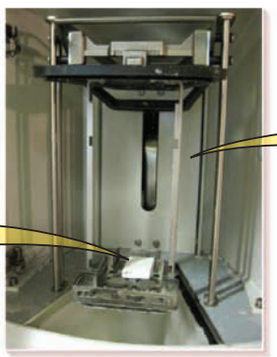
1 Preparation of tissues for histology

(a) Fixation

First the tissue is placed in fixative and allowed to fix

(b) Dehydration, clearing and wax impregnation

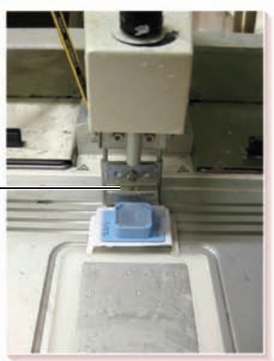
Next, the tissue is trimmed and placed in a cassette (the two halves of which are shown here)



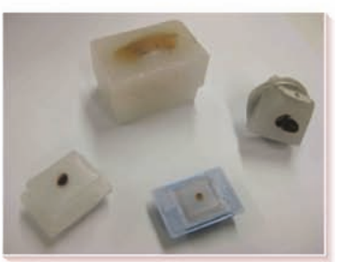
The holder is placed in a basket in the automatic processor

The processor transfers the tissue through a series of alcohol solutions of increasing strength, and then into a clearing agent (xylene) and finally into molten wax to complete the wax impregnation process

(c) Embedding

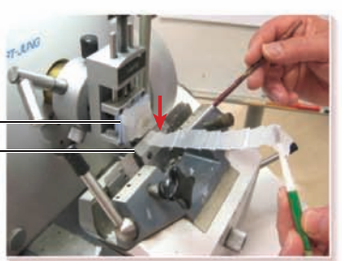


The tissue is transferred to a mould, and hot wax is dispensed into the mould

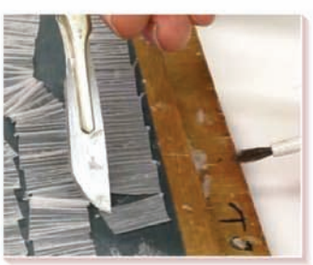


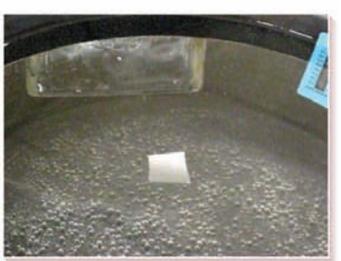
Blocks come in all shapes and sizes, depending on the size of the tissue

(d) Sectioning



The block is moved up and down (red arrow) and moved incrementally forward (toward the user) to cut sections. Serial sections emerge in a long ribbon, and are picked up with brushes





Single sections are picked up, floated on the surface of hot water, which removes the folds, and then transferred onto a glass slide

(e) Staining

The unstained section on the slide

The final slide after staining and mounting

Histology is the study of tissues and their appearance.

Histos is Greek for 'web or tissue', and ***logia*** is Greek for 'branch of learning'.

Anatomists first used the word 'tissue' to describe the different textures of parts of the body, as they were being dissected.

Today, histology and pathology (the study of diseased tissues) are routinely used in hospitals and research laboratories to study the organization of tissues and the cells within them.

Sectioning and preparing tissue for staining

To study the structures of cells and their organization within tissues, tissues have to be fixed and 'sectioned' (or cut), stained with dyes, and then observed with the light microscope. This is carried out in the following stages (see Fig. 1).

Fixation

A chemical solution containing a fixative at pH 7.0 is added to the tissue (Fig. 1a). The most commonly used fixative is formaldehyde at a concentration of 4%. (Commonly, dilutions are made from a stock of Formalin, i.e., 37% or 40% formaldehyde.) Formaldehyde binds to and cross-links some proteins, and denatures others, but does not interact well with lipids. The overall effect is to harden the tissue and inactivate enzymes, preventing the tissue from degrading.

