

A Simple Express Technique to Process Nematodes for Collection Slide Mounts

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Abstract: The new simple technique of hot fixation and a method for processing a mass quantity of nematodes to prepare nematode collection slides for taxonomic and ecological purposes, combined with an abrupt reduction of the set of procedures caused by the “cocktail” principle (a stratification of layers with a gradient of glycerol concentrations), are described.

Key words: collection slides, fixation, morphology, nematodes, technique.

The most popular technique to prepare nematode morphological collection slides among soil and plant nematologists is the methodology described by Seinhorst (1959, 1962), which includes hot fixation and stepwise transfer of worms to anhydrous glycerol. This technique (Seinhorst, 1959, 1962, 1966, 1974) and its following modifications (Netscher and Seinhorst, 1969; De Grisse, 1969; Ayoub, 1977; Huang et al., 1984; Hooper, 1986, 1990; Sulston and Hodgkin, 1988; Grewal, 1990; Poinar, 2010; De Ley et al., 2016; Hall, 1996; Ryss, 2003, 2015; Ferris, 2016) are perfect in the final morphological results. However, fine and high-qualified labor is required for transferring individual nematodes in a series of solutions. Or the protocols include several steps of the manual substitution of solutions. This paper is aimed to describe a simple and fast technique of processing mass nematode suspensions from a fixative to glycerol, with subsequent preparation of permanent collection slides. Special attention is given to high-quality nematode morphology.

MATERIALS AND METHODS

Fixation: The principle of Seinhorst (1962, 1966, 1974) is to add hot fixative (4% paraformaldehyde at 85°C or higher) to living nematodes, surrounded by a minimum amount of water, as quickly as possible (“killing” step). This stage of fixation ensures the quick access of the fixative into the inner tissues, thus overcoming the collagen–lipid cuticle protective barriers. This initial principle is used herein in the proposed modification, with 4% formalin or triethanolamine formalin (TAF) (2 ml triethanolamine, 10 ml 40% formalin, and distilled water added to a final volume of 100 ml). For this purpose, two Eppendorf tubes of 1.5 ml are prepared. One of them is filled with the fixative, whereas the nematode suspension is placed into the second tube. The latter is placed in a vertical position

for 10 min to settle nematodes to the bottom (or as alternative, using a centrifugation step of 3,000 rpm for 30 sec). Then the supernatant is removed, retaining water in 1/3 of the conical part of the tube (approximately 150 μ l); or, in case of mass nematode quantities exceeding 150 μ l volume, it is recommended to retain water at a level double the height of the nematode suspension. Both tubes are then placed into the “floats” (Fig. 1H). The “float” can be easily made from plastic screw caps of 500-ml polyethylene terephthalate bottles (diam. 28 mm). Within the cap, an 8-mm-diam. round hole is made, which is slightly less than the Eppendorf tube diameter to ensure tight contact and therefore good floating ability. The cap is to be turned bottom up and a tube is inserted tightly in the hole of cap. Before fixation, both tubes (with fixative and with the nematode suspension) are inserted into floats (Fig. 1H).

The tube with fixative is heated in a water bath. In field conditions, the water can be heated until boiling, and posteriorly poured into a 0.75- to 1.5-liter heat-resistant plastic container (for microwave oven). The floating tube with fixative is placed into a container with hot water for 3 (2–4) min (Fig. 1G). The tube is then removed and the heated formalin is transferred using a plastic pipette into the second tube containing the nematode suspension (Fig. 1H,I), which due to its low thermal conductive properties maintains the high temperature of fixative. The tube containing nematodes and fixative is then closed and transferred back into the water bath. To end the fixation process, it is necessary to keep the tube with nematodes in hot water for at least 1 hr (Fig. 1J). Then fixed nematodes may be kept in tubes for an indefinite period of time, either as collection material or nematodes may be processed to a slide preparation once the solution reaches room temperature.

