Chapter 3

Centrifugation

3.1 INTRODUCTION

The biochemical analysis of subcellular structures, supramolecular complexes and isolated macromolecules is of central importance for our understanding of the molecular biology of the cell. An important prerequisite for studying the biochemical and physiological properties of organelles and biomolecules is the preservation of their biological functions and properties during the separation of cellular components. A key technique for separating and analysing the various elements of a cellular homogenate is represented by centrifugation. The development of the first analytical ultracentrifuge by Svedberg in the late 1920s and the technical refinement of the preparative centrifugation technique by Albert Claude and colleagues in the 1940s positioned centrifugation technology at the centre of biological and biomedical research for many decades. Today, centrifugation techniques are an indispensable tool of modern biochemistry and employed in almost all invasive subcellular studies. While analytical centrifugation is concerned mainly with the study of purified macromolecules or isolated supramolecular assemblies, preparative centrifugation methodology is devoted to the actual separation of cells, subcellular structures, membrane vesicles and other particles of biochemical interest.

Most undergraduate students will be exposed to preparative centrifugation protocols during practical classes and might also experience a demonstration of analytical centrifugation techniques. This chapter is accordingly divided into a short introduction to the theoretical background of sedimentation, an overview of practical aspects of using centrifuges in the biochemical laboratory, an outline of preparative centrifugation and a description of the usefulness of ultracentrifugation techniques in the biochemical characterisation of macromolecules. To aid in the understanding of the basic principles of centrifugation, the general design of various rotors and separation processes is diagrammatically represented. Often the learning process of undergraduate students is hampered by a lack of a proper linkage between theoretical knowledge and practical applications. To overcome this problem, the description of preparative centrifugation techniques is accompanied by an explanatory flow chart and the detailed discussion of the subcellular fractionation protocol of a specific tissue preparation. Taking the isolation of

fractions from skeletal muscle homogenates as an example, the rationale behind individual preparative steps is explained. Since affinity isolation methods not only represent an extremely powerful tool in purifying biomolecules (see Section 11.8) but can also be utilised to separate intact organelles and membrane vesicles by centrifugation, lectin affinity agglutination of highly purified plasmalemma vesicles from skeletal muscle is described. Traditionally, marker enzyme activities are used to determine the overall yield and enrichment of particular structures within subcellular fractions following centrifugation. As an example, the distribution of key enzyme activities in mitochondrial subfractions from liver is given. However, most modern fractionation procedures are evaluated by more convenient methods, such as protein gel analysis in conjunction with immunoblot analysis. Miniature gel and blotting equipment can produce highly reliable results within a few hours, making it an ideal analytical tool for high throughput testing. Since electrophoretic techniques are introduced in Chapter 10 and are routine methods used in biochemical laboratories, the protein gel analysis of the distribution of typical marker proteins in affinity isolated plasmalemma fractions is graphically represented and discussed here.

Although monomeric peptides and proteins are capable of performing complex biochemical reactions, many physiologically important elements do not exist in isolation under native conditions. Therefore, if one considers individual proteins as the basic units of the proteome (see Section 8.5), protein complexes actually form the functional units of cell biology. This gives investigations into the supramolecular structure of protein complexes a central place in biochemical research. To illustrate this point, the sedimentation analysis of a high molecular mass membrane assembly, the dystrophin–glycoprotein complex of skeletal muscle, is shown and the use of sucrose gradient centrifugation explained.

3.2 BASIC PRINCIPLES OF SEDIMENTATION

From every-day experience, the effect of sedimentation due to the influence of the earth's gravitational field ($g = 981\ \mathrm{cm\,s^{-2}}$) versus the increased rate of sedimentation in a centrifugal field ($g > 981\ \mathrm{cm\,s^{-2}}$) is apparent. To give a simple, but illustrative example, sand particles added to a bucket of water travel slowly to the bottom of the bucket by gravitation, but sediment much faster when the bucket is swung around in a circle. Similarly, biological structures exhibit a drastic increase in sedimentation when they undergo acceleration in a centrifugal field. The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity. For a detailed description of the forces acting on biological particles suspended in a liquid medium the reader is referred to textbooks on centrifugation listed in Section 3.6. Below is a short description of equations used in practical centrifugation classes.

When designing a centrifugation protocol, it is important to keep in mind that:

- the more dense a biological structure is, the faster it sediments in a centrifugal field,
- the more massive a biological particle is, the faster it moves in a centrifugal field,

- the denser the biological buffer system is, the slower the particle will move in a centrifugal field,
- the greater the frictional coefficient is, the slower a particle will move,
- the greater the centrifugal force is, the faster the particle sediments,
- the sedimentation rate of a given particle will be zero when the density of the particle and the medium are equal.

Biological particles moving through a viscous medium experience a frictional drag, whereby the frictional force acts in the opposite direction to sedimentation and equals the velocity of the particle multiplied by the frictional coefficient. The frictional coefficient depends on the size and shape of the biological particle. As the sample moves towards the bottom of a centrifuge tube, its velocity will increase owing to the increase in radial distance. At the same time the particles also encounter a frictional drag that is proportional to their velocity. The frictional force of a particle moving through a viscous fluid is the product of its velocity and its frictional coefficient, and acts in the direction opposite to sedimentation.

From equation 3.1 for the calculation of the relative centrifugal field it becomes apparent that when the conditions for the centrifugal separation of a biological particle are described, a detailed listing of rotor speed, radial dimensions and duration of centrifugation has to be provided. Basically, the rate of sedimentation is dependent upon the applied centrifugal field (cm s^{-2}), G, that is determined by the radial distance, r, of the particle from the axis of rotation (in cm) and the square of the angular velocity, ω, of the rotor (in radians per second):

$$G = \omega^2 r \tag{3.1}$$

Example 1 CALCULATION OF CENTRIFUGAL FIELD

Question

What is the applied centrifugal field at a point equivalent to 5 cm from the centre of rotation and an angular velocity of 3000 rad s^{-1}?

Answer

The centrifugal field, G, at a point 5 cm from the centre of rotation may be calculated using the equation $G = \omega^2 r$

$G = (3000)^2 \times 5 \text{ cm s}^{-2} = 4.5 \times 10^7 \text{ cm s}^{-2}$

The average angular velocity of a rigid body that rotates about a fixed axis is defined as the ratio of the angular displacement in a given time interval. One radian, usually abbreviated as 1 rad, represents the angle subtended at the centre of a circle by an arc with a length equal to the radius of the circle. Since 360° equals 2π radians, one revolution of the rotor can be expressed as 2π rad. Accordingly, the angular velocity of the rotor can be expressed in terms of rotor speed in revolutions (rev) per minute:

$$\omega = \frac{2\pi \text{ rev min}^{-1}}{60} \tag{3.2}$$

and therefore the centrifugal field can be expressed as:

$$G = \frac{4\pi^2 (\text{rev min}^{-1})^2 r}{3600} \tag{3.3}$$

Example 2 CALCULATION OF ANGULAR VELOCITY

Question

For the pelleting of the microsomal fraction from a liver homogenate, an ultracentrifuge is operated at a speed of 40 000 r.p.m. What is the angular velocity, ω, in radians per second?

Answer

The angular velocity, ω, may be calculated using the equation:

$$\omega = \frac{2\pi \,\text{rev min}^{-1}}{60}$$

$\omega = 2 \times 3.1416 \times 40\,000/60 \text{ rad s}^{-1} = 4188.8 \text{ rad s}^{-1}$

The centrifugal field is generally expressed in multiples of the gravitational field, g (981cm s^{-2}). The relative centrifugal field, RCF, which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity, can be calculated from the following equation:

$$\text{RCF} = \frac{4\pi^2 (\text{rev min}^{-1})^2 r}{3600 \times 981} \tag{3.4}$$

RCF units are therefore expressed in '$\times g$' and revolutions per minute are usually abbreviated as 'r.p.m.' More simply RCF $= 1.12 \times 10^{-5}$ r.p.m.^{2} r.

Although the relative centrifugal force can be calculated easily, centrifugation manuals usually contain a nomograph for convenient conversion between relative centrifugal force and speed of the centrifuge at different radii of the centrifugation spindle to a point along the centrifuge tube. A nomograph consists of three columns representing the radial distance (in millimeters), the relative centrifugal force ($\times g$) and the rotor speed (in r.p.m.). For the conversion between relative centrifugal force and speed of the centrifuge spindle in r.p.m. at different radii, a straight line is drawn through known values in two columns, then the desired figure is read where the straight line intersects with the third column. See Fig. 3.1 for an illustration of the usage of a nomograph.

In a suspension of biological particles, the rate of sedimentation is dependent not only upon the applied centrifugal field but also on the nature of the particle, i.e. its density and radius, and also the viscosity of the surrounding medium. Stokes law describes these relationships for the sedimentation of a rigid spherical particle:

$$v = \frac{2}{9} \frac{r^2 (\rho_p - \rho_m)}{\eta} \times g \tag{3.5}$$

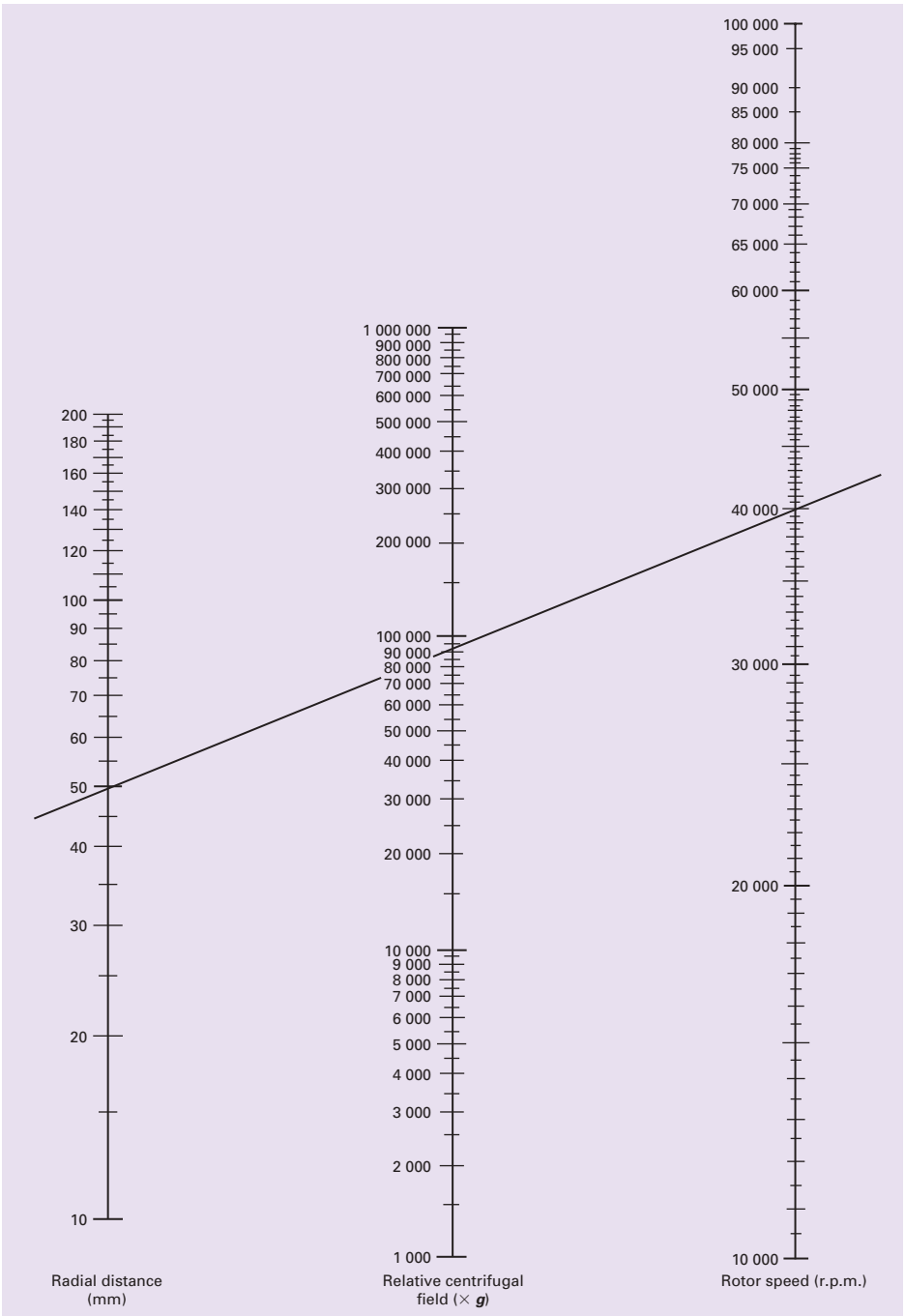

Fig. 3.1. Nomograph for the determination of the relative centrifugal field for a given rotor speed and radius. The three columns represent the radial distance (in mm), the relative centrifugal force (× ***g***) and the rotor speed (in r.p.m.). For the conversion between relative centrifugal force and speed of the centrifuge spindle in r.p.m. at different radii, draw a straight line through known values in two columns. The desired figure can then be read where the straight line intersects the third column. (Courtesy of Beckman–Coulter.)

where v is the sedimentation rate of the sphere, 2/9 is the shape factor constant for a sphere, r is the radius of the particle, ρ_p is the density of the particle, ρ_m is the density of the medium, $\boldsymbol{g}$ is the gravitational field, and η is the viscosity of the medium.

Example 3 CALCULATION OF RELATIVE CENTRIFUGAL FIELD

Question

A fixed-angle rotor exhibits a minimum radius, r_{min}, at the top of the centrifuge tube of 3.5 cm, and a maximum radius, r_{max}, at the bottom of the tube of 7.0 cm. See Fig. 3.2a for a cross-sectional diagram of a fixed-angle rotor illustrating the position of the minimum and maximum radius. If the rotor is operated at a speed of 20 000 r.p.m., what is the relative centrifugal field (RCF) at the top and bottom of the centrifuge tube?

Answer

The relative centrifugal field may be calculated using the equation:

$\text{RCF} = 1.12 \times 10^{-5}\ \text{r.p.m.}^2\ r$

Top of centrifuge tube:

$\text{RCF} = 1.12 \times 10^{-5} \times (20\,000)^2 \times 3.5\ \boldsymbol{g} = 15\,680\ \boldsymbol{g}$

Bottom of centrifuge tube:

$\text{RCF} = 1.12 \times 10^{-5} \times (20\,000)^2 \times 7.0\ \boldsymbol{g} = 31\,360\ \boldsymbol{g}$

This calculation illustrates that, with fixed-angle rotors, the centrifugal field at the top and bottom of the centrifuge tube might differ considerably, in this case approximately two-fold.

Accordingly, a mixture of biological particles exhibiting an approximately spherical shape can be separated in a centrifugal field based on their density and/or their size. The time of sedimentation (in seconds) for a spherical particle is:

$$t = \frac{9}{2}\frac{\eta}{\omega^2 r_p^2(\rho_p - \rho_m)} \times \ln\frac{r_b}{r_t} \tag{3.6}$$

where t is the sedimentation time, η is the viscosity of the medium, r_p is the radius of the particle, r_b is the radial distance from the centre of rotation to the bottom of the tube, r_t is the radial distance from the centre of rotation to the liquid meniscus, ρ_p is the density of the particle, ρ_m is the density of medium, and ω is the angular velocity of the rotor.

The sedimentation rate or velocity of a biological particle can also be expressed as its sedimentation coefficient (s), whereby:

$$s = \frac{v}{\omega^2 r} \tag{3.7}$$

Since the sedimentation rate per unit centrifugal field can be determined at different temperatures and with various media, experimental values of the sedimentation coefficient are corrected to a sedimentation constant theoretically obtainable in water at 20 °C, yielding the $S_{20,W}$ value. The sedimentation coefficients of biological macromolecules are relatively small, and are usually expressed (Section 3.5) as Svedberg units, S. One Svedberg unit equals 10^{-13} s.

3.3 TYPES, CARE AND SAFETY ASPECTS OF CENTRIFUGES

3.3.1 Types of centrifuge

Centrifugation techniques take a central position in modern biochemical, cellular and molecular biological studies. Depending on the particular application, centrifuges differ in their overall design and size. However, a common feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually suspended in a liquid buffer system contained in specific tubes or separation chambers that are located in specialised rotors. The biological medium is chosen for the specific centrifugal application and may differ considerably between preparative and analytical approaches. As outlined below, the optimum pH value, salt concentration, stabilising cofactors and protective ingredients such as protease inhibitors have to be carefully evaluated in order to preserve biological function. The most obvious difference between centrifuges are:

- the maximum speed at which biological specimens are subjected to increased sedimentation,
- the presence or absence of a vacuum,
- the potential for refrigeration or general manipulation of the temperature during a centrifugation run,
- the maximum volume of samples and capacity for individual centrifugation tubes.

Many different types of centrifuge are commercially available, including:

- large-capacity low speed preparative centrifuges,
- refrigerated high speed preparative centrifuges,
- analytical ultracentrifuges,
- preparative ultracentrifuges,
- large-scale clinical centrifuges,
- small-scale laboratory microfuges.

Some large-volume centrifuge models are quite demanding on space and also generate considerable amounts of heat and noise, and are therefore often centrally positioned in special instrument rooms in biochemistry departments. However, the development of small-capacity bench-top centrifuges for biochemical applications, even in the case of ultracentrifuges, has led to the introduction of these models in many individual research laboratories.

The main types of centrifuge encountered by undergraduate students during introductory practicals may be divided into microfuges (so called because they centrifuge small volume samples in Eppendorf tubes), large-capacity preparative centrifuges, high speed refrigerated centrifuges and ultracentrifuges. Simple bench-top centrifuges vary in design and are used mainly to collect small amounts of biological material such as blood cells. To prevent denaturation of protein samples, non-refrigerated microfuges are often used in cold rooms. Modern refrigerated microfuges are equipped with adapters to accommodate standardised plastic tubes for the sedimentation of 0.5–1.5 cm^3 volumes. They can provide centrifugal fields of approximately 10 000 ***g*** and sediment biological samples in minutes, making microfuges an indispensable separation tool for many biochemical methods. Microfuges can also be used to concentrate protein samples. For example, the dilution of protein samples eluted by column chromatography can often represent a challenge for subsequent analyses. Accelerated ultrafiltration with the help of plastic tube-associated filter units, spun at low ***g*** forces in a microfuge, can overcome this problem. Depending on the proteins of interest, the biological buffers used and the molecular mass cut-off point of the particular filters, a 10- to 20-fold concentration of samples can be achieved within minutes. Larger preparative bench-top centrifuges develop maximum centrifugal fields of 3000–7000 ***g*** and can be used for the spinning of various types of container. Depending on the range of available adapters, considerable quantities of 5–250 cm^3 plastic tubes or 96-well enzyme-linked immunosorbent assay (ELISA) plates can be accommodated. This gives simple and relatively inexpensive bench centrifuges a central place in many high throughput biochemical assays where the quick and efficient separation of coarse precipitates or whole cells is of importance.

High speed and supra-speed refrigerated centrifuges are absolutely essential for the sedimentation of protein precipitates, large intact organelles, cellular debris derived from tissue homogenisation and microorganisms. As outlined in Section 3.4, the initial bulk separation of cellular elements prior to preparative ultracentrifugation is performed by these kinds of centrifuge. They operate at maximum centrifugal fields of approximately 100 000 ***g***. Such centrifugal force is not sufficient to sediment smaller microsomal vesicles or ribosomes, but can be employed to differentially separate nuclei, mitochondria or chloroplasts. In addition, bulky protein aggregates can be sedimented using high speed refrigerated centrifuges. An example is the contractile apparatus released from muscle fibres by homogenisation, mostly consisting of myosin and actin macromolecules aggregated in filaments. In order to harvest yeast cells or bacteria from large volumes of culture media, high speed centrifugation may also be used in a continuous flow mode with zonal rotors. This approach does therefore not use centrifuge tubes but a continuous flow of medium. As the medium enters the moving rotor, biological particles are sedimented against the rotor periphery and excess liquid removed through a special outlet port.

Ultracentrifugation has decisively advanced the detailed biochemical analysis of subcellular structures and isolated biomolecules. Preparative ultracentrifugation can be operated at relative centrifugal fields of up to 600 000 ***g***. In order to

minimise excessive rotor temperatures generated by frictional resistance between the spinning rotor and air, the rotor chamber is sealed, evacuated and refrigerated. Depending on the type, age and condition of a particular ultracentrifuge, cooling to the required running temperature and the generation of a stable vacuum might take a considerable amount of time. To avoid delays during biochemical procedures involving ultracentrifugation, the cooling and evacuation system of older centrifuge models should be switched on at least an hour prior to the centrifugation run. On the other hand, modern ultracentrifuges can be started even without a fully established vacuum and will proceed in the evacuation of the rotor chamber during the initial acceleration process. For safety reasons, heavy armour plating encapsulates the ultracentrifuge to prevent injury to the user in case of uncontrolled rotor movements or dangerous vibrations. A centrifugation run cannot be initiated without proper closing of the chamber system. To prevent unfavourable fluctuations in chamber temperature, excessive vibrations or operation of rotors above their maximum rated speed, newer models of ultracentrifuges contain sophisticated temperature regulation systems, flexible drive shafts and an over-speed control device. Although slight rotor imbalances can be absorbed by modern ultracentrifuges, a more severe misbalance of tubes will cause the centrifuge to switch off automatically. This is especially true for swinging-bucket rotors. The many safety features incorporated into modern ultracentrifuges make them a robust piece of equipment that tolerates a certain degree of misuse by an inexperienced operator. See Sections 3.3.3 and 3.3.4 for a more detailed discussion of safety and centrifugation. In contrast to preparative ultracentrifuges, analytical ultracentrifuges contain a solid rotor that, in its simplest form, incorporates one analytical cell and one counterbalancing cell. An optical system enables the sedimenting material to be observed throughout the duration of centrifugation. Using a light absorption system, a Schlieren system or a Raleigh interferometric system, concentration distributions in the biological sample are determined at any time during ultracentrifugation. The Raleigh and Schlieren optical systems detect changes in the refractive index of the solution caused by concentration changes and can thus be used for sedimentation equilibrium analysis. This makes analytical ultracentrifugation a relatively accurate tool for the determination of the molecular mass of an isolated macromolecule. It can also provide crucial information about the thermodynamic properties of a protein or other large biomolecules.