Dehydration

In order for sections to be cut, the tissue has to be embedded in wax. However, wax is not soluble in water. Therefore, the water in the tissue has to be removed and eventually replaced with a medium in which wax *is* soluble. This is achieved by, first, sequentially replacing the water with alcohol, placing the tissue in a series of solutions that contain increasing concentrations of alcohol, ending at 100% (Fig. 1b). This process is carried out gradually in order to minimize tissue damage. The tissue must then be 'cleared' before it can be embedded in wax.

Clearing

Next, the section is placed in an organic solvent such as xylene or toluene, which replaces the alcohol. Wax is not soluble in alcohol. The clearing agents are so-called, because the tissue often looks completely clear when it is immersed in clearing agent. Finally, the tissue is impregnated with hot wax (Fig. 1b), which is soluble in this type of organic solvent.

Embedding

The tissue is placed in warm paraffin wax in a mould (Fig. 1c). On subsequent cooling, the wax hardens, and tissue slices can now be cut.

Sectioning

Sections (slices) about 10 to 20 microns (μm) thick are cut using a microtome (Fig. 1d).

Mounting

The wax sections are laid onto a glass microscope slide (Fig. 1e).

Staining

To see detail, the components of the tissue have to be stained. However, the stains that are used are all aqueous. Therefore, the wax has to be dissolved and replaced with water (rehydration), for the stains to be able to penetrate the tissue section. The sections are therefore placed in decreasing concentrations of alcohol, ending up at 0% alcohol (water).

A number of different stains can be used but the most common is hematoxylin & eosin (see Chapter 2).

Dehydration and mounting

The stained specimen is once again dehydrated, before placing it into mounting medium dissolved in xylene. Finally, a coverslip is placed on top of the sample to protect it, and the slide can be viewed on the microscope.

Other types of sectioning

Frozen sections

The tissue is rapidly frozen, fixed, and slices cut using a cryostat, before staining.

Semi-thin sections

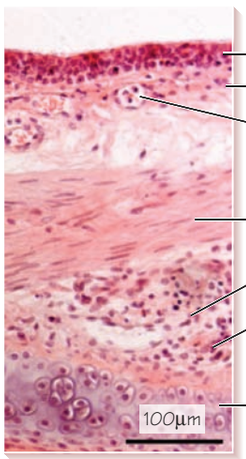
The tissue is embedded in epoxy or acrylic resin, which has different properties to wax, and allows thinner sections (less than 2 μm) to be cut.

Sections in electron microscopy

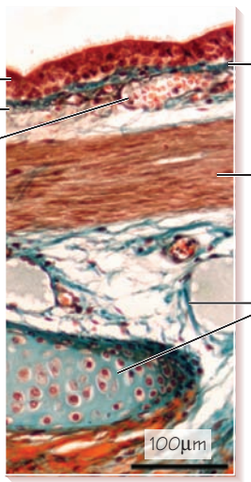
See Chapter 4.

2 Different types of histological stain

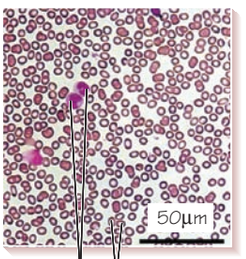
(a) Hematoxylin & eosin (H&E):
the most common stain



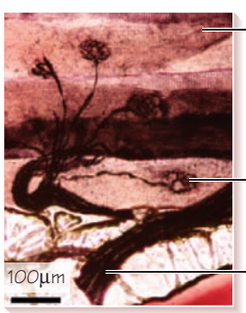
(b) Masson's trichrome:
a common alternative stain



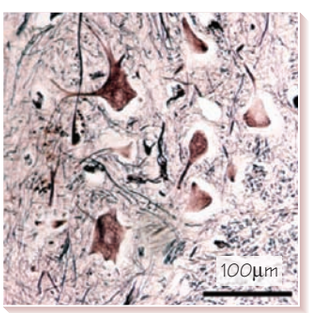
(c) Giemsa stain:
used for blood smears



(d) Silver stain:
used to stain nerves



(e) Cresyl violet

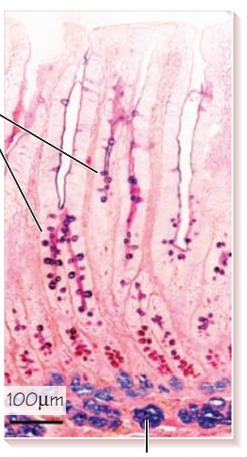


Cresyl violet stains rough endoplasmic reticulum strongly. Here cell bodies (perikarya) of neurons in the spinal cord are stained brown

