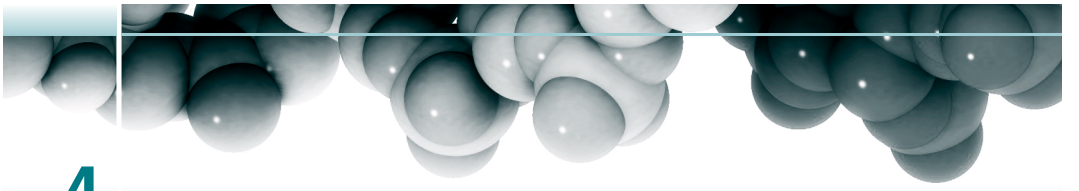

4 Microscopy

S. W. PADDOCK

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; 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 the best measure of a microscope, however. 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. Standard light microscopes have a lateral resolution limit of about 0.5 micrometers (μm) for routine analysis. In contrast, electron microscopes have a lateral resolution of up to 1 nanometer (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. Computer enhancement methods have improved upon the 0.5 μm resolution limit of the light microscope down to 20 nm resolution in some

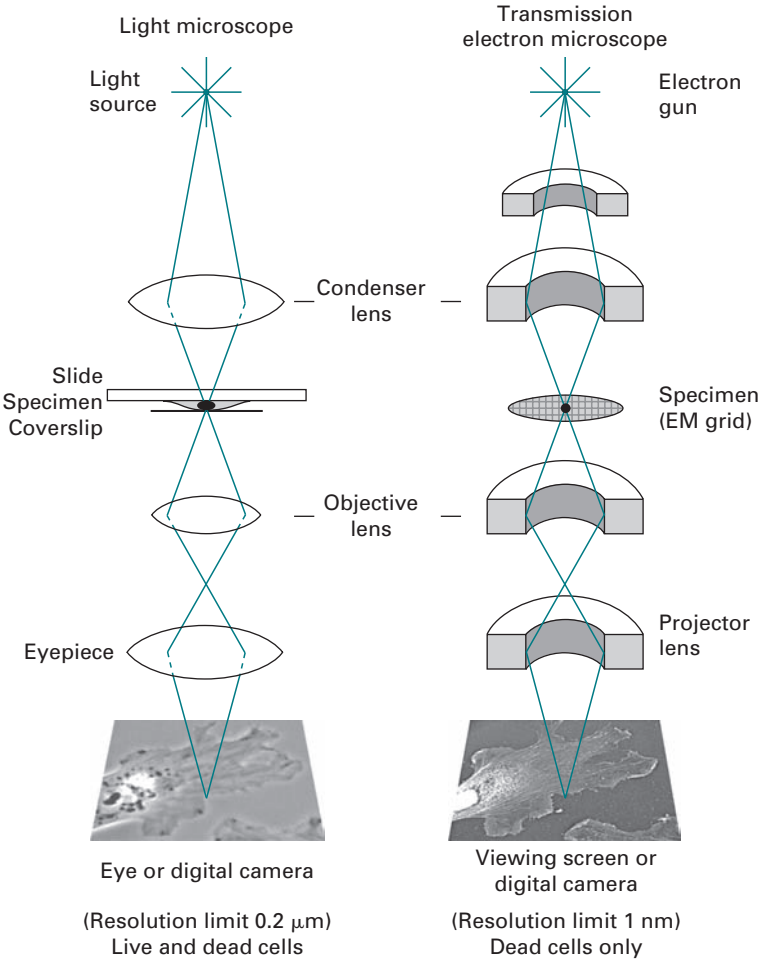

Fig. 4.1 Light and electron microscopy. Schematic that compares the path of light through a compound light microscope (LM) with the path of electrons through a transmission electron microscope (TEM). Light from a lamp (LM) or a beam of electrons from an electron gun (TEM) is focussed at the specimen 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 or gold 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 a phosphorescent viewing screen or digital camera in the TEM. (Light and EM images courtesy of Tatyana Svitkina, University of Pennsylvania, USA.)

specialised applications, for example using total internal reflection microscopy (TIRF) (Section 4.3.5).

Applications of the microscope in biomedical research may be relatively simple and routine; for example, a quick check of the status of a preparation or of the health of cells growing in a plastic dish 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

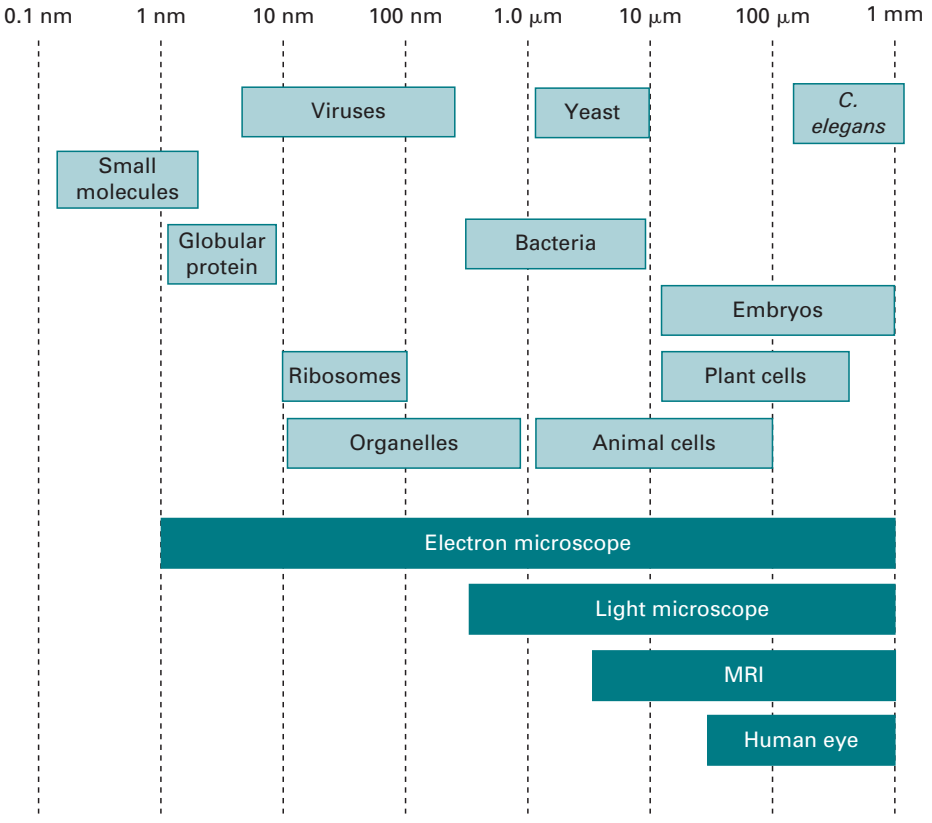

Fig. 4.2 The relative sizes of a selection of biological specimens and some of the devices used to image them. The range of resolution for each instrument is included in the dark bars at the base of the figure. MRI, magnetic resonance imaging.

over a millisecond timescale. Here a more advanced light microscope (often called an **imaging system**) is required.

Some microscopes are more suited to specific applications than others. There may be constraints imposed by the specimen. 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 (nm). The study of living cells may require time resolution from days, for example, when 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 many technological improvements 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. In addition, vast improvements have been made in the biological aspects of specimen preparation. These advancements on both fronts have fostered many more applications of the microscope in biomedical research.

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. Here the specimen requires very little preparation, and is usually held close to the eye in the hand. Focussing 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 artifacts and aberrations. For example, **chromatic aberration** occurs when different 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 van Leeuwenhoek and Hooke, for example. All modern lenses are now *corrected* to some degree in order to avoid this problem.

The main components of the compound light microscope include a **light source** that is focussed 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 focussed by the objective lens into the eyepiece lens. The image is either viewed directly by eye in the eyepiece or it 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 – an **upright** or an **inverted microscope** (Fig. 4.3). 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. Moreover, the condenser and light source can often be swung out of the light path. 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.

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, lasers or light-emitting diodes (LEDs).

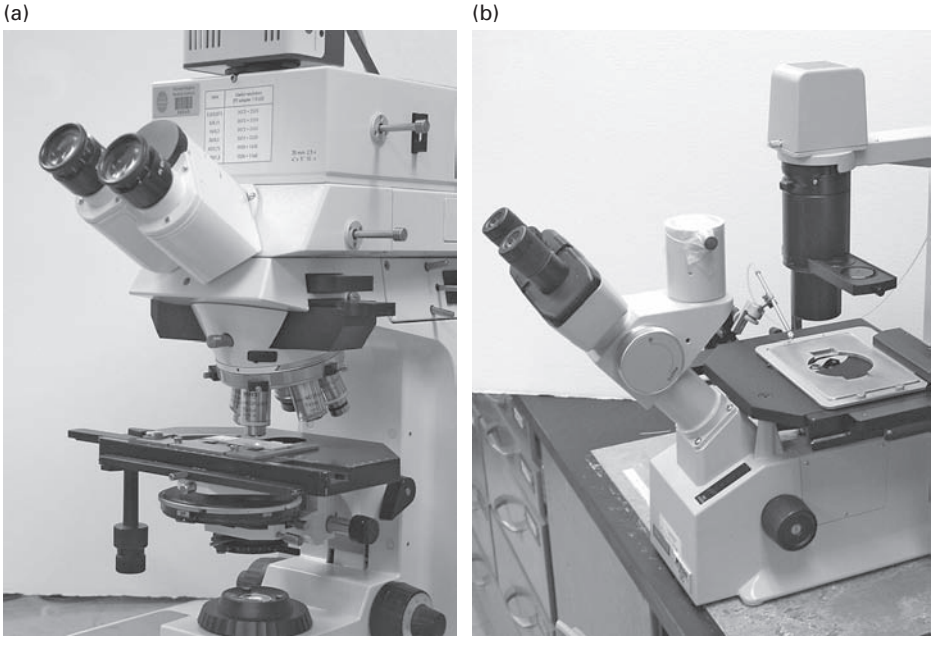

Fig. 4.3 Two basic types of compound light microscope. An upright light microscope (a) and an inverted light microscope (b). Note how there is more room available on the stage of the inverted microscope (b). This instrument is set up for microinjection with a needle holder to the left of the stage.

