Plant Microtechnique
Part Two

Clearing

Clearing is the process of removing depositions suspended in water or other liquids by using natural or chemical means. **Clearing** means transfer plant sample from the dehydrated solutions to a solutions dissolves paraffin waxes. if dehydrated solution does not dissolve the wax, for example, **Clearing** means removing of alcohol or any other substance within the sample tissues, which leads to difficulties in the cutting process. If the dehydrated solution melts the wax, it is unnecessary to the process of **Clearing** . samples can be directly transfer to Infiltration.

There are several types of **Clearing** media but a good solution is the one which has the following characteristics:

1 - Fast **Clearing** without distortion of the components of the sample tissues.
2 - Slow evaporation.
3 - Lack of a foul (bad)odor.
4 - Nontoxic.
5 - Not flameable.
6 - Easy to remove.
7 - Its ability to melt the infiltration media.

Clearing Reagents

1 - Xylene
2 – Chloroform
3 - Cider-wood oil
4 – Benzene
5 – Trichloroethylene
6 - Clove oil

Clearing Reagents

1 - Xylene (Xylol):

Samples must be completely dehydrated before being gradually brought into pure Xylene. the pure xylol needs to be changed several times. The transfer from Xylene to paraffin must also be gradual and is just as tedious a process. Every trace of xylol must be removed from the tissues befor the tissues embedded, otherwise the paraffin will crystallize. Tissues tend to become excessively hardened if left too long in Xylene. Xylene is still the prime reagent for clearing sections previous to mounting in balsam or other resinous medium. Xylene should always be free from water and acids.

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1Xylene: - الزايلين (الزايلون)

يعتبر الزايلين من أفضل أوساط الترويق الشائعة حتى الآن والمستخدمة في مختبرات التحضير المجهري، حيث أنه لا يتطاير بسرعة وأنه يضفي على النسيج شفافية تدل على إتمام عملية الترويق، كما يمكن نزعه بالتبخير من الأنسجة أثناء عملية التشريب، ودرجة غليانه حوالي 140 ^{5}م، كما أنه رخيص الثمن، ولكن من سلبيات الزايلين أنه يسبب انكماشاً للنسيج إذا ما ترك فيه لمدة طويلة.

2 - **Chloroform:** **Chloroform is the indicated clearing reagent for some kinds of material and occasionally had to be used in place of xylol before the butyl alcohols came into use. In the final stages of infiltration it is more easily removed than xylol but not so readily as the butyl alcohols. Equal parts of chloroform and carbon bisulphide sometimes constitute an excellent clearing agent. Chloroform hardens celloidin. It has been . accused of spoiling^delicate stain combinations, consequently one should avoid using it on stained slides. in trichloroethylene and mounted in balsam dissolved in this solvent dry far more quickly than those mounted with the balsam dissolved in xylol .**

3 - Clove Oil: The reagent most commonly used for clearing sections on the slid^before mounting in balsam is clove oil. Before the mounting is done, all traces of the oil should be removed by washing the slide in xylol, otherwise the stains are apt to fade. Clove oil renders tissues brittle if they remain in it for any length of time. Clove oil contains about 82% eugenol. While no data are on record, eugenol may conceivably be \ substituted for clove oil if necessary, but it is far more expensive.

4 – Trichloroethylene: This fluid is an excellent substitute for xylol. When used as a clearing agent, extraction of stains has not been observed. Paraffin is completely soluble in it, as is balsam. It has been claimed . That slides cleared

5 - Benzene:

These fluids work almost as well as "xylol. Great care must be taken when using these fluids because of their explosive | character.

Bergamot Oil.—This was a favorite with the older botanists. One • may begin with the material in 95% alcohol and, by adding a drop at a time, gradually replace the alcohol with the oil. Some oil will remain in the material after embedding, but this is sometimes more advantageous than otherwise. Bergamot oil does not affect coal-tar dyes if used in the . clearing of stained preparations.