3.3.2 Types of rotor

To illustrate the difference in design of fixed-angle rotors, vertical tube rotors and swinging-bucket rotors, Fig. 3.2 outlines cross-sectional diagrams of these three main types of rotor. Companies usually name rotors according to their type of design, the maximum allowable speed and sometimes the material composition. Depending on the use in a simple low speed centrifuge, a high speed centrifuge or an ultracentrifuge, different centrifugal forces are encountered by a spinning rotor. Accordingly, different types of rotor are made from different materials. Low speed rotors are made from steel or brass, while high speed rotors consist of

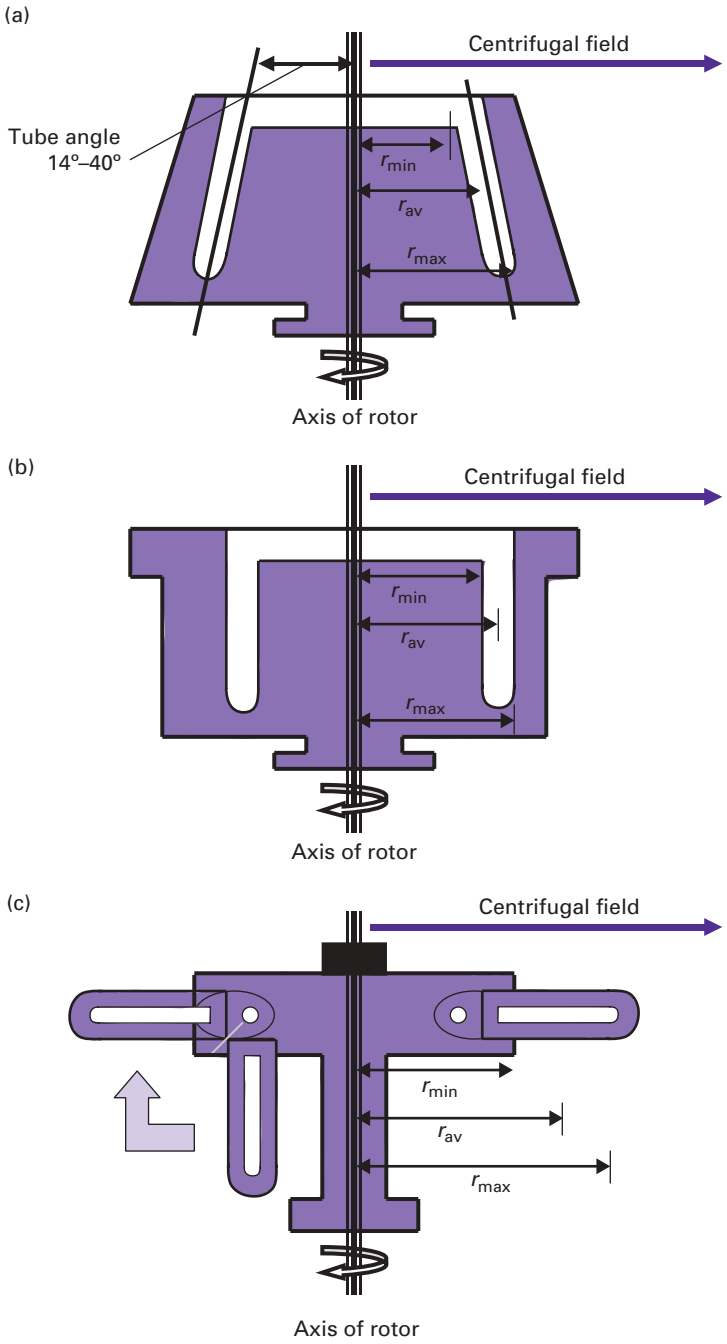

Fig. 3.2. Design of the three main types of rotor used in routine biochemical centrifugation techniques. Shown are cross-sectional diagrams of (a) a fixed-angle rotor, (b) a vertical tube rotor, and (c) a swinging-bucket rotor. A fourth type of rotor is represented by the class of near-vertical rotors.

aluminium, titanium or fibre-reinforced composites. The exterior of specific rotors might be finished with protective paints. For example, rotors for ultracentrifugation made out of titanium alloy are covered with a polyurethane layer. Aluminium rotors are protected from corrosion by an electrochemically formed tough layer of aluminium oxide. In order to avoid damage to these protective layers, care should be taken during rotor handling.

Fixed-angle rotors are an ideal tool for pelleting during the differential separation of biological particles whose sedimentation rates are significantly different; for example, when separating nuclei, mitochondria and microsomes. In addition, isopycnic banding may also be routinely performed with fixed-angle rotors. For isopycnic separation, centrifugation is continued until the biological particles of interest have reached their isopycnic position in a gradient. This means that the particle has reached a position where the sedimentation rate is zero because the densities of the biological particle and the surrounding medium are equal. Centrifugation tubes are held at a fixed angle of between 14° and 40° to the vertical in this class of rotor (Fig. 3.2a). Particles move radially outwards and, since the centrifugal field is exerted at an angle, they have only to travel a short distance until they reach their isopycnic position in a gradient using an isodensity technique or before colliding with the outer wall of the centrifuge tube using a differential centrifugation method. Vertical rotors (Fig. 3.2b) may be divided into true vertical rotors and near-vertical rotors. Sealed centrifuge tubes are held parallel to the axis of rotation in vertical rotors and are restrained in the rotor cavities by screws, special washers and plugs. Since samples are not separated down the length of the centrifuge tube, but across the diameter of the tube, isopycnic separation time is significantly shorter as compared with swinging-bucket rotors. In contrast to fixed-angle rotors, near-vertical rotors exhibit a reduced tube angle of 7°–10° and also employ quick-seal tubes. The reduced angle results in much shorter run times as compared with fixed-angle rotors. Near-vertical rotors are useful for gradient centrifugation of biological elements that do not properly participate in conventional gradients. Hinge pins or a crossbar is used to attach rotor buckets in swinging-bucket rotors (Fig. 3.2c). They are loaded in a vertical position and during the initial acceleration phase, rotor buckets swing out horizontally and then position themselves at the rotor body for support.

To illustrate the separation of particles in the three main types of rotor, Fig. 3.3 outlines the movement of biological samples during the initial acceleration stage, the main centrifugal separation phase, de-acceleration and the final harvesting of separated particles in the rotor at rest. In the case of isopycnic centrifugation in a fixed-angle rotor, the centrifuge tubes are gradually filled with a suitable gradient, the sample carefully loaded on top of this solution and then the tubes placed at a specific fixed-angle into the rotor cavities. During rotor acceleration, the sample solution and the gradient undergo reorientation in the centrifugal field, followed by the separation of particles with different sedimentation properties (Fig. 3.3a). The gradient returns to its original position during the deacceleration phase and separated particle bands can be taken from the tubes once the rotor is at rest. By analogy, similar reorientation of gradients and banding of particles occurs in a ver-

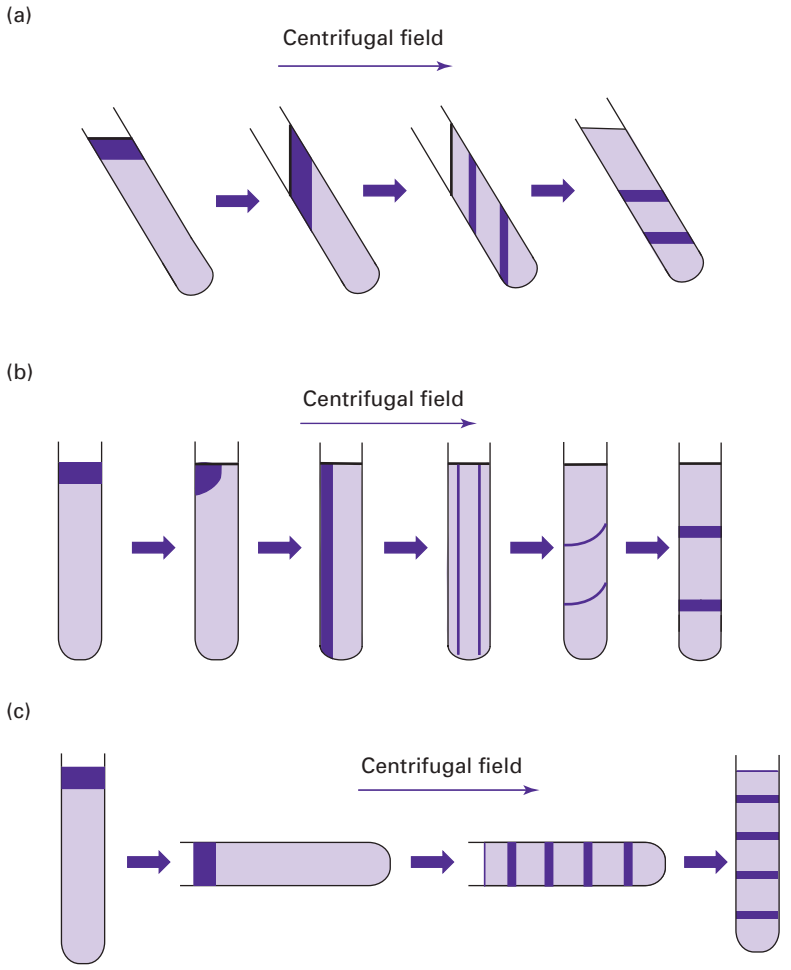

Fig. 3.3. Operation of the three main types of rotor used in routine biochemical centrifugation techniques. Shown are cross-sectional diagrams of a centrifuge tube positioned in (a) a fixed-angle rotor, (b) a vertical tube rotor, and (c) a swinging-bucket rotor (c). The diagrams illustrate the movement of biological samples during the initial acceleration stage, the main centrifugal separation phase, de-acceleration and the final harvesting of separated particles in the rotor at rest. Using a fixed-angle rotor, the tubes are filled with a gradient, the sample loaded on top of this solution and then the tubes placed at a specific fixed-angle into the rotor cavities. The sample and the gradient undergo reorientation in the centrifugal field during rotor acceleration, resulting in the separation of particles with different sedimentation properties. Similar reorientation of gradients and banding of particles occurs in a vertical tube rotor system. A great variety of gradients can be used with swinging-bucket rotors, making them the method of choice when maximum resolution of banding zones is required.

tical rotor system (Fig. 3.3b). Although run times are reduced and these kinds of rotor can usually hold a large number of tubes, resolution of separated bands during isopycnic centrifugation is less when compared with swinging-bucket applications. Since a greater variety of gradients exhibiting different steepness

can be used with swinging-bucket rotors, they are the method of choice when maximum resolution of banding zones is required (Fig. 3.3c), such as in rate zonal studies based on the separation of biological particles as a function of sedimentation coefficient.

3.3.3 Care and maintenance of centrifuges

Corrosion and degradation due to biological buffer systems used within rotors or contamination of the interior or exterior of the centrifuge via spillage may seriously affect the lifetime of this equipment. Another important point is the proper balancing of centrifuge tubes. This is not only important with respect to safety, as outlined below, but might also cause vibration-induced damage to the rotor itself and the drive shaft of the centrifuge. Thus proper handling and care, as well as regular maintenance of both centrifuges and rotors, is an important part of keeping this biochemical method available in the laboratory. In order to avoid damage to the protective layers of rotors, such as polyurethane paint or aluminium oxide, care should be taken in the cleaning of the rotor exterior. Coarse brushes that may scratch the finish should not be used and only non-corrosive detergents employed. Corrosion may be triggered by long-term exposure of rotors to alkaline solutions, acidic buffers, aggressive detergents or salt. Thus rotors should be thoroughly washed with distilled or deionised water after every run. For overnight storage, rotors should be first left upside down to drain excess liquid and then be positioned in a safe and dry place. To avoid damage to the hinge pins of swinging-bucket rotors, the rotor assembly should be dried with tissue paper following removal of biological buffers and washing with water. Centrifuge rotors are often not properly stored in a clean environment; this can quickly lead to the destruction of the protective rotor coating and should thus be avoided. It is advisable to keep rotors in a special clean room, physically separated from the actual centrifugation facility, with dedicated places for individual types of rotor. Some researchers might prefer to pre-cool their rotors prior to a centrifugation run by transferring them to a cold room. Although this is an acceptable practice and might, for example, keep proteolytic degradation to a minimum, rotors should not undergo long-term storage in a wet and cold environment. Regular maintenance of rotors and centrifuges by engineers is important for ensuring the safe operation of a centralised centrifugation facility. In order to properly judge the need for replacement of a rotor or parts of a centrifuge, it is essential that all users of core centrifuge equipment participate in proper book-keeping. Accurate record-keeping of run times and centrifugal speeds is important, since cyclic acceleration and deacceleration of rotors may lead to metal fatigue.

3.3.4 Safety and centrifugation

Modern centrifuges are not only highly sophisticated but also relatively sturdy pieces of biochemical equipment that incorporate many safety features. Rotor chambers of high speed centrifuges and ultracentrifuges are always enclosed in

heavy armour plating. Most centrifuges are designed to buffer a certain degree of imbalance and are usually equipped with an automatic switch-off mode. However, even in a well-balanced rotor, tube cracking during a centrifugation run might cause severe imbalance resulting in dangerous vibrations. Rotors must never be loaded with an odd number of tubes. When the rotor can only be partially loaded, the tubes must be located diametrically opposite each other in order that the load is distributed evenly around the rotor axis. This is not only important for ultracentrifugation with enormous centrifugal fields, but also for both small and large capacity bench centrifuges where the rotors are usually mounted on a more rigid suspension. When using swinging-bucket rotors, it is important always to load all buckets with their caps properly screwed on. Even if only two tubes are loaded with solutions, the empty swinging buckets have also to be assembled, since they form an integral part of the overall balance of the rotor system. In some swinging-bucket rotors, individual rotor buckets are numbered and should not be interchanged between their designated positions on similarly numbered hinge pins. To avoid the disturbance of delicate gradients, centrifugation runs with swinging-bucket rotors are usually initiated under fully established vacuum, and started and terminated at low acceleration and de-acceleration speeds, respectively. This practice also avoids the occurrence of sudden imbalances due to tube deformation or cracking and thus eliminates potentially dangerous vibrations.

Generally, safety and good laboratory practice are important aspects of all research projects and the awareness of the exposure to potentially harmful substances should be a concern for every biochemist. If you use dangerous chemicals, potentially infectious material or radioactive substances during centrifugation protocols, refer to up-to-date safety manuals and the safety statement of your individual department. Perform mock runs of important experiments in order to avoid the loss of precious specimens or expensive chemicals. As with all other biochemical procedures, experiments should never be rushed and protective clothing worn at all times. Centrifuge tubes should be handled slowly and carefully so as not to disturb pellets, bands of separated particles or unstable gradients. With respect to choosing the right kind of centrifuge tube for a particular application, the manufacturers of rotors usually give detailed recommendation of suitable materials. For safety reasons and to guarantee experimental success, it is important to make sure that individual centrifuge tubes are chemically resistant to the solvents used, have the right capacity for sample loading, can be used in the designated type of rotor and are able to withstand the maximum centrifugal forces and temperature range of a particular centrifuge. In fixed-angle rotors, large centrifugal forces tend to cause a collapse of centrifuge tubes making thick-walled tubes the choice for these rotors. In contrast, swinging-bucket rotor tubes are better protected from deformation and usually thin-walled polyallomer tubes are used. An important safety aspect is the proper handling of separated biological particles following centrifugation. In order to perform postcentrifugation analysis of individual fractions, centrifugation tubes often have to be punctured or sliced. For example, separated vesicle bands can be harvested from the pierced bottom of the centrifuge tube or are collected by slicing of the tube following

quick freezing. If samples have been pre-incubated with radioactive markers or toxic ligands, the contamination of the centrifugation chamber and rotor cavities or buckets should be avoided. If centrifugal separation processes have to be performed routinely with a potentially harmful substance, it makes sense to dedicate a particular centrifuge and accompanying rotors for this work and thereby eliminate the potential for cross-contamination.

3.4 PREPARATIVE CENTRIFUGATION

3.4.1 Differential centrifugation

Cellular and subcellular fractionation techniques are indispensable methods used in biochemical research. Although the proper separation of many subcellular structures is absolutely dependent on preparative ultracentrifugation, the isolation of large cellular structures, the nuclear fraction, mitochondria, chloroplasts or large protein precipitates can be achieved by conventional high speed refrigerated centrifugation. Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density. Crude tissue homogenates containing organelles, membrane vesicles and other structural fragments are divided into different fractions by the stepwise increase of the applied centrifugal field. Following the initial sedimentation of the largest particles of a homogenate (such as cellular debris) by centrifugation, various biological structures or aggregates are separated into pellet and supernatant fractions, depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles. To increase the yield of membrane structures and protein aggregates released, cellular debris pellets are often re-homogenised several times and then re-centrifuged. This is especially important in the case of rigid biological structures such as muscular or connective tissue, or in the case of small tissue samples as occurs with human biopsy material or primary cell cultures.

The differential sedimentation of a particulate suspension in a centrifugal field is shown diagrammatically in Fig. 3.4a. Initially all particles of a homogenate are evenly distributed throughout the centrifuge tube and then move down the tube at their respective sedimentation rates during centrifugation. The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant. However, during the initial centrifugation step smaller particles also become entrapped in the pellet, causing a certain degree of contamination. At the end of each differential centrifugation step, the pellet and supernatant fractions are carefully separated from each other. To minimise cross-contamination, pellets are usually washed several times by re-suspension in buffer and re-centrifugation under the same conditions. However, repeated washing steps may considerably reduce the yield of the final pellet fraction and are therefore omitted in preparations with limiting starting material. Resulting supernatant fractions are centrifuged at a higher speed and for a longer time to separate medium-sized and small-sized particles. With respect to the separation of

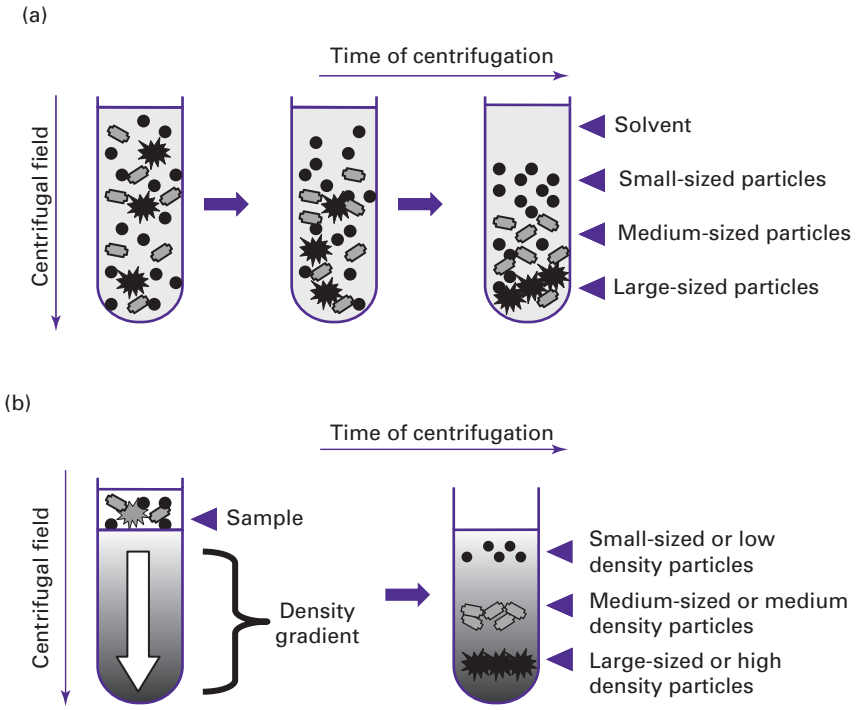

Fig. 3.4. Diagram of particle behaviour during differential and isopycnic separation. During differential sedimentation of a particulate suspension in a centrifugal field (a), the movement of particles is dependent upon their density, shape and size. For separation of biological particles using a density gradient (b), samples are carefully layered on top of a pre-formed density gradient prior to centrifugation. For isopycnic separation, centrifugation is continued until the desired particles have reached their isopycnic position in the liquid density gradient. In contrast, during rate separation, the required fraction does not reach its isopycnic position during the centrifugation run.

organelles and membrane vesicles, crude differential centrifugation techniques can be conveniently employed to isolate intact mitochondria and microsomes.

3.4.2 Density gradient centrifugation

To further separate biological particles of similar size but differing density, ultracentrifugation with pre-formed or self-establishing density gradients is the method of choice. Both rate separation or equilibrium methods can be used. In Fig. 3.4b, the preparative ultracentrifugation of low to high density particles is shown. A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation, is layered on top of a pre-formed liquid density gradient. Depending on the particular biological application, a great variety of gradient materials are available. Caesium chloride is widely used for the banding of DNA and the isolation of plasmids, nucleoproteins and viruses. Sodium bromide and sodium iodide are employed for the fractionation of lipoproteins and the banding of DNA or RNA molecules, respectively. Various companies offer a range

of gradient material for the separation of whole cells and subcellular particles, for example Percoll, Ficoll, Dextran, Metrizamide and Nycodenz. For the separation of membrane vesicles derived from tissue homogenates, ultrapure DNase-, RNase- and protease-free sucrose represents a suitable and widely employed medium for the preparation of stabile gradients. If one wants to separate all membrane species spanning the whole range of particle densities, the maximum density of the gradient must exceed the density of the most dense vesicle species. Both step gradient and continuous gradient systems are employed to achieve this. If automated gradient makers are not available, which is probably the case in most undergraduate practical classes, the manual pouring of a stepwise gradient with the help of a pipette is not that time-consuming or difficult. In contrast, the formation of a stable continuous gradient is much more challenging and requires a commercially available gradient maker. Following pouring, gradients are usually kept in a cold room for temperature equilibration and are moved extremely slowly in special holders so as to avoid mixing of different gradient layers. For rate separation of subcellular particles, the required fraction does not reach its isopycnic position within the gradient. For isopycnic separation, density centrifugation is continued until the buoyant density of the particle of interest and the density of the gradient are equal.

