Iceni Microscopy Study Group

4th March 2017: Presented by Peter Sunderland

Foreword: Regarding Xylene

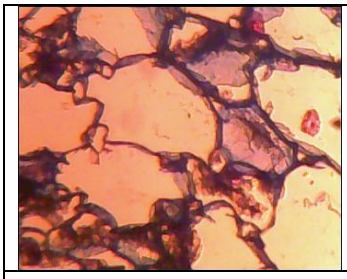
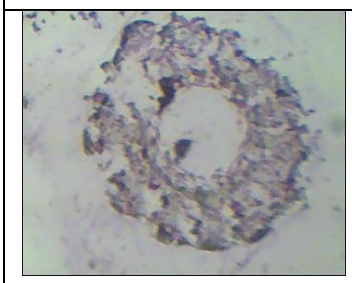
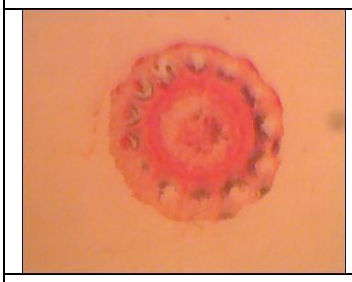
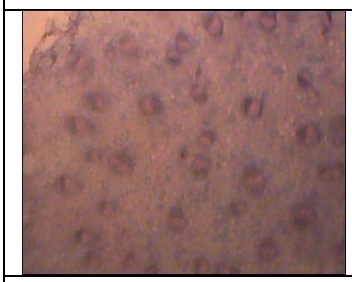
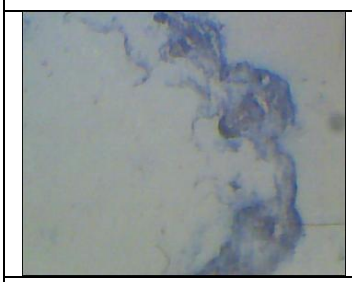
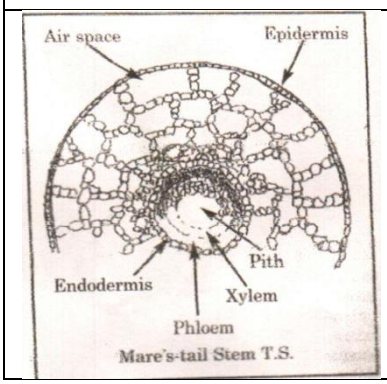
I have used the word xylene in the flow charts but in view of the committee's decision not to use xylene, please use and think histoclear in order to comply to the wishes of the committee. My personal view is that I will keep using xylene in a safer way. Let me give you the views of the famous Walter Dioni (Safe Microscopy Techniques 2014, page 42) and I quote "there are three dangerous products benzene is reputedly carcinogenic and like toluene and xylene (although not carcinogenic can cause serious damage to the nasal passage (epithelium), eye and brain cells if they are inhaled frequently). Xylene is the less dangerous."

How dangerous; let us go down the page the bottom half of page 42: We can use essence of turpentine as a balsam substitute for xylene, being made from the resin from coniferous trees its distillation gives a liquid form, although safer than all other solvents it is not totally safe. To a much lesser degree it produces the same problem as xylene. My personal view it is as dangerous as painting a house using turpentine in the paint and washing the paint brushes is the most dangerous of all, if using neat turpentine. I myself shall keep using xylene but will have a window open and instal a vent/fan to the outside air. John Blakesley has a set up using a four inch fan sitting behind his microscope/work area and he says it works well. I am thinking perhaps we should all go down that road at home, if we use xylene. Histoclear is the trade name for d-limonene which is the scientific name and it can be bought for a third of the price of Histoclear.