(f) Periodic acid Schiff (PAS) and alcian blue

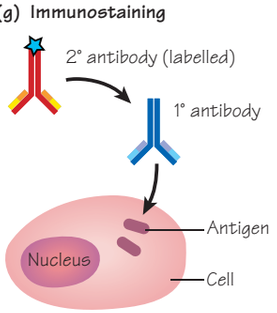
Goblet cells (mucous rich - PAS positive

PAS stains glycoproteins red. Alcian blue stains mucopolysaccharides and glycosoaminoglycans blue. Together, these stains show up goblet cells and mucous secreting glands in the gut as shown here



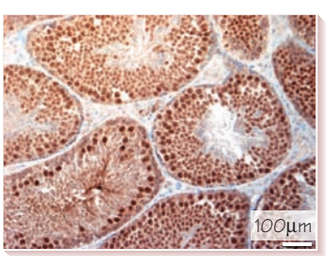
Glands (mucopolysaccharide and glycosoaminoglycan rich)

(g) Immunostaining



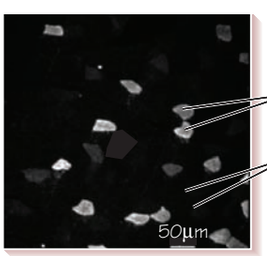
Primary (1°) antibody recognises and binds to the antigen (specific protein). Secondary antibody (2°) recognises and binds to the primary antibody and is labelled with a dye so it can be visualised.

TS testis stained for tubulin



1° antibody: anti-tubulin (raised in rabbit).
2° antibody: anti-rabbit conjugated with horse-radish peroxidase (HRP). HRP is visualized using 3,3'-diaminobenzidine (DAB)+ chromagen (results in the brown staining seen here).
Blue counterstain: Mayers haemotoxylin.
Courtesy of Mohammed Abdollahi, Leeds General Infirmary

TS of muscle cryosection stained for a myosin isoform



Muscle fibers that stain positively (contain antigen)

Muscle fibers that do not stain at all (negative, do not contain antigen)

1° antibody: anti-type I myosin (raised in mouse).
2° antibody: anti-mouse conjugated with a flurescent dye.
The section is visualized using epi-fluorescence microscopy

Cells are colorless and transparent, and it would be difficult to see much detail when observing them using a microscope. Therefore, stains have to be used to make the cells visible.

H&E (hematoxylin & eosin) is the most commonly used stain, but many additional stains are also used, a few of which are described here.

Hematoxylin & eosin

Hematoxylin is derived from the logwood tree (*Haematoxylum campechianum*), and can only be used as a dye in its oxidized form (hematein). It is a **basic** dye that binds to **acidic** structures in cells and stains them a purplish blue. These include:

- DNA in the nucleus, in heterochromatin and the nucleolus;
- RNA in the cytoplasm in ribosomes and rough endoplasmic reticulum;
- some extracellular materials (e.g., carbohydrates in cartilage).

Eosin is a negatively charged **acidic** dye. It binds to **basic** structures in cells and stains them red or pink. These include:

- most proteins in the cytoplasm;
- some extracellular fibers.

Cells in tissue stained with H&E (Fig. 2a) are therefore pink, with a purple nucleus.

Other types of histological stains

Connective tissue stains

Masson's trichrome method (Fig. 2b) uses three different dyes (hematoxylin, acid fuchsin, and methyl blue), resulting in three colors in the stained section.

- Nuclei are stained blue.
- Cytoplasm, red blood cells (erythrocytes), and keratin are stained bright red.
- Collagen in the basement membrane, connective tissue, and cartilage are stained green.

