**Freeze substitution**

Fast freezing by high pressure or other methods, freeze substitution (FS) is the most common way to process whole cells, tissues or organisms for electron microscopy. Freeze substitution acts to dehydrate then chemically fix samples at low temperatures in preparation for various treatments including embedding

Embedding permits cutting of thin sections, which is the most familiar way for most Electron Microscope researchers to evaluate cell ultrastructure.

Freeze-substitution is usually carried out at temperatures below which secondary ice crystals may grow (i.e., below -70°C). Once substitution is complete, samples may be warmed-up without recrystalization, as water is now absent from the sample.

Freeze-substitution is a physicochemical process in which biological specimens are immobilized and stabilized for microscopy. Water frozen within cells is replaced by organic solvents at subzero temperatures. Freeze-substitution is widely used for ultrastructural and immunocytochemical analyses of cells by transmission and scanning electron microscopy

By dehydrating and fixing cells at low temperature, some of the distortions that are common to conventional room temperature processing are avoided. However, as with conventional methods, different cells and tissues may require different FS processing methods to give optimal preservation of structure.

Less well recognized is its superiority over conventional chemical fixation in preserving labile and rare tissue antigens for immunocytochemistry by light microscopy.

Novel strategies will be needed to integrate knowledge of chemical structures of normal and abnormal macromolecules with the physiology and developmental biology of cells and tissues from whole organisms. This review summarizes the progress and future prospects of freeze-substitution for such explorations.

**The main advantages of this method are:**

1) Dehydration occurs at low temperature, thereby greatly reducing the occurrence of ultrastructural changes often seen from room temperature dehydrations;

2) Fixatives are uniformly distributed throughout the sample prior to warming, which is when their crosslinking activity begins, and

3) Sample intended for immunocytochemistry may be infiltrated with resin and polymerized at low temperature, thereby reducing the damaging effects that ambient-temperature organic solvents and heat polymerization may have on some epitopes.

The main variables to consider in designing a freeze subsitution protocol include:

1. Which **equipment** to use for processing. The usual options are :

1**)automatic freeze substitution (AFS) device**. The virtue of the AFS machines is that they can be programmed for any temperatures and rates of warming the operator desires. Several vendors offer these.

2) **Low temperature freezers (LTF).** This option is probably the least used. While freezers are common enough in most labs, you may need 2 or 3 dedicated LTFs at different temperatures to process samples. Also, most labs prefer not to mix volatile fixatives with the antibodies and other proteins that are typically stored in LTFs.

3)**Dry ice in an insulated box.** This is the low-cost option and is suitable for a wide variety of samples, but it lacks the reproducibility of the AFS and LTF approaches.

2. **The initial temperature and time**. Many FS methods start with a temperature of -90˚C if a liquid nitrogen cooling system is used, or -78˚C if dry ice is the coolant. How long the material is held at these initial temperatures varies from a few hours to several days or more.

3. **Choice of organic solvent and additives**. The most common FS fixative is 1-2% osmium tetroxide in acetone. A small percentage (0.1 – 0.5%) of uranyl acetate is often added. However, other solvents such as methanol may also be used . For some cells, such as yeast, water addition can have dramatic results on the visualization of

4. **Warm-up rates between temperatures.** Warm up rates between starting and ending (or intermediate) temperatures are typically in the range of 1-10˚C per hour.

**A) Equipment for Freeze-Substitution**

Some fine commercial devices for this method are available, while many homemade systems work equally well.

The basic design is a chamber which will maintain a sample in solution at -90°C for 2-3 days and can then be warmed-up slowly to a variety of graduated temperature points. Some examples follow.

1**) Commercial Models (Leica)**

These units are cooled by liquid nitrogen and are convenient as they may be programmed for warming. They are also equipped with a system for precooled solution exchanges for resin infiltration and, in addition, are fitted with an Ultraviolet lamp for low-temperature polymerization of resins used in cytochemistry.

2) Homemade LN2-cooled Basket

This type of system requires some machining and moderate expense. The samples are held in a metal block into which holes have been drilled to accommodate 2ml cryotubes (in which samples will reside). This metal block is12 wrapped with heater wire and hangs in a LN2 dewar. The level of liquid coolant (LN2) is kept below the hanging block, so that N2 gas is surrounding and cooling the block. The heater wire is connected to a temperature controller, and one cryovial contains a thermocouple to maintain the block at a set temperature (- 80°C). This unit can either warm-up passively when LN2 runs out, or can be reset for warmer temperatures for low temperature embedding.

