NEW WOODY TISSUE TECHNIQUES

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The preparation of microscope slides of woody tissues has always been attended by disappointing difficulties. For these reasons, considerable work has been done to develop better methods for this important type of technique.

Koehler, Gerry, and Weinstein[8] review most of the work done on this problem prior to 1927. Chowdhury[1] describes a method of softening hard tropical woods in a fraction of the time necessary by the orthodox method of soaking in hydrofluoric acid. He used hydrofluoric acid also, but applied it under pressure. Jeffrey[7] heated woody tissues for several hours in a dental vulcanizer at 320 F., and found softening to be more homogeneous and faster. Limbach[10] used dilute solutions of hydrofluoric acid, ethylene diamine, and ethanolamine under heat and pressure in a steam jacketed pressure tank. Later, comparable results (to be described in this paper) were obtained by immersing the wood blocks to be softened in five per cent ethylene diamine and placing in an autoclave for a period of time, depending on the hardness of the wood. Where an autoclave is not available, wood placed in a thermos bottle containing hot five per cent ethylene diamine also proved successful, although in this case, several days were necessary to accomplish the desired results.

THE TWO GENERAL METHODS OF TECHNIQUE USED

There are two general types of embedding media commonly used in microtechnique. Both are adaptable to woody tissues under certain circumstances. These media are paraffin and celloidin. While it is not the purpose of this paper to review these methods, it is believed advisable to give a brief review of the advantages of each, as the author sees them, so that the problems concerned in this report can be better understood.

Advantages of the Paraffin Method

Infiltration is faster and more thorough when the paraffin method is employed. This may be due to the ability of xylol and other paraffin solvents commonly used to penetrate better than ether-alcohol, the most common celloidin solvent, or it may be due to the inherent qualities of the matrices themselves. The fact remains that, not withstanding the use of heat, pressure, low-viscosity nitrocellulose,[6] and special solvents, material difficult to penetrate is less completely embedded in celloidin than in paraffin.

Sections can be cut more rapidly in paraffin, since it ribbons. Celloidin does not ribbon. Furthermore, a ribbon of sections is not as messy and time-consuming in handling as is an equal number of celloidin sections.

After paraffin sections are cut, they are normally cemented to slides, after which they are easily and readily handled. The constant handling of loose celloidin sections, besides being time-consuming, results in an increased and ever-present danger of delicate structures being broken.

Serial sections are kept in the proper sequence by the paraffin ribbon. When cutting celloidin sections, they are kept in serial order only with some inconvenience.

Thinner sections can be cut in paraffin than in celloidin. This observation may partially be due to the fact that material cut in celloidin has been, more often than not, of the type that ordinarily cannot be cut at all in paraffin. The author does not know how true this may be, not having cut material thatlends itself to thin paraffin sectioning (such as root tips) in celloidin. Better infiltration is possible with paraffin and this, as well as the inherent cutting qualities of paraffin itself, may have some influence on the results.

Any stains or combination of stains can be used on paraffin sections since the matrix is dissolved off and does not have to be taken into account. Since celloidin sections are normally handled singly or "en masse", however, the elimination of the matrix is impossible where elements of the section are discontinuous, and stains must be chosen that do not stain celloidin to an objectionable degree.

Where the celloidin can be removed, this objection is eliminated.

(Something further should be said, at this time, regarding staining. Celloidin sections are normally stained, singly or in bulk, by one of two methods. The sections may be retained in a containing vessel, such as a watch-glass, and the stains and other reagents poured into and drained out of this vessel. This procedure involves the danger of non-uniform staining, for the length of time it takes to pour off solutions without pouring off contained sections is quite unpredictable. Overlapping or sandwiched sections are not uniformly subjected to stains and other reagents, thus further increasing the danger of non-uniform staining.

The other method of transferring the sections through a series of reagents, involves some danger to the sections due to excessive handling, and is very time-consuming where many sections are to be stained.

Debris is difficult to eliminate when staining as above described, since the sections are in a horizontal position and are on the bottom of the vessels, during staining, where debris is usually to be found. The fact that Iron-alum haematoxylon is one of the common stains for celloidin sections, adds to the seriousness of the situation, for thorough washing is necessary if a precipitate is not to form. It might be mentioned here that besides the black precipitate that is formed by traces of iron-alum being present when staining with haematoxylon, there is also a yellow precipitate developed when sections fresh out of iron-alum are washed in hard water. This can be avoided by using distilled water or by acidifying the tap water slightly up to the point where no precipitate is formed. This point can be found by putting a drop of four per cent iron-alum in a test tube of the acidified water. When the mixture no longer becomes immediately cloudy, the water is suitable to use as wash water.)

Paraffin sections, when properly fastened to the slide, remain flat throughout the entire staining process. Celloidin sections stained "en masse" are very likely to curl badly. The process of uncurling may result in the injury to delicate portions of the section. Sometimes satisfactory uncurling is quite impossible. This curling can be diminished by staining between pieces of wire gauze, or something similar, that will hold the section flat until it is run into xylol. Once in xylol, curling takes place only slowly. Even when sections are relatively flat, however, internal shrinkages may be great enough and uneven enough to cause creases and folds when covering with the cover-glass. This may take place especially in the phloem region.

