Contents

1

Techniques in cell biology

B. King

MODERN VIEWS IN BIOLOGY

1.1 Introduction

During the past few decades there has been a tremendous growth in the biological sciences. There are many factors underlying this increase in our knowledge of living systems. The development of new techniques in the physical and chemical sciences has been important. It has been argued that the subsequent application of these methods in biology is the single most important factor in the increase of knowledge we have witnessed in the last 30 years.

This chapter is not intended to provide students with a comprehensive treatment of the techniques used in cell biology. It is designed to give the reader an understanding of the methods discussed in the following chapters of this volume.

1.2 Light microscopy

1.2.1 MAGNIFICATION AND RESOLUTION

The oldest and still the most widely used instrument for studying the structure of organisms and cells is the light microscope. Students of biology often regard the microscope as no more than a magnifying device and do not really appreciate *the vital property of resolution*. **Magnification** achieved by microscopy can be defined as the ratio of the apparent size of the object to the actual size of the structure. In practice, this can be calculated by multiplying the primary magnification of the objective lens by that of the eyepiece. It is possible to increase the magnifying power of the instrument by increasing the power of the objective or eyepiece or both. Above a certain level, simple enlargement *does not increase the amount of detail but only serves to increase the size of the image.*

The recognition of individual but closely spaced points is termed **resolution**. Each optical instrument has a maximum resolving power; for the eye it is reached when two points come to lie approximately 80 μm apart. Below this distance the eye will just see one point: use of a magnifying glass or microscope would, of course, resolve the points.

The absolute resolving power of the light microscope *is limited by the wave nature of light itself*. A single light ray is scattered by the specimen, the smaller the feature the greater the angle of scatter. The angle of rays gathered by the objective is important in the determination of resolution. This angle is expressed in terms of **numerical aperture**, NA (Fig. 1.1b):

$$\mathrm{NA} = n \sin u$$

where n is the refractive index of the medium between the specimen and the lens, and u is half the angle of light accepted by the objective.

Resolution is given approximately by the formula

$$\text{resolution} = \frac{0.61\lambda}{\mathrm{NA}}$$

where λ is the wavelength of light.

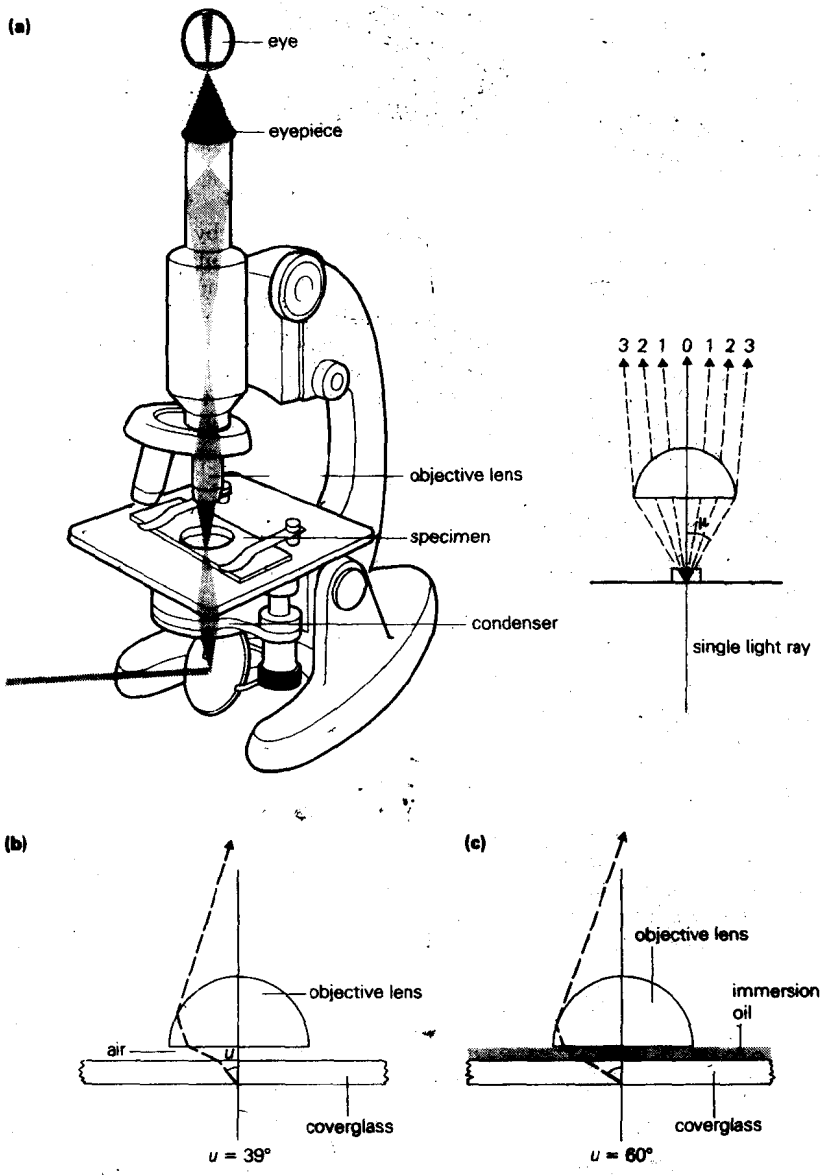

Figure 1.1 Light microscopy: (a) conventional ray pattern in a light microscope together with a single ray; (b) dry objective with a numerical aperture of 0.94; (c) oil immersion objective, numerical aperture increased to approximately 1.2.