A related stain also used to stain connective tissue is **Van Gieson**.

Giemsa stain

This type of stain is used for bone marrow and blood smears (Fig. 2c).

- Red blood cells are stained pink (they do not have nuclei).
- White blood cells: cytoplasm is stained pale blue and the nuclei are stained dark blue/purple.

Silver staining (for neurons)

Standard histological stains do not work well on neurons, mainly because their plasma membranes are rich in lipid. Moreover, nuclei are not detected, unless the sections include part of the central nervous system, where the majority of the nuclei are located. However, **silver staining** (Fig. 2d) does work well. Silver staining stains the nerves and nerve terminals (terminal boutons) black. An alternative method is Golgi-Cox (mercuric chloride, potassium chromate, and dichromate).

Cresyl violet

This stain is used to stain Nissl substance (rough endoplasmic reticulum; ER) in the cell bodies of neurons (Fig. 2e).

Staining carbohydrates and mucins

In the **periodic acid–Schiff (PAS) reaction,** periodic acid oxidizes carbohydrates and carbohydrate-rich molecules such as glycosaminoglycans, and the Schiff reagent stains the resultant oxidized molecules a deep reddish purple color. In the picture shown here (Fig. 2f), PAS has been combined with the dye, Alcian blue, which stains some mucins (glycosylated proteins) a deep blue color.

Goblet cells, which are rich in carbohydrates and mucin, are stained reddish purple.

Mucin-rich glands towards the bottom of the image shown here are stained a deep blue.

Stains for lipids

Lipid stains include **Oil Red O**, **Sudan black**, and **Nile blue**, and stain myelin sheaths of neurons brownish black (not shown here).

Immunocytochemistry

This technique is becoming much more widely used in histology, as it can detect specific proteins in a section. In this technique, an antibody is used that recognizes a specific antigen on the protein of interest (Fig. 2g). Usually, after incubating the section with the first antibody (primary antibody), a second antibody (secondary antibody) is added, which recognizes the primary antibody (indirect technique). The secondary antibody is commonly labeled using horseradish peroxidase, which turns brown when reacted with a chromogen substrate. This type of staining can be viewed on a normal brightfield microscope. A 'counterstain' is used to enable visualization of the overall organization of the cells in the tissue.

Alternatively, the secondary antibody is labeled with a fluorescent dye, in which case the sections have to be viewed using an epifluorescence (or confocal) microscope (see Chapter 4).

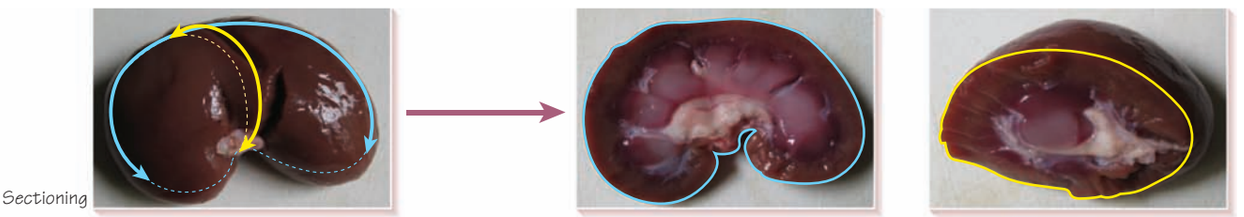
Fixing, dehydration, and wax embedding can destroy or mask antigens, which means the antibodies may not work. If this is the case, a number of different 'antigen retrieval' methods can be used, which unmask the antigens. These approaches commonly use pressure cookers or microwave ovens. Alternatively, cryosections can be used.