Advantages of the Celloidin Method

The celloidin method is used for material that is too hard to cut conveniently in paraffin. Satisfactory sections of Pinus, Tilia, and even Ginkgo can be made in paraffin, but at present, the celloidin method is the most reliable and convenient means of preparing sections of such hard, woody tissue.

In being run-up into celloidin, the material is not treated with reagents that cause it to become extremely hard and brittle, as is the case when running up into paraffin.

Ethyl-alcohol and ether-alcohol do not shrink (and consequently harden) woody tissue to the extent that xylol, chloroform, and butyl alcohol do. (Dioxan does not shrink woody tissue, but it does not penetrate very well either, so that while it can be used to run material up into paraffin, it has this disadvantage.)

The material is cut while in a wet, unshrunken condition, and is therefore softer than unsoaked paraffin-embedded material. This does not necessarily apply, however, when compared with soaked paraffin-embedded material. (Such soaking shall be discussed later under methods of cutting woody tissues in paraffin.)

Celloidin-embedded material is usually cut in a sliding microtome. In this case, it is possible to use a long slanting sweep in cutting the section thus utilizing the entire length of the blade rather than only a small fraction of the edge, as would be the case in cutting a paraffin ribbon. A shearing rather than a splitting action is therefore involved.

No heat need be used when employing the celloidin method. The harmful effects of heat on tissues is believed by some to be appreciable. Shrinking and hardening is supposed to result from too long exposures to oven temperatures. Thus, it is possible that more normal results may be obtained from the celloidin method. (While no heat need be used in the celloidin method, the usual procedure is to employ it to hasten the running-up process by keeping heavy celloidin solutions less viscous, and by creating a vapor pressure to aid in penetration. In this case, the possible advantage of obviating the use of heat is discounted.)

No telescoping of sections results when cutting material in celloidin. This is partly due to the fact that cutting in the sliding microtome does not develop the pressures on a section that cutting in a rotary microtome does, and partly due to the fact that paraffin is not as resilient as celloidin, and once compressed, tends to stay compressed, whereas celloidin springs back to its original shape.

Orientation of material is simple when celloidin is used, for it is perfectly transparent. On the other hand, paraffin material may be difficult to orientate because of the opaqueness of the paraffin. By staining lightly during the running-up process, this condition may be somewhat improved.

Sectioning in celloidin is not affected by conditions of static electricity or temperature. These factors may cause trouble in cutting paraffin ribbons but here, again, it is possible to largely eliminate the difficulties. Cooling in ice-water before cutting will insure good ribbons of paraffin material, and the soaking of such material in water overnight, will prevent static electricity troubles.

Another important reason for using the celloidin method for woody tissues should be mentioned. In the celloidin technique, the finished section is not allowed to dry as is the case with paraffin material, which is dried down in fastening it to the slide preparatory to staining. The maintaining of a moist condition prevents the cracking and splitting that is attendant with the drying down of a woody stem section.

IMPROVED TECHNIQUES EMPLOYED FOR STEMS

With these facts in mind, improved methods of preparing microscope slides of woody tissues were investigated. The chief concern was the preparation of slides of woody stems, and a description of the methods found most successful follows. The sectioning of commercial wood (lumber) will be described later.

Collecting the Material

A normal, healthy stem of the desired species, size, and age should be selected. After cutting the twig from the branch in the field, it is advisable to bring it into the laboratory where working conditions are more favorable, for further cutting. By putting the cut end of the stem into a bottle of water immediately after cutting, this can be done without harming the material. Once in the laboratory, the twig can be carefully cut into small pieces about one-half inch long. The stem should preferably be held in a pad of cheesecloth, and a "V" shaped chip cut out of one side down into the xylem. (Care must be taken to avoid crushing the phloem. It must be remembered that the harder the xylem, the more pressure will be exerted in holding the stem and the greater will be the danger of crushing the bark.) A new single-edged razor blade should be used for cutting. Further "V" shaped cuts into the xylem should be made so as to girdle the stem and finally cut it through. The end of the stem may then be trimmed flat, and the procedure repeated to cut off as many pieces of stem as desired.

Fixing the material

Woody stems can usually be fixed in F. A. A. with no fear of plasmolysis. After dropping the stem into the fixative, a suction pump should be applied to remove the air at least to the point where it sinks when returned to atmospheric pressure. This convenient fixative requires no washing, and material may remain stored in it indefinitely. It is also desirable because it has no appreciable hardening effect on wood. Material should be left in F. A. A. for two days at least, and then may be run up into celloidin. If the preparation of a "good looking" section is of secondary importance, and one is in a hurry, it is often possible to transfer the stem to glycerine alcohol for a few days, after which it may be cut in the sliding microtome without embedding.

Embedding

Better sections are made by resorting to the celloidin method and to do this the following schedule may be followed.

