Carleton's HISTOLOGICAL TECHNIQUE

FIFTH EDITION

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R. A. B. DRURY
M.A., D.M.(Oxon.), F.R.C.Path.
Plymouth General Hospital, Plymouth

E. A. WALLINGTON
F.I.M.L.S.
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Preface to the fifth edition

On 7 January 1926 Harry Carleton wrote for the introduction to the first edition 'the aim of this book is to give the chief methods employed in the microscopical examination of human and other animal organs'. Today, over fifty years and four editions later, there is no reason to change this objective, but it has become more difficult to achieve. A golden age of histological methodology in the later part of the last century, during which many of the general and specialized dye staining techniques were developed, has been followed by an even more productive period in the middle decades of the twentieth century. Alternative methods of demonstrating the structure, and especially the function, of tissues have evolved which include amongst others, histochemistry and immunohistology, whilst fluorescence and electron microscopy have become complementary to the use of the conventional light microscope. A wide range of non-dye staining methods have emerged in the mid-twentieth century, and these are best used in conjunction with the established dye staining techniques of the nineteenth century.

The modern histologist needs to be aware of all new techniques and will use many of them, but much of the work of a histology laboratory still consists of dye staining of tissue sections. A technologist must be expert in the classical methods of the past and use the artistry and flair that are needed for successful dye staining, but scientific accuracy and training are essential for the more exact methods such as those of enzyme histochemistry. The authors of the present edition face the same problem of incorporating much new methodology whilst preserving all that is valuable from the past. We have continued to emphasize the importance of understanding the rationale of each method as difficulties can only be overcome by the well informed bench worker. Dogma and mystery now have no place in the histology laboratory.

New material in this edition owes much to experts in specialized fields. Immunohistology has emerged from a research tool into routine techniques that can use fixed tissues and great help has been given by Mr J. Burns in the preparation of this chapter. The growing respectability of histology as a science has taken the microscopist out of a world of subjective qualitative impressions into one of objective quantitative facts and this has been a stimulus to include a new chapter on quantitative methods for which we thank Dr C. S. Foster. Dr Alison Smithies has revised diagnostic cytology with emphasis upon the need for the satisfactory collection and preparation of specimens; without these, subsequent staining and examination cannot be completely successful. The whole text has been revised and additional sections and methods have been added, but only a few techniques have been deleted. The methods for special organs and tissues have been redistributed amongst other chapters and dye staining of ultrastructural organelles is now replaced by enzyme histochemistry or electron microscopy. Techniques for electron microscopy remain outside the scope of this volume, but Dr P. G. Toner has assisted with

the introductory section on scanning electron microscopy. Many others have helped in the preparation of this edition and it is a pleasure to thank Professor H. A. Sissons, Mr W. Slidders, Miss Kathleen Page, Mr J. Chapman, and Mr R. A. Lambert for assistance with the text. Professor I. M. Roitt, Professor Deborah Doniach, Dr D. W. H. Barnes, Mr E. Lucas, Mr G. A. Harwood, and many commercial organizations provided illustrations, and our thanks are also due the editor of *Medical Laboratory Sciences*, Dr P. P. Anthony, and Messrs Churchill Livingstone for permission to publish some of the new figures. Others will recognize small details for which they were responsible; these are too numerous to list but are gratefully acknowledged. The completion of our work has been made possible by the active help and patient forbearance of our laboratory colleagues, by the secretarial assistance of Mrs Jacqueline West, and the guidance and support provided by the staff of the Oxford University Press.

Plymouth and London R.A.B.D.
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Contents

1 | The aims and methods of histology

The aims of histology

Histology is the study of tissues (from the Greek, *histos* = tissue; *logos* = word). Firstly, this involves the examination of the architecture and relationship of the different types of tissues, and secondly the detailed investigation of the structure of the individual cells—*cytology*. By combining this knowledge of microscopic anatomy and cellular composition much has been learnt about the physiological function of tissues and this has been amplified by more recent research into the histochemical reactions and ultrastructure of cells. Histology and cytology are closely interrelated, and in almost all histological preparations the components of individual cells will be studied. Ideally, microscopic examination of living tissues should be carried out, so that a true picture can be obtained, but only small primitive living animals can be examined in this way as most tissues are too thick or are not accessible for direct inspection. The majority of histological techniques are applied to killed tissues, preserved or 'fixed' in such a way as to retain the structure as closely as possible to that of the living tissues. It must be appreciated that the completed histological preparations of killed tissues show certain alterations of the cells and of the tissue as a whole; these changes, which we call '*artifacts*', may occur in any of the processes in the preparation of the specimen for microscopy, and the histologist must use methods which, for his particular purpose, cause the least tissue damage.

