



A review: Preparation of nematodes for electron microscopy: General principles and methods

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Abstract

This review provides information on the various procedures and techniques used for the preparation of plant parasitic and soil nematode samples for transmission and scanning electron microscopy studies regarding their ultrastructural information, morphogenesis, nematode-host interactions, responses to biotic and abiotic effects and as an important adjunct to taxonomic information.

Keywords: Nematode, technique, electron microscope

Introduction

Nematodes are difficult subjects for ultrastructural studies, partly because of the impermeability of their cuticle and partly because they are small and difficult to handle.

Two methods by which penetration of fixatives and embedding media can be increased are:

To cut the nematode during fixation or dehydration or;

To treat the nematode for 2-3 min with 15 per cent dimethyl sulphoxide to render the cuticle more permeable (Roggen *et al.*, 1967) [21].

It is not possible here to give complete details of the principles of fixing, dehydration and embedding. There are several works of reference in which this information is available, including Vickerman (1963) [27], Kay (1965) [15], Mercer and Birkbeck (1966) [18] and Sjöstrand (1967) [26]. In addition, methods of preparing nematodes for electron microscopy are reported by Bird (1964) [2], Bird and Rogers (1965) [3], Lee (1966) [16], Roggen *et al.*, (1967) [21], Rosenbluth (1965) [22], Wright (1965, 1968) [29, 30] and Wright and Jones (1965) [31]. Because of their small size, some species are more easily manipulated during the various procedures if they are first embedding in Noble agar or Ion agar, at 42 °C and then the small agar block is processed and embedded in resin. Agar embedding can be done as described by Mulvey (1955) [19], Hirschmann (1959) [13] or in the following way (Wright and Jones, 1965) [31].

Cover a microscope slide with a thin layer (1 mm) of 2 per cent agar. Make a small slit and place the nematode in position in it. If desired, several nematodes can be placed side by side in the slit, so that a number of nematodes are sectioned simultaneously. Place a small label on the agar near the specimen and cover the slit and the label with a drop of warm agar. Cut out a block of agar containing the specimen and its label. The width of the block should be slightly greater than the diameter of the gelatine capsule in which the final embedding will be done, so that when it is pushed into the capsule it will stay in position and not float, and then, the agar block should be trimmed (Chen and Wen, 1980) [8].

Many of the materials used in preparing specimens for electron microscopy are noxious; therefore, it is essential to carry out some of the procedures in the fume cupboard with an extractor fan and wash utensils in the fume cupboard (McClure and Stowell, 1978) [17].

Fixation

Several methods are in current use. The type of fixative used influences the condition of the tissues in the fixed specimen, some fixatives preserving certain cell constituents better than others. It may therefore be advisable to try several fixatives before attempting to interpret results. The quality of the results is also said to depend to some extent on the combination of fixative and embedding medium used (Schiff and Gennaro, 1979) [24].

It is essential that tissues are fixed as rapidly as possible to lessen artifactual changes in fine structure. For this reason, nematodes should not be relaxed and killed with heat before or during fixing, as is customary in some light microscopical studies. Live specimens should be plunged into fixative at 0-4 °C. If required, they can first be relaxed by narcotizing them in a 0.5-1 per cent aqueous solution of propylene phenoxetol or ethylene glycol monophenyl ether, or by cooling them to 5°C for ½ hour (Bird and Saurer, 1967) [4]. The most commonly used fixative is osmium tetroxide (OsO₄) and although some authors have stated that it penetrates more slowly than some other fixatives, it fixes many cell constituents excellently, especially phospholipid membranes. Prolonged treatment with OsO₄ more than 1 hr may remove some of the proteinaceous material but will increase the contrast of membranes. Best results are obtained if OsO₄ is used after aldehyde fixation in acrolein, glutaraldehyde or formaldehyde, all of which have a rapid rate of penetration and a capacity for cross-linking proteins. Tissues are first fixed in the aldehyde and then post-fixed in OsO₄. According to Wright (1968) [30], 1-2 per cent potassium permanganate (KMnO₄) may also be a useful fixative for some nematodes, fixing some structures better than aldehyde/OsO₄, though cytoplasmic particles with a high RNA content are not well preserved by KMnO₄. Some authors have stated that better results are obtained if fixation with KMnO₄ is preceded by aldehyde fixation.

Nematodes are usually fixed at 0-4 °C and at pH 7-7.5. Several buffers are commonly used, of which phosphate is excellent for most purposes. Buffer solutions should be freshly prepared just before use. Adequate rinsing in buffer solution is essential after aldehyde fixation if this is to be followed by OsO₄ because traces of aldehyde appear to react with OsO₄ producing a fine, dense precipitate.