As light travels through objects its speed may be slowed according to the refractive index of the medium. The use of immersion oil, which has an index of 1.5 (similar to that of glass), increases the numerical aperture of the objective lens (Fig. 1.1c). In addition, the distance between the specimen and the objective is reduced. In simple terms these two changes are effective in allowing a greater degree of information to be collected from the specimen, and hence a higher resolving power can be achieved.

1.2.2 STAINING

Direct observation of cells reveals a certain degree of information. However, the relatively transparent nature of cytoplasm means that there is little effect on light passing through the cells or tissues. The use of specific staining techniques greatly increases the usefulness of any microscope. In recent years, developments in the field of histochemistry have enabled scientists to stain specific molecules and hence cell organelles.

The staining of deoxyribonucleic acid (DNA) in cells by the technique of Feuglen and Rossenbeck is an example with which students may be familiar. The DNA in the nuclear material is treated with warm HCl. This causes partial hydrolysis, producing *deoxyribose components with exposed* aldehyde groups. These in turn react with Schiff's reagent, producing an insoluble purple compound.

Many of the stains used are toxic to living cells and hence only effective with dead tissue. Fortunately, there is another group of stains which can be incorporated into living cells without dramatically affecting the necessary functions of the tissue. These are termed **vital stains**.

In addition to staining techniques, a number of other methods that allow the examination of specimens have been developed. These can necessitate optical modifications to the microscope and some of the more important are described in the next section.

1.3 Contrast techniques

1.3.1 DARK GROUND MICROSCOPY

Although this method is not commonly used in schools it is an extremely valuable technique for studying certain types of cells and tissue. The optical system of the microscope is virtually reversed, *a bright image appearing against an essentially dark background*. A hollow cone of light is created as shown in Figure 1.2 by placing a central circular stop below the condenser.

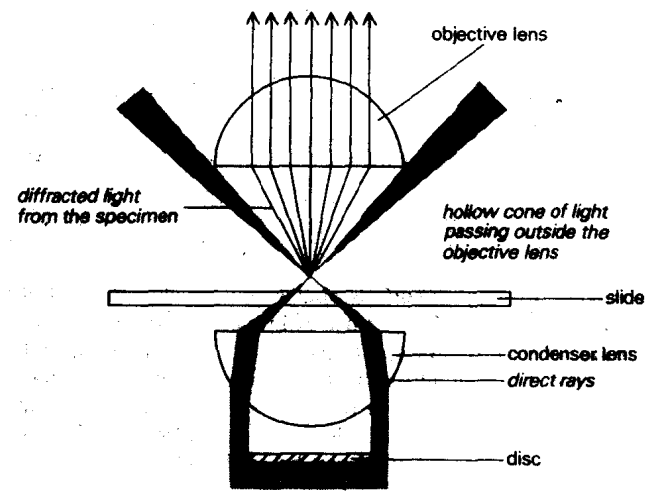

Figure 1.2 Optical arrangement used in dark ground illumination. The insertion of an opaque disc below the condenser prevents any of the direct light from the specimen entering the objective lens.

No direct light rays are gathered by the objective lens; the light rays diffracted from the specimen create a bright image on a black background. The use of dark ground illumination enables examination of details of the structure of aquatic organisms such as protozoa, rotifers and small crustaceans.

1.3.2 PHASE CONTRAST

In order to appreciate the usefulness of the technique of phase contrast a certain knowledge of the wave nature of light is required. The electromagnetic theory considers light to be associated with variations in electric and magnetic fields. This idea is illustrated in Figure 1.3a, together with several of the important parameters of light. The amplitude of a light wave is important as it determines the intensity of the light: the intensity is proportional to the square of the amplitude. The colour of the light is determined by its wavelength (λ). Light with a wavelength of 450 nm is detected as blue while a wavelength of around 660 nm gives red light.

Another important property of light is its **phase**. If two light waves are completely in phase (Fig. 1.3b), interference occurs between the waves; the resultant amplitude is greater and a much brighter light is seen. At the other extreme, if two waves are out of phase, the resultant wave has an amplitude of zero (Fig. 1.3c). In this case nothing is seen. When cells are viewed using normal light optics with no staining techniques, the indirect light waves passing through the cytoplasm are likely to be retarded. This amounts to around $\lambda/4$ relative to the direct rays (Fig. 1.3d) passing through the aqueous medium. Although interference has occurred it is usually insufficient to enable the eye to detect the detail within the cell, and the cytoplasm appears transparent.

