The Microtome

The structure of cells and their nature of arrangement etc. cannot be satisfactorily learnt by whole mounts. Therefore sectioning of materials is often resorted to. But all materials cannot be sectioned by the freehand method. Materials, especially unicellular forms and those that are not stiff to withstand the action of the knife, and those containing cavities, which would be crushed out of shape if sectioned, require a mechanical device, which cuts sections after surrounding and supporting such materials, with some substance like wax which impregnates them. Another drawback of freehand sectioning is that sections of uniform thickness cannot be produced by that method. Further, sections of less than 10 μm[1] thickness cannot be cut by the freehand method. These drawbacks are overcome by the use of the instrument called the microtome.[2] Microtome is defined as a device for advancing a block of tissue a given amount, cutting a slice from it, and then re-advancing it for the same amount, and so on. (Peter Gray)

Microtomes are broadly classified into two categories :

(*i*) those in which the block remains stationary and the knife moves across it; and

(*ii*) those in which the block moves across a stationary knife.

The first group includes several types known by names like : Bench Microtome, Clinical Microtome, Sliding Microtome, Sledge Microtome and so on. These are not useful in producing serial sections. They are, however, used in producing thin sections of wood and other materials, of which serial sections are not required.

Sledge Microtome

Sledge Microtome (Fig. 15) is a device by means of which a block of wood can be firmly held and presented to a moving knife which, as it passes across the block, removes a section of

[1] μm=micrometre=0.001 mm. This is better preferable to designate 1/1000 mm than is our conventionally used term μ or micron and is used throughout this book.

[2] For a full treatment of microtomes refer : "The Encyclopedia of Biological Sciences"—Peter Gray 1965 : Reinhold Pub. Corporation, New York.

predetermined thickness. The thickness at which sections are cut depends upon the work for which they are required. For general work sections of 20 μm thick are prepared.

Generally, in a Sledge Microtome, the block of wood of which the transverse face should have sides of not more than 1 cm. and the longitudinal face not more than 2 cm. long, is held in a Naples clamp, which is a device that enables the specimens to be accurately oriented in respect of the razor. The razor is a heavy knife of wedge shaped section and is clamped to the sledge, which slides along a groove on three bearing surfaces.

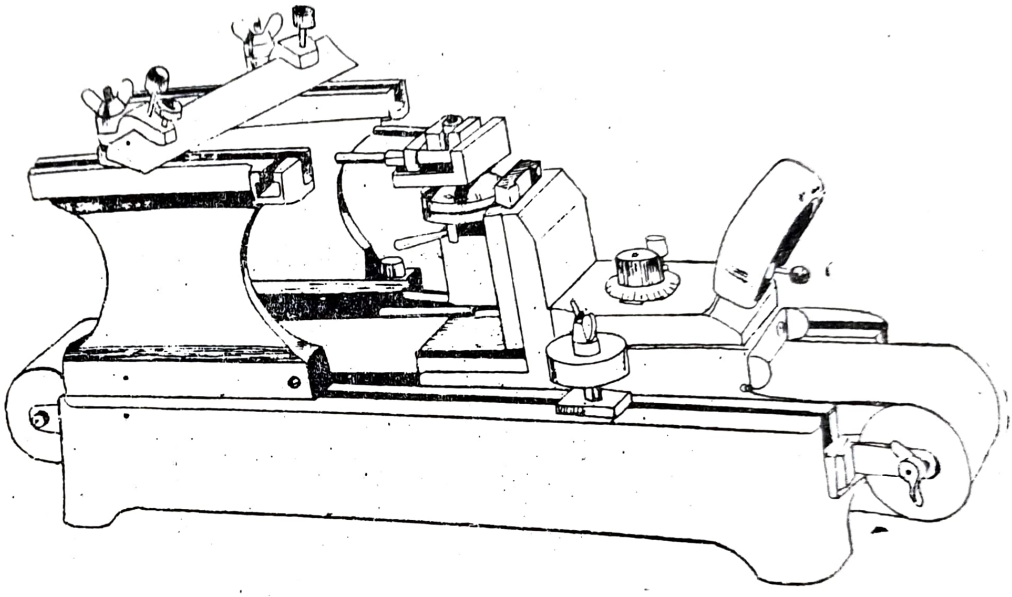


Fig. 15. Sledge microtome.

In some models the clamp is raised the thickness of the desired section by turning an accurately graduated head which, in rotating, turns a screw, which in turn moves the clamp. In others, the sledge, on returning to its starting point automatically moves the screw and thus raises the block through a previously determined increment.

The second group of instruments includes the Rocking Microtome and the Rotary Microtome. The Rocking Microtome (Fig. 16) is operated by moving a handle forward and backwards over a disc. The Rotary Microtome (Fig. 17) is operated by rotating a wheel. Both require no mechanical skill on the part of the operator and are easily manageable. The Rotary Microtome is the most advanced type and has all the mechanical improvements to produce good serial sections

in ribbon form. There are many models made by different

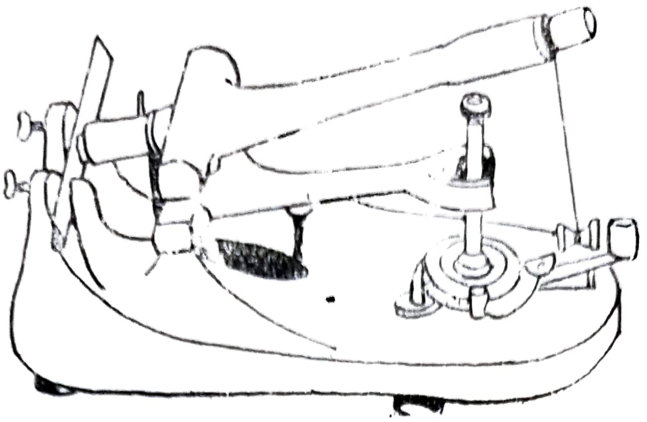


Fig. 16. Cambridge rocking microtome.

manufacturers, viz. Spencer Rotary Microtomes of the American Optical Co., Minot Microtomes of the R. Yung of Heidelberg; Rotary Microtomes of Leitz, Zeiss or Bausch & Lomb etc. The Spencer model is described below briefly.

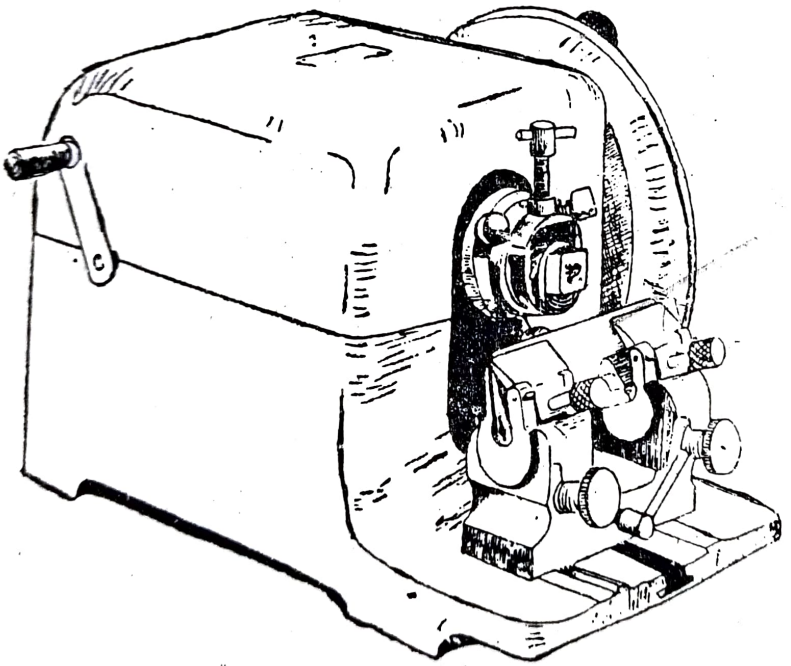


Fig. 17. Spencer rotary microtome.