3.4.3 Practical applications of preparative centrifugation

To illustrate practical applications of differential centrifugation, density gradient ultracentrifugation and affinity methodology, the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density is described (Fig. 3.5), the isolation of highly purified sarcolemma vesicles outlined (Fig. 3.6), and the subfractionation of liver mitochondrial membrane systems shown (Fig. 3.7). Skeletal muscle fibres are highly specialised structures involved in contraction and the membrane systems that maintain the regulation of excitation–contraction coupling, energy metabolism and the stabilisation of the cell periphery are shown diagrammatically in Fig. 3.5a. The surface membrane consists of the sarcolemma and its invaginations, the transverse tubular membrane system. The transverse tubules may be subdivided into the non-junctional region and the triad part that forms contact zones with the terminal cisternae of the sarcoplasmic reticulum. Motorneurone-induced depolarisation of the sarcolemma travels into the transverse tubules and activates a voltage-sensing receptor complex that directly initiates the transient opening of a junctional calcium release channel. The membrane system that provides the luminal ion reservoir for the regulatory calcium cycling process is represented by the specialised endoplasmic reticulum. It forms membranous sheaths around the contractile apparatus whereby the longitudinal tubules are mainly involved in the uptake of calcium ions during muscle relaxation and the terminal cisternae provide the rapid calcium release mechanism that initiates muscle contraction. Mitochondria are the site of oxidative phosphorylation and exhibit a complex system of inner and outer membranes involved in energy metabolism.

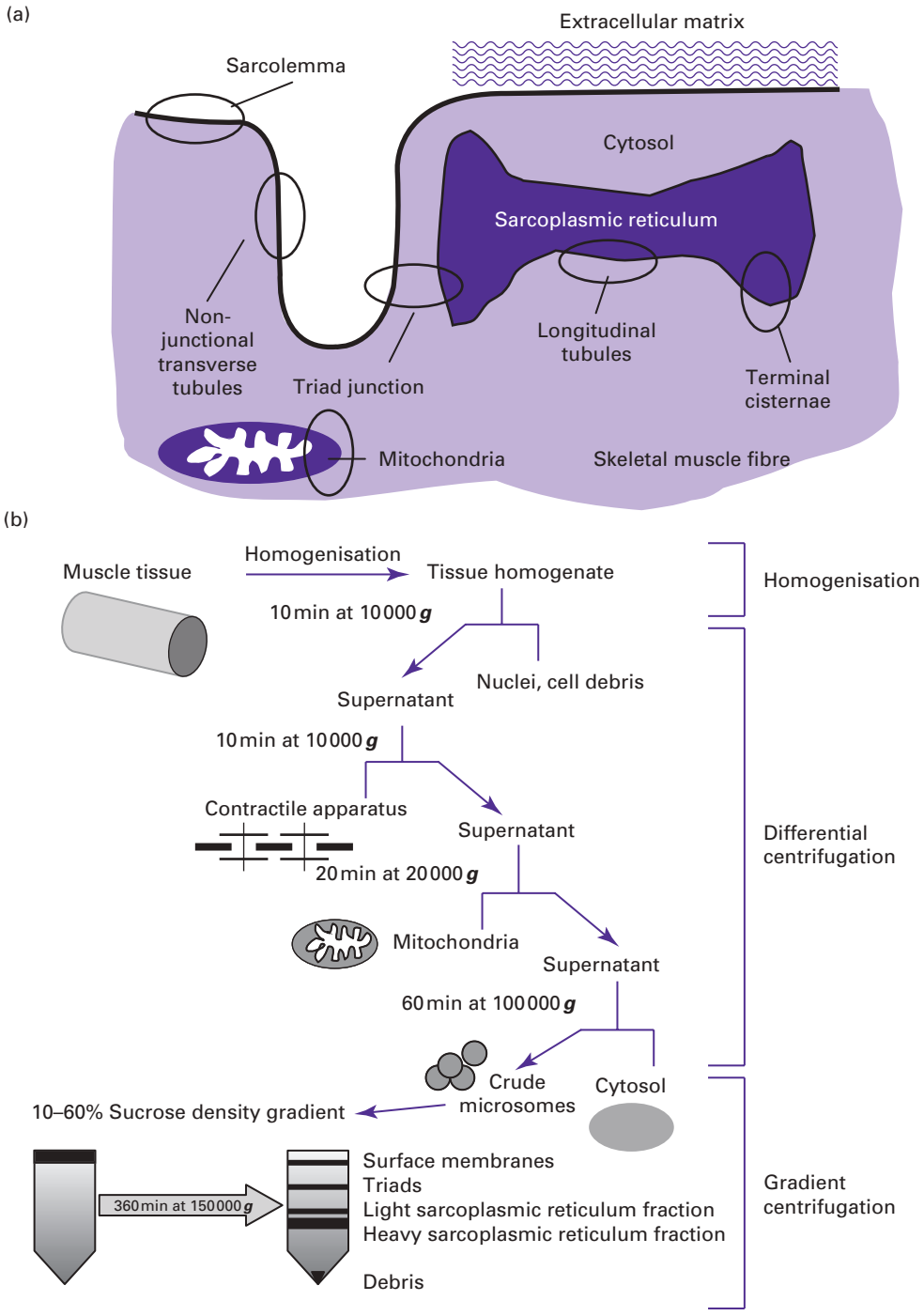

Fig. 3.5. Scheme of the fractionation of skeletal muscle homogenate into various subcellular fractions. Shown are (a) a diagrammatic presentation of the subcellular membrane system from skeletal muscle fibres and (b) a flow chart of the fractionation protocol of these membranes from tissue homogenates using differential centrifugation and density gradient methodology.

For the optimum homogenisation of tissue specimens, mincing of tissue has to be performed in the presence of a biological buffer system that provides the right pH value, salt concentration, stabilising cofactors and chelating agents. The optimum ratio between the wet weight of tissue and buffer volume, as well as the temperature (usually 4 °C) and presence of a protease inhibitor cocktail is also essential to minimise proteolytic degradation. Prior to the 1970s, neither protease inhibitors nor chelating agents were widely used in homogenisation buffers. This resulted in the degradation of many high molecular mass proteins. Since protective measures against endogenous enzymes have been routinely introduced into subcellular fractionation protocols, extremely large proteins have been isolated in their intact form, such as 427 kDa dystrophin, the 565 kDa ryanodine receptor, 800 kDa nebulin and the longest known polypeptide of 2200 kDa mass named titin. Commercially available protease inhibitor cocktails usually exhibit a broad specificity for the inhibition of cysteine proteases, serine proteases, aspartate proteases, metalloproteases and aminopeptidases. They are used in the micromolar concentration range and are best added to buffer systems just prior to the tissue homogenisation process. Depending on the half-life of specific protease inhibitors, the length of a subcellular fractionation protocol and the amount of endogenous enzymes present in individual fractions, tissue suspensions might have to be replenished with a fresh aliquot of a protease inhibitor cocktail. Protease inhibitor kits for the creation of individualised cocktails are also available and consist of substances such as trypsin inhibitor, E-64, aminoethylbenzenesulphonylfluoride, antipain, aprotinin, benzamidine, bestatin, chymostatin, ε-aminocaproic acid, *N*-ethylmaleimide, leupeptin, phosphoramidon and pepstatin. The most commonly used chelators of divalent cations for the inhibition of degrading enzymes such as of metalloproteases are EDTA and EGTA (ethylene glycol bis(aminoethylether)tetra-acetic acid).

3.4.4 Subcellular fractionation

A typical flow chart outlining a subcellular fractionation protocol is shown in Fig. 3.5b. Depending on the amount of starting material, which would usually range between 1 and 500 g in the case of skeletal muscle preparations, a particular type of rotor and size of centrifuge tube is chosen for individual stages of the isolation procedure. The repeated centrifugation at progressively higher speeds and longer centrifugation periods will fractionate the muscle homogenate into distinct fractions. Typical values for centrifugation steps are 10 min at 1000 ***g*** to pellet nuclei and cellular debris, 10 min at 10 000 ***g*** to pellet the contractile apparatus, 20 min at 20 000 ***g*** to pellet a fraction enriched in mitochondria, and 1 h at 100 000 ***g*** to separate the microsomal and cytosolic fractions. Mild salt washes can be carried out to remove myosin contamination of membrane preparations. Sucrose gradient centrifugation is then used to further separate microsomal subfractions derived from different muscle membranes. Using a vertical rotor or swinging-bucket rotor system at a sufficiently high ***g*** force, the crude surface membrane fraction, triad junctions, longitudinal tubules and terminal cisternae

membrane vesicles can be separated. To collect bands of fractions, the careful removal of fractions from the top can be achieved manually with a pipette. Alternatively, in the case of relatively unstable gradients or tight banding patterns, membrane vesicles can be harvested from the bottom by an automated fraction collector. In this case, the centrifuge tube is pierced and fractions collected by gravity or slowly forced out of the tube by a replacing liquid of higher density. Another method for collecting fractions from unstable gradients is the slicing of the centrifuge tube after freezing. Both latter methods destroy the centrifuge tubes and are used routinely in research laboratories.

Cross-contamination of vesicular membrane populations is an inevitable problem during subcellular fractionation procedures. The technical reason for this is the lack of adequate control in the formation of various types of membrane during tissue homogenisation. Membrane domains originally derived from a similar subcellular location might form a variety of structures including inside-out vesicles, right-side-out vesicles, sealed structures, leaky vesicles and/or membrane sheets. In addition, smaller vesicles might become entrapped in larger vesicles. Different membrane systems might aggregate non-specifically or bind to or entrap abundant solubilised proteins. Hence, if highly purified membrane preparations are needed for sophisticated cell biological or biochemical studies, affinity separation methodology has to be employed. The flow chart and immunoblotting diagram in Fig. 3.6 illustrates both the preparative and analytical principles underlying such a biochemical approach. Modern preparative affinity techniques using centrifugation steps can be performed with various biological or chemical ligands. In the case of immunoaffinity purification, antibodies are used to specifically bind to their respective antigen.

3.4.5 Affinity purification of membrane vesicles

In Fig. 3.6a is shown a widely employed lectin agglutination method. Lectins are plant proteins that bind tightly to specific carbohydrate structures. The rationale behind using purified wheat germ agglutinin (WGA) lectin for the affinity purification of sarcolemma vesicles is that the muscle plasmalemma forms mostly right-side-out vesicles following homogenisation. By contrast, vesicles derived from the transverse tubules are mostly inside-out and thus do not expose their carbohydrates. Glycoproteins from the abundant sarcoplasmic reticulum do not exhibit carbohydrate moities that are recognised by this particular lectin species. Therefore only sarcolemma vesicles are agglutinated by the wheat germ lectin and the aggregate can be separated from the transverse tubular fraction by centrifugation for 2 min at 15 000 ***g***. The electron microscopical characterisation of agglutinated surface membranes reveals large smooth sarcolemma vesicles that have electron-dense entrapments. To remove these vesicular contaminants, originally derived from the sarcoplasmic reticulum, immobilised surface vesicles are treated with low concentrations of the non-ionic detergent Triton X-100. This procedure does not solubilise integral membrane proteins, but introduces openings in the sarcolemma vesicles for the release of the much smaller sarcoplasmic reticulum

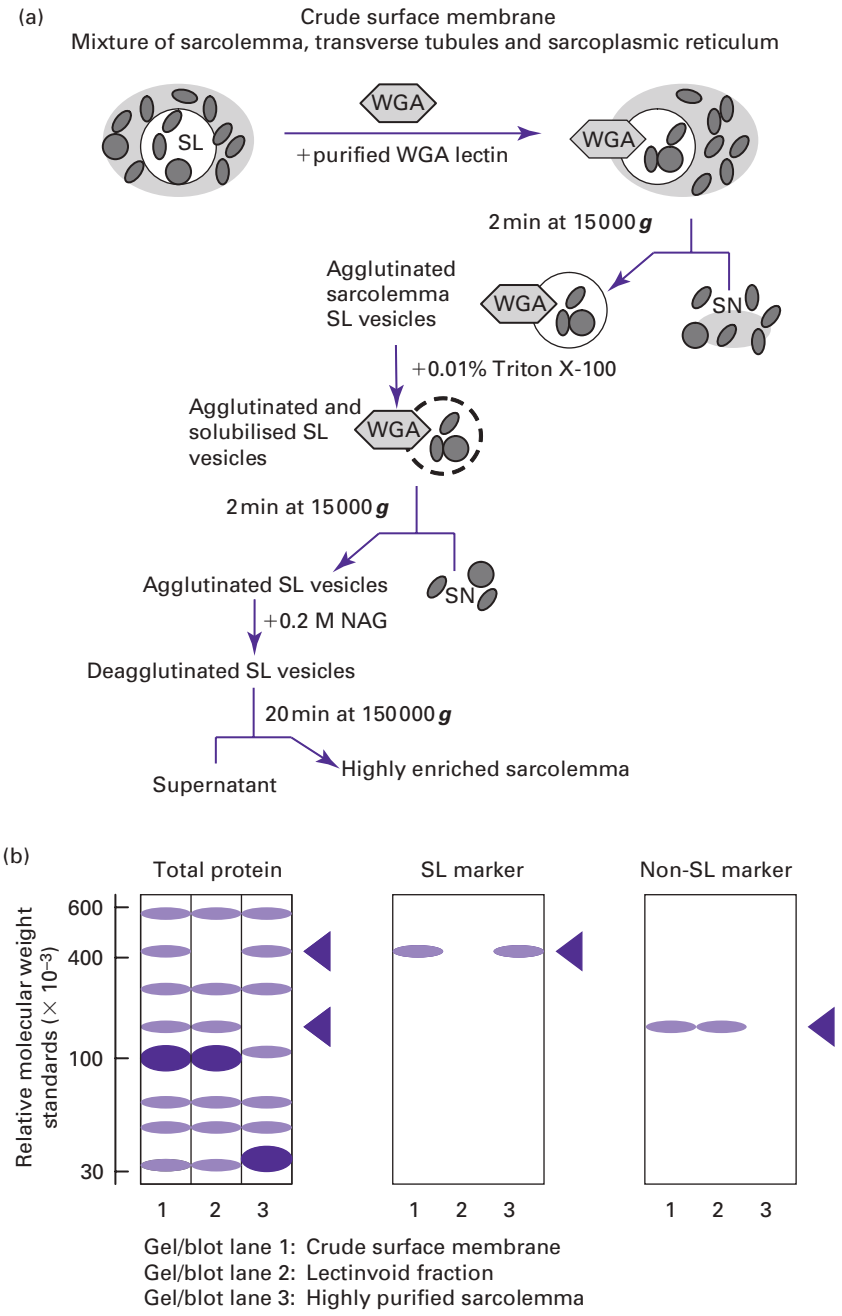

Fig. 3.6. Affinity separation method using centrifugation of lectin agglutinated surface membrane vesicles from skeletal muscle. Shown are (a) a flow chart of the various preparative steps in the isolation of highly purified sarcolemma (SL) vesicles and (b) a diagram of the immunoblot analysis of this subcellular fractionation procedure. The SL and non-SL markers are surface-associated dystrophin of 427 kDa and the transverse-tubular α_{1S}-subunit of the dihydropyridine receptor of 170 kDa, respectively. NAG, *N*-acetylglycosamine; WGA, wheat germ agglutinin; SN, supernatant.

vesicles. Low ***g*** force centrifugation is then used to separate the agglutinated sarcolemma vesicles and the contaminants. To remove the lectin from the purified vesicles, the fraction is incubated with the competitive sugar *N*-acetylglucosamine, which eliminates the bonds between the surface glycoproteins and the lectin. A final centrifugation step for 20 min at 150 000 ***g*** results in a pellet of highly purified sarcolemma vesicles. A quick and convenient analytical method to confirm whether this subcellular fractionation procedure has resulted in the isolation of the muscle plasmalemma is immunoblotting with a mini electrophoresis unit. Fig. 3.6b shows a diagram of the protein and antigen banding pattern of crude surface membranes, the lectin void fraction and the highly purified sarcolemma fraction. Using antibodies to markers of the transverse tubules and the sarcolemma, such as the α_{1S}-subunit of the dihydropyridine receptor of 170 kDa and dystrophin of 427 kDa, respectively, the separation of both membrane species can be monitored. This analytical method is especially useful for the characterisation of membrane vesicles, when no simple and fast assay systems for testing marker enzyme activities are available.

In the case of the separation of mitochondrial membranes, the distribution of enzyme activities rather than immunoblotting is used routinely for determining the distribution of the inner membrane, contact zones and the outer membrane in density gradients. Binding assays or enzyme testing represents the more traditional way to characterise subcellular fractions following centrifugation. Fig. 3.7a outlines diagrammatically the microcompartments of liver mitochondria and the associated marker enzymes. While monoamino oxidase (MAO) is enriched in the outer membrane, the enzyme succinate dehydrogenase (SDH) is associated with the inner membrane system and a representative marker of contact sites between both membranes is glutathione transferase (GT). Membrane vesicles from intact mitochondria can be generated by consecutive swelling, shrinking and sonication of the suspended organelles. The vesicular mixture is then separated by sucrose density centrifugation into the three main types of mitochondrial membrane (Fig. 3.7b). The distribution of marker enzyme activities in the various fractions demonstrates that the outer membrane has a lower density compared to the inner membrane. The GT-containing contact zones are positioned in a band between the inner and outer mitochondrial membrane and contain enzyme activities characteristic for both systems (Fig. 3.7c). Routinely used enzymes as subcellular markers would be the Na^+/K^+-ATPase for the plasmalemma, glucose 6-phosphatase for the endoplasmic reticulum, galactosyl transferase for the Golgi apparatus, SDH for mitochondria, acid phosphatase for lysosomes, catalase for peroxisomes and lactate dehydrogenase for the cytosol.

3.5 ANALYTICAL CENTRIFUGATION

3.5.1 Applications of analytical ultracentrifugation

As biological macromolecules exhibit random thermal motion, the earth's gravitational field does not significantly affect their relatively uniform distribution in

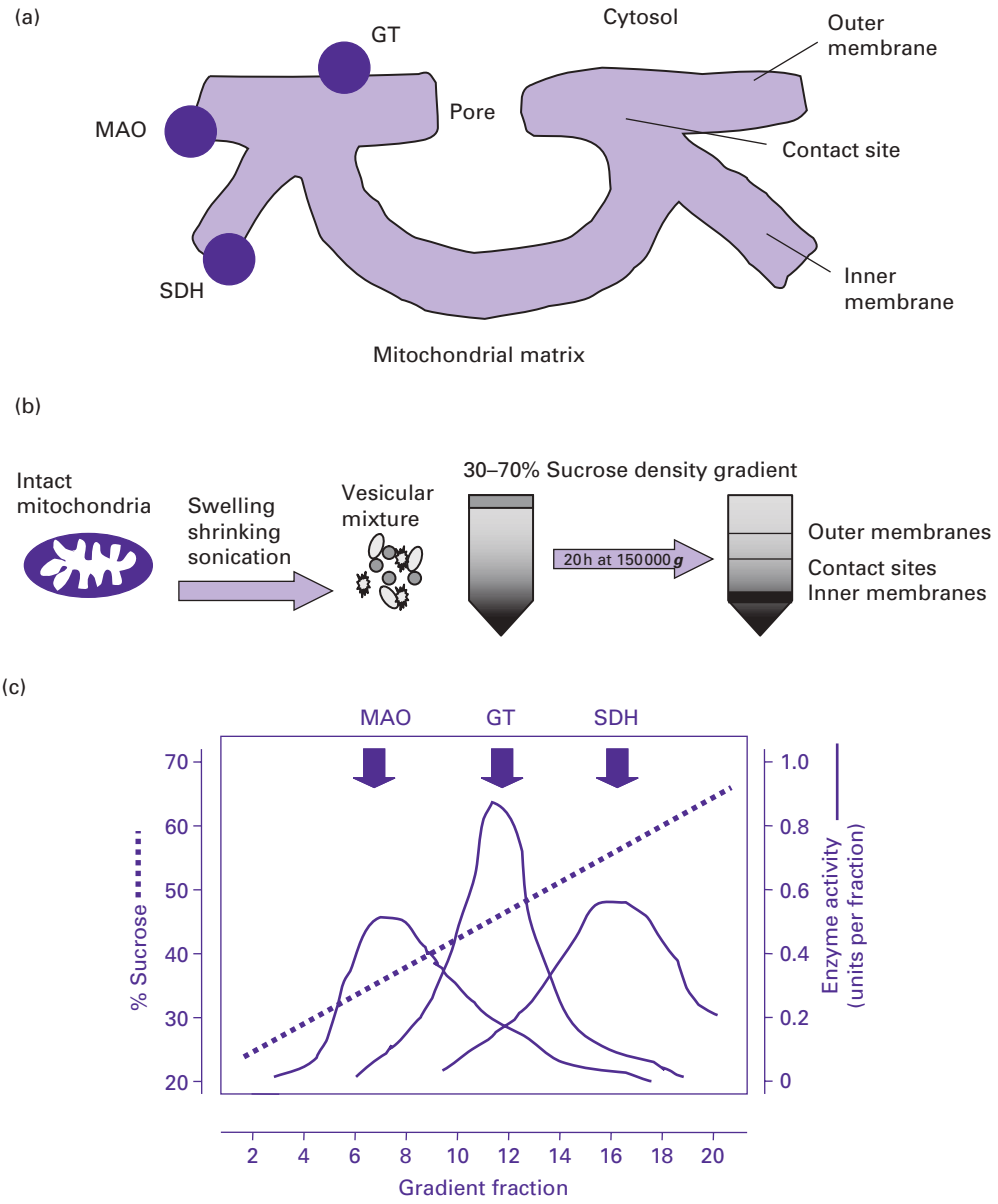

Fig. 3.7. Scheme of the fractionation of membranes derived from liver mitochondria. Shown are: (a) the distribution of marker enzymes in the microcompartments of liver mitochondria (MAO, monoamino oxidase; SDH, succinate dehydrogenase; GT, glutathione transferase); (b) the separation method to isolate fractions highly enriched in the inner cristae membrane, contact zones and the outer mitochondrial membrane and (c) the distribution of mitochondrial membranes after density gradient centrifugation.