The principle behind phase contrast microscopy lies in the *further retardation of these indirect waves*. If the difference between these two different waves can be increased to approximately $\lambda/2$ then the interference created between the two sets of waves will be sufficient to reveal details of the cytoplasm. This is achieved by the insertion of a phase plate within the objective lens (Fig. 1.4a). The plate is a glass disc in which an annular groove has been cut. The light waves which have been diffracted by the specimen are forced through the central region of the phase plate (Fig. 1.4b). This area is thicker and retards these light rays by $\lambda/4$. Since the waves are already out of phase with the direct rays, a total phase shift of $\lambda/2$ is achieved. This phase shift is ensured by placing an annular diaphragm before the condenser, which forces the majority of the direct light waves through the inner part of the phase plate.

Since the separation of direct and diffracted light rays can never be perfect, a small proportion of the diffracted light will in fact pass through the groove in the phase plate. This leads to a 'halo' appearing around the object. This effect can be seen quite clearly in Figure 7.15a. The use of phase microscopy has enabled such processes as cell division and endocytosis to be studied in living cells.

1.3.3 FLUORESCENCE MICROSCOPY

Certain compounds absorb short wavelength radiation and then re-emit energy as light of a longer wavelength. This phenomenon is known as **fluorescence** and is now routinely used in microscopy. The fluorescent microscope (Fig. 1.5) is essentially an ordinary optical instrument which has

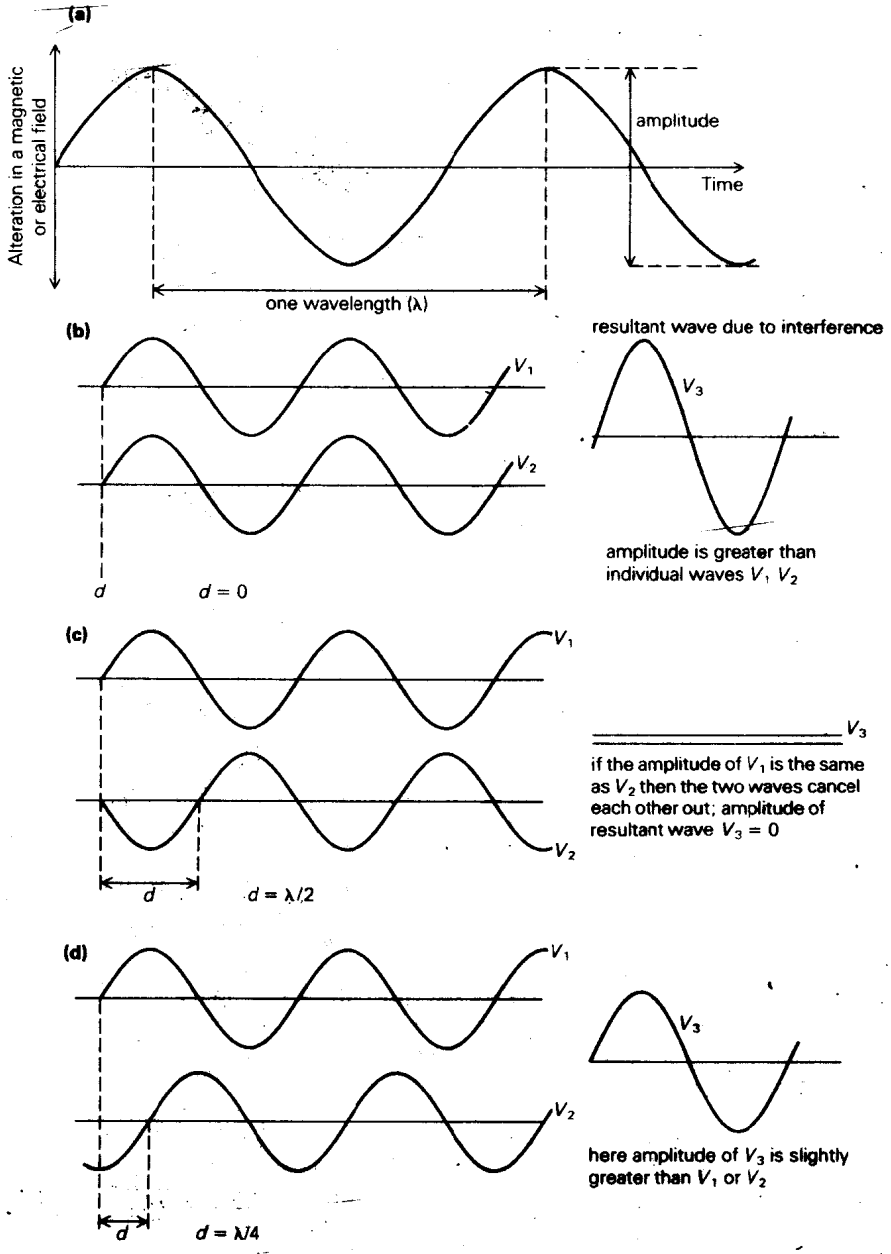

Figure 1.3 (a) Wave form of light. (b), (c) and (d) Resultant waves produced after interference between waves. The degree to which the waves are cut of phase (d) affects the amplitude of the resultant wave.