The Rotary Microtome

There is a large wheel, the rotation of which causes the block holder to move up and down across the knife. The material mounted on the block holder is fixed to the rotating object clamp, a standard ball and flange type with screws to

provide means for orienting the object to any desired position. The object holder works on a horizontal feed mechanism consisting of a feedscrew and a notched rachet wheel, actuated by a pawl which turns the feedscrew, automatically advancing the specimen on the upward stroke. The force of the feedscrew is transmitted to the specimen through an inclined plane. The amount of feed equivalent to the thickness of sections to be cut, in micrometres, is shown on the side near the wheel, and can be set by a knurled button. A crank at the end of the feedscrew provides a convenient means of adjusting the object to the knife and of returning the object clamp to the beginning of the feedscrew. When object clamp reaches the extreme forward position, the feed mechanism automatically ceases to work. The knife is held in a rigid holder, which can be tilted through a wide range of cutting angles and is adjustable to and from the object.

The working of the rotary microtome is best learnt by observing an experienced worker after gathering a general knowledge of the 'anatomy' of the instrument. The most important factor in the production of good sections is the quality of the knife used. The perfect knife edge[1] is a must for good sectioning. A solid knife supplied by the manufacturer is ideal for perfect cutting edge, though use of safety razor blade edge attached to the knife is resorted to by some workers. The steps in the operation of the microtome is given in chapter 8 (2).

[1] The knife edge seen theough 'Low power' of a microscope must be a perfectly straight line, free of any nicks. The way in which the knife is sharpened to produce the correct cutting facet is described by Peter Gray in "Handbook of Basic Microtechnique", McGraw Hill, New York, 1958.

2. Killing and Fixation

These are two essential requisites for the processing of cells and tissues. Both are usually performed by a single fluid (called *fixative*), which may be a combination of usually more than one chemical reagent. The term *Killing* means the sudden stoppage of the life processes with respect to the entire organism and more specifically individual cells of which tissues are made up of. *Fixation* is commonly the preservation of all structural and cellular elements in as near their original state as possible, or a good fixative is one that changes the cell chemistry the least and preserves the cell structure the best (Schiller 1930).

The goals of killing and fixation are the following:

(*i*) To preserve cell structures and contents in as natural a form as possible.

(*ii*) modify the refractive index of some of the cellular elements so that they are made better distinguishable under the microscope.

(*iii*) To make materials resistant and hard to reactions during further treatment in processing.

(*iv*) To prepare the material to improve upon effects of certain stains.

Reagents in Killing and Fixation

No single substance has been found to meet the requirements of successful fixation. So combinations of many reagents are often used. The general principle involved in the combining of reagents to form a fixing fluid is to secure a balance between all the properties of the reagents involved : (*i*) A substance which tends to shrink cytoplasm must be mixed with one which tends to swell cytoplasm. Two reagents having identical disadvantages should never be combined. (*ii*) Substances

which are easily oxidised should not as a rule be combined with reagents that are powerful reducers. Although there are many chemical reagents recommended for killing and fixing, the most common reagents, their general properties and specific uses are briefly discussed here, so that students can familiarise themselves with them and can recognise them.

1. ETHYL ALCOHOL[1]

Miscible with water. Boiling point 78.3°C. inflammable. A reducing agent. Dissolves fats and phospholipids. Very rapid penetrability. Shrinks tissues and hardens very much. Precipitates albumen and globulin, nucleic acids and glycogen. Makes tissues difficult to stain.

2. FORMALIN

Formalin is the trade name given to an aqueous solution of formaldehyde, 40% by weight. Miscible with water. A reducing agent. No effect on fats. Slow penetrability. Causes no shrinkage; but causes shrinkage in alcohol. Very great hardening effect. Makes tissues difficult to stain, except with acid dyes. Fumes are extremely irritating to mucous membranes.

3. ACETIC ACID

Miscible with water. Very rapid penetrating agent. No hardening effect. Does not fix cytoplasm and fats. Makes tissues soft and incapable of being hardened by alcohol. No action on staining. Precipitates nucleoproteins.

4. PICRIC ACID (crystals)

Miscible with water. No effect on lipids. Fairly rapid penetration. Causes great shrinkage. Very little hardening effect. Precipitates all proteins and makes them insoluble in water.

6. POTASSIUM BICHROMATE

Miscible with water. An oxidiser. Should not be used with alcohol or formalin. Slow penetration. Fixes cytoplasm and mitochondria. Causes little shrinkage. Slow hardening.

7. PROPIONIC ACID

Miscible with water. Modifies hardening and shrinkage effects of alcohol and formalin. Preserves nuclear details. Good penetration. Tends to swell tissues. Tends to soften also. Fixes nucleoproteins. Reduces staining of cytoplasm.

8. MERCURIC CHLORIDE

A rapid fixer and a powerful precipitant of proteins and nucleic acids. But it tends to shrink tissues. Transparent tissues are rendered opaque as soon as fixed. Aquous solutions must always be based upon distilled water. Thorough washing is essential.

9. IODINE

Penetration rapid. Excellent for fixing microscopic plant forms.

KILLING AND FIXING FLUIDS[1]

Fixatives fall into many groups according to their ingredients. Some formulae are stable and can be prepared and stored for use at any time. Some, however, must be made up immediately before use. There are a few basic formulae, the number of which has been increased from time to time by changes in combination of ingredients or modifications in amounts thereof. Some of the fluids are known by the names of the investigators who first devised the type of formula. Some are known by names of the major ingredients.

The most important formulae are the following :

[1] For an exhaustive list refer "Plant Science Formulae" McLean and Ivimey Cook, 1965, MacMillan & Co. Ltd., London.

1. Acetic Acid—Alcohol Mixtures

(*i*) Farmer's Formula

Glacial acetic acid	5 ml
Absolute alchol	15 ml

Recommended for root tips and anthers for cytological preparations. Fixation time : 15 minutes for root tips and 1 hour for anthers. Wash and store in 70% alcohol

(*ii*) Carnoy's Formula

Absolute alcohol	10 ml
Chloroform	15 ml
Glacial acetic acid	5 ml

Recommended for root tips and anthers for cytological preparations. Fixation time : 10—15 minutes. Wash in 85% alcohol and store in same.

2. Formalin—Acetic Acid – Alcohol Mixtures

(*i*) Rawlin's Formula/F. A. A.

Ethyl alcohol 95%	50 ml
Glacial acetic acid	5 ml
Formalin	10 ml
Water	35 ml

Recommended for algae and materials for histological studies. Has good hardening action and materials may be stored in this for years even. A lower % of alcohol may be used while fixing delicate objects. For hard woody materials decrease acid and increase formalin. Fixation time : 18 hours. Wash in alcohol and store in same.

(*ii*) Chicago

3. Dehydration

Water will not mix with the usual media in which stained and sectioned materials may be finally preserved. The paraffin with which the materials are impregnated for serial sectioning is also not miscible with water. The purpose of dehydration is, therefore, to remove all traces of water from the cells and tissues before either impregnating or finally mounting.

Dehydration consists of treating the material with a series of solutions containing progressively decreasing concentrations of water and a progressively increasing concentration of the dehydrant.

The dehydrating agents are changed by decanting them from materials and immediately flooding the material with the next grade of fluid. The material should not be allowed to become dry at any stage. The interval in each grade must be determined by the size, nature of the material and the solubility of the residual reagents left in the materials. Two maxims that are helpful in judging the interval are :

(*i*) Long intervals in low concentrations make tissues soft and

(*ii*) Long intervals in high concentrations make materials brittle.

