# Plectological techniques for Larger Fungi

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Abstract. For larger fungi the recommended procedure is: fixation in cold and buffered, clean formaldehyde (or osmic acid for laticiferous hyphae), single-step dehydration in cold methoxyethanol, embedding in a soft Spurr's resin mixture. Sections cut with a steel knife are stretched by floating them on hot water, then stained, if necessary, by floating then on the staining solution. For mounting a specially fast curing Spurr resin is proposed to avoid wrinkling of the sections. Alternative mounting in a noil mixture of appropriate refractive index is described.

Preamble: Since fungal trama differ fundamentally from tissues of higher plants, I prefer to designate them with terms ending on "-plect", e. g. ixoplect for a gelatinized trama. Therefore "fungal histology" becomes plectology.

Classical histological techniques, mostly using acidified fixing fluids and paraffin embedding, have repeatedly been applied to agarics and other large fungi (e. g. Kühner 1938; Reijnders 1963; Disbrey and Watling 1967; Moore 1965). Although many results have been obtained this way, there can be no doubt that most of the classical formulas are inadequate for good fixation of inflated and thinwalled hyphae, and even hyphal tips rich in cytoplasm are poorly fixed. Delicate fungal trama made mainly from thinwalled and loosely arranged hyphae is in many cases simply destroyed by paraffin embedding. Very similar observations have been made by Mai (1975) working with pyrenomycetes.

In this paper several years of experimentation of handling delicate fungal trama are discussed, and a description of a method is presented. The results are very encouraging even with the most delicate structures found in agarics.

Experimentation with plectological techniques was initiated by the outstanding paper of Feder and O'Brien (1968). Their conclusions are perfectly correct and directly applicable to large fungi. They used methacrylates as an embedding medium, but these resins have several disadvantages, such as sensibility to oxygen, shrinkage during polymerisation, short potlife of the complete monomere mixture and the frequent need to remove stabilizers before use.

Using slow curing, low viscosity epoxy resins it becomes very easy to infiltrate uniformly large blocks of fungal trama. Sectioning is no problem, even at great thickness and large cutting plane, but staining is more difficult than with methacrylates.

## 1. Fixation

Classical, coagulating fixation results in destruction of fungal vacuoles and dramatic condensation of nuclei. In most instances the fixation solution is by far to highly concentrated, giving badly deformed and collapsed cells. In dense cells, such as young basidia or asci, the over all form is better preserved, but artificial granulosity of the cytoplasm masks details, and fine structures have been destroyed.

Non coagulating fixation solutions, when applied correctly, give excellent results preserving even the finest details without any artificial granulosity and without deformation of cells, even if these are highly vacuolated. Two of them, formaldehyde and osmium tetroxide, have been in use for over a century. Formaldehyde is cheep and penetrates quickly, so that large blocks of trama can be fixed. However, a few precautions have to be taken to assure good results:

a) Formaldehyde solutions quickly become unsuitable for fixation by accumulation of formic acid and other substances. It is therefore essential not to use commercial formaldehyde solutions, even if they are labelled analytical grade.

For each fixation a small amount of formaldehyde has to be prepared, and it should be used the same day. This can easily be done by adding potassium hydroxide to a warm suspension of paraformaldehyde in distilled water.

b) The formaldehyde solution has to be buffered, and total ionic strength must be low for fleshy fungi. A small amount of calcium chloride has proven to be beneficial. It probably facilitates penetration and prevents swelling of the organelles.

There are a number of buffers available for fixation solutions. Two of the best are potassium phosphates and cacodylate-hydrochloric acid buffers. Phosphate solutions may form precipitations with calcium and other substances occurring in fungi, and they quickly get spoiled by submerged growth of molds. Cacodylate solutions are stable for years and performed nicely in my laboratory.