- Cedar Oil:

Paraffin Infiltration

Paraffin wax is the most common infiltration and embedding medium. Paraffin wax is miscible with xylene as well as isopropanol, but is not miscible with alcohols or aqueous fixatives. A wide variety of infiltration media is available to suit every tissue type and application. During Microtomy, factors such as wax hardness, stickiness, and brittleness should be considered with the technique and tissue in mind. Most common paraffins have a melting point between 55-58° C and the paraffin is typically kept at 2-4° C above melting point on the processor. During this last processing step. Heat and vacuum are both utilized for optimal infiltration of paraffin. However. Too much heat and time in liquid paraffin may harden tissue and create Microtomy problems. As with the previous processing steps, the optimal infiltration time for each tissue type/size must be determined for optimal results.

Embedding :

1 - Paraffin wax Embedding :The actual embedding takes place when the paraffin- infiltrated tissue is placed in fresh paraffin and the latter allowed to cool. It is important to remember that the xylol and other solvents will dissolve the fats of the tissues unless they are fixed by some special chemical such as osmic acid.

2 - Celloidin Embedding

Celloidin is dissolved in equal parts of absolute alcohol and ether. The tissue is dehydrated in alcohol in the same way as for paraffin except that it is transferred from absolute alcohol to a dilute solution of celloidin. As the alcohol and ether evaporate, they are replaced by more concentrated celloidin. It is finally hardened in chloroform and stored in 80 percent alcohol. It is a much longer process than paraffin but causes much less shrinkage and distortion. It is rarely used in planr sample embedding.

3 - Paraplast Embedding

4 - Gelatin Embedding

5 - Carbowax Embedding

6 - Synthetic resins

Microtomy :

- Is the means by which tissue can be sectioned and attached to a surface for further microscopic examination.

Microtome:

- Basic instrument used in microtomy.
- Mechanical device for cutting thin uniform slices of tissue – sections.

Types of microtomes

- There are 5 basic types of microtomes named according to the mechanism-

- Rocking microtome
- Rotary microtome
- Base sledge microtome
- Sliding microtome
- Freezing mictotome.

Rocking microtome:

- Name derived from the rocking action of the cross arm.
- Oldest in degisn, cheap , simple to use.
- Extremely reliable.
- Very minimum maintenance.

Mechanism of action:

- Knife is fixed, the block of the tissue moves through an arc to strike the knife.
- Between strokes the block is moved towards the knife for the required thickness of sections by means of a ratchet operated micrometer thread.
- Steady backward and forward movement of the handle gives ribbons of good sections.

Disadvantage:

- Size of the block that can be cut is limited.
- Sections are cut in a curved plane:
  (Microtomes designed to cut perfectly flat sections; the block moving through an arc at right angles to the knife edge are available.)
- Light instrument : advisable to fit it into a tray which is screwed to the bench , or to place it on a damp cloth to avoid movement during cutting.

Rotary microtome

- First machine designed by Professor Minot,hence often referred to as the "Minot Rotary".

- Manual (completely manipulated by the operator).
- Semi-automated (one motor to advance either the fine or coarse hand –wheel)
- Fully automated (two motors that drive both the fine and the coarse advace hand-wheel)
- Mechanism of block advancement: retracting or non retracting.
- Retracting action moves the tissue block away from the knife on upstroke, producing a flat face to the tissue block.

- Originally designed for cutting sections of very large blocks of tissue (eg. whole brains)
- Used primarily for
- Large blocks,hard tissues,whole mounts.
- Especially useful in neuropathology and ophthalmic pathology.

Mechanism of action:

- The block holder is mounted on a steel carriage which slides backwards and forwards on guides against a fixed horizontal knife.

Advantages:

- Ability to cut thin 2-3 mm sections.
- Easy adaptation to all types of tissues (hard, fragile, or fatty) sectioning.
- Ideal for cutting serial sections: large number of sections from each block.
- Cutting large blocks
- Cutting angle of knife is adjustable.
- Large and heavier knife used-less vibration when cutting hard tissue.
- Heavier and more stable .