Microtome

A **microtome** (from the Greek *mikros*, meaning "small", and *temnein*, meaning "to cut") is a tool used to [cut](http://en.wikipedia.org/wiki/Cutting) extremely thin slices of material, known as sections. Important in science, microtomes are used in [microscopy](http://en.wikipedia.org/wiki/Microscopy), allowing for the [preparation of samples](http://en.wikipedia.org/wiki/Microscope_slide) for observation under transmitted [light](http://en.wikipedia.org/wiki/Visible_light) or[electron](http://en.wikipedia.org/wiki/Electron) radiation.

Microtomes use steel, glass, or diamond blades depending upon the specimen being sliced and the desired thickness of the sections being cut. Steel blades are used to prepare sections of animal or plant tissues for [light microscopy](http://en.wikipedia.org/wiki/Light_microscopy) [histology](http://en.wikipedia.org/wiki/Histology).

Glass knives are used to slice sections for light microscopy and to slice very thin sections for [electron microscopy](http://en.wikipedia.org/wiki/Electron_microscopy).

Industrial grade diamond knives are used to slice hard materials such as bone, teeth and plant matter for both light microscopy and for electron microscopy. Gem quality [diamond knives](http://en.wikipedia.org/wiki/Diamond_knife) are used for slicing thin sections for [electron microscopy](http://en.wikipedia.org/wiki/Electron_microscopy).

Microtomy is a method for the preparation of thin sections for materials such as bones, minerals and teeth, and an alternative to [electropolishing](http://en.wikipedia.org/wiki/Electropolishing) and [ion milling](http://en.wikipedia.org/wiki/Focused_ion_beam). Microtome sections can be made thin enough to section a human hair across its breadth, with section thickness between 50 [nm](http://en.wikipedia.org/wiki/Nanometre) and 100 [µm](http://en.wikipedia.org/wiki/Micrometre).

Applications

The most common applications of microtomes are:

* **Traditional**[**Histology**](http://en.wikipedia.org/wiki/Histological)**Technique**: tissues are hardened by replacing water with [paraffin](http://en.wikipedia.org/wiki/Paraffin_wax). The tissue is then cut in the microtome at thicknesses varying from 2 to 50 µm. From there the tissue can be mounted on a microscope slide, stained with appropriate aqueous dye(s) after prior removal of the paraffin, and examined using a light microscope.
* [**Cryosectioning**](http://en.wikipedia.org/wiki/Frozen_section_procedure)**Technique:** water-rich tissues are hardened by freezing and cut in the frozen state with a reezing microtome or microtome-[cryostat](http://en.wikipedia.org/wiki/Cryostat); sections are stained and examined with a light microscope. This technique is much faster than traditional histology (5 minutes vs 16 hours) and is used in conjunction with medical procedures to achieve a quick diagnosis.
* [**Electron Microscopy**](http://en.wikipedia.org/wiki/Electron_Microscopy)**Technique**: after embedding tissues in epoxy resin, a microtome equipped with a glass or gem grade diamond knife is used to cut very thin sections (typically 60 to 100 nanometer). Sections are stained with an aqueous solution of an appropriate heavy metal salt and examined with a [transmission electron microscope](http://en.wikipedia.org/wiki/Transmission_electron_microscope). This instrument is often called an *ultramicrotome*.
* **Botanical Microtomy Technique**: hard materials like wood, bone and leather require a sledge microtome. These microtomes have heavier blades and cannot cut as thin as a regular microtome.
* [**Spectroscopy**](http://en.wikipedia.org/wiki/Spectroscopy)**(especially**[**FTIR**](http://en.wikipedia.org/wiki/FTIR)**or**[**Infrared spectroscopy**](http://en.wikipedia.org/wiki/Infrared_spectroscopy)**) Technique:** thin polymer sections are needed in order that the infra-red beam will penetrate the sample under examination. It is normal to cut samples to between 20 and 100 µm in thickness. For more detailed analysis of much smaller areas in a thin section, FTIR [microscopy](http://en.wikipedia.org/wiki/Microscopy) can be used for sample inspection.

Microtome types

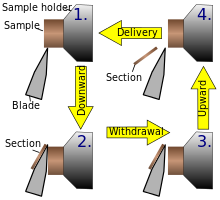
**Sledge microtome**

A sledge microtome is a device where the sample is placed into a fixed holder (shuttle), which then moves backwards and forwards across a knife.

 By adjusting the angles between the sample and the microtome knife, the pressure applied to the sample during the cut can be reduced.[[12]](http://en.wikipedia.org/wiki/Microtome#cite_note-Lang-12) Typical applications for this design of microtome are of the preparation of large samples, such as those embedded in paraffin for biological preparations. Typical cut thickness achievable on a sledge microtome is between 1 and 60 µm.

