Microtomy

A few cell types are thin enough to be viewed directly in a microscope (algae, protozoa, blood, tissue cultures), but most tissues (kidney, liver, brain) are too thick to allow light to be transmitted through them. The tissues can be sliced into very thin sections provided they are first processed to prevent cell damage. The processing involves a series of steps; **fixation, dehydration, embedment** and subsequent sectioning with a **microtome**. These steps, explained in detail later in this chapter, are time consuming and often alter the cell structure in subtle ways. Fixing cells with formaldehyde, for example, will preserve the general organelle structure of the cell, but may destroy enzymes and antigens which are located in the cell.

Pathologists routinely examine tissues which have been fixed in formaldehyde and embedded in paraffin wax prior to sectioning. The process requires a minimum of 24 hours, and usually more if automated equipment is not available. This time delay can be crucial when a diagnosis of benign or malignant cancer is at stake. Valuable time can be saved by skipping the fixation and dehydration steps required for paraffin embedding, and freezing the tissue in a modified microtome, the **cryostat**. Sections can be prepared within minutes and diagnoses made while the patient remains on the operating table. Additionally, frozen sections will more often retain their enzyme and antigen functions. The use of frozen sections can reduce the processing time, but it is not a panacea. Freezing is not adequate for long term preservation of the tissues and the formation of ice crystals within the cells destroys subcellular detail. Frozen sections are also thicker since ice does not section as thin as paraffin. This results in poor microscopic resolution and poor images of what subcellular structures remain. If time or enzyme function is critical frozen sections are the preferred process. If subcellular detail is important, other procedures must be used. Selection of the correct procedure depends on what the cell biologist is looking for and to a point, becomes an art form. The histologist must choose among hundreds of procedures to prepare tissues in a manner that is most appropriate to the task at hand.

Fixation

Since cellular decomposition begins immediately after the death of an organism, biologists must fix the cells to prevent alterations in their structure through decomposition. Routine fixation involves the chemical cross-linking of proteins (to prevent enzyme action and digestion) and the removal of water to further denature the proteins of the cell. Heavy metals may also be used for their denaturing effect.

A typical laboratory procedure involves the use of an aldehyde as the primary fixative. Glutaraldehyde is used for transmission electron microscopy (TEM), and formaldehyde

is used for routine light microscopy. The formaldehyde solution most often employed was originally formulated by Baker in 1944.

Baker's Formalin Fixative contains:

calcium chloride	1.0 g
cadmium chloride	1.0 g
formalin, concentrated	10.0 ml
distilled water	100.0 ml

Blocks of tissue (liver, kidney, pancreas, etc.) of approximately 1 cm^3 are rapidly removed from a freshly killed organism and placed in the fixative. They are allowed to remain in the fixative for a minimum of four hours but usually overnight. The longer the blocks remain in the fixative, the deeper the fixative penetrates into the block and the more protein cross--linking occurs. The fixative is therefore termed progressive. Blocks may remain in this fixative indefinitely, although the tissues will become increasingly brittle with long exposures and will be more difficult to section. While it is not recommended, sections have been cut from blocks left for years in formalin.

Formalin has lately been implicated as a causative agent for strong allergy reactions (contact dermatitis with prolonged exposure) and may be a carcinogen ---- it should be used with care and always in a well ventilated environment. Formalin is a 39% solution of formaldehyde gas. The fixative is generally used as a 10% formalin or the equivalent 4% formaldehyde solution. The key operative term here is gas -- formaldehyde should be handled in a hood, if possible. As a gas, it is quite capable of fixing nasal passages, lungs and corneas.

Dehydration

Fixatives, such as formaldehyde, have the potential to further react with any staining procedure which may be used later in the process. Consequently, any remaining fixative is washed out by placing the blocks in running water overnight or by successive changes of water and/or a buffer. There are myriad means of washing the tissues (using temperature, pH and osmotically controlled buffers), but usually simple washing in tap water is sufficient.

If the tissues are to be embedded in paraffin or plastic, all traces of water must be removed: water and paraffin are immiscible. The removal of water is **dehydration**. The dehydration process is accomplished by passing the tissue through a series of increasing alcohol concentrations. The blocks of tissue are transferred sequentially to 30%, 50%, 70%, 80%, 90%, 95%, and 100% alcohols for about two hours each. The blocks are then placed in a second 100% ethanol solution to ensure that all water is removed. Note

that ethanol is hydroscopic and absorbs water vapor from the air. Absolute ethanol is only absolute if steps are taken to ensure that no water has been absorbed.