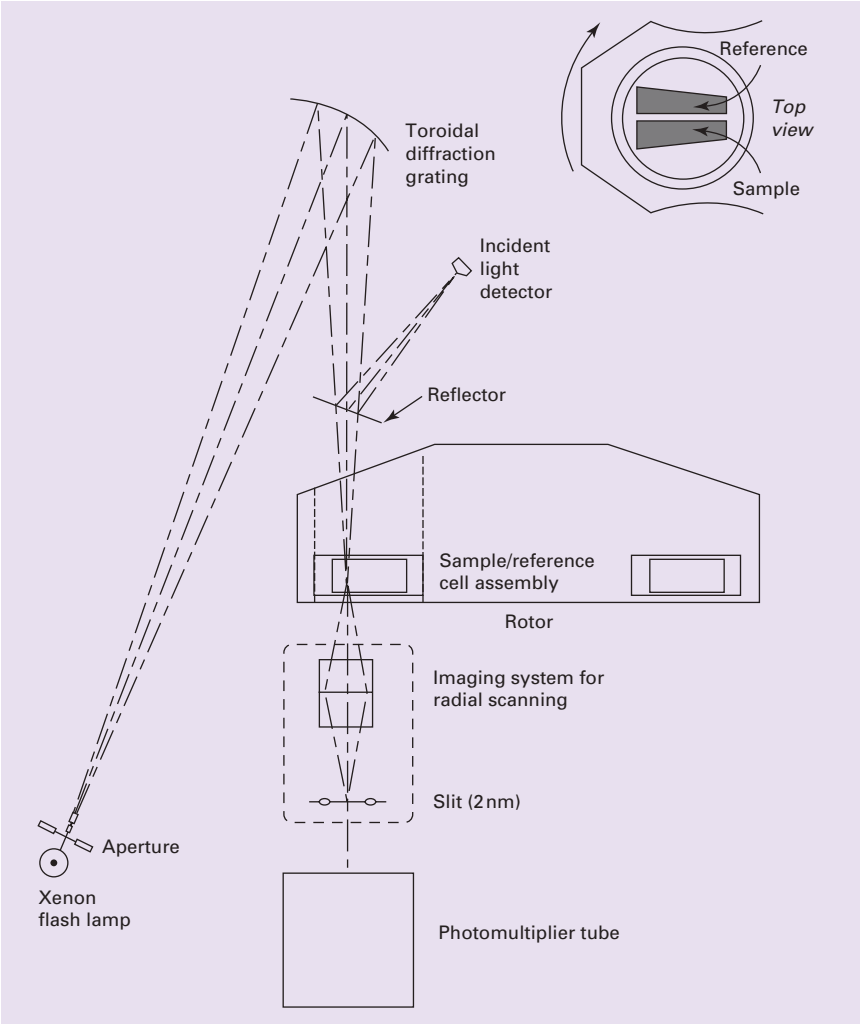

Fig. 3.8. Schematic diagram of the optical system of an analytical ultracentrifuge. The high intensity xenon flash lamp of the Beckman Optima XL-A analytical ultracentrifuge shown here allows the use of wavelengths between 190 nm and 800 nm. The high sensitivity of the absorbance optics allows the measurement of highly dilute protein samples below 230 nm. (Courtesy of Beckman–Coulter).

an aqueous environment. Isolated biomolecules in solution exhibit distinguishable sedimentation only when they undergo immense accelerations, for example in an ultracentrifugal field. A typical analytical ultracentrifuge can generate a centrifugal field of 250 000 ***g*** in its analytical cell. Within these extremely high gravitational fields, the ultracentrifuge cell has to allow light passage through the biological particles for proper measurement of the concentration distribution. The schematic diagram shown in Fig. 3.8 outlines the optical system of a modern analytical ultracentrifuge. The availability of high intensity xenon flash lamps and the advance in instrumental sensitivity and wavelength range has made the

accurate measurement of highly dilute protein samples possible below 230 nm. Analytical ultracentrifuges such as the Beckman Optima XL-A allow the use of wavelengths between 190 and 800 nm. Sedimentation of isolated proteins or nucleic acids can be useful in the determination of the relative molecular mass, purity and shape of these biomolecules. Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a sedimentation velocity approach or sedimentation equilibrium methodology. The hydrodynamic properties of macromolecules are described by their sedimentation coefficients and can be determined from the rate that a concentration boundary of the particular biomolecules moves in the gravitational field. Such studies on the solution behaviour of macromolecules can give detailed insight into the properties of large aggregates and thereby confirm results from biochemical analyses on complex formation. The sedimentation coefficient can be used to characterise changes in the size and shape of macromolecules with changing experimental conditions. This allows for the detailed biophysical analysis of the effect of variations in the pH value, temperature or cofactors on molecular shape.

Analytical ultracentrifugation is most often employed in:

- the determination of the purity of macromolecules,
- the determination of the relative molecular mass of solutes in their native state,
- the examination of changes in the relative molecular mass of supramolecular complexes,
- the detection of conformational changes,
- ligand-binding studies (Section 16.4.1).

The sedimentation velocity method can be employed to estimate sample purity. Sedimentation patterns can be obtained using the Schlieren optical system. This method measures the refractive index gradient at each point in the ultracentrifugation cell at varying time intervals. During the sedimentation velocity analysis, a homogeneous preparation forms a single, sharp, symmetrical, sedimenting boundary. This demonstrates that the biological macromolecules analysed exhibit the same relative molecular mass, shape and size. However, one cannot assume that the analysed particles exhibit an identical electrical charge or biological activity. Only additional biochemical studies using electrophoretic techniques and enzyme/bio-assays can differentiate between these minor subtypes of macromolecules with similar molecular mass. The great advantage of the sedimentation velocity method is that smaller or larger contaminants can be recognised clearly as shoulders on the main peak, asymmetry of the main peak and/or additional peaks. For a list of references please consult the review articles listed in Section 3.6. In addition, manufacturers of analytical ultracentrifuges have made available a large range of excellent brochures on the theoretical background of this method and its specific applications. These introductory texts are usually written by research biochemists and are well worth reading to become familiar with this field.

3.5.2 Relative molecular mass determination

For the accurate determination of the relative molecular mass of solutes in their native state, analytical ultracentrifugation presents an unrivalled technique. The method requires only small sample sizes (20–120 mm^3) and low particle concentrations (0.01–1 g dm^{-3}) and biological molecules with a wide range of relative molecular masses can be characterised. In conjunction with electrophoretic, chromatographic, crystallographic and sequencing data, the biochemical properties of a biological particle of interest can be determined in great detail. As long as the absorbance of the biomolecules to be investigated (such as proteins, carbohydrates or nucleic acids) is different from that of the surrounding solvent, analytical ultracentrifugation can be applied. At the start of an experiment using the boundary sedimentation method, the biological particles are uniformly distributed throughout the solution in the analytical cell. The application of a centrifugal field then causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the centre of rotation. The solvent that has been cleared of particles and the solvent still containing the sedimenting material form a sharp boundary. The movement of the boundary with time is a measure of the rate of sedimentaion of the biomolecules. The sedimentation coefficient depends directly on the mass of the biological particle. The concentration distribution is dependent on the buoyant relative molecular mass. The movement of biomolecules in a centrifugal field can be determined and a plot of the natural logarithm of the solute concentration versus the squared radial distance from the centre of rotation (ln c versus r^2) yields a straight line with a slope proportional to the monomer molecular mass. Alternatively, the relative molecular mass of a biological macromolecule can be determined by the band sedimentation technique. In this case, the sample is layered on top of a denser solvent. During centrifugation, the solvent forms its own density gradient and the migration of the particle band is followed in the analytical cell. Relative molecular mass determination by analytical ultracentrifugation is applicable to values from a few hundred to several millions. It is therefore used for the analysis of small carbohydrates, proteins, nucleic acid macromolecules, viruses and subcellular particles such as mitochondria.

3.5.3 Sedimentation coeffcient

Biochemical studies over the last few decades have demonstrated clearly that biological macromolecules do not perform their biochemical and physiological functions in isolation. Many proteins were shown to be multifunctional and their activity is regulated by complex interactions within homogeneous and heterogeneous complexes. Cooperative kinetics and the influence of microdomains have been recognised to play a major role in the regulation of biochemical processes. Since conformational changes in biological macromolecules may cause differences in their sedimentation rates, analytical ultracentrifugation is an ideal experimental tool for the determination of such structural modifications. For example, a macromolecule that changes its conformation into a more compact structure decreases its frictional resistance in the solvent. In contrast, the frictional resistance increases

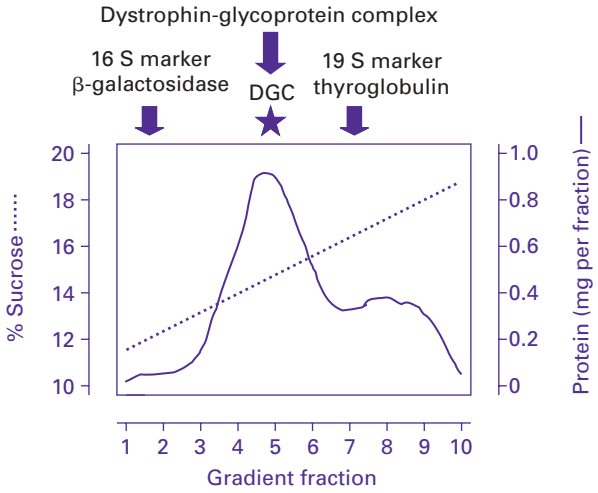

Fig. 3.9. Sedimentation analysis of a supramolecular protein complex. Shown is the sedimentation of the dystrophin–glycoprotein complex (DGC). Its size was estimated to be approximately 18 S by comparing its migration to that of the standards β-galactosidase (16 S) and thyroglobulin (19 S). Since the sedimentation coefficients of biological macromolecules are relatively small, they are expressed as Svedberg units (S), where 1 Svedberg unit equals 10^{-13} s.

when a molecular assembly becomes more disorganised. The binding of ligands (such as inhibitors, activators or substrates) or a change in temperature or buffering conditions may induce conformational changes in subunits of biomolecules that in turn can result in major changes in the supramolecular structure of complexes. Such modifications can be determined by distinct differences in the sedimentation velocity of the molecular species. Sedimentation equilibrium experiments can be used to determine the relative size of individual subunits participating in complex formation, the stoichiometry and size of a complex assembly under different physiological conditions and the strength of interactions between subunits.

When a new protein species is identified that appears to exist under native conditions in a large complex, several biochemical techniques are available to evaluate the oligomeric status of such a macromolecule. Gel filtration analysis, blot overlay assays, affinity chromatography, differential immunoprecipitation and chemical cross-linking are typical examples of such techniques. With respect to centrifugation, sedimentation analysis using a density gradient is an ideal method to support such biochemical data. For the initial determination of the size of a complex, the sedimentation of known marker proteins is compared with the novel protein complex. Biological particles with a different molecular mass, shape or size migrate with different velocities in a centrifugal field (Section 3.1). As can be seen in equation 3.7, the sedimentation coefficient has dimensions of seconds. The value of Svedberg units ($S = 10^{-13}$ s) lies, for many macromolecules of biochemical interest, typically between 1 and 20, and for larger biological particles such as ribosomes, microsomes and mitochondria between 80 and several thousand. The prototype of a soluble protein, serum albumin of apparent relative molecular mass 66 000, has a sedimentation coefficient of 4.5 S. Fig. 3.9 illustrates

the sedimentation analysis of the newly discovered dystrophin–glycoprotein complex (DGC). The size of this complex was estimated to be approximately 18 S by comparing its migration with that of the standards β-galactosidase (16 S) and thyroglobulin (19 S). When the membrane cytoskeletal element dystrophin was first identified, it was shown to bind to a lectin column, although it does not exhibit any carbohydrate chains. This suggested that dystrophin might exist in a complex with surface glycoproteins. Sedimentation analysis confirmed the existence of such a DGC and centrifugation, following various biochemical modifications of the protein assembly, led to a detailed understanding of its composition. Alkaline extraction, acid treatment or incubation with different types of detergent causes the differential disintegration of the DGC. It is now known that dystrophin is tightly associated with at least 10 different surface proteins that are involved in membrane stabilisation, receptor anchoring and signal transduction processes. The successful characterisation of the DGC by sedimentation analysis is an excellent example of how centrifugation methodology can be exploited quickly to gain biochemical knowledge of a newly discovered protein.

3.6 SUGGESTIONS FOR FURTHER READING

FINDLAY, J. B. C. and EVANS, W. H. (1987). *Biological Membranes*. Published in the Practical Approaches in Biochemistry Series, IRL Press, Oxford/Washington, DC. (Contains two extensive chapters on differential centrifugation procedures used in the subcellular fractionation of animal and plant cells.)

FISHER, D., FRANCIS, G. E. AND RICKWOOD, D. (eds.) (1998). *Cell Separation*. Published in the Practical Approaches in Biochemistry Series, IRL Press, Oxford/Washington, DC. (Outlines fractionation of cells by sedimentation methodology.)

GRAHAM, J. M. AND RICKWOOD, D. (eds.) (1997). *Subcellular Fractionation*. Published in the Practical Approaches in Biochemistry Series, IRL Press, Oxford/Washington, DC. (Provides a description of essential subcellular fractionation techniques.)

LAUE, T. M. (2001). Biophysical studies by ultracentrifugation. *Current Opinion in Structural Biology* **11**, 579–583. (Provides an excellent synopsis of the applicability of ultracentrifugation to the characterisation of macromolecular behaviour in complex solution.)

LAUE, T. M. and STAFFORD, W. F. III (1999). Modern applications of analytical ultracentrifugation. *Annual Reviews in Biophysics and Biomolecular Structures* **28**, 75–100. (Provides an overview of available information on analytical ultracentrifugation and how this analytical technique can be used in contemporary applications.)

MURRAY, B. E. and OHLENDIECK, K. (2000). Chemical cross-linking analysis of Ca^{2+}-ATPase from rabbit skeletal muscle. *Biochemical Education* **28**, 41–46. (Description of an undergraduate student experiment dealing with the subcellular fractionation of skeletal muscle homogenate.)

RALSTON, G. (1993). *Introduction to Analytical Ultracentrifugation*. Beckman Instruments, Fullerton, CA. (Describes the different types of experiment that can be performed with an analytical ultracentrifuge.)

Chapter 4

Microscopy

4.1 INTRODUCTION

Biochemical analysis is frequently accompanied by microscopic examination of tissue, cell or organelle preparations. Such examinations are used in many different applications; for example, to evaluate the integrity of samples during an experiment, to map the fine details of the spatial distribution of macromolecules within cells, or to directly measure biochemical events within living tissues.

There are two fundamentally different types of microscope: the light microscope and the electron microscope (Fig. 4.1). Light microscopes use a series of glass lenses to focus light in order to form an image whereas electron microscopes use electromagnetic lenses to focus a beam of electrons. Light microscopes are able to magnify to a maximum of approximately 1500 times whereas electron microscopes are capable of magnifying to a maximum of approximately 200 000 times.

Magnification is not, however, the best measure of a microscope. Rather, resolution, the ability to distinguish between two closely spaced points in a specimen, is a much more reliable estimate of a microscope's utility. Light microscopes have a resolution limit of about 0.5 micrometres (μm) for routine analysis. In contrast, electron microscopes have a resolution of up to 1 nanometre (nm). Both living and dead specimens are viewed with a light microscope, and often in real colour, whereas only dead ones are viewed with an electron microscope, and never in real colour. Recent advancements have improved upon the 0.2 μm resolution limit of the light microscope for some special applications (Section 4.8).

Applications of the microscope in biochemistry may be relatively simple and routine; for example, a quick check of the status of a cell preparation or of cells growing in tissue culture. Here, a simple bench-top light microscope is perfectly adequate. On the other hand, the application may be more involved, for example measuring the concentration of calcium in a living embryo over a millisecond time scale. Here a more advanced light microscope (often called an imaging system) is required. Alternatively, the application may require the location of macromolecules in membrane-bound compartments inside the cell. Here the electron microscope will be chosen for imaging, since it is the only instrument that is capable of the nanometre resolution required for such images.

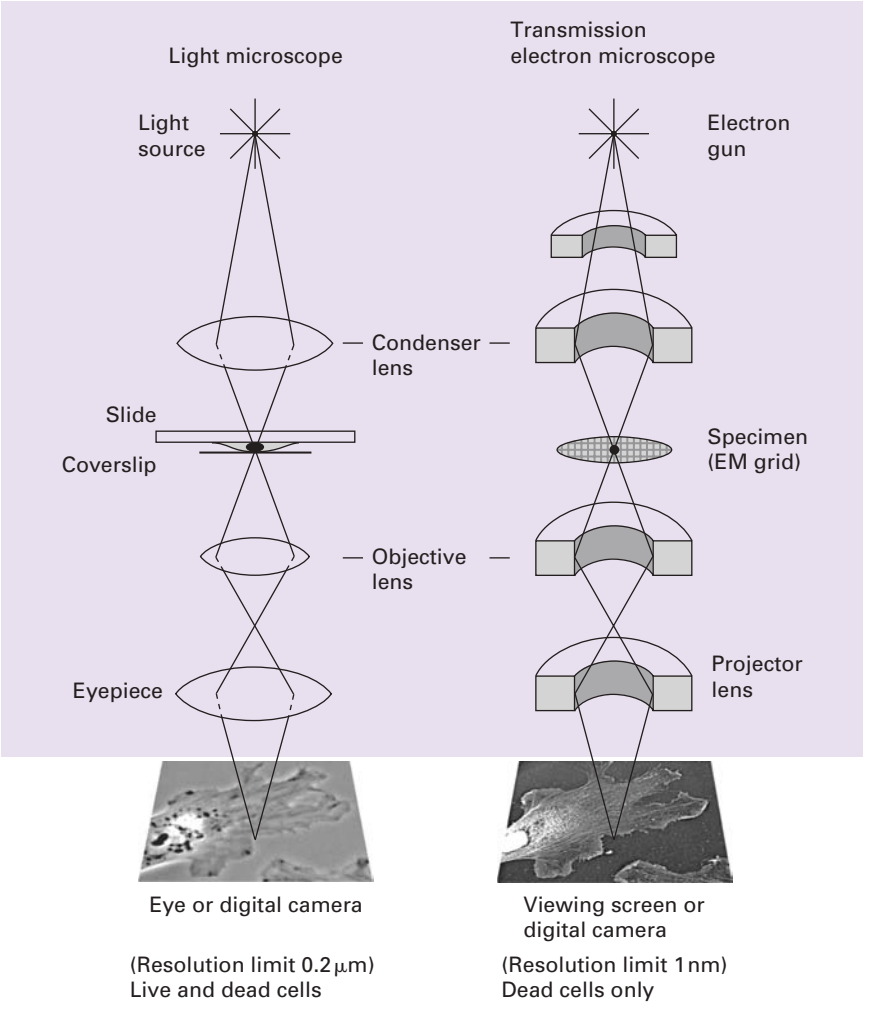

Fig. 4.1. Light and electron microscopy. Schematic that compares the path of light through a compound light microscope (LM) with the path of electrons through a transmission electron microscope (TEM). Light from a lamp (LM) or a beam of electrons from an electron gun (TEM) is focused at the specimen by a glass condenser lens (LM) or electromagnetic lenses (TEM). For the LM the specimen is mounted on a glass slide with a coverslip placed on top, and for the TEM the specimen is placed on a copper electron microscope grid. The image is magnified with an objective lens, glass in the LM and electromagnetic lens in the TEM, and projected onto a detector with the eyepiece lens in the LM or the projector lens in the TEM. The detector can be the eye or a digital camera in the LM or phosphorescent viewing screen or a digital camera in the TEM. (Light and EM images courtesy of Tatyana Svitkina.)

Some microscopes are more suited to specific applications than others. Images may be required from specimens of vastly different sizes and magnifications (Fig. 4.2); for example, for imaging whole animals (metres), through tissues and embryos (micrometres), and down to cells, proteins and DNA (nanometres). The study of living cells may require time resolution from days, for example when

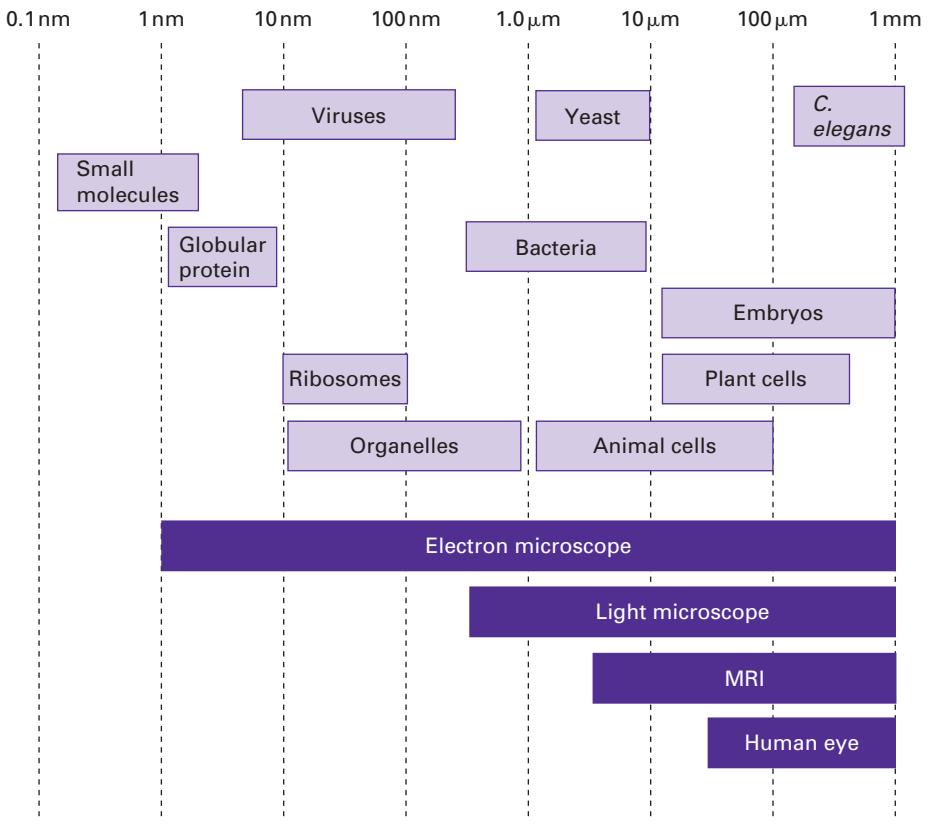

Fig. 4.2. The relative sizes of a selection of biological specimens and some of the devices used to image them. The range of resolution for each instrument is included in the dark purple bars at the base of the figure.

imaging neuronal development or disease processes, to milliseconds, for example when imaging cell signalling events.

The field of microscopy has undergone a renaissance over the past 20 years, with the addition of various technological advancements to the instruments. Most images produced by microscopes are now recorded electronically using digital imaging techniques – digital cameras, digital image acquisition software, digital printing and digital display methods. These advancements have allowed many more applications of the microscope in biochemistry, ranging from routine observations of cells and cell extracts to specialised techniques for directly measuring biochemical events in cells.

