microscope without first staining the specimen. Many **chemical stains** bind to biological molecules; for example, **hematoxylin** binds to the basic amino acids Arg and Lys in proteins, and **eosin** binds to acidic molecules (such as DNA and the side-chains of the amino acids Asp and Glu). Another way of visualizing specific structures within cells is **cytochemical staining** in which an enzyme catalyzes the production of many molecules of a localized, colored reaction product from a colorless precursor. The colored product can then be seen in the light microscope wherever the enzyme is present. For example, peroxisomes can be visualized by using a cytochemical stain for catalase (Section A2).

Phase-contrast microscopy

When light passes through a living cell, the phase of the light wave is changed according to the **refractive index** of the cell: light passing through a relatively thick or dense part of the cell, such as the nucleus, is retarded; consequently, its **phase** is shifted relative to light that has passed through an adjacent thinner region of the cytoplasm. Both **phase-contrast microscopy** and, in a more complex way, **differential interference contrast microscopy** (or Nomarski interference microscopy), exploit the interference effects produced when the two sets of light waves recombine, thereby creating an image of the cell's structure. As these types of microscopy do not require specimens to be fixed or stained they are useful for examining the structure of larger organelles (nucleus, mitochondria, etc.) in living cells. **Video-enhanced differential interference contrast microscopy** can be used to visualize the movement of organelles within cells, for example along microtubules (Section A2).

Fluorescence microscopy

In fluorescence microscopy, the light microscope is adapted to detect the light emitted by a **fluorescent compound** that is used to stain selectively components within the cell. A chemical is said to be fluorescent if it absorbs light at one wavelength (the **excitation wavelength**) and then emits light at a longer wavelength (the **emission wavelength**). Two commonly used compounds in fluorescent microscopy are **rhodamine** and **Texas red**, which both emit red light, and **fluorescein**, which emits green light. First, an antibody against the antigen of interest (so-called **primary antibody**; Section B6) is added to the specimen. A fluorescent compound is chemically coupled to a **secondary antibody** that recognizes the primary antibody. Then the fluorescently tagged secondary antibody is added to the tissue section or permeabilized cell, and the specimen is illuminated with light at the exciting wavelength (Figure 2). The structures in the specimen to which the antibody has bound can then be visualized.

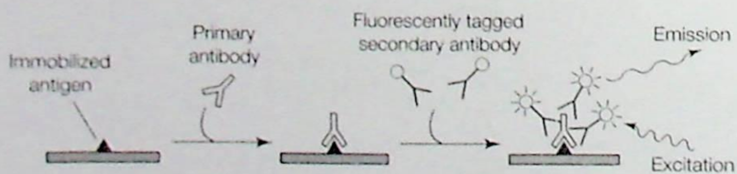

Figure 2. Labeling of protein with a fluorescently tagged antibody for fluorescent microscopy. The primary antibody recognizes the antigen of interest and binds to it in the specimen. Several molecules of the secondary antibody bind to the primary antibody providing amplification of the signal. The secondary antibody is covalently coupled to a fluorescent dye that emits light when illuminated at its excitation wavelength.

Confocal microscopy is a refinement of normal fluorescence microscopy, which produces clearer images of whole cells or larger specimens. In normal fluorescence microscopy, the fluorescent light emitted by the compound comes from molecules above and below the **plane of focus**, blurring the image and making it difficult to determine the actual three-dimensional molecular arrangement. With the confocal microscope, only molecules in the plane of focus fluoresce due to the use of a focused **laser beam** at the exciting wavelength. The laser beam is moved to different parts of the specimen, allowing a series of images to be taken at different depths through the sample. The images are then combined by a computer to provide the complete three-dimensional image. **Deconvolution microscopy** achieves the same image-sharpening effect as confocal microscopy but through a different process.