Peter Gray (1954) recommends that the materials to be dehydrated should be suspended towards the top of a tall cylinder of dehydrant, since water is heavier than most of the dehydrants.

In the last but one grade of dehydrant a very small quantity of "eosin" powder may be added to colour the material dehydrated for embedding in paraffin. This helps in locating

materials, otherwise rendered transparent, in the finished paraffin blocks prepared for sectioning.

Reagents in Dehydration

Many reagents have been recommended for use as dehydrants. Some of them are merely water removing, while others act both as water removers and solvents for paraffin with which materials are impregnated before sectioning and the resinous media in which materials are mounted. Some of the most important dehydrants are treated below.

1. Ethyl Alcohol/Isopropyl Alcohol

The most commonly used dehydrating agent is ethyl alcohol. Isopropyl alcohol can be used exactly in the same manner as ethyl alcohol, is cheap and easily procured. Methyl alcohol has been recommended by some textbooks, but rarely used with plant materials due to its high toxicity and drastic dehydrating action that damages structures.

After ascertaining the strength of alcohol supplied prepare with distilled water, about 200 ml of 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80 per cent. alcohol by volume, to be stored in glass stoppered reagent bottles. Next in the series will be 95% and absolute alcohol.[1] After the materials are washed decide upon which percentage of alochol is to be started with for dehydration. Materials fixed in such fluids that require no washing may be directly dehydrated. Begin with a grade that has approximately the same percentage of water as the fixing fluid. After washing in water begin from 5% alcohol.

Normally the time for which materials are left in each grade shall be 30 minutes for root tips and small pieces of leaf and 12 hours for large blocks of wood, upto 70% and 1 hour and 24 hours respectively for 80, 95 and 100%.

2. Acetone[2]

The series to be prepared with distilled water are : 7.5, 10,

[1] Absolute alcohol is 100% ethyl atcohol. Can be prepared by distilling rectified spirit or purchased as a chemical—"Alcoholum Anhydratum" without licence.

[2] Acetone is highly volatile, and operations must be quicker.

15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 per cent. Intervals can be less than one hour in each grade. The procedure is exactly the same as with ethyl or isopropyl alcohol.

3. GLYCERINE (GLYCEROL)

Glycerine is a thick, colourless, sweet-tasting liquid and is an excellent reagent for dehydrating delicate materials like filamentous algae and fungi. It has a high boiling point (290°C) and so water can be removed by evaporation. If the progressive dehydration is done slowly plasmolysis is avoided.

The material is washed well and transferred to a large volume of 5% glycerine, taken in a wide-mouthed jar. Leave this jar open but well protected from dust in a room until the water evaporates leaving the material covered by glycerine. Evaporation can be hastened by keeping the jar in an oven kept at 35°C. After a nearly anhydrous condition is reached give a change of pure glycerine and proceed for infiltration by paraffin method or whole mount.

4. Both normal butyl alcohol and tertiary butyl alcohol are useful as dehydrants. These alcohols have one additional advantage that they can be directly followed by paraffin impregnation since they are also solvents of paraffin. The different grades are prepared in combination with ethyl alcohol and are tabulated below :

Series No.	Ethyl alcohol 95% in ml	Normal butyl alcohol in ml	Distilled water in ml
1	20	10	70
2	25	15	60
3	30	25	45
4	30	40	30
5	25	55	20
6	20	70	10
7	15	85	0
8	0	100	0

Table 2. Normal butyl alcohol series (*after Sass*)

After an aqueous fixative, wash in water and transfer to alcohol br nging upto 30% and then pass through the above

in the rest.

The series with teriary butyl alcohol is given below:

Series No.	Absolute alcohol in ml	95% Ethyl alcohol	Tertiary butyl alcohol in ml	Distilled water in ml
1	0	50	10	40
2	0	50	20	30
3	0	50	35	15
4	0	50	50	0
5	25	0	75	0

Table 3. Tertiary butyl alcohol series (*after Sass*)

Dehydrate first in ethyl alcohol upto 50% and then pass through the above. Make three changes of pure tertiary butyl alcohol.

5. DIOXAN

Dioxan (diethylene dioxide) has been given many attributes by experienced workers. It does not require any other reagents to be mixed with it for dehydration. It has no drastic plasmolysing effect. It has very rapid penetrating capacity. It does not make tissues brittle. Further it reduces the number of separate steps in the process of dehydration. In spite of these advantages Peacock (1966) warns that dioxan vapour may have long term effects, not yet known, upon humans.

The following series are recommended for materials washed in water fixing. Make two more changes of pure dioxan.

Series No.	Dioxan in ml	Distilled water in ml	Duration in each in hr
1	35	65	4—12
2	65	35	4—12
3	100	0	4—12

Table 4. Dioxan series 1.

For materials fixed in any fluid containing about 50% alcohol the series can be the following one. Finally give two more changes of pure dioxan.

Series No.	Dioxan in ml	Distilled water in ml	Duration in each in hr
1	50	50	4—12
2	65	35	4—12
3	100	0	4—12

Table 5. Dioxan series 2.

Clearing or Dealcoholization

The transfer of materials after dehydration in the reagents that are not solvents of wax (like the ethyl and isopropyl alcohols) to solvents of wax is customarily called clearing. The clearing action is merely incidental to the function of the reagent to serve as a solvent of wax. Clearing is not an essential step in processing plant materials, but becomes necessary when materials are dehydrated in reagents that are not miscible with wax used in paraffin method of preparing serial sections or the resinous media in which sections or preparations are permanently mounted.

Reagents in Clearing

1. Xylene (xylol)[1]

Xylene, better known as Xylol, is the conventional reagent used for dealcoholization before materials are transferred to solvents of paraffin. A graded series is preferred.

Series No.	Ethyl alcohol in ml	Xylene/tricholoro-ethylene in ml	Duration in hr
1	75	25	3
2	50	50	3
3	25	75	3
4	0	100	3

Table 6. Xylene/Trichloroethylene series for large possible of tissue

[1] Trichloroethylene is a good substitute for Xylene and can be used exactly the same way. It is non-toxic and non-inflammable.

4. Stains and Staining

Staining means the use of dyes to render various tissue constituents visible and distinct from one another. Different parts of cells or tissues react differently to colours due to their chemical and/or physical differences. Thus they become easily visible. The preparation, experimenting and uses of stains form a separate science in itself.

The now widely accepted theory of staining is the view advanced by Witt (1876). The presence of colour in a chemical is by the presence of groups or radicals called *chromophors*. The material that contains these are called *chromogens*. The power of imparting this colour to other substances is given to a chromogen by the presence of an *auxochrome*. Auxochromes are either acid or alkali radicals, which also are responsible for the solubility of stains.

There are many ways in which a colour may be caused to remain on a particular structure.

(*i*) The ionized stain becomes precipitated upon materials by surface adsorption.

(*ii*) A definite chemical combination is entered into between the dye and the tissue

(*iii*) The saturation of a material with a dye and the subsequent precipitation of the dye in place by the advantageous solvents for dehydrating in which the dye is not so possible infusion,

(*iv*) The tissue is first caused differentially to absorb substance with which the dye subsequently makes an insoluble compound. These substances are called mordants.

Stains are classified into many based on varied principles.

The coal tar dyes are many. They are grouped into ... depending upon their chemical nature. Picric acid, Orange G, Alizarin, Methylene blue, Safranin, Light Green, Fast Green, Basic Fuchsin, Erythrosin, Eosin are some of the coal tar dyes.

The more important stains, their preparation and uses are given below:

1. ANILIN BLUE/COTTON BLUE /CHINA BLUE/SPIRIT BLUE

Both aqueous and alcoholic solutions are used.