After removing the steam from F. A. A. place it in:

50% alcohol	12 hrs.
70% alcohol	12 hrs.
absolute alcohol	12 hrs.
ether-alcohol	12 hrs.

From ether-alcohol, the steam is put into a 12 per cent solution of low-viscosity nitro-cellulose in ether-alcohol. (Parlodion may be used in which case a two per cent solution is of comparable viscosity. It is expensive but of a somewhat tougher consistency than cellulose-nitrate. Cellulose-nitrate, while being more brittle, is thoroughly suitable, and has the advantage of being very inexpensive.) A tall vial or bottle should be used, and it should be well filled with the nitro-cellulose solution. The purpose in filling the vial is to avoid the possibility of the further concentration, and consequent diminution of volume, of the celloidin, from uncovering the stem lying on the bottom of the vial.

Place the vial in a pint size Mason jar, and clamp the jar cover down tightly. (The vial may be put inside a small bottle with a broad base, to keep it from tipping over.) It is a good idea to use a new rubber on the jar to prevent leakage of ether-alcohol vapor. Place the jar in a paraffin oven at 50° - 60° C. for 12 hours. After this time, remove the jar from the oven and allow it to cool. When cool, remove the cover, take out the vial, and pour out the ether-alcohol that has distilled over into the Mason jar. Replace the bottle and repeat the procedure until the celloidin is the consistency of very thick syrup. If a pressure tank is available, the vial with the material should be put under 100 lbs. pressure for about 12 hours. Release the pressure very slowly (over a span of about 1/2 hour) to avoid the boiling over of the celloidin. Then remove the stem from the celloidin with a pair of tweezers and drop it into chloroform, in which it should remain for one day. After this time has passed, transfer the stem to a mixture of equal parts of glycerine and 95 per cent alcohol. Remember not to cork the containing bottle, for the chloroform must be allowed to escape. Shaking the bottle at intervals will aid in dispersing this chloroform. After several days, the stem should be ready to cut. If not, change the glycerine-alcohol solution, and try again after a few more days. The material may be allowed to remain indefinitely in glycerine-alcohol, no oversoftening taking place with this type of tissue.

In no instance has the author found it necessary to further soften stems not exceeding one-fourth inch in diameter, regardless of age. (*Quercus*, *Fraxinus*, *Platanus*, and *Ulmus* were cut as well as *Tilia*, *Salix*, and *Aristolochia*.) Material softened in 30 per cent hydrofluoric acid also cut well, and in cases where softening might be necessary, this method would probably suffice.. If not necessary, however, it is advised that hydrofluoric acid not be resorted to since it may make the phloem too soft to be conveniently cut. It is also dangerous to handle, and can cause bad burns.

Knife sharpening

Regardless of how well prepared the material to be sectioned may be, good sections cannot be made with a knife that is not sharp. All too often, difficulty in sectioning is blamed on the material, when a dull knife is in reality the source of the trouble.

There is no "one and only" way to sharpen a knife. A knife may be properly sharpened in many ways.[11] A method, used by the author, that has proven both effective and fast, is described.

Some equipment is necessary besides the knife with its handle and knife-back. A blue slate hone and a leather strop of the type used by barbers, are all that are necessary to keep a good edge on the knife.

A new knife, or a newly reground knife should be procured. If the knife is a newly reground one, the edge should be checked for straightness. If it is crooked, the knife will sharpen only at the high spots. Such a knife should be sent back to the company that did the grinding. It will be reground free of charge, if the company is a reputable one.

To insure making a fresh start with the knife free from a wire edge, the rubbing stone of the blue slate should be held loosely between the fingers and drawn over the edge of the knife. If the knife is then drawn over the thumbnail, it will be noticed that it slides smoothly without digging in. Next, the slate should be wet with water, and a good lather worked up with the rubbing stone. It is the lather that is to do the sharpening, and a little more time spent working up a good lather, will save much labor later. Probably a better way to state the matter is to say that without a good lather, it is impossible to get a sharp knife. When a good lather is worked up, the knife is passed over the hone, edge foremost, with slight pressure from the fingers. The stroke is started at one end of the knife, which is then drawn obliquely over the hone in the customary manner, and finished at the other end. Turning the knife over, it is then pushed obliquely in the opposite direction in like manner. Fifty strokes in each direction should prove sufficient, provided the correct pressure is applied to the knife while sharpening. If at this time, the edge of the knife digs into the thumbnail sufficiently to cause the loosely-held thumb to jerk slightly back and forth as the knife is drawn over the thumbnail, the knife is ready to strop. What has happened here is, of course, that the formerly flattened down edge has been brought to a finely-serrate edge by the newly ground bevel on each side. The knife should be honed until this point has been reached, but should not be honed further, since such further honing not only would be a waste of time, but also might cause a more deeply serrate edge than desired.