4.2 THE LIGHT MICROSCOPE

4.2.1 Basic components of the light microscope

The simplest form of light microscope consists of a single glass lens mounted in a metal frame – a magnifying glass (Fig. 4.3). Here the specimen requires very little

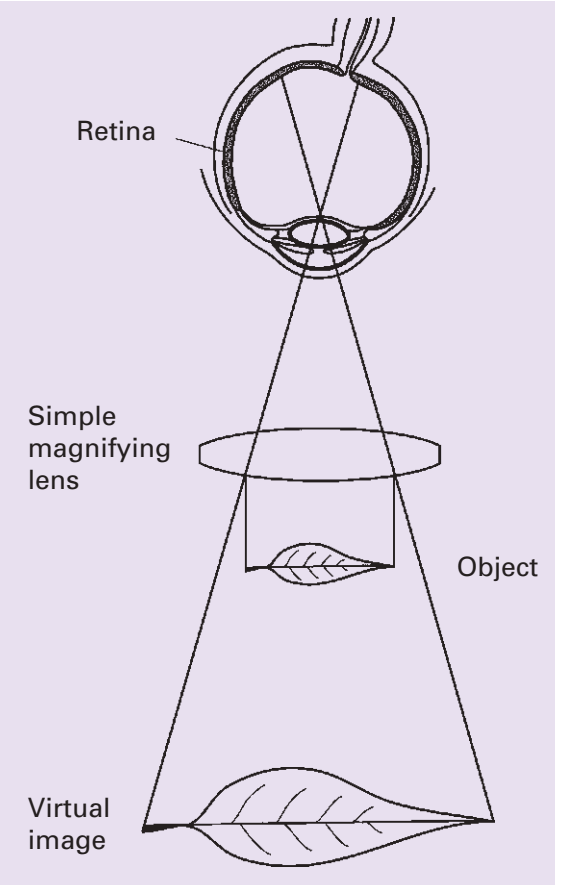

Fig. 4.3. Magnification in a simple microscope – a glass magnifying lens. The lens of the eye focuses light onto the retina – a collection of light-sensitive cells located at the back of the eye. The specimen is found by placing the convex lens between it and the eye. Magnification of the image of the specimen is achieved by the lens spreading the visual angle on the retina. Here the image appears as if it is on the same side of the lens as the specimen – this is called a virtual image.

preparation, and is usually held close to the eye in the hand. Focusing of the region of interest is achieved by moving the lens and the specimen relative to one another. The source of light is usually the sun or ambient indoor light. The detector is the human eye. The recording device is a hand drawing or an anecdote.

Compound microscopes

All modern light microscopes are made up of more than one glass lens in combination. The major components are the condenser lens, the objective lens and the eyepiece lens, and, such instruments are therefore called compound microscopes (Fig. 4.1). Each of these components is in turn made up of combinations of lenses, which are necessary to produce magnified images with reduced artefacts and aberrations. For example, chromatic aberration occurs when different

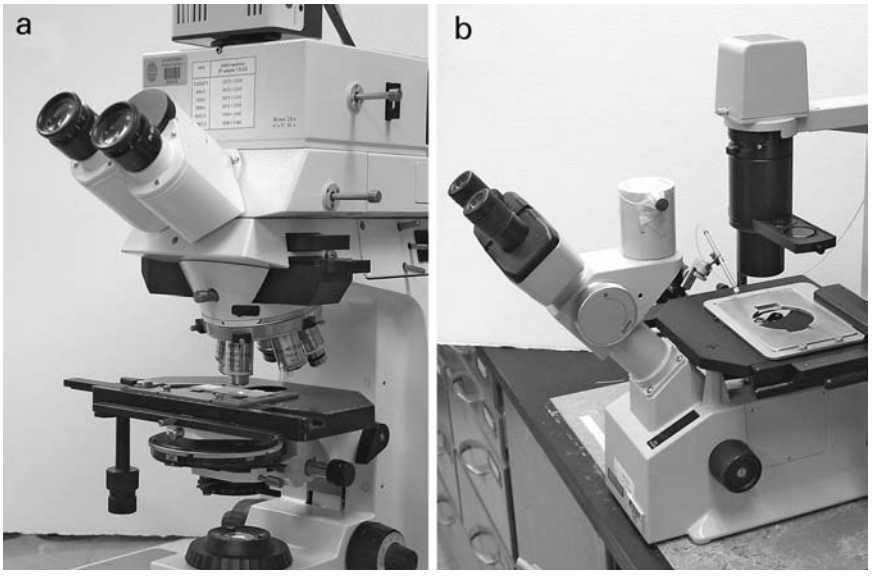

Fig. 4.4. Two basic types of compound light microscope. (a) An upright light microscope and (b) an inverted light microscope. Note how there is more room available on the stage of the inverted microscope (b). This instrument is set up for microinjection with a needle holder to the left of the stage.

wavelengths of light are separated and pass through a lens at different angles. This results in rainbow colours around the edges of objects in the image. This problem was encountered in the early microscopes of Antonie Van Leeuwenhoek and Robert Hooke, for example. All modern lenses are corrected to some degree in order to avoid this problem.

The main components of the compound light microscope include a light source that is focused at the specimen by a condenser lens. Light that either passes through the specimen (transmitted light) or is reflected back from the specimen (reflected light) is focused by the objective lens into the eyepiece lens. The image is either viewed directly by eye in the eyepiece or is most often projected onto a detector, for example photographic film or, more likely, a digital camera. The images are displayed on the screen of a computer imaging system, stored in a digital format and reproduced using digital methods.

The part of the microscope that holds all of the components firmly in position is called the stand. There are two basic types of compound light microscope stand – upright and inverted microscopes (Fig. 4.4). The light source is below the condenser lens in the upright microscope and the objectives are above the specimen stage. This is the most commonly used format for viewing specimens. The inverted microscope is engineered so that the light source and the condenser lens are above the specimen stage, and the objective lenses are beneath it. This allows additional room for manipulating the specimen directly on the stage, for example for the microinjection of macromolecules into tissue culture cells, for *in vitro* fertilisation of eggs, or for viewing developing embryos over time.

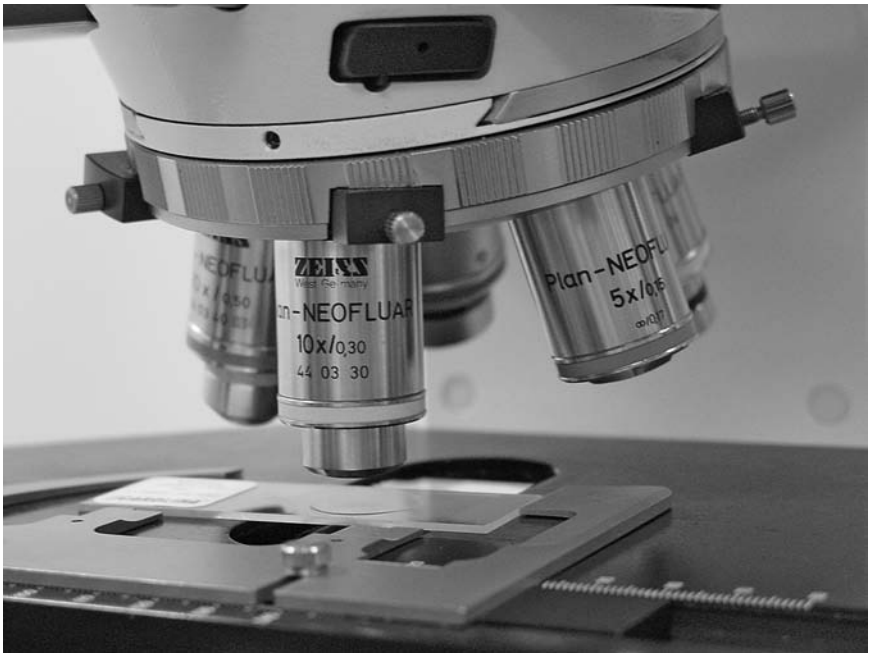

Fig. 4.5. The objective lens. A selection of objective lenses mounted on an upright research grade compound light microscope. From the inscription on the two lenses in focus they are relatively low magnification 10× and 5× of numerical aperture 0.3 and 0.16, respectively. Both lenses are Plan Neofluar, which means they are relatively well corrected. The 10× lens is directly above a specimen mounted on a slide and coverslip, and held in place on the specimen stage.

The correct illumination of the specimen is critical for achieving high quality images and photomicrographs. This is achieved using a light source. Typically light sources are mercury lamps, xenon lamps or lasers. Light from the light source passes into the condenser lens, which is mounted beneath the microscope stage in an upright microscope (and above the stage in an inverted microscope) in a bracket that can be raised and lowered for focusing (Fig. 4.4). The condenser focuses light from the light source and illuminates the specimen with parallel beams of light. A correctly positioned condenser lens produces illumination that is uniformly bright and free from glare across the viewing area of the specimen (Köhler illumination). Condenser misalignment and an improperly adjusted condenser aperture diaphragm are major sources of poor images in the light microscope.

The specimen stage is a mechanical device that is finely engineered to hold the specimen firmly in place (Fig. 4.5). Any movement or vibration will be detrimental to the final image. The stage enables the specimen to be moved and positioned in fine and smooth increments, both horizontally and transversely, in the *X* and the *Y* directions, for locating a region of interest. The stage is moved vertically in the *Z* direction for focusing the specimen or for inverted microscopes, the objectives themselves are moved and the stage remains fixed. There are usually coarse

and fine focusing controls for low magnification and high magnification viewing, respectively. The fine focus control can be moved in increments of 1 μm or better in the best research microscopes. The specimen stage can either be moved by hand or be controlled more accurately by a computer via stepper motors attached to the fine focus control of the microscope.

The objective lens is responsible for producing the magnified image, and can be the most expensive component of the light microscope (Fig. 4.5). Objectives are available in many different varieties, and there is a wealth of information inscribed on each one. This may include the manufacturer, magnification (4×, 10×, 20×, 40×, 60×, 100×), immersion requirements (air, oil or water), coverslip thickness (usually 0.17 mm) and often with more-specialised optical properties of the lens (Section 4.2.3). In addition, lens corrections for optical artefacts such as chromatic aberration and flatness of field may also be included in the lens description. For example, words such as fluorite, the least corrected (often shortened to 'fluo'), or plan apochromat, the most highly corrected (often shortened to 'plan' or 'plan apo'), may appear somewhere on the lens.

Lenses can either be dry or immersion lenses, and as a rule of thumb, most objectives below 40× are air (dry) objectives and those of 40× and above are immersion objective (oil, glycerol or water). Should the objective be designed to operate in oil it will be labelled 'OIL' or 'OEL'. Other immersion media include glycerol and water, and the lens will be marked to indicate this. Many lenses are colour coded to a manufacturer's specifications.

The numerical aperture (NA) is always marked on the lens. This is a number usually between 0.04 and 1.4. The NA is a measure of the ability of a lens to collect light from the specimen. Lenses with a low NA collect less light than those with a high NA. Resolution varies inversely with NA, which infers that higher NA objectives yield the best resolution. Generally speaking, the higher power objectives have a higher NA and better resolution than the lower power lenses with lower NAs. For example, 0.2 μm resolution can only be achieved using a 60× or a 100× plan-apochromat oil immersion lens with a NA of 1.4. Should there be a choice between two lenses of the same magnification, then it is usually best to choose the one of higher NA.

The objective lens is also the part of the microscope that can most easily be damaged by mishandling. Many lenses are coated with a protective coating but, even so, one scratch on the front of the lens can result in serious image degradation. Therefore great care should be taken when handling objective lenses. Objective lenses must be cleaned using a protocol recommended by the manufacturer, and only by a qualified person. A dirty objective lens is a major source of poor images.

The resolution of the lens measures the ability to distinguish between two objects in the specimen. The shorter the wavelengths of illuminating light, the higher is the resolving power of the microscope (Fig. 4.6). The limit of resolution for a microscope that uses visible light is about 300 nm with a dry lens (in air) and 200 nm with an oil immersion lens. By using ultraviolet light as a light source the resolution can be improved to 100 nm because of the shorter

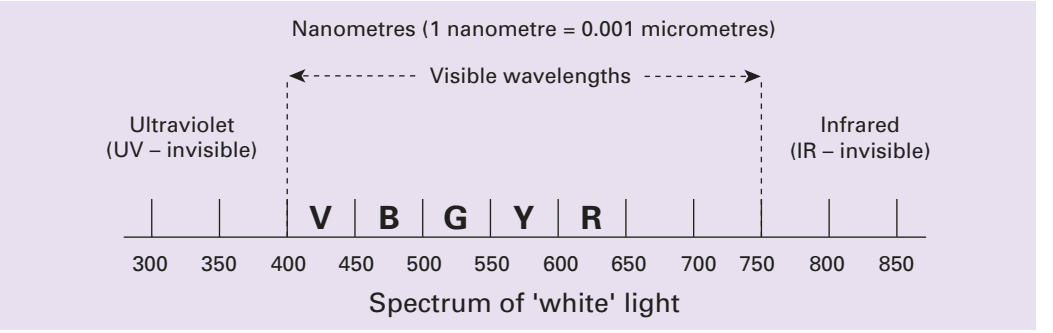

Fig. 4.6. The visible spectrum – the spectrum of white light visible to the human eye. Our eyes are able to detect colour in the visible wavelengths of the spectrum; usually in the region between 400 nm (violet) and 750 nm (red). Most modern electronic detectors are sensitive beyond the visible spectrum of the human eye. UV, ultraviolet; IR, infrared; V, violet; B, blue; G, green; Y, yellow; R, red.

wavelength of the light (200–300 nm). These limits of resolution are often difficult to achieve practically because of aberrations in the lenses and the poor optical properties of many biological specimens. More specialised research microscopes can now attain a resolution of around 20 nm in non-routine applications (Section 4.8).

The eyepiece (sometimes referred to as the ocular) works in combination with the objective lens to further magnify the image, and allows it to be detected by eye or more usually to project the image into a digital camera for recording purposes. Eyepieces usually magnify by 10×, since an eyepiece of higher magnification merely enlarges the image, with no improvement in resolution. There is an upper boundary to the useful magnification of the collection of lenses in a microscope. For each objective lens the magnification can be increased above a point where it is impossible to resolve any more detail in the specimen. Any magnification above this point is often called empty magnification. The best way to improve magnification is to use a higher magnification and higher NA objective lens. Should sufficient resolution not be achieved using the light microscope, then it will be necessary to use the electron microscope (Section 4.6).

In addition to the human eye and photographic film there are two types of electronic detector employed on modern light microscopes. These are area detectors that form an image directly, for example video cameras and charge-coupled devices (CCDs). Alternatively, point detectors can be used to measure intensities in the image; for example photomultiplier tubes (PMTs) and photodiodes. Point detectors are capable of producing images in scanning microscopy (Section 4.3).

4.2.2 The specimen

The specimen (sometimes called the sample) can be the entire organism or a dissected organ (whole mount), an aliquot collected during a biochemical protocol

Table 4.1 Generalised indirect immunofluorescence protocol

1.	Fix in 1% formaldehyde for 30 min
2.	Rinse in cold buffer
3.	Block buffer
4.	Incubate in primary antibody, e.g. mouse anti-tubulin[a]
5.	Wash 4 times in buffer
6.	Incubate in secondary antibody, e.g. fluorescein-labelled rabbit anti-mouse[a]
7.	Wash 4 times in buffer
8.	Incubate in anti-fade reagent, e.g. Vectashield[a]
9.	Mount on slide with a coverslip
10.	View using epifluorescence microscopy

[a] The incubation times vary with the tissue type, from 30 min for tissue culture cells (thin samples) to overnight for whole embryos (thick samples).

for a quick check of the preparation, or a small part of an organism (biopsy) or smear of blood or spermatozoa. In order to collect images from it, the specimen must be in a form that is compatible with the microscope. This is achieved using a published protocol. The end-product of a protocol is a relatively thin and somewhat transparent piece of tissue mounted on a piece of glass (slide) in a mounting medium (water, tissue culture medium or glycerol) with a thin square of glass mounted on top (coverslip).

Coverslips are graded by their thickness. The thinnest ones are labelled no. 1, which corresponds to a thickness of approximately 0.17 mm. The coverslip side of the specimen is always placed closest to the objective lens. It is essential to use a coverslip that is matched to the objective lens in order to achieve optimal resolution. This is critical for high-magnification imaging because if the coverslip is too thick it will be impossible to achieve an image using a high magnification objective lens.

Specimen preparation protocols can be relatively simple or they may involve many steps that take several days to complete (Table 4.1). A simple protocol would be to take an aliquot of a biological preparation, for example living spermatozoa, place a drop on a slide and put a coverslip onto the drop. The coverslip is sealed to the glass slide in some way, for example using nail polish or beeswax, so that it does not move. Shear forces from the movement of the coverslip over the glass slide can cause damage to the specimen or the objective lens.

Many specimens are too thick to be mounted directly onto a slide, and these are cut into thin sections using a device called a microtome. The tissue is usually mounted in a block of wax and cut with the knife of the microtome into thin sections (between 100 μm and 500 μm in thickness). The sections are then placed onto a glass slide, stained and sealed with mounting medium with a coverslip. Some samples are frozen and cut on a cryostat, which is basically a microtome that can keep a specimen in the frozen state, and produce frozen sections more suitable for immunolabelling (Section 4.2.3).

Prior to sectioning, the tissue is usually treated with a chemical agent called a fixative to preserve it. Popular fixatives include formaldehyde and glutaraldehyde,

which act by cross-linking proteins, and alcohols, which act by precipitation. All of these fixatives are designed to maintain the structural integrity of the cell. After fixation the specimen is usually permeabilised in order to allow a stain to infuse the entire tissue. The amount of permeabilisation (time and severity) depends upon several factors; for example, the size of the stain or the density of the tissue. These parameters are found by trial and error for a new specimen, but are usually available in published protocols. The goal is to infiltrate the entire tissue with a uniform staining.

4.2.3 Contrast in the light microscope

Most cells and tissues are colourless and almost transparent, and lack contrast when viewed in a light microscope. Therefore to visualise any details of cellular components it is necessary to introduce contrast into the specimen. This is achieved either by optical means using a specific configuration of microscope components, or by staining the specimen with a dye or, more usually, using a combination of optical and staining methods. Different regions of the cell can be stained selectively with different stains.

Optical contrast

Contrast is achieved optically by introducing various elements into the light path of the microscope and using lenses and filters that change the pattern of light passing through the specimen and the optical system. This can be as simple as adding a piece of coloured glass or a neutral density filter into the illuminating light path, by changing the light intensity, or by adjusting the diameter of a condenser aperture. Usually all of these operations are adjusted until an acceptable level of contrast is achieved for imaging.

The most basic mode of the light microscope is called brightfield (bright background), which can be achieved with the minimum of optical elements. Contrast in brightfield images is usually produced by the colour of the specimen itself. Brightfield is therefore used most often to collect images from pigmented tissues or histological sections or tissue culture cells that have been stained with colourful dyes (Figs. 4.7a and 4.8b).

Several configurations of the light microscope have been introduced over the years specifically to add contrast to the final image. Darkfield illumination produces images of brightly illuminated objects on a black background (Figs. 4.7b and 4.8a). This technique has traditionally been used for viewing the outlines of objects in liquid media such as living spermatozoa, microorganisms, cells growing in tissue culture or for a quick check of the status of a biochemical preparation. For lower magnifications, a simple darkfield setting on the condenser will be sufficient. For more critical darkfield imaging at a higher magnification, a darkfield condenser with a darkfield objective lens will be required.

Phase contrast is used for viewing unstained cells growing in tissue culture and for testing cell and organelle preparations for lysis (Fig. 4.7c,d). The method

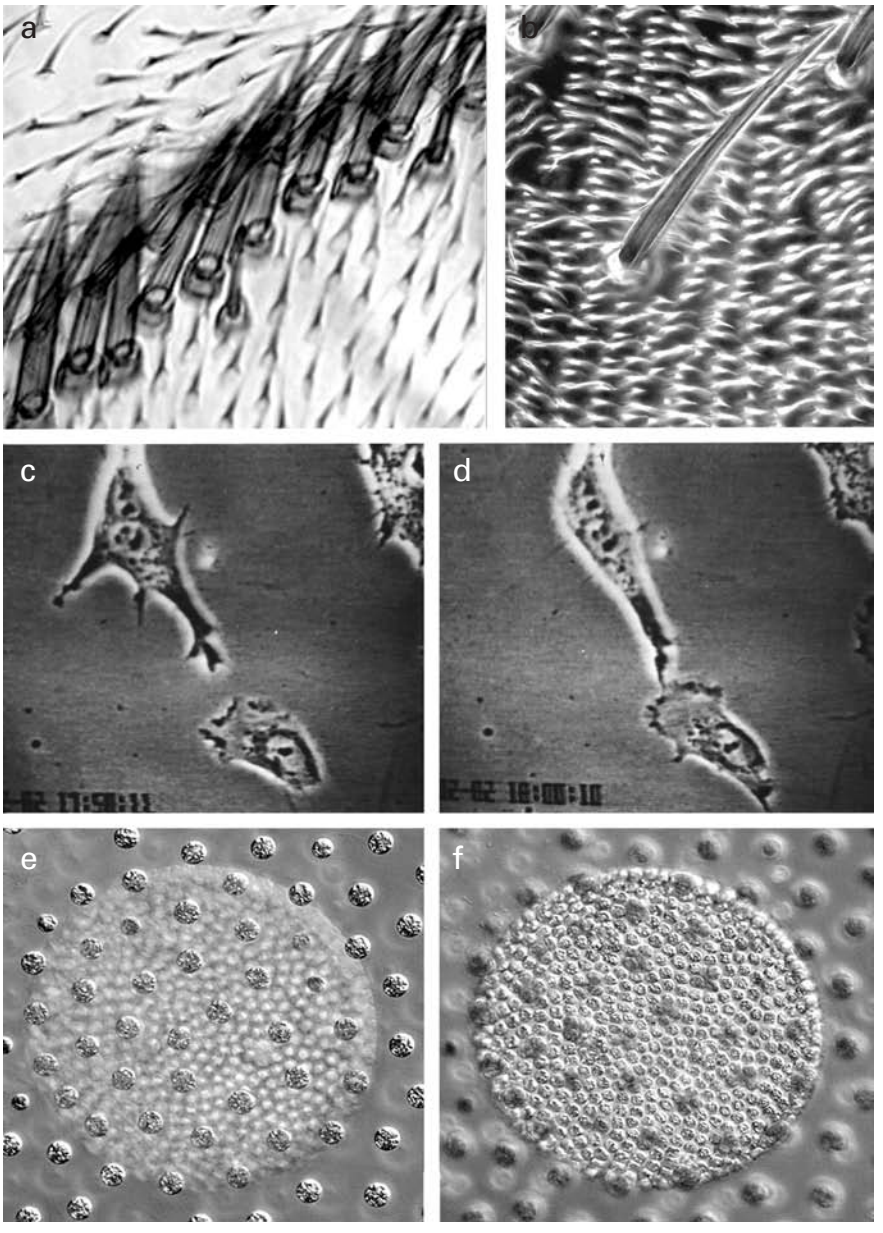

Fig. 4.7. Contrast methods in the light microscope. (a and b) A comparison of (a) brightfield and (b) darkfield images. Here the sensory bristles on the surface of a fly appear dark on a white background in the brightfield image (a) and white on a black background in a darkfield image (b). The dark colour in the larger bristles in (a) is produced by pigment. (c and d) Phase contrast view of cells growing in tissue culture. Two images extracted from a time-lapse video sequence (time between each frame is 5 min). The sequence shows the movement of a mouse 3T3 fibrosarcoma cell and a chick heart fibroblast. Note the bright 'phase halo' around the cells. (e and f) Differential interference contrast image of two focal planes of the multicellular alga *Volvox*. (Images (e) and (f) courtesy of Michael Davidson.)