**Rotary microtome**

This instrument is a common microtome design. This device operates with a staged rotary action such that the actual cutting is part of the rotary motion. In a rotary microtome, the knife is typically fixed in a horizontal position.

[](http://en.wikipedia.org/wiki/File:Microtome_principle.svg)

In the figure to the left, the principle of the cut is explained. Through the motion of the sample holder, the sample is cut by the knife position 1 to position 2), at which point the fresh section remains on the knife. At the highest point of the rotary motion, the sample holder is advanced by the same thickness as the section that is to be made, allowing for the next section to be made.

**Cryomicrotome**

For the cutting of frozen samples, many rotary microtomes can be adapted to cut in a liquid nitrogen chamber, in a so-called cryomicrotome setup. The reduced temperature allows for the hardness of the sample to be increased, such as by undergoing a glass transition, which allows for the preparation of semi-thin samples.[[12]](http://en.wikipedia.org/wiki/Microtome#cite_note-Lang-12) However the sample temperature and the knife temperature must be controlled in order to optimise the resultant sample thickness

**Ultramicrotome**

An ultramicrotome is a main tool of [ultramicrotomy](http://en.wikipedia.org/wiki/Ultramicrotomy). It can allow for the preparation of extremely thin sections, with the device functioning in the same manner as a rotational microtome, but with very tight tolerances on the mechanical construction. As a result of the careful mechanical construction, the linear thermal expansion of the mounting is used to provide very fine control of the thickness.[[12]](http://en.wikipedia.org/wiki/Microtome#cite_note-Lang-12)

**Embedding**

It is the casting or blocking of tissue section, which involves the enclosure of the tissue in the infiltration medium used for processing, and then allowing the medium to solidify. The infiltrating medium is selected according to the embedding media that will be used.

**EMBEDDING MEDIA**

Infiltrating and embedding media must fill all spaces within the tissue to support cellular

components adequately during microtomy. Density of the hardened medium should approach

that of the densest tissue component otherwise section deformation will result. The matrix must

be elastic enough to recover sectioning deformation, and plastic enough to facilitate thin

sectioning. Tissue-medium adhesion is enhanced if the embedding matrix has a fine uniform

1 2

crystalline morphology which intimately contacts the tissue. Viscosity and melting point of the

infiltration medium partly determine the duration and temperature of processing conditions.

Embedding tissues in paraffin wax

Tissues are embedded by placing them in a mold filled with melted embedding medium which is

then allowed to solidify. Embedding requirements and procedures are essentially the same for all

waxes, and only the technique for paraffin wax is provided here in detail. At the completion of

processing, tissues are held in clean paraffin wax which is free of solvent and particulate matter.

Requirements for embedding are as follows:

• a supply of clean, filtered paraffin wax held at 2-4°C above its melting point.

• a cold plate to rapidly cool the wax.

• a supply of molds in which to embed the tissues.

These elements are conveniently combined in commerciall y available embedding stations.

Otherwise a wax dispenser, embedding oven and ice will suffice. There are four main mold

systems and associated embedding protocols presently in use: traditional methods using paper

boats; Leuckart or Dimmock embedding irons or metal containers; the Peel-a-way system using

disposable plastic molds and; systems using embedding rings or cassette-bases which become an

integral part of the block and serve as the block holder in the microtome.

General Embedding Procedure

METHOD

1) Open the tissue cassette, check against worksheet entry to ensure the correct number of

tissue pieces are present.

2) Select the mold, there should be sufficient room for the tissue with allowance for at least

a 2 mm surrounding margin of wax.

3) Fill the mold with paraffin wax.

4) Using warm forceps select the tissue, taking care that it does not cool in the air; at the

same time.

5) Place the tissue in the mold according to the side to be sectioned. This side should be

facing down against the mold. A small amount of pressure may be used in order to have

more even embedding.

6) Chill the mold on the cold plate, orienting the tissue and firming it into the wax with

warmed forceps. This ensures that the correct orientation is maintained and the tissue

surface to be sectioned is kept flat.

7) Insert the identifying label or place the labeled embedding ring or cassette base onto the

mold.

8) Add more paraffin into the mold to fill the cassette and mold.

9) Cool the block on the cold plate.

10)Remove the block from the mold.