After the knife is properly honed, it must be stropped. To do this, pull the strop tight with one hand and draw the knife, with the knife-back still in place, over the strop, this time knife-back foremost. Fifty times in each direction again should be sufficient if the correct pressure is used. If the knife will cut a hair held between the fingers at a point one-eighth inch from the fingers, it can be assumed to be sharp, providing the foregoing procedure has been followed. The entire edge of the knife must be tested in this way, since it is perfectly possible to have only a portion of the knife sharp. This test obviously does not insure against nicks in the knife, but the precaution of obtaining a new or newly reground knife should take care of this point. If the knife is nicked in use, the nick can be avoided if the knife is to be used in the rotary microtome. If the knife is to be used in the sliding microtome, however, where the full edge of the knife is employed, every nick will result in a scratch on the sections cut. Small nicks can be removed with a Belgian hone. To remove the nick, the edge of the knife must be ground down to the bottom of the nick. Where the nick is deep, the bevel on the knife is appreciably widened during this process. The wider this bevel, the more strokes will be necessary to rehone the knife, hence when the bevel becomes so wide that rehoning becomes too time-consuming, it is adviseable to have the knife reground. This is easily done if one has the equipment at hand, otherwise it must be sent to a commercial house specializing in such work.

It is not necessary to draw the rubbing stone over the edge of the knife every time the knife is sharpened. Stropping puts a smooth edge on the knife that feels exactly the same to the thumbnail as the edge over which the stone has been drawn. This means that the same test for a digging-in of the knife-edge on the nail can be applied to a knife sharpened for the second, third, or fourth time, etc. In these cases, the knife edge has been made smooth by the strop, and rehoning is not complete until a new serrate edge is formed.

Sectioning

The actual process of sectioning is comparatively simple provided the material has been properly prepared and the knife is sharp. A sliding microtome of the type having a knife holding block (such as the Spencer) that cannot be lifted off the slide will be found to be preferable. The knife is placed in the block and the block adjusted so that the bevel of the knife is parallel with the table top. This angle may be found readily by placing the knife-back over one end of the clamped knife and holding a soft piece of wood, such as a match against the knife-back and the edge. This match may then be lined up with the table top until it is in a parallel position. A very slight tilting in the direction that will provide a little clearance for the rear edge of the bevel will be found to be advantageous provided it is only a very slight tilt.

Next, the stem to be cut is removed from glycerine-alcohol and the excess celloidin trimmed off. If the celloidin can be peeled off the epidermis of the stem without harming the epidermis, sectioning will be simplified to the extent that the sections will be more easily handled. Rather than to mount the portion of stem to be cut on a block with celloidin, it is preferable to clamp the stem itself in the microtome. This provides a more solid mounting and eliminates much difficulty that might otherwise be due to a "rubbery" mounting, which often results in either sections thin on one edge and thick on the other, or in sections too thick on one stroke and too thin on the next. To keep the bark from breaking away from the xylem along the cambium when clamping the stem in the microtome, it is necessary to cut away some of the bark at the bottom of the stem before clamping it, so that it may be clamped by solid xylem only. The stem should be oriented so as to give a perpendicular cross section, and the angle of the knife adjusted so that the entire edge will be utilized in making the section. The stem should be trimmed down in 25 micron steps until a complete section is cut. If after examination under a microscope, this section is found to have tiny holes here and there, trim the stem further until holes no longer are present. (These holes, if present, are due to some tissue being torn out when cutting the stem into small pieces with a razor blade.)

Now resharpen the knife and replace it in the microtome block in as nearly the same position as possible. A little further trimming may be necessary before a full section is cut, and this should be done at 15 micron intervals. When a complete section comes off the knife, the sections may be saved for further treatment.

In sectioning, the knife and stem should be kept flooded with glycerine-alcohol. (If the mixture recedes from the edge of the knife, add more alcohol to it until it flows evenly to the edge of the knife at all places.) The glycerine-alcohol can be applied most conveniently with a camel's hair brush (the ordinary water-color paint-brush that is sold in five-and-ten-cent-stores is suitable). This brush should be used to keep the section flat as it is being cut. The phloem is best preserved in stem sections if the sections are not allowed to curl, since in subsequent uncurling much harm may be done. A sharp knife will cut flat sections; a dull knife will not. One must be careful not to tear the thin section with the ends of the bristles of the brush, both while cutting and in subsequent handling. Stem sections seem to cut best at 10 or 15 microns. Thicker sections have a greater tendency to curl, while thinner sections often are not perfect.

Treatment of the cut celloidin sections

This is one of the most important of the problems concerned with, and a brief discussion should not be out of order at this time.

It was noted that many of the advantages of the paraffin method were due to the fact that the sections could be fastened to slides. This fact alone makes for sections that are flatter, more uniformly stained, free from matrix, and more conveniently handled. Thus, it was decided to find some way to stick celloidin sections to the slide.

After reading all available literature on the subject, no described method for fastening woody sections was found. Many methods were noted for fastening celloidin sections of animal tissues to the slide,[9, 11] but every one of these was found to be unsuitable because of the fact that woody sections have a strong tendency to curl when dehydrated. This tendency was strong enough so that in all of these methods the sections curled off the slide because they were not held down firmly enough.