Phosphate buffer pH 7.2

0.1 M NaH₂PO₄...28 ml

0.1 M Na₂HPO₄...72 ml

Mix and add 10 drops of 1 per cent CaCl₂

This buffer vapour is harmful and use in a fume cupboard. Some authors recommended adjusting the toxicity of the buffer solutions with 0.2 M sucrose (7g per 100 ml buffer), or with salt solutions (Mercer and Birkbeck, 1966) [18].

Glutaraldehyde

Beware of vapour is important and use only in a fume cupboard. Take 15 ml glutaraldehyde (usually as 25 per cent aqueous solution) and add to it BaCO₃ to excess, until a precipitate remains (a small amount on the end of a spatula is usually sufficient). This removes any glutaric acid which may be present. After filtration, alternatively purified glutaraldehyde, free from glutaric acid

Acid-free glutaraldehyde...10 ml

(Final concentration, 5 per cent)

Buffer solution...40 ml

(Check the pH of the fixative solution)

The following procedure should take place at 0-4 °C, in an ice bath. Fix for 1-24 hr. Alternatively, cut the nematode after 1-2 hr in fixative and embed in agar, returns to fixative for at least a further hour. Rinse in buffer, 1 hr. Change buffer 6 times at ½ hour intervals. Post-fix with OsO₄.

Acrolein

Beware of vapour is important and use only in a fume cupboard. Acrolein polymerizes if exposed to heat and light. It should be stored in a dark, cool place, preferably in a refrigerator. It should be colourless or pale yellow. If darker than this, polymerization has occurred and it should be safely disposed of. Prepare a 3-5 per cent solution of acrolein in buffer. Fix as for glutaraldehyde. Post-fix with OsO₄.

Formaldehyde

Add 2 g paraformaldehyde to 25 ml distilled water and heat to 60 °C, stirring. Add 0.1 N NaOH dropwise until the solution clears. Cool and make up to 50 ml with buffer. (Final concentration of formaldehyde, 4 per cent). Fix as for glutaraldehyde, 4 per cent formaldehyde can also be used mixed with 3-5 per cent glutaraldehyde (Karnovski, 1965) [14].

Osmium tetroxide

Beware of vapour is important and use only in a fume cupboard. Osmium tetroxide is usually supplied in ampoules containing 0.25 or 0.5 g. A convenient method of making a solution of OsO₄ in buffer is as follows: Take the intact ampoule and carefully liquefy the contents in a beaker of water at 60 °C. Remove the ampoule from the water and rotate it until the OsO₄ recrystallizes evenly around the inside of the ampoule. Rinse in distilled water. Wrap the ampoule in clean paper and crush it, taking care not to pierce the paper. Tip the fragments into a flask and make up to the required volume with buffer to give a 1-2 per cent solution. Osmium tetroxide is slow to dissolve, so allow to stand for at least an hour before use. Fix at 0-4 °C for 1-16 hr.

Dehydration

This should take place in the cold and can be quite rapid (Rossner and Perry, 1975) [23]. Take the specimen through an ethanol series; say 20, 30, 50, 70 and 90 per cent, and

absolute ethanol, at ¼-½ hr intervals, followed by three or four changes of absolute ethanol over 2 hr at room temperature. If possible, redistilled absolute ethanol should be used for the last two changes. Bird (1964) [2] recommends the water-soluble poly-epoxide 'Durcupan A' as an alternative dehydrating agent.

Infiltration of embedding resin

Replace the ethanol with propylene oxide (use in a fume cupboard) and cap the vial, giving two changes at 10-minute intervals. Then, to the vial containing the propylene oxide, add dropwise in gradually increasing quantities 8 ml of a 1:1 mixture of propylene oxide and embedding resin monomer. This should take about 2 hr. Replace the cap between additions of the mixture to limit evaporation at this stage. Then leave the vial uncapped for 24 hr for the propylene oxide to evaporate. Remove the specimens from the resin, blot them on absorbent paper and replace them in freshly made resin. Change the resin in this way, daily for 2-3 days, stirring two the three times a day and leave in a dry place to ensure good infiltration. If necessary, place under vacuum.

Embedding

Embedding media in current use include 'Epon', 'Araldite' and Maraglas 655', all of which are epoxy resins, and 'Vestopal' (Kay, 1965) [15], which is a polyester resin. Of these, Araldite and Epon are probably the most use. 'TAAB Resin' is an epoxy resin of low viscosity which may be useful for infiltrating nematodes. These materials consist of several components-the resin, a hardener, a diluent and an accelerator- which are mixed together just before use. There are several components and mixes for each of these resins, and it is important to obtain the correct ingredients.

