Theory and Practice of Histological Techniques

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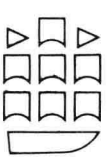
SECOND EDITION



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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 cells, 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

I.M.P.D.

Preface to the Second Edition

In the five or so years since the preparation of the first edition of this book, there have been many advances in histotechnology, not only in the development of techniques, but also in their wider application to research and diagnostic problems. Some measure of this increase can be appreciated by the fact that we have found it necessary to include seven new chapters in this edition, although some are expanded derivatives of chapters in the first edition. Techniques which were confined to specialised centres five years ago are now being used in many routine diagnostic laboratories; an example is the now common use of immunohistochemical methods, the subject of Chapter 20, providing more accurate and simple identification of cellular substances than has been achieved before. New chapters have been added on Resin Embedding (Ch. 22), Histometry (Ch. 27), Safety in the Laboratory (Ch. 31) and the APUD system (Ch. 14), and the treatment of electron microscopy has been re-organised and expanded to include two new chapters. A section on the principles of polarising microscopy has been added to Chapter 1, and some hints on photomicrography have been incorporated into the section on Museum and other Demonstration Techniques.

Where relevant, the uses of the techniques in solving specific diagnostic problems have been outlined, to provide help and guidance to pathologists in the selection of suitable special methods in difficult cases.

J.D.B.

Nottingham, 1982 A.S.

Preface to the First Edition

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.B.

Nottingham, 1977 A.S.

Acknowledgements

It is particularly difficult in a book such as this, with many contributors from different parts of Great Britain, to acknowledge realistically and accurately all the assistance received, as every contributor owes gratitude to many people.

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Contents

1

Light microscopy

ROGER 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 utilise 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 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 nanometers. 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.

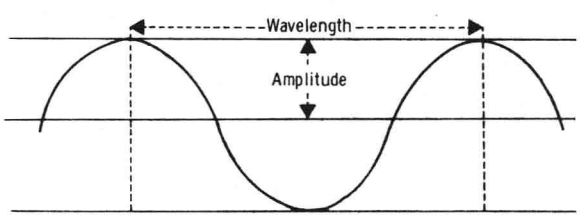

Fig. 1.1 Representation of a light ray showing wavelength and amplitude.

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

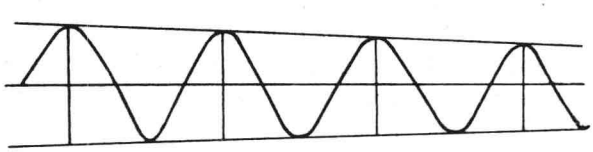

Fig. 1.2 Amplitude diminishes as it gets further from the source by absorption in the media through which it passes.

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).

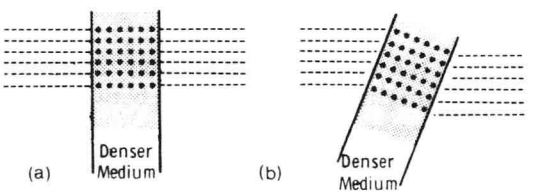

Fig. 1.3 (a) Rays passing from one medium to another, perpendicular to its interface, are slowed down at the same moment. (b) Rays passing at any other angle to the interface are slowed down in the order that they cross the interface and are deviated.

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 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

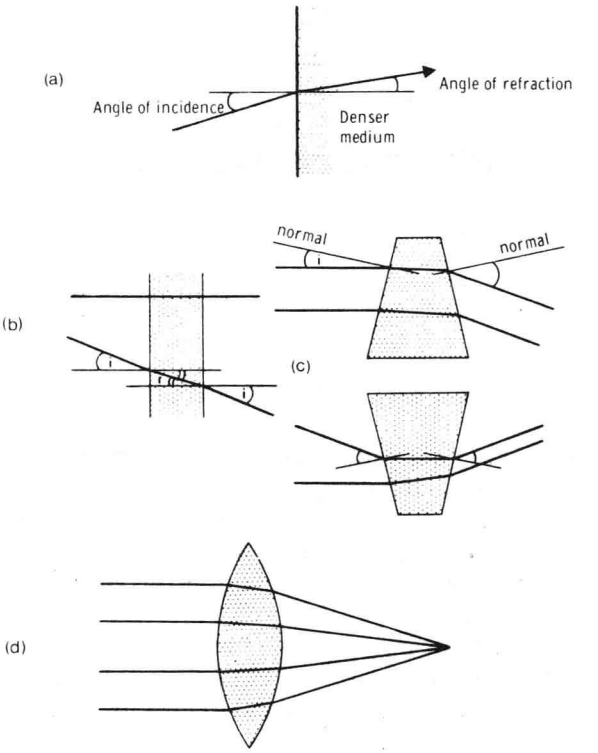

Fig. 1.4 Behaviour of light rays: (a) Angles of incidence and refraction. (b) Light rays passing through glass resume their original direction on emergence if the surfaces are parallel. (c) By shaping the glass, rays can be directed in predictable directions. (d) A lens can be computed by plotting the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