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 focussing (Fig. 4.3). The condenser focusses 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 (**Koehler 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. 4.4). 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 focussing the specimen or for inverted microscopes, the objectives themselves are moved and the stage remains fixed. There are usually coarse and fine focussing 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 by a stepper motor attached to the fine focus control of the microscope, and controlled by a computer.

The objective lens is responsible for producing the magnified image, and can be the most expensive component of the light microscope (Fig. 4.4). Objectives are available

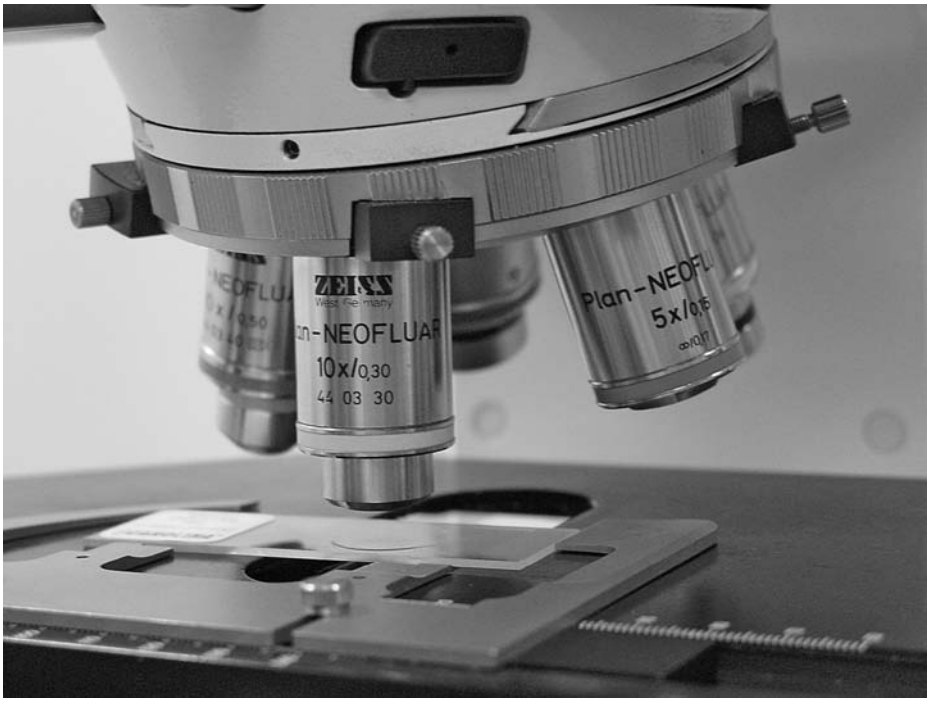

Fig. 4.4 The objective lens. A selection of objective lenses mounted on an upright research grade compound light microscope. From the inscription on the two lenses in focus they are relatively low magnification 10× and 5× of numerical aperture (NA) 0.3 and 0.16 respectively. Both lenses are Plan Neofluar, 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.

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 more-specialised optical properties of the lens (Section 4.2.3). In addition, lens corrections for optical artifacts 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.

Objective lenses can either be **dry** (glass/air/coverslip) or **immersion** lenses (glass/oil or water/coverslip). As a rule of thumb, most objectives below 40× are air (dry) objectives, and those of 40× and above are immersion (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. Dipping lenses are specially designed to work without a coverslip, and are dipped directly into water or tissue culture medium. These are used for physiological experiments.

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.

Table 4.1 **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

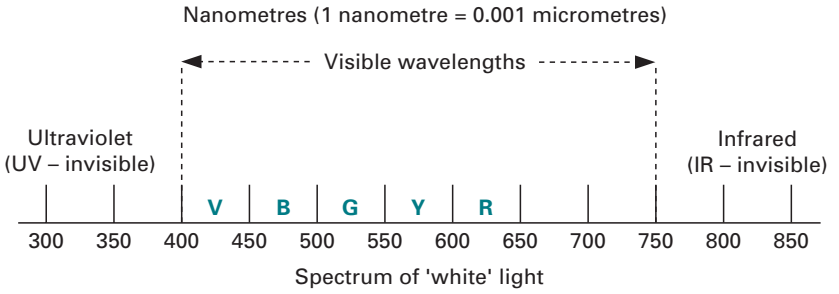

Fig. 4.5 The visible spectrum – the spectrum of white light visible to the human eye. Our eyes are able to detect colour in the visible wavelengths of the spectrum, usually in the region between 400 nm (violet) and 750 nm (red). Most modern electronic detectors are sensitive beyond the visible spectrum of the human eye.

Resolution varies inversely with NA, which implies 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 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 achieved by a lens is a measure of its ability to distinguish between two objects in the specimen. The shorter the wavelengths of illuminating light the higher the resolving power of the microscope (Fig. 4.5). 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 (UV) as a light source the resolution can be improved to 100 nm because of the shorter 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. The lateral resolution is usually higher than the axial resolution for any given objective lens (Table 4.1).

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

Fig. 4.6 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 fruit flies. The large objective lens above the specimen can be rotated to zoom the image.

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 detectors employed on modern light microscopes. These are area detectors that actual 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).

Stereomicroscopes

A second type of light microscope, the **stereomicroscope**, is used for the observation of the surfaces of large specimens (Fig. 4.6). The microscope is used when 3D

information is required, for example for the routine observation of whole organisms, for example for screening through vials of fruit flies. Stereomicroscopes are useful for micromanipulation and 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 (Section 4.2.3). These different light angles serve to add contrast or shadow relief to the images.

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 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 #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 optically 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.

The goal of a specimen preparation protocol is to render the tissue of interest into a form for optimal study in the microscope. This usually involves placing the specimen in a suitable medium on a glass slide with a coverslip over it. Such protocols can be relatively simple or they may involve a lengthy series of many steps that take several days to complete (Table 4.2). An example of a simple protocol would be taking an aliquot of a biological preparation, for example, isolating living spermatozoa into a balanced salt solution, placing an aliquot of it onto a slide and gently placing a clean coverslip onto the top. The entire protocol would take less than a minute. The coverslip is sealed to the glass slide in some way, for example, using nail polish for dead cells or perhaps a mixture of beeswax and Vaseline for living cells. Shear forces from the movement of the coverslip over the glass slide can cause damage to the specimen or the objective lens. In order to keep cells alive on the stage of the microscope, they are usually mounted in some form of chamber, and if necessary heated.

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

Table 4.2 **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
5. Wash 4× in buffer
6. Incubate in secondary antibody e.g. fluorescein-labelled rabbit anti-mouse
7. Wash 4× in buffer
8. Incubate in anti-fade reagent e.g. Vectashield
9. Mount on slide with a coverslip
10. View using epifluorescence microscopy

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, or 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 infiltrate 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, 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, 4.8a). This technique has traditionally been used for viewing the outlines of objects in liquid media such as living spermatozoa, microorganisms or 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 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.

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 since it gives superior signal-to-noise ratios (typically white on a black background) for many applications (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, and more recently lasers and LED sources, which emit from the UV into the red wavelengths (Fig. 4.5). A specific wavelength of light is used to excite a fluorescent

