METHODS: MICROSCOPY

Bruce D. Ryan

Refraction

By adjusting the rings under the condenser, you can get refraction by the transparent parts of the section, or a dark background.

Hertel (1986, pers. comm.)

Kolen Method (for achieving sharp image)

For each objective, turn diaphragm to minimum, move condenser until ring is sharp, then open diaphragm until it fills the field.

Colors

The apparent color ("color temperature") of objects under a microscope changes color with the voltage. Artificial light is +/ yellowish, so bluegreen appears yellowgreen. Therefore, use a blue filter to make the light more like that of sunlight.

To compare colors, use sections of constant thickness.

Polarized Light

The color of crystals in polarized light indicates different substances. There is a special polarizer that makes a blue background and makes the crystals orange.

Excipulum Structure

To examine excipulum structure, soak the apothecia for several acetone (room temperature or warm), then rehydrate them.

Mycobiont/Phycobiont Contacts

To see contacts between fungal hyphae and algal cells, use interference contrast.

Walls of spores, paraphyses, asci, etc.

To see the walls of spores, etc., use interference contrast, on

squash mounts.

Chemistry of granules in epihymenium, cortex, etc.

Try dissolving granules in acetone. If there is no effect from acetone or reagents using the bibulous paper method, try applying the reagent directly to a dry section, and then adding water as a drop on the underside of the coverslip.

Blotting

Toilet paper is handy for blotting up excess liquids.

Cotton Blue

The lactophenol in cotton blue may dissolve crystals, especially after some time.

Freehand sectioning

Break doubleedged razor blades in half; check the edges under a dissecting scope to see if they are straight and sharp.

Soak the specimen in water for a while, then blot dry before sectioning.

Ascus structure

After squashing, add a drop of water or lift up the coverslip to release the pressure.

Pycnidial structures (fulcra, etc.)

Use 0.5% erythrosin in 100 ml of 10% ammonia.

TIPS ON SECTIONING OF LICHENS

Bruce Ryan

Nov. 1987

Some microscopic observations, e.g. observations of spores, can be made on squash mounts. However, many critical features can be clearly seen only in sections, and with large, hard or fragile structures, it is often necessary to use reasonably thin sections even to make good squash mounts.

Brodo (1984) and others have recommended that in studying apothecia, sections should be made of the central, portion of mature, healthy ones. If the sections are not roughly median through the apothecia, the often deeply conical base of the hypothecium will be missed. It is important not to cut away the tissues of the apothecial margin because they often contain useful features of the cortex.

In most cases, sections should be made vertically (perpendicular to the upper surface of the structure). However, horizontal sections can sometimes also be useful, e.g., in measuring the diameter of the lower parts of the paraphyses (Hertel?, pers. comm.). In sectioning lobes, sections made longitudinally in the direction in which the lobes radiate may show a structure different from that in sections made perpendicular to the radial direction.

The sections need to be flattened out and separated from each other (using a probe or brush under a dissecting scope, if necessary) before being fanned out under the cover slip.

Sectioning by Hand

To get the lower cortex, pull lobe or apothecium off, turn it upside down, and slice. Even if the medulla falls apart and the section doesn't include the entire thickness of the structure, the lower cortex will be included. (With apothecia, the upside down position is also more stable for making sections through the entire structure).

To get thin enough sections, one can make each slice at a slight angle to the previous one, such that one end of the section will be very thin. However, this may produce some distortion in the

appearance of the tissues.

To section thalli or apothecia (especially hard or brittle material), soak the structure (usually while still attached to the thallus) in water, then blot up the excess water (Hertel, pers. comm.). Some experimentation is needed to determine what degree of wetness is best for sectioning a given structure. If a structure is too dry it may pop off the thallus or crumble; if itis too wet, it may become too soft and flexible.

Unless it is desirable to examine sections made parallel to the lateral surface, it is generally useful to make an initial cut tangential to the structure and discard the thickish curved outer part before making thin sections.

Slice the structure from top to the very base with a rapid slicing motion, discard or set aside the outer sections, and cut the middle sections (four or five) free, to be examined microscopically (Brodo, 1984).

The outer few sections (or imperfect sections) can be saved for making chemical tests.

Be sure the razor blades are sharp, to avoid destruction of characters amd prevemt carring of granules from one part to another (Brodo, 1984). Use a fresh razor blade after every five or ten specimens, or more frequently when the slices are partly through rock or when very thin sections are critical. Use doubleedged razor blades, since they are usually sharper than the singleedged ones (Rhoades, pers. comm.). Break them diagonally, and use the pointed part for slicing (Hertel, 1986, pers. comm.). Examine razor blades under the dissecting scope to see if they are straight and sharp (Hertel, 1986, pers. comm.)

Wet the razor blade to pick up the slices. When removing the slices, try not to drag them across surfaces where they might pick up small particles (e.g., pruina or particles of dirt).

Bland (1971) recommended placing the thallus between two small pieces of balsa wood which are pressed close together, and cutting sections through both the balsa wood and the lichen. Sipman (1983) used Sambucus pith for this purpose.