There are many different techniques for the preparation of tissues and many methods of examination. Most involve the preparation of *sections* that are examined by various types of microscopes, but no single method of examination, and certainly no single staining technique, will display all the cells of the tissue equally well, nor all the individual components within the cells. An advantage of histological techniques is that only small amounts of tissue are required and it may be possible to demonstrate morphology by dye staining, functional characteristics by histochemical or immunological methods, and ultrastructure by electron microscopy, all from the same specimen. Staining methods sometimes have no fully understood scientific basis, but an increasing number with a known chemical reaction are now being used; these histochemical techniques are not to be regarded as something distinct, only a more scientific and exact part of histology. Every histologist should know the reason why a technique is used in histology and how it works, in addition to remembering the details of the method. In a few cases we admit that we do not know, but in almost all histological techniques there are easily understood scientific reasons for what we do and how we do it. Recognition of the reasons behind the methods makes the whole subject more interesting and less of a mystery. Histological techniques remain something of an art, but are becoming

more and more a science, and have not been superseded by the development of other biological scientific methods. They are capable of displaying beautifully the cellular details and some of the functional activities of the tissue, but it must be realized that histology has its weaknesses and not a few pitfalls.

The aim of the histologist is to obtain, within the time available, the greatest possible amount of information from the tissue. This will be achieved if workers appreciate that their techniques can cause alterations within the tissue; must be carried out with care, skill, and empathy; and need accurate and critical interpretation.

The methods of histology

METHODS OF PREPARATION

FRESH CELLS AND TISSUES

Cells that are suspended in a fluid, such as blood or lymph, may be seen by *direct examination* in a drop of the fluid. The fluid may require dilution with an isotonic solution such as normal saline. Cells that are grouped together in a loose tissue, as in subcutaneous connective tissue, may also be examined directly if the tissue is thin. If the tissue is thick, or in the case of a solid organ, cells can be separated from one another in a fluid medium like normal saline by *dissociation* or *teasing*. Fresh preparations show cells in their natural state, but suffer from difficulty in examination due to lack of contrast. This can be overcome by phase-contrast microscopy or vital staining.

Vital staining is a method of giving contrast and colour to the cytoplasm of cells. Ehrlich (1887, 1894) showed that certain parts of the living cell can be stained if the cells are dissociated in the staining solution (supra-vital staining) or by the injection of the dye into the living organism (intra-vital staining). These methods are ideal in that they demonstrate parts of living cells, but vital staining is limited by the fact that only certain cytoplasmic elements can be demonstrated. The nucleus is resistant to vital stains, and the permeability of the nuclear membrane towards dyes appears to be an indication of cell death. Vital staining has been widely used in experimental work, and can be a valuable control of cytological methods using fixed material but is now partly replaced by phase-contrast microscopy. Features of this method are described in greater detail in Chapter 6 [p. 108].

CYTOLOGICAL TECHNIQUES

Fluids containing cells, or tiny fragments of tissue such as aspirated bone marrow, are smeared upon a microscope slide, and the adherent cells are fixed in order to preserve their appearance. Organs or tissues can be smeared upon slides in the same way, and a number of the cells, depending upon the consistency and structure of the tissue, will adhere to the slide. The smears are stained to demonstrate cell structure, and finally mounted in a medium that gives a suitable optical refractility to the tissue. The examination of stained smears is the standard method in exfoliative cytology [p. 335]. The finding of atypical cells in body fluids and secretions may be suggestive or diagnostic of malignancy, and the recent great increase in diagnostic cytology is largely due to the development of the method by Papanicolaou (1943), although examina-

tion of body fluids for the detection of carcinoma cells dates back to the nineteenth century (Beale 1860).

For some organs, such as spleen or bone marrow, the *impression method* is used. This is done by touching the cut surface of the organ or piece of tissue with a slide; in this way, a little of the architectural arrangement of the tissue may be preserved in the imprint. Soft tumours may be rapidly studied by this method, which is still of value in the diagnosis of malignancy (Tribe 1973).