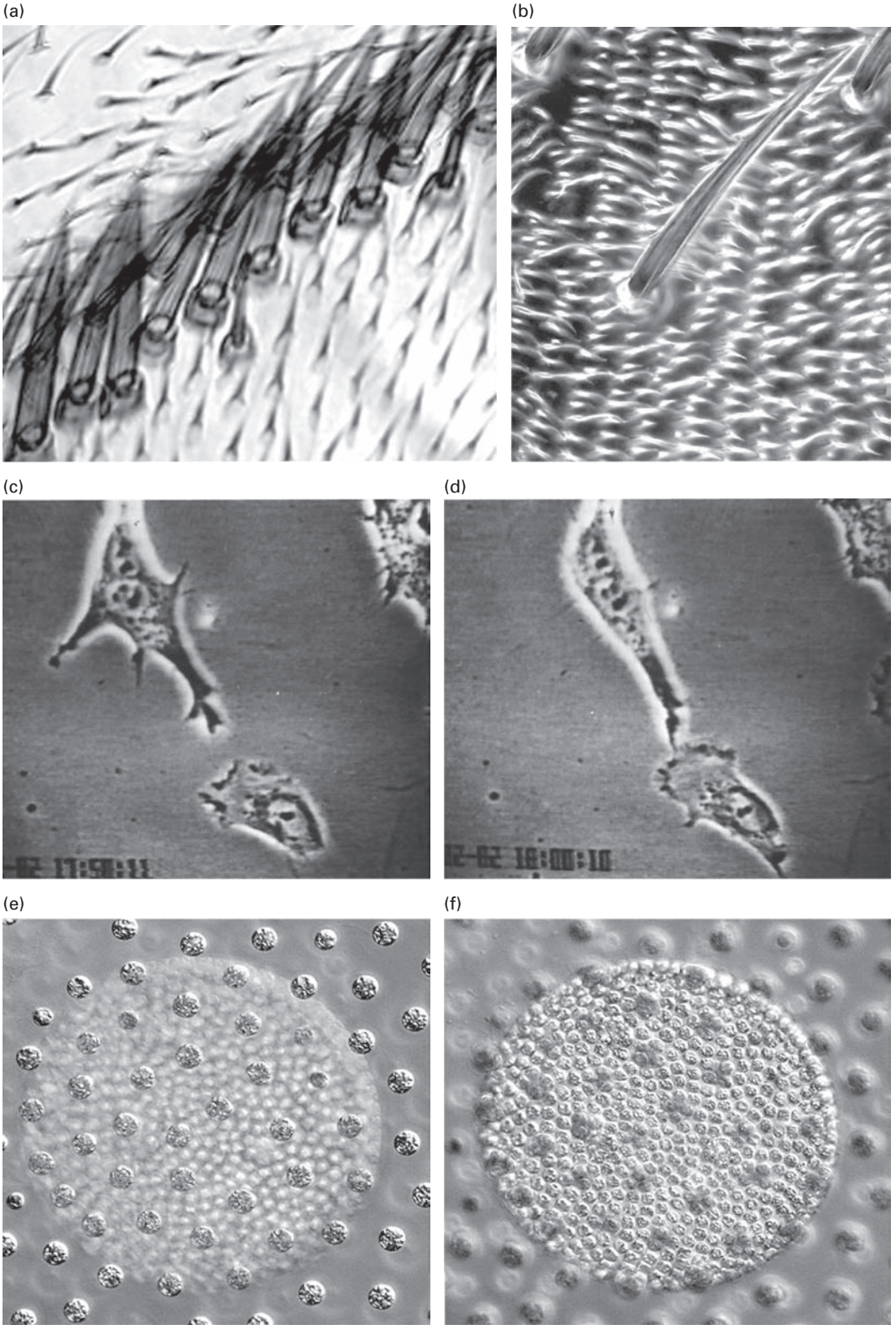

Fig. 4.7 Contrast methods in the light microscope. (a) and (b) A comparison of brightfield (a) and darkfield images (b). Here the sensory bristles on the surface of the fly appear dark on a white background in the bright

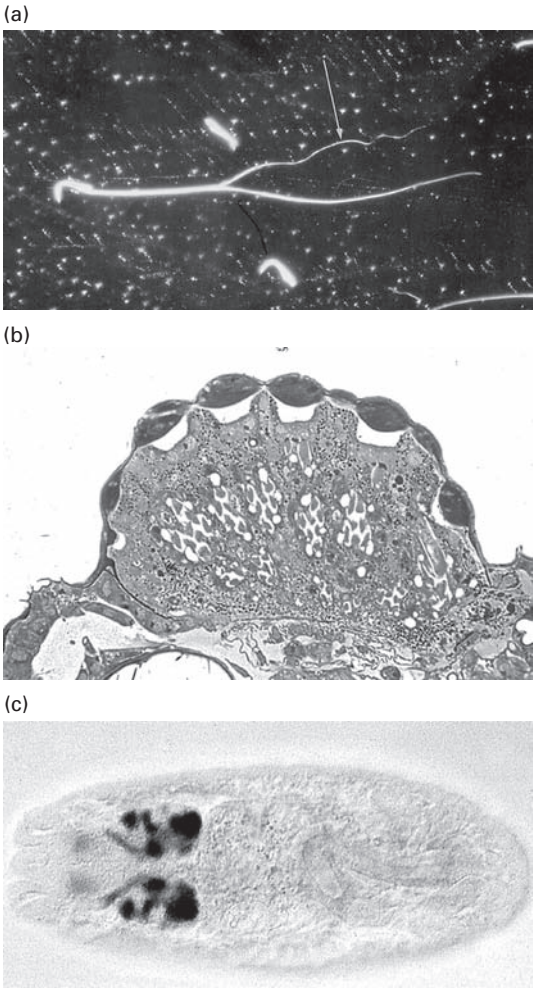

Fig. 4.8 Examples of different preparations in the light microscope. (a) Darkfield image of rat sperm preparation. An aliquot was collected from an experimental protocol in order to assess the amount of damage incurred during sonication of a population of spermatozoa. Many sperm heads can be seen in 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 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 context of the anatomy.

Caption for Fig. 4.7 (*cont.*)
field image (a) and white on a black background in a dark field 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 (DIC) image of two focal planes of the multicellular alga *Volvox*. (Images (e) and (f) courtesy of Michael Davidson, Florida State University, USA.)

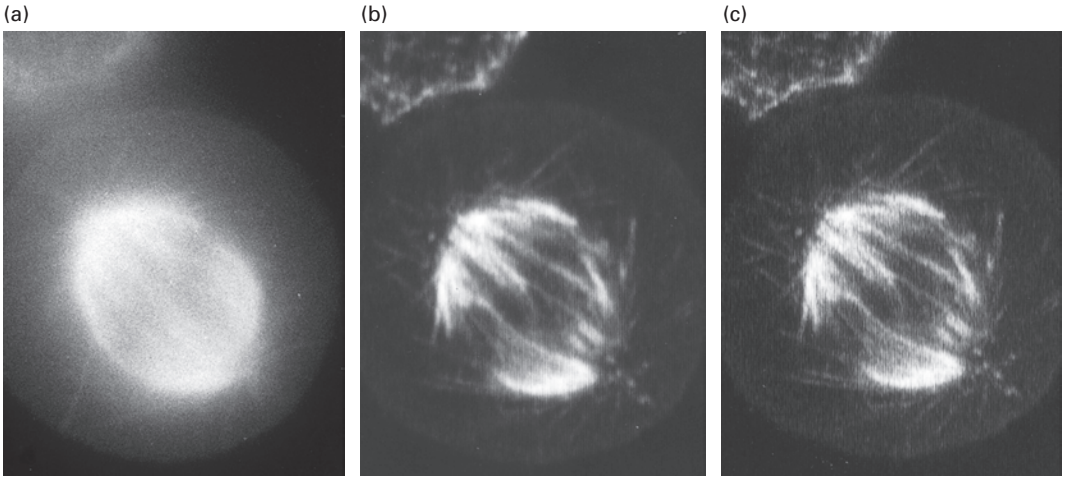

Fig. 4.9 Fluorescence microscopy. Comparison of epifluorescence and confocal fluorescence imaging of a mitotic spindle labelled using indirect immunofluorescence labelling with anti-tubulin (primary antibody) and a fluorescently labelled secondary antibody. The specimen was imaged using (a) conventional 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). (Images kindly provided by Brad Amos, University of Cambridge, UK.)

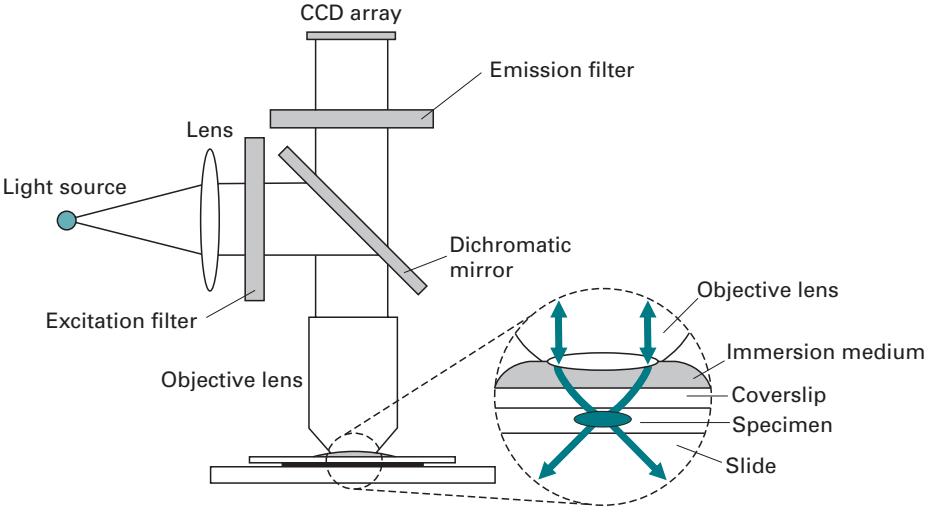

Fig. 4.10 Epifluorescence microscopy. Light from a xenon or mercury arc lamp (Light source) passes through a lens and the excitation filter and reflects off the dichromatic mirror into the objective lens. The objective lens focusses the light at the specimen via the immersion medium (usually immersion oil) and the glass coverslip (see insert). 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 only allows light of the specific emission wavelength of the fluorochrome of interest to pass through to the CCD array, where an image is formed.

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**, a **dichromatic mirror** (often called a **dichroic**) and a **barrier** filter, mounted in a single 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 4.3).

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 exciter 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.

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.

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 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, one can use 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 4.2).

Table 4.3 **Table of fluorophores**

Dye	Excitation max. (nm)	Emission max. (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
Feulgen	570	625
Calcium indicators		
Fluo-3	506	526
Calcium green	506	533
Reporter molecules		
CFP (cyan fluorescent protein)	443/445	475/503
GFP (green fluorescent protein)	395/489	509
YFP (yellow fluorescent protein)	514	527
DsRed	558	583
Mitochondria		
JC-1	514	529
Rhodamine 123	507	529

Additional methods are available for amplifying the fluorescence signal in the specimen, for example using the tyramide amplification method or at the microscope, for example by using a more sensitive detector.