Processing of the fixed nematodes until concentrated glycerol: It is recommended to start the slide preparation from previously heated and fixed nematode suspension only after they have reached room temperature (15–25°C). For best results, it would be good to keep nematodes in fixative for a minimum of 48 hr or more. During this period, the process of tissue and collagen hardening takes place.

To saturate bodies of nematode with glycerol in mass nematode suspensions, it is necessary to create

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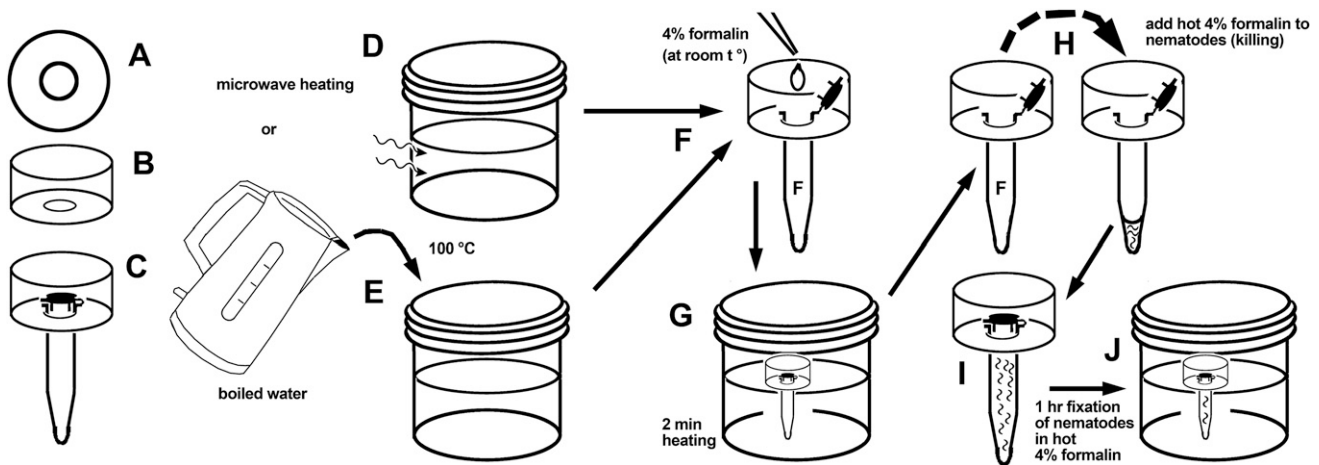


FIG. 1. Hot fixation of nematodes. A–C. Float for the 1.5-ml Eppendorf tube: A, upper view with central hole for a tube; B, 3D view of the float (plastic bottle cap); C, the float with the Eppendorf tube inside, tightly pressed. D, E. Water heating (use any way of these two): D, heating in microwave oven; E, heating in electric boiler and then pouring to the 1-liter heat-resistant microwave pot. F. Pouring of 4% formalin (at room temperature) into 1.5-ml Eppendorf tube inside the float. G. Eppendorf tube with the 4% formalin inside, 100°C for 2 min. H. Transfer of hot 4% formalin into the Eppendorf tube with alive nematodes inside a drop of water (“killing”). I. Nematodes in hot 4% formalin. J. Eppendorf tube with nematodes killed in 4% formalin: further 1-hr fixation in a 90°C water bath.