Araldite

Resin MY 740...19 ml

Hardener HY 964 (DDSA dodecenylsuccinic anhydride) ...25 ml

Diluent DY 022...2 ml

Accelerator DY 064 (DMP 30; 2, 4, 6-tri dimethylaminomethyl-phenol) ...0.3 ml

Mix together the resin and hardener, using a mechanical stirrer, for about 10 min; then, while stirring, add the diluent. When thoroughly mixed after a further 10-15 min, add the accelerator. Continue mixing until all streaks disappear. Air bubbles may appear if stirring is rapid. These usually disappear on standing of after a brief period in an oven at 60 °C.

Epon

Mixture A Epikote 812...31 ml, Hardener HY 964 (DDSA dodecenylsuccinic anhydride) ...50 ml

Mixture B Epikote 812...50 ml, Diluent MNA (methyl nadic anhydride) ...45 ml

These two mixtures can be stored separately in a refrigerator for several months. Take care to avoid contamination by water condensation. When required for use, mix together equal volumes of A and B and add 1.5-2 per cent accelerator DY 064. Ratios of A: B may be varied to give blocks of varying hardness.

These resins are hardened by polymerizing at 60-80 °C for several days. Embedding is most conveniently carried out in gelatine capsules size 00. It is sometimes easier to orientate the specimen on a flat blank of ready-polymerized resin

with one drop in the bottom of the capsule. Polyethylene Beem capsules are also available. These are pre-shaped with a tapered base so that the block requires less trimming. Place a few drops of resin in the capsule, allow excess resin to drain from the specimen on to absorbent paper and then place it in the capsule, allowing it to sink; then fill the capsule to just below the brim with resin, inserting a label if necessary. Leave the capsule open and place in an oven at 60-80 °C to harden. During polymerization the capsules may be held conveniently in a wooden block in which cavities of the appropriate size have been drilled, or in a cardboard box lid in which holes have been punched with a cork-borer.

Microtomy

The art of cutting thin sections is best learnt by practical training from an experienced microtome. The very thin sections (200-1000 Å) necessary for observation in an electron microscope are cut on an ultramicrotome, using a glass or diamond knife. Many of the current models of ultramicrotome operate on a thermal advance system, so that the thickness of the section depends partly on the rate of heating of the advance mechanism and partly on the speed of the cutting cycle. In most, therefore a motor drive is used to produce a steady and adjustable cutting rate.

Glass knives are fairly easily prepared (Mercer and Birkbeck, 1966) [18] from specially selected plate glass, although some practice is required at first. Knife-making machines, now available, simplify the procedure greatly. The main advantages of glass knives are cheapness and disposability. Diamond knives are expensive but with careful use they last for six or more months and can be re-sharpened.

The ultra-thin sections are floated on water as they are cut and diamond knives are usually supplied with a water bath attached. Glass knives are easily fitted with a water bath made from waterproof adhesive tape and sealed to the knife with paraffin wax applied with a hot needle.

The block containing the specimen is trimmed under a stereomicroscope. The initial rough trimming of 'Araldite' blocks is often done with a jeweller's saw and the fine trimming with a razor blade or scalped blade. The final cutting face should be of the order of 0.2 mm².

The sectioning procedure is watched through a stereomicroscope with incident illumination over the knife and water bath, specially positioned to show the interference colours of the sections by which their thickness is judged. The following table after Mercer and Birkbeck, (1966) [18] gives the estimated thickness of differently coloured sections.

Grey: 600 Å
Silver: 600-900 Å
Gold: 900-1500 Å
Purple: 1500-1900 Å
Blue: 1900-2400 Å
Green: 2400-2800 Å

For most purposes' sections should be grey or silver, although for low-magnification surveys pale-gold sections may suffice. The shape of the water meniscus at the knife edge is important if the interference colours are to be seen easily. If the water bath is too full the illumination will not be correct and in addition, the block face will draw water over the knife edge.

Providing the block face is carefully trimmed with the upper and lower surfaces parallel, and the block is correctly orientated to the knife edge, the sections should form a ribbon on the surface of the water. Araldite sections usually become compressed during cutting. They can be expanded by holding a small piece of filter paper soaked in chloroform just above the surface of the water in the vicinity of the ribbon of sections.

Small grids, usually of copper or copper alloy are used to support the sections for viewing in the electron microscope. These are obtainable in many sizes and shapes of mesh and the type selected depends on the size and thickness of the section and the nature of the specimen. Large meshed or slot holed grids are coated with collodion, formvar or carbon to support the sections. To pick up the sections, grid is held with fine forceps and lowered on to the sections as they float on the water. The sections adhere to the grid which can then be lifted off the water and dried by touching the edge with filter paper. The grid is then placed, section side up, on filter paper in a Petri dish, which should then be covered to exclude dust. Sections can be stored indefinitely in this way. If difficulty is experienced in getting the sections to adhere to the grid, it should first be soaked in CCl₄ (carbon tetrachloride) to remove the anti-corrosive coating, then rinsed with water and dried. Alternatively, the grid can be brought up from below the sections so that they come to rest on top of it.