Green fluorescent protein

Visualization of proteins in living cells has been revolutionized by the discovery of a naturally fluorescent protein found in the **jellyfish** *Aequorea victoria*. In this 238 amino acid protein, called **green fluorescent protein** (GFP), certain amino acid side-chains have spontaneously cyclized to form a green-fluorescing chromophore. Using recombinant DNA techniques (Section I1), the DNA encoding GFP can be tagged on to the DNA sequences encoding other proteins, and then introduced into living cells in culture or into specific cells of a whole animal. Cells containing the introduced gene will then produce the **protein tagged with GFP**, which will fluoresce green under the fluorescence microscope. The localization and movement of the GFP-tagged protein can then be studied in living cells in real time. Multiple variations of GFP have been engineered, which emit light at different wavelengths, e.g. cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), allowing several proteins to be visualized simultaneously in the same cell.

Fluorescence resonance energy transfer (FRET)

Interactions between one protein and another can be monitored by **fluorescence resonance energy transfer** (FRET). The two proteins of interest are each labeled with a **different fluorochrome** (tagged with different variants of GFP, see above), chosen so that the emission spectrum of one fluorochrome overlaps with the excitation spectrum of the other (Figure 3a). If the two proteins come into very **close proximity** (closer than 2 nm), the energy of the absorbed light can be transferred directly from one fluorochrome to the other (Figure 3c). Thus, when the sample is illuminated at the excitation wavelength of the first fluorochrome, light is emitted at the emission wavelength of the second. If the two proteins fail to come into close proximity then no transfer of fluorescence occurs (Figure 3b).

Fluorescence recovery after photobleaching (FRAP)

The **lateral movement** of proteins in a membrane can be visualized through the use of the technique **fluorescence recovery after photobleaching** (**FRAP**). In this technique, a small region of interest in the cell expressing a GFP-labeled protein is exposed to a very intense light pulse from a laser, which destroys (**bleaches**) the fluorescent molecules in that area. The fluorescence of this bleached region is subsequently monitored as a function of time, as other fluorescently labeled proteins move into the bleached region of the membrane. The rate of recovery of the fluorescence depends on the lateral mobility of the fluorescently labeled protein.

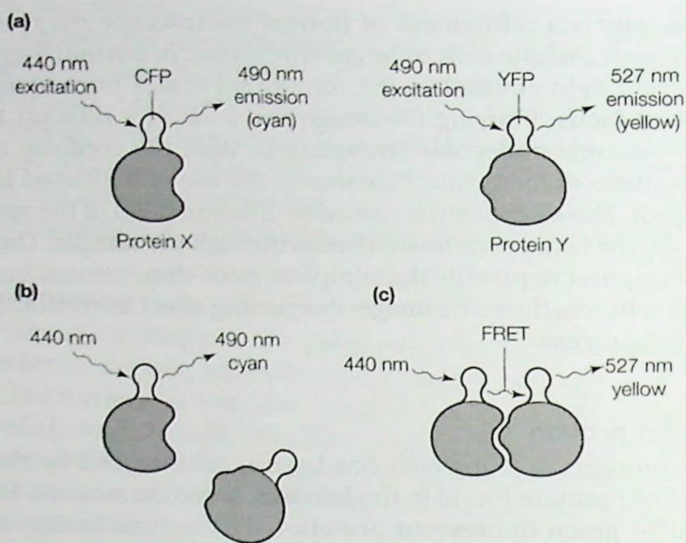

Figure 3. FRET. To determine whether two proteins interact inside the cell, the proteins are first tagged with two different variants of GFP. (a) In this example, protein X is coupled to CFP, which is excited at 440 nm and emits blue light at 490 nm, while protein Y is coupled to YFP, which is excited at 490 nm and emits yellow light at 527 nm. (b) If protein X and Y do not interact, illuminating the sample at 440 nm yields fluorescence at 490 nm from CFP only. (c) When protein X and Y interact, FRET now occurs. Illuminating the sample at 440 nm excites CFP, whose emission in turn excites YFP, resulting in the emission of yellow light at 527 nm.