the stratified “cocktail” of glycerol–water solutions containing different ratios of glycerol to water. The heaviest solution in specific gravity is anhydrous glycerol, the most floaty is water itself, and the water–glycerol solutions may be diluted in between depending on the glycerol content. If we add concentrated glycerol directly to fixed nematodes, the body structures will be irreversibly damaged. Therefore, the saturation of a nematode with glycerol must be slow and gradual, and the stratified column of glycerol “cocktail” is the best tool to achieve this. To create the stratified column, it is necessary to limit the lens-like drop of liquid from spreading on glass surface; a hydrophobic beeswax–paraffin ring is good for this purpose. For a ring, the histology mixture of beeswax and paraffin (1:5) with a 65°C melting point is to be used. A ring is to be attached to a microscopic slide glass. To form a ring, the cotton or silk thread coiled in 5 hanks on a plastic 20-mm cylinder is used (Fig. 2A). It is better to moisten the thread for easy coiling. The thread ring is a frame for a beeswax–paraffin ring. The former is to be placed in the center of the slide. Then two pieces of beeswax–paraffin mixture are placed at the ring in superposition (Fig. 2B,C). The slide with a ring and wax pieces is heated on a hot plate (85°C). The wax mixture will melt and flow along the thread ring, thus forming an accurate hydrophobic ring attached to a slide surface (Fig. 2D,E). After cooling to room temperature, the slide with the ring may be used to process nematodes to glycerol. It may be used repeatedly for processing.

After the wax mixture cooling and solidifying, two glycerol drops are placed on the bottom of the ring; the drops are to be spread on the glass surface by cotton stick to form a thin glycerol layer (Fig. 3C). Then three drops of distilled water are placed above the glycerol,

thus forming a layer that will protect nematodes from direct contact with the concentrated glycerol (Fig. 3D).

Fixed nematodes are washed with distilled water three times, thus removing excess fixative. At the end, nematodes are retained in a water layer of ½ height of the conical part of a 1.5-ml Eppendorf tube (approximately 170 µl). Water with fixed nematodes is shaken and slowly poured from above on the combination of glycerol and water inside the wax ring (Fig. 3E). To maximize the number of nematodes collected from the tube, the nematode tube can be washed twice more with a drop of distilled water. As a result, a high lens of liquid is formed within the hydrophobic wax ring stratified in the increasing density of glycerol from top to bottom. Nematodes are above in the water layer, while the thin layer of glycerol is at the bottom (Fig. 3E). This “cocktail” lens will be kept overnight in open

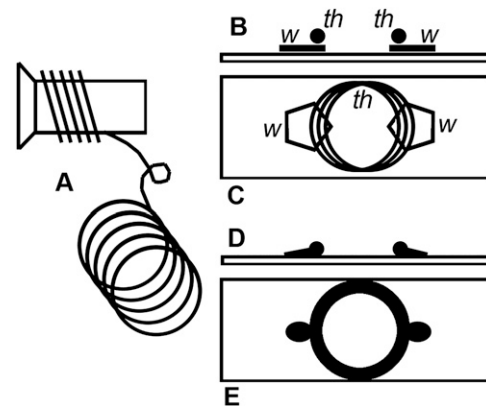


FIG. 2. Preparation of armed wax ring slide. A. Coiling of the thread on the plastic cylinder (five hanks). B, C. Ring of thread (th) on slide, supported with wax chips (w): B, lateral view; C, upper view. D, E. Slide with thread ring after heating on a hot plate, wax melted forming wax ring with thread inside: D, lateral view; E, upper view.