Knowledge is Power
Unless you try to do something beyond what you have already mastered, you will never grow
Foreword from Walter Dioni's book Safe Microscopy Techniques for Amateurs

oooOooo

Today's meeting is to show you how to make and cut sections, stain and mount them. Let us deal with cutting first, we need a subject it can be animal or vegetable, let's take today's example; a slice of mares tail (Hippuris Vulgaris L.- see image 3.). Stem T.S. a section of squash plant leader (Cucurbita – see images 2 and 5), a section of stalk from a rhubarb plant (Red Ken – see images 1 and 4). What can these sections show us after fixing and treatments? See below:-

	1) This section shows clearly the cell walls are stained and they show the dark stain in the walls. Other parts are stained red. You can only do this if the sections are cut really thin. In order to get enhanced detail you need to cut them thin enough to fit in the depth of field of your chosen microscope (I will show you an example in a book) **RHUBARB SECTION**
	**2)** This section shows a section through a squash plant, although it has stained the epidermis it is not a rich or bold stain. Likewise the cell detail which should have been red is very weak. The reason being following details in a book the recommended time was five minutes per stain, but further testing showed you need to have at least half an hour in each stain. **SQUASH PLANT LEADER**
	3) This section shows mares tail, the epidermis in this case is stained red and the endodermis is red, as is the pith. But the xylem is stained the same as the background, i.e. it has not stained at all? **MARES TAIL**
	4) This section through a rhubarb stem when viewed at 400 clearly shows the phloem tubes. They need to be stained with red much longer than my five minutes. Tests afterwards found we needed as least half an hour. **RHUBARB SECTION**
	5) This is an edge through a squash leader or stem showing that the epidermis on the outside has stained well but the cellular cells have stained weakly as has the phloeum tubes. **SQUASH PLANT STEM**
	6) This is a drawing I got from a book of a mares tail stem.

Staining

I have found there are old stains and bold stains, but never any bold old stains, my old stains have degraded and gone poor, but when I mixed up a small amount of safranin stain the pick up was 100% better, I have no idea why. Like most people I used to think that stains lasted for ever, this is not the case. Most "die" after three or four years. We may as a group have to buy in some powder to make some fresh stains on the day. Some mixed stains I bought in 2009 for big money are now past it, they were trichrome stains for nosema and bacteria, they stain red but no longer give the halo rings – I wish I could understand why.

The other slides show that they stain much the same as the mares tail but by controlling stains different colours can be made: red or blue or part stained. The rhubarb shows clearly the sap tubes in red/pink. I think the time in the stain equals the depth of colour, I think this one is under-stained. The mares tail with the spoilt stain is caused by the aqueous stain under an aqueous mountant, it caused it to run or a form of differentiation affect. (Note: it may in time correct itself, like a stain in jelly mounted pollen stain, where the aqueous stain is in the jelly). I will keep this slide for a year or two and let you know what happens to it.

Last: the lateral cut rhubarb slide is very, very thin in section (beware if you remove the wax it will look as if nothing is there, but there is). I found I could stain it but it had to have a long time in the stain bath, four hours minimum. Also, note a slight red hue in the background all over this is caused by the Haupts picking up the stain by being organic based, the trick is to stain before removing the wax, where you will find it does not penetrate under the wax. You can then mount, cover glass when dry clean off all exposed surfaces with Jif/toothpaste with a little alcohol to a polished finish and when dry ring and label.

<u>Things to help you mount</u>

1. Stain times quoted in books are often too short
2. Staining when waxed up means you can give more treatments, i.e. differentiation plus washes without losing the section by dropping off, as it is held by the wax
3. Haupts will background stain in its jelly form
4. Ernie's Haupts: I have no idea but it should be the same as the jelly form. Pears glue is chitin, polysaccharides and collagen, being based on hoof and hair, it is organic based
5. Washing off stains by water (distilled) is best carried out using four or five bottles and going through them . Water with a little bit of colour will leave stain on the Haupts base.
6. The cleaning of the slide before Haupts is most important, there are many ways to clean. I clean mine using detergent then rinse with water under the tap, letting it dry while covered to stop dust sticking
7. To make sure I keep the surface clean I put on a label marked TOP but you could use a diamond or a marker pen
8. When dewaxing do not rinse off hard or the specimen will fold over at the edge. An easy way is to lay the slide face up in a Petri dish with support under one end to stop the slide sticking to the bottom

9. For small specimens you can stain in a Petri dish, de-wax in a Petri dish onto the centre of a cleaned cover slip with mountant and lower the slide down onto it for a perfect position of the subject
10. After the treatment you can keep finished specimens stored between glass thirds and store until you have the desire to mount them. The liquid you store them in must be miscible with the mountant used. They should never be allowed to dry out, or you will get bubbles which you cannot get rid of.