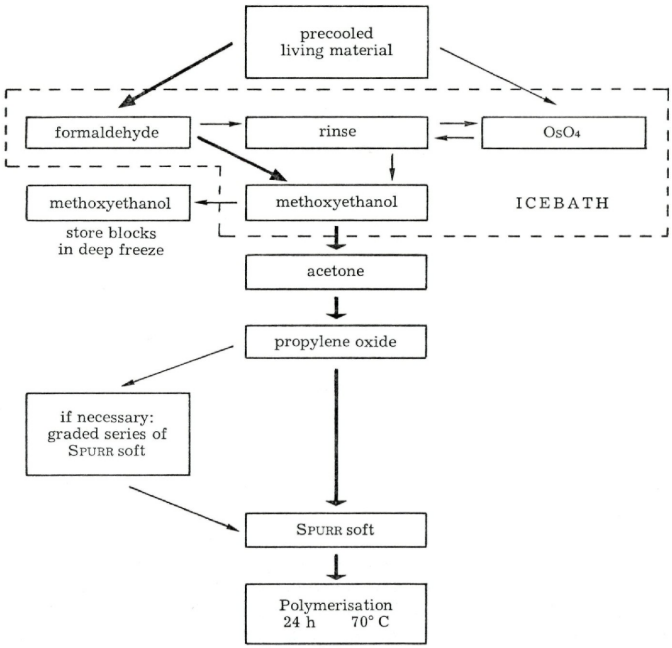
For fleshy fungi, mainly agarics, the optimum pH is about 6.9, and the buffer concentration should not be higher than 0.05 m.

c) It is important to fix at low temperatures, preferrably in an ice bath. Small fungus fragments can be immersed directly into the cold fixation solution, larger blocks should be precooled. Cooling effectively prohibits collapse of delicate cells and fine cytoplasmic

strands between large vacuoles, probably by increasing the viscosity of the cytoplasm.

d) Maximum fixation time should not exceed 24 hours.

Problems may arise when the fungus contains trapped air. Air content can be minimized by not allowing the fungus to dry even slightly. Keep it in a moist chamber or plunge the base of its stipe in tap water for a few hours to saturate the trama with water. Never




soak the whole fruit body. The hymenium of Basidiomycetes is very quickly damaged by liquid water. Remaining air should be removed quickly by applying a moderate vacuum several times for a few seconds. Keep the recipient in the ice bath during evacuation. This prevents the water or the fixation solution from boiling which would destroy fine details.

Buffer stock solution, concentrated 20 times:

| | |
|---|---|
| Distilled water | 87.4 ml |
| sodium cacodylate | 21.40 g |
| HCl 1 n | 12.60 ml |

Formaldehyde solution:

| | |
|---|---|
| Paraformaldehyde powder | 2 g |
| distilled water | 25 ml |

Heat the suspension over a flame. Thermometer control is not necessary. When the suspension gets to hot for being held in the palm of the hand, the required temperature range is reached with sufficient precision.

Add two drops of 5% KOH, stopper and agitate. The solution will clear within a few minutes.

Cool under running water. Add 25 mg of anhydrous $CaCl_2$ (or 33 mg of $CaCl_2.2\ H_2O$).

Fixation solution:

| | |
|---|---|
| formaldehyde solution | 25 ml |
| buffer stock solution | 2.5 ml |
| distilled water | 22.5 ml |

This will result in a clean fixation fluid containing approximatively 4% of fresh formaldehyde, having a pH of 6.9 and a buffer concentration of 0.05 m.

Fix for 2—24 hours in a ice bath.

Aldehydes do not fix the content of laticiferous hyphae of Russula, Lactarius, Lentinellus and other fungi, but osmium tetroxide does. Use it at a concentration of 2% in the cacodylate buffer, in an ice bath and fix for 12—24 hours, or use osmium tetroxide vapors for the same period of time.

## 2. Dehydration

After fixation with formaldehyde the fungus material is transferred directly into cold and undiluted 2-methoxyethanol (synonyms: methyl cellosolve, ethylene glycol monomethyl ether). It is important that the fungal block comes from ice cold fixation solution and that the methoxyethanol is precooled, but it is not necessary to rinse with buffer prior to dehydration.