SECTIONING BY MICROTOME

Very critical or detailed anatomical studies require the use of a microtome, to prepare thin sections. Timdal (1984) used sections 820 um thick.

Brodo (1986, pers. comm.)

Mount specimen in mixture of gum arabic and phenol crystals.

Ice should be just hard enough to dent slightly with your fingernail.

Soak specimen (on slide, with label) in PhotoFlo, then put sections in watchglass of water.
Put 5 sections on each slide.

Lift sections from watchglass, put into cotton blue on slide, with brush.

Clean coverslip in water before putting on top of cotton blue; put coverslip on slowly with tweezers, with one end touching slide first, and try to avoid bubbles; use tiny drop of cotton blue, so it doesn't go past the coverslip. For permanent slid, wipe cotton blue with moist towlette, and seal twice with clear nail polish.

Hertel (1986, pers. comm.)

Ice should not be too cold (25oC is about right).

Soak specimen in alcohol first (prevents rupturing of tissues, but may dissolve substances).

Use thin, flexible tweezers, which will not squash the apothecia.

Attach disc side of apothecia to sloping surface of the ice.

Make ice flat by running a finger across it before the ice is completely frozen; add water in tiny amounts to avoid washing away the specimen.

Make at least 4 slides per specimen, at least 2 with cotton blue.

Put cotton blue on slide before putting specimen on. Leave sections in cotton blue for a while (at least several hours)_

before putting on the coverslip; this will stain the tissues better.

Adjust knife angle, experimenting until the knife neither shatters the ice nor glides over it, but actually slices it.

Section several apothecia of a specimen at a time, but remove each 34 sections, and put each set of 34 in a different drop on a slide.

Immediately after sectioning, before putting the coverslip on, and before the sections dry out, flatten and separate the sections and remove sand grains, using a needle under a dissecting scope.

From Roger Anderson

Put an insect pin inside a hypodermic needle and bend it to make a probe for manipulating ascocarps to be sectioned.

METHODS: FREEZING MICROTOME

Brodo (1986, pers. comm.)

Mount specimen in mixture of gum arabic and phenol crystals.

Ice should be just hard enough to dent slightly with your fingernail.

Soak specimen (on slide, with label) in PhotoFlo, then put sections in watchglass of water.

Put 5 sections on each slide.

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METHODSMICROSCOPYMISC.

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Select a full grown but not large apothecium for sectioning. (Sipman 1983).

Soak the apothecia first in 90% ethanol to expell the air, then in tap water. Sipman (1983)

After making measurements and other observations in water and letting the sections air dry, wash away secondary metabolites, by adding and evaporating drops of acetone at room temperature. (Sipman 1983, following the second method described by Thomson 1967, p. 16). Use the acetone extracts for TLC, then resoak the sections in ethanol and then in tap water. Then transport the sections into lactophenol cotton blue.

Observe the semipermanent mounts made in lactophenol cotton blue after one day, and note changes (Sipman 1983).

Moisten the ascocarps with water containing a wetting agent (Harris, 1973; this may be necessary mainly for pyrenolichens).

Asci

Separate asci from paraphyses by gentle pressing with the points of a pair of forceps under the microscope with low magnification. (Sipman 1983; this may be feasible only with large asci).

Cut off about one third of the ascocarp and dissect out a portion of the hymenium, which then may be gently squashed on a slide without the interference of parts of the substrate or carbonized wall fragments (Harris 1973).

Soredia

Examine and measure soredia under a compound microscope at low magnification, with incumbent light and in airdried state. (Sipman 1983).

Crystals

The nature of the crystals of calcium oxalate can be proved by their solubility in concentrated acetic acid and/or diluted

hydrochloric acid. In the first they do not dissolve, in the second they do without effervescence. Experiments to remove the crystals before sectioning, to obtain better sections, are unsuccessful (Sipman 1983).

Stains and Mounting Media

Lugol's Iodine usually provides sufficient contrast to makedetails visible without chemically distorting them. The iodine can then be cleared by pulling 1020% KOH under the coverslip and drawing the iodine out the other side (Harris 1973).

Aqueous phloxine (12% in water) gives good contrast for seeing septation in spores and hyphae, and can be cleared with KOH, leaving only the protoplasm deeply stained (Harris 1973). Spore ornamentation can be seen by staining with acetic cotton blue (Chadefaud, 1969cited by Harris 1973) bu heating just to the boiling point over a flame.

Algae can be seen in endophloic thalli by mounting thin sections in lactophenolcotton blue and warming gently (Harris 1973); the algae will stain deep blue, while the bark cells will be relatively unaffected (Harris 1973). A mixture of aqueous phloxine and 1520% KOH can also be used for this purpose (Harris 1975).

Chlorazol Black (12 percent in water), mixed with KOH on a microscope slid immediately before use is the most satisfactory stain fro the chitinoid ring in the ascus tip of Porina and Trichothelium (Harris 1975).

Use orseillein in lactophenol cotton blue. Orseillin stains the cytoplasm red; when observing with a blue filter the cytoplasm appears dark bluish. Orseillin is a more satisfactory stain than cotton blue and should be used routinely (Anderson 1970).