The first method that held the sections firmly to the slide consisted of placing the section (washed free from all glycerine-alcohol by several changes of 70 per cent alcohol) on a slide, covering it with a drop of celloidin and pressing it in place with the finger through a piece of toilet tissue. The tissue, when removed, left the section flattered. The section was stained with Iron-alum haematoylon and safranin.

Destaining with acid alcohol removed most of the stain from the celloidin, but the excess celloidin covering the section did not destain completely, and was thick enough to interfere with the covering of the section with a cover-glass. Most of this celloidin was successfully removed by placing the slide in ether-alcohol, being careful not to dissolve off too much lest the section fall off the slide. The main objections to this method were that all of the celloidin could not be dissolved off without the section falling off, and that the ether-alcohol continued to destain the safranin in the section as long as the section remained in the ether-alcohol. The first objection was eliminated by generously smearing the slide with gelatine fixative (Haupt's fixative) and allowing it to dry, after which the section was fastened down as before. This was found to hold the section firmly in place even after all the celloidin was dissolved off. The celloidin covering is kept in place during staining, and it is at this time that the gelatine becomes plastic (in the aqueous solutions), fastens the sections to the slide and hardens (in the alcoholic solutions). The second objection, that of the ether-alcohol destaining the section while dissolving off the celloidin, was overcome by using amyl-acetate to dissolve the celloidin. This did as good a job as the ether-alcohol, and did not destain the safranin.

It was found necessary to have the sections in a normal unshrunken condition when fastening them to the slide, and this meant that subsequent dehydration, involving a slight shrinkage, caused the section to pull apart at its weakest points leaving unsightly cracks. While amyl-acetate causes comparatively little shrinkage, it was found that dioxan caused no shrinkage at all, and since it also dissolved celloidin very well and safranin very little, it was found to be preferable to amyl-acetate for use in dissolving the celloidin from the slide.

The covering of the section is as important as any of the other steps. If the balsam, or other mounting medium used, is dissolved in a reagent that shrinks wood, splitting of the section will result either immediately or after a period of time. This depends on the degree with which the reagent shrinks wood. The best solvent to use for the mounting reagent, for the reasons aforementioned, was found to be dioxan. This reagent dissolves Canada balsam readily. It was found not to dissolve Clarite in all proportions, the mixture becoming saturated at room temperature before a suitable consistency was reached. When making up the balsam, it is necessary to make it up from dry balsam so as to avoid any xylol or turpentine being present in the covering medium. The presence of xylol or turpentine would cause splitting of the section, for both shrink wood considerably.

Dioxan has not been conclusively proven to be entirely safe for laboratory use, and under these conditions, it is best to avoid its constant excessive use. For this reason it is advised that dioxan be used only as a solvent of the mounting medium (balsam) and that amyl-acetate be used to dissolve off the celloidin from the slides. This practice results in a thoroughly presentable slide free of splits. As a matter of fact, amyl-acetate may also be used as a solvent of the mounting medium (and here we may use Clarite, if we wish), resulting in a slide that will split very slightly if at all, and then only after a considerable period of time. One advantage in the use of amyl-acetate balsam following the dissolving off of the celloidin with amyl-acetate, is that no further loss of safranin results following the covering of the slide. When dioxan balsam is used to cover a slide fresh out of amyl-acetate, a slight destaining of safranin results for a day or so, due possibly to a small carrying over of safranin in the slow exchange from amyl-acetate in the section to a mixture of amyl-acetate, dioxan, and balsam.

One further point should be mentioned. The index of refraction of dioxan and amyl-acetate is not as high as that of xylol or balsam, and the freshly covered slide may not look thoroughly cleared because of this fact. However, after a day or two, most of the solvent evaporates and the section clears up nicely.

The complete process that is being used at present to fasten woody sections to the slide is as follows:

1. Wash freshly cut section several times in 70 per cent alcohol to remove glycerine. (assuming that sections were cut with a knife lubricated with glycerine-alcohol.)
2. Transfer the section to a slide that has been previously smeared with Haupt's fixative. The fixative must be dry before this is done.
3. Dry the section with toilet tissue. The important thing to remember here is that the section must be superficially dry, but not dry enough internally to cause it to curl.
4. Place a drop of thin celloidin (12 per cent cellulose-nitrate) over the section. Be sure that the whole section is covered with celloidin, otherwise it will not be fastened, in the finished slide, at such uncovered portions. In such instances, bubbles under the section are difficult to eliminate.
5. Lay a fresh piece of toilet tissue over the celloidin.
6. Put several drops of ether-alcohol on the paper in the spot over the celloidin.
7. Flatten the section under the paper by gentle rubbing pressure. Use a circular motion so as to rub the excess celloidin off to one side of the section.

8. Remove the sheet of paper and place the slide in water to harden the celloidin. The celloidin must not be allowed to dry in the air. (If the section comes off with the paper, either the gelatine was not dry, the section not dry enough before putting the celloidin over it, or not enough celloidin was left remaining over the section.)