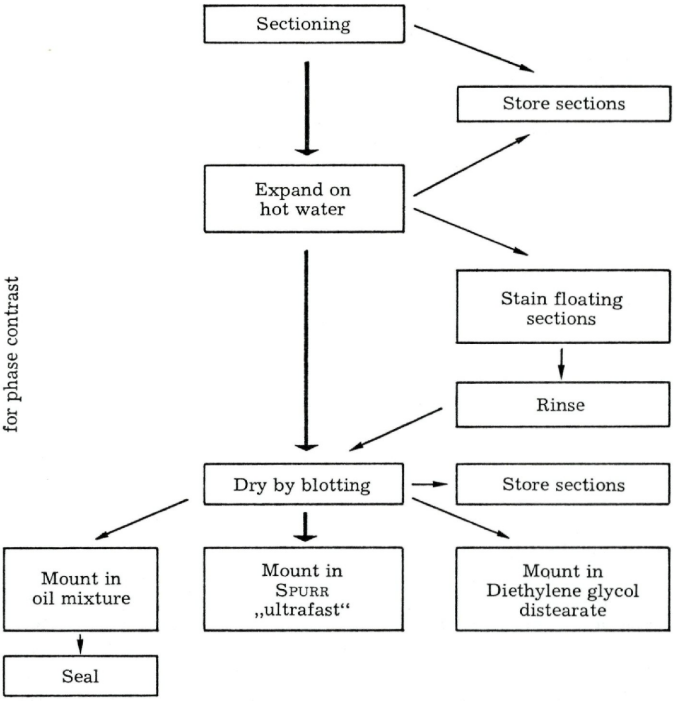
After fixation with osmium tetroxide the block should be rinsed two or three times for several minutes with cold buffer.

Small blocks (up to about 3 mm) are dehydrated after half an hours, larger blocks need several hours. Water diffusing out of the fungal trama will spread only slowly throughout the dessiccant, so gently swirl the vial from time to time. Large blocks are left in the methoxyethanol over night.

The ungraded dehydration looks quite shocking to the histologist

used to work with acetone or ethanol, but actually it is very gentle, and typically neither the gross morphology nor the fine cytological details suffer from it.

After initial dehydration it might be useful to store the material away for later processing. To do so replace the methoxyethanol by




two changes of dry methoxyethanol at room temperature (do not use cold dessiccant, or it will take up traces of water by condensation from the air). Completely dessiccated fungal trama blocks will keep for months and years in methoxyethanol when placed in the deep freezer at —20 to —30° C.

Chemical dehydration using acidified 2,2-dimethoxypropane (MULLER and JACKS, 1975) did not give satisfactory results with fungi.

### 3. Infiltration and embedding

Paraffin frequently destroys fungal trama beyond recognition and works only moderately well with compact cells. Synthetic waxes work a little better but still are not good.

Using plastics as embeeding material a few conditions must be met with:

a) The infiltration mixture must be the complete monomere mixture used for polymerisation. Infiltration without catalist or starter will result in poor block quality.

b) The complete infiltration mixture must have low viscosity, even at low temperatures (refrigerator).

c) Useful potlife (the time the mixture remains reasonably liquid) should be as long as possible, up to 3 weeks in the refrigerator to allow for long infiltration times.

d) Polymerisation must be accomplished by heat, not by UV light. Dark or big blocks are not penetrated by UV and polymerisation is bad and heterogenous.

e) During polymerisation the volume should change as little as possible.

Methacrylates fail to meet conditions a, c and e, but with small blocks still give good results.

All conditions can easily be fulfilled by using SPURR's low viscosity embedding medium (SPURR, 1969). Probably other low viscosity epoxy resins with a long potlife can be used as well.

SPURR's medium does mix with methoxyethanol, but it polymerizes slowly to a sticky and rubbery mass when containing small amounts of that dessiccant carried over with the fungal material. The best intermediate solvent is propylene oxide which mixes readily with methoxyethanol. Two changes are used, but to lower the cost the first change can be acetone. This gives results in no way inferior to using propylene oxide alone. Care should be taken to let the first dessiccant come to room temperature before changing into acetone.