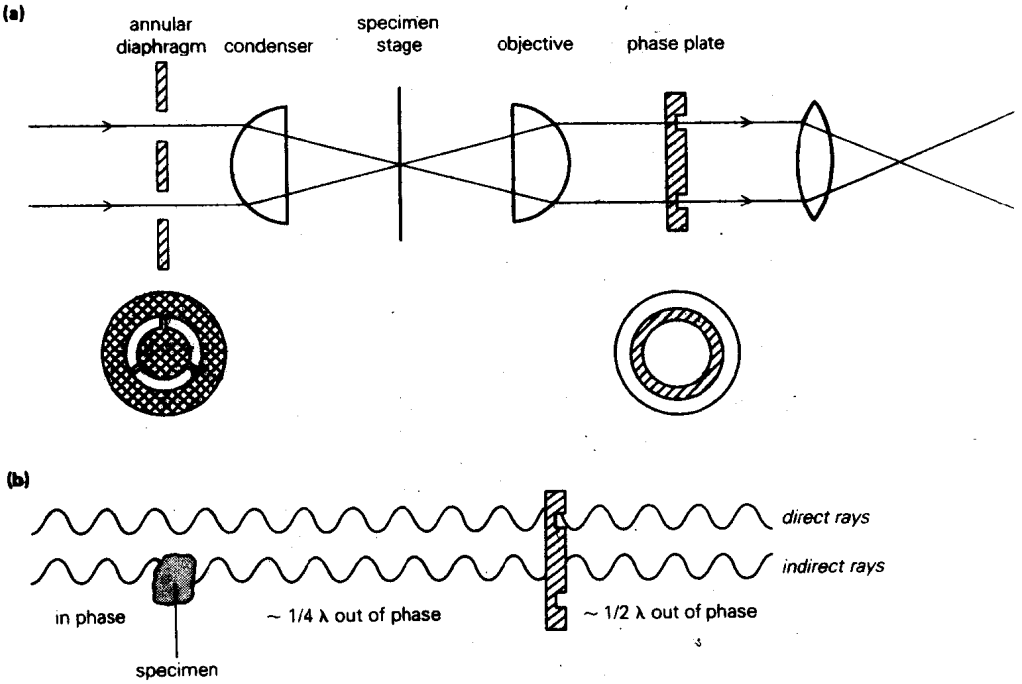

Figure 1.4 (a) Optical system of a phase contrast microscope. (b) Enhanced contrast ensured by the phase plate.

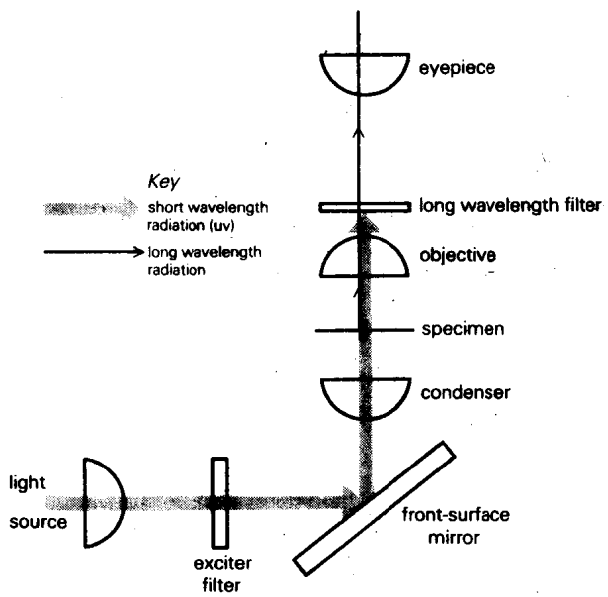

Figure 1.5 Fluorescent light microscopy. The pathway of the short wavelength light is shown by the thick line. Longer wavelength fluorescence produced by the specimen is shown by the thinner line.

been modified by incorporation of special filters. The light source is usually a high-intensity lamp which emits radiation in the blue-violet or ultra-violet region of the spectrum. This radiation passes through an 'exciter' filter which allows only the required wavelengths to pass. This light is capable of causing fluorescence in the specimen. The placing of a second filter beyond the objective lens removes any of the short exciting radiation while allowing the longer wavelength fluorescence to form an image. Since the image is formed entirely by light emanating from the specimen, fluorescing objects appear as *intensely bright images in a uniformly dark background.*

Compounds which naturally fluoresce exist in most biological tissues, for instance collagen and chlorophyll. It is also possible to induce fluorescence in certain compounds by chemical means. Amines can be modified by formaldehyde treatment so that they then exhibit fluorescence. This has enabled substances such as dopa, dopamine and andrenalin to be located within nerve cells. Fluorescent dyes '**fluorochromes**' are now available and these can be introduced into cells. An important application of such dyes has been in the investigation of chromosome behaviour.