Advantages:

- Heavy , very stable, not subject to vibration.
- Knife large(24 cm in length) and usually wedge shaped –less vibration .
- Adjustable knife holding clamps allow tilt and angle of the knife to the block to be easily set
  – used for cutting celloidin sections by setting the knife obliquely
  - paraffin wax embedded sections are more easily cut .

Disadvantages

- Slower in use than rocker or rotary microtome- true only when change from one instrument to another is made .
- With practice, sections from routine paraffin blocks can be cut as quickly as on any other type of microtome.

Sledge microtome

Sliding microtome

- Designed for cutting celloidin-embedded tissue blocks.
- The knife or blade is stationary, specimen slides under it during sectioning.
- Also used for paraffin –wax embedded sections.

Freezing microtome

- Gives best results for cutting frozen sections.
- Machine is clamped to the edge of a bench and connected to a cylinder of CO_2 by means of a specially strengthened flexible metal tube.

Freezing microtome

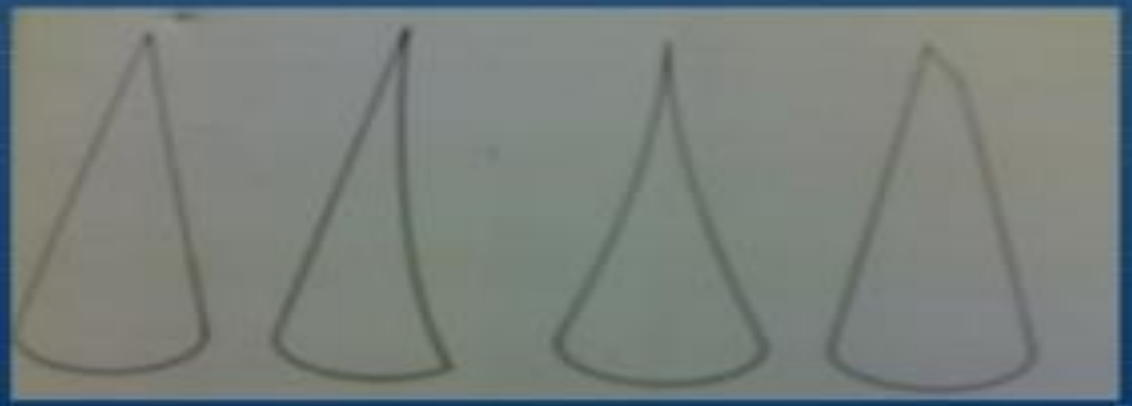
- Knife freezing attachment is supplied with most machines.
- Separately controlled flow of CO2 on the edge of the knife - to delay the thawing of sections on the knife and make it possible to transfer them directly from knife to slides.
- Sections thickness gauge is graduated in units of 5 micrometer instead of 1micrometer.

Microtome knives

- Developed to fit specific types of microtomes and cope with different degrees of hardness of tissues and embedding media.
- Paraffin-wax embedded tissues knives are made of steel.
- Resin-embedded tissue is normally cut using glass knives.