USE OF POLARIZED LIGHT IN MICROSCOPIC STUDY OF LICHENS

Bruce Ryan

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The use of polarized light, (with one filter between the light source and the specimen and one between the specimen and the eye) has been described by Kofler (1956).

As pointed out by Brodo (1984), "crossedNichols" illumination, which renders crystalline or refractive material bright against a dark background, is essential for distinguishing between minute oil droplets and granules, or between air pockets and large colorless crystals in the amphithecium, and for seeing granules in a strongly pigmented epihymenium.

Use polarized light to see the algal layer, cortex, and other tissues in thick sections.

Use polarized light to distinguish the subhymenium, hypothecium, etc. from each other.

Sometimes the paraphyses and asci refract, appearing to glow in polarized light. This is a common phenomenon (Brodo, pers. comm.) In many cases where this phenomenom occurs, for one reason or another there are no spores (or at least no welldeveloped ones) in the hymenium.

Some types of refraction may occur in areas where the section changes in thickness (e.g., at the edge of the structure, or at the boundary between two tissues) or bends out of the plane of the section (Nash, pers. comm.). Comparison of different sections will usually show that such patterns are artifacts.

The degree or appearance of refraction is affected by the thickness of the section; thin areas usually refract less than thick areas.

METHODOLOGY

Descriptions of various techniques and refinements are scattered through a large number of books and articles on lichens or other organisms, and most workers have their own special methods acquired through much experience and trial and error. It is desirable to make a larger portion of this extremely diffused information available in a single place, with comparisons of alternative variations, and to contribute towards eventual standardization of some of the techniques.

EXAMINATION OF LICHENS THROUGH A DISSECTING SCOPE

Wetting the surface of the thallus will often make spermagonia easier to locate. It will also help to see the color and surface structure under pruina.

To examine the undersides of lobes or squamules and determine how they are attached to the rock, carefully detach and lift the structures using a probe needle.

MEASUREMENTS

Measurements given in mm are made under a binocular scope on dry material. Those given in um are made under a compound scope. The latter are usually made in water unless stated otherwise. Timdal (1984) made all microscopic measurements on material mounted in LCB.

Timdal (1984) generally based descriptions of the dimensions of spores or conidia on fifty or measurements (per species). It is desirable (though not always feasible) to measure at least 10 spores (or spermatia, etc.) on each specimen. The largest one and the smallest one should be sought; otherwise the search can be either systematic or haphazard (by moving from each measured one to the closest one to it?).

Measurements can be recorded or expressed in various ways. Timdal (1984) recorded the two extreme values to the nearest 0.5 um (0.5 mm could be used for features measured under the dissecting scope), and calculated the arithmetic mean to the nearest 0.1 um (or less precisely for structures only with mean dimensions under 1.5 um)

STUDYING SPORES

Do not assume that scattered spores belong to the species you are studying, unless similar ones occur in the asci. Also watch out for parasymbionts.

It is important to distinguish between mature and immature spores. In general, immature spores have granules and oil drops; mature ones often do not (Nearing, the Lichen Book).
When measuring thinwalled spores, it is important not to exert pressure on the coverslip, which can increase the spore lenght up to onethird more (Swinscow, freshwater Verrucaria).

MISCELLANEOUS METHODS

Eucortex and Pseudocortex can often be distinguished in rather thick sections if they are in KOH.

To see paraphyses or other tissues clearly, let the sections absorb cotton blue for several hours, then rinse with water (Hertel, pers. comm.).

SemiPermanent Slides

Semipermanent slides, which can be rewet whenever needed, have the advantage of being easy and inexpensive to prepare, and they do not distort hyphae and tissues the way that the permanent mounting media do (Poelt, pers. comm.).

Semipermanent slides can be prepared by simply glueing one or two edges of the coverslip to the slide, and then when a dry slide needs to be reexamined, simply putting water under the coverslip again. Poelt (pers. comm.) uses "UHU Alleskleber" for this purpose; various kinds of model cement or clear nail polish also work well.

Fixing Agents

According to McWhorter (1921), fungal elements fix well in chromacetic acid, while algal elements fix well in hot bichloride of mercury.

A solution of 7% formalin or F.A.A. will preserve lichens (Sass, 1951; recipie on card).

Epihymenium Chemistry

For determination of the actual chemical contents of the

epihymenium, Leuckert (pers. comm.) recommends moistening the apothecia, blotting with a paper towel, scraping with a fine scalpel (carefully avoiding getting the margin) and doing HPLC or (if large numbers of apothecia are available) TLC, on the scrapings.

Reagents

Ferrous (ic?) Chloride ($FeCl_3$)

TLC

Anisaldehyde in ethanol, mixed with sulfuric aciddetects usnic acids, which turn deep violet (Leuckert, pers. comm.).
When gyrophoric acid is present, at least traces of lecanoric acid are usually also present (Leuckert, pers. comm.).

LITERATURE ON METHODS IN LICHENOLOGY (MISCELLANEOUS)

Bruce Ryan

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(* = information incorporated into my files on wp)

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