The most sophisticated application of fluorescent microscopy so far

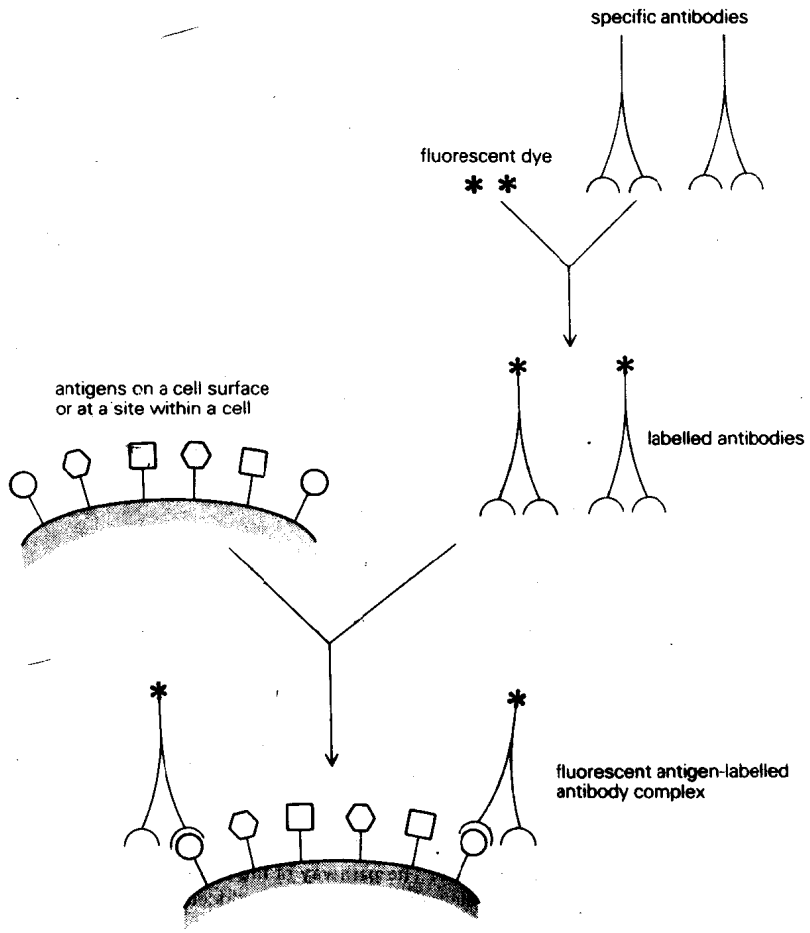

Figure 1.6 Stages in the technique of fluorescent antibody labelling.

developed has been in the field of **immuno-fluorescent antibody labelling**. Antibodies can be prepared against particular cell proteins. The attachment of a fluorochrome to an antibody creates a very powerful analytical tool (Fig. 1.6): using this method the exact location of certain cellular proteins has now been found. One of the most dramatic discoveries using this technique has been that of the **cytoskeleton** (see §7.3 & Fig. 7.10). Antibodies to actin are covalently labelled with a fluorescent molecule such as fluoresein. The addition of this complex to cells in culture followed by fluorescent microscopy reveals a highly ordered array of filaments. Using similar techniques the cellular location of the protein vinculin is revealed in Figure 7.17.

1.4 Electron microscopy

Although the light microscope with all its variation and modifications remains a powerful research tool, the fundamental wave properties of light impose a *limitation on the resolving power* of the instrument. The development of the electron microscope (EM) enabled a much greater resolving power to be achieved. A beam of electrons exhibits many of the properties similar to those shown by light waves. In particular, the electrons have a wavelength that is dependent upon their energy. This wavelength means a resolution of 0.5 nm becomes theoretically possible. In fact this resolution remains unattainable for a variety of reasons, one of the most important being the relatively inefficient nature of the magnetic lenses needed to focus the electron beams.

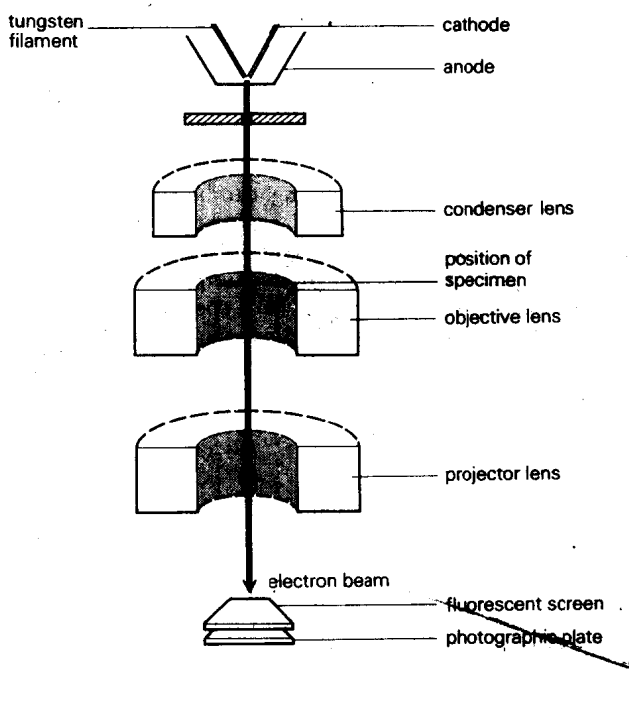

Figure 1.7 The transmission electron microscope.