For years I used to infiltrate the fungal blocks gradually by using solutions of approximately 25%, 50% and 75% of SPURR's medium in propylene oxide before bringing them into pure resin. But then I found that direct immersion into pure resin gives just as good results. At first the fungal material floats near the surface of the resin. With progressing infiltration it sinks to the bottom. This makes periodical mixing unnecessary, and large blocks or dense trama can be left in the vial for days or weeks in the refrigerator without need for frequent handling. Medium size blocks (about up to 6 mm) are infiltrated at room temperature, preferrably on a slow rotary mixer. Agarics are completely infiltrated after 1 or 2 hours this way, dense material may take 10—12 hours.

The resin mixture used to infiltrate the material may be used to cast the blocks, the propylene oxide does not interfere with the polymerisation. However, it is possible that the resins get charged with substances extracted from the fungus, and these substances may results in a general stainability of the embedding material, giving an unpleasant background to the stained sections. If the sections are to be stained it is better to use fresh resin to cast the blocks.

The recommended resin mixture is Spurr's soft medium, modified to give longer pot life:

| | |
|---|---|
| ERL | 10 g |
| DER | 8 g |
| NSA | 26 g |
| S-1 | 0.2 g |

Polymerisation at 70° C for 24 hours.

## 4. Sectioning epoxy resin blocks

In light microscopy epoxy resins are mostly used to cut thin sections, but in plectology of larger fungi thick sections in the range of 15 to 30 μm, or even thicker are needed. With SPURR's soft medium I find it very easy to cut large blocks using a Minot type rotary microtome equipped with a knife designed to cut hard materials. These are either tungsten carbide knives or steel knives with a D-profile. Both work nicely, but steel knives require frequent resharpening, whereas tungsten carbide knives are very expensive.

The knife must be adjusted to a minimum cutting angle, but this angle is not very critical.

The block need not be trimmed to rectangular shape, and even if this is done, the sections will not form ribbons. They become highly charged with static electricity during sectioning causing them to stick to the knife surface. This is very convenient, since the sections can be stored sequentially on the knife's face. When the sections are picked up immediately after sectioning they roll and become difficult to handle. If they are parked on the knife, where they may be pushed around, they will stiffen within a few minutes. With a fine forceps pick them up and transfer them into a Petri dish in due sequence. The sections will adhere to the glass surface electrically and will not get mixed up during transport, but they can easily be picked up again with the forceps. Sections may be stored in the Petri dish for very long periods.

Using steel knives or tungsten carbide knives the sections look more or less milky due to surface markings that can hardly be avoided. These markings are microscopic arches with the main orientation parallel to the cutting edge. The surface of the sections look somewhat like sand dunes. These markings evidently are not due to knife

imperfections which leave longitudinal scratches. The surface markings do not harm or interfere with observation when the sections are mounted properly. However, they may stain with certain dyes, even if the main mass of the plastic does not.

### 5. Stretching the sections

During sectioning the material is more or less compressed. This is of no harm, and the sections may even be stored that way.

When sections are immersed in organic solvents they soften and usually over-expand very rapidly, often almost explosively. Over-expansion is considerable and the sections sometimes almost double their original surface (i. e. the surface of the cutting face of the block) within a few seconds. They become very soft and totally unsuited for handling. Over-expansion also affects the fungal material and deforms it. On drying the sections shrink again, but usually become badly wrinkled and folded.

Water soluble organic solvents can be mixed with water to moderate over-expansion. Diluted ethanol works well, but it is difficult to control the expansion to restore exactly the original size.

Epoxy resins are thermoplastics. SPURR's medium softens enough at moderate temperatures to allow stretching to original size. Float the sections on distilled water in a crystallizing dish and place it in an oven at 70° C. The sections stretch to exactly original size and shape in a few minutes, but leaving them for many hours does not harm them. They never over-expand. Lower temperatures work equally well (e. g. 50° C), but it takes longer.