It is important to distinguish between dehydration and drying. Tissues should **NEVER** be allowed to air dry. Dehydration involves slow substitution of the water in the tissue with an organic solvent. For comparative purposes, consider the grape. A properly dehydrated grape would still look like a grape. A dried grape is a raisin. It is virtually impossible to make a raisin look like a grape again, and it is equally impossible to make a cell look normal after you allow it to dry.

Embedding

After dehydration, the tissues can be embedded in paraffin, nitrocellulose or various formulations of plastics. Paraffin is the least expensive and therefore the most commonly used material. More recently, plastics have come into increased use, primarily because they allow thinner sections (about 1.5 microns compared to 5--7 microns for paraffin).

Paraffin

For paraffin embedding, first clear the tissues. Clearing refers to the use of an intermediate fluid that is miscible with ethanol and paraffin, since these two compounds are immiscible. Benzene, chloroform, toluene or xylol are the most commonly used clearing agents, although some histologists prefer mixtures of various oils (cedarwood oil, methyl salicylate, creosote, clove oil, amyl acetate or Cellosolve). Dioxane is frequently used and has the advantage of short preparation times. It has the distinct disadvantage of inducing liver and kidney damage to the user and should only be used with adequate ventilation and protection.

Be wary of all organic solvents. Most are implicated as carcinogenic agents. Heed all precautions for the proper use of these compounds.

The most often used clearing agent is toluene. It is used by moving the blocks into a 50:50 mixture of absolute ethanol:toluene for two hours. The blocks are then placed into pure toluene and then into a mixture of toluene and paraffin (also 50:50). They are then placed in an oven at 56 - 58° C (the melting temperature of paraffin).

The blocks are transferred to pure paraffin in the oven for 1 hour and then into a second pot of melted paraffin for an additional 2--3 hours. During this time the tissue block is completely infiltrated with melted paraffin.

Subsequent to infiltration, the tissue is placed into an embedding mold and melted paraffin is poured into the mold to form a block. The blocks are allowed to cool and are then ready for sectioning.

Plastic

More recent developments in the formulation of plastic resins have begun to alter the way sections are embedded. For electron microscopy that requires ultrathin sections, paraffin is simply not suitable. Paraffin and nitrocellulose are too soft to yield thin enough sections.

Instead, special formulations of hard plastics are used, and the basic process is similar to that for paraffin. The alterations involve placing a dehydrated tissue sample of about 1 mm^3 into a liquid plastic which is then polymerized to form a hard block. The plastic block is trimmed and sectioned with an ultramicrotome to obtain sections of a few hundred Angstroms. Table 2.1 presents a comparison of paraffin embedding with the typical Epon embedment for TEM.

Softer plastics are also being used for routine light microscopy. The average thickness of a paraffin-sectioned tissue is between 7 and 10 microns. Often this will consist of two cell layers and, consequently lack definition for cytoplasmic structures. With a plastic such as Polysciences JB--4 it is possible to section tissues in the 1--3 micron range with increased sharpness. This is particularly helpful if photomicrographs are to be taken. With the decrease in section thickness, however, comes a loss of contrast, and thin sections (1 micron) usually require the use of a phase contrast microscope as well as special staining procedures. The sharp image makes the effort worthwhile.

Table	Light	Electron
Sample Size	1 cm^3	1 mm^3
Fixative	Formaldehyde	Glutaradehyde
Post-Fixation	None	Osmium Tetroxide
Dehydration	Graded Alcohol	Alcohol or Acetone
Clearing Agent	Xylol/Toluene	Propylene Oxide
Embedding Material	Pfaffin	Various Plastics
Section Thickness	5-10µ	60-90 nm
Stains	Colored dyes	Heavy Metals

Table 2.1 Light and electron microscopy preparations.

These soft plastics can be sectioned with a standard steel microtome blade and do not require glass or diamond knives, as with the harder plastics used for EM work.