In smears or films the cells flatten and are effectively in two, rather than three, dimensions. They are larger than the same cells in tissue sections and cellular details are more easily seen. These are valuable features and smears can supplement sectional methods, thereby obtaining both cellular structural detail and architecture of the tissue. With tissue cultures and leucocyte cultures for chromosomes the use of films and smears is particularly appropriate.

SECTIONAL METHODS

These involve cutting the specimen into very thin translucent slices or sections, and have the advantage (in contrast to the methods mentioned above) that the architecture of the tissue—the relation of the cells to one another—is preserved. Three-dimensional tissues are deliberately converted into sections approximately one cell thick, and to see the cellular structure these are stained in various ways. The interpretation of thin sections requires experience, especially if the section is not a vertical cross-section. Oblique or tangential sections may give surprising appearances, and these are represented in FIGURE 1.1, which shows the shape of some of the sections that can be obtained by slices through a curved cylindrical object such as a banana in its skin. The accuracy of the various sectional methods is sufficiently great that most research and routine work in histology is carried out by one of them. An accurate reconstruction of a small piece of the tissue can be obtained by the examination of *serial sections*. The whole specimen is sectioned, and the sections retained in their correct order; the numbered, stained preparations are examined in sequence. With larger specimens serial sections may be an impossibility, but alternate sections, or sections retained at regular intervals (e.g. every tenth section) may give a good indication of the structure of the whole specimen; this is known as *step sectioning* [see p. 94].

Thick sections of fresh or fixed tissue may be cut freehand with a sharp knife or razor and by limiting staining to the surface, the histological structure can be seen. This technique (Terry 1928) has been of some value in rapid diagnostic work, and is still a quick and easy way of identifying tissues (Westwood and Hunt 1973), but is now superseded by sections cut on a microtome. Most sectional methods are based upon the conversion of the tissue into a uniform consistency suitable for sectioning. This alteration of tissue consistency may be done by *freezing*, though more commonly this is brought about by infiltrating and embedding the specimen with *paraffin wax*, *celloidin*, or *synthetic resins*. Frozen sections can be cut from fresh tissue in a cryostat refrigerated microtome, but tissue which is infiltrated and embedded in wax or other solid materials is 'fixed' to preserve the tissue, prevent diffusion from it and make it possible to prepare and stain suitable sections.

Most histological sections are 4–7 μm thick. The micrometre (μm) is a standard unit of measurement in histology and 1 μm equals one thousandth of a

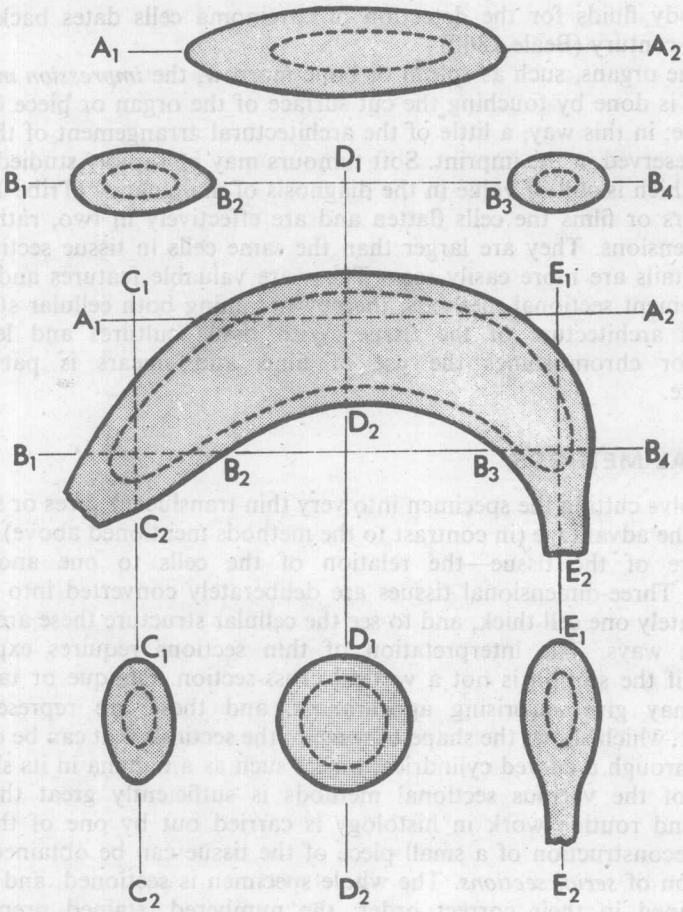

FIG. 1.1. Sections taken through a banana in its skin, showing variations in shape in different planes of section.