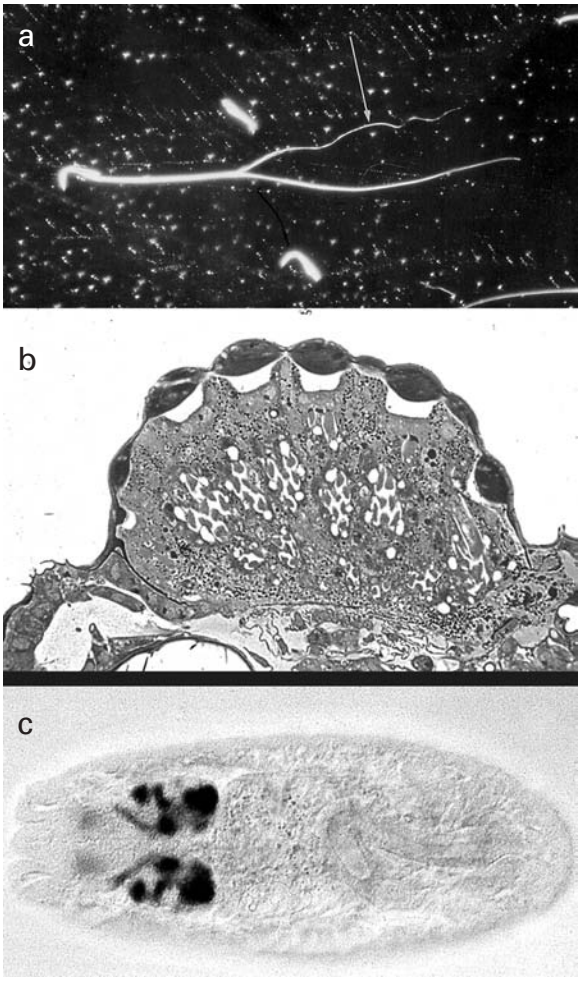

Fig. 4.8. Examples of different preparations in the light microscope. (a) Darkfield image of rat sperm preparation. An aliquot was collected from an experimental protocol in order to assess the amount of damage incurred during sonication of a population of spermatozoa. Many sperm heads can be seen in the preparation, and the fibres of the tail are starting to fray (arrowed). (b) A brightfield image of total protein staining on a section of a fly eye cut on a microtome, and stained with Coomassie Brilliant Blue. (c) DIC image of a stained *Drosophila* embryo – the DIC image shows the outline of the embryo with darker regions of neuronal staining. The DIC image of the whole embryo provides structural landmarks for placing the specific neuronal staining in the context of the anatomy.

images differences in the refractive index of cellular structures. Light that passes through thicker parts of the cell is held up relative to the light that passes through thinner parts of the cytoplasm. It requires a specialised phase condenser and phase objective lenses (both labelled 'ph'). Each phase setting of the condenser lens is matched with the phase setting of the objective lens. These are usually numbered as phase 1, phase 2 and phase 3, and are found on both the condenser and the objective lens.

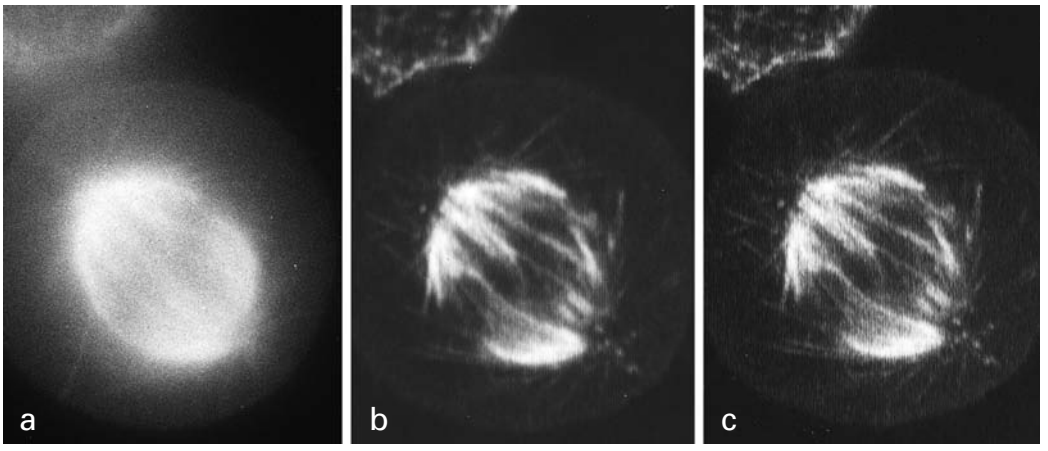

Fig. 4.9. Fluorescence microscopy. Comparison of epifluorescence and confocal fluorescence imaging of a mitotic spindle labelled using indirect immunofluorescence labelling with anti-tubulin (primary antibody) and a fluorescently labelled secondary antibody. The specimen was imaged using (a) conventional epifluorescence light microscopy or (b and c) using laser scanning confocal microscopy. Note the improved resolution of microtubules in the two confocal images (b) and (c) as compared with the conventional image (a). (b) and (c) represent two different resolution settings of the confocal microscope. Image (b) was collected with the pinhole set to a wider aperture than (c). (Image kindly provided by Brad Amos.)

Differential interference contrast (DIC) is a form of interference microscopy that produces images with a shadow relief (Fig. 4.7e,f). It is used for viewing unstained cells in tissue culture, eggs and embryos, and in combination with some stains. Here the overall shape and relief of the structure is viewed using DIC and a subset of the structure is stained with a coloured dye (Fig. 4.8c).

Fluorescence microscopy is currently the most widely used contrast technique (Fig. 4.9). The most commonly used fluorescence technique is called epifluorescence light microscopy, where 'epi' simply means 'from above'. Here, the light source comes from above the sample, and the objective lens acts as both condenser and objective lens (Fig. 4.10).

Fluorescence is popular because of the ability to achieve highly specific labelling of cellular compartments. The images usually consist of distinct regions of fluorescence (white) over large regions of no fluorescence (black), which gives excellent signal-to-noise ratios.

The light source is usually a high pressure mercury or xenon vapour lamp, which emits from the ultraviolet into the red wavelengths (Fig. 4.6). A specific wavelength of light is used to excite a fluorescent molecule or fluorophore in the specimen (Fig. 4.10). Light of longer wavelength from the excitation of the fluorophore is then imaged. This is achieved in the fluorescence microscope using combinations of filters that are specific for the excitation and emission characteristics of the fluorophore of interest. There are usually three main filters: an excitation filter, a dichromatic mirror (often called a dichroic) and a barrier filter, mounted in a single

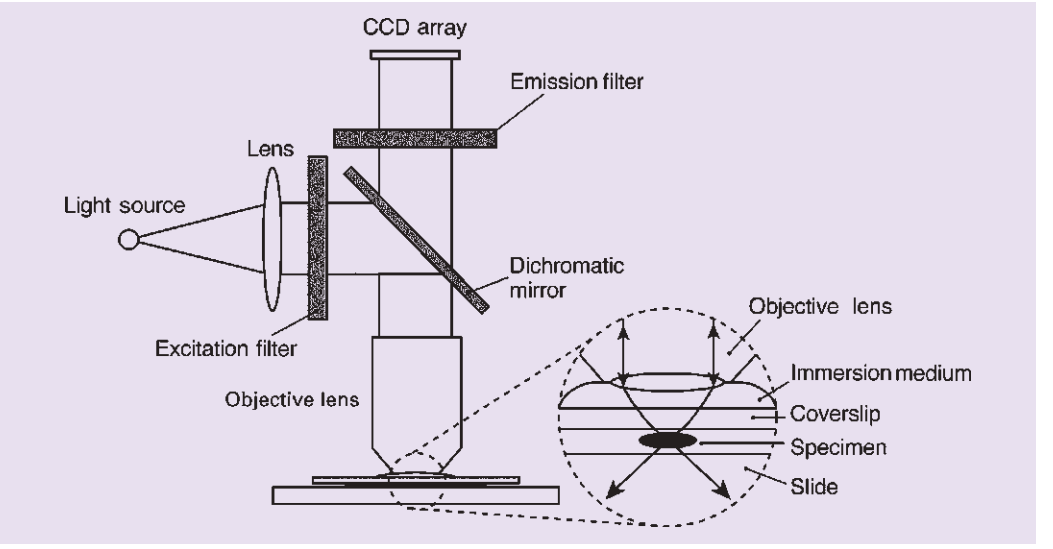

Fig. 4.10. Epifluorescence microscopy. Light from a xenon or mercury arc lamp (Light source) passes through a lens and the excitation filter and reflects off the dichromatic mirror into the objective lens. The objective lens focuses the light at the specimen via the immersion medium (usually immersion oil) and the glass coverslip (see exploded section). Any light resulting from the fluorescence excitation in the specimen passes back through the objective lens and, since it is of longer wavelength than the excitation light, it passes through the dichromatic mirror. The emission filter allows only light of the specific emission wavelength of the fluorochrome of interest to pass through to the detector , in this case a CCD array, where an image is formed.

housing above the objective lens. For example, the commonly used fluorophore fluorescein is optimally excited at a wavelength of 488 nm, and emits maximally at 518 nm (Table 4.2).

A set of glass filters for viewing fluorescein requires that all wavelengths of light from the lamp be blocked except for the 488 nm light. A filter is available that allows a maximum amount of 488 nm light to pass through it (the excitation filter). The 488 nm light is then directed to the specimen via the dichromatic mirror. Any fluorescein label in the specimen is excited by the 488 nm light, and the resulting 518 nm light that returns from the specimen passes through both the dichromatic mirror and the barrier filter to the detector. The emission filters only allow light of 518 nm to pass through to the detector, and ensure that only the signal emitted from the fluorochrome of interest reaches it. (For further details of fluorescence, see Sections 12.5 and 16.3.2.)

Chromatic mirrors and filters can be designed to filter two or three specific wavelengths for imaging specimens labelled with two or more fluorochromes (multiple labelling). The fluorescence emitted from the specimen is often too low to be detected by the human eye or it may be out of the wavelength range of detection of the eye, for example in the far-red wavelengths (Fig. 4.6). A sensitive digital camera easily detects such signals, for example a CCD or a PMT.

Table 4.2 Table of flurophores

Dye	Excitation maximum (nm)	Emission maximum (nm)
Commonly used fluorophores		
Fluorescein (FITC)	496	518
Bodipy	503	511
CY3	554	568
Tetramethylrhodamine	554	576
Lissamine rhodamine	572	590
Texas Red	592	610
CY5	652	672
Nuclear dyes		
Hoechst 33342	346	460
DAPI	359	461
Acridine Orange	502	526
Propidium iodide	536	617
TOTO3	642	661
Ethidium bromide	510	595
Ethidium homodimer	528	617
Feulgen	570	625
Calcium indicators		
Fluo-3	506	526
Calcium Green	506	533
Reporter molecules		
Green fluorescent protein (GFP)	395/489	509
DsRed	558	583
Mitochondria		
JC-1	514	529
Rhodamine 123	507	529

DAPI, 4′,6′-diamidino-2-phenylindole.

Specimen stains

Contrast can be introduced into the specimen using one or more coloured dyes or stains. These can be non-specific stains, for example a general protein stain such as Coomassie Brilliant Blue (Fig. 4.8), or a stain that specifically labels an organelle, for example the nucleus, mitochondria, etc. Combinations of such dyes may be used to stain different organelles in contrasting colours. Many of these histological stains are usually observed using brightfield imaging. Other light microscopy techniques may also be employed in order to view the entire tissue along with the stained tissue. For example, using DIC to view the entire morphology of an embryo and a coloured stain to image the spatial distribution of the protein of interest within the embryo (Fig. 4.8).

More specific dyes are usually used in conjunction with fluorescence microscopy. Immunofluorescence microscopy is used to map the spatial distribution of

macromolecules in cells and tissues. The method takes advantage of the highly specific binding of antibodies to proteins. Antibodies are raised to the protein of interest and labelled with a fluorescent probe. This probe is then used to label the protein of interest in the cell and can be imaged using fluorescence microscopy. In practice, cells are usually labelled using indirect immunofluorescence. Here the antibody to the protein of interest (primary antibody) is further labelled with a second antibody carrying the fluorescent tag (secondary antibody). Such a protocol gives a higher fluorescent signal than using a single fluorescently labelled antibody (Table 4.1).

A related technique, fluorescence *in situ* hybridisation (FISH) employs the specificity of fluorescently labelled DNA or RNA sequences. The nucleic acid probes are hybridised to chromosomes, nuclei or cellular preparations. Regions that bind the probe are imaged using fluorescence microscopy. Many different probes can be labelled with different fluorochromes in the same preparation. Multiple colour FISH is used extensively for clinical diagnoses of inherited genetic diseases. This technique has been applied to rapid screening of chromosomal and nuclear abnormalities in inherited diseases, for example Down's syndrome.

There are many different types of fluorescent molecules that can be attached to antibodies, DNA or RNA probes for fluorescence analysis (Table 4.2). All of these reagents, including primary antibodies, are available commercially or often from the laboratories that produced them. An active area of development is the production of the brightest fluorescent probes that are excited by the narrowest wavelength band and that are not damaged by light excitation (photobleaching). Traditional examples of such fluorescent probes include fluorescein and rhodamine. More modern examples include the Alexa range of dyes and the cyanine dyes. A recent addition to the list of fluorescent probes for imaging is the quantum dot. Quantum dots do not fluoresce but are nanocrystals that glow in different colours in laser light. The colours depend on the size of the dots, and they have the advantage that they are not photobleached.

4.3 OPTICAL SECTIONING

Many images collected from relatively thick specimens produced using epifluorescence microscopy are not very clear. This is because the image is made up of the optical plane of interest together with contributions from fluorescence above and below the focal plane of interest. Since the conventional epifluorescence microscope collects all of the information from the specimen, it is often referred to as a wide-field microscope. The 'out-of-focus fluorescence' can be removed using a variety of optical electronic techniques to produce optical sections (Fig. 4.9).

The term 'optical section' refers to a microscope's ability to produce sharper images of specimens than those produced using a standard wide-field epifluorescence microscope by removing the contribution from out-of-focus light to the image and, in most cases, without resorting to physical sectioning of the tissue. Such methods have revolutionised the ability to collect images from thick and

fluorescently labelled specimens such as eggs, embryos and tissues. Optical sections can also be produced using high resolution DIC optics (Fig. 4.7e,f).

4.3.1 Laser scanning confocal microscopes

Optical sections are produced in the laser scanning confocal microscope (LSCM) by scanning the specimen point by point with a laser beam focused in the specimen and using a spatial filter, usually a pinhole (or a slit), to remove unwanted fluorescence from above and below the focal plane of interest (Fig. 4.11). The power of the confocal approach lies in the ability to image structures at discrete levels within an intact biological specimen.

There are two major advantages of using the LSCM in preference to conventional epifluorescence light microscopy. Glare from out-of-focus structures in the specimen is reduced and resolution is increased both laterally in the *X* and the *Y* directions (0.14 μm) and axially in the *Z* direction (0.23 μm). Image quality of some relatively thin specimens, for example chromosome spreads and the leading lamellipodium of cells growing in tissue culture (<0.2 μm thick), is not dramatically improved by the LSCM whereas thicker specimens such as fluorescently labelled multicellular embryos can only be imaged using the LSCM. For successful confocal imaging, a minimum number of photons should be used to efficiently excite each fluorescent probe labelling the specimen, and as many of the emitted photons from the fluorochromes as possible should make it through the light path of the instrument to the detector.

The LSCM has found many different applications in biomedical imaging. Some of these applications have been made possible by the ability of the instrument to produce a series of optical sections at discrete steps through the specimen (Fig. 4.12). This *Z* series of optical sections collected with a confocal microscope are all in register with each other, and can be merged together to form a single projection of the image (*Z* projection) or a three-dimensional (3D) representation of the image (3D reconstruction).

Multiple label images can be collected from a specimen labelled with more than one fluorescent probe, using multiple laser light sources for excitation (Fig. 4.13, see colour section). Since all of the images collected at different excitation wavelengths are in register, it is relatively easy to combine them into a single multicoloured image. Here, any overlap of staining is viewed as an additive colour change. Most confocal microscopes are able to image three or four different wavelengths simultaneously, and some more modern instruments are able to detect and separate up to 32 different wavelengths.

4.3.2 Spinning disc confocal microscopes

The spinning disc confocal microscope employs a scanning system different from that of the LSCM. Rather than scanning the specimen with a single beam, multiple beams scan the specimen simultaneously and optical sections are viewed in real time. Modern spinning disc microscopes have been improved

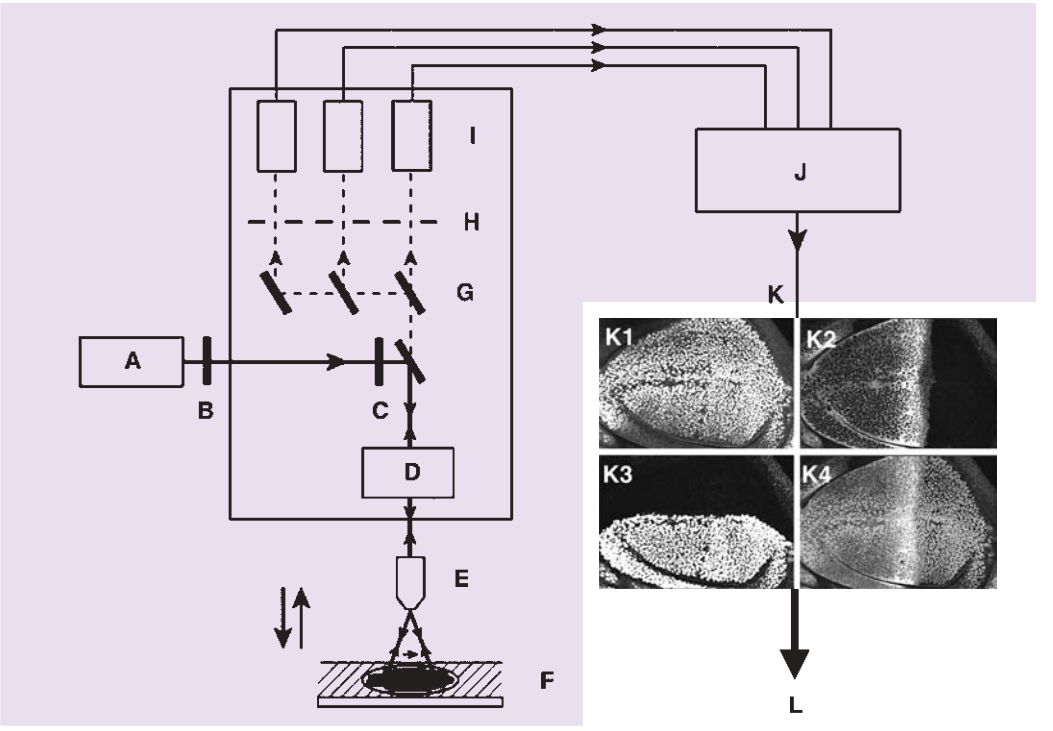

Fig. 4.11. Information flow in a generic laser scanning confocal microscope. Light from the laser (A) passes through a neutral density filter (B) and an excitation filter (C) on its way to the scanning unit (D). The scanning unit produces a scanned beam at the back focal plane of the objective lens (E) which focuses the light at the specimen (F). The specimen is scanned in the *X* and the *Y* directions in a raster pattern and in the *Z* direction by fine focusing (arrows indicate the movement of objective lens E). Any fluorescence from the specimen passes back through the objective lens and the scanning unit and is directed via dichromatic mirrors (G) to three pinholes (H). The pinholes act as spatial filters to block any light from above or below the plane of focus in the specimen. The point of light in the specimen is confocal with the pinhole aperture. This means that only distinct regions of the specimen are sampled. Light that passes through the pinholes strikes the PMT detectors (I) and the signal from the PMT is built into an image in the computer (J). The image is displayed on the computer screen (K) often as three greyscale images (K1, K2 and K3) together with a merged colour image of the three greyscale images (K4) and (Fig. 4.13a, see colour section). The computer synchronises the scanning mirrors with the build-up of the image in the computer framestore. The computer also controls a variety of peripheral devices. For example, the computer controls and correlates movement of a stepper motor connected to the fine focus of the microscope with image aquisition in order to produce a *Z* series. Furthermore the computer controls the area of the specimen to be scanned by the scanning unit so that zooming is easily achieved by scanning a smaller region of the specimen. In this way, a range of magnifications is imparted to a single objective lens so that the specimen does not have to be moved when the magnification is changed. Images are written to the hard disc of the computer or exported to various devices for viewing, hard copy production or archiving (L).