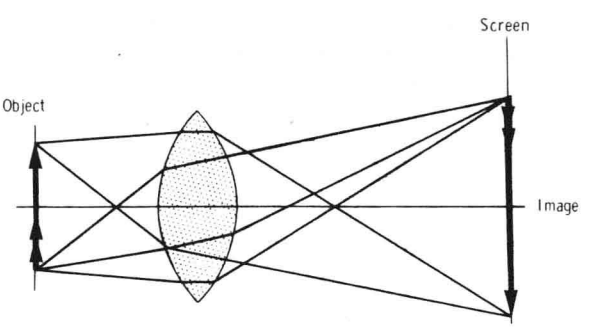

Fig. 1.5 A real image is formed by rays passing through the lens from the object, and can be demonstrated on a screen placed at the focus of the lens.

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).

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.

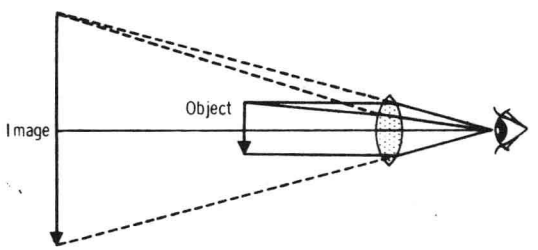

Fig. 1.6 A virtual-image is viewed through the lens. It appears to be on the object side of the lens. If the lens is convex the image is enlarged.

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 different glass and differing shape.

THE COMPONENTS OF A MICROSCOPE

Light source

Light of course is an essential part of the system; at one time sunlight was the usual source. In many countries sunlight 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,

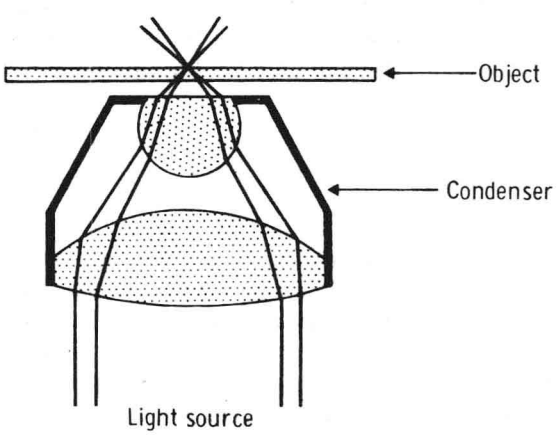

Fig. 1.7 The function of the condenser is to concentrate, or focus, the light rays at the plane of the object.

the more light at the specimen, the better is the resolution of the image, resolution being the ability to separate minute adjacent details.

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.

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 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.

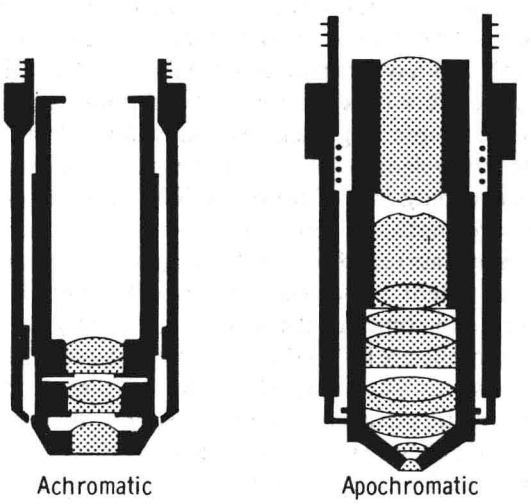

Fig. 1.8 Diagram of achromatic and apochromatic objectives. Some examples of the latter may have as many as fourteen separate lens elements.

