

# Manual of Agricultural Nematology

edited by

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## Part 3. Preparation of Nematodes for Transmission Electron Microscopy

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### I. INTRODUCTION

The basic steps for processing nematodes for transmission electron microscopy (TEM) are the same as for other biological specimens. These steps include fixation, dehydration, infiltration and embedding, sectioning, and staining. The microscopic size of most nematodes and the fact that it is often difficult for fixatives to penetrate the cuticle may create some additional problems.

General TEM references (Hayat, 1981, 1986; Lee, 1983; Aldrich and Mollenauer, 1986; Robinson et al., 1987; Crang and Klomparens, 1988) should be consulted for selecting methods for specific needs not well represented in the nematological literature. An excellent summary of TEM methods with one protocol for nematode processing was recently published (Shepherd and Clark, 1988). A detailed presentation of TEM histochemistry (McClure, 1981) is available and will not be addressed in this discussion.

This portion of the chapter provides an overview of and comment on various TEM methods for resolving morphology in different nematode systems. Different methods are often needed for different nematode genera, life stages, and parts of the body. The best possible fine-structural morphology is especially important for organ reconstructions, detecting phenotypic variations in mutant nematodes, and describing host-parasite associations.

### II. FIXATION

For best general preservation, glutaraldehyde (sometimes with other aldehydes) is followed by osmium tetroxide after thorough rinsing to avoid undesirable fixative complexes. Glutaraldehyde fixes proteins and nucleic acids. Osmium tetroxide fixes many unsaturated lipids, acts as an electron stain, and enhances lead poststaining. Note that aldehydes can penetrate latex gloves, whereas gloves containing neoprene give better protection.

#### A. Chemical Combinations Used in Nematode Fixation

The basic fixation strategy involves fast and nearly simultaneous killing and fixation to minimize autolytic changes. This goal is not easily achieved with many terrestrial parasitic nematodes. In these nematodes chemical fixation may be difficult due to a relatively impermeable cuticle and high turgor pressure.

Glutaraldehyde followed by osmium, always with puncture in glutaraldehyde, is a standard fixation method that works well in many nematodes (Bird, 1971; Baldwin and Hirschmann, 1973; Ward et al., 1975; Perkins et al., 1986). The author has found this method to work fairly well in the head region of heteroderid plant parasites, perhaps because of the many sensory organ openings, but detail was poor in the phasmidial sheath cell in tails (Fig. 5).

Some of the earliest fixation methods employed osmium alone with *Xiphinema index* (Roggen et al., 1965), *Heterodera schachtii* (Wisse and Daems, 1968), and *Caenorhabditis elegans* (Ward et al., 1975; Sulston et al., 1980). Although it penetrates more slowly



FIGURE 5 Glutaraldehyde: 2%, 25°C; osmium tetroxide: 2%, 25°C. *Meloidodera floridensis*, tail: phasmid sheath cell enclosing neuron, x.s. Bar = 1  $\mu$ m.

than aldehydes, osmium fixes faster. As a consequence, it takes less time before puncture can be completed than with aldehydes. Unlike glutaraldehyde, osmium tetroxide destroys the semipermeability of membranes, but it allows leaching of proteins. The sensillar matrix material in nematodes is not fixed by osmium alone (Perkins et al., 1986).

Osmium followed by glutaraldehyde was effective in fixing tails of *Scutellonema brachyurus* (Wang and Chen, 1985). It was also fairly effective in juvenile and male tails of heteroderids, and very good for *C. elegans*, but some leaching of cytoplasmic ground substance can occur (Carta, unpublished observations). The method was not effective in fixing female *Heterodera schachtii* (Cordero, pers. commun.).