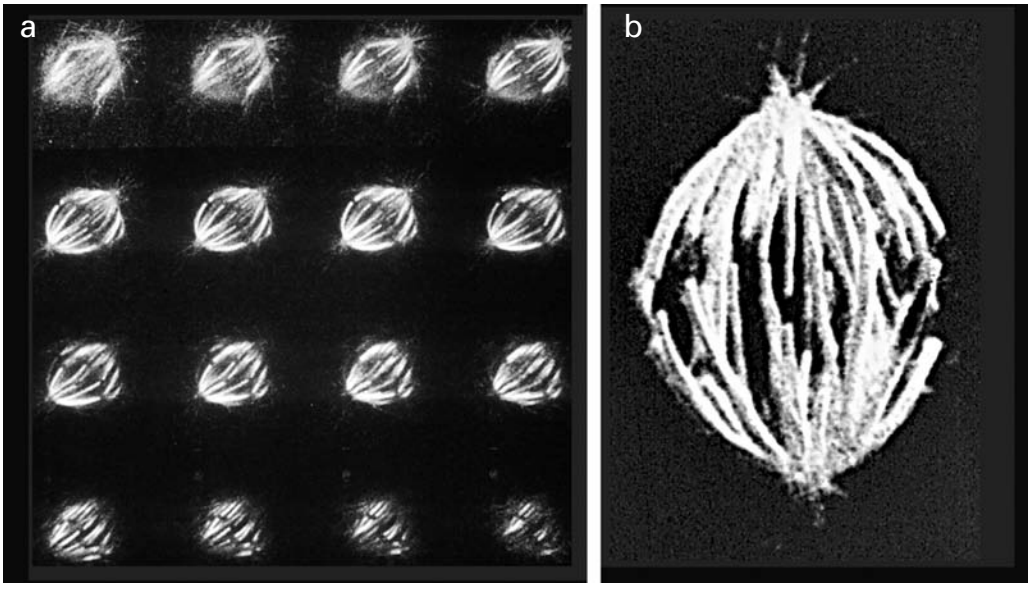

Fig. 4.12. Computer 3D reconstruction of confocal images. (a) Sixteen serial optical sections collected at 0.3 μm intervals through a mitotic spindle of a PtK1 cell stained with anti-tubulin and a second rhodamine-labelled antibody. Using the *Z* series macro program, a pre-set number of frames can be summed and the images transferred into a file on the hard disk. The stepper motor moves the fine focus control of the microscope by a pre-set increment. (b) Three-dimensional reconstruction of the data set produced using computer 3D reconstruction software. Such software can be used to view the data set from any specified angle or to produce videos of the structure rotating in 3D.

significantly by the addition of laser light sources and high quality CCD detectors to the instrument. Spinning disc systems are generally used in experiments where high resolution images are collected at a fast rate (high spatial and temporal resolution), and are used to follow the dynamics of fluorescently labelled proteins in living cells.

4.3.3 Multiple photon imaging

The multiple photon microscope has evolved from the confocal microscope. In fact, many of the instruments use the same scanning system as the LSCM or the spinning disk systems. The difference is that the light source is a high energy pulsed laser with tunable wavelengths, and the fluorochromes are excited by multiple rather than single photons. Optical sections are produced simply by focusing the laser beam in the specimen, since multiple photon excitation of a fluorophore occurs only where energy levels are high enough – statistically confined to the point of focus of the objective lens (Fig. 4.14).

Since red light is used in multiple photon microscopes, optical sections can be collected from deeper within the specimen than can those collected with the

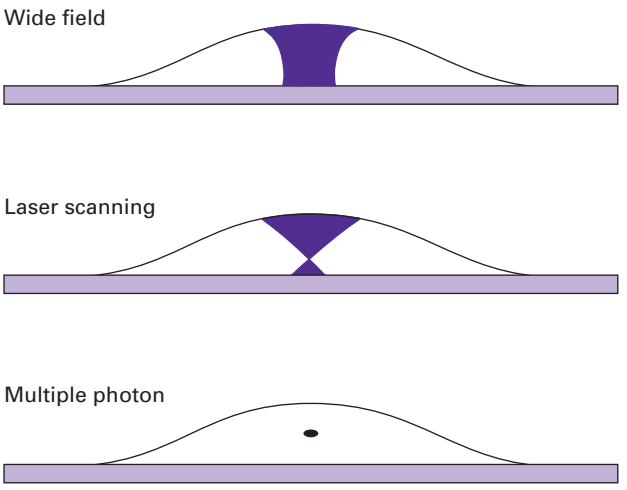

Fig. 4.14. Illumination in wide-field, confocal and multiple photon microscopes. The diagram shows a schematic of a side view of a fluorescently labelled cell on a microscope slide. The dark purple areas in each cell represent the volume of fluorescent excitation produced by each of the different microscopes in the cell. Conventional epifluorescence microscopy illuminates throughout the cell. In the LSCM, fluorescence illumination is throughout the cell but the pinhole in front of the detector excludes the out-of-focus light from the image. In the multiple photon microscope, excitation occurs only at the point of focus where the light flux is high enough.

LSCM. Multiple photon imaging is generally chosen for imaging fluorescently labelled living cells because red light is less damaging to living cells than are the shorter wavelengths usually employed in confocal microscopes. In addition, since the excitation of the fluorophore is restricted to the point of focus in the specimen, there is less chance of overexciting (photobleaching) the fluorescent probe and causing photodamage to the specimen itself.

4.3.4 Deconvolution

Optical sections can be produced using an image-processing method called deconvolution to remove the out-of-focus information from the digital image. Such images are computed from conventional wide-field microscope images. There are two basic types of deconvolution algorithm: deblurring and restoration. The approach relies upon knowledge of the point spread function of the imaging system. This is usually measured by imaging a point source, for example a small subresolution fluorescent bead (0.1 μm), and imaging how the point is spread out in the microscope. Since it is assumed that the real image of the bead should be a point, it is possible to calculate the amount of distortion in the image of the bead imposed by the imaging system. The actual image of the point can then be restored using a mathematical function, which can be applied to any subsequent images collected under identical settings of the microscope.

Early versions of the deconvolution method were relatively slow; for example, it could take some algorithms in the order of hours to compute a single optical section. Deconvolution is now much faster using today's fast computers and improved software, and the method compares favourably with the confocal approach for producing optical sections. Deconvolution is practical for multiple label imaging of both fixed and living cells, and excels over the scanning methods for imaging relatively dim and thin specimens, for example yeast cells. The method can also be used to remove additional background from images that were collected with the LSCM, the spinning disk microscope or a multiple photon microscope.

4.4 IMAGING LIVING CELLS AND TISSUES

There are two basically different approaches to imaging biochemical events over time. One strategy is to collect images from a series of fixed and stained tissues at different developmental ages. Each animal represents a single time point in the experiment. Alternatively, the same tissue can be imaged in the living state during its development. Here, the events of interest are captured directly. The second approach, imaging living cells and tissues, is technically more challenging than the first approach.

4.4.1 Avoidance of artefacts

The only way to eliminate artefacts from specimen preparation is to view the specimen in the living state. Many living specimens are sensitive to light, and especially those labelled with fluorescent dyes. This is because the excitation of fluorophores can release cytotoxic free radicals into the cell. Moreover, some wavelengths are more deleterious than others. Generally, the shorter wavelengths are more harmful than the longer ones and near-infrared light rather than ultraviolet light is preferred for imaging (Fig. 4.6). The levels of light used for imaging must not compromise the cells. This is achieved using extremely low levels of light, relatively bright fluorescent dyes and extremely sensitive photodetectors. Moreover, the viability of cells may also depend upon which cellular compartment has been labelled with the fluorochrome. For example, imaging the nucleus with a dye that is excited with a short wavelength will cause more cellular damage than imaging in the cytoplasm with a dye that is excited in the far red.

Great care has to be observed in order to maintain the tissue in the living state on the microscope stage. A live cell chamber is usually required for mounting the specimen on the microscope stage. This is basically a modified slide and coverslip arrangement that allows access to the specimen by the objective and condenser lenses. It also supports the cells in a constant environment and, depending on the cell type of interest, the chamber may have to provide a constant temperature, humidity, pH, carbon dioxide and/or oxygen levels. Many chambers have the facility for introducing fluids or perfusing the preparation with drugs for experimental treatments.

4.4.2 Time-lapse imaging

Time-lapse imaging continues to be used for the study of cellular dynamics. Here images are collected at predetermined time intervals (Fig. 4.13c–h, see colour section). Usually a shutter arrangement is placed in the light path so that the shutter is open only when an image is collected in order to reduce the amount of light energy impacting on the cells. When the images are played back in real time, a video of the process of interest is produced, albeit speeded up from real-time. Time-lapse is used to study cell behaviour in tissues and embryos and the dynamics of macromolecules within single cells. The event of interest and also the amount of light energy absorbed and tolerated by the cells govern the time interval used. For example, a cell in tissue culture moves relatively slowly and a time interval of 30 s between images might be used. Stability of the specimen and of the microscope is extremely important for successful time-lapse imaging. For example, the focus should not drift during the experiment.

All forms of light microscopy can be used for time-lapse imaging (Section 4.2.3 and Section 4.3). Phase contrast was the traditional choice for imaging cell movement and behaviour of cells growing in tissue culture. DIC or fluorescence microscopy is generally chosen for imaging the development of eggs and embryos. Computer imaging methods can be used in conjunction with DIC to improve resolution. Here, a background image is subtracted from each time-lapse frame and the contrast of the images is enhanced electronically. In this way microtubules assembled *in vitro* from tubulin in the presence of microtubule-associated proteins can be visualised on glass. These images are below the resolution of the light microscope. Such preparations have formed the basis of motility assays for motor proteins, for example kinesin and dynein.

4.4.3 Fluorescent stains of living cells

Relatively few cells possess any inherent fluorescence (autofluorescence) although some endogenous molecules are fluorescent and can be used for imaging, for example NAD(P)H. Relatively small fluorescent molecules are loaded into living cells using many different methods, including diffusion, microinjection, bead loading or electroporation. Relatively larger fluorescently labelled proteins are usually injected into cells, and after some time they are incorporated into the general protein pool of the cell for imaging.

Many reporter molecules are now available for recording the expression of specific genes in living cells using fluorescence microscopy (Table 4.2). The green fluorescent protein (GFP) is a very convenient reporter of gene expression because it is directly visible in the living cell using epifluorescence light microscopy with standard filter sets. The GFP gene can be linked to another gene of interest so that its expression is accompanied by GFP fluorescence in the living cell. No fixation, substrates or coenzymes are required. The fluorescence of GFP is extremely bright and is not susceptible to photobleaching. Spectral variants of GFP and additional reporters such as DsRed are now available for multiple labelling of living cells.

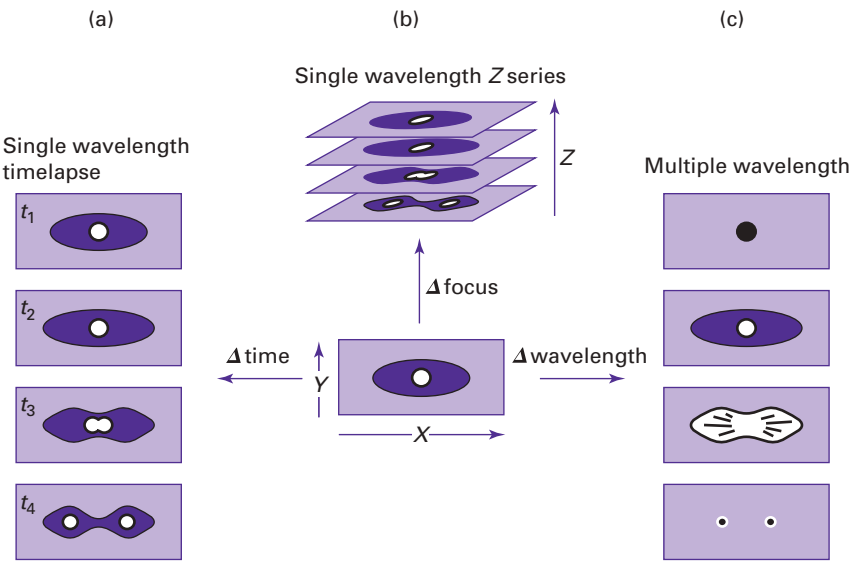

Fig. 4.15. Multidimensional imaging. (a) Single wavelength excitation over time or time-lapse *X*, *Y* imaging; (b) *Z* series or *X*, *Y*, *Z* imaging. The combination of (a) and (b) is 4D imaging. (c) Multiple wavelength imaging. The combination of (a) and (b) and (c) is 5D imaging.

These probes have revolutionised the ability to image living cells and tissues using light microscopy.

4.4.4 Multidimensional imaging

The collection of *Z* series over time is called four-dimensional (4D) imaging where individual optical sections (*X* and *Y* dimensions) are collected at different depths in the specimen (*Z* dimension) at different times (the fourth dimension), i.e. one time and three space dimensions (Fig. 4.15). Moreover multiple wavelength images can also be collected over time. This approach has been called 5D imaging. Software is now available for the analysis and display of such 4D and 5D data sets. For example, the movement of a structure through the consecutive stacks of images can be traced, changes in volume of a structure can be measured, and the 4D data sets can be displayed as series of *Z* projections or stereo videos. Multidimensional experiments can present problems for handling large amounts of data since gigabytes of information can be collected from a single 4D imaging experiment.

4.5 THE STEREOMICROSCOPE

A second type of light microscope, the stereomicroscope is used for the observation of the surfaces of large specimens (Fig. 4.16). The microscope is used when 3D information is required, for example for the observation of whole organisms (Fig. 4.17, see colour section). Stereomicroscopes are useful for micromanipulation and

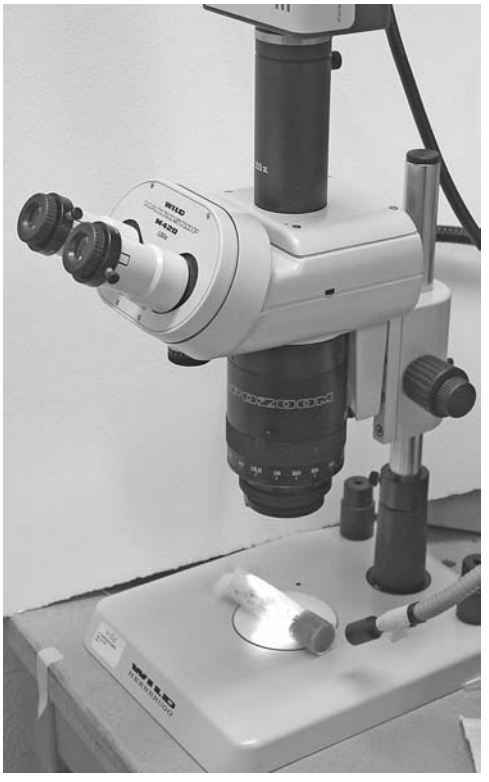

Fig. 4.16. A research grade stereomicroscope. Note the light source is from the side, which can give a shadow effect to the specimen – in this example a vial of flies. The large objective lens above the specimen can be rotated to zoom the image.

dissection where the wide field of view and the ability to zoom in and out in magnification is invaluable. A wide range of objectives and eyepieces are available for different applications. The light sources can be from above, from below the specimen, encircling the specimen using a ring light or from the side, giving a darkfield effect. These different light angles serve to add contrast or shadow relief to the images. Fluorescent stereomicroscopes are also available and are used for screening transgenic animals labelled with GFP and its variants.

4.6 THE ELECTRON MICROSCOPE

4.6.1 Principles

Electron microscopy is used when the greatest resolution is required. The images produced in an electron microscope (EM) reveal the ultrastructure of cells. There are two different types of electron microscope: the transmission electron microscope (TEM) and the scanning electron microscope (SEM). In the TEM, electrons that pass through the specimen are imaged. In the SEM, electrons that are

reflected back from the specimen (secondary electrons) are collected, and the surfaces of specimens are imaged.

The equivalent of the light source in an electron microscope is the electron gun. When a high voltage of between 40 000 and 100 000 V (the accelerating voltage) is passed between the cathode and the anode, a tungsten filament emits electrons (Fig. 4.1). The negatively charged electrons pass through a hole in the anode forming an electron beam. The beam of electrons passes through a stack of electromagnetic lenses (the column). Focusing of the electron beam is achieved by changing the voltage across the electromagnetic lenses. When the electron beam passes through the specimen some of them are scattered whilst others are focused by the projector lens onto a phosphorescent screen or recorded using photographic film or a digital camera. The electrons have limited penetration power, which means that specimens must be thin (50–100 nm) to allow them to pass through.

Thicker specimens can be viewed by using a higher accelerating voltage, for example in the high voltage electron microscope (HVEM), which uses 1 000 000 V accelerating voltage or in the intermediate voltage electron microscope (IVEM), which uses an accelerating voltage of around 400 000 V. Here, stereo images are collected by collected two images at 8° to 10° tilt angles. Such images are useful in assessing the 3D relationships of organelles within cells when viewed in a stereoscope or with a digital stereoprojection system.

4.6.2 Preparation of specimens

Contrast in the EM depends on atomic number: the higher the atomic number the greater the scattering and the contrast. Thus heavy metals are used to add contrast in the EM, for example uranium, lead and osmium. Labelled structures appear black or electron dense in the image (Fig. 4.18).

All of the water has to be removed from any biological specimen before it can be imaged in the EM. This is because the electron beam can be produced and focused only in a vacuum. The major drawback of EM observation of biological specimens therefore is the non-physiological conditions necessary for their observation. Nevertheless, the improved resolution afforded by the EM has provided much information about biological structures and biochemical events within cells that could otherwise not have been collected using any other microscopical technique.

Extensive specimen preparation is required for EM analysis, and for this reason there can be issues of interpreting the images because of artefacts from specimen preparation. For example, specimens have been traditionally prepared for the TEM by fixation in glutaraldehyde to cross-link proteins, followed by osmium tetroxide to fix and stain lipid membranes. This is followed by dehydration in a series of alcohols to remove the water, and then embedding in a plastic such as Epon for thin sectioning (Fig. 4.18).

Small pieces of the embedded tissue are mounted and sectioned on an ultramicrotome using either a glass or a diamond knife. Ultrathin sections are cut to a thickness of approximately 60 nm. The ribbons of sections are floated onto the surface of

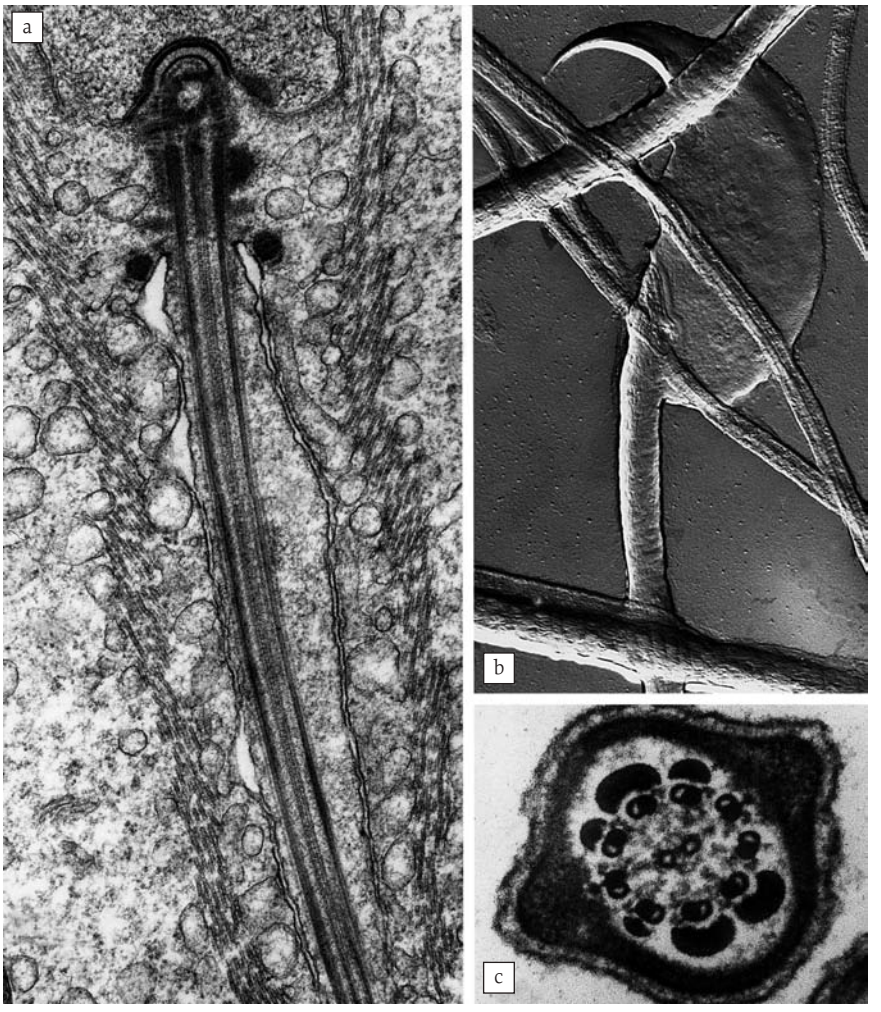

Fig. 4.18. Transmission electron microscopy. (a and c) Ultrathin Epon sections (60 nm thick) of developing sperm cells stained with uranyl acetate and lead citrate. (b) Carbon surface replica of a mouse sperm.

water and their interference colours are used to assess their thickness. The desired 60 nm section thickness has a silver/gold interference colour on the water surface. The sections are then mounted onto copper or gold EM grids, and are subsequently stained with heavy metals, for example uranyl acetate and lead citrate.

For the SEM, samples are fixed in glutaraldehyde, dehydrated through a series of solvents and dried completely either in air or by critical point drying. This method removes all of the water from the specimen instantly and avoids surface tension in the drying process, thereby avoiding artefacts of drying. The specimens are then mounted onto a special metal holder or stub and coated with a thin layer of gold before being viewed in the SEM (Fig. 4.17, see colour section). Surfaces can also be viewed in the TEM using either negative stains or carbon replicas of air-dried specimens (Fig. 4.18).