First of all we must fix the sample. What is fixing and why do we do it?

Most biological specimens are examined in a non-living state. Such material must be stablized, or fixed, for the following reasons:-

1. Cellular and structural components must be kept as close as possible to the living state, and the tissue protected against osmotic shock, distortion and undue shrinkage.
2. Solution and loss of proteins, carbohydrates and other cell constituents must be minimised during subsequent processing.
3. Autolysis, fungal and bacterial decay must be arrested to avoid unacceptable artefacts.
4. The tissue must then be chemically prepared for differential staining to improve contrast and component demonstration.
5. The tissue must be hardened to allow subsequent processing and sectioning with the minimum of damage. In this case a decision must be made to "trade-off" chemical alteration against mechanical damage.
6. Fixation alters the refractive index of tissue components, improving contrast if they are to be examined without staining.

A good way to fix is to submerge the subject in FAA which will fix the specimen, this method is suitable for vegetation and small and moderate beetles up to size; i.e. hornet size you may need to remove the intestines. Larger animals, i.e. a mouse or a bat will need other preparations. FAA will fix the specimen and it will keep for a very long time, without appreciable hardening. The next stop is to dehydrate (removing H2O) to less than 1%. For dehydration Isopropanol (Propanol-2-Ol) does not harden tissue to the same extent as ethanol.

It is a saving of time to have a portable wooden rack made to carry a series of small bottles/tubes, or a printed work-sheet upon which you can stand marked jars, containing the following alcohols: 30%, 50%, 70%, 90% , 100% x 2 absolute.

While certain tissues may be transferred direct from (say) water to absolute alcohol without damage to their structure, this is by no means usual. Most tissues when transferred direct from water to alcohol (100%) are too rapidly plasmolysed, i.e. the water is removed from them too rapidly, and as a consequence they shrink and may be distorted. Shrinkage and distortion are avoided by gradual dehydration with strengths of alcohol gradually increased, and this should be accepted as a standard method whatever the tissue, unless an exception is specifically made.

Start the dehydration with the strength of alcohol immediately above that in which the tissue was last placed. (FAA is normally 70%, stains are 50-70% (normally), but there are a few exceptions).

This is the way we are going to make the slides.

1. Let's call it method number 1, through alcohols
2. We could use method number 2, which uses butyl alcohol
3. Or method number 3, which uses cellosolve or methanol
4. Method number 4, which uses dioxin (diethylene dioxide)

I only say this so that you understand that there are many ways to remove water from specimens: animal or vegetable.

We are using a method which is safe and now we must work out to which level of percentage of water we need to dehydrate down to. Most stains are approximately 50-70% alcohol so we can go down to the 50-70% jar. If we have a specimen fixed to the slide we can stain now, or if we wish or we can stain after the wax is removed, or not using a staining station. Slide mailers are set up in a carousel shape each containing a different percentage of alcohol and stain. Also at this stage you can if you wish take any insects that you have put into the FAA, small mites, bee parts, small parts of vegetative matter. You can now take them out and using a watch glass you can now stain your subjects. If you take it down to 50-70% which is the level of FAA. FAA is the chemical mix that fixes the specimens, animal and vegetable (formalin, alcohol, acetic = FAA, all three fix specimens and are very commonly used and are good for long term storing; the three chemicals fix in different ways: dehydrating, destroying bacteria and destroying fungi details on how to make up are on the bottom of page 11). Small insects flies or bits of bees after cleaning and arranging on a 1/3rd of a slide, can be squashed between an additional 1/3rd plate and clipped, then submerged into two changes of absolute alcohol, which will finally set the subject in position and take away all the water. I personally would give it another soaking in Histoclear II and then mount it in practamount. Some people say you can go straight from the alcohol absolute to practamount, I have never tried it myself. **Please refer to the diagram on page 6.**