Acrolein is well known for its rapid penetration of tissues, but it gave very poor resolution when used alone and followed by osmium (Zeikus and Aldrich, 1975). However,

acceptable results are reported when 1 or 10% acrolein is added to glutaraldehyde and followed by osmium (Wright and Jones, 1965). This method works better in marine than soil nematodes. Very short (1-min) exposure of acrolein with glutaraldehyde and propylene phenoxetol allowed earlier puncture and better overall preservation than standard methods in *C. elegans* (Chalfie and Thomson, 1979). This method gave acceptable preservation of *H. schachtii* second-stage juveniles. However, greater exposure to acrolein for 2 hr with 1.5% acrolein, 1.5% paraformaldehyde, and 3% glutaraldehyde (Hayat, 1981) followed by osmium distorted the cuticle, inflated membranes, and generally obscured cellular detail so that only nuclei were discernible (Carta, unpublished observations). A similar fixation mix (3% acrolein, 3% glutaraldehyde) was used on *Paratrichodorus porosus* (G. W. Bird, 1970) with better results. Fixation in this relatively permeable plant parasite was generally good except for poorly preserved muscle fibers.

Simultaneous double fixation with glutaraldehyde and osmium at 4°C worked well in preserving the insects *Periplaneta americana* and *Musca domestica* larvae (Singh et al., 1985). The same method was ineffective with the nematodes *Meloidodera floridensis* (Carta, unpublished observation.) and *Romanomermis culicivora* (S. El-Din, pers. commun.). Tissues were fixed but very poorly resolved.

Cryofixation was first demonstrated with *Xiphinema index* and *Caenorhabditis briggsae* and compared with a standard glutaraldehyde-formaldehyde-osmium fixation. It gave relatively poor resolution of myofilaments and uterine double membranes and slightly better cuticle preservation. Mitochondrial cristae were poorly resolved with both techniques (Himmelhoch and Zuckerman, 1982). Poor membrane preservation and "gummy" phasmid secretion were noted in nematodes immersed in liquid nitrogen and slowly thawed in glutaraldehyde followed by osmium (Fig. 6) (Carta, unpublished observation).

Formaldehyde when added to glutaraldehyde followed by osmium has given superior results over other methods for a number of authors, either without heat (Wharton and Barrett, 1985; Himmelhoch and Zuckerman, 1982) or with 70°C heat (Zeikus and Aldrich, 1975; Byard et al., 1986). This method worked extremely well with hydrogen peroxide (Byard et al., 1986) and 60°C heat in heteroderid tails (Carta and Baldwin, 1989) and females of *H. schachtii* (Cordero, pers. commun.). Membranes and ground cytoplasm were especially well fixed in phasmidial sense organs (Fig. 7).

## B. Fixation Additives

Hydrogen peroxide has been reported to improve the fixation of glutaraldehyde, presumably by increasing the rate of cross-linkage to proteins. Note that it must not be added to fixatives with paraformaldehyde or an explosive mixture may result (Byard et al., 1986).

Tannic acid (TA) (1% in glutaraldehyde) has been very effective in resolving microtubules in nematodes where total size and number of protofilaments is critical to accurate interpretation (Chalfie and Thompson, 1982). It should be remembered that apparent microtubule size may increase in proportion to TA concentration (Hayat, 1981).

En block staining with aqueous or alcoholic uranyl acetate is often used to enhance contrast, stabilize membranes, and reduce shrinkage from dehydration (Lee, 1983).

## C. Timing

Nematodes killing can be very fast, but it may take much longer before the specimen body is well enough fixed that it can be cut to allow further penetration. Plant parasitic nematodes can be killed in 5–6 min at 4°C and 2 sec at 70°C with 3% formaldehyde (Zeikus and



FIGURE 6 Glutaraldehyde: 2%,  $-60^{\circ}\text{C}$  thawed to  $25^{\circ}\text{C}$ ; osmium tetroxide: 2%,  $25^{\circ}\text{C}$ . *Meloidodera floridensis*, tail: phasmid sheath cell enclosing neuron, x.s. Bar =  $1\ \mu\text{m}$ .

Aldrich, 1975). Yet it may take 30 min to 2 hr before they are sufficiently fixed for puncture. Complete aldehyde fixation may take hours beyond that.