Sectioning

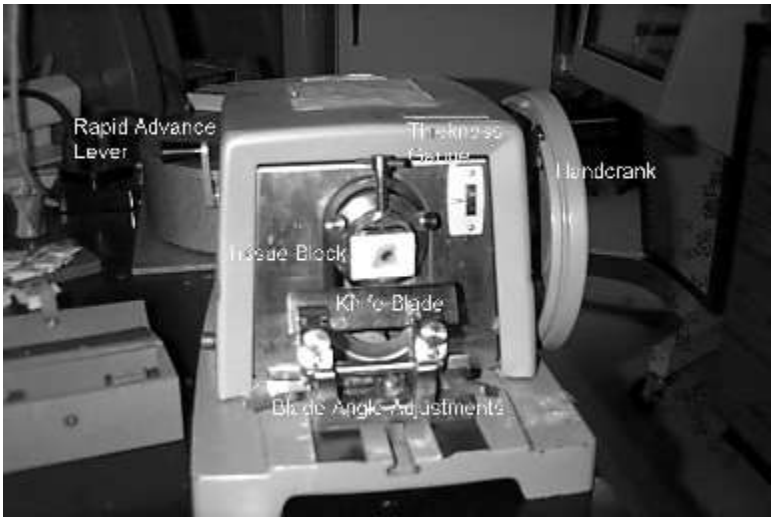

Figure 2.1 microtome for paraffin sectioning

Figure 2.1 presents a photograph of the AO standard microtome. This device is found universally in cell biology laboratories and remains a fundamental instrument for histology.

This rather simple device consists of a stationary knife holder/blade and a specimen holder which advances by pre-set intervals with each rotation of the flywheel mounted on the right hand side. In operation, it is similar to the meat and cheese slicers found within delicatessans. A control knob adjusts internal cams which advance the paraffin block with each stroke. It is relatively easy to section paraffin at 10 microns but requires a lot of skill and practice to cut at 5 microns. Since each section comes off of the block serially, it is possible to align all of the sections on a microscope slide and produce a serial section from one end of a tissue to the other.

While virtually anyone can cut a section within minutes of being introduced to the microtome, proper use of the microtome is an art form and requires practice and inventiveness. Many a cell biology research project has depended on the skills inherent in the use of this instrument. A microscope is nearly useless without a good thin, flat, and undistorted section from properly fixed, dehydrated and embedded tissue.

The Ultramicrotome

Figure 2.2 Sorvall ultramicrome

Figure 2.2 presents a view of an ultramicrotome. In principle, it is the offspring of the standard microtome, in that it also is a mechanical device that involves a stationary knife (glass or diamond) and a moving specimen. The specimen, or block, is a plastic embedded tissue that advances in nanometers rather than microns.

Operationally, the only difference is that smaller samples are handled, which in turn requires a binocular dissecting microscope mounted over the blade. The tissue sections are too thin to see their thickness with the naked eye, one usually estimates thickness by the color of the diffraction pattern on the section as it floats off the knife onto the surface of a water bath. The sections are also too thin to be handled directly, and they are therefore transferred with wire loops, or picked off the water directly onto an EM grid.This process requires a good light source mounted to cast the light at just the correct angle to see the color pattern.

Since the plastics are hard enough to break steel knives, freshly prepared glass knives or commercially available diamond knives are used. A glass knife costs several dollars each, while a good diamond knife will cost in excess of $3,000. Either can be permanently damaged with a single careless stroke by the operator. Diamond knives are used in research laboratories by trained technicians because they have the advantage of a consistent knife edge (unlike glass which varies with each use) and can last for years if treated properly. They can usually be resharpened several times before discarding.

To minimize vibrations (which lead to uneven sections) ultramicrotomes are cast in heavy metal, are mounted on shock absorbent tables and, preferrably, kept in draft free environments of relatively constant temperature. To further minimize vibrations, some manufacturers have replaced the block's mechanical advance mechanism with a thermal bar, which advances the tissue by heating a metal rod. This can be exquisitely precise and is the ultimate in thin sectioning. Of course with this advancement comes increased cost and maintenance, and decreased ability to withstand rough treatment. The mechanically advanced ultramicrotome remains as the workhorse of the cell biology laboratory.