Electron microscopy

In contrast with light microscopy where optical lenses focus a beam of light, in electron microscopy **electromagnetic lenses** focus a beam of **electrons**. Because electrons are absorbed by atoms in the air, the specimen has to be mounted in a **vacuum** within an evacuated tube. The resolution of the electron microscope with biological materials is at best 0.10 nm. In **transmission electron microscopy**, a beam of electrons is directed through the specimen and electromagnetic lenses are used to focus the **transmitted electrons** to produce an image either on a viewing screen or on photographic film (Figure 4a). As in standard light microscopy, thin sections of the specimen are viewed. However, for transmission electron microscopy the sections must be much thinner (50–100 nm thick). Since electrons pass uniformly through biological material, unstained specimens give very poor images. Therefore, the specimen must routinely be stained in order to scatter some of the incident electrons, which are then not focused by the electromagnetic lenses and so do not form the image. **Heavy metals** such as gold and osmium are often used to stain biological materials. In particular, **osmium tetroxide** preferentially stains certain cellular components, such as membranes, which appear black in the image. The transmission electron microscope has sufficiently high resolution that it can be used to obtain information about the shapes of purified proteins, viruses and subcellular organelles. Three-dimensional views of structures with resolutions of 2–10 nm can be obtained using the technique of **electron tomography**, which generates three-dimensional images by computer analysis of multiple two-dimensional images obtained over a range of viewing directions. **Cryoelectron microscopy**, in which the sample is rapidly frozen at very low temperatures, is used to determine the three-dimensional structures of proteins (Section B2).

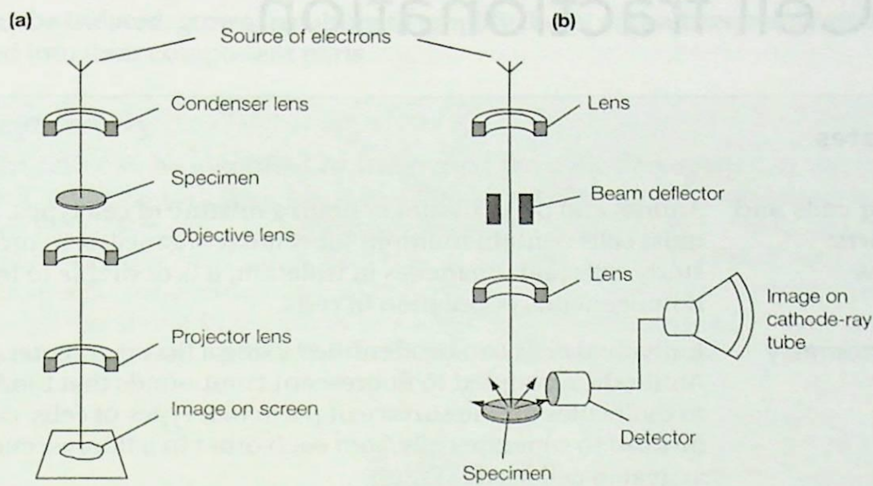

Figure 4. Principal features of (a) a transmission electron microscope and (b) a scanning electron microscope.

Antibodies can be tagged with electron-dense gold particles in a similar way to being tagged with a fluorescent compound in fluorescence microscopy, and then bound to specific target proteins in the thin sections of the specimen. When viewed in the electron microscope, small dark spots due to the gold particles are seen in the image wherever an antibody molecule has bound to its antigen (Section C4) and so the technique can be used to localize specific proteins.

In **scanning electron microscopy**, an (unsectioned) specimen is fixed and then coated with a thin layer of a **heavy metal** such as **platinum**. An electron beam then **scans** over the specimen, exciting molecules within it that release secondary electrons. These secondary electrons are focused on to a detector and the resulting image displayed (Figure 4b). The scanning electron microscope produces a **three-dimensional image** because the number of secondary electrons produced by any one point on the specimen depends on the angle of the electron beam in relation to the surface of the specimen. The resolution of the scanning electron microscope is only about 10 nm.