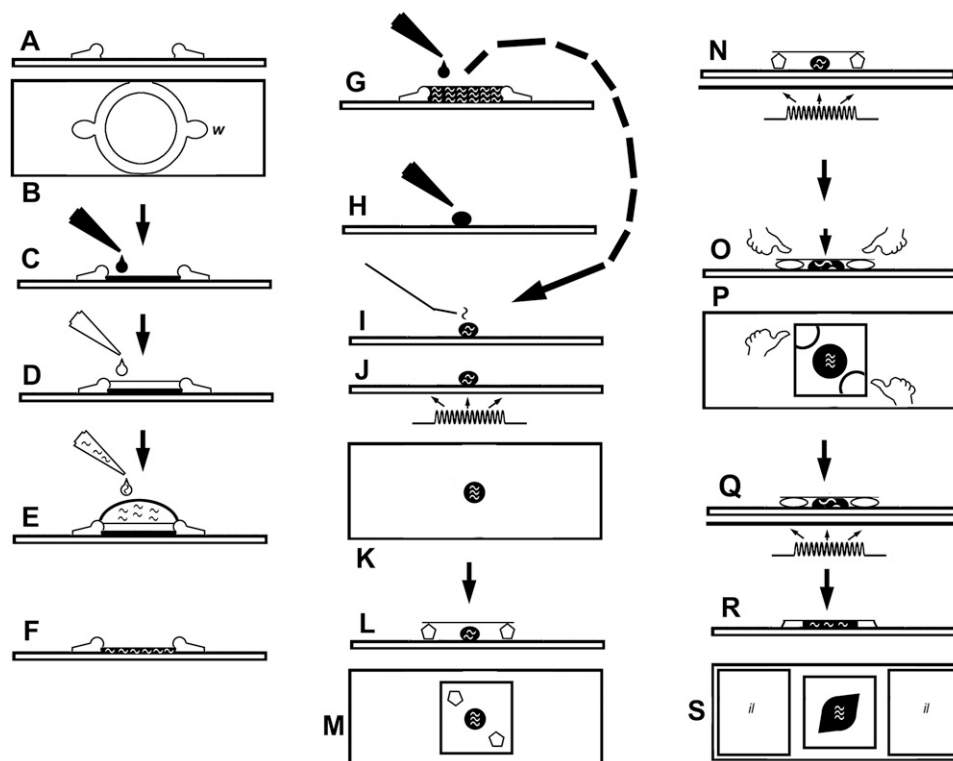


FIG. 3. Processing of nematodes. A, B. Wax ring on slide (*wr*: beeswax–paraffin mix): A, lateral view; B, upper view. C. Glycerol input of the bottom of ring. D. Water input above glycerol layer. E. Input of the fixed nematode suspension to prepare 3-layered “cocktail.” F. After 12-hr water evaporation, nematodes are in thin film of glycerol. G. Input of anhydrous glycerol to fill the wax ring. H. A minute anhydrous glycerol drop in the center of glass slide. I, J. Transfer of nematodes with needle, one by one, into a glycerol drop: I, lateral view; J, heating to evaporate water from nematode suspension in glycerol drop and to better saturation of nematodes with anhydrous glycerol. K. Nematode orientation in line order on the bottom of the glycerol drop, with a needle, upper view. L, M. Placing of the coverslip on the beeswax–paraffin supporters, above the center of a glycerol drop with nematodes. N. Slight heating of the slide at 85°C (on a hot plate) for 3 to 5 sec to soften wax supporters. O, P. Soft pressing of the coverslip with fingers till touching the top surface of the glycerol drop with a coverslip; indication of the places to press the coverslip borders, above the heat-softened wax supporters. Q. New heating on a 85°C hot plate till melted wax surrounds the glycerol drop with nematodes. It is necessary to cool a slide to room temperature immediately to finish a preparation. R, S. Ready collection slide with nematodes in glycerol: central spindle-shaped area surrounded by wax edging under coverslip and two inventory labels (*il*) at sides.

air at room temperature, covered from dust, allowing water evaporation. During this time, a gradual increase in glycerol concentration takes place, saturating the nematode tissues. Nematodes will slowly sink down into a more concentrated glycerol layer. After 12 hr, nematodes are in concentrated glycerol within the ring. At this stage, they may be studied under a light microscope (Fig. 3F). Such nematode suspensions in glycerol may be kept as collection samples and supplied with inventory labels. However, for transport and long-term storage, permanent slides should be prepared, which include (i) slides for ecology purposes (the nematode suspensions in glycerol) and (ii) true collection taxonomic slides with nematode specimens in glycerol surrounded by wax edging.