millimetre or one millionth of a metre. For techniques demonstrating large structures such as fat droplets, nerve fibres, and blood vessels, thicker sections in the range 10–25 μm are more successful. Sections 1 μm thick can be cut from tissues embedded in synthetic resins and these show more detail than thicker wax sections; they are intermediate between the conventional histological section and the 50–100 nm (0.05–0.1 μm) thick sections which are cut on the ultramicrotome for electron microscopy. In contrast, 300–400 μm thick sections of whole lungs or other organs designed for naked-eye examination can be cut on a large microtome after gelatin impregnation (Gough and Wentworth 1949), and are used for demonstration or teaching [p. 482]. Whilst most tissues are soft and require support before they can be sectioned, some are too hard, and bone usually needs decalcification [p. 200] before sections can be cut. Sections of undecalcified bone may be prepared from small specimens in order to determine if calcification of the matrix is normal. The use of dense embedding media and heavy microtomes has made this possible [p. 208].

The *staining of tissue sections* is required as tissues need contrast and colour

for microscopical examination and the completed preparation must have a suitable refractive index. 'Staining' techniques will be considered in general in Chapter 7 [p. 125] and in detail in many of the other chapters. In brief, staining may be carried out by dyes, which may be coloured or induce fluorescence; by chemical reactions that produce coloured end-products; or by making tissue components opaque by metallic impregnation.

Non-staining techniques for tissue sections are used in addition to the traditional 'staining' methods. Modern histological techniques include fluorescent immunological methods, autoradiography, microincineration, and microradiography.

Fluorescent immunohistological methods are based on the use of fluorochrome-labelled antibodies [p. 323]. These have a high degree of specificity and can be used to demonstrate immune complexes and a wide range of cellular structures in tissues. The preparations are examined with the fluorescence microscope, which is capable of showing very small amounts of the fluorochrome.

Autoradiography demonstrates radioactive materials following the introduction of a labelled radioactive element into the tissue. This may be quickly incorporated into cells of the tissues and autoradiography will demonstrate the sites of radioactive isotopes by their ability to reduce the silver salts in a photographic emulsion. The technique was described by London in 1904, but the method in general use is that of Pelc (1947) and Doniach and Pelc (1950). In this, the photographic emulsion is stripped from special plates and applied to sections [p. 469]; after appropriate development, fixation, and mounting, the sites and approximate quantity of the radioactive isotope can be seen. All those who work with radioactive materials must remember that radioactivity is harmful, and special precautions are necessary when handling or disposing of radioactive substances. Radioactivity decays steadily, but may never completely disappear, so the duration of the radioactivity of an isotope is expressed as its 'half-life'; this is the time necessary for one half of the number of unstable atoms to disintegrate, after which the amount of radioactivity in the material is reduced by 50 per cent. The half-life of radioactive isotopes in biological use varies from a few hours to an enormous number of years.

Micro-incineration is believed to have been first carried out by Raspail in 1833. A section of tissue is mounted on a slide and heated in an electric furnace. The temperature is raised, slowly at first (Scott 1950), until all the organic matter has been burnt off, leaving a mineral skeleton of the tissue on the slide. This can be examined by reflected light or by dark ground illumination, and the sites of mineral deposition compared with a control section that has not been incinerated. Micro-incineration is reviewed by Glick (1949) and Horning (1951). Techniques such as histospectrography (Policard 1933) enable quantitative estimations of very small amounts of mineral materials to be made.

Microradiography is used to study the structure of histological sections by their absorption of X-rays, and it is also possible to obtain information on the actual chemical composition of the tissues concerned (Engström 1962).