Anilin blue	1 gm
Distilled Water or 85% alcohol	100 ml

This is excellent for filamentous algae and fungi. Stains the cell walls and achromatic figure used in combination with safranin.

Cotton blue in Lactophenol is prepared with

Phenol	100 ml
Glycerine	100 ml
Lactic acid	100 ml
Distilled water	100 ml

This is used for fungi in whole mounts.

2. BASIC FUCHSIN/FEULGEN STAIN

Basic fuchsin	1 gm
95% alcohol	100 ml
Distilled water	100 ml

This solution is used to stain bacteria as well as the vascular system in higher plants. The Feulgen stain is prepared in the following way.

Bring 100 ml of distilled water to boiling. Remo... of heat and immediately dissolve 1 gm. of basic fu... sadvan- solution cools to 60°C, filter and add to filterate ... possible bisulphite and 10 ml of 1N HCl Stopper and sto... nfusion, the dark at room temperature for 24 hours. Filte...

Feulgen stain prepared in the following way is f... ideal : Dissolve 1 gm. Basic fuchsin and 1.9 gm. sodium ... bi- sulphite in 100 ml 0.15 N HCl in a conical flask and shake

...may be purchased

3. Bismark brown

Bismark brown	2 gm
Alcohol 70%	100 ml

Stains cellulose cell walls and plant mucin. This is recommended as a single stain for Xylaria stroma, Sargassum conceptacle etc.

4. Congo red / Dianil red

Congo red	1 gm
Distilled water	100 ml

This is specially recommended for rusts, after acetic-alcohol fixation. Colours walls of parasite but not of host. Preparations stained with Congo red must be well washed before mounting in balsam.

5. Crystal violet / Gentian violet

Crystal violet	1 gm
Clove oil	100 ml

This solution works well, since aqueous or alcoholic violets get washed away during dehydration. Nuclear stain. Stains cutinised and lignified cell walls. A good counter stain for safranin in anatomical sections.

6. Carmine / Acetocarmine

...arly 1ml. of 45% acetic acid in a flask fitted with a reflux ...al add a small pinch of carmine powder from a ...hequantity of 2 gm. Remove source of heat and add ...inin, dissolve, cool and filter. Add a few drops of ...ead ... dissolved in glacial acetic acid until the colour is ...ti... Store in a refrigerator. This stain is customarily ...used ... cytological preparations.

7. CARBOL FUCHSIN

Dissolve 0.3 gm of basic fuchsin in 100 ml of 5% carbolic acid.

This is used for staining bacterial smears.

8. ERYTHROSIN

Erythrosin	1 gm
Alcohol 95% or clove oil	100 ml

Stains cell walls. Excellent counter stain for Delafield's hematoxylin. Stains gelatinous sheaths of blue green algae. Apply only for 10 seconds if in clove oil.

9. FAST GREEN FCF

Fast green	1 gm
Clove oil	75 ml
Absolute alcohol	25 ml

Filter when sufficient time has been allowed for the stain to dissolve completely. The stain acts on non-lignified tissues and on spindle fibres. Good counterstain for safranin. Fresh stain is not to be used. Does not fade.

10. HEMATOXYLINS

Hematoxylin has a great affinity for metals. Most formulae include a mordant containing either aluminium or iron, or potassium alum. The colour resulting from staining depends on the mordant used. Hematoxylin is of great use in histology nd cytology. There are several formulae named after many rkers.

i) HEMATOXYLIN—HARRIS'

Hematoxylin crystals	2 gm
Aluminium chloride	1 gm
Alcohol 50%	1000 ml

Heat on a waterbath until dissolved. Add 6 gm oxide and filter. Add to the filterate 1 ml conc. hy acid.

sadvan- possible nfusi on,

(ii) HEMATOXYLIN DELAFIELD'S (Modified)

Dissolve 1 gm of hematoxylin crystals in 6 m l absolute

alcohol. Add this drop by drop to 100 ml of a saturated solution of ammonia alum. Expose to light in open bottle for one week. Filter, and add 2.5 ml glycerine and 2.5 ml methyl alcohol. Pour into a shallow dish and expose to a quartz mercury vapour lamp for 2 hours. Otherwise the above solution takes two months to ripen.

(iii) HEMATOXYLIN / IRON-ALUM-HEMATOXYLIN : HEIDENHAIN'S

This formula includes separate mordanting solution, but hematoxylin stain does not require long ripening time.

Mordant :

Iron alum 4%	500 ml
Glacial acetic acid	5 ml
Sulphuric acid 10%	6 ml

Stain :

Hematoxylin	1 gm
Distilled water	200 ml
Sodium bicarbonate	very small quantity.

Measure out the water and add the sodium bicarbonate. Bring water to boiling point, remove source of heat and add the dye. Cool and store in a refrigerator. Dilute stain before use.

Another type of stock solution is made by bringing 100 ml of distilled water to simmering and then add 1 gm of hematoxylin after removing source of heat. Add 5 ml of phenol. Cool and store in a refrigerator for one day. The stain can be used without dilution.

11. LIGHT GREEN SF / ACID GREEN

Light green SF	1 gm
Clove oil	75 ml
Absolute alcohol	25 ml

good cytoplasmic stain. Stains cellulose walls too. staining agent, but soon fades. Recommended for mentous algae. Light green SF reduces safranin, should not be allowed to react for more time.

MALACHITE GREEN / EMERALD / LIGHT GREEN

Malachite green	0.5 gm

Alcohol 95% or	100 ml
Clove oil or Distilled water	

Stains cell walls. Endodermis, bast, cytoplasm, nuclei and chloroplasts. Provides good combination with safranin for staining pathlogical specimens.

13. METHYLENE BLUE

Methylene blue	0.3 gm
Alcohol 95%	30 ml
Distilled water	100 ml

Dissolve the dye in alcohol and then dilute with the distilled water. This is a bacteriological stain. Used to stain yeasts also.

14. ORANGE G

Orange G	1 gm
Alcohol 100%	100 ml
Clove oil	100 ml

Dissolve stain in alcohol and add clove oil. Allow alcohol to evaporate. This is a most useful cytoplasmic counter stain for sections previously stained red, blue or green.

15. SAFRANIN O

Safranin O	2.25 gm
Alcohol 95%	225 ml

Dissolve dye in alcohol and dilute to required concentration with distilled water. Stains lignified, cutinised, and suberised structures as well as chromosomes, nucleoli and centromeres. Excess stain should be always washed away. A good combination with Fast green FCF.

The principal botanical uses for common stains.

CELLULOSE CELL WALLS

Hematoxylin
Fast green FCF
Aniline blue
Bismark brown

LIGNIFIED CELL WALLS

Safranin
Malachite green

/ Gentian violet

CHROMOSOMES

Iron hematoxylin
Crystal violet
Carmine
Orcein

FILAMENTOUS FUNGI IN HOSTS

Iron hematoxylin
Safranin
Fast green FCF

CYTOPLASM

Fast green FCF
Orange G
Congo red
Erythrosin

BACTERIA

Carbol fuchsin
Methylene blue

Methods of Staining

1. *Progressive staining*

This is a method useful for beginners. The tissues are deliberately understained first. Then gradually more stain is added until the desired intensity is attained. The tissue is frequently or a... arly ...nder the microscope.[1]

...ect staining interval for a given subject must be ... by trial. Only an experimental worker can make a ...or a trial slide and make correction for next slides. ...ovary sections fixed in Bouin's solution may require

A square piece of glass is cut to the size of the stage of the microscope and placed over it for examining wet slides. This prevents damage of the stage by flooding reagents.

only 10 minutes for hemalum, but another set fixed in Craf may require one hour in the same stain. To determine the correct time requirement in stain, stain 3 slides of a material for three durations, say 10 minutes, 20 minutes and 40 minutes respectively. Mark the slides. After staining rinse the slides with distilled water and wash with 0.1% sodium carbonate solution, then examine them under a microscope, without condenser under 10× objective. The tissues should not become dry during this quick examination. Nuclei should be blue black, cellulose cell walls black, whereas lignified cell walls should be colourless. Plastids must be pale blue and cytoplasm pale grey. If these are not achieved transfer the slide from water to hemalum for another interval. Rinse and give a sodium carbonate wash, repeat observation until satisfactory results are obtained.