For a quick glance in a hurry work an individual section can be floated on water on a microscope slide and heated over a flame. It works nicely and rapidly. Boiling does not harm the sections, but do not allow the section to dry onto the glass.

Prior to picking up the sections from the water surface, they must be cooled. Sections are placed on a filter paper to blot off excess water. Pick them up again and dry them between two filter papers. Use clean and hard paper to avoid lint. Stretched and dried sections can be cleaned with a soft brush. Antistatic devices help but are not essential.

### 6. Staining epoxy resin sections

A word of caution is indicated here: never dry the sections onto a microscope slide! Large sections adhering to the slides are lost because it is impossible to mount them correctly. The mounting medium will not penetrate uniformly between the section and the glass.

I most strongly recommend to observe the sections unstained

with the phase contrast. Only when specific reactions are intended or when no phase contrast is available should they be stained.

Epoxy resins are dense and allow only limited access to the material inclosed. However, given enough time many substances dissolved in water do penetrate even thick sections. This is greatly accelerated by higher temperatures.

It may seem tempting to remove epoxy resins from a section affixed to a slide using the method of MAYOR et al. (1961). The result is disasterous. The hyphae, lacking any mechanical support, change orientation and distort. The trama collapses and the sections detach from the glass.

Sections are best stained by floating them on the surface of the staining solution. Since organic solvents over-expand the sections, only water may be used to dissolve the dyes and all other necessary substances. Penetration is slow, and staining times are long for most dyes. Higher temperatures shorten staining times, but frequently the epoxy resin also stains under these conditions.

After staining the sections are floated once or twice on distilled water. Finally they are dried between blotting paper.

Most dyes stain better in buffered solutions of well defined pH, but I found that for alcaline solutions 1% borax (pH 9) and for acidic solutions 5% potassium aluminum sulfate (alum, pH 3) work fine.

Table 1 lists some dyes that give good results in my laboratory. They are all progressive single dye stains. Experiments with complex procedures are in progress and will be published later.

Staining time must be tried out. At room temperature most stains need 12 to 24 hours to penetrate a 20 µm section, but floating the sections for 3 or 4 days may enhance the quality of the result, usually with no or only very weak staining of the plastic. At 70° C staining time is reduced to 1—3 h but sometimes the plastic staines also somewhat.

## 7. Mounting the sections

For optimal observation conditions the sections have to be mounted in a medium of the same refractive index as that of the embedding plastic. This becomes imperative when unstained sections are to be viewed in phase contrast.

The most obvious way is to use SPURR's resin as a mounting medium. This works well with very small sections up to about $1 \times 1$ mm in size, but it does not with large ones. Just as the sections over-expand in organic solvents, they swell and over-expand in SPURR's medium, though at a slower rate. Over-expansion makes it impossible to mount the sections in a resin solution such as Permount, Rhenohistol, Entellan or similar products. In effect, between slide and cover glass,

the sections wrinkle and fold in a spectacular way in a few hours at room temperature or in a few minutes in the embedding oven when mounted in SPURR's medium or in a resin solution. Keeping them flat with a heavy weight results in large cracks and mostly the sections wrinkle anyway.

The answer is to use SPURR's medium with a heavy overdose of S-1. The medium hardens so quickly at 70° C that it gets very viscous within a few minutes, before the sections have the time to wrinkle. After 15—20 minutes the medium, here called "Spurr ultra", is completely cured.

Table 1. Suitable stains for progressive one-dye staining

a) Solvent: 5% alum in distilled water (pH = 3).

| Dye | % | Preference for (if any) |
|---|---|---|
| aniline green | 1 | walls, gelatinized trama |
| aniline red | 1 | same |
| diamond phosphine | 1 | same |
| fuchsine acid | 0.005 | same |
| methyl violet | 1 | same |
| crystal violet | 0.1—1 | stains all |
| fuchsine basic | 0.005 | same |
| methyl blue | 1 | same |
| orseilline | 1 | same |
| rhodamine B | 1 | same |
| trypan blue | 1 | same |
| xylidine red | 1 | same |

b) Solvent: 1% borax in distilled water (pH = 9).