Microradiography has particularly been applied to the study of calcified tissues (bone, cartilage, enamel, and dentine) when the presence of mineral material (hydroxyapatite) is responsible for virtually all the absorption of X-rays. If a thin section of bone is placed in close contact with a fine grain photographic emulsion and exposed to a beam of soft X-rays, the resultant

picture shows the distribution of mineral material. A preparation of this type is known as a 'contact microradiograph', and is examined with an ordinary light microscope. Instruments for 'projection microradiography' have been developed, where the production of a magnified X-ray shadow allows direct enlargement of the negative. The measurement of the amount of mineral present in a given area of section is also possible. Bone specimens for microradiographic examination are usually prepared by sawing or by grinding and polishing after embedding in methyl methacrylate (Jowsey 1955). Relatively soft (20 kV) X-rays are used, and special photographic emulsions with a very fine grain are required.

Microradiographs of sections of soft tissues can give information on the protein content of tissues and the 'dry mass' of cells (Greulich 1960). For this work, very soft (5–10 kV) X-rays are needed. Microradiography is sometimes used to demonstrate the arrangement of blood vessels in a tissue, following their injection with radiopaque material.

METHODS OF EXAMINATION

MACROSCOPICAL METHODS

Histologists can learn much from the naked-eye examination of tissues. This is essential in the case of organs which are not uniform, because histological examination can only be made of parts of a large specimen and the appropriate selection of tissue blocks may be critical. The preparation of specimens for museum display (Kaiserling 1899; Wentworth 1947) and the demonstration of blood vessels or other anatomical structures by injection techniques (Tompsett 1970), are methods that supplement those of microscopic histology, whilst large paper-mounted sections of whole organs [p. 482] are intermediate links between gross and microscopic methods.

MICROSCOPICAL METHODS

The examination of intact tissue specimens with a low-power stereoscopic *dissecting microscope*, using reflected or transmitted light, will identify tissues and some of their components, such as the villi of the mucosa of the small intestine. The *phase-contrast microscope* converts differences of refractive index into patterns of light and shade and plays an important part in the examination of fresh and unstained cells and tissues. The *interference microscope* can be used to visualize cell details of living, unstained cells and by using it as an optical balance the weight of intracellular structures can be determined.

Smears and sections of tissues are translucent and are usually examined with a *conventional binocular microscope* using transmitted light. A wide range of magnification ($\times$ 20 to $\times$ 1000) is needed and it is an advantage if the microscope can also be used for some of the specialized types of light microscopy. High-intensity illumination is necessary for the indirect visualization of objects by *dark-ground microscopy* [p. 20] or for the examination of tissues that have a polarized or crystalline structure by the *polarized-light microscope* [p. 27]. A *fluorescence microscope* [p. 22] is used for sections that have been stained with fluorochrome dyes or have been treated with fluorescent-labelled antibodies in immunohistological techniques [p. 323]; these are methods which have a high degree of sensitivity.

Electron microscopes use a short wavelength beam of electrons that produce high resolution with high magnification. The *scanning electron microscope* [p. 32] demonstrates the surfaces of tissues and cells at low and high magnification and is a sophisticated extension of the examination of intact tissues by light microscopy using reflected illumination. *Transmission electron microscopes* [p. 29] examine ultra-thin sections with a highly magnified transmitted electron beam and have largely superseded light microscopy methods of stained sections for demonstrating the larger intracellular organelles such as mitochondria and the Golgi apparatus. A development of the transmission electron microscope is the *electron probe microanalyser* which gives a quantitative assessment of the chemical composition of tissues by passing a narrow electron beam through them, using a spectrometer to detect the X-rays that are given off (Carroll 1967).

METHODS OF RECORDING HISTOLOGICAL OBSERVATIONS

The final stage is reached when the histologist records the observations. Traditionally this has been in *qualitative* terms, using words to describe the structure of the tissues examined. With the development of colour photomacrography and photomicrography [p. 462] permanent *photographic records* can be kept or used for demonstration and teaching. *Quantitative* methods [p. 436], by which the numbers, sizes, and areas of histological structures can be expressed in absolute or comparative terms, are a more exact way of recording microscopical observations and can be applied to tissue sections. A feature of stained sections and tissue embedded in paraffin blocks is their permanence, and most histology laboratories will preserve blocks and sections for long periods of time.