The source of electrons is a heated tungsten filament. These electrons are then accelerated towards a positively charged anode (Fig. 1.7). The application of a potential difference of up to 100 kV produces a beam of electrons with an extremely short wavelength. The beam passes through a hole in the centre of the anode and then follows a pathway similar to that in a conventional optical microscope (see Fig. 1.1). The whole of the electron microscope column operates at a high vacuum which prevents collisions between the electrons and gas molecules. Such collisions would scatter the beam, so reducing the number of electrons which might pass through the specimen and eventually contribute to an image. However, the vacuum means that *living cells cannot be observed in a conventional EM*. This problem can be at least partly solved by the use of a high voltage EM (see §1.4.5).

The poor penetrative power of electrons also means that thin specimens must be prepared for the EM. The standard procedure for the preparation of specimens entails fixation, dehydration, staining and sectioning. This is similar to the techniques used in the preparation of sections for the light microscope, the most significant difference being the need for ultra-thin sections. Depending upon the tissue and type of investigation being performed, the sections can be between 10 and 100 nm. To obtain such sections, the tissue blocks are sectioned on an **ultramicrotome** using a glass or diamond knife. Typical examples of electron micrographs are shown in Figures 2.1 and 7.2.

1.4.1 STAINING TECHNIQUES

As has already been mentioned, electrons are scattered by collisions with atoms. The extent to which scattering occurs depends upon the size and concentration of atoms encountered. The larger the atom (higher the atomic number), the greater the probability of collisions. The elements present in biological material are relatively light, e.g. hydrogen, carbon, nitrogen, phosphorus and sulphur. In order to differentiate between regions of the cell it is often necessary to introduce heavy metal stains into the section.

1.4.2 NEGATIVE STAINING

Although microscopy is usually thought to be concerned with the investigation of the internal organisation of a structure, very often the shape and surface features are of importance. This is clearly true in the case of viruses and isolated macromolecules. In the technique of negative staining a heavy metal stain is allowed to cover the specimen (Fig. 1.8a). The stain accumulates around the object and adheres to it on drying. The electron beam will be scattered by the stain while passing largely unaffected through the specimen. This produces a light object on a dark background.

1.4.3 SHADOWING

This technique is really an adaptation of negative staining. Once again heavy metal stains are used. However, here a metallic element is selected rather than a metal salt. The element is evaporated by high-temperature treatment and the metal atoms allowed to coat the specimen. If the stream of evaporated metal hits the object at an angle the stain will not be deposited in the 'shadow' of the specimen (Fig. 1.8b). The technique is commonly used to reveal details of

the external structure of an object. In addition, it is possible to calculate the dimensions of a structure if the *angle of shadowing is known*: the length of the shadow is directly related to the height of the object.

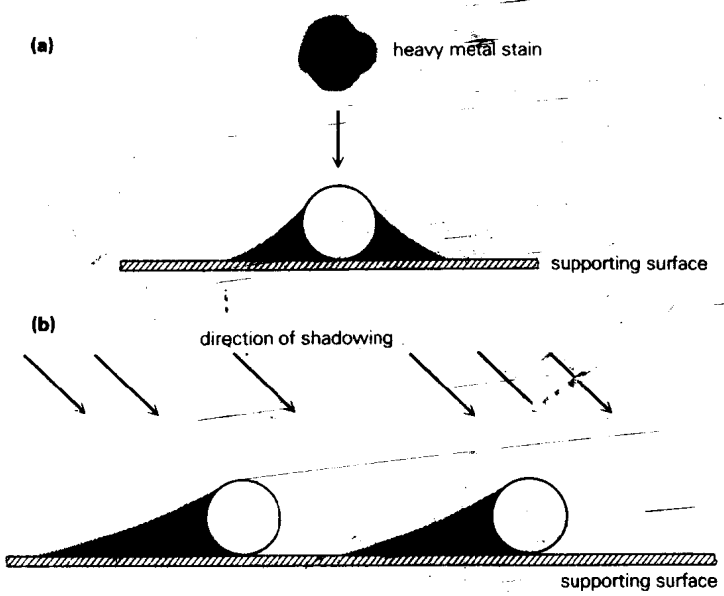

Figure 1.8 (a) Negative staining: the specimen appears light against a dark background. (b) Shadowing technique: evaporated metal is deposited across the specimen.