Free-living nematodes are penetrated by fixatives more readily than most plant or animal parasites. When free-living *C. elegans* is placed in 2% glutaraldehyde in M9 buffer, the majority of worms cease movement, and explosive release of internal tissue does not occur when punctured after 3–5 min. With Heteroderidae, the time increases to between 70 and 90 min (Carta, unpublished observations). Zeikus and Aldrich (1975) advise that tylenchid plant parasites being fixed by aldehyde should not be punctured before 2 hr. Puncture time is about 30 min for second-stage *Meloidogyne javanica* at  $4^{\circ}\text{C}$  (Bird, 1967) and fourth-stage *Ditylenchus dipsaci* (Wharton and Barrett, 1985). Third-stage trichostrongyle juveniles survive at least 30 hr in 6.5% glutaraldehyde (Eckert and Schwarz, 1965). Initial

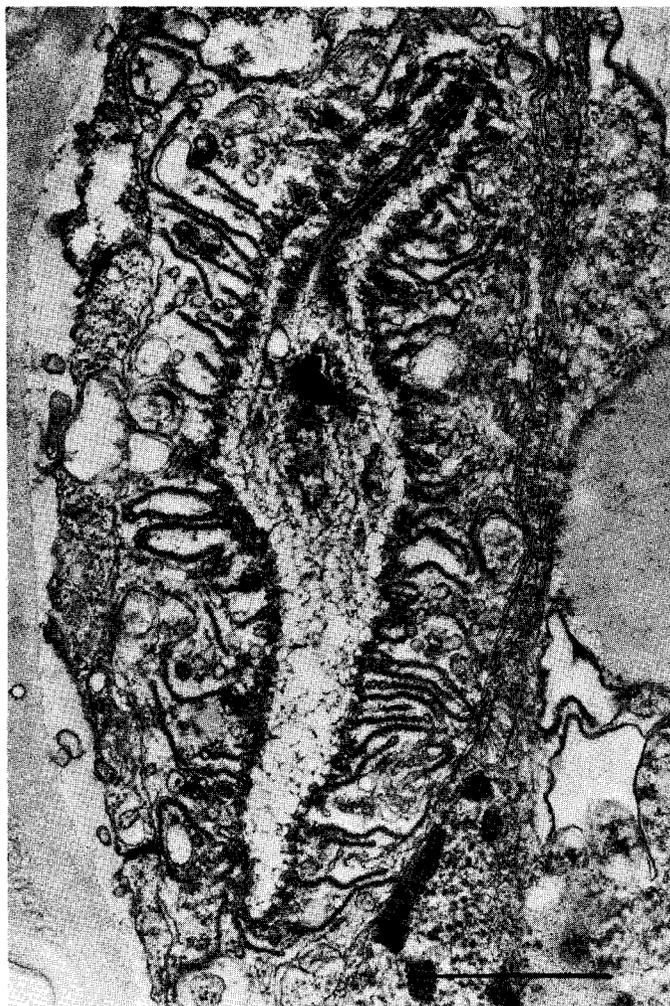


FIGURE 7 Glutaraldehyde: 2%, and paraformaldehyde: 1.5%, 60°C cooled to 25°C; osmium tetroxide: 2%, 25°C. *Meloidodera floridensis*, tail: phasmid sheath cell enclosing neuron, x.s. Bar = 1  $\mu$ m.

fixative penetration sufficient to inactivate turgor pressure, and total fixation time varies among genera. Males of *Heterodera glycines* needed up to 8 hr more glutaraldehyde fixation than *Meloidogyne incognita* (Baldwin and Hirschmann, 1975).

Nematodes may have different osmolarity requirements for good fixation results, but no systematic studies have been published to determine optimum ranges. It is especially important to determine the effective osmolarity in different buffer systems on organisms with different membrane properties during early fixation when the greatest amount of distortion can occur to surface cells (Hayat, 1981).