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 4.3). 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, rhodamine, the Alexa range of dyes and the cyanine dyes. A recent addition to the extensive list of probes for imaging is the **quantum dot**. Quantum dots do not fluoresce per se but they rather are nanocrystals of different sizes 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 and 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 physically sectioning 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), micro computerised tomography (CT) scanning or optical projection tomography. However, currently by far the most prevalent method is using some form of confocal or associated microscopical approach.

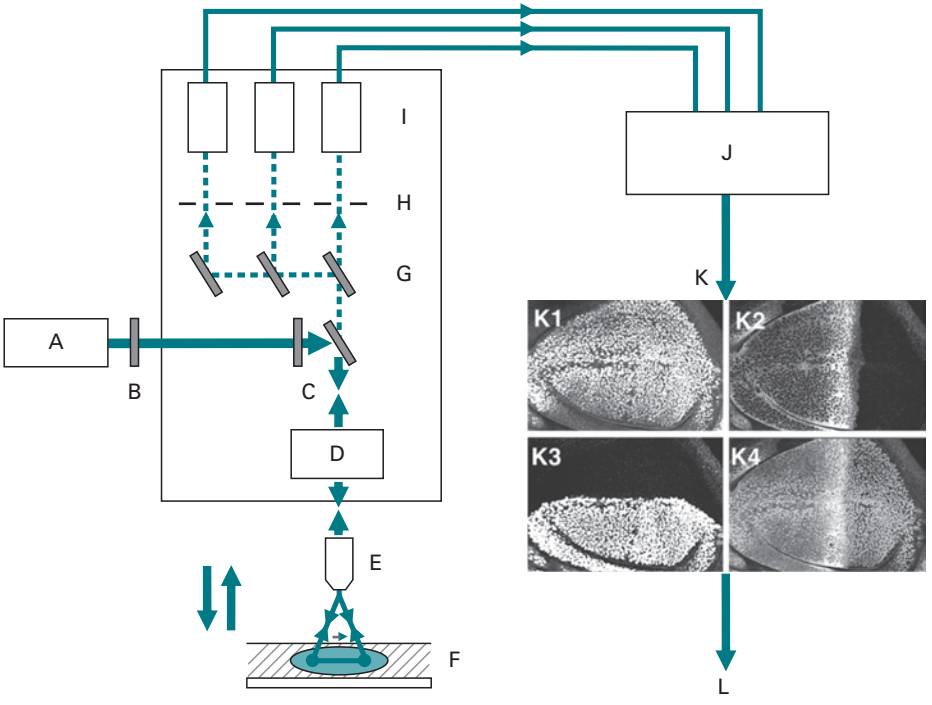

Fig. 4.11 Information flow in a generic LSCM. Light from the laser (A) passes through a neutral density filter (B) and an exciter filter (C) on its way to the scanning unit (D). The scanning unit produces a scanned beam at the back focal plane of the objective lens (E) which focusses the light at the specimen (F). The specimen is scanned in the *X* and the *Y* directions in a raster pattern and in the *Z* direction by fine focussing (arrows). 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 acquisition 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 changing magnification. Images are written to the hard disk of the computer or exported to various devices for viewing, hardcopy production or archiving (L).

4.3.1 Laser scanning confocal microscopes (LSCM)

Optical sections are produced in the **laser scanning confocal microscope** by scanning the specimen point by point with a laser beam focussed 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

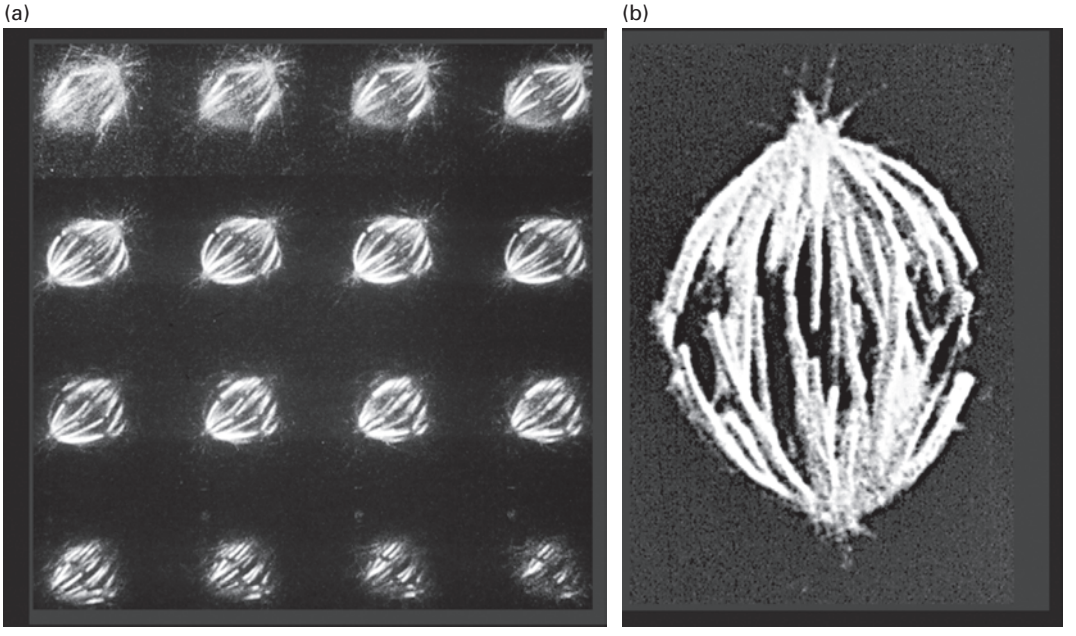

Fig. 4.12 Computer 3D reconstruction of confocal images. (a) Sixteen serial optical sections collected at 0.3 μm intervals through a mitotic spindle of a PtK1 cell stained with anti-tubulin and a second rhodamine-labelled antibody. Using the *Z*-series macro program a preset number of frames can be summed, and the images transferred into a file on the hard disk. The stepper motor moves the fine focus control of the microscope by a preset 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 movies of the structure rotating in 3D.

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 3D representation of the image (**3D reconstruction**).

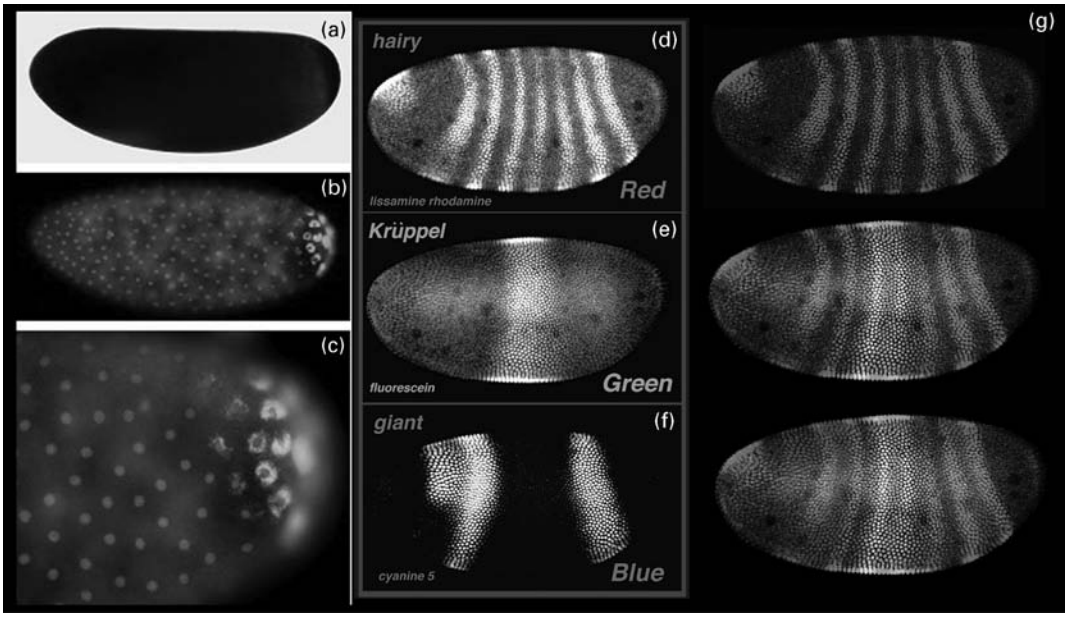

Fig. 4.13 Optical sectioning. Optical sections produced using laser scanning confocal microscopy. Comparison of alkaline phosphatase (a) and tyramide-amplified detection of mRNAs (b,c). Staining patterns obtained using DIG-labelled antisense probes directed against the CG14217 mRNAs, through conventional AP-based detection (a) or tyramide signal amplification (b), using tyramide–Alexa Fluor 488 (green fluorescence). Close-up images of tyramide-amplified samples are also shown (c). In (b) and (c), nuclei were labelled in red with propidium iodide. (d, e, f, g) Triple-labelled *Drosophila* embryo at the cellular blastoderm stage. The images were produced using an air-cooled 25 mW krypton argon laser which has three major lines at 488 nm (blue), 568 nm (yellow) and 647 nm (red). The three fluorochromes used were fluorescein (exc. 496 nm; em. 518 nm), lissamine rhodamine (exc. 572 nm; em. 590 nm) and cyanine 5 (exc. 649 nm; em. 666 nm). The images were collected simultaneously as single optical sections into the red, the green and the blue channels respectively, and merged as a three-colour (red/green/blue) image (Fig. 4.11). The image shows the expression of three genes: *hairy* (in red), *Krüppel* (in green) and *giant* (in blue). Regions of overlap of gene expression appear as an additive colour in the image, for example, the two yellow stripes of *hairy* expression in the *Krüppel* domain (g). (Images (a), (b) and (c) were kindly provided by Henry Krause, University of Toronto, Canada.) (See also colour plate.)