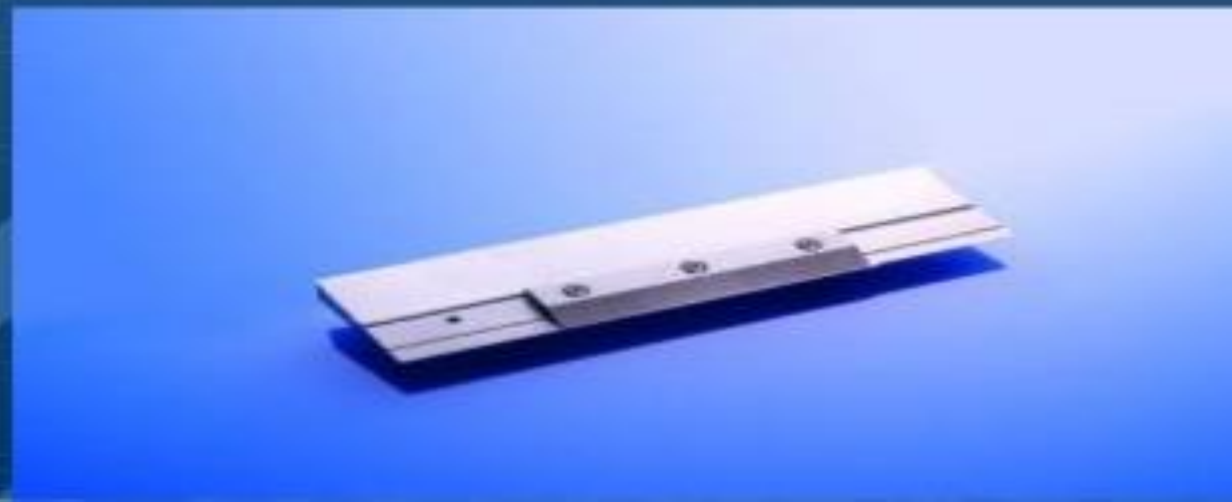
Knives are classified according to their shape when viewed in profile as:

- Wedge.
- Planoconcave.
- Biconcave.
- Tool edge or D profile.



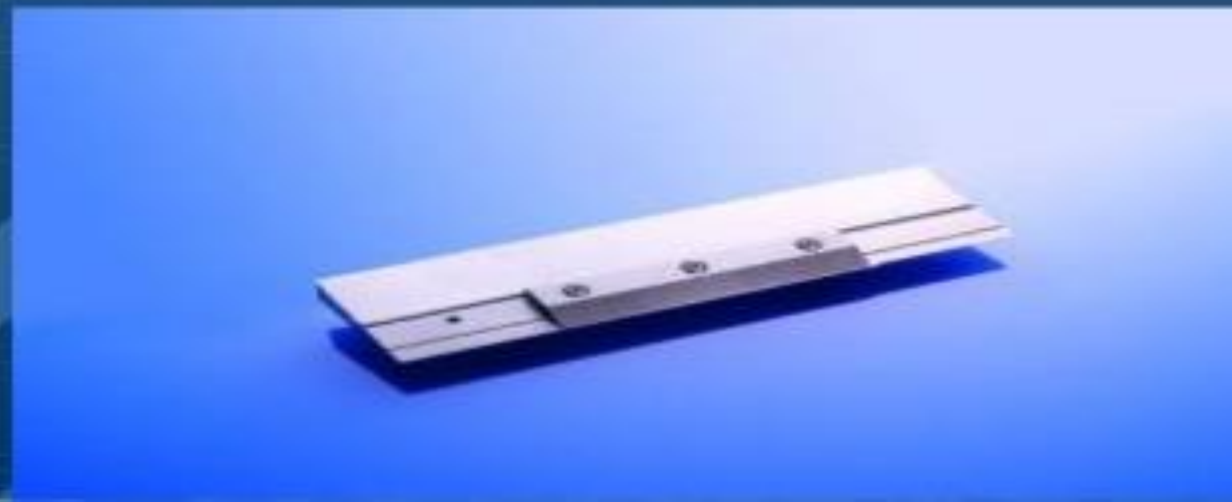
Disposable blades

- Used for routine microtomy and cryotomy.
- Provide a sharp cutting edge, produce flawless 2-4 mm sections.
- Disposable blade holders incorporated into the microtome or an adapter.



Disposable blades

- Blade is coated with PTFE (polytetrafluoroethylene) allowing ribbons to be sectioned with ease.
- Over-tightening the disposable blade in the clamping device may cause cutting artifact such as thick and thin sections.