Empirical evaluation from osmometer readings of osmolarities can sometimes be found in the nematological literature. Otherwise an approximation can be derived from osmolarity tables (Hayat, 1981) to determine the range of osmolarities (for different nema-

tode fixation protocols). Osmometer readings were used to show that *Xiphinema americanum* is well fixed in 150 mosm glutaraldehyde in cacodylate (Wright and Carter, 1980) while animal parasitic trichuroid nematodes were fixed in 350 mosm (Wright, 1971). Plant parasitic *Ditylenchus dipsaci* was fixed in about 1800 mosm aldehyde fixative (derived from Wharton and Barrett, 1985), and *Dolichodorus heterocephalus* was fixed (Zeikus and Aldrich, 1975) in approximately 1100 mosm.

A given hypertonic solution may alter the body volume of different stages of *Panagrellus silusiae* to different degrees (Prencipe et al., 1984). It might be necessary to select the osmolarity of buffer and fixative for different nematode stages as was done with insect stages (Beaulaton and Gras, 1980).

Prehatch nematodes show better resolution and less shrinkage from the cuticle than posthatch, but many structures are developmentally different. The cuticle, hypodermis, and sensory organs are especially variable just before hatch (Carta and Baldwin, 1990). Therefore fixation quality may have to be sacrificed to developmental accuracy. It is very important to indicate whether nematodes have been naturally hatched or artificially hatched by physical or chemical release from the egg. Even hatching induction by  $ZnCl_2$  in heteroderids seems to have an adverse effect on the physiological health and perhaps tissue preservation of newly hatched juveniles.

#### D. Relaxation

Nematodes will appear coiled or twisted unless properly relaxed and straightened before or during fixation. This makes orientation of nematodes easier for sectioning. Cooling to 4 or 5°C for 30 min is done most commonly before or during initial fixation (Bird, 1971; Wisse and Daems, 1968; Shepherd and Clark, 1988).

Propylene phenoxetol (1% for 1 min) has been used with acrolein and glutaraldehyde for 1 min to straighten nematodes at the beginning of fixation (Chalfie and Thompson, 1979). It also facilitates entrance of aldehydes and preserves flexibility of tissues (Steedman, 1976). This compound is more difficult to obtain than sodium azide and has mildly toxic effects during recovery. Sodium azide has generally replaced propylene phenoxetol in narcotization of *C. elegans* for light microscopy. No reports of successful nematode fixation with sodium azide in comparison with other means of narcotization are available to evaluate. However, in other organisms sodium azide also improves fixation of mitochondria in deeper regions of tissue (Minassian and Huang, 1979).

Fifteen minutes of 0.001 N iodine at room temperature was used to relax third- and fourth-stage *Haemonchus contortus* before fixation with osmium (Ross, 1967).

Mild heat of molten agar (45–60°C) straightens nematodes before fixation without any noticeable impairment of fine structure (Endo, 1979). Heat also has other properties important for nematode preservation. These properties to be discussed include chemical fixative penetration, physical fixation, and cuticle permeabilization.

#### E. Puncture and Penetration

Many investigations have shown that puncture improves penetration of fixatives and the quality of fine structure (Bird, 1971; Zeikus and Aldrich, 1975). This is especially true with terrestrial parasites. Cuticle puncture may under some circumstances cause the pulling of body contents from cuticle at some distance from the cut. However, it is this author's experience that cold temperature without puncture showed considerable tissue pulling compared to standard fixation with puncture. If tissue distortion occurs, osmolarity during fixation and dehydration should be considered as well as puncture.

Eye knives are popular instruments for cuticle puncture (Baldwin and Hirschmann, 1973; Zeikus and Aldrich, 1975). A lancet was used to cut nematodes in buffer on slides during osmium fixation (Wisse and Daems, 1968). A stone-ground, three-sided sewing needle point (A. H. Bell, pers. commun.), and electrochemically sharpened tungsten needle (Hubel, 1957) have given the author reliable results during glutaraldehyde fixation.

Dimethylsulfoxide (DMSO) can increase the rate of penetration of fixatives, but its potential for artifacts has not been well assessed (Hayat, 1981). It was first used in *Xiphinema index* to aid in the penetration of osmium tetroxide as a primary fixative (Roggen et al., 1966), and was added to speed penetration of glutaraldehyde after treatment with hot formaldehyde-glutaraldehyde (Zeikus and Aldrich, 1975).