A5 Cell fractionation

Key Notes

Isolating cells and their parts: overview	Animal and plant tissues contain a mixture of cell types, and most cells contain multiple subcellular organelles. In order to study cells and organelles in isolation, it is desirable to have a homogeneous population of cells.
Flow cytometry	Individual cells can be identified using a flow cytometer. Antibodies, coupled to fluorescent compounds that bind to molecules on the surface of particular types of cells, can be used to separate cells from each other in a fluorescence-activated cell sorter (FACS).
Subcellular fractionation	Subcellular fractionation is the breaking open of a cell (e.g. by homogenization) and the separation of the various organelles from one another, usually by centrifugation. Differential centrifugation separates the subcellular organelles on the basis of their size and density. An ultracentrifuge is used to generate powerful forces to separate the various organelles, which pellet to the bottom of the centrifuge tube. At lower forces, nuclei, mitochondria, chloroplasts and lysosomes pellet, whereas higher forces are needed to pellet the endoplasmic reticulum, Golgi apparatus and plasma membrane. Equilibrium density-gradient centrifugation uses a gradient of a dense solution (e.g. sucrose solution) to separate out subcellular organelles on the basis of their density.
Marker proteins	A convenient way of determining the purity of an organelle preparation is to measure the activity of a marker protein or enzyme in the various subcellular fractions. A marker protein is one that is found within only one particular compartment of the cell.
Related topics	(A2) Eukaryotic cells; (A4) Cell imaging; (C2) Gel electrophoresis; (C4) Immunodetection; (D1) Introduction to enzymes

Isolating cells and their parts: overview

Most animal and plant tissues contain a mixture of cell types, and most cells contain multiple **subcellular organelles** (Section A2). Although microscopy techniques (Section A4) can be used to visualize organelles and large molecules inside cells, many studies on cell structure and function require samples of a particular type of cell, subcellular organelle or components within them. Most biochemical procedures require obtaining large numbers of cells and then physically disrupting them to isolate their components. **Tissue samples** will often provide large quantities of material but will contain a heterogeneous mix of cells. Techniques have been developed whereby **homogeneous populations of**

cells can be isolated, grown in culture to amplify them, and subsequently studied or fractionated into their component parts.

Flow cytometry

Different cells can be identified by measuring the light they scatter, or the fluorescence they emit, as they pass a laser beam in a **flow cytometer**. In a **fluorescence-activated cell sorter** or **FACS** (Figure 1), an instrument based on flow cytometry, cells can be identified and separated from each other. The cells of interest are first labeled with an **antibody**, which is specific for a particular cell surface molecule. The antibody is coupled to a fluorescent dye (Section A4), such that when the individual cells pass a laser beam in single file in a narrow stream, the fluorescence of each cell is measured. A vibrating nozzle then