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 also 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 routinely image three or four different wavelengths simultaneously.

The scanning speed of most laser scanning systems is around one full frame per second. This is designed for collecting images from fixed and brightly labelled fluorescent specimens. Such scan speeds are not optimal for living specimens, and laser scanning instruments are available that scan at faster rates for more optimal live cell imaging. In addition to point scanning, swept field scanning rapidly moves a μm-thin beam of light horizontally and vertically through the specimen.

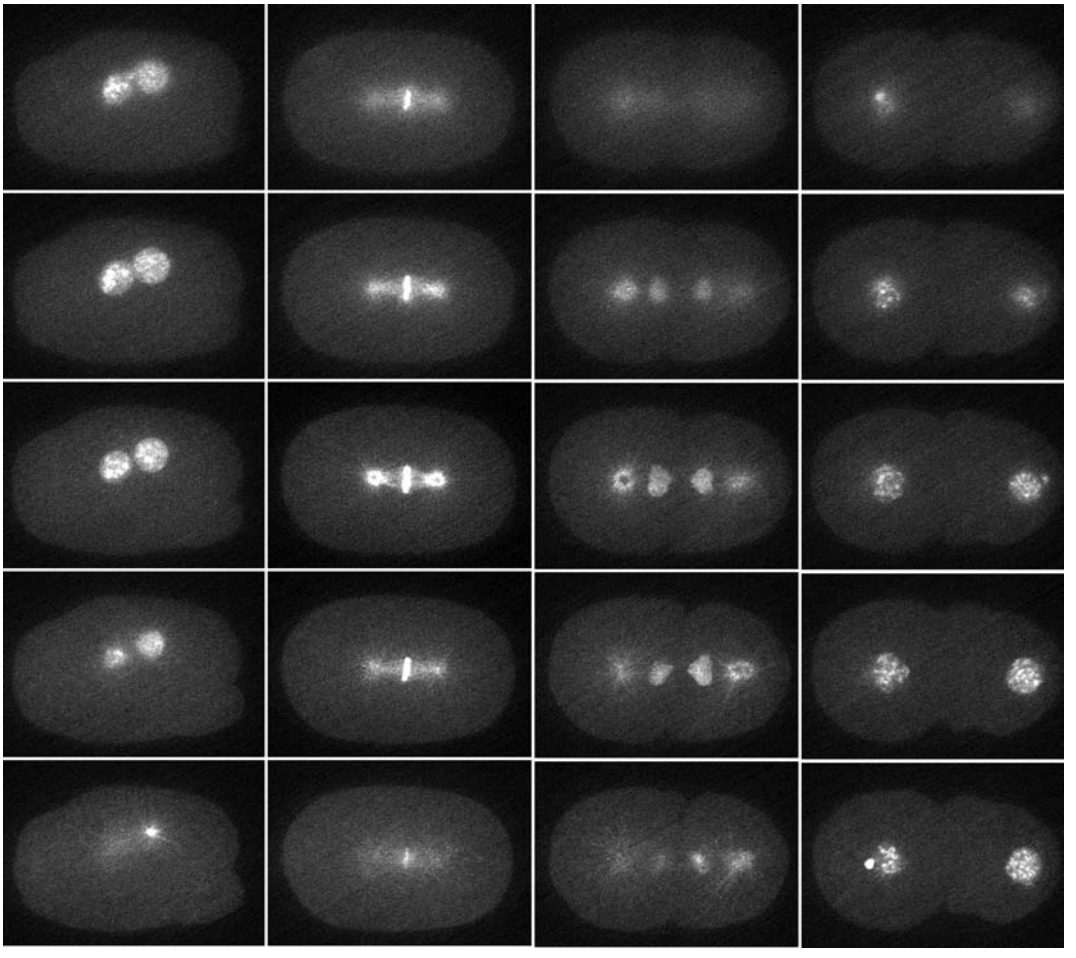

Fig. 4.14 Time-lapse imaging of *Caenorhabditis elegans* development. *Z*-series were collected every 90 s of a developing *C. elegans* embryo genetically labelled with GFP-histone (nuclear material) and GFP-alpha tubulin (microtubules – cytoskeleton) and imaged with a spinning disk confocal microscope. Each column consists of six optical sections collected 2 μm apart, and the columns are separated by 90 s increments of time. (Image kindly provided by Dr Kevin O'Connell, National Institutes of Health, USA.)

4.3.2 Spinning disk confocal microscopes

The **spinning disk confocal microscope** employs a different scanning system from 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 disk microscopes have been improved significantly by the addition of laser light sources and high-quality CCD detectors to the instrument. Spinning disk 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 (Fig. 4.14).

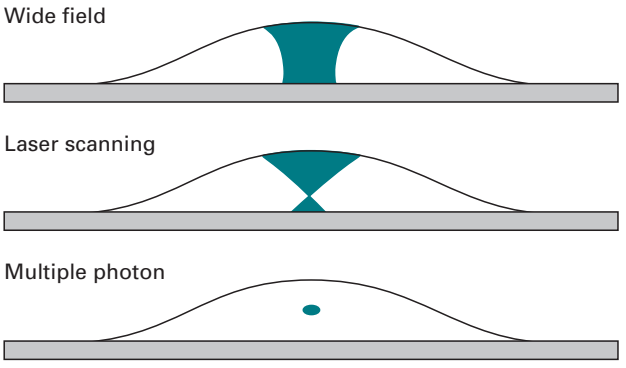

Fig. 4.15 Illumination in a wide field, a confocal and a multiple photon microscope. The diagram shows a schematic of a side view of a fluorescently labelled cell on a coverslip. The shaded green areas in each cell represent the volume of fluorescent excitation produced by each of the different microscopes 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 only occurs at the point of focus where the light flux is high enough.

4.3.3 Multiple photon microscopes

The **multiple photon microscope** has evolved from the confocal microscope. In fact, many of the instruments use the same scanning system as the LSCM. 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 focussing the laser beam in the specimen since multiple photon excitation of a fluorophore only occurs where energy levels are high enough – statistically confined to the point of focus of the objective lens (Fig. 4.15).

Since red light is used in multiple photon microscopes, optical sections can be collected from deeper within the specimen than those collected with the LSCM. Multiple photon imaging is generally chosen for imaging fluorescently labelled living cells because red light is less damaging to living cells than the shorter wavelengths usually employed by 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 over exciting (**photobleaching**) the fluorescent probe and causing photodamage to the specimen itself (Fig. 4.15).

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

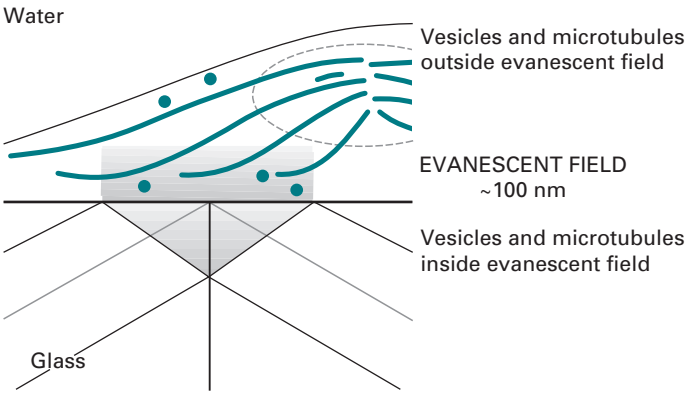

Fig. 4.16 Total internal reflection microscopy (TIRF). A 100-nm thick region of excitation is produced at the glass–water interface when illumination conditions are right for internal reflection. In this example only those vesicles and microtubules within the evanescent field will contribute to the fluorescence image at 100 nm *Z*-resolution.

measured by imaging a point source, for example, a small sub-resolution fluorescent bead (0.1μm), 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.

4.3.5 Total internal reflection microscopy

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. 4.16), 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.

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. 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 artifacts

The only way to eliminate artifacts 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. 4.5). The levels of light used for imaging must not compromise the cells. This is achieved using extremely low levels of light, using relatively bright fluorescent dyes and extremely sensitive photodetectors. Moreover, the viability of cells may also depend upon the cellular compartment that 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. 4.14). Usually a shutter arrangement is placed in the light path so that the shutter is only open when an image is collected in order to reduce the amount of light energy impacting the cells. When the images are played back in real time, a movie 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.

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 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 including viewing whole transgenic animals using fluorescence stereomicroscopes (Table 4.3). 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 co-enzymes 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. These probes have revolutionised the ability to image living cells and tissues using light microscopy (Fig. 4.17, see also colour section).