Using 60–70°C heat, mechanical puncture may be unnecessary when hot formaldehyde-glutaraldehyde is followed by glutaraldehyde–hydrogen peroxide and osmium in *C. elegans*. Puncture was not required for good fixation under these conditions (Byard and Sigurdson, 1986). When considering the need for puncture, it must be remembered that this free-living nematode loses its capacity for internal displacement very quickly after fixation compared to many plant and animal parasites.

The use of heat to aid in penetration of chemical fixatives as well as fixation itself is not recommended for routine electron microscopy (Hayat, 1981). However, when fixing nematodes, 60–70°C heat seems to provide a significant improvement in fine structure where other methods have failed (Zeikus and Aldrich, 1975; Byard and Sigurdson, 1986; Carta and Baldwin, 1990).

When heat was used for straightening nematodes, 70°C was the most reliable temperature for doing this without noticeably affecting the fine structure in tylenchids (Zeikus and Aldrich, 1975). However, it was suggested that this temperature may be too high for obtaining the best preservation in all nematodes or stages. First- and second-stage juveniles of *C. elegans* were not well fixed compared to good fixation in third through fifth (adult) stages, perhaps because of differences in heat resistance (Byard and Sigurdson, 1986). However, osmolarity problems had not been ruled out as a contributing cause.

Mild heat (50°C) has been helpful in fixing eggs of *Meloidogyne javanica* (Bird and McClure, 1976). The use of mild heat as a protein fixative in other systems has been carefully examined, indicating that tissue shrinkage is no greater than with aldehydes (Hopwood et al., 1984).

It seems that heat may affect more than simply the activity of the fixative, or reduction in turgor from relaxation or death. A recent study on cuticular permeability to water and osmium showed that heat between 40 and 50°C applied to *Ditylenchus dipsaci* caused a phase change in the cuticular lipid. A further increase in permeability between 55 and 70°C was believed to be due to the death of the nematode (Wharton et al., 1988). Whether this phase change could be translated into faster penetration of glutaraldehyde into other nematodes was recently tested with *C. elegans* and *Verutus volvingentis*, a heterodermid plant parasite. Juveniles and adults were subjected to 2% glutaraldehyde in M9 buffer at 45°C. This allowed puncture after 10 min rather than the usual 90 min for *Verutus*. No noticeable improvement was seen within the 3-min control time for *C. elegans* (Carta, unpublished observations). Any potential artifacts from heat as part of the fixation process must be weighed against generally more severe postmortem artifacts (Sjostrand, 1967).

Various potential cuticle solvents have been tried with variable success. A brief (4-min) immersion in 10% bleach (0.5% NaOCl) before a standard fixation showed poor resolution of structures with the TEM in *Heterodera schachtii*. However, 2% bleach for 15 min enhanced fixative penetration in *C. elegans* (Sulston et al., 1983) and *Romanomermis culicivorax* (Platzer and Platzer, 1988).

Difficulties in penetrating eggs with needles and knives have been overcome with finer tools made from broken coverslips (Bird and McClure, 1976), and more easily controlled pieces of cellulose acetate sheeting (Perry et al., 1982). Lasers (von Ehrenstein et al., 1981) and chitinase enzymes (Albertson and Thomson, 1982) have also been effective in puncturing eggs for enhanced penetration of fixatives.

## F. Buffers

Phosphate buffer is one of the most physiologically compatible buffers available (Hayat, 1981). It has been effective at pH 6.8 (Endo, 1979), pH 7.2 (Baldwin and Hirschmann, 1973), pH 7.3 (Bird, 1971), to pH 7.4 (Ward et al., 1975). Artifactual deposits may sometimes result with phosphate buffer, particularly when used above 0.1 M concentrations (Hayat, 1986).

For optimal physiological functioning nematodes require certain salts in the surrounding medium (Wright and Newall, 1980). The M9 buffer which contains these salts is commonly used with *C. elegans* (Sulston and Hodgkin, 1988) and has been used successfully in nematode fixation (Byard and Sigurdson, 1986). This buffer comes closest to the physiological ideal but has not been compared to stronger phosphate buffers for general preservation or artifactual deposits.