2. *Regressive or retrogressive stainings*

The tissue is deliberately overstained first and then destained or differentiated until the desired intensity is attained. The usual destaining agent is 70% alcohol to which 1% acetic acid or 0.5% conc. nitric acid or hydrochloric acid is added. After proper differentiation the tissues are immediately washed.

3. *Counterstaining*

This involves staining certain part or parts of cells or tissues with a particular dye and the other parts with a contrasting colour afterwards. But in fact both the dyes colour all the parts, but by differential displacement the two colours finally appear at different parts. (See Method 11 in Chapter 8)

4. *Double, triple and quadruple staining*

The use of two, three and four contrasting colours used simultaneously is known by double, triple and quadruple staining respectively.

Double stain : Eg. Safranin O and Fast green FCF

Triple stain : Eg. Safranin O, Gentian violet and Orange G.

Quadruple stain : Eg. Safranin O Methyl violet, Fast green FCF and Orange G.

(See Method 24 in Chapter 8 for triple staining).

6. Whole Mounts, Cytological Methods and Maceration

WHOLE MOUNTS

Whole mount preservation is adopted for a wide range of materials like whole plants, stems, leaves, flowers and fruits. This helps to retain the natural colour, form, and shape and also prevents decay.

Microscopic materials like museum specimens are best preserved in ethyl alcohol or formaldehyde solution. Both of them have the disadvantage that they cause shrinkage and loss of colour. A slightly modified preservative contains :

Water	93 ml
Formaldehyde	5 ml
Glacial acetic acid	2 ml

This is found to be ideal for algae. The following solution is even better.

Water	72 ml
Formaldehyde	5 ml
Glacial acetic acid	3 ml
Glycerine	20 ml

This is excellent for preserving filamentous algae. The following formula is suggested for retention of colour in Algae.

Copper sulphate	0.2 gm
Water	35 ml

When the copper sulphate has completely dissolved, add

Glacial acetic acid	-5 ml
Formaldehyde	10 ml
Ethyl alcohol (95 %)	50 ml

Penetration of the solution is hastened if the materials are subjected to a vacuum pump or aspirator.

For preserving the colours of fruits the following solution is suggested (Hessler)

Water	1000 ml
Zinc chloride	50 gm
(Dissolve in boiling water and filter)	
Formaldehyde	25 ml
Glycerine	25 ml

McWhorter and Waier (1936) give the following solution for preserving filamentous algae, fungi and such delicate materials. The solution may be used for temporary mounting as well.

Dioxan	50 ml
Formalin	6 ml
Acetic acid	5 ml
Water	50 ml

Temporary and Semi-permanent Slides

A very simple method of preserving small filamentous algae and the like is placing it in a drop of 10% glycerine and covering with a cover slip. But this is purely temporary.

An improved preservative and mounting medium is the Lactophenol mountant. Filamentous algae *Spirogyra*, *Ulothrix* etc. and fungi like *Rhizopus*, *Aspergillus*, *Penicillium*, *Phytophtora* and *Pythium* may be mounted in this medium.

Aman's Lactophenol

Phenol (melted)	20 ml
Lactic acid	20 ml
Glycerine	40 ml
Water	20 ml

Prior to mounting in this medium the material may be stained in cotton blue or aniline blue (Page 39). The excess stain may be removed by giving a wash in the liquid itself.

Glycerine Jelly

This medium has some merits over pure glycerine since it possesses properties of preservation as well.

Gelatin	
Water	5 gm
Glycerine	30 ml
	35 ml

Phenol dissolved in 10 drops of water 5 gm.

The gelatin is dissolved in luke-warm water. The other components are added to it and then filtered hot. It is kept in a closed bottle.

Materials like filamentous algae and fungi are stained in Haematoxylin (Page 41) and then dehydrated before mounting in the glycerine jelly. For dehydration, the glycerine evaporation method is suggested (Page 33).

A small quantity (as big as match head) of glycerine jelly is placed on a slide and melted. Then the material to be mounted is removed from the glycerine and transferred into the warm jelly on the slide. A clean cover-slip is now put over the jelly and pressed gently to extrude the excess jelly. After cooling, the excess jelly around the cover slip is wiped off. The mount is then sealed off with any of the quick drying sealing compounds (Refer Appendix).

Permanent Whole Mounts

The previously mentioned methods are all temporary and liable to be spoiled on handling. Constant handing demands the preparation of a permanent nature. The method is outlined below :

Filamentous algae like *Spirogyra*, *Oedogonium* or *Batrachospermum* or the like are first killed in any fixing solution. Then the excess killing solution is washed off with water and the material stained with a self-mordanting haematoxylin (Page 42) for about ½ to 1 hour. The stain is now washed off and the material transferred to a destaining solution of 0.1% hydrochloric acid in a cavity block. Stir well and then drain out, wash in tap water and examine with a microscope. The washing is repeated until the nucleus and pyrenoids alone retain the colour.

VENETIAN TURPENTINE METHOD

The material is stained and dehydrated by the glycerine evaporation method. It is then mounted permanently as descri

7. Sections

1. FREE HAND SECTIONS

Except in the case of small, thin transparent bodies, almost all plant and animal materials are opaque or thick enough to prevent light from passing through. The necessity of sectioning a material is to allow enough light to pass through it making it possible to see clearly.

If the material is sufficiently hard to withstand the pressure of sharp cutting instrument, a free hand section can be taken. No embedding will be necessary then. Extremely thin sections, as thin as 10 μm can be cut by experience and skill. The method consists of firmly holding a piece of fresh or preserved material between the left hand thumb and fore-finger and cutting with a sharp razor (Fig. 25). The razor must be of shaving sharpness and preferably plano-concave. As and when it becomes blunt it may be stropped.

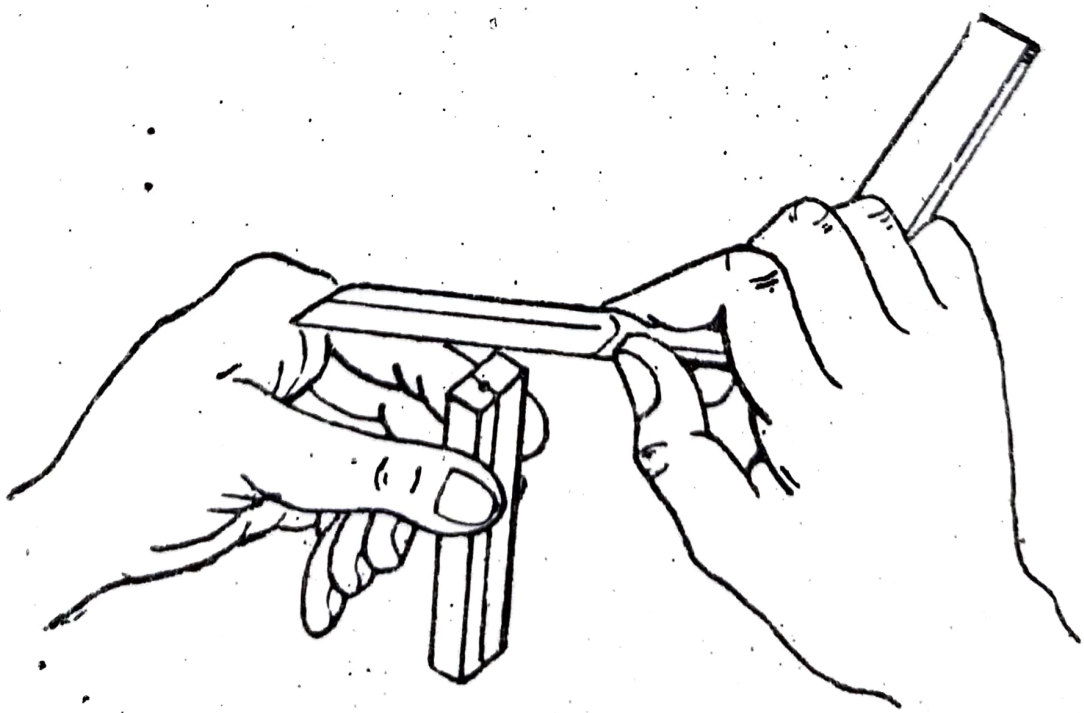


Fig. 25. Free hand sectioning.