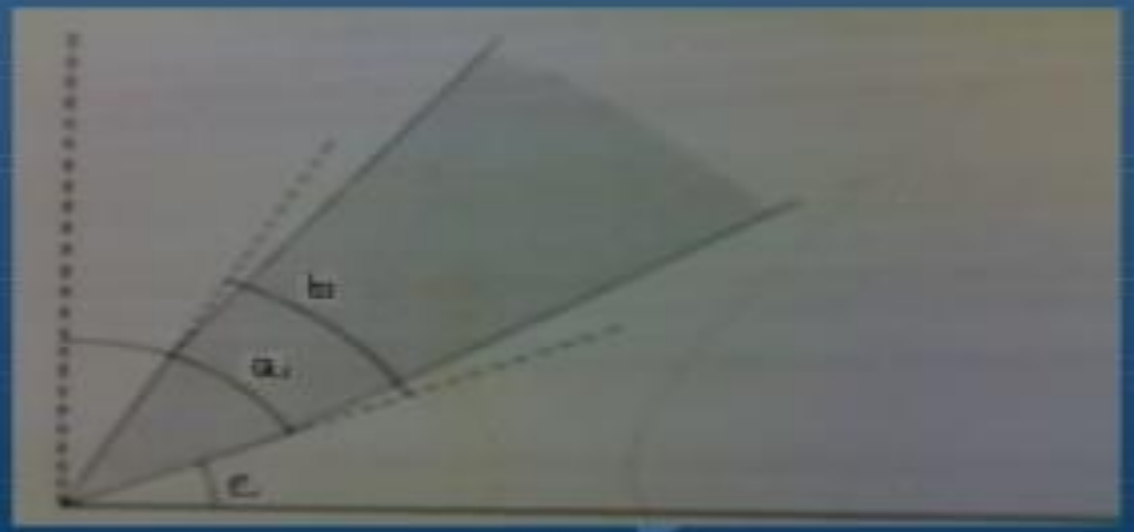


Glass and diamond knives

- Used in electron microscopy and with plastic resin-embedded blocks.

Knife angles

- Clearance angle: angle formed by a line drawn along the block surface and the lower bevel of the knife.
- Rake angle: angle between the upper bevel of the knife and a line at 90 degrees to the block surface.



Angles asociated with the knife edge.
A:rake angle; b:bevel ; c:clearance angle.

Abrasives

- Aluminium oxide(alumina)
- Iron oxide(Jeweller's rouge)
- Silicon carbide
- Diamond

Automatic knife sharpners

Automatic knife sharpners

- Two basic designs available.

  1) knife is held vertically with revolving sharpening wheels grinding the cutting edge.

  2) knife is held horizontally against the surface of a slowly rotating flat plate.

Automatic knife sharpners

- Plates – glass , copper or bronze charged with an abrasive.
- Glass plates need to be roughened before use to allow the abrasive particles to be held more easily in place.
- Copper and bronze plates used in conjunction with diamond paste, 6micrometer particle size being most appropriate for rough sharpening, and 1 micrometer for fine polishing.

Stropping Technique:

- Knife is laid on the near end of the strop with the cutting edge towards the operator (opposite direction to that used in honing.)
- Knife held with forefinger and thumb to facilitate easy rotation at end of each stroke.
- Action is exact opposite to that used in honing,using full length of the strop and stropping evenly the whole of the blade.

Staining:

Staining is the use of dyes to render distinct colours to different constituents of the tissues under investigation. Some of the stain or dyes used in biological studies are given in the Table (Important Stains and their properties) next slide.