Cacodylate buffer used by various authors (Webster, Wharton, Carta, Shepherd, Wright) participates with the fixative in the killing of nematodes. It does not lead to artifactual precipitates and it may also reduce osmotic shrinkage with glutaraldehyde as compared with phosphate buffer (Lee, 1984).

PIPES buffer has been used with good results in some plant and animal tissue (Hayat, 1981). However, even with careful buffer washes, this buffer badly obscured the cytoplasmic ground substance in *Meloidodera floridensis* (Carta, unpublished observation). HEPES buffer has been used for fixing *C. elegans* (Sulston and Hodgkins, 1988).

## III. TRANSFER

Transfer of specimens is important in fixation as well as dehydration. Nematodes may be transferred through solutions either in agar or unenveloped.

One of the most common methods of safe transfer of individual specimens involves tucking them into a shallow agar groove and overlaying with a drop of 45°C agar. The agar should be made up in salt solution of similar osmolarity to fixatives or shrinkage may result (Wright and Jones, 1965). Commonly, the agar is cut into oblong sheets tapered at one end, or in blocks. Timing of placement in agar may vary. Specimens may be immobilized or killed in agar just before fixation (Endo, 1979), after puncture in the middle of glutaraldehyde fixation (Shepherd and Clark, 1988), before osmium (Zeikus and Aldrich, 1975), or after osmium (Baldwin and Hirschmann, 1973).

Transfer of unenveloped individuals generally saves time during the osmotically fragile steps of fixation. Unenveloped processing would also be necessary when using flat embedding procedures (Reymond and Pickett-Heaps, 1982) for precise longitudinal orientation or correlative LM and EM.

Vehicles for transfer of unenveloped specimens include standard-sized centrifuge tubes (Wisse and Daems, 1968), small polypropylene microcentrifuge tubes (Hong and Barta, 1986), and Bureau of Plant Industry (BPI) dishes for transfer of specimens individually (Zeikus and Aldrich, 1975). Alternatively, small sieves or baskets made from 5-100- $\mu$ m fabric mesh fitted to modified Beem R\* capsules (Eisenback, 1985) or glass tubes and Tef-

lon rings (De Grisse, 1977) may be used. If the mesh is appropriate for the size and activity of the specimens, they will not be lost from the open end unless pipetting or immersion is very rough. The sieves rely on diffusion of solvent through the mesh, which may be slow and require blotting. A more elegant apparatus employs a syringe connected to the longer of two opposed, 10- $\mu$ m mesh-covered Teflon tubes. The Teflon tubes create a chamber when concentrically shoved within a glass tube. The syringe allows for complete and rapid solution transfers (McClure and Stowell, 1978).

#### IV. ORIENTATION

The positioning of agar sheets (Wright and Jones, 1965) or blocks containing carefully oriented specimens within a gelatin capsule or other mold is one of the most common methods of orientation of nematodes. However, manipulations in the molds can be difficult and imprecise. Sandwiching nematodes between sheets of epoxy (Wisse and Daems, 1968) or on a prepolymerized plate below small epoxy drops (DeGrisse, 1977) provides more precise orientation within both the agar and the mold. However, using a jeweler's saw to shape specimen blocks is tedious, and the epoxy dust may be dangerous. A safer and more precise method involves placing worms in a thin layer of epoxy on a slide treated with a releasing agent (McClure, pers. commun.). Individual cells may be observed with the LM, scored with a diamond scribe, and glued to resin stubs for sectioning (Reymond and Pickett-Heaps, 1982).

Eggs or larvae can be oriented into a distal, compact pellet at the end of a small rod. This can be done by microcentrifuging in a microcapillary containing a tiny polyethylene tube with specimens in unpolymerized epoxy. After polymerization the specimens in the rod can be positioned close to the tip of a larger mold (Hong and Barta, 1986).

