhler illumination.

shows the main difference between Critical and Köhler systems.

DARK GROUND ILLUMINATION

So far we have discussed the microscope as suitable for the examination of stained preparations. Staining aids the formation of images by absorbing part of the light (some of the wavelengths) and producing an image of amplitude differences and colour. Occasions arise when staining processes cannot be performed. Either they are difficult and too lengthy, or distortion or destruction of important parts of the specimen is likely, or if it is necessary to keep specimens in their living state. Many transparent cells when left unstained are very difficult to see by normal bright field methods, for their component parts, nucleus and cytoplasm for instance, differ optically only in the Refractive Index of their substance.

One method of rendering these objects more visible is by the use of darkground illumination, in which special condensers are used, which, instead of leading the light rays directly to the focal point at the object and through to enter the objective, direct the light obliquely from the periphery in a hollow cone glancing the edges of the structures and missing the front lens of the objective, (see Fig. 1.12). Only some of the light reflected and diffracted from the structures is able to enter the objective. The effect is to

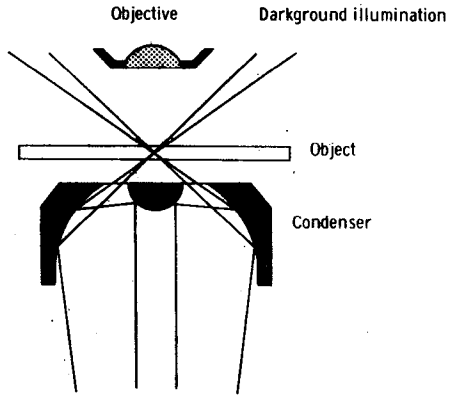

Fig. 1.12 In darkground illumination no direct rays enter the objective. Only scattered rays from the edges of structures in the specimen form the image.

illuminate the edges and other structures within the cells which shine brightly against a dark background. It is a very useful method for searching for spirochaetes and other fine structures that would otherwise need long and capricious staining methods.

Dark ground condensers may be for either dry, low power objectives, or for oil immersion high power objectives. Whichever is used, the objective must have a lower Numerical Aperture than the condenser (in brightfield illumination, optimum efficiency is obtained when the N.A.'s of both objective and condenser are matched). In order to obtain this condition it is sometimes necessary to use objectives with a built in iris diaphragm or, more simply, by inserting a funnel stop into the objective. Perfect centering of the condenser is essential, and with the oil immersion systems it is necessary to put oil between the condenser and the object slide in addition to the oil between the slide and the objective, sometimes a very messy procedure.

PHASE CONTRAST

A method widely used for examining unstained specimens is phase contrast, which can provide a large amount of object detail and information. When two or more rays of light from the same source are emitted with the same frequency, we refer to them as being coherent. If we consider them again in their wave form we can demonstrate diagramatically some of their important properties (Fig. 1.13). Two coherent rays of equal amplitude in the same

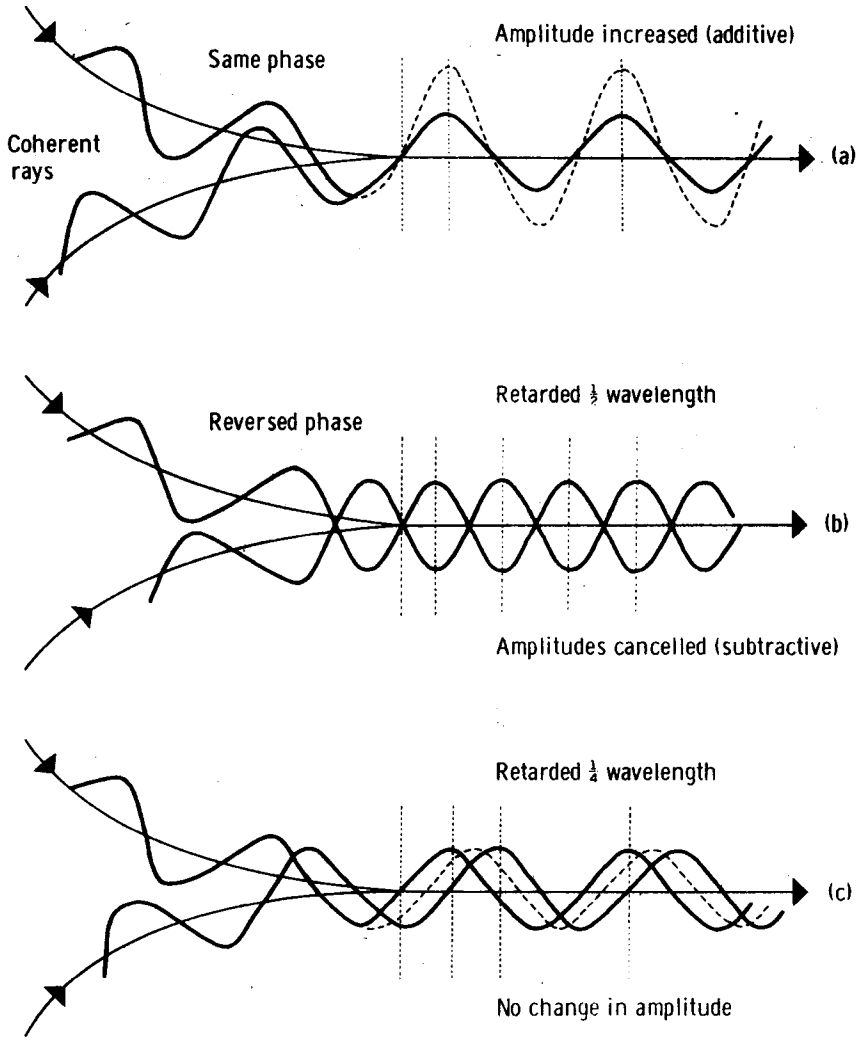

Fig. 1.13(a) (b) (c) Behaviour of coherent rays, from the same source, of the same wavelength, and of the same frequency, but of different phases. (See text below)

phase (that is to say, their amplitude peaks coincide) are able to combine or interfere. The result of this combination is that the amplitude doubles, or, to put it another way, the light is twice as bright (see Fig. 1.13a). If, however, they are in reverse phase (the peak of one coincident with the trough of the other); they cancel each other, and the result is no light. Simply stated, rays in the same phase are additive, rays in reverse phase subtractive (see Fig. 1.13b), both of these conditions being visible to the eye. The difference between the two conditions is half a wavelength and appears as differences in amplitude. Phase differences of less than half a wavelength however, show no amplitude difference and cannot be seen (see Fig. 1.13c).

With brightfield illumination, stained specimens are made visible by amplitude differences and by wavelength absorption (see Fig. 1.14a). Unstained specimens are practically invisible because only small differences in phase occur when the rays pass through material with minutely differing refractive indices. In order to change these small phase differences into visible images we convert the phase shift by slowing down some of the light by a quarter of a wavelength by use of a 'phase plate' (*see below*, and Fig. 1.14b). Thus conditions are brought closer to the position where the phase difference is half a wavelength, and therefore evident as amplitude differences.

In the phase condenser is an annular stop, so positioned