Staining in electron microscopy

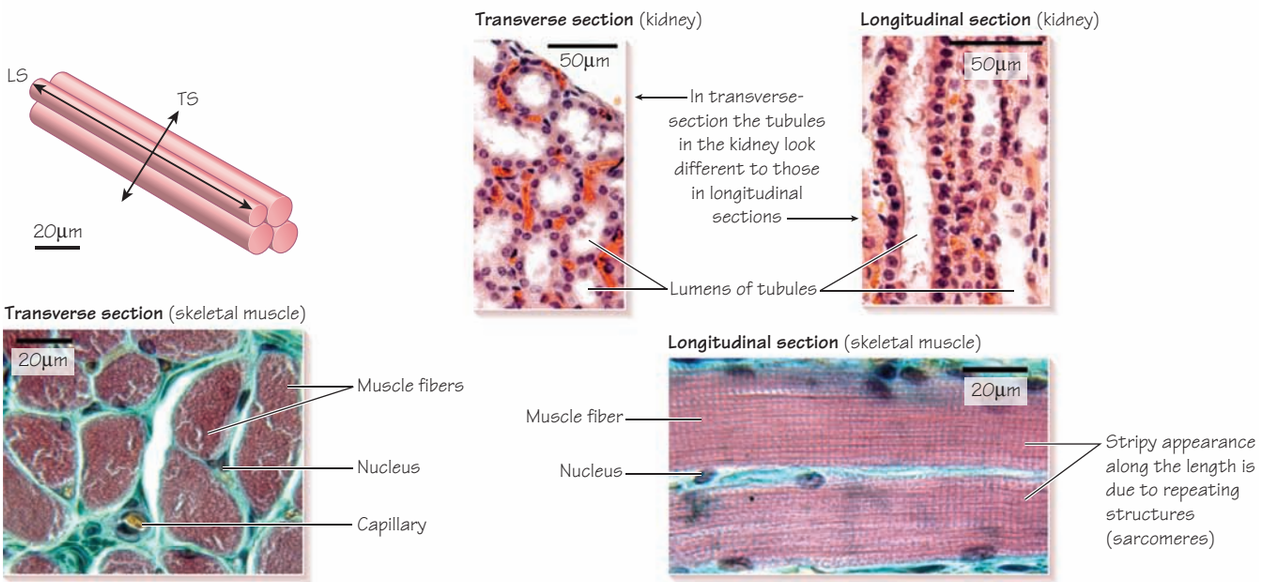
See Chapter 4.

3 Sectioning and appearance of sections in the light microscope

(a) Longitudinal and transverse sections

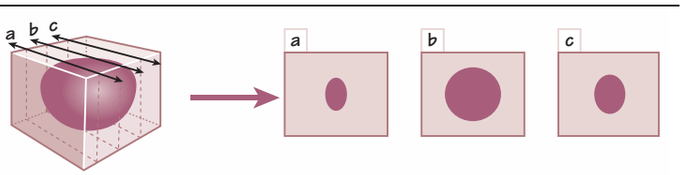


The direction of sectioning is important. Depending on whether a transverse or a longitudinal section is cut, the end result can look quite different, as shown here for the LS and TS through a kidney (above) and skeletal muscle (below). The kidney is full of long tubular structures, and skeletal muscle is full of long muscle fibers. These can either seen 'end on' (in cross or transverse sections) or along their lengths (in longitudinal sections) or something in between (oblique sections)

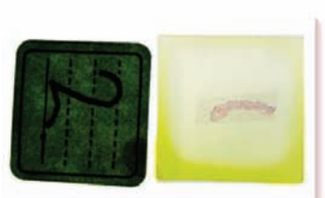


(b) Serial sectioning

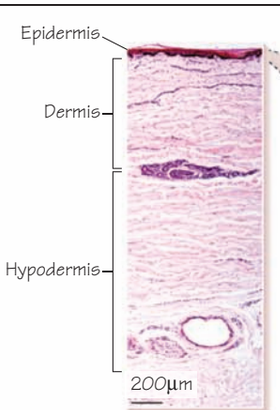
Serial sectioning will also make a difference to the appearance of the final sections. Here serial sections result in a nucleus that is apparently different in size between sections



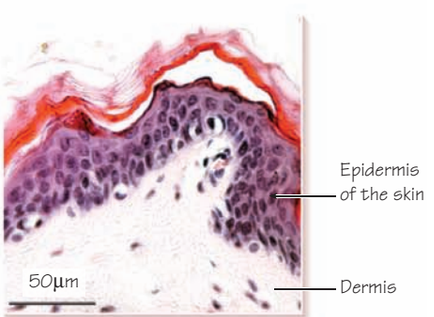
(c) Magnification



By eye - the stained slide (a section through the skin)



Using a low power lens (x2.5) gives a general idea of the structure of the tissue, but little detail



Viewing part of the section with a high power lens (x40) gives a detailed view of the cells and how they are organized

Tissues are thick, therefore the organization of cells within tissues cannot easily be visualized in the microscope. To see the detailed structure, sections have to be cut and stained and then visualized in the microscope.