Collection slides preparation

Preparation of suspension slides for ecological purposes: The wax ring is removed carefully from the glass slide using a scalpel. Anhydrous glycerol is then added until it covers evenly all nematodes on the area that is identical to a 24 × 24-mm coverslip. A slide is heated 10 min on a hot plate regulated for 85°C to remove residual water

and accelerate the glycerol infusion into nematode bodies. In field conditions, a hot plate may be replaced with an iron turned bottom up and regulated for 85°C, “silk mode.” After cooling the slide to room temperature, the glycerol area is covered with a 24 × 24-mm coverslip. At corners of the coverslip, four pieces of beeswax–paraffin mix (1:5, melting point 65°C) are added and then the slide is heated on a hot plate (85°C) until the wax melts and flows, thus surrounding the coverslip to form an edging. The slide is ready after labeling.

Sometimes it is necessary to pick nematode specimens from a suspension slide and to prepare the standard collection taxonomic slides. This can be achieved by opening the slide using a scalpel along the wax edges. Two drops of glycerol are to be added at edges of 24 × 24-mm coverslip. The coverslip will float up and may be easily moved away. Nematodes may be picked up by a needle from the glycerol suspension to prepare standard collection slides.

Preparation of standard collection slides: A petite drop of glycerol is placed on a clean microscopic slide (Fig. 3H);

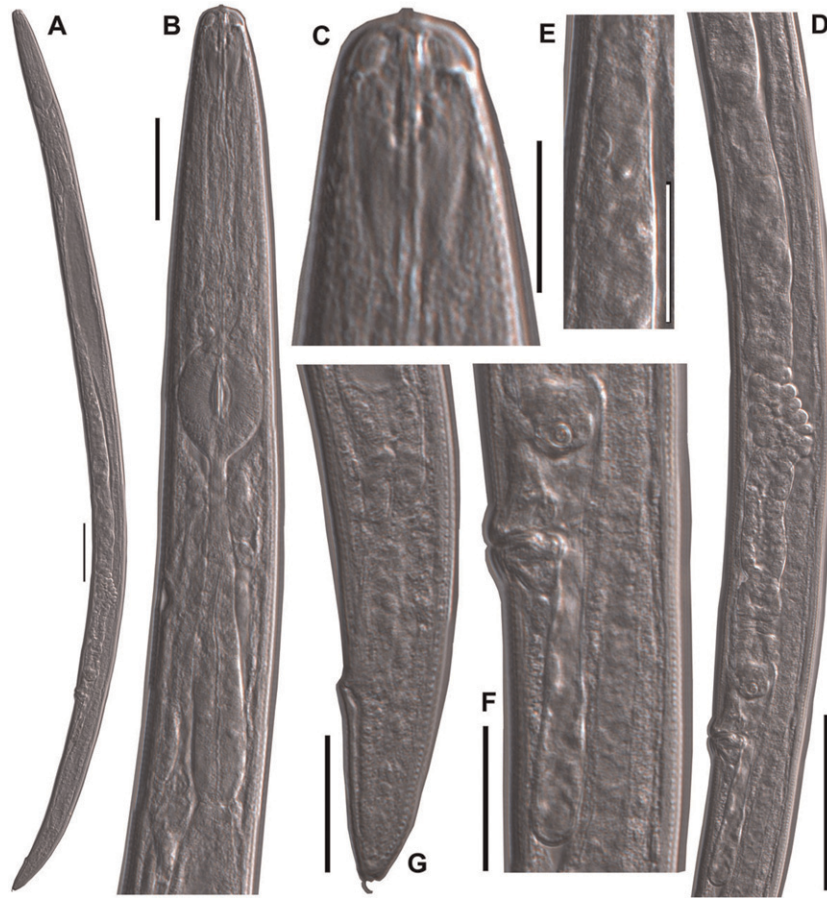


FIG. 4. Photographs of nematode specimens, prepared with the express technique. *Paraphelenchus pseudoparietinus*, female: A, body outline; B, anterior part; C, head; D, genital system; E, anterior end of ovary; F, vulva, vagina, and uterus; G, tail. Scale 50 μm for A and D; 20 μm for B, E, F, and G; and 10 μm for C.