| Dye | % | Preference for (if any) |
|---|---|---|
| Azure A, B or C | 0.002 | walls, gelatinized trama |
| pyronine 2G<br>pyronine Y | 1 | same |
| brilliant phosphine | 1 | stains all |
| safranine O | 0.005 | same |

The proportions of ERL, DER and NSA are critical for the correct refractive index, but apparently the S-1 is not. The resin mixture without S-1 is stable for about a month at room temperature, and much longer when refrigerated. Therefore a small amount of mounting medium is prepared without S-1:

Mounting medium (stock):

| | |
|---|---|
| ERL | 1.00 g |
| DER | 0.80 g |
| NSA | 2.60 g |

To mount a section, put a medium size drop on a glass slide. Plunge the first 15—20 mm of a dissecting needle into S-1, withdraw,

and without hesitation add the S-1 on the needle to the drop and mix well. With the same needle pick up the section and place it on the drop, spread the mounting medium onto the section and apply a cover slip. Air bubbles may be squeezed out by gentle pressure without great danger for the section. Put the section on a hot plate of 65—75° C and add a weight of 5—20 g.

Polymerize for 20 minutes. If the plate is to hot, or if the preparation is left for too long a time, the medium will turn yellow to amber.

After cooling excess medium may be scraped off with a strong razor blade.

If there is a great excess of medium, it will wet the upper surface of the cover glass and flow under the weight.

If many sections are to mounted within 2—3 hours, then add 0.5 g of S-1 to the stock mounting medium as defined above. The mixture instantly turns dark yellow and heats considerably, but within a few minutes will cool again to room temperature. It then has the viscosity of light syrup. On the hot plate the medium will get much less viscous and flow to the edges of the cover glass. Useful potlife is about 3 hours, but the medium will get more and more viscous, so the danger of including air bubbles increases steadily.

It is important that a weight is applied during curing. The sections usually are not perfectly flat. The heat of the hot plate softens the section and the weight flattens it against the glass. Too heavy a weight will cause cracks.

The components of SPURR's medium are very reactive chemicals and good solvents. Some dyes are affected or partially extracted from the sections during the mounting process, and nothing is known about the durability of stained material over a period of many years.

Some authors recommend to mount the sections in immersion oil for better conservation of the staining. However, the refractive index is not correct, so the surface markings are clearly visible even in bright fleld. A better liquid mounting medium probably conserving the stains for a long time is obtained by mixing paraffin oil with immersion oil. The exact proportions depend on the quality of the paraffin oil. In my laboratory the following mixture is used:

| | |
|---|---|
| paraffin oil | 4.50 g |
| synthetic immersion oil | 10.0 g |

There are brands of immersion oil that do not mix with paraffin oil.

Preparations mounted with this oil mixture must be sealed, but nail polish or similar solutions do not work. I found the paste of DU NOYER (1918) very useful and of easy application. Small excess of mounting oil mixture on the slide are tolerated and do not harm the sealing.

First the 4 corners of the cover glass are fixed with small drops of the paste, then seal the edges with plenty of paste. Avoid pressure but flatten the seal as much as possible. The paste is applied with a hot 15 Watt soldering iron constantly branched to the current.

Paste of DU NOYER:

| | |
|---|---|
| Anhydrous lanoline | 20 g |
| Colophony | 80 g |

Melt the lanoline, add small portions of colophony. Let degas every time before adding more resin.

For rapid mounts in an inert solid medium the synthetic wax diethylene glycol distearate (Polyscience, Inc., Warrington PA, USA) gives good results. Its refractive index is sufficiently close to that of SPURR's medium for undisturbed observation in bright field. Melt a flake of the wax on a slide, add the dry section and put another flake on top of it. Heat gently, apply a cover slip and hold it down until the wax has solidified.