The appearance of the sections depends on how the sections are cut (Fig. 3).

Longitudinal and transverse sections

A tissue cut longitudinally looks different to a tissue cut transversely (Fig. 3a).

A longitudinal section through kidney tubules shows long linear structures, with a central lumen, whereas a transverse section or cross-section through the same tubules shows round structures with a central lumen.

A longitudinal section through muscle tissue, in which the muscle fibers are long and thin, shows the repeating striated pattern along the length of the muscle fiber.

A transverse section through muscle tissue shows the polygonal shape of the fibers, with the nuclei at the edges; no striations are apparent.

Sections can also be cut obliquely, in which case the appearance is part-way between a longitudinal section and a cross-section.

Serial sections

Sections are cut in series through a tissue. The appearance of the cell and tissue will depend on where the section is cut (Fig. 3b).

Sections cut through the middle of cells look different from those cut through the edges.

Magnification

Once the section of tissue has been cut, stained, and mounted it is examined in a light microscope, using a range of different lenses, with different magnifications (Fig. 3c).

Viewing the slide by eye, does not show how the cells are organized in the tissue, but gives an overall impression of the tissue itself.

Examining the overall morphology of the section on a slide by eye usually gives a good idea of which tissue the section has been cut from.

To investigate the organization of the sectioned tissue in more detail, the slide is usually viewed in stages, starting with a low-power objective such as ×1.6 or ×5 or ×10 to obtain an overall impression of the organization of the tissue. Finally, a high-power objective, ×20, ×40, ×63 or ×100, is used to examine the cells in detail.

Resolving power of the light microscope

The resolution of a microscope determines how close together two objects can be before they can no longer be distinguished as two separate objects.

High-power objectives tend to have a higher numerical aperture, collect more light and therefore have a higher resolving power.

The spatial resolution of a lens is determined by the resolving power of the microscope (d) in the following equation:

$$d = 0.61 \times \lambda / NA$$

where λ is the wavelength of the light in µm, and NA is the numerical aperture of the objective lens. This equation holds for microscopes where the numerical aperture of the condenser is greater than or equal to the numerical aperture of the objective.

Brightfield illumination, used to examine histology slides, commonly employs a tungsten lamp, which produces white light over a broad range of wavelengths (from 400–500 nm to 700–800 nm).

The resolving power of a ×40 oil immersion lens, with an NA of 1.3, at a wavelength of 600 nm (0.6 µm), is $0.61 \times 0.6/1.3$, which is equal to 0.28 µm (280 nm).

Two objects that are closer together than this distance will not be resolved at this wavelength.

The resolving power of a low-power lens, which works in air, with an NA of 0.12 (for example) is only 3.05 µm at 600 nm.