then the nematodes that were fixed and processed to anhydrous glycerol are picked up with a fine needle. They are transferred individually into the drop of glycerol. For nematodes belonging to the same species, the recommended number of specimens placed on one collection slide can vary from 1 to 10 (preferably 4–5), combining adult specimens of both sexes. The slide is heated on a hot plate (85°C) for 10 min to remove the residual water, to accelerate an infusion of glycerol into nematode bodies, and to eliminate slight deformations of nematodes (Fig. 3I,J). Then, under a stereomicroscope, the nematodes are immersed with a needle down on the bottom, and arranged in one or two rows (Fig. 3K). The positioning of nematodes on the bottom is critical, to prevent them floating away to the wax edging during the next stage of preparation. The wax is then heated briefly to make it soft, and two small wax spots are placed diagonally with a scalpel (Fig. 3L,M). An 18 × 18-mm coverslip is placed above the glycerol drop on the wax spots, thus serving as the diagonal supports of the coverslip. The center of coverslip is to be set exactly above a glycerol drop (Fig. 3M). The slide is slightly heated (1–5 sec) on a 85°C hot plate to soften the wax supports. The edges of the

coverslip are then gently pressed with fingers to allow the glycerol drop to touch the coverslip, forming a wide transparent meniscus, within which a row of nematodes may be seen (Fig. 3O,P). Handling should be done quite carefully, otherwise nematodes may change their positions, moving to the periphery and later to the wax edging. After the first contact of the coverslip and a drop, the slide is to be heated again until the wax melts and flows around glycerol drop (Fig. 3Q). After the slide cools down, the collection slide preparation is finished, with one or two rows of nematodes inside of the glycerol drop surrounded with wax edging (Fig. 3R,S). Two inventory labels are then attached to the slide at side areas (Fig. 3S, *il*). One label containing information on the nematode species identification: the Latin name of the nematodes, the name of identifying taxonomist, and the date of species identification. The second label includes the collection data: the locality and date of collection, the collector name, as well as the slide inventory number. Some data on this label may be substituted by codes used in the collection database. It is recommended to include the GPS data in the second label or in the collection database. Photographs

of nematode specimens mounted in collection slides using this technique are given in Fig. 4.

For nematodes with the body diameter more than 40 μm , it is recommended to add coverslip supports of proportionate diameter (glass rods, nylon fibers, and beads) near two wax spots before placing the coverslip. Otherwise the coverslip may cause morphological deformations resulting in flattened specimens. After wax melting, these additional hard supports will be embedded into wax edging.

Collection slides with nematodes mounted between two coverslips: Sometimes it is very useful to prepare the collection slide with nematodes mounted between two coverslips. The user may turn a slide viewing a nematode morphology from two opposite sides. To make such slide, two coverslips are used, one large (24 \times 24 mm) can be used as a base and the second small one (18 \times 18 mm) is used as a cover. The large coverslip is to be attached to the Cobb aluminum frame or to the routine microscopic slide with sticky labels or bands. On the large coverslip, the small drop of glycerol is to be placed. The fixed and glycerol-processed nematode specimens belonging to the same species are transferred into a drop with a needle. Further slide preparation stages are the same as described above for the preparation of the standard collection slides.

Lifetime of collection slides: According to the author's experience, the morphology of the nematodes mounted on slides prepared in 2010 is still as good as in a day of preparation. The quality of the nematode morphology depends mostly on duration of the fixation procedure. The 2-hr fixation allows a good quality of morphology. However, in nematode specimens that are fixed for two or more days, the resulting hardened inner tissues give better quality of morphology.

Another risk is when specimens become flattened over the long lifetime of slides. This may occur if paraffin without beeswax is used as a sealant. Paraffin has a porous crystal-like structure at room temperature. After several years, glycerol may gradually be sucked out from the central zone of a slide due to capillary action of the paraffin edging. Nematodes may become dry or flattened in slide mounts. To avoid this "filter paper" effect, the amorphous beeswax-paraffin mixture (1:5, melting point 65°C) should be used as a sealant and not paraffin itself.