Embedding in Wax

Alcohol or water cannot mix with wax but xylene and histoclear are misible with alcohol. So that last bath would be in absolute alcohol absolute, two changes, to remove 99%+ water content. When this is done we can now lower our specimen into the wax. The xylene or histoclear will now exchange fluids for wax. After a few hours change the wax for new clean wax, this act will remove the chemical histoclear or xylene in the wax which if left in will cause problems when cutting sections (soft spots). The composition of the wax is important too, soft waxes cleave point to point (parallel fashion) and the ambient temperature has a greater effect on the cutting qualities of soft than hard mixtures. Hard mixtures are therefore better for cutting thin sections and the degree of compression is less. Therefore, sections that roll into a tight roll are cut too thick. Therefore, cut them thinner. Very thin sections compress so much more because of the effect of local warming, a cold knife will

dissipate the heat generated by friction and so will further reduce compression as will a reduced rake angle (tip: brush the blade with cold water with a dash of absolute alcohol to cool it), or put the block in the fridge to cool it to make it harder. Tip: for me I find that genuine beeswax (ester based wax) is harder than the paraffin wax at the 48-55 melting degrees range. The Royal Microscopical Society recommend that you re-use old wax and re-filter the hot wax; it then undergoes a change in that the wax crystals are smaller the more times you use it. When making blocks with virgin wax put the specimen off centre when using new wax because when the wax cools large crystals are formed in the middle of the block, and are hard to cut and less prone to infiltrate the specimen with wax.

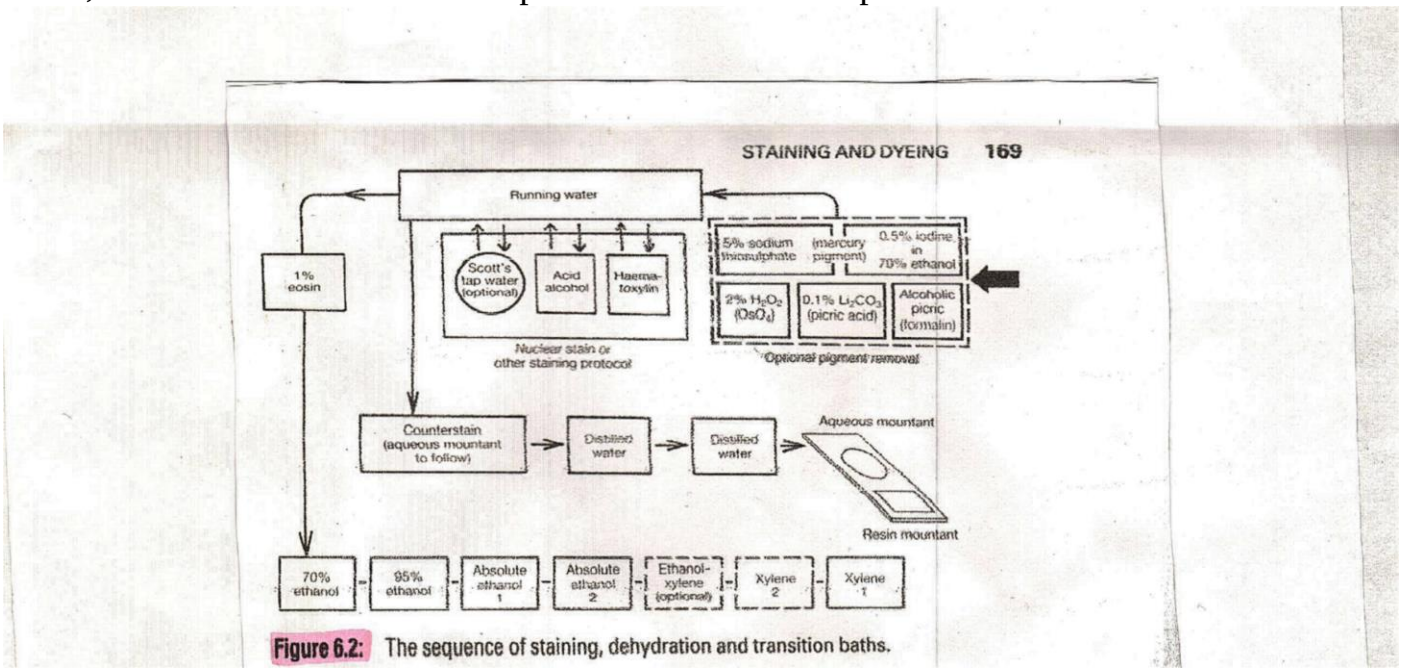


Figure 6.2: The sequence of staining, dehydration and transition baths.