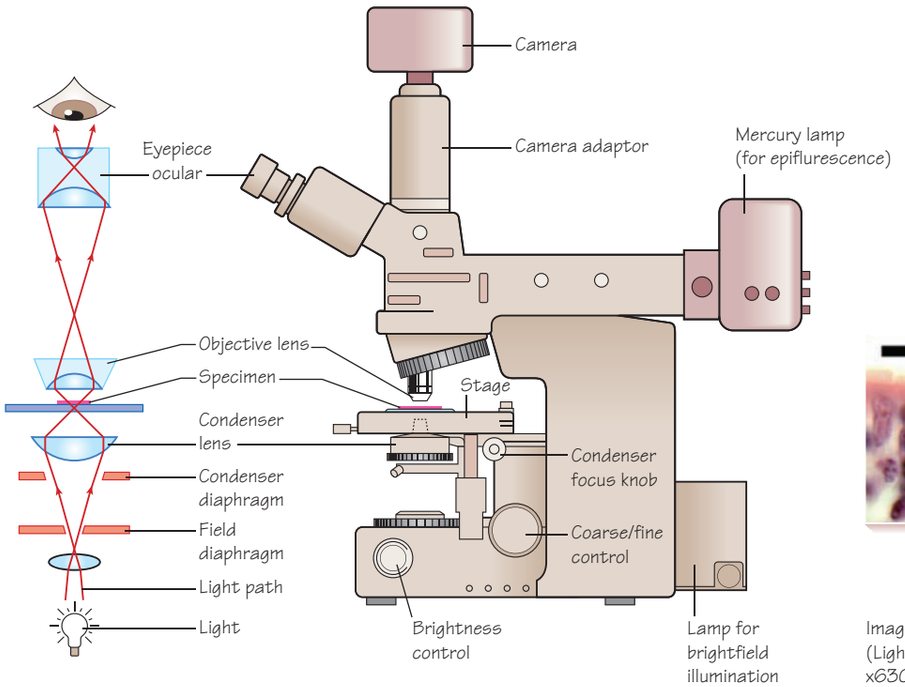
This means that much less detail is visible at low magnification than at high magnification.

The overall magnification of the specimen depends on the magnification of the objective lens and the magnification of the eyepieces. It is important to determine this overall magnification from a calibration graticule for each objective–eyepiece lens combination that is used.

Electron microscopy gives the highest resolution, because it uses an electron beam that has a much shorter wavelength (about 0.1 nm) than visible light (see Chapter 4).

4 Light and electron microscopes

(a) The light microscope and the light path



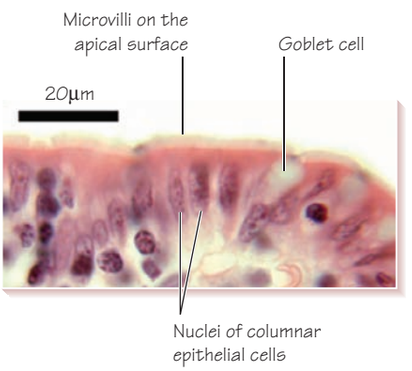
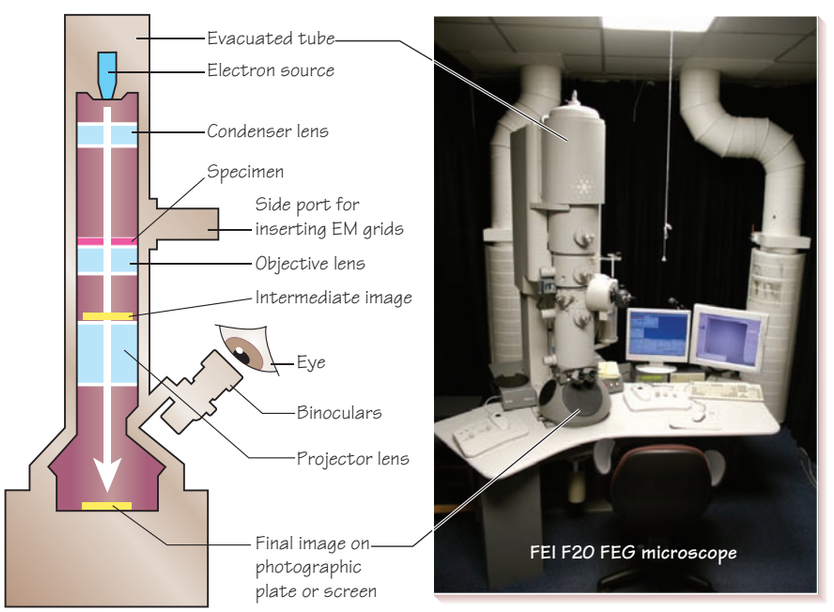


Image of the epithelium of the small intestine (Light microscope using 63x objective lens; x630 total magnification)