1.4.4 FREEZE FRACTURE

In recent years the technique of freeze fracture has added a great deal of information about the internal structure of cells. In particular, a great wealth of knowledge has been learnt about the surfaces of organelles. In this technique cells are frozen quickly in liquid nitrogen (−196 °C). If the cells are now exposed to a razor blade they tend to fracture along lines of weakness. The fracture plane *often follows the internal membrane system*. An analogous situation occurs when a piece of wood is trapped in the frozen surface of a pond. If the ice is cracked then the fracture plane will invariably pass along the wood – ice interface. One can imagine the surface of the nucleus offering a similar path to the advancing fracture plane. A replica of the surface is then prepared after shadowing with a platinum and carbon mixture. The application of carbon directly from above strengthens the structure and allows it to be removed for observation under the EM. This technique was used in the preparation of the cell membrane seen in Figure 4.8. The so-called intramembrane proteins can be clearly seen.

1.4.5 HIGH VOLTAGE ELECTRON MICROSCOPY

In the conventional transmission EM a potential of around 100 kV acts as an accelerating voltage. In the early 1960s the so-called high voltage EM was developed for scientific work. This form of EM uses a potential of approximately 3×10^3 kV. The electrons produced have sufficient kinetic energy to *penetrate thick specimens or whole cells*. There is no need for ultra-thin sections, and in addition the instrument provides high degree of

resolution and a considerable depth of field. Use of this instrument has enabled such features as the cytoskeleton and mitosis to be examined in detail.

1.4.6 SCANNING ELECTRON MICROSCOPY

In recent years the development of the scanning EM has provided biologists with another extremely powerful means of examining surfaces. A relatively low-energy beam of electrons is allowed to strike the surface of the specimen. The electrons are scattered by the specimen and they also cause secondary electrons to be emitted from the specimen. These secondary electrons are gathered by a **Faraday cage**. This instrument contains a **scintillant** which produces photons as a result of collisions with electrons. A system of coils within the column moves the primary beam across the specimen, producing a series of images created by the secondary electrons. The scanning primary beam is synchronised with the scanning of the cathode ray viewing screen. In this way a detailed picture is created. The fibroblasts in Figures 7.13, 7.14 and 7.15b were all photographed using this technique.

1.5 Cell and tissue separation techniques

The eukaryotic cell is a highly ordered structure. Membrane systems of various types essentially create a spatial separation of molecules, allowing a vast number of biochemical reactions within the cell to occur in a controlled manner. Cells exhibit a varying degree of differentiation and this itself creates another order of complexity. Prokaryotic cells, while not approaching this level of organisation, do possess a significant degree of order.

The investigation of cellular processes often involves the isolation of a specific organelle or molecule from this complex and dynamic cellular system. While the cell is intact and living *organelles and metabolites are maintained in their natural state*. The disruption of cells, which must inevitably occur if components are to be isolated, may lead to the *alteration or destruction* of many of these subcellular components.

1.5.1 HOMOGENISATION OF CELLS AND TISSUES

There are many methods routinely in use to homogenise cells and tissues. Just as there is really no typical cell, so no standard method of cell disruption can be said to exist. Cells are most commonly disrupted by some mechanical means. This may be in a glass homogeniser or Waring blender or by forcing cells through a small aperture. Exposure of cells to an osmotic stress or high-frequency sound can also cause disruption.

Regardless of the method used, the destruction of cellular components must be kept at a minimum. Homogenisation is routinely carried out at 4 °C in an effort *to reduce the enzymatic breakdown of cell components*. An osmotic buffer is normally included in the isolation medium if intact organelles are required. Sucrose often fulfils this role, although other chemicals such as sorbitol, ficol or manitol can be used instead. The pH of the isolation medium is also maintained at a specified level. The actual pH depends upon the nature

of the final product. Isolation of the enzyme acid phosphatase is normally carried out at pH 5.0 and ribosome isolation medium is usually buffered at approximately pH 7.5.

1.5.2 CENTRIFUGATION

Once the cells or tissues have been disrupted the isolation of purified fractions is most commonly carried out by *centrifugation*. In a centrifuge, particles sediment at different rates when an accelerating force is applied. The rate depends upon the size, density and shape of the particles. A typical cell fractionation scheme is shown in Figure 1.9.

As the particle size decreases, the centrifugation speed must clearly increase to effect the sedimentation of the organelle. Intact nuclei can be separated in a bench centrifuge of the type often found in school laboratories. The isolation of microsomes and ribosomes requires an accelerating force of around 500 000g; such forces are generated in the ultra-centrifuge. The development of this particular form of centrifuge was possible due to the work of the Swedish scientist Theodor Svedberg. The sedimentation coefficients of biological molecules are expressed in Svedberg units (S). A range of sedimentation values is shown in Table 1.1.

Table 1.1 Sedimentation coefficients.