Staining

It is possible to improve the contrast of the specimen, when viewed in the microscope, by increasing the electron density of certain tissues using heavy-metal stains. This can be done either during the preparation of the specimen for embedding, or by staining the sections on the grid.

Lead compounds, usually lead citrate, are useful for staining sections as follows:

Lead Citrate

Pb(NO₃)₂ (lead nitrate)...1.35 g

Na(C₆H₅O₇). 2H₂O (sodium citrate) ...1.76 g (Reynolds, 1963) [20]

Add to 30 ml distilled water in a 50 ml volumetric flask. Stopper and shake vigorously for 1 min. Allow to stand for 30 min, shaking at intervals. Add 8 ml 1N NaOH. This should give a completely clear solution. Make up to 50 ml with distilled water and mix by inversion. Any remaining turbidity is removed by centrifugation. Store in a refrigerator. Place drops of stain solution on a sheet of dental wax in the bottom of a Petri dish. It is important, if lead stains are used, to exclude CO₂, which causes the precipitation of PbCO₃ on the section. Place pellets of NaOH in the Petri dish around the dental wax, and place a filter paper soaked in 0.02N NaOH in the lid, to absorb the CO₂. Float the grid on a drop and cover the dish. The stain is taken up rapidly and 5-10 min a normally long enough. Remove the grid from the stain and rinse in 0.02N NaOH, then rinse thoroughly with a jet of freshly boiled and cooled distilled water from a wash bottle. Place the grid on filter paper or an unglazed tile to dry.

Other stains are uranyl acetate and less commonly phosphotungstic acid. These, particularly uranyl acetate are often used in addition to lead salts, staining first with uranyl acetate or phosphotungstic acid followed by lead.

Uranyl acetate

1 per cent aqueous solution. A staining time of 15-60 min may be necessary.

Phosphotungstic acid

1 per cent solution in absolute ethanol for 15-60 min. Phosphotungstic acid and uranyl acetate may also be incorporated at the 70 per cent ethanol stage of dehydration, at a concentration of 1 per cent. Specimens should be stained for 1-2 hr.

Preparation of nematodes for observing with a scanning electron microscope (SEM)

This microscope scans the outer surface of specimens with a focussed beam of electrons in an evacuated chamber. It gives a picture with great depth of field which shows up the surface structure of nematodes admirably (Green, 1967) [12]. The surface of the specimen must be electrically conducting and this is often ensured by coating it with metal, e.g. aluminium (Wergin, 1982) [28]. However, with biological material, e.g. nematodes processed to glycerol, better results are obtained without coating but using low kV (Clark and Stone, 1975) [9]. As the specimens are exposed to high vacuum in the instrument, volatile liquids must be removed during preparation, without distorting the cuticle (Black, 1974) [5].

The following methods have given good results, though they may require modification with some specimens.

Glycerol Method

Fix the specimens in TAF (Courtney *et al.*, 1955) [11] and impregnate with glycerol by Seinhorst's glycerol-ethanol method (Seinhorst, 1959) [25] or Baker's rapid method (Baker, 1953) [1]. Drain off excess glycerol by warming slightly and lifting the nematode from the warm liquid. Place the nematode on a tiny spot of fresh nail varnish, 'Durofix' or a similar quick-drying adhesive, on a 9 mm circular coverslip. The specimen should be examined before the adhesive hardens, under the highest-power dry objective of an optical microscope, for possible distortion and check that the orientation is correct. Several specimens can be mounted on a coverslip providing their positions are noted. Stick the coverslip, with the specimens uppermost, on to one of the mounting stubs provided for the scanning microscope. This method leaves the specimens flaccid and not all structural features are equally well exposed (Boyde and Wood, 1969; Boyde, 1978) [6, 7].

Polyethylene glycol method

This compound is more difficult to handle than glycerol but gives better results. Fix the specimen in TAF and impregnate with polyethylene glycol 400, using the same schedule as for glycerol but substituting the following two solutions for the glycerol mixtures.

Solution I ethanol (96%) ...20 ml, polyethylene glycol 400...1 ml distilled water...79 ml

Solution II ethanol (96%) ...95 ml, polyethylene glycol 400...5 ml

Put a tiny drop of nail varnish or another adhesive on a 9 mm circular coverslip and allow it partially to dry. Remove the specimen from the solution, which has been slightly warmed and drain off any excess polyethylene glycol (Cohen, 1979) [10]. Lay the nematode on the nail varnish, exposing the required area. The glycol partially dissolves

the varnish and so the specimen should not be manoeuvred once in contact with it.

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