DISCUSSION

The novelty of this technique allows a mass processing of different types of nematode samples in suspensions, with an abrupt reduction of time and sets of procedures, facilitated by stratification of layers composed of a gradient of glycerol-to-water concentrations with different densities (a "cocktail"). The basic inner principles of hot fixation are kept unaltered, but the processing stages are reduced to one step. The proposed wax ring slides with

nematode suspensions may be kept as the collection units. They are ready for microscopic study any time without additional procedures. They are useful to confirm reliability of the published ecological data based on nematode materials (Fig. 5).

The hot (85°C) TAF and hot 4% formalin were used as fixatives. Both are suitable for the glycerol-processed technique described here. The fixative FA (mixture of 1% acetic acid and 4% formaldehyde in distilled water) was excluded due to unstable results: acetic acid cannot be washed completely from the internal structures, and during the long-term storage, the nematode structures become more and more transparent. In addition, acetic acid makes the structures too soft, and during processing of mass nematode suspensions via glycerol-water mixtures, some nematodes may be deformed in a final slide mount. TAF seems to be the best fixative compared with hot 4% formalin in one additional feature: after at least 3 yr in glycerol slide, the TAF-fixed specimens may be taken out from the slide and stained with nucleic stains (such as acetic orcein), whereas 4% formalin-fixed nematodes cannot be stained. However, specimens fixed in hot 4% formaldehyde are more rigid than those fixed in hot TAF. To avoid possible shrinkage of some thick nematodes during fast glycerol-water processing of multispecies nematode suspensions, it would be safe to use hot 4% formalin as a fixative.

In the preparation technique described here, ethanol is not used. Usually in fixation and preparation techniques ethanol has two functions: (i) it may be an additional fixative if 5% ethanol-glycerol-water mixture added just after very short killing with hot 4% formalin or TAF and (ii) it allows quicker infusion of glycerol into nematode tissues, thus replacing inner residual water. In fast processing of mass nematode suspensions, the smallest nematodes may shrink during addition of ethanol-containing solutions. Therefore

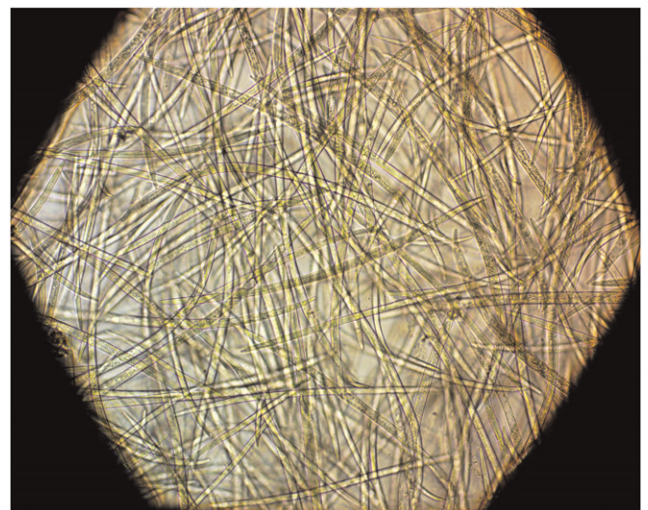


FIG. 5. Mass nematode suspension in glycerol, on the ring slide.

ethanol is excluded. But 10-min heating at 85°C is used in the proposed technique to evaporate residual water from fixed nematodes and to accelerate an infusion of glycerol into nematode bodies. Heating is used when nematode specimens are placed in a small drop of glycerol on a slide glass. In the “ecological” suspension slides, the beeswax–paraffin ring is removed with a scalpel, and then the slide with a total nematode suspension is heated on a 85°C hot plate for 10 min before a coverslip mounting and sealing.

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