The Cryostat

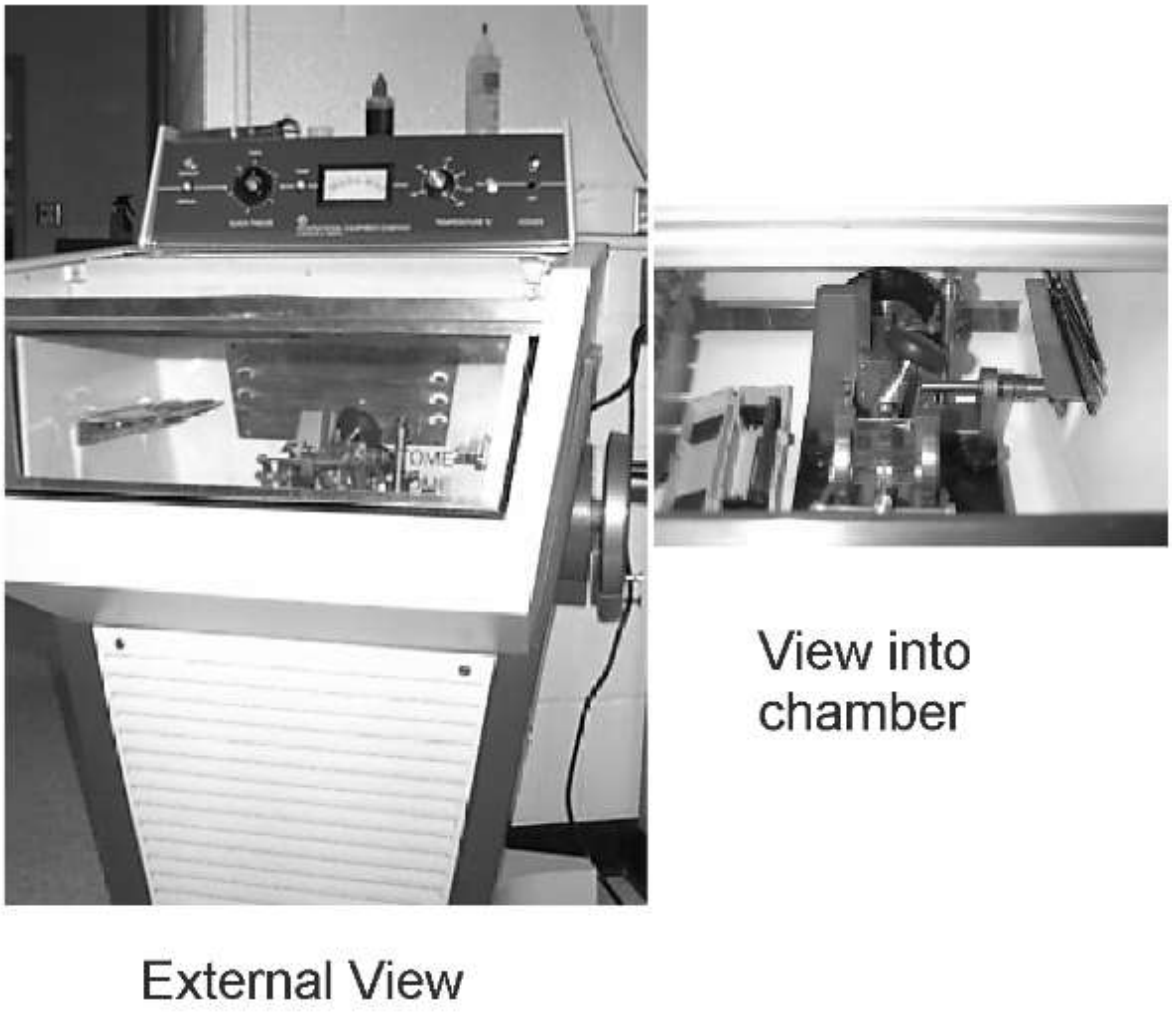

Figure 2.3 Modern cryostat

Whether the sectioning is performed with a microtome or an ultramicrotome, one of the major delays in preparing a tissue section is the time required to dehydrate and embed the tissue. This can be overcome by direct sectioning of a frozen tissue. Typically a piece of tissue can be quick frozen to about -15 to -20 °C (for light microscopic work) and sectioned immediately in a device termed a cryostat. The cryostat is merely a microtome mounted within a freezer box. Figure 2.3 presents a modern cryostat.

A piece of tissue is removed from an organism, placed onto a metal stub and covered with a viscous embedding compound to keep it in a form convenient for sectioning. The stud and tissue are placed within the cryostat and quick frozen.

This method has the advantage of speed, maintenance of most enzyme and immunological functions (fixation is unnecessary) and relative ease of handling (far fewer steps to manipulate). It has the disadvantage that ice crystals formed during the freezing process will distort the image of the cell (bursting vacuoles and membranes for example) and the blocks tend to freeze-dry or sublimate. Thus, the blocks must be used immediately and great care must be taken to guard against induced artifact from the freezing process.

When temperature-sensitive (or lipid-soluble) molecules are to be studied, or where speed is of the essence (such as pathological examination during an operation) this is the preferred method. Sectioning operation with the cryostat is similar to that of the microtome, with the exception that one handles single frozen sections and thus all operations must be handled at reduced temperatures.