Drawing shows dehydration, on the bottom series, to xylene. The middle series shows re-hydration for use with aqueous stains. (At this stage you can mount in an aqueous mount).

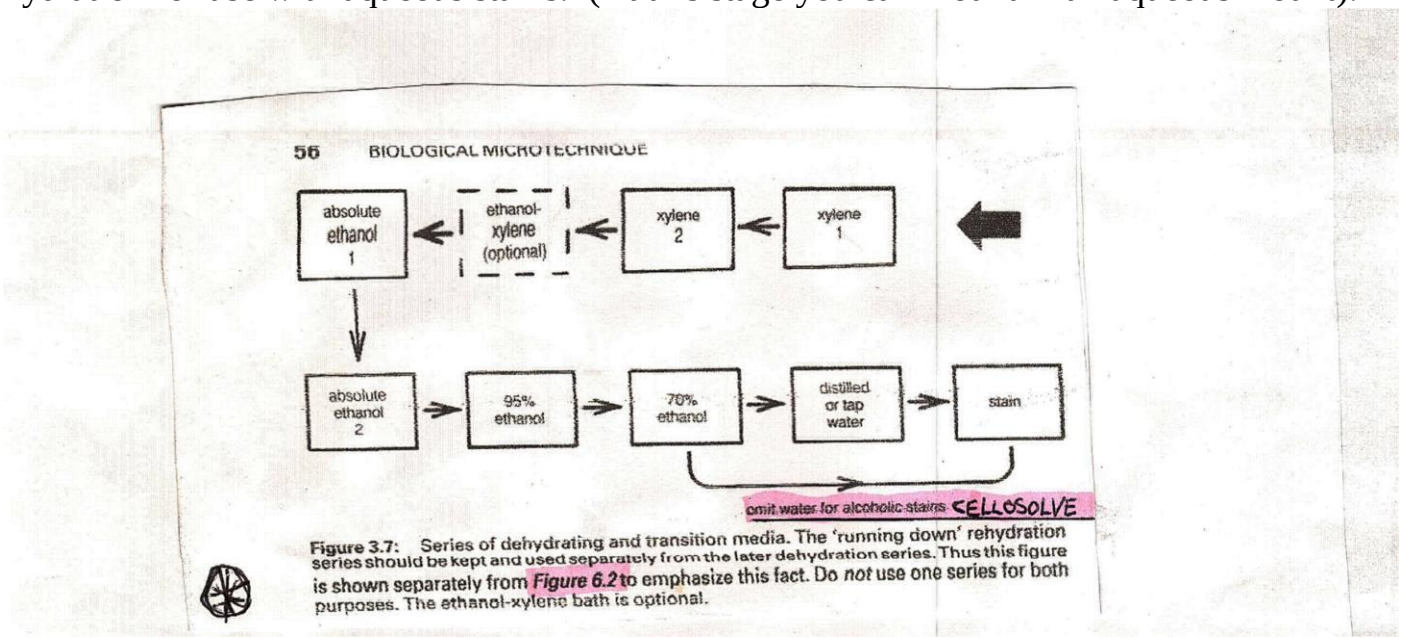


Figure 3.7: Series of dehydrating and transition media. The 'running down' rehydration series should be kept and used separately from the later dehydration series. Thus this figure is shown separately from *Figure 6.2* to emphasize this fact. Do *not* use one series for both purposes. The ethanol-xylene bath is optional.

This statement relates to the drawing above (see *above)

Running down rehydration to stain, then reverse to dehydrate to xylene/histoclear bottles in series. New bottles; do not use original series of bottles for fear of contamination. NB You have to know the difference between dehydration and rehydration through the alcohols. On the day I will have other examples to show you, which may simplify it.