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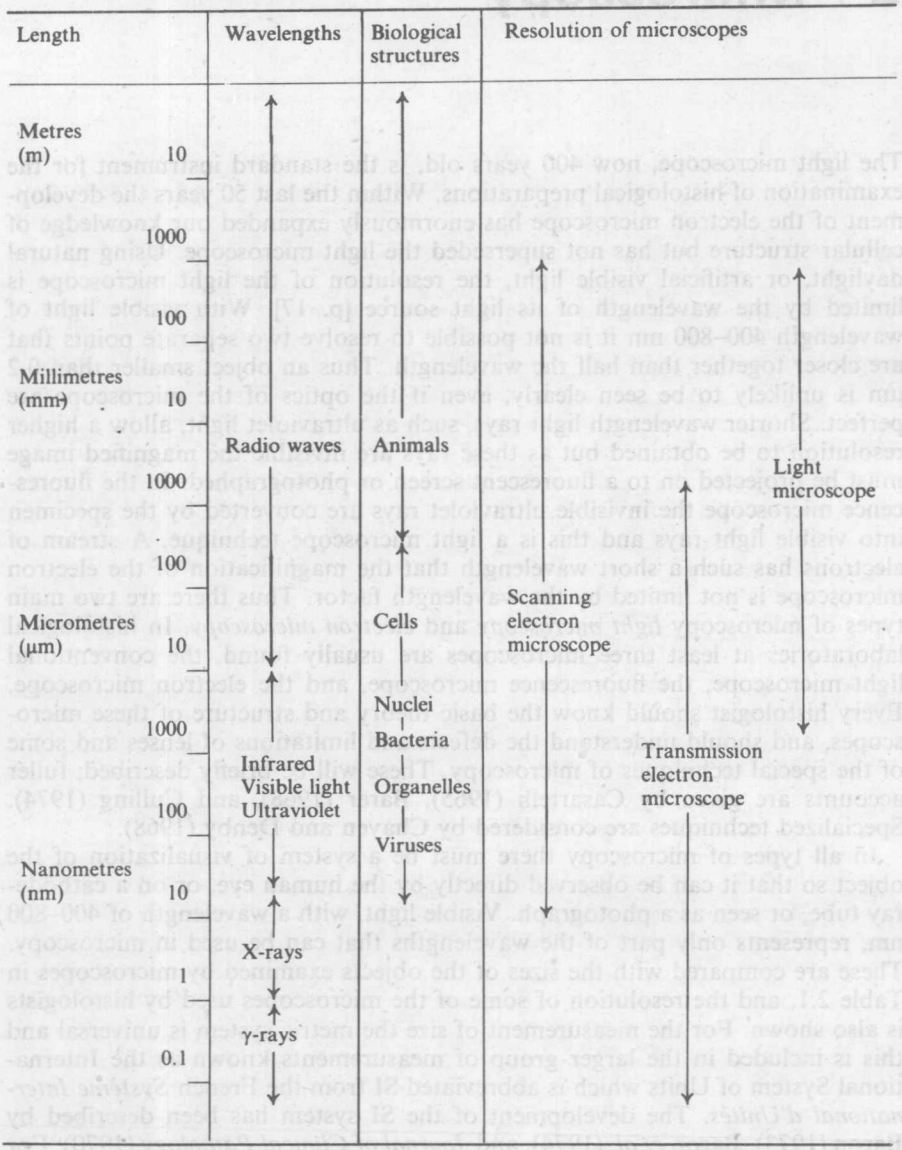
2 | Microscopy

The light microscope, now 400 years old, is the standard instrument for the examination of histological preparations. Within the last 50 years the development of the electron microscope has enormously expanded our knowledge of cellular structure but has not superseded the light microscope. Using natural daylight, or artificial visible light, the resolution of the light microscope is limited by the wavelength of its light source [p. 17]. With visible light of wavelength 400–800 nm it is not possible to resolve two separate points that are closer together than half the wavelength. Thus an object smaller than 0.2 μm is unlikely to be seen clearly, even if the optics of the microscope are perfect. Shorter wavelength light rays, such as ultraviolet light, allow a higher resolution to be obtained but as these rays are invisible the magnified image must be projected on to a fluorescent screen or photographed. In the fluorescence microscope the invisible ultraviolet rays are converted by the specimen into visible light rays and this is a light microscope technique. A stream of electrons has such a short wavelength that the magnification of the electron microscope is not limited by the wavelength factor. Thus there are two main types of microscopy *light microscopy* and *electron microscopy*. In histological laboratories at least three microscopes are usually found, the conventional light microscope, the fluorescence microscope, and the electron microscope. Every histologist should know the basic theory and structure of these microscopes, and should understand the defects and limitations of lenses and some of the special techniques of microscopy. These will be briefly described; fuller accounts are given by Casartelli (1965), Barer (1968), and Culling (1974). Specialized techniques are considered by Chayen and Denby (1968).