Several types of objectives 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 equalised 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:

$$\text{N.A.} = n \times \sin u$$

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

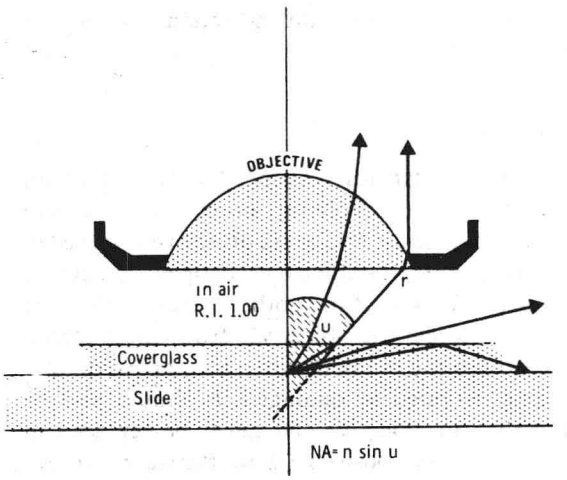

Fig. 1.9 The Refractive Index of the medium between the coverglass and the surface of the objective's front lens (in this case air, R.I. = 1.00), and the sine of the angle (u) between the optical axis and the widest accepted ray (r), gives the Numerical Aperture (see text below).

from this point 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

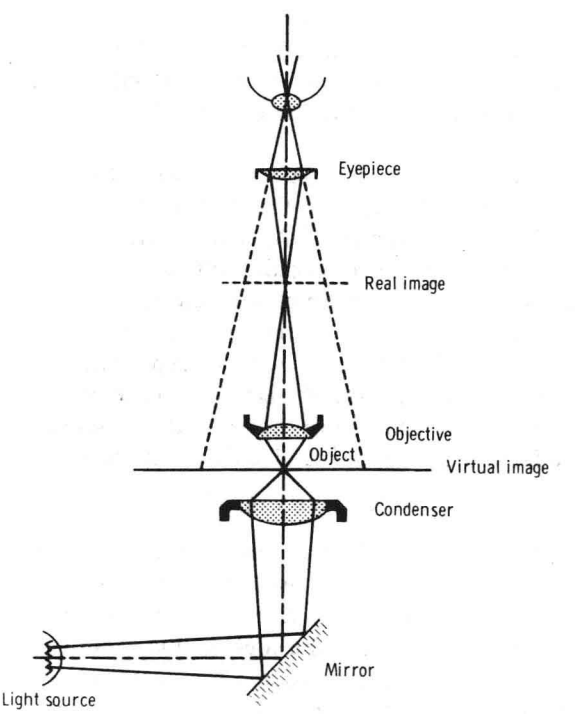

Fig. 1.10a Ray path through the microscope. The eye sees the magnified virtual image of the real image, produced by the objective.

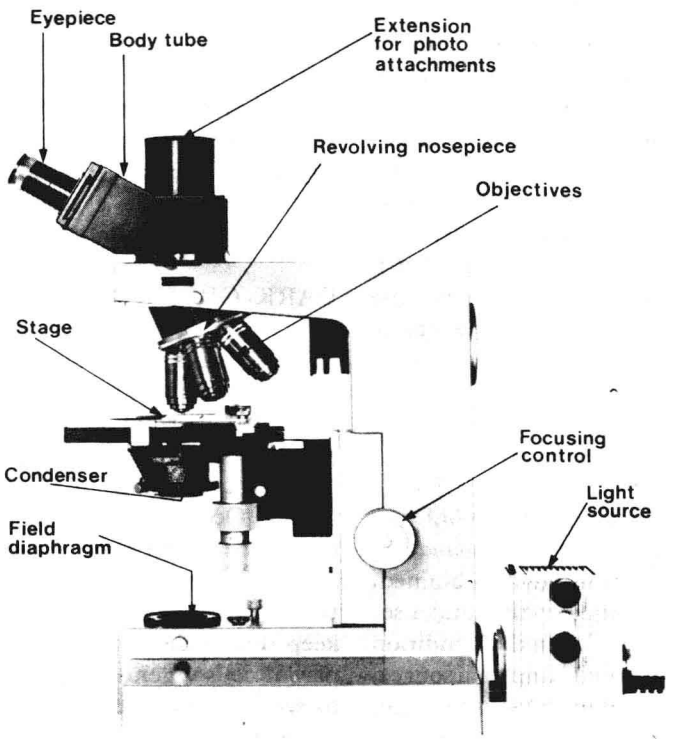

Fig. 1.10b A modern microscope.

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 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 $40 \times 10 \times 1.25 = 500$. The factors involved are usually to be found engraved 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 magnification, and here the N.A. of the objective will be of value in deciding how high the total magnification