The next step is to cut the sections if things work you may have ribbons, if not merging ribbons take off one at a time with a wet brush and lower into a warm water bath in which they will unroll. We have our own water bath and I propose to demonstrate this on the day, in a temperature controlled water bath which belongs to the Iceni. It would be handy if everyone can practice this on the day, it is a handy thing to know.

Wax Embedding

There are several ways in which paraffin and beeswax blocks can be cast, or a mixture of both.

1. Using a re-useable metal or plastic mould in an embedding centre, I have seen plastic plant plug trays used for this purpose because the sections are approx. one inch square
2. Using a disposable paper or metal foil mould (aluminium cooking foil). I propose to demonstrate this on the day.
3. Using leuckhart "L" pieces
4. Using a watch glass for very small or delicate specimens
5. Re-using empty night light containers

Wax needs to be held at about 1-2 degrees C above its melting point, if the wax is kept too hot it will oxidize forming a yellowish mixture and losing much of its crystal structure. First class embedding needs two or even three changes of wax. Tip: paper boxes produce the best cubes of wax because the paper can flex to accommodate the considerable shrinking of the cooling wax, 10% approximately, so obviating localised pressure regions. The maximum reasonable size for such blocks is approximately 45 x 20 x 20mm. Of course, there are many wax plugs which are only 10mm in diameter, the best way to obtain these is to start with a one inch cube to form a pyramid which serves three purposes: 1) the bigger diameter is easier to fix onto the machine 2) the bigger block will give you better crystallisation 3) the height of the block will also make the cutting action more visual and it will not be shielded by the apparatus.

Cutting Wax Ribbons

Ribbons will usually form from friction alone as the block is cut, but in a low-melting point wax, or mixture of beeswax and embedding wax, may be melted by friction on a thick layer onto a leading edge of the cutting face. This also assists cutting and manipulating the ribbons when mounting the sections later. As the sections ribbon upon cutting, support the weight of the ribbon without stretching, using forceps to grasp the edge of the leading section, but some microtomes have a conveyor belt system which does it automatically. Once the ribbon is the right length, come underneath it with a brush or seeker and gently twist the rear section to one side to peel the trailing edge of the next section. Leave two to three sections on the knife edge to start the next ribbon. It often helps to breathe ("huff") onto each section as it is cut to prevent curling.

Ribbon difficulties:

1. Sections curl from hard, refractile or brittle tissue – soak or hold the first section down with a brush as it comes off the knife.
2. Reduce static
3. The room atmosphere may be too cold for the wax in use – employ a harder formulation, with a higher melting point, in warm weather and vice versa. Alternatively, the wax may simply be too hard a formulation.
4. Wax accretion may have built up on the back of the knife, especially if there was difficulty in getting the first section to cut – clean the back of the knife with xylene and dry thoroughly, while still in place
5. Embedding may be faulty.
6. The sections may be too thick.
7. The knife is blunt, tearing minute fibres from the tissue surface.
8. The knife tilt may be too great or small – try this adjustment last in this list.

Mounting Onto Slides

The water-bath method

The water bath should be thermostatically controlled, and held at a temperature about 10 degrees C below that of the melting point of the wax. As a rough guide the water should be hot to the touch. If the water is too hot the wax is liable to melt and over stretch or break up when the section is laid on the water surface, and the morphological arrangement or parts of the section may be lost. If, on the other hand, the water is not sufficiently hot, the section will retain its wrinkles and compressed shape. The same parameters apply to the hot-plate. The water should not be taken straight from the tap, but boiled and allowed to stand until it reaches the right temperature. In this way, bubbles that could become trapped under the section on the slide will be avoided.

Where tissue is liable to expand at different rates within the section, use a cooler water bath and tease out the wrinkles with forceps and/or seekers. Care should be taken at this stage, as the warm section can very easily stick to the forceps and wrap itself around them as they are lifted free! The problem mainly occurs with cartilage and mucus; blocks with large amounts of these substances should be well-trimmed of free wax before cutting (i.e. bees are lateral cut or similar).