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. 4.18). 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

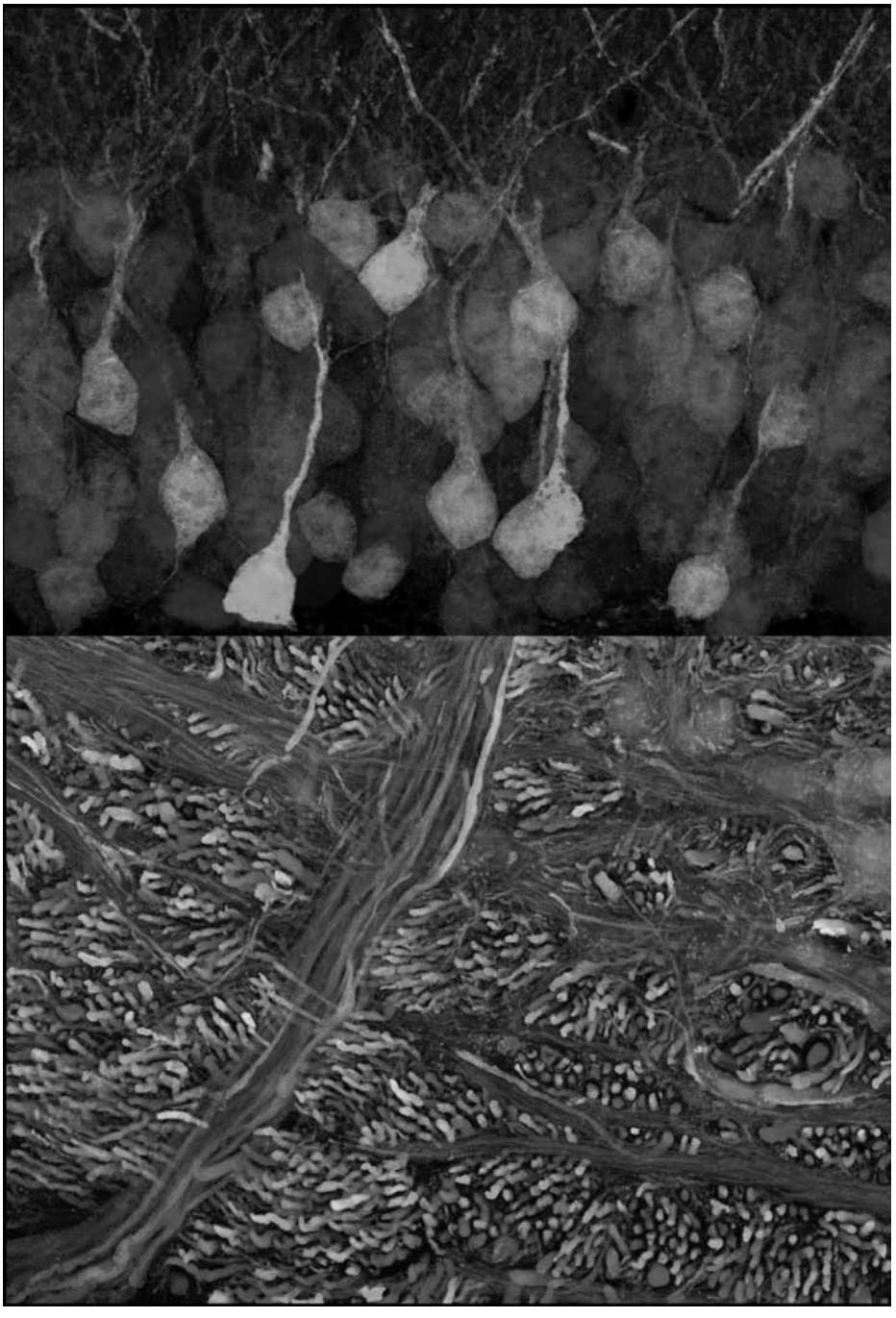

Fig. 4.17 Multiple labelling in a living mouse brain using the 'Brainbow' technique. Unique colour combinations in individual neurons are achieved by the relative levels of three or more fluorescent proteins (XFPs). The images are collected using a multi-channel laser scanning confocal microscope. Up to 90 different colours (neurons) can be distinguished using this technique. Top image, hippocampus; bottom image, brainstem. (Image courtesy of Jeff Lichtman, Harvard University, USA.) (See also colour plate.)

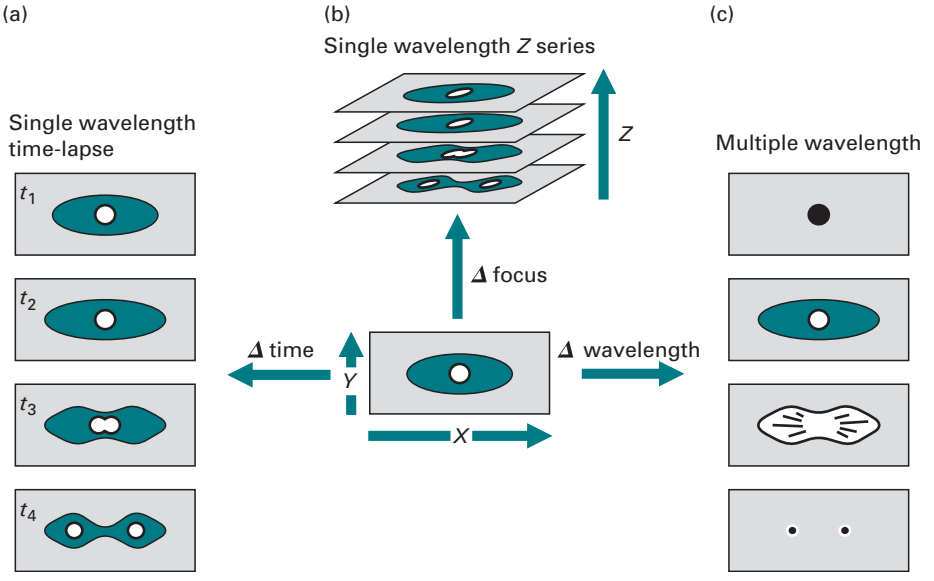

Fig. 4.18 Multidimensional imaging. (a) Single wavelength excitation over time or time-lapse *X*,*Y* imaging; (b) *Z*-series or *X*,*Y*,*Z* imaging. The combination of (a) and (b) is 4D imaging. (c) Multiple wavelength imaging. The combination of (a) and (b) and (c) is 5D imaging.

displayed as series of *Z*-projections or stereo movies. 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 MEASURING CELLULAR DYNAMICS

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 qualitatively viewing 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 were 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.

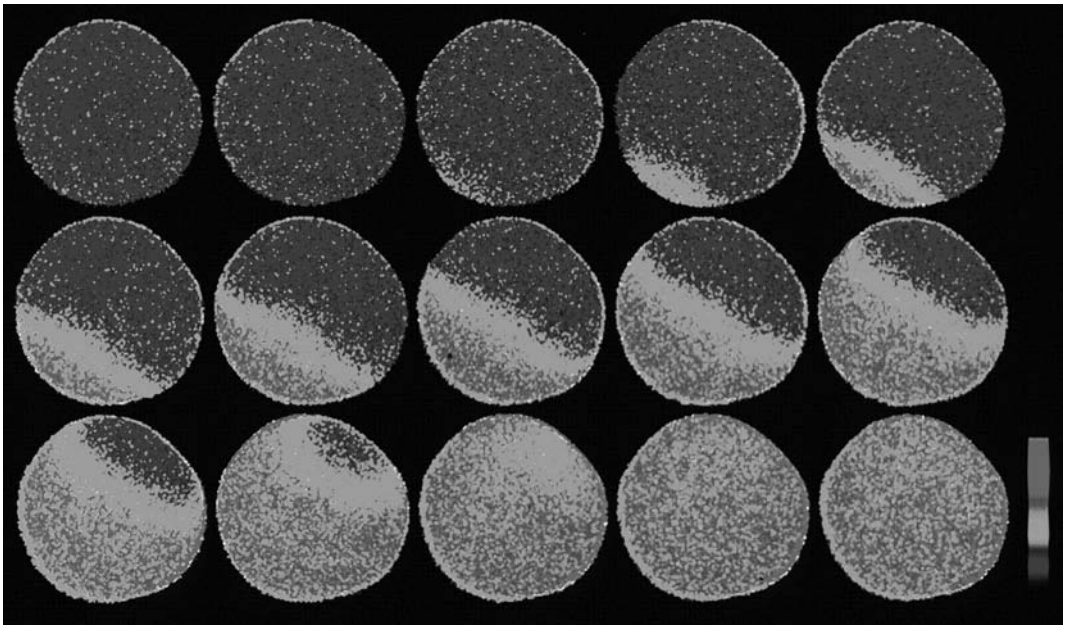

Fig. 4.19 Calcium imaging in living cells. A fertilisation-induced calcium wave in the egg of the starfish. The egg was microinjected with the calcium-sensitive fluorescent dye fluo-3 and subsequently fertilised by the addition of sperm during observation using time-lapse confocal microscopy with a 40× water immersion lens and a LSCM. An optical section located near the egg equator was collected every 4 s using the normal scan mode accumulated for two frames, and afterwards the images were corrected for offset and ratioed by linearly dividing the initial pre-fertilisation image into each successive frame of the time-lapse run. The ratioed images were then prepared as a montage and outputted with a pseudocolour look-up table in which blue regions represent low ratios and free calcium levels, and red areas depict high ratios and free calcium levels. Note that the wave sweeps through the entire ooplasm, rather than being cortically restricted. (Image kindly provided by Steve Stricker, University of New Mexico, USA.) (See also colour plate.)

The rapid development of fluorescence microscopy together with digital imaging and, above all, the development of new fluorescent probes of biological activity have brought a new level of sophistication into quantitative imaging. Most of the measurements are based on the ability to measure accurately the brightness of and the wavelength emitted from a fluorescent probe within a sample using a digital imaging system. This is also the basis of **flow cytometry**, which measures the brightness of each cell in 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**.