#### V. EMBEDDING

The cuticle represents a barrier to good epoxy resin infiltration (Wright and Jones, 1965). As a result, a relatively long time (2–3 days) is required for infiltration of nematode tissue compared to tissue such as rat liver (hrs). Slow dropwise infiltration of 50% epoxy-acetone over 1 1/2 hr was effective for vermiform plant parasites and *C. elegans*; 1/2 hour was insufficient in the author's experience. The possibility that puncture of the cuticle may significantly aid in the penetration of embedding media seems to be a logical prediction. However, even the tougher plant parasites did not require puncture for good infiltration of low-viscosity Spurr's epoxy resin with a slow infiltration schedule (Endo, 1979). No puncture was necessary for *C. elegans* with either Spurr's or Epon. Both epoxies showed equally good infiltration and staining (Byard et al., 1986). A number of undescribed methods were tested for the especially difficult infiltration of egg shells. The final reported method involved infiltration of unaccelerated Epon CY212 resin for 3 days at room temperature and in accelerated resin for 12 hr at 37°C (Preston and Jenkins, 1984). Very fresh epoxy is of some importance in obtaining the best quality sections possible, and should be kept no longer than three months to a year (Endo, pers. commun.).

Ultra low-viscosity LR white acrylic resin shows promise for nematode embedding (Hussey, pers. commun.). However, curing the resin to the desirable consistency for sectioning is not always reliable, and some report UV radiation to be better than heat. Because of the sensitivity to oxygen, a gelatin capsule must be used rather than flat embedding

molds. In this capsule, orientation in agar can be done by the pointed block configuration of Wright and Jones (1965).

## VI. SECTIONING

The process of trimming a block in preparation for sectioning may be more exacting with microscopic nematodes than with larger organisms and tissues. This is an advantage since the smaller the block face, the greater the ease of sectioning. However, the consistency of the epoxy-infiltrated tissue and that of the surrounding epoxy are generally different. Fibrous, heterogeneous tissues such as cuticle are difficult to section (Hayat, 1986). This can create problems with nematodes, especially swollen parasitic females, falling out of the surrounding sections.

Grids coated with formvar or parlodion are necessary for obtaining serial sections. Although image contrast is lowered and formvar may tear, the specimen itself may be protected from falling out of the section. This is not uncommon even with good tissue infiltration (Baldwin, pers. commun.). When tissue of different consistency is sectioned, the consistency of the embedding media should approach the hardest cuticular tissue for easiest sectioning. Adjustment in the epoxy components, orientation of specimens near block surfaces, and extra polymerization time will help to create the desired hardness (Wright and Jones, 1965).

Although Epon may section with difficulty on glass knives (Wright and Jones, 1965), softer Spurr's resin may have a greater tendency to hold a static charge making sectioning difficult on either glass or diamond.

Excessive static electricity may occasionally make the cutting of serial ribbons an impossible task. Antistatic guns, ionizing polonium bars, a grounded copper wire in the boat, and humidifiers are sometimes helpful in reducing static charge which can build up on a block during sectioning. However, a diamond knife may also contribute to a problem. In our lab a drop of metal paint was used to connect and ground the diamond to its conductive metal boat. Diamond knives are generally mounted in nonconductive epoxy (Koen, pers. commun.).

## VII. STAINING

Staining with uranyl acetate and lead citrate is one procedure for which there is the least variation from standard methods. En block staining with uranyl acetate (aqueous or alcoholic) is commonly used in nematode studies to improve preservation and increase contrast when combined with poststaining of uranyl acetate. When this is done it is important that any cacodylate or phosphate buffer be well washed out before addition of uranyl acetate to avoid precipitation (Hayat, 1986). Sometimes heat is used with uranyl acetate to enhance staining after sectioning (Ward et al., 1975; Hope, 1988).

The likelihood of lead contamination by carbon dioxide can be reduced by inserting grids at the base of the drop rather than on its surface (Aldrich and Mollenhauer, 1986).

Staining times for uranyl acetate and lead citrate vary somewhat with nematodes. In our lab *Meloidogyne* requires less staining time than *Heterodera*.