(b) The electron microscope and its light path (light source is electrons)



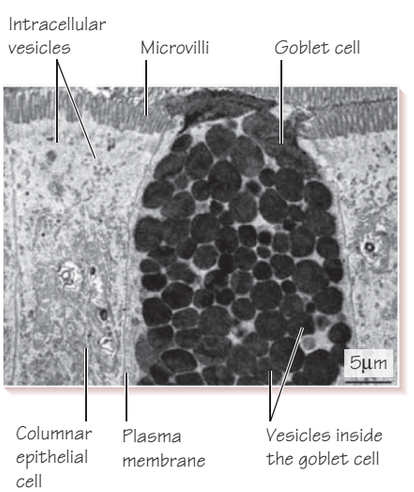


Image of a section through the epithelium of the small intestine taken with the electron microscope (x 20,000 magnification)

The light microscope

In the light microscope (Fig. 4a), illumination is provided by a tungsten lamp with a wavelength of about 400–800 nm.

Light is focused on the specimen, which is placed on the microscope stage.

The image is formed in the eyepiece by the combination of the objective lens and the eyepiece lens.

The total overall magnification depends on the magnification of both the eyepiece and the objective lens. For example, the total magnification for a ×10 eyepiece lens and a ×20 objective lens is ×200.

To obtain a clear, evenly illuminated image, it is important to set up Koehler illumination of the specimen.

In this type of illumination, all the light from the lamp is focused at the front aperture of the condenser.

Koehler illumination

Koehler illumination is achieved by:

1 focusing on the specimen;
2 closing the field diaphragm;
3 adjusting the position of the condenser to bring an image of the aperture of the field diaphragm into sharp focus;
4 opening the aperture of the diaphragm until the edges just disappear from view.

This process should be repeated each time the objective lens is changed, to ensure and bright and even illumination of the specimen.

As explained in Chapter 3, the resolution of the image depends on the lens that is used.

The very best resolution obtainable from a standard light microscope is about 0.2 µm.

Cells are about 20–40 µm in diameter, and therefore can be seen by light microscopy.

However, intracellular vesicles are usually smaller than 0.2 µm and individual vesicles cannot normally be seen by light microscopy.

The electron microscope

To investigate tissues in more detail, the electron microscope is used (Fig. 4b).The electron microscope uses an electron beam as the source of illumination, which has a much shorter wavelength than light (0.004 nm, compared to ~600 nm for light).

Electromagnetic coils are used to focus the beam, instead of lenses,

The effective numerical aperture of the electron microscope is 0.012. Therefore the theoretical resolving power (d) = $0.61 \times 0.004/0.012$ nm, or 0.2 nm.

In practice, the resolving power is less than this, due to imperfections in the electromagnetic lenses.

Usually the resolution is closer to 1 or 2 nm, and the greatest magnification is about ×50 000.

However, this means that a lot more detail can be seen by electron microscopy than by light microscopy, such as intracellular vesicles and protein filaments within cells.

The tube that the electrons move through is evacuated to reduce scatter of the electrons. This means that the samples have to be fixed before viewing in the electron microscope.

Sectioning for electron microscopy

The process of generating sections for electron microscopy is similar to that for light microscopy, but with some key differences.

1 Tissues are normally fixed with glutaraldehyde, rather than paraformaldehyde.
2 Tissues are postfixed in osmic acid.
3 As with light microscopy, the tissues are dehydrated via a series of increasing ethanol concentrations.
4 Tissues are then transferred to propylene oxide (not wax), which enables impregnation of the tissue with resin, which is allowed to harden.
5 Sections are then cut from the block using an ultramicrotome and either a glass or diamond knife. The thickness of the sections is much smaller than that for light microscopy, ranging from 60 to 100 nm thick.
6 Finally, cut sections are stained with heavy metal salts such as osmium, uranyl acetate, and lead to increase the contrast of the image, as these stains scatter the electrons.

As with light microscopy, cryosections can also be used in the electron microscope, and the sections can be immunostained, but in this case antibodies are labeled using gold, so that they are visible in the electron microscope.