Organelle/molecule	*Sedimentation coefficient (S)*	*Mass (k dal)*
bacterial ribosomal RNA	23	1.2×10^6
	16	0.55×10^6
	5	3.6×10^4
transfer RNA	4	2.5×10^4
bacterial ribosome	70	2.7×10^6
eukaryotic ribosome	80	4×10^6

The isolation of a purified mitochondrial fraction in the scheme shown in Figure 1.9 involves sucrose density-gradient centrifugation. The development of this technique was a significant advance in cell fractionation, cytology and molecular biology in general. Two types of gradient can in fact be formed. A continuous gradient is one in which a mixing device creates a smooth gradient, with the bottom being the most concentrated and the top being the lightest. In a discontinuous gradient, a series of discrete density bands are formed by carefully layering one on top of the other.

In the simplest form of density-gradient work the extract is layered on top of a gradient in which the density is less than the particles to be isolated. The application of an accelerating force causes the particles to *migrate in discrete bands according to their density*. The density gradient serves to prevent the spreading of the bands, thus allowing relatively pure fractions to be isolated.

This technique can be modified by centrifuging particles in a gradient which includes the density value of the particle. This is often termed **equilibrium-density** or **isopynic centrifugation**. At equilibrium the particles will be located at a point which is around their own **buoyant density**. In their classic experiment Meselson and Stahl (see §5.3) used a caesium chloride gradient to determine the buoyant density of various DNA strands.

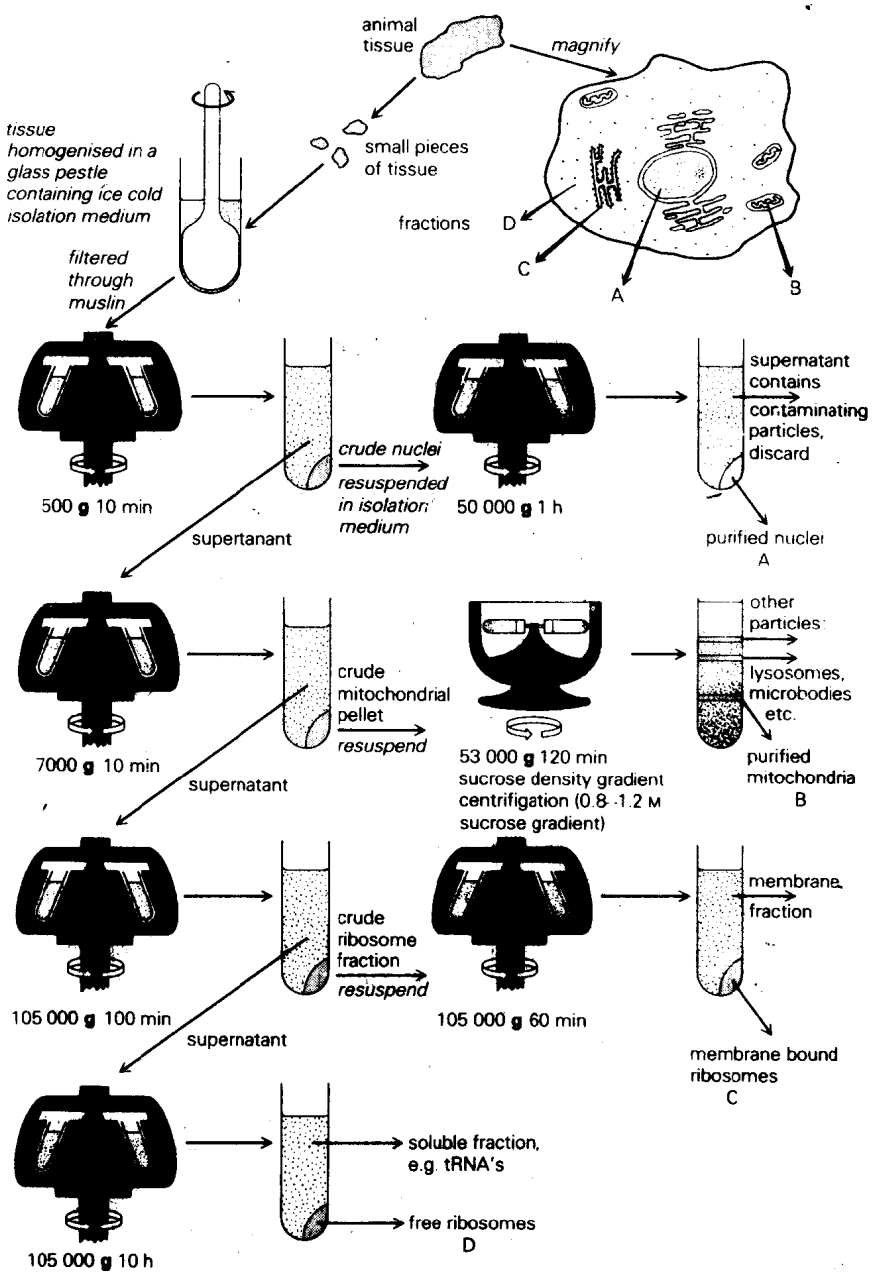

Figure 1.9 A typical cell fractionation scheme. All the steps are carried out at 0–4 °C.