9. The following staining schedule is then used:

Iron-alum (4% aqueous)	15 min.
Wash in running tap water	15 min.
Dilute haematoxylon (add enough stock solution of haematoxylon to distilled water to approximate the color of the iron-alum.)	until sufficiently stained (about 2 min.)
Wash in running tap water	15 min.
Safranin (1% in 50% alcohol)	overnight
Rinse in water	several seconds
Acid alcohol (1 drop HCl per 10 cc. 95% alcohol)	15 sec.
Alkaline alcohol (1 drop NH_4OH per 100 cc. 95% alcohol)	15 sec.
Absolute alcohol	30 sec.
Absolute alcohol	30 sec.
Amyl-acetate	5 min.
Amyl-acetate	5 min.

10. Cover the slide with dioxan balsam and a cover glass.

IMPROVED TECHNIQUES EMPLOYED FOR COMMERCIAL-WOODS

This segration includes the fully mature xylem taken from the main stem (the commercial part) of tree species. The harder of these woods must be softened to be sectioned, even when the sliding microtome is used.

Collecting

Fresh material is preferable, but it is often impossible or impractical to get it. It is not good practice to cut down a tree to get one cubic inch of the heartwood. Sometimes it is possible to cut out a piece of heartwood with a hammer and chisel. Where this can be done, the wound in the tree should be painted over with a neutral creosote paint similar to that used by tree surgeons.

More often, one must resort to using dried material, and this, in most cases, is perfectly suitable if the sample of wood is carefully selected. It will be found advantageous to cut three blocks of wood of the size desire, rather than only one. It should be remembered that the larger the size of the block, the more difficult will be the embedding. Pieces 1 cm. square or smaller should be about right. If the cross-section shows several annual rings, that is all that is necessary as far as size is concerned. Use one block of wood for transverse sections, one for radial sections, and one for tangential sections. This will be found to be more convenient than trying to make all sections from one block. This procedure also insures against sectioning of material previously compressed in the microtome clamp.

Fixing

Unless living sapwood or cambium is to be cut, no fixing is necessary, since heartwood is dead tissue whether it be taken from the standing tree or the lumber pile. Where living material is to be cut, however, F.A.A. will be found to be suitable.

Softening

Where cambium sections are to be made, the most convenient method of softening is to first boil the material and then immerse it in 30% hydrofluoric acid for an indefinite period of time. The length of time it should remain in acid is dependant on the size and hardness of the block. When, with a sharp razor blade, the block cuts like hard rubber, the desired softness has probably been achieved. One can best learn by experience when this point has been reached. One week in acid will be necessary in any event, and if the block is tested at regular intervals after that time, it will be caught at the right stage. Before testing the block, the acid should be at least superficially washed from the block, and the block handled with rubber gloves to avoid burning the hand with traces of acid.

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The softening effects of hydrofluoric acid, from the author's experience, does not seem to be very pronounced, and for this reason, as well as for the dangerous potentialities of the acid, other reagents have been resorted to. The most successful of these is ethylene diamine. This reagent, when used in even very dilute solutions, has a strong softening effect on wood. Ethylene diamine should not be used to soften wood from which bark has not been removed, for reasons that will be explained later. For softening heartwood, however, it is much superior to hydrofluoric acid.

The original procedure of employing ethylene diamine was as follows: First the air was removed from the wood by boiling or by a vaccuum pump. Then the wood block was immersed in a 100 cc. bottle full of two per cent ethylene diamine, which was then put into a steam-jacketed pressure tank. Heat (155° C.) and high pressures (100-200 lbs. per square inch) were applied and within a period of one and one-half hours, the wood was soft enough to cut.

The disadvantage of this method of using ethylene diamine is the necessity of having a steam-jacketed pressure tank, so other ways of employing it were devised. Where an autoclave is available, the bottle holding the ethylene diamine and the wood block can be put under a stream pressure of 15 lbs., and, with a slighly stronger ethylene diamine (five per cent), the hardest woods will soften sufficiently in several hours.

Where an autoclave is not available, it is still possible to use this reagent to soften wood. If the block, from which the air has been removed, is placed in a thermos bottle of boiling five per cent ethylene diamine, it will soften in from one to several days. The block should be removed and tested every day, at which time fresh boiling five per cent ethylene diamine should be used if further softening is found to be necessary.

Ethylene diamine has a tendency to swell wood. Upon being washed free of ehtylene diamine, the wood returns to its original size. Bark and wood are swelled to different degrees, however, and it is for this reason that ethylene diamine cannot be used for softening wood blocks that are to be cut with the bark on. The excessive swelling of the bark tears it from the wood at the cambium.

After softening has been completed, either by hydrofluoric acid or ethylene diamine, the reagent must be washed from the blocks in running water. When hydrofluoric acid is used, one must be careful to remove all traces of the acid. When the blocks have no sour taste to the tip of the tongue, they are free of acid. One does not have to be so careful in removing all traces of ethylene diamine, but the blocks should be washed for a day at least. (Ethylene diamine does not attack glass nor the steel of the microtome knife to the extent that hydrofluoric acid does).