Lay the section *matt side up* on the surface of the water bath. The action must be smooth, with the trailing edge of the ribbon touching the water first. The slight dragging that results assists in removing wrinkles. If the layering action is too fast the sections will be overstretched; if too slow the sections will fold up as they are laid down; folds rather than wrinkles are impossible to remove. It is best to cut three or four sections in a ribbon and discard the outer ones once the ribbon has been flattened.

If there is a problem with floating-out, the section can also be picked up by a wet paint brush and laid dry on a microscope slide, which is then flooded with 20% alcohol underneath, and floated onto the water bath where it is picked up in the manner just described. The section to be mounted can then be selected out of the ribbon, and carefully prised apart at the joins

using forceps. Move the section to a free edge of the water bath, take a clean grease-free slide and insert the slide into the water at an acute angle of about 60 degrees, a centimetre or so from the section. Do not do this too near the section, or the surface tension of the water as the slide enters will drag the section down into the water. Manoeuvre the slide under the section, and withdraw the slide smoothly in one movement, again at an acute angle, allowing the section to adhere to the slide and be withdrawn.

Shake off the surplus water, and lightly blot with damp Postlip blotting paper if required or use damp tissue roll press down and fix using your broad thumb, which may leave blue fibres on but can be easily removed; dry tissue will destroy the sections. Moist wax is opaque, while dried sections have transparent wax borders. Label the slide (usually with a diamond marker pen) and dry at 60 degrees C, or just below the melting point of the wax, for 10-30 min on a hot-plate or in the wax oven before dewaxing and storing, can also be stored in the waxed state. Alternatively, the section can be dried off at 37 degrees C overnight (this is good for neural tissue). Other aids to adhesion are: adding gelatine and potassium dichromate separately, from 1% stock solutions, at concentrations of 0.002% to the water bath; using a slide smeared with a touch of 1% glycerine albumen solution, using slides previously coated with adhesive. Go to Adhesive, Mayers/Haupts, Ernie Ives's section further down this paper on page 11.

The water bath

We have in our club a water bath, to set up as a guide temperature it should be as hot as a cup of tea. There is a knack in placing a section on the slide, I will show you how. After the section is on the slide either dry off on a hot plate or air dry. Then you are left with a wax section fixed onto the slide for staining (a discussion on adhesives later), see above. First you must run down the specimen rehydrate to 50-70% through alcohol to match the 50-70% cellosolve level in the stain. Some are 50% (check). Remember cellosolve is misible with water xylene and histoclear (I think so? _____), and also a common solvent for certain stains.

If you use water aqueous stains you need to rehydrate down to 50%, 40%, 30%, 20% alcohol and then distilled water. Then stain aqueous. After staining dehydrate 30%, 40%, 50%, 70%, 90%, 100% absolute alcohol. Last bath: histoclear mount/practamount, or from 100% alcohol absolute to an aqueous mountant (see bottom diagram on page 6). Test for stain running you may have to wash off four or five times, when no more comes off then you can mount it. Ring and label as for a finished slide. It is generally about a minute or two minutes in each bath. It is important that you keep your specimen wet until you mount, if you let it dry you will never get rid of the bubbles.

Attachment of Wax-Impregnated Sections to Slide

For ease of manipulation after sectioning, the wax-impregnated sections are attached to a glass slide or, in some instances, to a glass cover slip, or floated off and placed in a watch glass, assisted possibly with a wet brush.

The adhesive used must be sufficiently strong to retain the section(s) attached to the slide, and must not dissolve off the slide during subsequent operations. On the other hand, it must

in no way interfere with the final preparation. The substance that used to be in most common use is egg-albumen, either alone or with glycerine; but human saliva is cheaper and very effective, but today we use Adhesives, Mayers or Haupts (Ernie's special Haupts). These are tried and tests methods and can be relied upon.

After the sections have been attached to the slide, the wax with which they were impregnated must be removed. This is done by dissolving it is xylene or histoclear. There are more chemicals but histoclear is the safest of the two chemicals.

Technique of Attachment of Wax-Impregnated Sections to Slide

1st Smear a clean grease-free slide very sparingly and evenly with egg-albumen or saliva and Mayers or Haupts (The edge of the palm of the hand drawn from end to end of the slide produces an even smear). Or alternatively use your fat thumb print to go from one end to the other.