11)Cross check block, label and worksheet

ORIENTATION OF TISSUE IN THE BLOCK

Correct orientation of tissue in a mold is the most important step in embedding. Incorrect

placement of tissues may result in diagnostically important tissue elements being missed or

damaged during microtomy. In circumstances where precise orientation is essential tissue should

be marked or agar double embedded. Usually tissues are embedded with the surface to be cut

facing down in the mold. Some general considerations are as follows:

• elongate tissues are placed diagonally across the block

• tubular and walled specimens such as vas deferens, cysts and gastrointestinal tissues are

embedded so as to provide transverse sections showing all tissue layers

• tissues with an epithelial surface such as skin, are embedded to provide sections in a

plane at right angles to the surface (hairy or keratinized epithelia are oriented to face the

knife diagonally)

• multiple tissue pieces are aligned across the long axis of the mold, and not placed at

random. During cooling, paraffin wax shrinks up to 15%, causing compression in tissues. This

compression is almost fully recovered when sections are floated on a warm waterbath;

compression resulting from microtomy is only partially recovered.

Alternative embedding media and processing methods

Alternative embedding media may provide optimum support for tissues in applications for which

paraffin waxes are unsuited, for example when:

• tissue components are heat or reagent labile

• hard or dense tissues are inadequately supported

• adhesion between specimen and wax is poor

• very thick or very thin sections are required

• sectioning whole organs such as lung or brain.

• Resin embedding methods are now used for many of these applications. Non-resinous

embedding media include those listed below

AQUEOUS MEDIA

Agar has a high melting point and low gelling temperature of agar make it ideal for double

embedding multiple small tissue fragments. Agar is generally unstained by overnight stains, but

will stain with alcian blue.

Gelatin is used for simple embedding in a similar manner to agar. However the low melting

point of gelatin (35-40°C) makes it unsuitable for double embedding. It is used in Gough and

Wentworth's whole-organ sectioning method and its variants, or simply to support large tissue

blocks for 1 mm thick sectioning and subsequent three-dimensional reconstruction. In

phospholipid and enzyme studies tissues may be infiltrated and embedded in gelatin and the

resulting blocks sectioned on a freezing microtome. This technique has now largely been

superseded by other media used for cryotomy.

SPECIAL TECHNIQUES IN PROCESSING

A. Decalcification

Some tissues contain calcium deposits which are extremely firm and which will not

section properly with paraffin embedding owing to the difference in densities between

calcium and paraffin. Bone specimens are the most likely type here, but other tissues

may contain calcified areas as well. This calcium must be removed prior to embedding

to allow sectioning. A variety of agents or techniques have been used to decalcify tissue

and none of them work perfectly. Mineral acids, organic acids, EDTA, and electrolysis

have all been used. Strong mineral acids such as nitric and hydrochloric acids are used with dense cortical

bone because they will remove large quantities of calcium at a rapid rate. Unfortunately,

these strong acids also damage cellular morphology, so are not recommended for

delicate tissues such as bone marrow.

Organic acids such as acetic and formic acid are better suited to bone marrow, since

they are not as harsh. However, they act more slowly on dense cortical bone. Formic 5

acid in a 10% concentration is the best all-around decalcifier. Some commercial

solutions are available that combine formic acid with formalin to fix and decalcify tissues

at the same time.

EDTA can remove calcium and is not harsh (it is not an acid) but it penetrates tissue

poorly and works slowly and is expensive in large amounts.

Electrolysis has been tried in experimental situations where calcium had to be removed

with the least tissue damage. It is slow and not suited for routine daily use. After

decalcification tissue should be rinsed thoroughly in running tap water before

proceeding with the processing steps.

End of decalcification:

1. Mechanical

a. Bending the tissue for flexibility

b. Using a pin to feel softness

c. Scraping the section surface

2. Chemical

a. 5 mL of used decalcification solution mixed with 5 mL of concentrated

Ammonium hydroxide, and then 5 mL of saturated ammonium oxalate.

Let stand for 30 minutes. If a precipitates form then the decalcification

is not complete.

3. Radiography

a. X-rays is the more accurate for verification of calcium salts removal.

B. Frozen Sections

At times during performance of surgical procedures, it is necessary to get a rapid

diagnosis of a pathologic process. The surgeon may want to know if the margins of his

resection for a malignant neoplasm are clear before closing, or an unexpected disease

process may be found and require diagnosis to decide what to do next, or it may be

necessary to determine if the appropriate tissue has been obtained for further workup of

a disease process. This is accomplished through use of a frozen section. The piece(s)

of tissue to be studied are snap frozen in a cold liquid or cold environment (-20 to -70

Celsius). Freezing makes the tissue solid enough to section with a microtome Frozen sections are performed with an instrument called a cryostat. The cryostat is just

a refrigerated box containing a microtome. The temperature inside the cryostat is about

-20 to -30 Celsius. The tissue sections are cut and picked up on a glass slide. The

sections are then ready for staining.