Wood can be cut without embedding, or it can be run up into celloidin to be cut. The chief advantage of celloidin is its ability to hold longitudinal sections together. When cut without embedding, these longitudinal sections may fall apart wherever a vessel cuts across them. (Tipping the block so as to prevent any vessel from traversing the length of the section can be resorted to in unembedded material).

Before embedding such material, it is advised that the wood be cut unembedded, and then, if the sections are unsatisfactory, embedding be resorted to. If an attempt is to be made to cut the wood without embedding, it should be placed in glycerine alcohol for a day or two subsequent to washing. If then it is decided to embed the wood, the excess glycerine alcohol should be washed from the wood by boiling it in water for a few minutes. The remainder of it will come out in the running up process.

Embedding

Exactly the same method of embedding is used here as was given under woody stems.

Sectioning

Sections are cut in a manner similar to that described under woody stems, with the exception that here we must cut longitudinal sections in most cases. When cutting longitudinal sections (and this applies to stem sections also), it is advised that the section be allowed to curl until it is almost cut through. The section should then be flattened out on the knife, and the remaining corner cut through. This procedure will be found to keep longitudinal sections in better condition than they would be if they were kept flat throughout the sectioning.

Fifteen microns is the usual thickness of wood sections, but if curling causes much difficulty, ten micron sections will be found to be more manageable.

Treatment of the cut sections

Wood sections may be either fastened to the slide and stained, as described under woody stem treatment, or they may be stained singly or "en masse". If they are to be stained loose, curling troubles may develop, and in this case, placing the sections between wire screens during staining will be found helpful. The screens may be removed once the sections are in xylol, since they curl only very slowly after this.

There are many schedules used for staining wood, but the following will be found to be as good as most.

4% Iron alum	15 min.
Wash in running water	15 min.
Dilute haematoxylon (add enough stock solution to distilled water to give it the same color as the iron alum).	Until stain intense enough (1-5 min.)
Wash in running water	15 min.
1% safranin in 50% alcohol	15 min. - several hours

Rinse in water	Several seconds
Acid alcohol	Several seconds
Alkaline alcohol	Several seconds
95% alcohol	30 sec.
95% alcohol	30 sec.
Absolute alcohol	30 sec.
Absolute alcohol	30 sec.
Xylol	5 min.
Xylol	5 min.
Xylol	5 min.

Cover with balsam and a cover glass.

TECHNIQUES EMPLOYED FOR CUTTING WOODY TISSUES IN PARAFFIN

Dr. G. H. Conant cuts hard woody stems and fern rhizomes in paraffin as a matter of commercial routine. *Pinus* and *Tilia* stems cut perfectly, and even the harder steams such as *Ginkgo* and *Quercus* can be successfuly cut by his method.

The essentials of the method are these. The material is run up using a slow butyl alcohol series,[5] and them embedded in a rubber paraffin. The slow butyl alcohol series insures good penetration with a minimum hardening of tissues. The importance of rubber paraffin will be explained later. After the material is embedded, one edge of the paraffin block is trimmed away so as to expose the end of the stem or rhizome. This is then soaked in water, making sure that the exposed end of the material is submerged.[2] This soaking softens the material so that it can be cut readily in the rotary microtome. Immediately before cutting, the block is cooled in ice-water so as to keep the matrix as firm as possible. Used in conjunction with a sharp knive, this method is capable of almost unbelievable results.

Contrary to popular opinion, paraffin embedding is never so complete that the continuity of the cell walls is interrupted to the extent that it prevents the conduction of moisture through its elements. It is for this reason that soaking in water will soften such embedded material over a period of time. _Pinus_ stems cut nicely after only one day in water, but _Tilia_ may require months of soaking before it is soft enough to cut readily.

Such soaking will swell material somewhat, and in ordinary unadulterated paraffin this swelling breaks the material loose from the paraffin. This results in a ribbon from which the sections fall out. The use of a rubber paraffin prevents this from happening. Rubber paraffin seems to have the ability to adhere better to soaked material. The exceptional ribboning qualities of this type of paraffin make it doubly desirable. Soaking takes a little longer with rubber paraffin, but its advantages far outweigh this disadvantage.

Where material must remain in water for more than a day or so, it is necessary to add something to the water that will prevent molds from growing and attacking the material. A few crystals of phenol or of copper sulfate serve this purpose well. Blocks can be soaked in glycerine alcohol or 50 per cent alcohol as well as in water, and in these cases one need add no chemical to discourage molds.

Dr. Conant[3,4,5] has suggested the use of one per cent safranin in 50 per cent alcohol especially for use in soaking stems and resistant tree buds. The safranin shows the extent of the softening that has taken place, and may also have some chemical effect on the tissues. Cutting is not attempted on a bud until it is completely soaked, as shown by the safranin. Dr. Conant says that alcoholic safranin is more rapid in its softening action on woody tissues than is water.