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, for example fluo-3, whose fluorescence increases in proportion to the amount of free calcium in the cell (Fig. 4.19, see also 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

artifacts 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 incorporate into macromolecular structures over time. This makes the structures accessible to time-lapse imaging using fluorescence microscopy. Such methods can lead to high backgrounds, and can be difficult to interpret. In addition to optical sectioning methods 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 observation and recording of the subsequent movement of undamaged fluorophores into the bleached zone gives a measure of molecular mobility. This enables biochemical analysis within the living cell. A second technique related to FRAP, **photoactivation**, uses a probe whose fluorescence can be induced by a flash of short wavelength (UV) light. The method depends upon 'caged' fluorescent probes that are locally activated (uncaged) by a pulse of UV light. Alternatively variants of GFP can be expressed in cells and selectively photoactivated. 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 while microinjecting fluorescently labelled proteins into living cells. Basically, when a really low concentration of fluorescently labelled protein is injected into cells, 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. 4.20). 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 only occur when two fluorophore molecules are very close to one another, at a distance of 6 nm or less.

An example of a FRET experiment would be to use spectral variants of GFP (Fig. 4.20). 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 only be observed under the excitation conditions of CFP 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.

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 a method used for detecting multiple fluorophores with different fluorescent lifetimes and overlapping emission spectra.

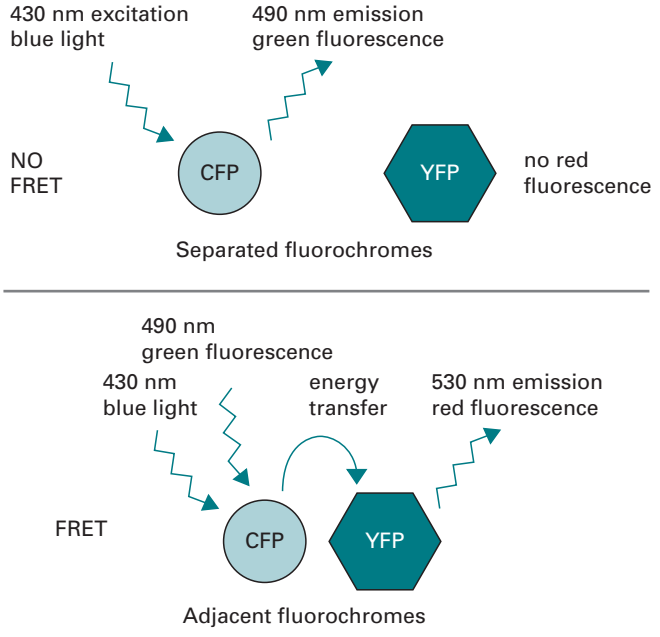

Fig. 4.20 Fluorescence resonance energy transfer (FRET). In the upper example (NO FRET) the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) are not close enough for FRET to occur (more than 60 nm separation). Here excitation with the 430 nm blue light results in the green 490 nm emission of the CFP only. In contrast, in the lower example (FRET), the CFP and YFP are close enough for 'energy transfer' or FRET to occur (closer than 6 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.

4.6 THE ELECTRON MICROSCOPE (EM)

4.6.1 Principles

Electron microscopy is used when the greatest resolution is required, and when the living state can be ignored. The images produced in an electron microscope 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 volts (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**). Focussing of the electron beam is achieved by changing the voltage across the electromagnetic lenses. When the electron beam passes through the specimen some of the electrons are scattered while others are focussed 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 made by collecting two images at 8–10° tilt angles. Such images are useful in assessing the 3D relationships of organelles within cells when viewed in a stereoscope or with a digital stereo projection 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. 4.21).

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 only be produced and focussed 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 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 artifacts 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. 4.21).

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

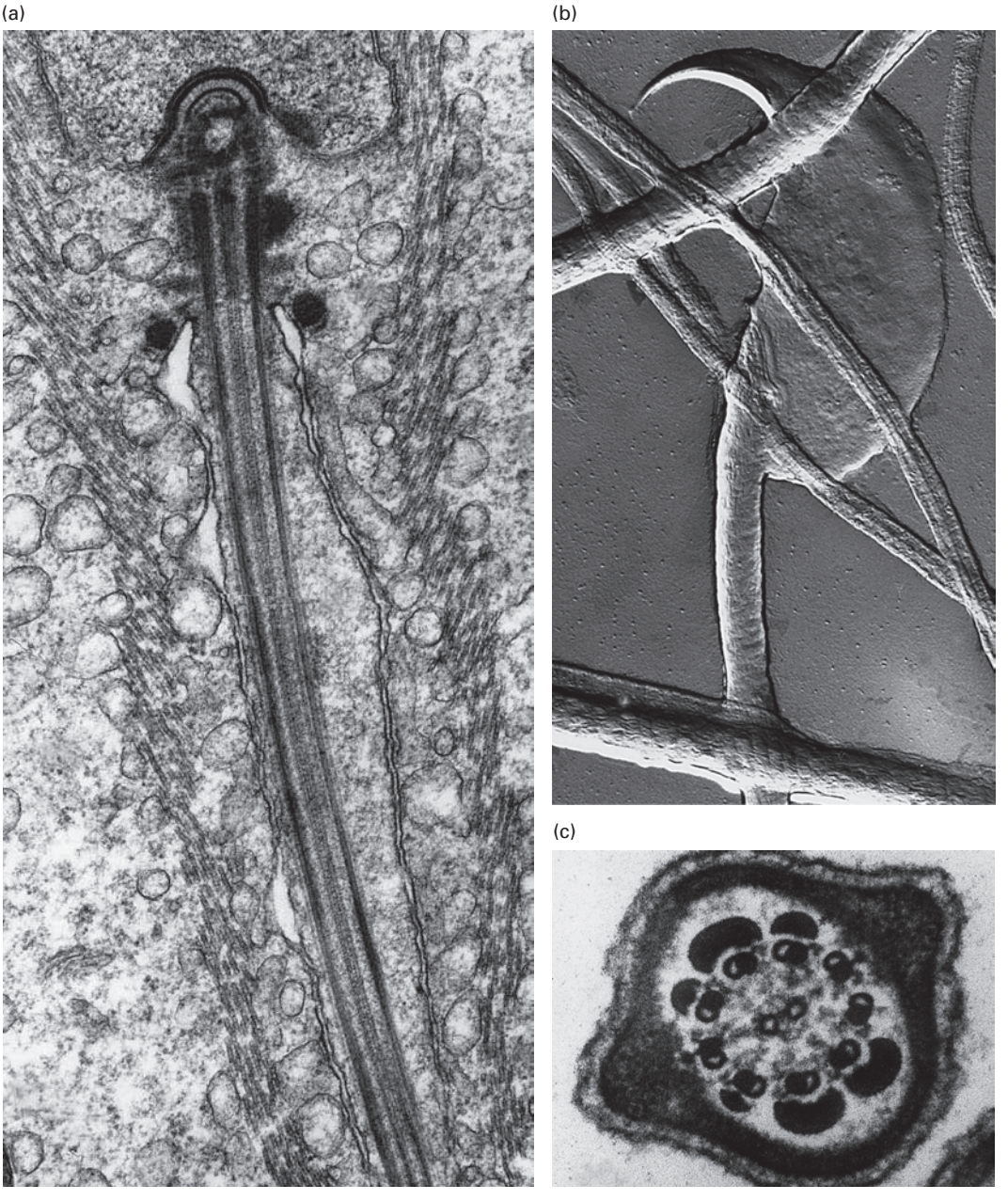

Fig. 4.21 Transmission electron microscopy (TEM). (a) and (c) Ultrathin Epon sections (60 nm thick) of developing rat sperm cells stained with uranyl acetate and lead citrate. (b) Carbon surface replica of a mouse sperm preparation.

of approximately 60 nm. The ribbons of sections are floated onto the surface of 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.

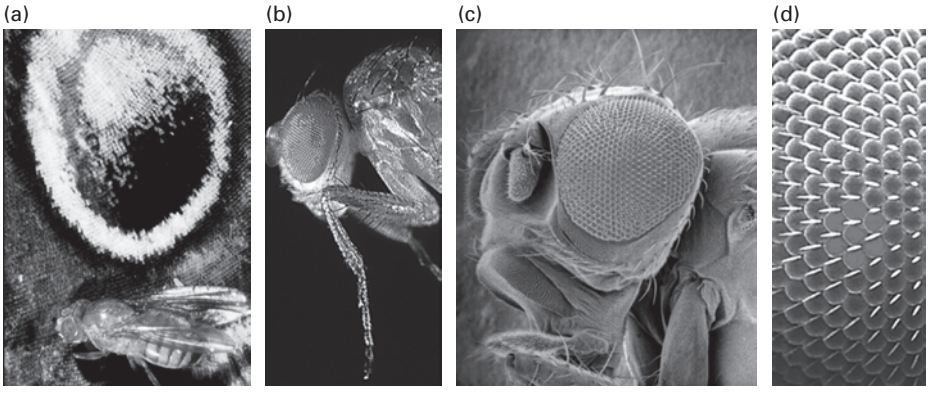

Fig. 4.22 Imaging surfaces using the light microscope (stereomicroscope) and the electron microscope (scanning electron microscope). Images produced using the stereomicroscope (a) and (b) and the scanning electron microscope (c) and (d). A stereomicroscope view of a fly (*Drosophila melanogaster*) on a butterfly wing (*Precis coenia*) (a) zoomed in to view the head region of the red-eyed fly (b). SEM image of a similar region of the fly's head (c) and zoomed more to view the individual ommatidia of the eye (d). Note that the stereomicroscope images can be viewed in real colour whereas those produced using the SEM are in greyscale. Colour can only be added to EM images digitally (d). (Images (b), (c) and (d) kindly provided by Georg Halder, MD Anderson Medical Centre, Houston, USA.) (See also colour plate.)