2nd Pass the prepared slide under the ribbon (or portion of ribbon, if it has been divided) and draw it on to the slide with the aid of surface tension. The final manipulation with a wet brush – float it to the centre of the slide.

3rd Remove excess water by gently covering with a damp tissue not a dry tissue, a dry tissue will remove it from the slide and push down to fix wax to glue with thumb, then slowly remove damp tissue.

Adhesives, Mayers or Haupts
Ernie Ives's Special and Why

Ernie used a special Haupts made using pearl glue, i.e. the old wood working glue, heated in a glue pot made by melting down squares of gelatinous material made from fish bones and hoofs. It comes in squares like chocolate and you render it down with water over heat. He then mixed it with Haupts gelatine which made a glue which held better with aqueous stains. Pure Haupts gelatine will let go with water stains. Ernie's mix could take an aqueous stain, plus five or six washes in distilled water and not let go. Also Ernie's trick on a double stain was first to stain aqueous, second stain cellosolve based which did not let go or wash out the glue which held the specimen in place. Staining most of the time in the waxed state. Why do we stain on the slide? If we do something like a flower or a seed head there are gaps between the specimen the glue holds them in the correct ratio to nature. If this was not so you would find lots of little bits floating about and you would not be able to assemble them in the correct order. For the future I will try to track down some pearl glue and make some for all of us. All the above is from personal communication with Ernie in about 2010.

Staining and Counterstaining
(Differential staining)

The phenomenon of selectivity is frequently used in the processes of counterstaining, when first one part of the cell or tissue is stained with a suitable dye and the other parts are afterwards treated with a stain of contrasting colour. It should be understood that in many instances the process of counterstaining really involves the displacement of one dye by another. The dye first used (e.g. safranin O) stains all the tissues. The dye next used (e.e. Delafield's haematoxylin) will also stain all the tissues, but the two dyes operate together by differential displacement till, finally, for example, the lignin is stained red and the cellulose

blue. I did try this by mixing two stains on my carousal, I got mixed results but should have been more patient and left them in longer and I did not have the ratio 50:50 which I should have done, nor did I have fresh stains.

Yet another, less well known, method of differential substitution staining involves the use of free dye-acids and dye-bases. The principles involved are as follows:

The acid dyes eosin Y and erythrosine bluish in their usual states are the coloured sodium salts of the acids tetrabromofluorescein and trataiodofluorescein respectively. The dyes are soluble in water but not in most organic solvents. The free acids can be separated from an aqueous solution of the dyes by treating the aqueous solution with hydrochloric acid which combines with the sodium and leaves the free dye-acid as a solid. The free dye-acids give colourless solutions in ether or xylene. From these colourless solutions colour is again produced by the addition of either alkalis (e.g. sodium hydroxide given the coloured sodium salt). (Copied from Peacock Dyes and Stains).

I know very little about stains but reading up on the subject I find some have a shelf life of approximately three to four years only. Secondly a lot of cellosolve stains are made up with 50% alcohol, 50% cellosolve and 1% stain. This is the average but some types have a 95% alcohol, as in orange O, these types of stains help us when staining waxed sections on slides without unsticking the water based glue. If we used a water based stain there is a risk of lifting the section off the slide. I have many stains which I have had more than 10 plus years and I think as a group we should examine our stains and test by trial and error, I will bring Ernie's stock of stains with me and hand them out at the meeting.

I think we should have a talk about stains, but we may have to buy in some expertise. Is there anyone here willing to do a talk on stains? Could we recruit someone from the Quekett on stains, we could prepare subjects to be stained as a group before the meeting? Any ideas?

FAA
(Formo-acetic-alcohol) (FAA)

Alcohol	70%	88%	by volume
Acetic Acid	100%	7%	by volume
Formalin	40%	5%	by volume

FA
(Formalin-alcohol)

Alcohol	70%	94%	by volume
Formalin	40%	6%	by volume

HISTO-O-CLEAR (trade name)
REAL NAME D.Limonene C10 H16
(water insoluble) 0%

Xylene CH32 C6 H4
Practically insoluble in water 1%