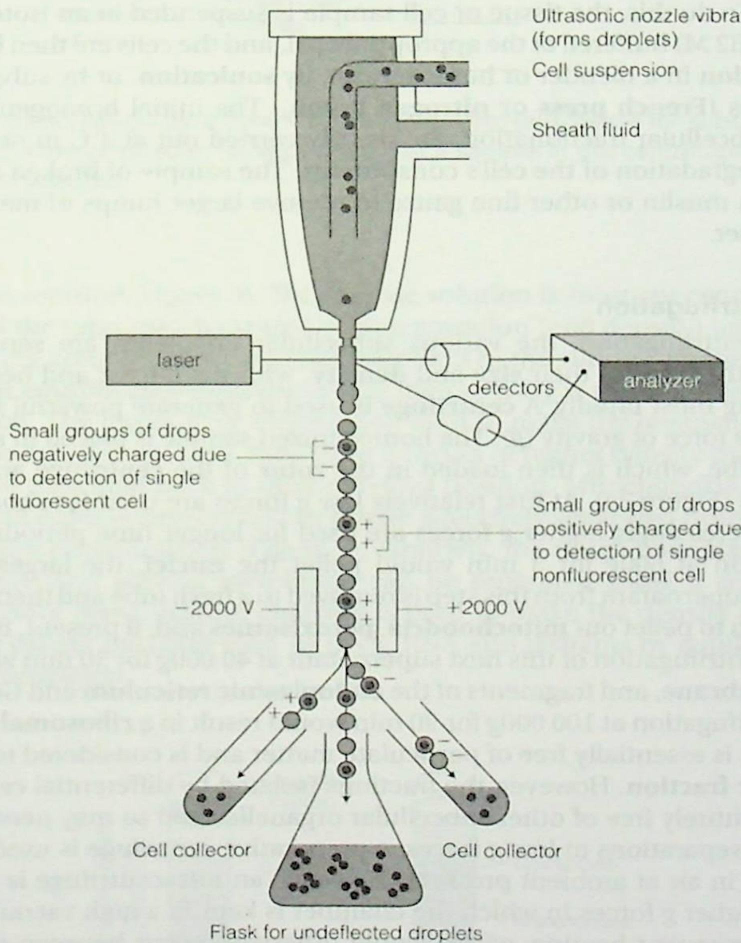

Figure 1. A fluorescence-activated cell sorter. An antibody specific for a particular cell surface protein is linked to a fluorescent molecule and then added to a mixture of cells. When the individual cells pass through a laser beam they are monitored for fluorescence. Droplets containing single cells are given a positive or negative charge, depending on whether the cell has bound the fluorescently tagged antibody or not. The droplets containing a single cell are then deflected by an electric field into collection tubes according to their charge. The cell concentration is such that most droplets contain no cells and flow through undeflected to a waste container together with any clumps of cells.

forms tiny droplets, each containing a single cell, which are given a positive or negative charge depending on whether the cell they contain is fluorescing. A strong electric field deflects the different charged droplets into separate containers so that each container eventually has a **homogeneous population of cells** with respect to the cell surface molecule tagged with fluorescent antibody. These homogeneous populations can then be used for biochemical analysis or grown in culture. The DNA and RNA content of a cell can also be measured by flow cytometry.

Subcellular fractionation

In order to study macromolecules and metabolic processes within cells, it is often helpful to isolate one type of **subcellular organelle** (Section A2) from the rest of the cell contents by subcellular fractionation. Initially, the plasma membrane (and cell wall if present) has to be ruptured. To do this, the tissue or cell sample is suspended in an isotonic sucrose solution (0.25–0.32 M) buffered at the appropriate pH, and the cells are then broken open by **homogenization** in a blender or homogenizer, by **sonication**, or by subjecting them to high pressures (**French press** or **nitrogen bomb**). The initial homogenization, and the following subcellular fractionation, are usually carried out at 4°C in order to minimize enzymic degradation of the cell's constituents. The sample of broken cells is often strained through muslin or other fine gauze to remove larger lumps of material before proceeding further.

Differential centrifugation

In differential centrifugation, the various subcellular organelles are separated from one another on the basis of their **size** and **density**, with the largest and heaviest structures sedimenting most rapidly. A **centrifuge** is used to generate powerful forces; up to 100 000 times the force of gravity (g). The homogenized sample is placed in an appropriate centrifuge tube, which is then loaded in the **rotor** of the centrifuge and subjected to centrifugation (Figure 2a). At first relatively low g forces are used for short periods of time but then increasingly higher g forces are used for longer time periods. For example, centrifugation at 600g for 3 min would pellet the **nuclei**, the largest organelles (Figure 2b). The supernatant from this step is removed to a fresh tube and then centrifuged at 6000g for 8 min to pellet out **mitochondria**, **peroxisomes** and, if present, **lysosomes** or **chloroplasts**. Centrifugation of this next supernatant at 40 000g for 30 min will pellet out the **plasma membrane**, and fragments of the **endoplasmic reticulum** and **Golgi apparatus**. A final centrifugation at 100 000g for 90 min would result in a **ribosomal pellet** and a supernatant that is essentially free of particulate matter and is considered to be the true soluble **cytosolic fraction**. However, the fractions isolated by differential centrifugation are not usually entirely free of other subcellular organelles and so may need to be purified further. For separations at low g forces, a preparative centrifuge is used, which has a rotor spinning in air at ambient pressure. However, an ultracentrifuge is required for separations at higher g forces in which the chamber is kept in a high vacuum to reduce friction, and subsequent heating, which would otherwise occur between the spinning rotor and air.