Sectioning can be perfected by placing the material in a vertically split piece of pith (*Aeschynomene aspera* root or tapioca stem). The following procedure may be adopted :

1. Trim a good piece of pith into a rectangular block of about 5 cm.
2. Split it vertically to ½ length.
3. Place the given material vertically in the slit and hold firmly in the left hand between the thumb and fore-finger. (If a longitudinal section is desired, the material must be placed horizontally in between the lobes of the pith.)
4. After placing a drop of water on the concave surface, hold the razor exactly horizontal, handle directed away from you at level with the pith.
5. Draw the razor towards the body in a long, sliding movement. Repeat until thin, good and complete sections are got.
6. Care should be taken to see that the cut surface of the material and the pith are not oblique lest sections become oblique.
7. Transfer the sections from the razor with a brush to a watch glass containing water.
8. Select only thin, complete sections for observation.
9. The sections may be stained suitably.

For staining procedure refer Selected Methods.

2. SERIAL SECTIONS

The Paraffin Method

Where objects are cut into a series of sections, each of which is mounted in consecutive order on a slide, the preparation is known as a serial section. Serial sections have certain advantages over free-hand sections. From serial sections of an organ is possible to reconstruct the external or internal structure of the organ, of a tissue system, or even a single cell. Further, the course of orientation of vascular supplies, the intact cellular organisation of a tissue, modes and levels of differentiation of organs, structural relationships between hosts and parasites etc. can be studied in the correct perspective only from serial sections.

The most convenient, reliable and widely used method in microtechnique to produce serial sections, is the *paraffin method*. Objects that are not stiff, and that contain cavities, intercellular spaces etc. are covered by some substance which will also impregnate the material and preserve its shape and form at the same time resisting it against the impact of cutting action of the knife. Paraffin[1] wax is the substance chosen by all to accomplish this task. Wax is preferred to other substances like nitrocellulose (known by commercial names such as celloidin, parlodion) because wax readily passes from a solid to liquid state at temperatures that do not spoil the materials and it is somewhat sticky so that ribbons of sections can be made.

Preparation of sections by paraffin method involves many complex operations. To introduce paraffin into parts of cells and tissues is not an easy task. Paraffin is introduced as minute particles after all the water contained in cells and tissues is withdrawn. This withdrawal is accomplished very gradually during dehydration (Refer page 32). The whole process of making sections by paraffin method consists of the following steps :

(*i*) Fixation

(*ii*) Dehydration

(*iii*) Removal of dehydrant if not miscible with wax (clearing)

(*iv*) Soaking of material in molten wax (infiltration)

(*v*) Casting of wax impregnated material into blocks.

(*vi*) Attachment of the block to the holder of a microtome.

(*vii*) Cutting of serial sections (microtomy).

(*viii*) Affixing the ribbon in well arranged rows on glass slides.

(*ix*) Removal of wax.

(*x*) Staining and mounting.

Fixing, dehydration, and clearing have been already described.

[1] Paraffin is a colourless, odourless, tasteless mineral wax, a mixture of hydrocarbons, chiefly of methane series, many of which are obtained from petroleum. Wax strictly is beeswax. The term "Paraffin wax" is

Infiltration and Embedding in Paraffin

Infiltration consists of dissolving the paraffin in the solvent containing the tissues, gradually increasing the concentration of paraffin and decreasing the concentration of the solvent. The solvent is then completely eliminated by decanting or both.

The paraffin used for infiltration must have a known and constant melting point, appropriate hardness, smooth even texture and must be free from particles of dirt, including water and volatile or oily components. Paraffin of melting points between 50—55°C is ideal for botanical work[1]. The bulk paraffin purchased is first sampled by melting and casting a test block which on cooling must not contain any bubbles, opaque spots, streaks or internal fractures. On storing for 24 hours at room temperature it should not show any opaque crystalline spots.

The preliminary steps in the technique of infiltration for embedding varies with the use of different solvents. They are outlined in the following table.

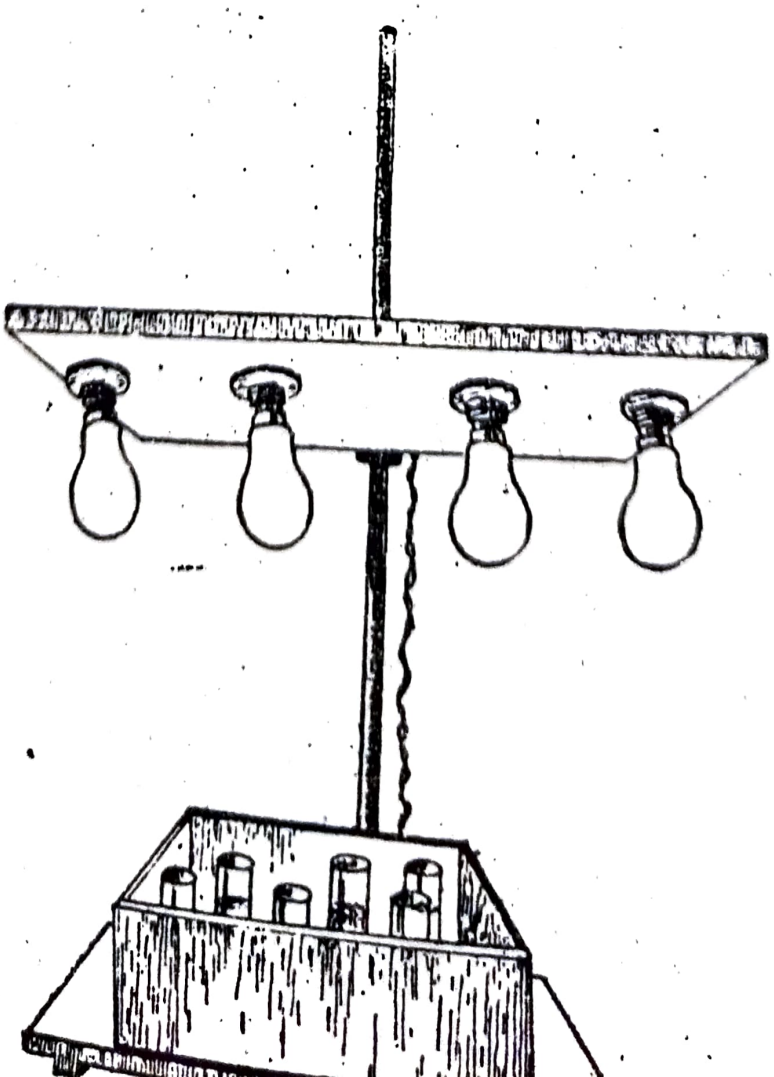
Dehydration and/or clearing in	Technique of infiltration
1. Xylene/Trichloroethylene	Melted paraffin[2] is gradually poured into the tube containing material in xylene so that paraffin forms a suspended layer on top. Replace cork.
2. Chloroform/ Benzene	Add small chips of paraffin to tube containing material in chloroform or benzene. Let the chips float. Cork the tube.