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 artifacts of drying. The specimens are then mounted onto a special metal holder or **stub** and coated with a thin layer of gold before viewing in the SEM (Fig. 4.22, see also colour section). Surfaces can also be viewed in the TEM using either negative stains or carbon replicas of air-dried specimens (Fig. 4.21).

Immuno-EM methods allow the localisation of molecules within the cellular microenvironment for TEM and on the cell surface for SEM (Fig. 4.23). 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 (10 nm) are used. Multiple labelling can be achieved using different sizes of gold particles 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 Electron tomography

New methods of fixation continue to be developed in an attempt to avoid the artifacts 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 preserved. Many of these frozen hydrated samples can be observed directly in the EM or they can be chemically

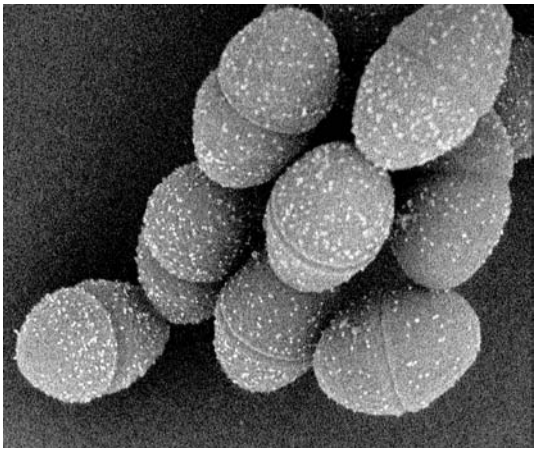

Fig. 4.23 Immunoelectron microscopy. Scanning electron microscope (SEM) image of microbes *Enterococcus faecalis* labelled with 10 nm collidal gold for the surface adhesion protein 'aggregation substance'. This protein facilitates exchange of DNA during conjugation. The gold labels appear as white dots on the surface of the bacteria. (Image kindly provided by the late Stan Erlandsen, University of Minnesota, USA.)

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 typically rapidly frozen, fixed by freeze substitution and embedded in epoxy resin. Thick 200 nm sections are cut and imaged in the TEM equipped with a tilting stage. A typical tilt series of 100 or so images is collected in a digital form and exported to a computer reconstruction program for analysis. By using electron tomography, a 2D digital EM image is converted into a high-resolution 3D representation of the specimen (Fig. 4.24, see also colour section). The method is especially useful for imaging the fine connections within cells especially the cytoskeleton and nuclear pores and elucidating the surface structures of viruses.

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. 4.25). The integrated approach also addresses the problem of artifacts. Probes are now available that are fluorescent in the light microscope and are electron dense in the EM.

4.7 IMAGE ARCHIVING

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

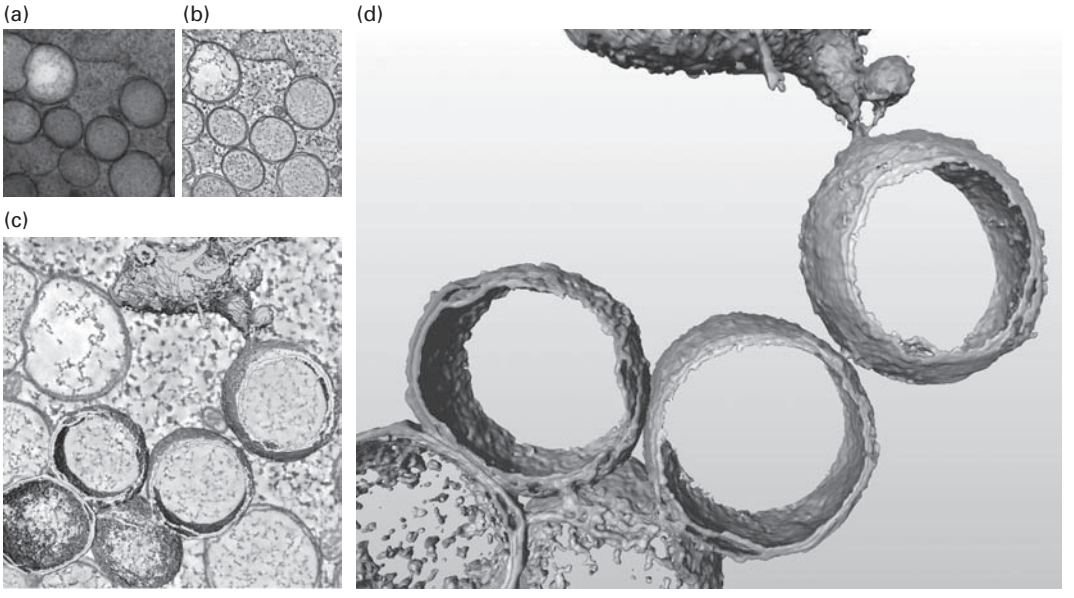

Fig. 4.24 Electron tomography revealing the interconnected nature of SARS–*Coronavirus*-induced double-membrane vesicles. Monkey kidney cells were infected with SARS–*Coronavirus* in a biosafety level-3 laboratory and pre-fixed using 3% paraformaldehyde at 7 h post-infection. Subsequently, the cells were rapidly frozen by plunge-freezing and freeze substitution was performed at low temperature, using osmium tetraoxide and uranyl acetate in acetone to optimally preserve cellular ultrastructure and gain maximal contrast. After washing with pure acetone at room temperature, the samples were embedded in an epoxy resin and polymerised at 60 °C for 2 days. Using an ultramicrotome, 200-nm thick sections were cut, placed on a 100 mesh EM grid, and used for electron tomography. To facilitate the image alignment that is required for the final 3D reconstruction, a suspension of 10 nm gold particles was layered on top of the sections as fiducial markers (a). Scale bar represents 100 nm. Images were recorded with an FEI T12 transmission electron microscope operating at an acceleration voltage of 120 kV. A tilt series consisted of 131 images recorded using 1° tilt increments between −65° and 65°. For dual-axis tomography, which improves resolution in the *X* and *Y* directions, the specimen was rotated 90° around the *Z*-axis and a second tilt series was recorded. To compute the final electron tomogram, the dual-axis tilt series were aligned by means of the fiducial markers using the IMOD software package. A single tomogram slice through the 3D reconstruction with a digital thickness of 1.2 nm is shown in (b). The 3D surface-rendered reconstruction of viral structures and adjacent cellular features (c) was made by thresholding and subsequent surface rendering using the AMIRA Visualization Package (TGS Europe). The final 3D surface-rendered model (d) shows interconnected double-membrane vesicles (outer membrane, gold; inner membrane, silver) and their connection to an endoplasmic reticulum stack (depicted in bronze). (Images kindly provided by Kevin Knoops and Eric Snijder, Leiden University, The Netherlands.) (See also colour plate.)

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. One such effort is the Open Microscopy Environment (OME). There is a trend for modern microscopes to produce more and more data, especially when multi-dimensional datasets are generated. This trend is continuing with the need to develop automated methods of image analysis for large scale screening of gene expression data from genomic screens.

Table 4.4 **Websites of interest**

http://www.microscopyu.com/
http://www.microscopy.fsu.edu/
http://www.microscopy-analysis.com/
http://www.msa.microscopy.org
http://www.rms.org.uk/index.shtml
http://www.peachpit.com/articles/article.aspx?p=1221827
http://www.openmicroscopy.org
http://swehsc.pharmacy.arizona.edu/exppath/micro/index.html
http://www.itg.uiuc.edu/
http://rsb.info.nih.gov/ij/
http://www.openmicroscopy.org

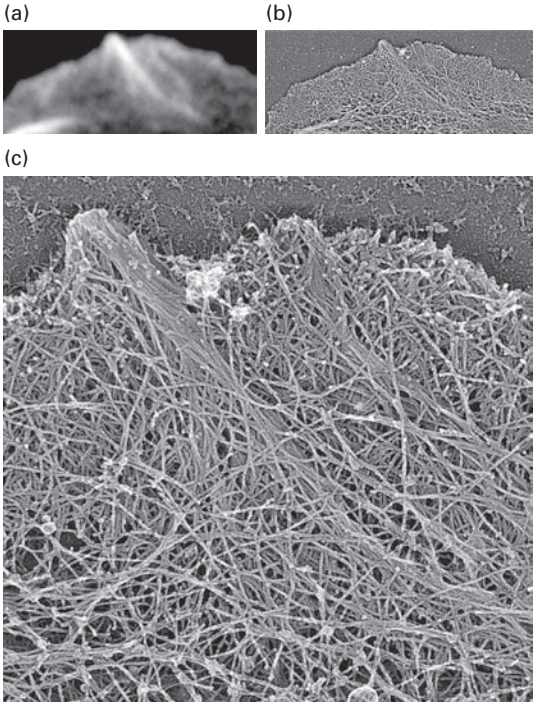

Fig. 4.25 Integrated microscopy. (a) Epifluorescence image and (b) and (c) whole mount TEM at different magnifications of the same cell. The fluorescence image is labelled with rhodamine phalloidin, which stains polymerised actin. A stress fibre at the periphery of the cell appears as a white line in 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, University of Pennsylvania, USA.)

More detailed information on any of the microscopes and their applications in biochemistry and molecular biology can accessed on the World Wide Web. Several websites have been included as starting points for further study (Table 4.4). Should any of these listed websites become out of date, more information on any topic can be accessed using a web search engine. In addition, a comprehensive reference list has been provided for more detailed information (Section 4.8). The field of microscopy continues to be advanced but the basic principles and practices of light and electron microscopy remain unchanged.

4.8 SUGGESTIONS FOR FURTHER READING

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