In all types of microscopy there must be a system of visualization of the object so that it can be observed directly by the human eye, or on a cathode-ray tube, or seen as a photograph. Visible light, with a wavelength of 400–800 nm, represents only part of the wavelengths that can be used in microscopy. These are compared with the sizes of the objects examined by microscopes in Table 2.1, and the resolution of some of the microscopes used by histologists is also shown. For the measurement of size the metric system is universal and this is included in the larger group of measurements known as the International System of Units which is abbreviated SI from the French *Système International d'Unités*. The development of the SI system has been described by Baron (1973), Baron *et al.* (1974), and *Journal of Clinical Pathology* (1970). For the measurement of length in histology we use the millimetre (mm), which is one thousandth part of a metre; the micrometre (μm), one thousandth part of a millimetre; and the nanometre (nm), one thousandth part of a micrometre (Table 2.2). The micrometre (μm) is the same length as the micron (μ), and the nanometre (nm) is the same as the millimicron (mμ). The Ångstrom unit (Å) is one tenth of a nanometre. Microns, millimicrons, and Ångstrom units have now been superseded and their use is being discontinued as the SI system becomes widely accepted.

Table 2.1 Wavelengths, sizes of biological structures, and resolution of microscopes



The light microscope

GENERAL PRINCIPLES OF LIGHT MICROSCOPY

THE FORMATION OF THE IMAGE

The simple microscope may be considered to have two convex lenses, one as the objective and the other as the eyepiece. The path of light through these is shown in FIGURE 2.1. The objective (O) magnifies the illuminated specimen SP

Table 2.2. Units of length (SI units)

SI Unit	Length in metres	Equivalent
Metre (m)	1	
Millimetre (mm)	0.001 (1×10^{-3})	
Micrometre (μm)	0.000001 (1×10^{-6})	Micron (μ)
Nanometre (nm)	0.000000001 (1×10^{-9})	Millimicron (mμ)
	0.0000000001 (1×10^{-10})	Ångstrom (Å)

to produce a real inverted image S_1P_1; to do this, the specimen must be farther away from the objective than the focal length of the objective lens. The eyepiece (E) is close to the image S_1P_1, within the focal length of the eyepiece lens; a magnified upright virtual image of S_1P_1 is produced by the eyepiece and will be seen by the observer's eye at S_2P_2. Thus the specimen has been magnified twice but inverted only once for visual examination.

These basic principles apply to all light microscopes, and the condenser and light source are only used for the satisfactory illumination of the specimen.

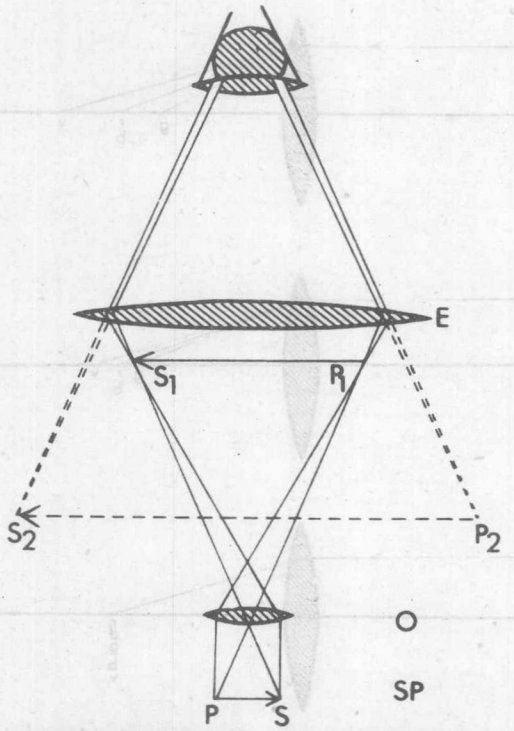


FIG. 2.1. The path of light through a simple microscope. E = eyepiece; O = objective; SP = specimen.