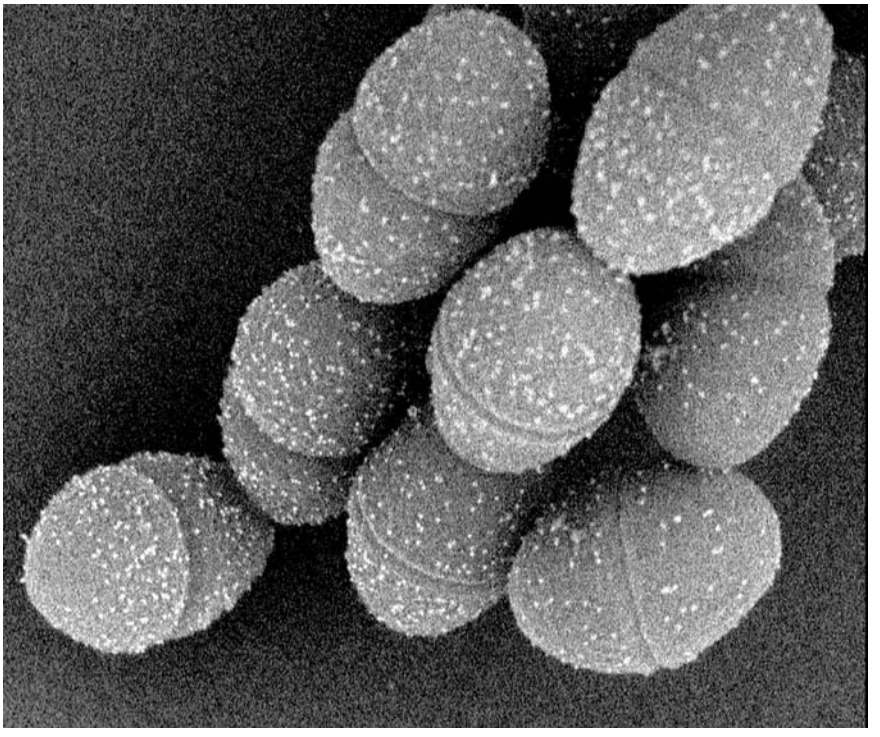

Fig. 4.19. Immunoelectron microscopy. Scanning electron microscope (SEM) imaging of microbes *Enterococcus faecalis* labelled with 10 nm colloidal gold for the surface adhesion protein 'aggregation substance'. This protein facilitates exchange of DNA during conjugation. The gold labels appear as white dots on the surface of the bacteria. (Image kindly provided by Stan Erlandsen.)

Immuno-EM methods allow the localisation of molecules within the cellular microenvironment for TEM and on the cell surface for SEM (Fig. 4.19). Cells are prepared in a similar way to indirect immunofluorescence, with the exception that, rather than a fluorescent probe bound to the secondary antibody, electron-dense colloidal gold particles are used. Multiple labelling can be achieved using different sizes of gold particles (10 nm) attached to antibodies to the proteins of interest. The method depends upon the binding of protein A to the gold particles, since protein A binds in turn to antibody fragments. Certain resins, for example Lowicryl and LR White, have been formulated to allow antibodies and gold particles to be attached to ultrathin sections for immunolabelling.

4.6.3 Recent developments in EM methods

New methods of fixation continue to be developed in an attempt to avoid the artefacts of specimen preparation and to observe the specimen more closely to its living state. Specimens are rapidly frozen in milliseconds by high pressure freezing. Under these conditions the biochemical state of the cell is more likely to be

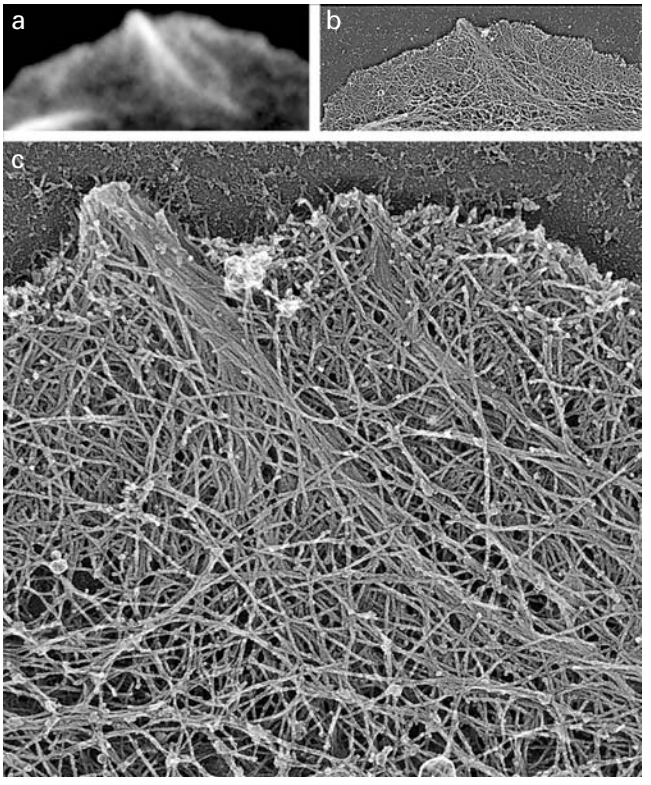

Fig. 4.20. Integrated microscopy. (a) Epifluorescence image and (b and c) whole mount TEM at different magnifications of the same cell. The fluorescence image is labelled with rhodamine phalloidin, which stains polymerised actin. A stress fibre at the periphery of the cell appears as a white line in the fluorescence image (a), and when viewed in the TEM the stress fibres appear as aligned densities of actin filaments. The TEM whole mount was prepared using detergent extraction, chemical fixation, critical point drying and platinum/carbon coating. (Image kindly provided by Tatyana Svitkina.)

preserved. Many of these frozen hydrated samples can be observed directly in the EM or they can be chemically fixed using freeze substitution methods. Here, fixatives are infused into the preparation at low temperature, after which the specimen is slowly warmed to room temperature.

Using cryo-electron tomography (Cryo-ET) the 3D structure of cells and macromolecules can be visualised at 5–8 nm resolution. Cells are frozen and mounted in an apparatus that moves the specimen through a range of tilt angles. A 2D digital EM image is collected at each one of these tilt angles, and, using computer software, a 3D representation of the specimen can be constructed.

4.6.4 Integrated microscopy

The same specimen can be viewed in the light microscope and subsequently in the EM. This approach is called integrated microscopy. The correlation of images of

the same cell collected using the high temporal resolution of the light microscope and the high spatial resolution of the EM gives additional information to imaging using the two techniques separately (Fig. 4.20). The integrated approach also addresses the problem of artefacts. Probes are now available that are fluorescent in the light microscope and electron dense in the EM.

4.7 IMAGING AND BIOCHEMISTRY

Understanding the function of proteins within the context of the intact living cell is one of the main aims of contemporary biological research. The visualisation of specific cellular events has been greatly enhanced by modern microscopy. In addition to qualitative viewing of the images collected with a microscope, quantitative information can be gleaned from the images. The collection of meaningful measurements has been greatly facilitated by the advent of digital image processing. Subtle changes in intensity of probes of biochemical events can be detected with sensitive digital detectors. These technological advancements have allowed insight into the spatial aspects of molecular mechanisms.

Relatively simple measurements include counting features within a 2D image or measuring areas and lengths. Measurements of depth and volume can be made in 3D, 4D and 5D data sets. Images can be calibrated by collecting an image of a calibration grid at the same settings of the microscope as was used for collecting the images during the experiment. Many image-processing systems allow for a calibration factor to be added into the program, and all subsequent measurements will then be comparable.

The rapid development of fluorescence microscopy together with digital imaging and, above all, the development of new fluorescent probes of biological activity, have added a new level of sophistication to quantitative imaging. Most of the measurements are based on the ability to accurately measure the brightness of a fluorescent probe within a sample using a digital imaging system. This is also the basis of flow cytometry (Section 16.3.2), which measures the individual brightness of a population of cells as they pass through a laser beam. Cells can be sorted into different populations using a related technique, fluorescence-activated cell sorting (FACS).

The brightness of the fluorescence from the probe can be calibrated to the amount of probe present at any given location in the cell at high resolution. For example, the concentration of calcium is measured in different regions of living embryos using calcium indicator dyes (e.g. fluo-3) whose fluorescence increases in proportion to the amount of free calcium in the cell (Fig. 4.21, see colour section). Many probes have been developed for making such measurements in living tissues. Controls are a necessary part of such measurements, since photobleaching and various dye artefacts during the experiment can obscure the true measurements. This can be achieved by staining the sample with two ion-sensitive dyes and comparing their measured brightness during the experiment. These measurements are usually expressed as ratios (ratio imaging) and control for dye loading problems, photobleaching and instrument variation.

Fluorescently labelled proteins can be injected into cells where they are incorporated into macromolecular structures over time. This makes the structures accessible to time-lapse imaging using fluorescence microscopy. Such methods can lead to high background dye levels and can be difficult to interpret. In addition to optical sectioning methods (Section 4.3), several methods have been developed for avoiding high backgrounds for fluorescence measurements of biochemical events in cells.

Fluorescence recovery after photobleaching (FRAP) uses the high light flux from a laser to locally destroy fluorophores labelling the macromolecules to create a bleached zone (photobleaching). The subsequent movement of undamaged fluorophores into the bleached zone gives a measure of molecular mobility. This enables biochemical analysis within the living cell (Section 16.3.2). A second technique related to FRAP, photoactivation, uses a probe whose fluorescence can be induced by a pulse of short wavelength light. The activated probe is imaged using a longer wavelength of light. Here the signal-to-noise ratio of the images can be better than that for photobleaching experiments.

A third method, fluorescence speckle microscopy, was discovered as a chance observation but is now popular for imaging macromolecular dynamics inside living cells. Basically, a lower concentration of fluorescently labelled protein is injected into cells so that the protein of interest is not fully labelled inside the cell. When viewed in the microscope, structures inside cells that have been labelled in this way have a speckled appearance. The dark regions act as fiduciary marks for the observation of dynamics.

Fluorescence resonance energy transfer (FRET) is a fluorescence-based method that can take fluorescence microscopy past the theoretical resolution limit of the light microscope allowing the observation of protein–protein interactions *in vivo* (Fig. 4.22). FRET occurs between two fluorophores when the emission of the first one (the donor) serves as the excitation source for the second one (the acceptor). This will occur only when two fluorophore molecules are very close to one another, at a distance of 60 Å (6 nm) or less. An example of a FRET experiment would be to use spectral variants of GFP. Here the excitation of a cyan fluorescent protein (CFP)-tagged protein is used to monitor the emission of a yellow fluorescent protein (YFP)-tagged protein. YFP fluorescence will be observed under the excitation conditions of CFP only if the proteins are close together. Since this can be monitored over time, FRET can be used to measure direct binding of proteins or protein complexes (Section 16.3.2).

A more complex technique, fluorescence lifetime imaging (FLIM), measures the amount of time a fluorophore is fluorescent after excitation with a 10 ns pulse of laser light. FLIM is used for detecting multiple fluorophores with different fluorescent lifetimes and overlapping emission spectra.

4.8 SPECIALISED IMAGING TECHNIQUES

Technical advancements continue to impact on the field of microscopy and imaging, and enable yet more experimental approaches. Instruments continue to

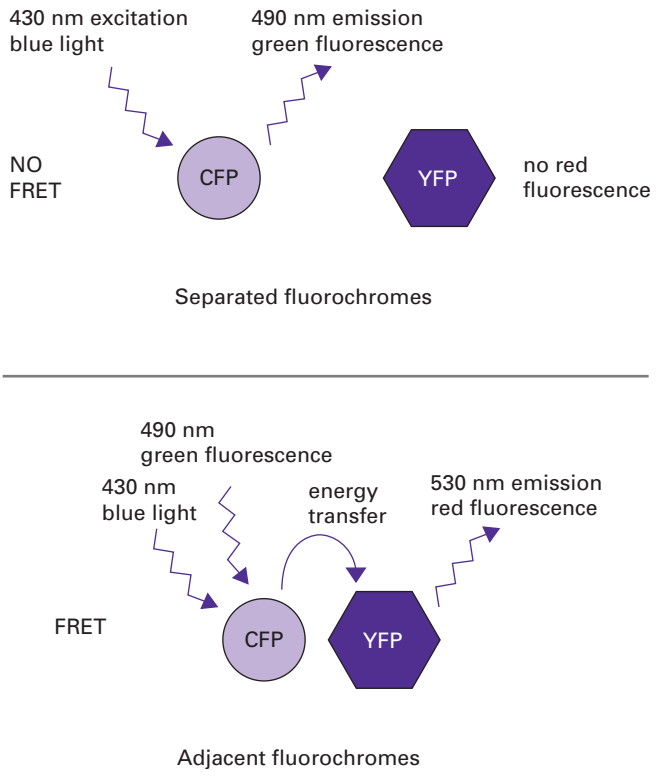

Fig. 4.22. Fluorescence resonance energy transfer (FRET). In the upper example (NO FRET) the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) are not close enough for FRET to occur (more than 60 Å (6 nm) separation). Here excitation with the 430 nm blue light results in the green 490 nm emission of the CFP only. Whereas, in the lower example (FRET), the CFP and YFP are close enough for 'energy transfer' or FRET to occur (closer than 60 nm). Here, excitation with the 430 nm blue light results in fluorescence of the CFP (green) and of the YFP (red).

Example 1 LOCATING AN UNKNOWN PROTEIN TO A SPECIFIC CELLULAR COMPARTMENT

Question

You have isolated and purified a novel protein from a biochemical preparation. How might you determine its subcellular distribution and possible function in the cell?

Answer

Many fluorescent probes are available that label specific cellular compartments. For example, TOTO3 labels the nucleus and fluorescent phalloidins label cell outlines. An antibody to your protein could be raised and used to immunofluorescently label cells. Using a multiple labelling approach and perhaps an optical sectioning technique such as laser scanning confocal microscopy the distribution of the protein in the cell relative to known distributions can be ascertained. For higher resolution immuno-EM or FRET studies could be performed.

be developed for specialised, non-routine applications in biochemistry. Most developments are designed to image more efficiently living cells and tissues at improved resolution.

Magnetic resonance imaging (MRI), whilst well developed for collecting 3D information from large structures such as whole body anatomy, has recently been adapted for the collection of relatively high resolution (10 μm) images from deep into otherwise opaque microscopic tissues; for example, for the observation of the 3D anatomy of living embryos as they develop. Contrast agents are becoming available that can be used for both fluorescence microscopy and MRI of the same tissue. The MRI technique is able to penetrate the entire volume of the tissue whereas the fluorescence images can be collected from only a few micrometres within the embryo (see also Section 13.4.3).

Another area of active research is in the development of single molecule detection techniques. For example total internal reflection microscopy (TIRF) uses the properties of an evanescent wave close to the interface of two media (Fig. 4.23), for example the region between the specimen and the glass coverslip. The technique relies on the fact that the intensity of the evanescent field falls off rapidly so that the excitation of any fluorophore is confined to a region of just 100 nm above the glass interface. This is thinner than the optical section thickness achieved using confocal methods and allows the imaging of single molecules at the interface.

The atomic force microscope, rather than using a lens, probes the surface of a specimen with a sharp tip that is several micrometres in length and less than 10 nm in diameter at the point (near field imaging). The tip is at the end of a lever some 100–200 μm in length. As the tip moves across the specimen, forces between the two cause the lever to bend. The movement of the lever is detected using a computer, and an image is built up of the surface of the specimen from the minute deflections of the tip. The method produces images of surfaces at very high resolution (Table 4.3).

4.9 IMAGE ARCHIVING, PRESENTATION AND FURTHER INFORMATION

Most images produced by any kind of modern microscope are collected in a digital form. In addition to greatly speeding up the collection of the images (and experiment times), the use of digital imaging has allowed the use of digital image databases and the rapid transfer of information between laboratories across the World Wide Web. Moreover there is no loss in resolution or colour balance from the images collected at the microscope as they pass between laboratories and journal web pages.

International image databases are under development for the storage and access of microscope image data from many different locations. There is a trend for modern microscopes to produce more and more data, especially when multidimensional data sets are generated. This trend is continuing with the need to develop automated methods of image analysis of gene expression data from genomic screens.

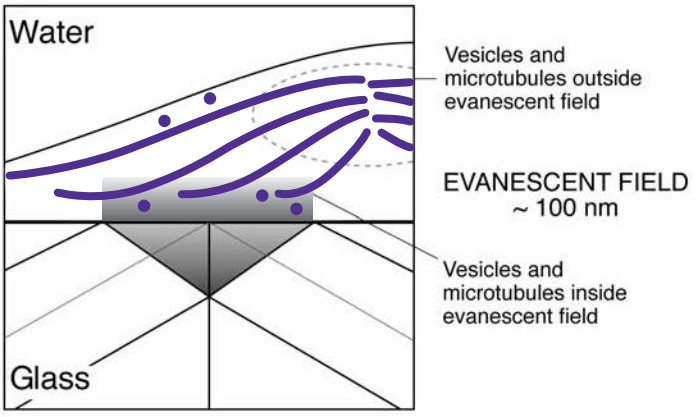

Fig. 4.23. Total internal reflection microscopy (TIRF). A 100 nm thick region of excitation is produced at the glass–water interface when illumination conditions are right for internal reflection. In this example, only those vesicles and microtubules within the evanescent field will contribute to the fluorescence image at 100 nm *Z* resolution.

Table 4.3 Resolution in optical imaging

	XY	*Z*
Standard microscope	0.5 μm	1.6 μm
Confocal/multiple photon	0.25 μm	0.7 μm
TIRF – evanescent wave	0.5 μm	0.3 μm
Atomic force – near field	0.05 μm	0.01 μm

TIRF, total internal reflection microscopy.

More detailed information on any of the microscopes and their applications in biochemistry can be accessed on the World Wide Web. Several websites have been included as starting points for further study (see Section 4.10). Should any of these listed websites become out of date, more information on any topic can be accessed using a Web search engine. The field of microscopy is moving very fast but the basic principles of light and electron microscopy remain unchanged.

4.10 SUGGESTIONS FOR FURTHER READING

ABRAMOWITZ, M. (2003). *Microscope Basics and Beyond*. Olympus of America Inc., Melville, NY. (Good, well-illustrated primer on all aspects of basic light microscopy – also available online as a pdf file.)

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. and WALTER, P. (2002). *Molecular Biology of the Cell*, 4th edn. Garland Science, New York. (Good introduction to all forms of microscopy and live cell imaging for the cell biologist.)

ANDREWS, P. D., HARPER, I. S. and SWEDLOW, J. R. (2002). To 5D and beyond: quantitative fluorescence microscopy in the postgenomic era. *Traffic*, **3**, 29–36. (Review of multidimensional imaging, methods of coping with large data sets and international image databases.)

BARUCH, A., JEFFERY, D. A. and BOFYO, M. (2004). Enzyme activity – it's all about image. *Trends in Cell Biology*, **14**, 29–35. (Recent review of biochemical experiments using fluorescence.)

BRAGA, P. C. and RICCI, D. (2003). *Atomic Force Microscopy – Biomedical Methods and Applications*. Methods in Molecular Biology, vol. 242. (Protocols-based methods and applications of atomic force microscopy.)

FRANKEL, F. (2002). *Envisioning Science: The Design and Craft of the Science Image*. MIT Press, Cambridge, MA. (Popular work on imaging with some great tips and trips for the stereomicroscope.)

HAUGLAND, R. (2003). *Handbook of Fluorescent Probes and Research Products*, 9th edn. Molecular Probes Inc., Eugene, OR. (A compendium of all modern fluorescent probes together with protocols and references – constant updates on the web.)

HURTLEY, S. M. and HELMUTH, L. (2003). Biological imaging. *Science*, **300**, 75–145. (Very good collection of papers on contemporary imaging topics.)

INOUE, S. and SPRING, K. (1997). *Video Microscopy, The Fundamentals*, 2nd edn. Plenum Publishing Corp., New York. (Excellent primer on live cell imaging, video microscopy and general microscopy.)

ISHIJIMA, A. and YANAGIDA, T. (2001). Single molecule nanobioscience. *Trends in Biochemical Sciences*, **26**, 438–444. (Review of single molecule imaging.)

LEWIS, A., TAHA, H., STRINKOVSKI, A., MANEVITCH, A., KHATCHATOURIANTS, A., DEKHTER, R. and AMMANN, E. (2003). Near-field optics: from subwavelength illumination to nanometric shadowing. *Nature Biotechnology*, **21**, 1378–1386. (Review of near-field optics and contemporary developments.)

MCINTOSH, J. R. (2001). Electron microscopy of cells: a new beginning for a new century. *Journal of Cell Biology*, **153**, F25–F32. (A good and brief start on modern EM techniques.)

PERIASAMY, A. (2001). *Methods in Cellular Imaging*. Oxford University Press, Oxford. (An excellent collection of advanced light microscopy including confocal, multiple photon and FRET.)

SCHULDT, A. and SMALLRIDGE, R. (2003). *Imaging in cell biology*. Supplement to Nature Cell Biology, **6**. (Recent overview of modern imaging techniques.)

SEDGEWICK, J. (2002). *Quick PhotoShop for Research: A Guide to Digital Imaging for PhotoShop*. Kluwer Academic/Plenum Publishing, New York. (Hands-on recipe based protocols for presentation of digital images from microscopes.)

SHAPIRO, H. M. (2003). *Practical Flow Cytometry*, 4th edn. John Wiley and Sons, New York. (Wonderfully written book on basic fluorescence and flow cytometry.)

SPECTOR, D. L. and GOLDMAN, R. D. (2004). *Live Cell Imaging: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (A good introduction to contemporary methods of imaging living cells.)

VAN ROESSEL, P. and BRAND, A. H. (2002). Imaging into the future: visualizing gene expression and protein interaction with fluorescent proteins. *Nature Cell Biology*, **4**, E15–E20. (Good primer on GFP and FRET.)

WALLACE, W., SCHAEFER, L. H. and SWEDLOW, J. R. (2001). Working person's guide to deconvolution in light microscopy. *BioTechniques*, **31**, 1076–1097. (Review of the deconvolution technique.)

ZHANG, J., CAMPBELL, R. E., TING, A. Y. and TSIEN, R.Y. (2002). Creating new fluorescent probes for cell biology. *Nature Reviews in Molecular Cell Biology*, **3**, 906–918. (Review of the development of fluorescent probes of biological activity especially reporter molecules.)

Websites of interest

General microscopy

<http://www.microscopyu.com/>
<http://www.microscopy.fsu.edu/>

<http://www.microscopy-analysis.com/>
<http://www.msa.microscopy.com/>
<http://www.rms.org.uk/>
<http://www.ou.edu/research/electron/mirror/web-subj.html>
<http://www.ou.edu/research/electron/www-vl/>

Fluorescent probes

<http://www.bdbiosciences.com/clontech/>
<http://www.qdots.com/>
<http://www.probes.com>
<http://www.jacksonimmuno.com/>

Image processing

<http://www.apple.com/quicktime/qtvr/>
<http://rsb.info.nih.gov/nih-image/>
<http://rsb.info.nih.gov/ij/>
<http://www.uiowa.edu/~dshbwww/>
<http://www.lemkesoft.de/en/index.htm>

Database

<http://www.openmicroscopy.org>