Very recently, the author has found it possible to cut even the kiln-dried heartwood of many tree species (_Pinus_, _Tilia_, _Juglans_, _Populus_, and _Catalpa_) by softening the wood somewhat more than necessary (or desirable) with the celloidin method, prior to running it up into paraffin. This material, when later soaked, was found to cut well and to give sections of a quality approaching those procurable with the celloidin method. Results, while very promising, have not as yet warranted a report of standardized methods. Much more reasearch must be done in this field before a definite method can be assembled.

Paraffin-cut sections can be stuck to the slide by the method described in this report if the paraffin is dissolved off first. Dioxan is the best reagent for this purpose since it keeps the tissues softer than other reagents, and causes no shrinkage. Consequently the bark is not torn off stem sections by virtue of the xylem shrinking away from the phloem (as is the case in some stems when xylol is used to dissolve the paraffin from the sections.) After the paraffin has been removed, the dioxan should be washed out in 70 per cent alcohol, and from this point the sections may be stuck to the slide with celloidin, as aforementioned.

The advantage of this procedure over the fastening of the paraffin sections to the slide in the orthodox way (by floating the sections on water) is that splits are avoided. When paraffin sections of woody tissues are floated down on the slide and allowed to dry, they shrink and crack in drying. This does not happen when the sections are treated as outlined in this report under the discussion of stems.

SUMMARY

1. Collecting, fixing, celloidin embedding, knife sharpening, sectioning, and treatment of stem sections are discussed.

2. A method of fastening celloidin woody stem sections to the slide is discussed. The advantages of this procedure over the orthodox method of handling this type of section "en masse" are several:

   a. Sections are more easily handled. Time is saved and delicate portions of sections are less liable to be harmed.

   b. The celloidin matrix is removed leaving a cleaner-looking slide.

   c. Sections are perfectly flat. For this reason they "cover" better and are more readily examined under the microscope.

   d. Sections are free from splits and folds so common in celloidin stem sections.

3. Collecting, fixing, celloidin embedding, sectioning, and treatment of commercial-wood sections are discussed.

4. The sectioning of woody tissues in parafin is discussed.

REAGENTS

Haupt's adhesive

Gelatin	1 g.
Distilled water	100 cc.
Phenol crystals	2 g.
Glycerine	15 cc.

(Dissolve gelatin in water, dissolve phenol, add flycerine, filter)

When used with the paffin method, a drop is smeared on the slide, allowed to dry, a drop of 3% formalin added and the section loated on this.

When used with the celloidin method, several drops of the adhesive are smeared on the slide forming a very thick layer which may take several minutes to dry. Formalin is not used in conjunction with this method, but the sections are fastened in place with a layer of celloidin.

F.A.A.

There are a number of F.A.A. mixtures in use. The one used by the author has the following consistency.

Formalin	6.5 c.c.
Acetic acid	2.5 c.c.
50% alcohol	100.0 c.c.

Ether alcohol

Equal parts of ether and absolute alcohol.

Glycerine alcohol

Equal parts of glycerine and 95% alcohol.

Acid alcohol

1 drop of HCl per 10 cc. of 95% alcohol.

Alkaline alcohol

1 drop of NH_4OH per 100 cc. of 95% alcohol.
(This is used to neutralize slides destained in acid alcohol).

Haematoxylon (stock solution)

Haematoxylon crystals	10 g.
Absolute alcohol	100 cc.

BIBLIOGRAPHY

1. Chowdhury, K. A. 1934. An improved method of softening hard woody tissues in hydrofluoric acid under pressure. Ann. Bot. 48: 308-310.

2. Conant, G. H. 1936. Paraffin sectioning. Triarch Topics, April, 1936.

3. Conant, G. H. 1936. Sectioning woody materials in paraffin. Triarch Topics, August, 1936.

4. Conant, G. H. 1936. More information on woody sections in paraffin. Triarch Topics, December, 1936.

5. Conant, G. H. 1940. Helps for histologists. Triarch Botanical Products Catalog No. 5. 1940: 25-27.

6. Davenport, H. A. and R. L. Swank. 1934. Embedding with low-viscosity nitrocellulose. Stain Tech. 9: 137-139.

7. Jeffrey, E. C. 1928. Improved method of softening hard tissues. Bot. Gaz. 86: 456-458.

8. Koehler, A., E. Gerry, and A. I. Weinstein. 1927. Preparing woody tissues for making microscopic mounts. U. S. D. A. For. Prod. Lab. Bull. March 16, 1927.

9. Lee, A. Bolles. 1937. The microtomist's Vade-Mecum. 10th ed. Blakiston. Philadelphia.

10. Limbach, J. P. 1938. Softening woods for micro-sectioning with heat and pressure. Unpublished report to Forestry Department, Mich. State Coll., E. Lansing, Mich. June 1938.

11. Mallory, F. B. 1938. Pathological technique. W. B. Saunders Co. Philadelphia.

12. Sass, J. E. 1940. Elements of botanical microtechnique. McGraw-Hill Book Co. Inc. New York.