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Important Stains and their properties

Table 6.1. Important stains and their properties

S.No.	*Stain*	*Colour*	*Properties*
1.	Acetocarmine	Bluish-red	It stains chromosomes.
2.	Acid fuchsin	Brilliant magenta	It stains cortex, cellular walls and also mitochondria.
3.	Aniline blue	Blue magenta	It used to stain fungal hyphae and spores.
4.	Basic blue	Magenta red	It is a nuclear dye and also used to stain mucin and cellulose.
5.	Crystal violet	Violet	It is a good bacterial stain used in gram staining.
6.	Eosine	Pink	It is a good cytoplasmic stain for animal tissues.
7.	Fast green	Green	It is used to stain cellulose and lignified cell walls.
8.	Fuelgen's stain	Purple of red	Used to stain DNA and for observing chromosomes during cell division.
9.	Hematoxylin	Violet or blue	Used to stain cellulose cell wall.
10.	Iodine solution	Blue	It stain starch grains.
11.	Janus green	Greenish Blue	Used to stain fungi and mitochondria.
12.	Methylene Blue	Blue	It stain golgi complex and yeasts.
13.	Phloroglucinol + HCH	Red	It stain lignified walls.
14.	Ruthenium Red	Red	It stains Pectin of middle lamellae.
15.	Safranin	Red	It stains lignified Tissues as well as nuclei.
16.	Sudan (II) or (IV)	Red	It stain oil, cutin and suberin
17.	Sudan black	Black	It is a specific stain for fatty substances.
18.	Toludine blue	Blue	Used to stain RNA.

Mounting:

Adequately thin and properly stained section of the biological material is required to mounted to glass slides for investigation under the microscope. A drop of mounting medium is put on sections and gently cover slip is lowered on the sample with the help of tweezers.
Air bubbles trapped on the material is gently removed when cover slip is on the material. Extreme care should be taken when handling newly covered slides as it is easy for cover slip to dislodge and damage the tissue. Freshly covered slides should not be examined with microscope, as is possible to get a drop of "wet" mounting media on the objective.

Mounting media

The mounting medium is the solution in which the specimen is embedded, generally under a cover glass. Simple liquids like water or glycerol can be considered mounting media, though the term generally refers to compounds that harden into a permanent mount. Popular mounting media include Permount, glycerol jelly, and Hoyer's mounting medium. Properties of a good mounting medium include:

1 -Having a refractive index close to that of glass (1.518).

2 - Non-reactivity with the specimen.

3 - Stability over time without crystallizing.

4 - Darkening, or changing refractive index.

5 - Solubility in the medium the specimen was prepared in (either aqueous or non-polar such a xylene or toluene).

6 - not causing the specimen stain to fade or leach.

Examples of mounting media

Aqueous

Popularly used in immunofluorescent cytochemistry where the fluorescence cannot be archived. The temporary storage must be done in a dark moist chamber. Common examples are:

1. Glycerol-PBS (9:1) with antiquench e.g. any of the following 1.p-phenylenediamine

2. propyl gallate

3. 1,4-Diazabicyclo (2,2,2)-octane (DABCO) (very popular)

4. Ascorbic acid

5. Mowiol or Gelvatol

6.Gelatin

7. Mount™

8. Vectashield

9. Prolong Gold

10. CyGEL / CyGEL Sustain (to immobilize living, unfixed cells and organisms)

Non-Aqueous

Slide of 60-year-old holotype specimen of a flatworm (Lethacotyle fijiensis) permanently mounted in Canada balsam

Used when a permanent mount is required

1.Canada balsam

2.DPX (Distrene 80 - a commercial polystyrene, a Plasticizer e.g. dibutyl phthalate and Xylene)

3.DPX new (with Xylene but free of carcinogenic Dibutyl phthalate)

4.Entellan™ (with Toluene)

5.Entellan™ new

6.Neo-Mount™ (compatible with aliphatic Neo-Clear® but not compatible with aromatic solvents like Xylene)

Euparal: This mounting medium was invented in 1904 by Prof. G. Gilson, Professor of Zoology at Louvain University, Belgium. It contains the substances sandarac, eucalyptol, paraldehyde, camphor, and phenyl salicylate. Euparal possesses a nice odor (but don't smell it anyway), due to the natural oils that are included. Euparal is commonly used to mount histological specimens and insects. One big advantage of Euparal is, that the specimens can be transferred directly from the alcohol in which they are stored. Do not embed specimens which contain water, this may result in a clouding of the mounting medium.