Equilibrium density-gradient centrifugation

Equilibrium density-gradient centrifugation is often used to purify further organelles following their partial separation by differential centrifugation. In this procedure, the organelles are separated on the basis of their **density**. The impure organelle fraction is loaded at the top of a centrifuge tube that contains a gradient of a **dense solution**

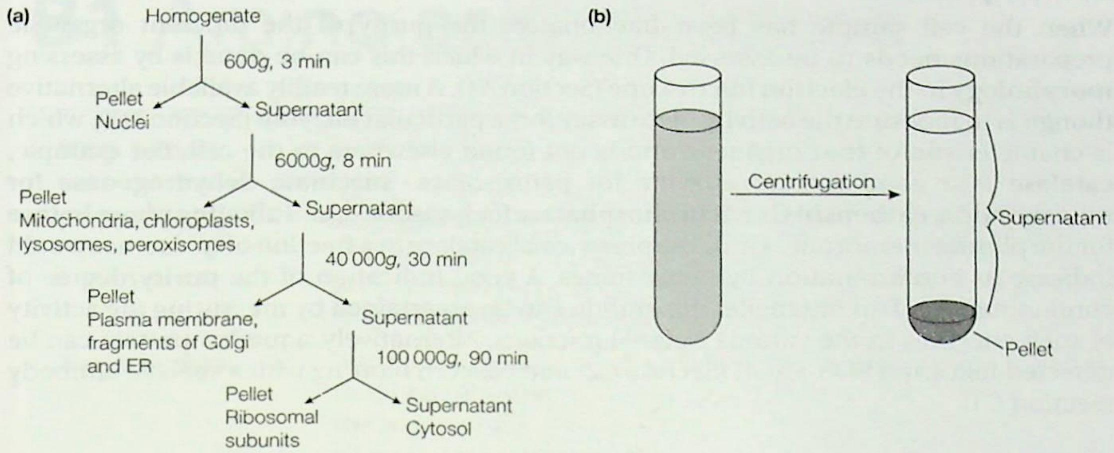

Figure 2. Cell fractionation by differential centrifugation: (a) scheme for subcellular fractionation of a tissue sample; (b) appearance of a sample in the centrifuge tube before and after centrifugation.

(e.g. a sucrose solution; Figure 3). The sucrose solution is most concentrated (dense) at the bottom of the tube, and decreases in concentration (and density) towards the top of the tube. During centrifugation (e.g. 160 000*g* for 3 h) the various organelles move down the tube to an **equilibrium position**, where their density is equal to that of the sucrose at that position. The forces of sedimentation tend to make the organelles move further down the tube but, if they do so, they enter a region of higher density than the organelle density and so they float back to their previous position. Mitochondria, lysosomes and peroxisomes all differ in density and so can be effectively separated from one another by density-gradient centrifugation (Figure 3). Similarly, the rough endoplasmic reticulum, Golgi apparatus and plasma membrane can be separated using a gradient of lower density. The more dense **cesium chloride** is used to make the density gradient for the separation of denser particles such as DNA, RNA and proteins by equilibrium density-gradient centrifugation.

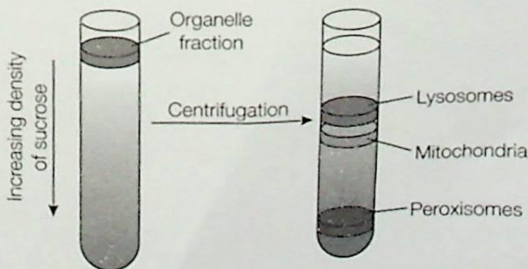

Figure 3. Separation of organelles by equilibrium density-gradient centrifugation.

Marker proteins

When the cell sample has been fractionated, the purity of the different organelle preparations needs to be assessed. One way in which this can be done is by assessing **morphology** in the electron microscope (Section A4). A more readily available alternative though is to measure the activity of (to assay for) a particular **enzyme** (Section D1), which is characteristic of that organelle and is not found elsewhere in the cell. For example, **catalase** is a good marker enzyme for peroxisomes, **succinate dehydrogenase** for mitochondria, **cathepsin C** or **acid phosphatase** for lysosomes, and **alkaline phosphatase** for the plasma membrane. Thus, the presence of catalase in a fraction of lysosomes would indicate its contamination by peroxisomes. A good indication of the **purity**/degree of contamination of an organelle preparation can be ascertained by measuring the activity of such enzymes in the various isolated fractions. Alternatively, a marker protein can be detected following SDS-PAGE (Section C2) and Western blotting with a specific antibody (Section C4).