## 8. Conclusions and comparisons

Some agarics with very delicate trama have been used to test the fixation and embedding methods just described and to compare them with other procedures often used in mycology. Among the fungal test material were young carpophores of *Amanita rubescens* whose universal veil contains large, inflated, thinwalled cells, *Agaricus bisporus* whose partial veil is a delicate, loosely arranged web of cylindrical, easily damaged hyphae, and young primordia of *Termitomyces* from a termites' nest.

The method described above always gave best results. The other fixations tested all gave granular cytoplasm or even resulted in collapsed hyphae and distorted trama. They are, in order of decreasing quality: formaldehyde with 1% $HgCl_2$ added, FLEMMING, BOUIN, NEWCOMER and CARNOY.

Three other embedding media have been tested. All were inferior to SPURR's medium. Comparatively good results were obtained with a mixture of methacrylate and paraffin, as proposed by ENGEN and WHEELER (1978), but this medium is not easily handled, and big inflated cells are frequently deformed. Very unsatisfactory results were obtained by embedding in diethylene glycol distearate (TALEPOROS, 1974), and paraffin destroyed most of the test material.

Plate 1 illustrates some of the excellent results obtained with procedures described in this paper.

Flow charts 1 and 2 summarize the method.

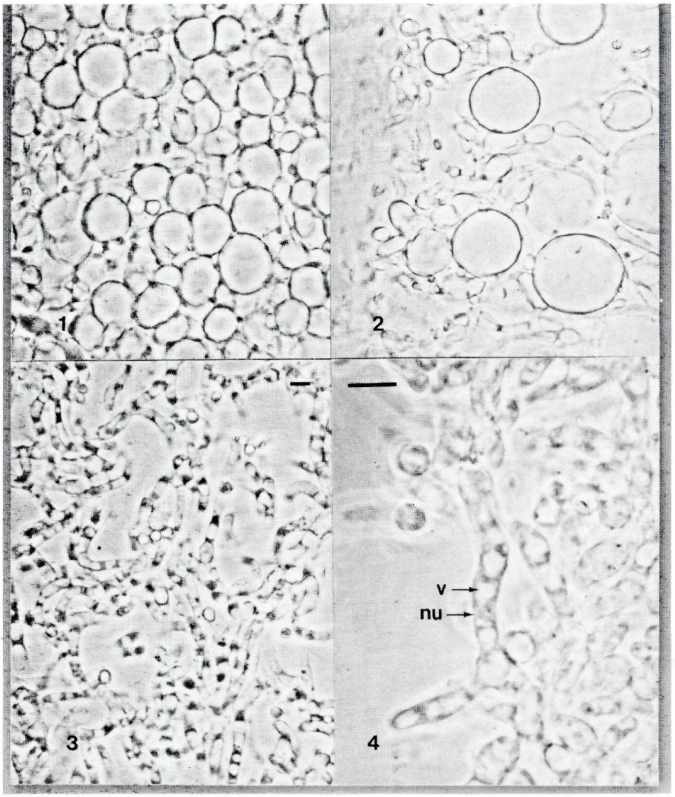



Fig. 1. Amanita rubescens, transverse section through the stipe. Numerous inflated cells and some filamentous hyphae.

Fig. 2. Amanita rubescens, transverse section through the universal veil. Gelatinized cuticle of the cap is at left. Very loose arrangement of filamentous hyphae and large spherocysts.

Fig. 3. Agaricus bisporus, tangential section through partial veil composed solely of filamentous hyphae loosely arranged in a cotton-like web. Note conservation of vacuoles.

Fig. 4. Termitomyces spec., transverse section of a primordium from a termites' nest, superficial hyphae. Note cytological detail. V = vacuole, nu = 2 nuclei, one nucleolus visible at left.

Bar equals 10 µm. Figures 1, 2 and 3 photographed with a fluotar len 40/0.75, figure 4 photographed with an apochromatic objective 63/1.40.

Fixation with freshly prepared, buffered and cold formaldehyde, dehydration in 2-methoxyethanol, rinsed with acetone, transferred from propylene oxide into Spurr's medium. Unstained sections observed in phase contrast.

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