1 Commercial paraffins available are many—BDH, Merck, Fisher etc. Fisher tissue—mat of Fisher Scientific Co. of USA is superior to paraffin and will not crumble or crack. BDH-paraffin is cheap, but used alone does not give good result. This may be mixed with beewax in the proportion 100 : I for general use.

2 Solid paraffin sinks in xylene due to lesser density of xylene.

Table 10. Initial steps in infiltration with paraffin.

The solid paraffin gradually dissolves and diffuses downwards into the material. When the paraffin completely disappears add more. If the tube becomes full, decant some and repeat the process until a thin layer of undissolved paraffin remains on top of the solution. This may take 2-4 days, but tissues are not damaged by this. The process may be speeded up if the tubes are placed in a warm place (35°C) preferably about 15 cm away from a lighted electric bulb of 250 volts. Now the tubes



are uncorked and transferred to a temperature equal to that of the melting point of the paraffin used. Well equipped laboratories can use the thermostatically controlled ovens for this purpose. But a most simple and convenient device will be using a series of electric bulbs fixed to a horizontal plank that can be moved up and down on a vertical column (Fig. 26). The distance between the burning bulbs and the tubes must be adjusted by trials so that paraffin remains just in the molten state. The tubes may be placed in a wooden box and covered at the top by a wire net to prevent nocturnal insects visiting the tubes and getting drowned in the paraffin.

The impregnation of paraffin may take a few hours to several days. One or two changes of fresh molten paraffin may aid in removing the solvent from the tissue. It is important that all the solvent be removed from the tissue. This can be tested by the 'button test' of Sass (1958). Cast a button of paraffin by pouring some molten paraffin from the tubes into a pan of cold water. (Promptly replace the specimen tube into the oven !). Allow the test disk to cool thoroughly. The cooled test button should not be greasy. Chew this piece to test traces of solvents. If all solvents are removed, make two changes of paraffin. Now proceed for casting into blocks.

The technique of enclosing of the tissue in a block of solidified paraffin is termed *embedding*. Many methods are employed in this. Some use metallic 'L' blocks and others the iid of the coplin jars, smeared with glycerin. Paper 'trays' or 'boats' excel all the other devices. A very easy method of making paper trays or boats described by Johansen (1940) is as follows : Using the diagram (Fig. 27) as a guide first fold over along CC′ and DD′, the width of the fold being governed by the thickness of the material to be embedded. This should be about 2 or 3 mm. more than actually needed because the layer of paraffin, when cooled, is somewhat thicker along the periphery than in the centre. Next fold over AA′ and BB′, the width being twice that of CC′ or DD′. Then fold back along the middle of each of these two flaps, as indicated by aa′ and bb′. Hold the paper in the fingers, and by using the nail of the thumb, make the short diagonal creases. To complete the folding, bring one end and one side perpendicular, with the fold at the short

diagonal crease, and turn the resulting flap back of the end wall. Bring up the opposite side wall, and fold its flap back. Fold down, backward, the upper flap of the end wall, thus securely locking the entire end. Follow the same procedure for the opposite end.

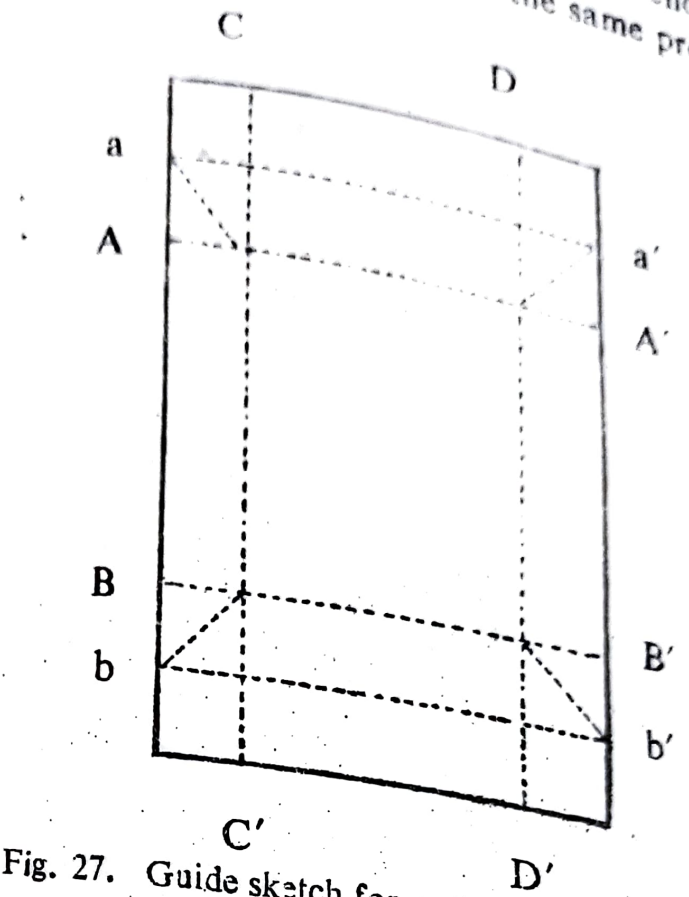


Fig. 27. Guide sketch for making paper boats

Technique of Embedding

Assemble the following articles on a table near the place when materials are left for infiltration.

(*i*) a large tray of cold water

(*ii*) a source of heat like a spirit lamp

(*iii*) a copper sheet table of 10×15 cm. and 10 cm. high (Fig. 28)

(*iv*) a pair of needles and forceps

(*v*) sufficient number of paper boats

Keep the metal table (Fig. 28) warm by the lighted spirit lamp. The tubes containing the infiltrated materials are placed on the metal table (never above the flame !). Wet the underside of the bottom of the paper boat and press it into contact on the surface of the flat work table. The molten paraffin reserved for embedding is poured into the paper boat to fill three-fourths

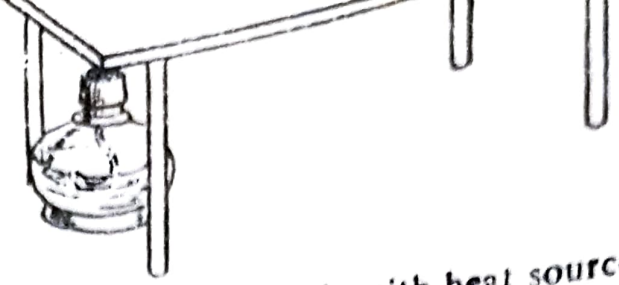


Fig. 28. Metal table with heat source.

of it. Wait for a few moments until a thin skin forms on the surface. By this time a layer of hardened wax will have been formed at the bottom of the boat. Then the tissues are transferred to the boat either by slowly whirling and then emptying the entire contents of the tube or by a warmed eye dropper if tissues are too small. Pour more molten paraffin to fill the boat upto its brim, if required. Warmed needles or forceps are used to orient[1] tissue in the appropriate position in which it is required to lie in the finished block. Wait till the wax has sufficiently been solidified to enable one to pick up the boat without disturbing the arrangement and float it on cold water in the tray. Blow on the surface until the paraffin is sufficiently firm enough to be immersed in water by keeping a heavy scalpel over the boat. The boat may be left in water for considerable length of time. It is taken out, the paper peeled off and stored dry in properly labelled packets. Blocks stored dry for a long time should always be soaked in a 5% solution of glycerine in 70% alcohol for at least a day before sectioning.