Summary: Advantages of Euparal include the possibility to directly transfer specimens from alcohol to Euparal without the need of toxic solvents. A disadvantage is the relatively long drying time of a few days to weeks.

Canada Balsam: This is a natural mounting medium obtained from the balsam fir tree (*Abies balsamea*). The optical properties are nearly identical with those of glass. For this reason, Canada Balsam was used for many years as a kit to hold optical lenses in place. Meanwhile, synthetic lens kits have replaced Canada Balsam, it is still used as a mounting medium for microscopy, however. Canada Balsam has the advantage that its optical properties do not deteriorate with age. Permanent slides mounted with Canada Balsam have been stored for a century and are still useful.

The disadvantage of Canada balsam is, that the specimen must be placed into xylene (toxic!) before embedding. Wet specimens must first be dehydrated in alcohol and then transferred to xylene. Transferring specimens directly from alcohol to Canada balsam won't work, because the alcohol won't dissolve the Canada balsam.

Summary: The advantage of Canada balsam is the long storage ability of the slides. Other, modern, mounting media may have a similar storage ability, but with Canada balsam there is historic experience. A disadvantage is the need for toxic solvents when preparing the specimen. Apparently, it is also not very cheap to obtain.

Eukitt and other resin-based media: Eukitt is a very fast drying general-purpose resin-based mounting medium. Eukitt will solidify within about 20 minutes. The specimens must be free of water and placed first in alcohol and then in xylene prior to mounting. The use of xylene is a disadvantage, as it is harmful when inhaled. Eukitt itself can also be diluted by xylene to adjust it viscosity.

Besides Eukitt, a range of other resin-based mounting media are commercially available, such as Diatex, Entellan, Malinol, Rhenohistol and Depex. They differ in their refractive index. All of these mounting media require the specimen to be first dehydrated in alcohol and then transferred to xylene. Some of these resins shrink significantly during the drying process.

Summary: The advantage of Eukitt is that it is a fast drying mounting medium. The disadvantage is the need for toxic solvents to prepare the specimen.

Clear nail polish: Nail polish can be used to seal the sides of the coverslip when using aqueous mounting media. It can also be used directly as a mounting medium. The specimens must first be dehydrated in alcohol and can then be directly mounted (without xylene) in nail polish.

Summary: The advantage of nail polish is, that it is readily available and that it avoids the use of toxic organic solvents to treat the specimens. One disadvantage is, that it seems to shrink a lot when making very thick mounts (such as whole insects).

Glycerol jelly: The advantage of Glycerol jelly is that it s water-based and that this avoids the need of alcohol dehydration (which possibly deforms the specimens), and other toxic organic solvents. Some specimens can only be satisfactorily mounted in Glycerol jelly. It also does not shrink. The disadvantages include the need for a potentially toxic antiseptic in the jelly, the difficulty of mounting the specimens and the need to seal the cover slip with nail polish.

Glycerol: It is possible to make a permanent mounts by embedding the specimen either in pure liquid glycerol or a specified glycerol-water mixture. The glycerol-water mixture can be adjusted to an appropriate refractive index. Adding more water lowers the refractive index. It is also possible to use pure water alone (for some delicate algae, for example).

Algae and other water organisms can be embedded this way. Algae that are embedded in pure glycerol may shrink because the glycerol withdraws water from the cells. If the algae shrink too much, then the glycerol should be more diluted with water. A high concentration of glycerol should be maintained, however, otherwise there is a risk of fungal growth in the medium.

The advantage of glycerol is, that fungi and algae do not shrink as much as with other mounting media. It is also not necessary to treat the specimens with alcohol or organic solvents, which may introduce artifacts and remove pigments. The disadvantage is, that it is difficult to prepare slides that are truly permanent in nature. A proper sealing of the cover slip corners is absolutely necessary if one wants to store the slides over extended periods.