Sectioning / Microtomy

Cutting sections by a microtome is better demonstrated than described.

The block is divided into separate pieces, each piece containing the tissue to be sectioned. After deciding which face of the tissue is to be cut, the piece is trimmed roughly into a rectangu-

[1] Spacing of tissues depends upon size of tissues. Root tips and such small pieces of stem, flowerbuds etc. spaced 1 cm apart, and very slender needles or root tips may be in groups of 3 or 4 placed parallely.

lar prism. This rough block is then put on a block holder, and stuck to it by use of a heated scalpel handle which melts the paraffin against the block holder. The holder with block is cooled under water. Wipe off water and neatly trim the block with a razor blade. The face across which the cuts are to be made should have its upper and lower sides parallel. Then the block holder is affixed to its clamp on the rotary microtome. Manipulate the screws of the clamp until the forward face of the block is parallel to the knife edge. The knife carrier is then moved forward and the tissue carrier downward until the material almost touches the knife. Set the desired thickness. Check the cutting angle of the knife and the set screws. Now turn the operating wheel slowly so that each downward stroke removes a complete section and proceed with the cutting at moderate speed. The speed[1] should be such that there is on compression of sections and successive sections adhere to form a ribbon. The ribbon is held by a neat pencil brush and removed to a clean paper or the ribbon tray at convenient lengths. A very useful account of the defects appearing during cutting, their reasons and remedies appears in "Microtomists' Formulary and Guide" by Peter Gray (1954).

Affixing Ribbon on Slides

Slides (7.5 cm. × 2.5 cm.) are cleaned well with soap and water. They are then rinsed with 95% alcohol and dried by evaporation.

A tiny drop of adhesive (see Appendix) is placed on the dry slide and smeared over it by the fore-finger. The slide is left flat on the table and flooded by a dropper, with distilled water to which a few drops of formalin are added. The ribbon is cut into convenient lengths and placed on the slide with the glistening side down. The pieces of the ribbon are handled with a clean needle and a camel hair brush. The length of the ribbon pieces is to be determined by the size of the coverglass one is to use for mounting. At least 25 to 30 mm of space should be left at one end of the slide for labelling. The slide is then warmed

[1] Excessive speed makes violent impact between the pawl and the rachet wheel and spoils the feed mechanism, let alone the inexcusable but avoidable excessive wear and tear of the instrument.

The slide is marked and ness can be gauged by the fact that a moist the wax to be more or less opalescent, while on a properly dried slide it is almost glass clear. They are stored for the next operations.

Staining and Mounting

Considered below are the general procedure for staining and mounting paraffin sections.

The first step is the removal of paraffin from sections. The slide is dropped into a coplin jar containing xylene or benzene. A specimen tube just over 2.5 cm diameter which maintains a single slide in an upright position without the necessity of using large quantities of fluids usually required in coplin jars, can be easily adopted for this purpose. It is absolutely necessary now onwards to know which side of the slide carries the sections, otherwise the sections are likely to be rubbed off at some stage or other. The wax has to be completely removed from sections, for proceeding any further. After about a dozen slides have been dewaxed, replace with fresh fluids. Transfer the slide now to a mixture of absolute alcohol and xylene, then to absolute alcohol, and to water. If a smoky precipitate appears in absolute alcohol, replace it. After the slide remains in water for sufficiently long time to remove the alcohol, it should be examined carefully to make sure whether all wax has been completely removed.[1]

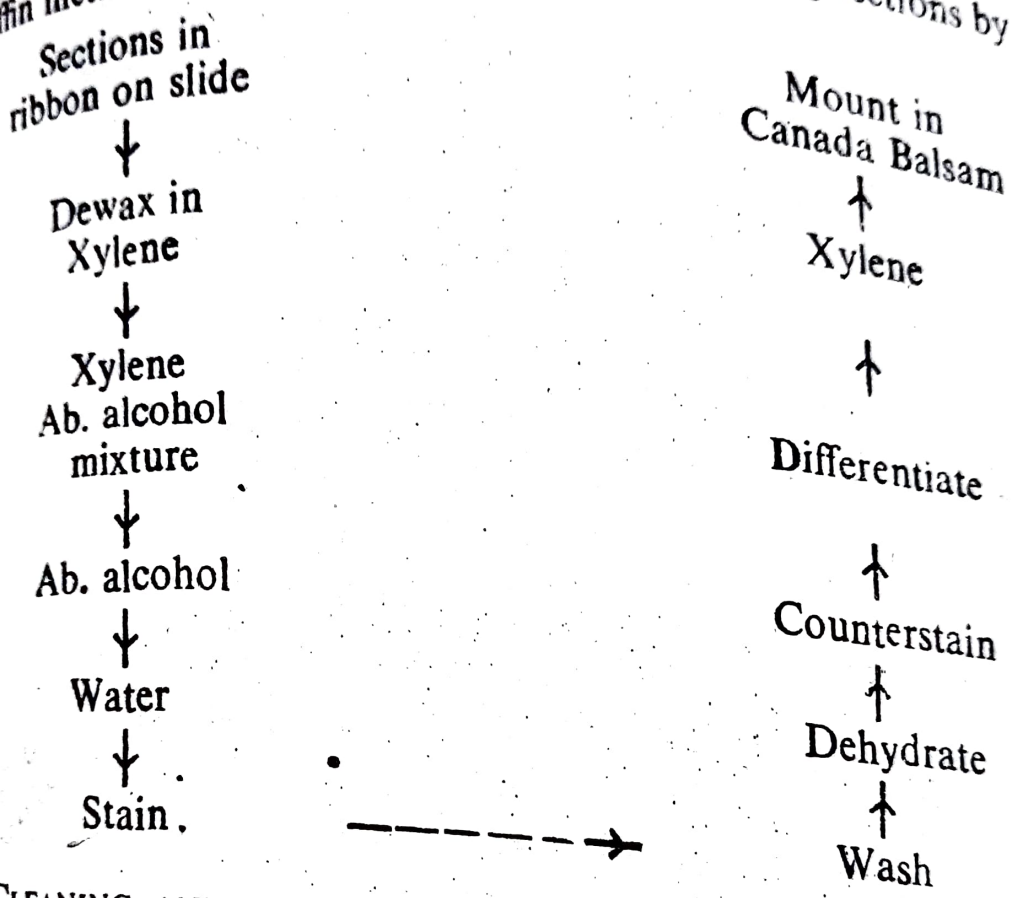
Now the sections are stained in the desired combinations, passed through the required dehydrants (see Selected Methods) and brought to xylene. They are ready for mounting. Place a thin coat of Canada balsam[2] on top of the slides and leave the solvent to evaporate from this, on the surface of a hot

[1] The sure test is this : if water flows evenly over the surface including the sections they are free from traces of wax.

[2] A resin obtained from the bark of the tree *Abies balsamea* much used as the Christmas tree in Canada.

plate. The balsam acts as a varnish and prevents the slides from getting dried out. On the next day a coverslip is placed on the surface. The slide becomes hardened as soon as it is cooled. Generally, soon after the dilute balsam is placed on top of the sections, a clean coverslip is passed over a flame and pressed over the slide, the slide is left to dry then.

Chart showing general outline of processing sections by paraffin method :



CLEANING AND LABELLING

When the slides are completely dried, the surface balsam should be carefully removed with a blunt knife and the slide left overnight. If the freshly cut edge appears to be sticky, the slide is not yet ready. If not, the whole slide is dipped in 90% alcohol and rubbed briskly to remove excess balsam. It is immediately rinsed in a strong soap solution and then polished.

Write the serial number of the slide and some indication of its nature on the glass with a diamond pencil and attach the label written in water proof India ink.